

ENCYCLOPEDIA OF IMMUNOLOGY

SECOND EDITION



EDITED BY
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VOLUME ONE



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PREFACE

This encyclopedia provides the largest integrated reference source of immunological knowledge that is available. In the six years since the first edition of the Encyclopedia of Immunology was published, there have been astounding advances in many areas of the subject. The second edition of the encyclopedia seeks to encompass these advances and to provide a more up-to-date coverage of the subject as a whole than is now provided by the original text. The large number of the topics covered will be of direct interest not only to experimental and clinical immunologists but also to scientists from many other disciplines, including microbiologists, veterinary surgeons, protein chemists, physiologists, hematologists, and so forth. In addition to being a primary reference source, the encyclopedic format readily lends itself to casual reading with most entries being only two or three pages long.

The encyclopedia has been expanded to four volumes, with entries given in alphabetic order; Volume 1 covers ABO Blood Group System to Cooperation, Mechanisms of Cellular; Volume 2 covers Copper and the Immune System to Idiotype Network; Volume 3 covers $I\alpha/I\beta$ (CD79a/CD79b) to Nude (Athymic) Mice; Volume 4 covers Nutrition and the Immune System to Zinc and the Immune System. A complete list of entries is given at the beginning of each volume. Any topic that does not have its own separate entry will be quickly located by reference to the index.

The vast majority of the articles have been totally rewritten from those that appeared in the first edition and the overall production time of the Encyclopedia has been speeded up in an attempt to make the work as current as possible. To reflect important advances there are 64 completely new entries. These cover fields such as second signals for lymphocyte activation, apoptosis, anergy, dendritic cells, nucleic acid immunization, MHC-peptide binding specificity, bacteriophage display of antibody fragments and humanization of antibodies. All areas that were in their infancy when the first edition was produced, but are now central to immunology. Other entries reflect the fact that many cytokines and cell surface molecules have been discovered in the intervening six years. Individual entries are contributed by researchers widely acknowledged as experts in their field. There are a total of 630 entries, and 1200 authors have contributed to this work. Because each entry has been written to be self-contained it would be impossible for there not to be some repetition between entries. Whilst this has in most cases been kept to a minimum, we make no apologies for such overlap as it is felt that the alternative would require the irritating practice of having to flick back and forth between several different entries for the uninitiated reader to fully interpret a particular entry. Inevitably, size limitations have not permitted each entry to be exhaustive; entire books could (and have!) been written with titles similar to individual entry headings. Rather, the aim has been to provide the most pertinent information for each topic. The space allocation imposed upon each contributor has also meant that contributors have not been able to cite by name the work of every individual who has made a significant contribution to their field, but rather they have tried to provide the essential core of the available data.

Readers should note that whilst convention dictates that genes are identified using italicized characters and their protein products referred to using plain text, we have generally only adopted this procedure in entries dealing with nomenclature. Otherwise we have stayed with the approach used by the majority of the contributors, namely using plain text also for the genes whilst making it clear that it is the gene that is being referred to.

The reader will find a list of further reading at the end of each entry, comprising a selection of review articles and perhaps some key papers in each area. Furthermore, at the end of each article there is a cross-referencing '*See also*' section where the reader is directed to other entries within the encyclopedia which contain additional and/or related information.

We have introduced color plate sections into the second edition, not only to add to the attractiveness of these volumes but also to present information, such as computer generated images derived from X-ray crystallographic data, that is otherwise difficult to visualize. Also new for the Second Edition is a glossary providing concise definitions of commonly encountered immunological terms.

An electronic version of the Encyclopedia is available online to purchasers of the print edition (see page iii) which will add to the value of this edition. The user of the electronic version will enjoy extensive hypertext linking and advanced search tools.

We have ourselves, as the first individuals able to consult the completed work, again found it extremely useful as a reference source, a teaching aid, and a text for general interest. We trust others will find this Second Edition of the Encyclopedia of Immunology equally valuable.

April 1998

PJD
IMR

ACKNOWLEDGEMENTS

The major credit for these volumes clearly rests with the authors of each entry. They are all recognized as being amongst those pre-eminent in their field. We wish to thank each of these authors for their excellent contribution and for their willingness to produce entries to tight deadlines. An additional 'thank you' is also due to the many contributors who provided suggestions and constructive criticism regarding the encyclopedia.

Our task as editors was helped immeasurably by the participation of our outstanding Editorial Advisory Board. Their ability to 'name names', their comments regarding additions or modifications to the list of entries and their expert reviewing of manuscripts established a solid foundation upon which the encyclopedia could be built. We would particularly like to welcome Ian McConnell, Fritz Melchers, Fred Rosen, Emil Unanue and Harald von Boehmer to the Editorial Advisory Board for the second edition of the Encyclopedia.

We greatly thank the following members of Academic Press for the publishing services of editorial development support and production of both print and electronic versions:

Carey Chapman, Lorraine Parry, Sara Gorman, Heather Burroughs, Manjula Kariyawasam, Ed Pentz, Emma Parkinson, Helen Knapp, Debi Kruse, Peter Lord, and also to picture researcher Emma Krikler and indexer Jan Ross.

Finally, thanks to Jane, Joe, Tom and Jess for the inevitable sacrifices that they had to make during the preparation of this second edition of the encyclopedia.

Plate 1

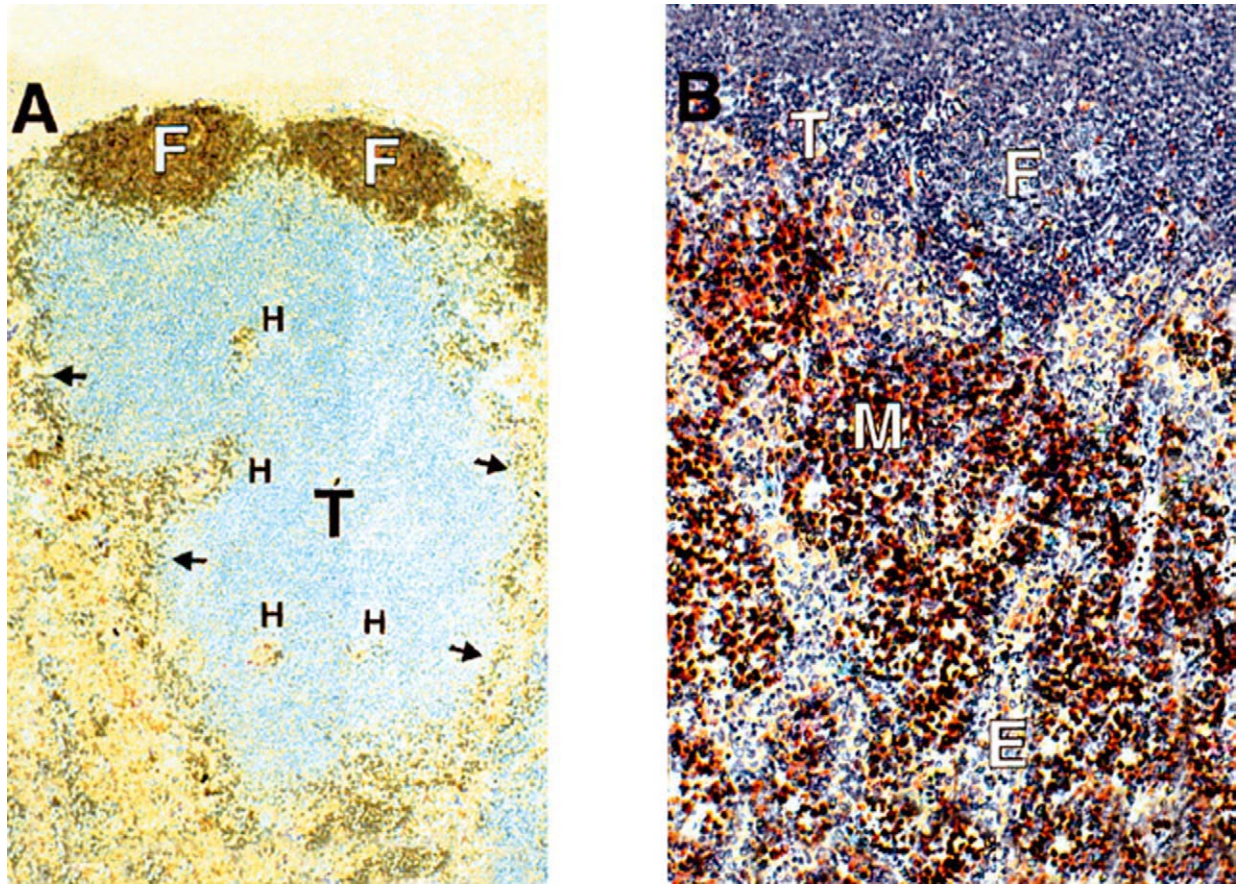


Plate 1 Lymph node. (A) Section of mouse lymph node showing recirculating IgD^+ B cells around their portal of entry. The high endothelial venules (H) located in the T zone (T) are stained blue. IgD^+ cells are also seen in the walls of the intranodal lymphatics on the edge of the T zone and in the follicles (F). En route to the follicles recirculating B cells travel along the walls of the intranodal lymphatics (arrows) where encounter with antigen from the lymph may occur. (B) Section of a formalin-fixed rat lymph node demonstrating large numbers of plasma cells in the medullary cords (M). These are stained brown with anti-Ig. Formalin-fixation has destroyed the surface Ig on the B cells. T - T zone; F - follicle; E - efferent lymphatic. (Kindly provided by Ian MacLennan, University of Birmingham Medical School, UK. Reproduced from MacLennan et al., 1997, *Immunological Reviews* **156**, 53-66 with permission.)

Plate 2

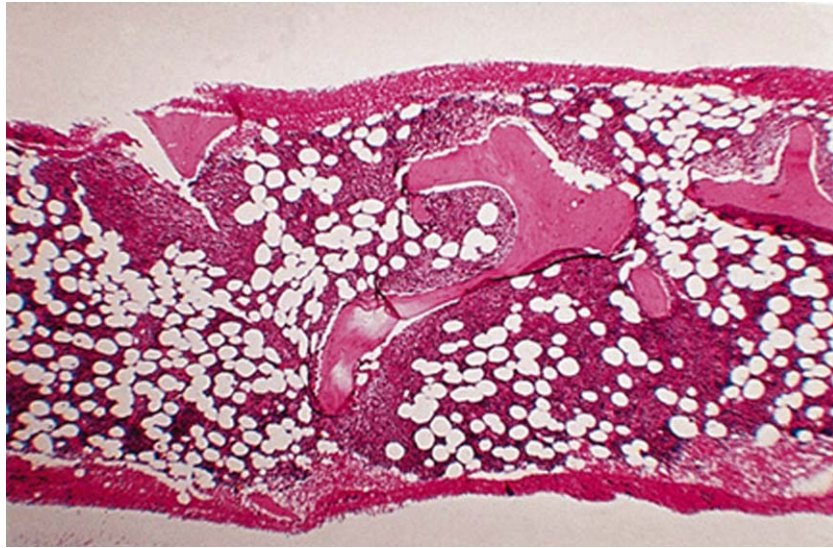


Plate 2 Bone marrow. Trephine biopsy from a 45 year old man (Haematoxylin and eosin stain) Trephine biopsy examination of bone marrow is used commonly to diagnose both hematological and nonhematological conditions. (Kindly provided by Michael Watts, University college Hospitals, London, UK with permission.)

Plate 3

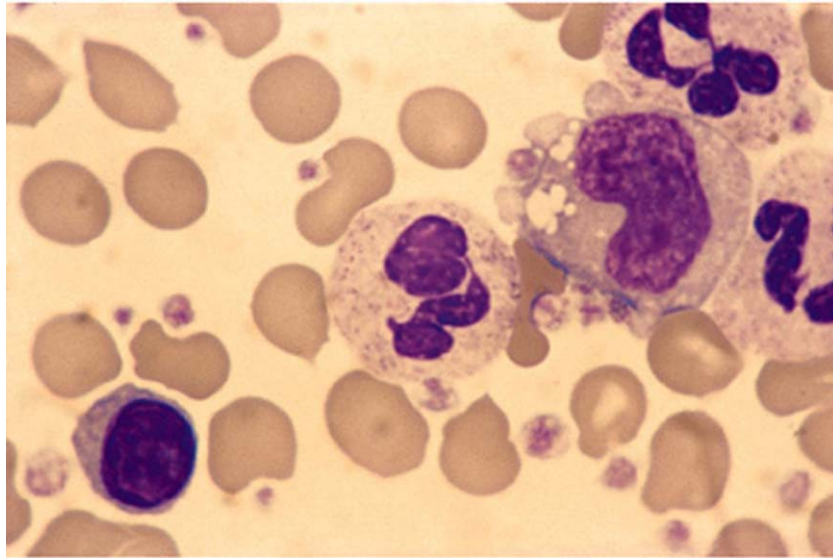


Plate 3 Lymphocyte, neutrophil and monocyte. Bottom left is a small lymphocyte, center is a polymorphonuclear neutrophil and top right is a monocyte showing the characteristic 'horseshoe-shaped' nucleus and moderately abundant pale cytoplasm. Two additional neutrophils are partly in the field of view at the top right. Romanowsky stain. (Kindly provided by the Michael Watts, University College Hospitals, London, UK with permission.)

Plate 4

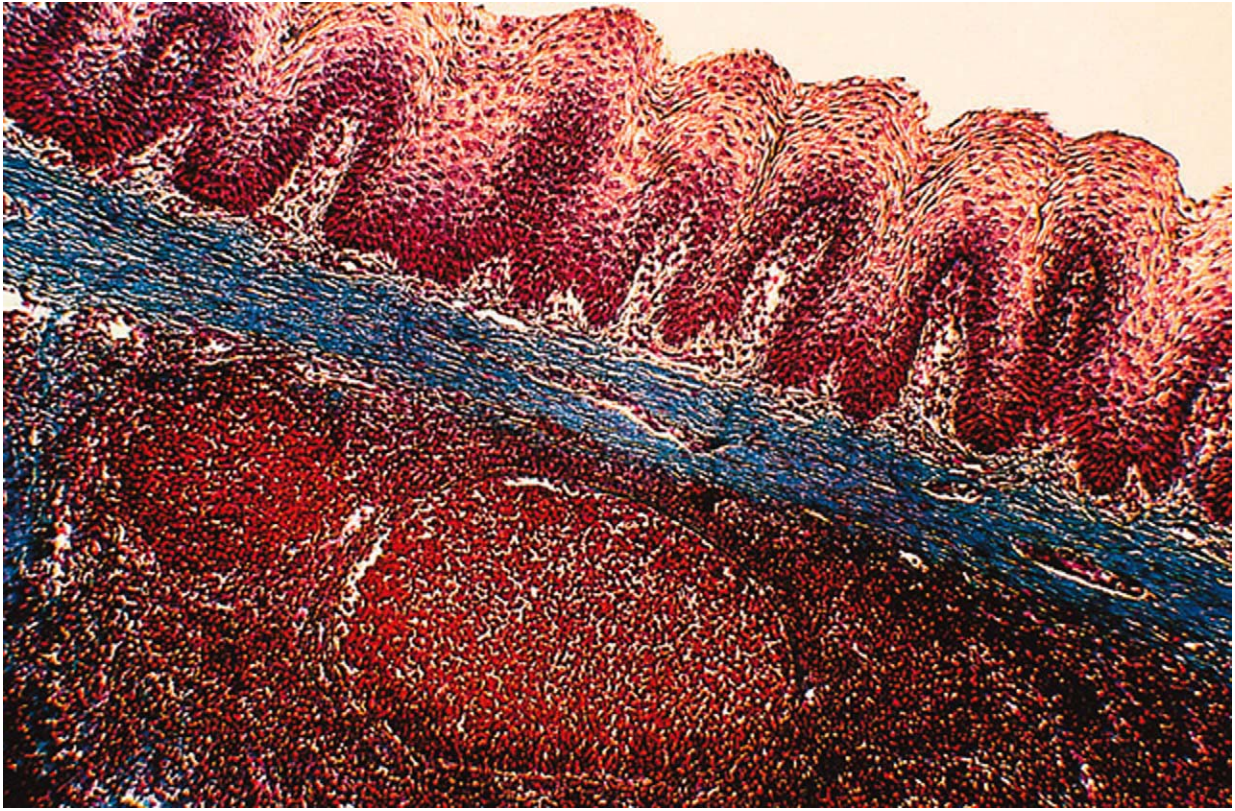


Plate 4 Tonsil. Section of a normal human palatine tonsil. At upper right (pink) is the stratified squamous epithelium of the surface of the tonsil. This epithelium is highly folded. Beneath it is a collagen fiber layer (blue). At lower center is the lymphoid tissue (red). Apart from the pair of palatine tonsils, there is a pair of lingual tonsils and a pair of pharyngeal tonsils (adenoids) which contribute to the Waldeyer's ring of lymphoid tissue. (With permission from Photo Science Library.)

Plate 5

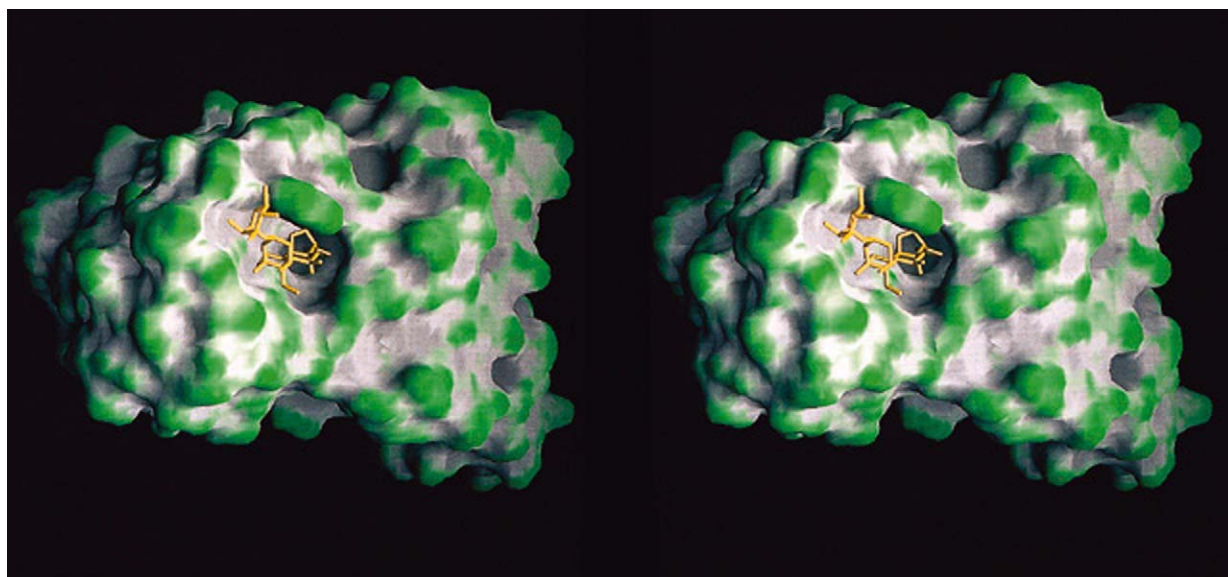


Plate 5 Antibody binding to a hapten. Stereo view of the 40-50 antibody combining site with V_L on the left and V_H on the right and colored to display concave (dark gray) and convex (green) molecular surfaces. The 40-50 binding site is a groove mostly on the surface of the light chain. The bound hapten, ouabain, is shown in yellow. (Kindly provided by Steven Sheriff, Bristol-Myers Squibb Pharmaceuticals Research Institute, Princeton, USA. Reproduced from Jeffrey *et al.*, 1995, *Journal of Molecular Biology* **248**, 344-360 with permission.)

Plate 6

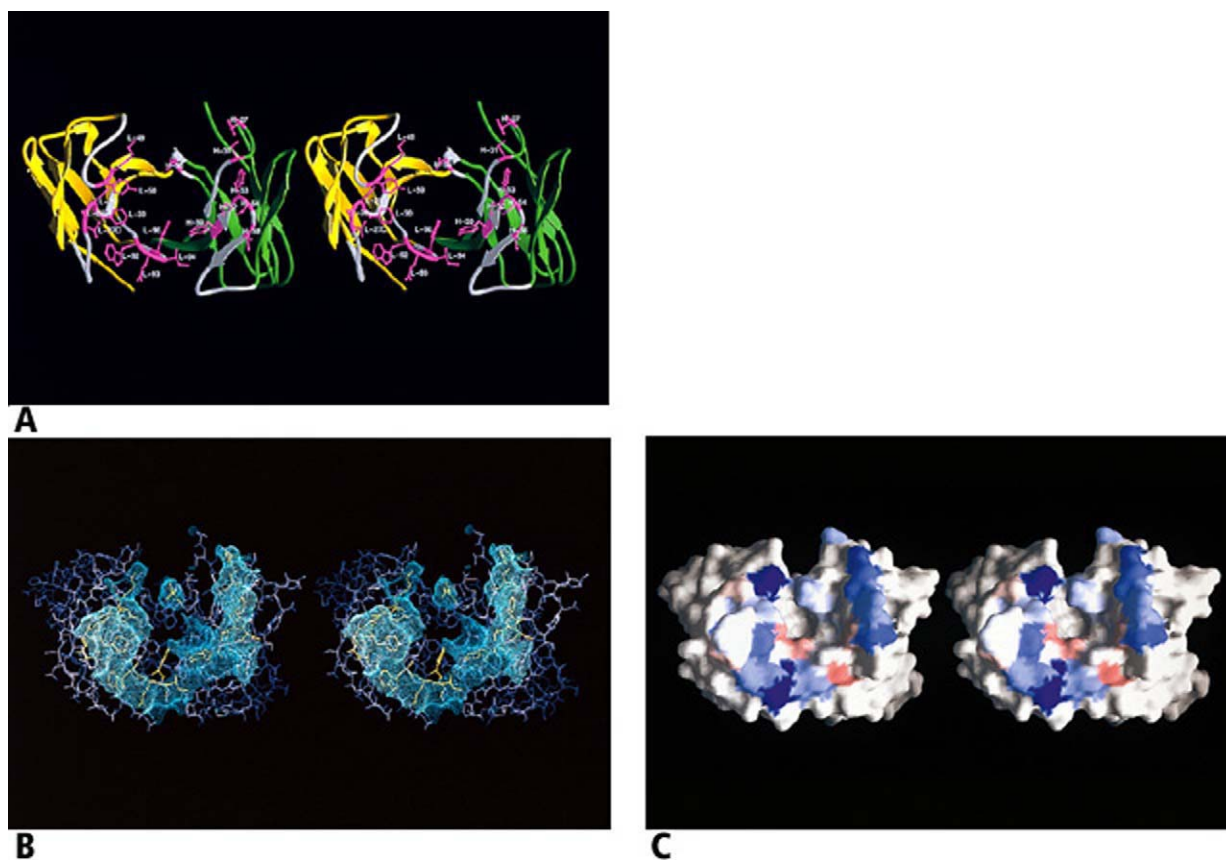
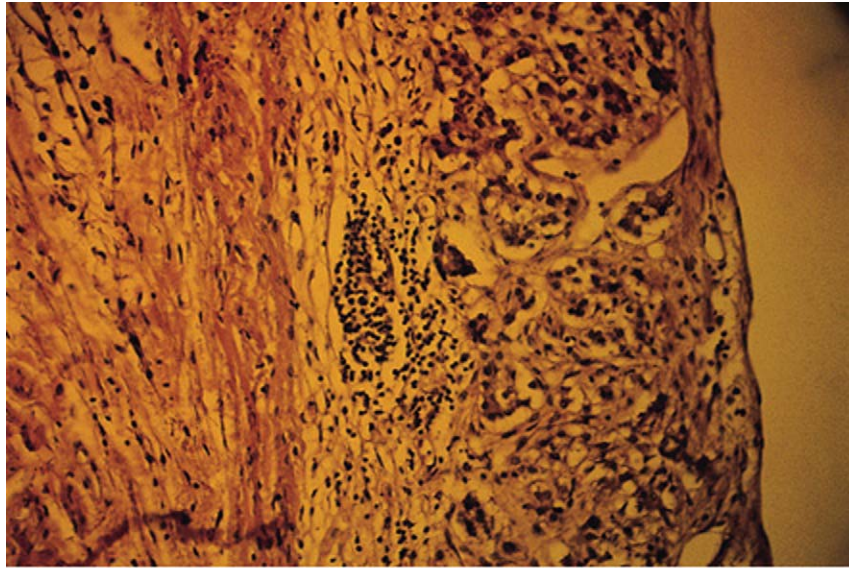
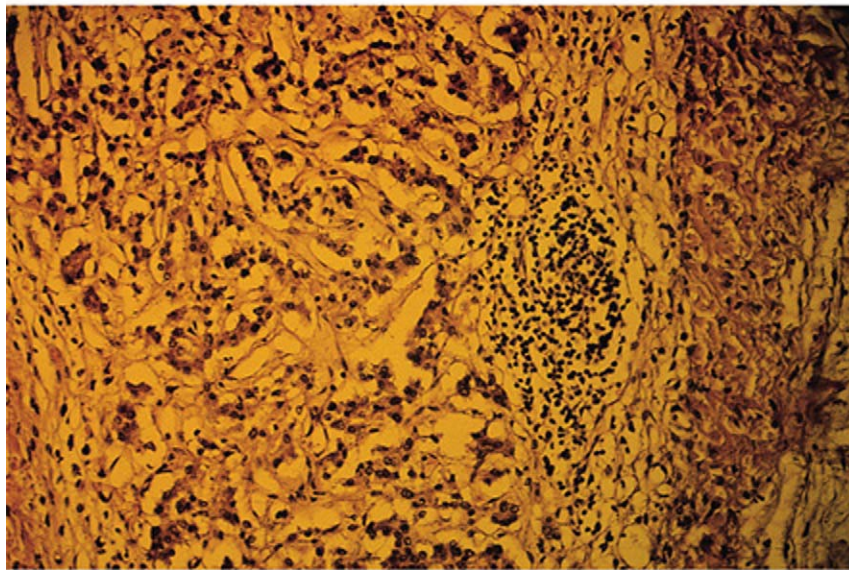


Plate 6 Antibody paratope. (A) Stereo diagram of a ribbon structure of the $V_H: V_L$ domain showing the side-chains of the contact residues that comprise the paratope of the N10 anti-Staphylococcal nuclease antibody. The heavy chain is colored green, the light chain yellow, CDR regions silver and contact residues (with side-chains shown) are magenta. Figure produced with RIBBONS (Carson 1991). (B) Contact molecular surface (cyan dots) of the N10 antibody (blue with contact residues in yellow) calculated by the method of Connolly (1983) and displayed using GRASP (Nicholls et al 1991.) Note the U-shape of the contact surface with the single heavy chain CDR3 contact residue Asn H-96 located just inside the open end of the U. (C) The N10 antibody paratope with the light chain on the left. Surface representation of the calculated ΔG residue contribution to binding by the N10 antibody and the Staphylococcal nuclease antigen residues. A color scale was constructed of the ΔG residue values, from blue (-2.0 kcal/mol) to red ($+2.0$ kcal/mol). Thus, blue colors represent negative ("attractive") residue contributions, red colors represent positive ("repulsive") contributions. Figure produced with GRASP (Nicholls et al 1991). (Kindly provided by Steven Sheriff, Bristol-Myers Squibb Pharmaceuticals Research institute, Princeton, USA. Reproduced from Bossart-Whitaker *et al.*, 1995, *Journal of Molecular Biology* **253**, 559-575 with permission.)

Plate 7



A



B

Plate 7 (A) Addison's Disease. Adrenal gland from patient with Addison's disease, showing atrophy of the cortex and infiltration with mononuclear cells. (B) Another area from same adrenal gland, photographed at higher magnification. (Kindly provided by P E Bigazzi, University of Connecticut Health Center, USA with permission.)

Plate 8

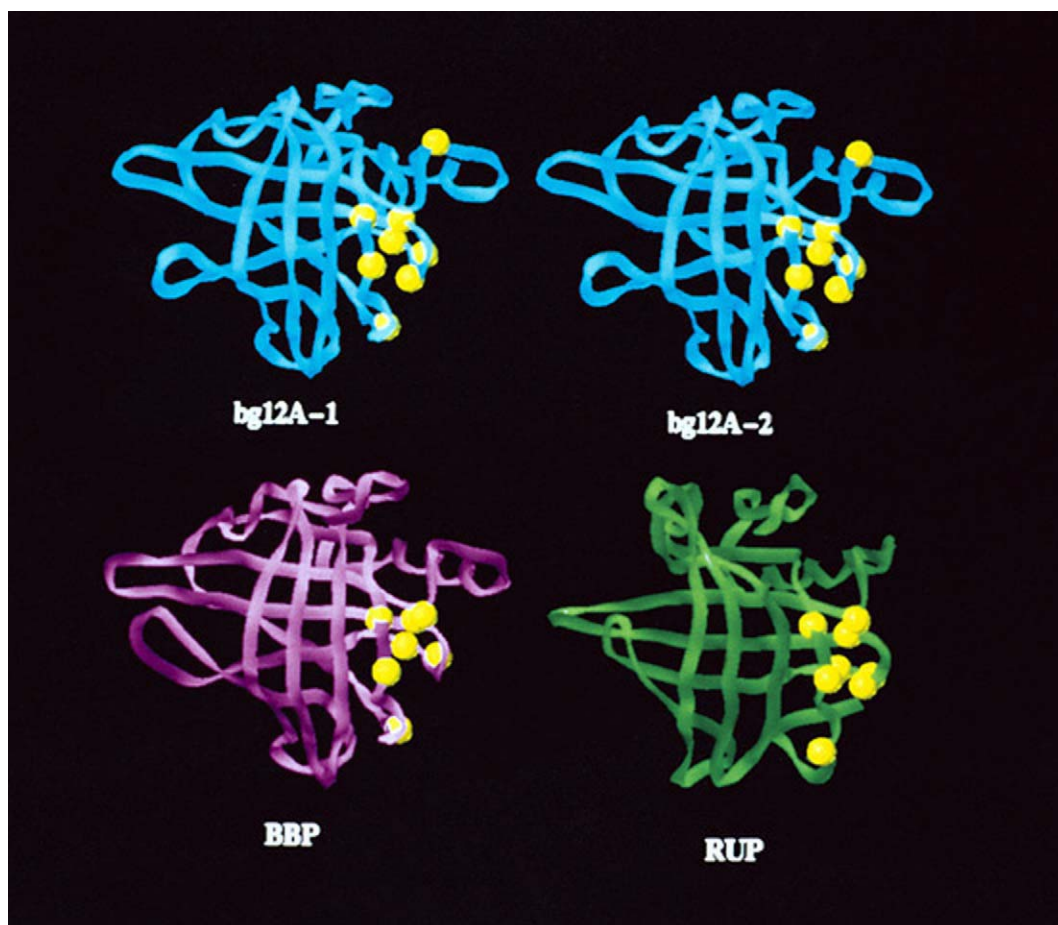


Plate 8 Allergens. Molecular modeling of the cockroach calycin allergen, Bla g 4. The C α backbone structures for two models of Bla g 4 (designated bg12A-1 and bg12A-2) were modeled on the X-ray crystal coordinates for butterfly bilin-binding protein (BBP) and are compared with rat urinary protein allergen (RUP), for which the X-ray crystal structure has also been determined. The yellow spheres are conserved amino acid residues that form motifs which define the ligand-binding proteins, or calycins. (Kindly provided by M D Chapman, University of Virginia, USA with permission.)

Plate 9

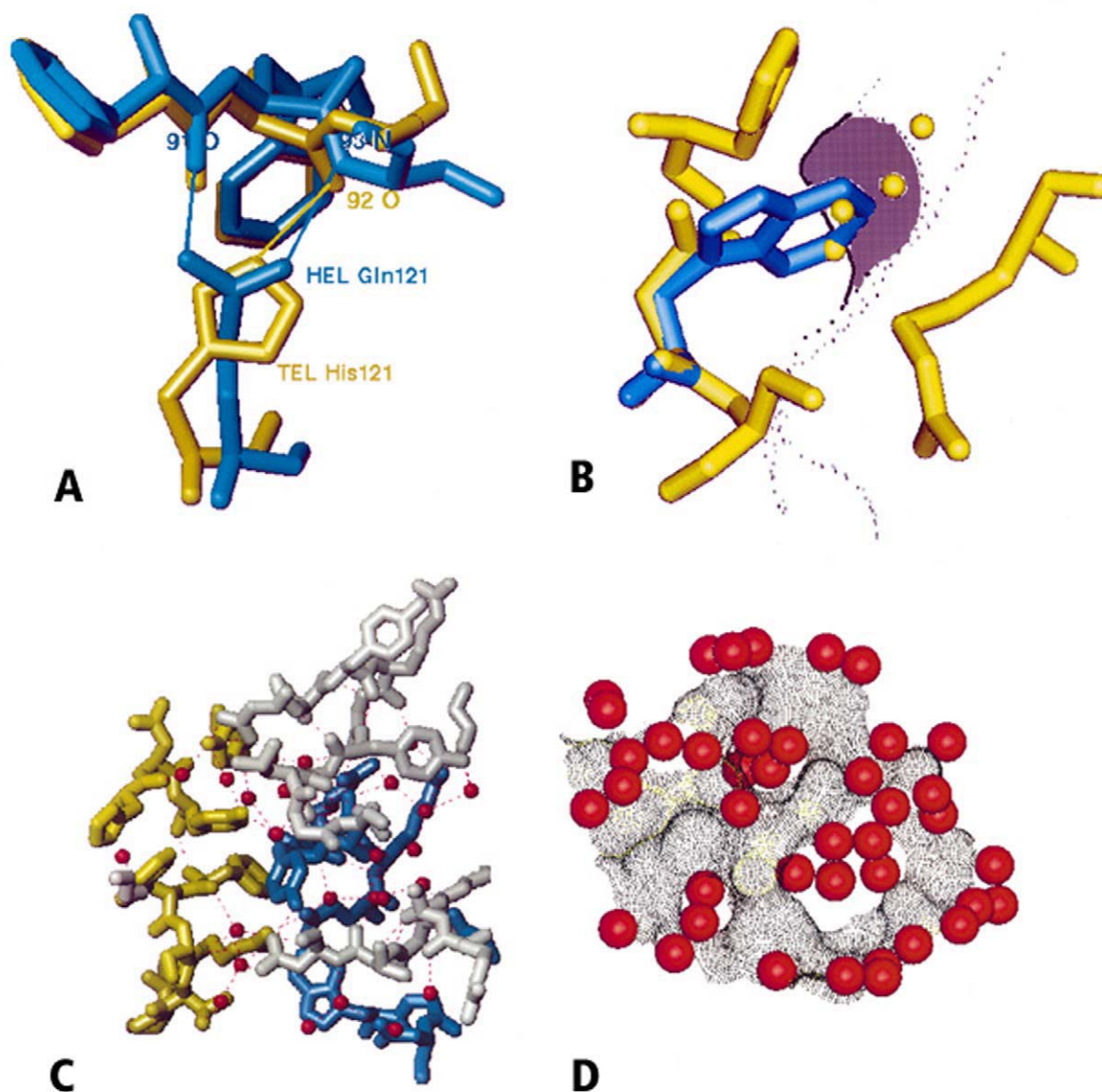


Plate 9 Antibody-antigen interaction. (A) Conformational differences in antibody D1.3 V_L CDR3 induced by differing antigen side-chains. Hen egg lysozyme (HEL) residue Gln121 makes two main-chain hydrogen bonds; to the carbonyl oxygen of D1.3 V_L Phe91 and the amide nitrogen of D1.3 V_L Ser93. Replacement of the glutamine by histidine in Turkey egg lysozyme (TEL) induces a conformational change in the backbone of V_L CDR3 which allows the formation of a hydrogen bond between the histidine and the carbonyl oxygen of V_L Trp92. (B) The effect of a Trp-Asp mutation on the interaction surface area of the D1.3-HEL complex (dots) and the mutant D1.3-HEL surface (solid line) demonstrates the loss of interaction surface area in the mutant complex. A 150 Å² loss in surface area accounts for the reduction in affinity of the mutant D1.3-HEL reaction. (C) Hydrogen bonding network of the D1.3-HEL interface mediated by bound solvent molecules; 25 water molecules form hydrogen bonds linking the antibody and antigen, directly or through other water molecules. (D) Water molecules in contact with the D1.3-HEL buried surface. Including the 25 bridging water molecules, nearly 50 solvent sites are in contact with the buried surface defined by the D1.3-HEL interface. Many of these water molecules fill internal cavities, further stabilizing the complex. (Kindly provided by B C Braden and R J Poljak, Center for Advanced Research in Technology, USA with permission.)

Plate 10

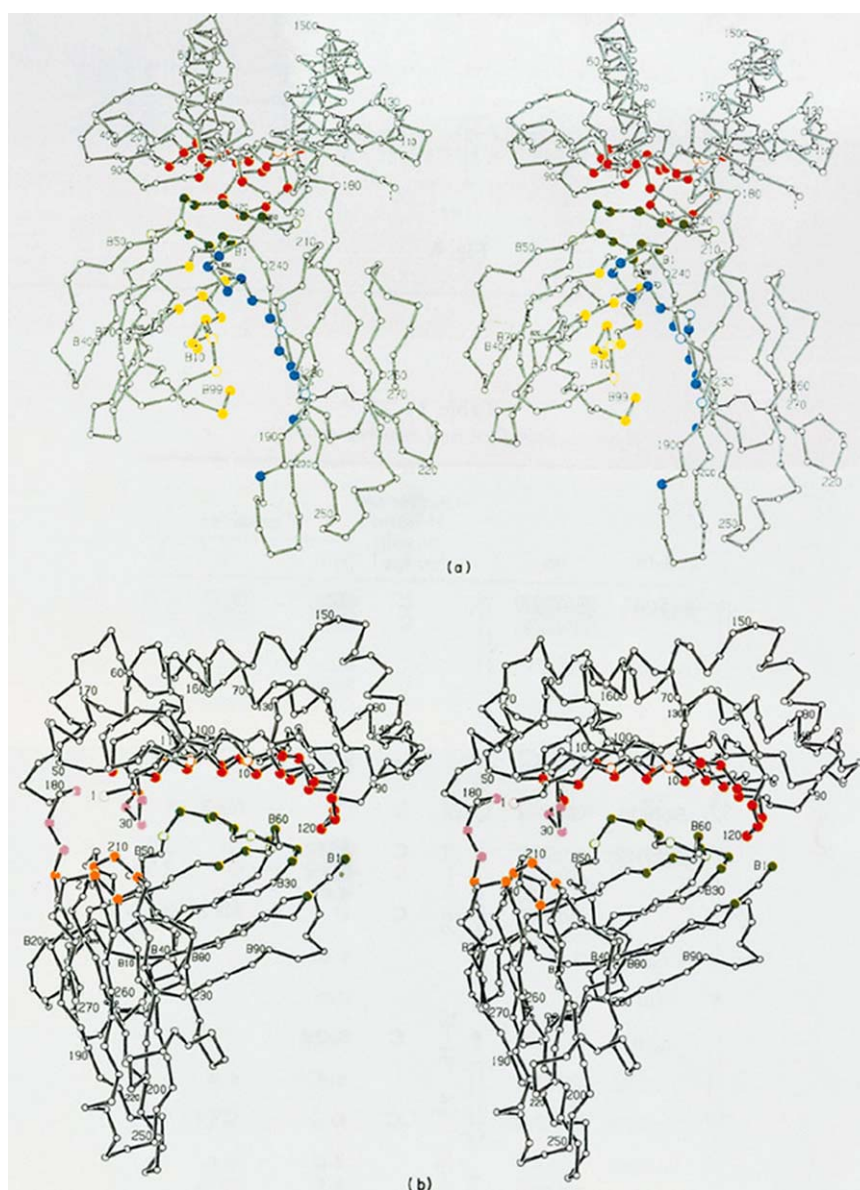


Plate 10 HLA-A2. The residues forming the domain interfaces are highlighted in color on the C_α backbone stereogram. Filled and colored C_α atoms make contacts ≤ 4 Å. Red, α_1 , α_2 residues in interface with β_2M ; green, β_2M residues in interface with $\alpha_1\alpha_2$; blue α_3 residues in interface with β_2M ; yellow, β_2M residues in interface α_3 ; pink, $\alpha_1\alpha_2$ residues in interface with α_3 ; orange, α_3 residues in interface with $\alpha_1\alpha_2$. (A) A view perpendicular to $\alpha_1\alpha_2$ pseudodyad with the binding cleft viewed end-on ($\alpha_1\alpha_2$ - α_3 interface not shown). (B) A side view with the molecule rotated 90° about the pseudodyad (α_3 - β_2M interface not shown). (Reprinted with permission from Saper MA, Bjorkman PJ and Wiley DC (1991) Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *Journal of Molecular Biology* **219**: 277-319.)

Plate 11

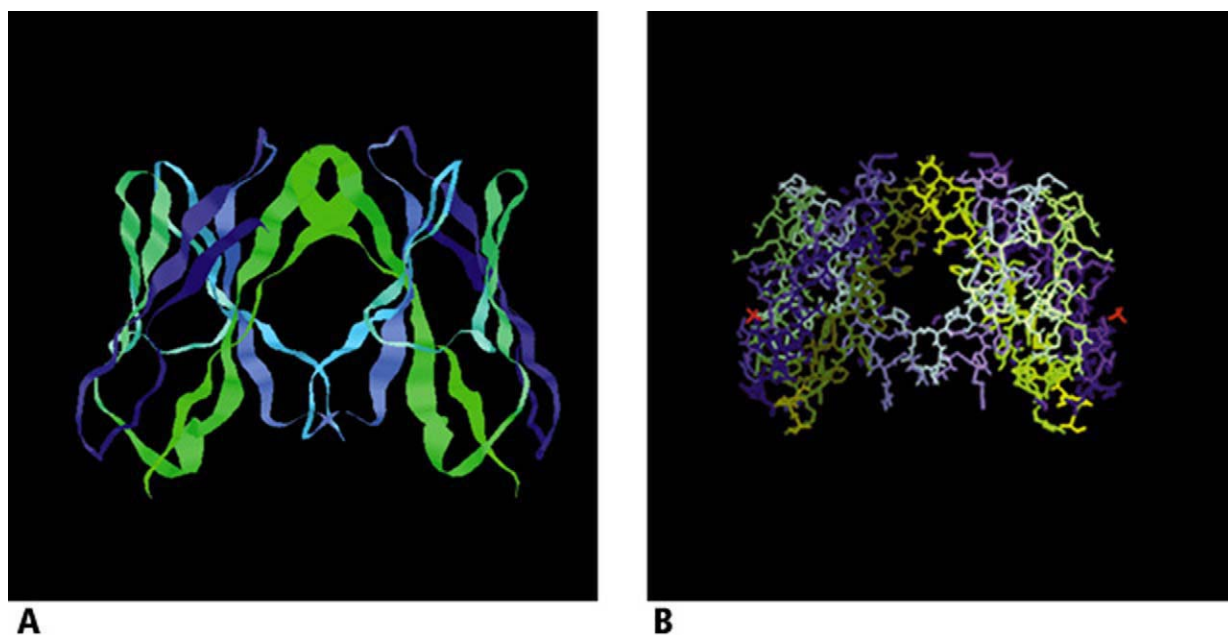


Plate 11 CD8. Three-dimensional molecular model of the N-terminal region of a CD8 homodimer consisting of two α chains; (A) ribbon presentation, (B) stick presentation. The structural data for the N-terminal 113 amino acids are based on crystallography (protein data base: 1CD8). The three-dimensional model for each monomer was generated with RasMol version 2.5 (Roger Sayle, Greenford, Middlesex, UK), and the two monomers were combined as described (Leahy *et al.* (1992) *Cell* **68**: 1145). The color coding represents groups; blue corresponds to the CDR1-like loop, light blue to the CDR2-like loop, and lime to the CDR3-like loop. The monomers are distinguished in brightness of color. (Kindly provided by GF Weber and H Cantor, Dana-Faber Cancer Institute, USA with permission.)

Plate 12



Plate 12 Dendritic cell. Colored scanning electron micrograph. (With permission from Photo Science Library.)

Plate 13

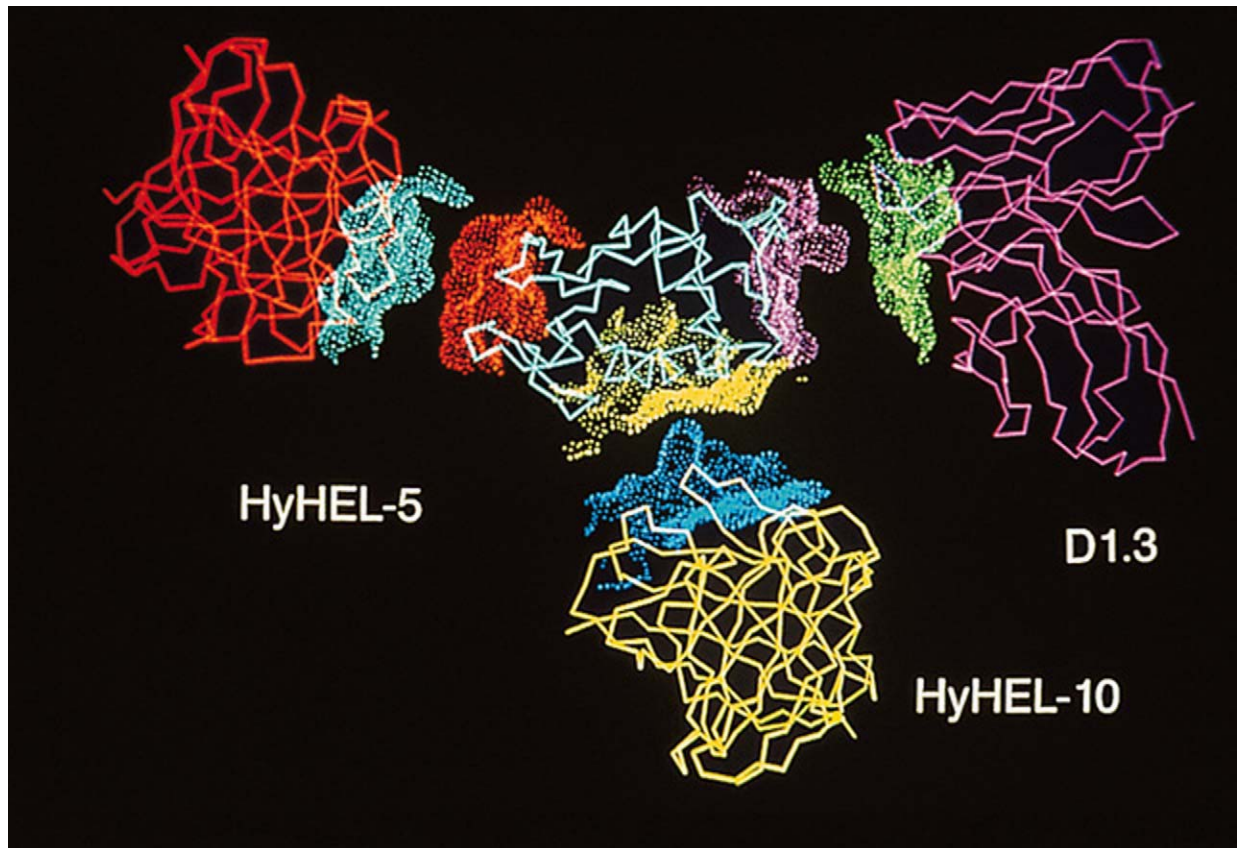


Plate 13 Monoclonal antibodies to lysozymes. Hen egg white lysozyme in complex with the Fab fragments of monoclonal antibodies raised against it. An exploded-view collage indicating how three different anti-lysozyme Fab's interact with lysozyme (center) in the x-ray structures of their respective complexes. The proteins are represented by their C_α chains and their interacting surfaces are outlined by juxtaposed dot surfaces. Note that the three crystal structures on which this diagram is based each contain only one Fab species; the Fabs do not crystallize together. (Photograph courtesy of Steven Sheriff and David Davies, NIH, USA.)

Plate 14

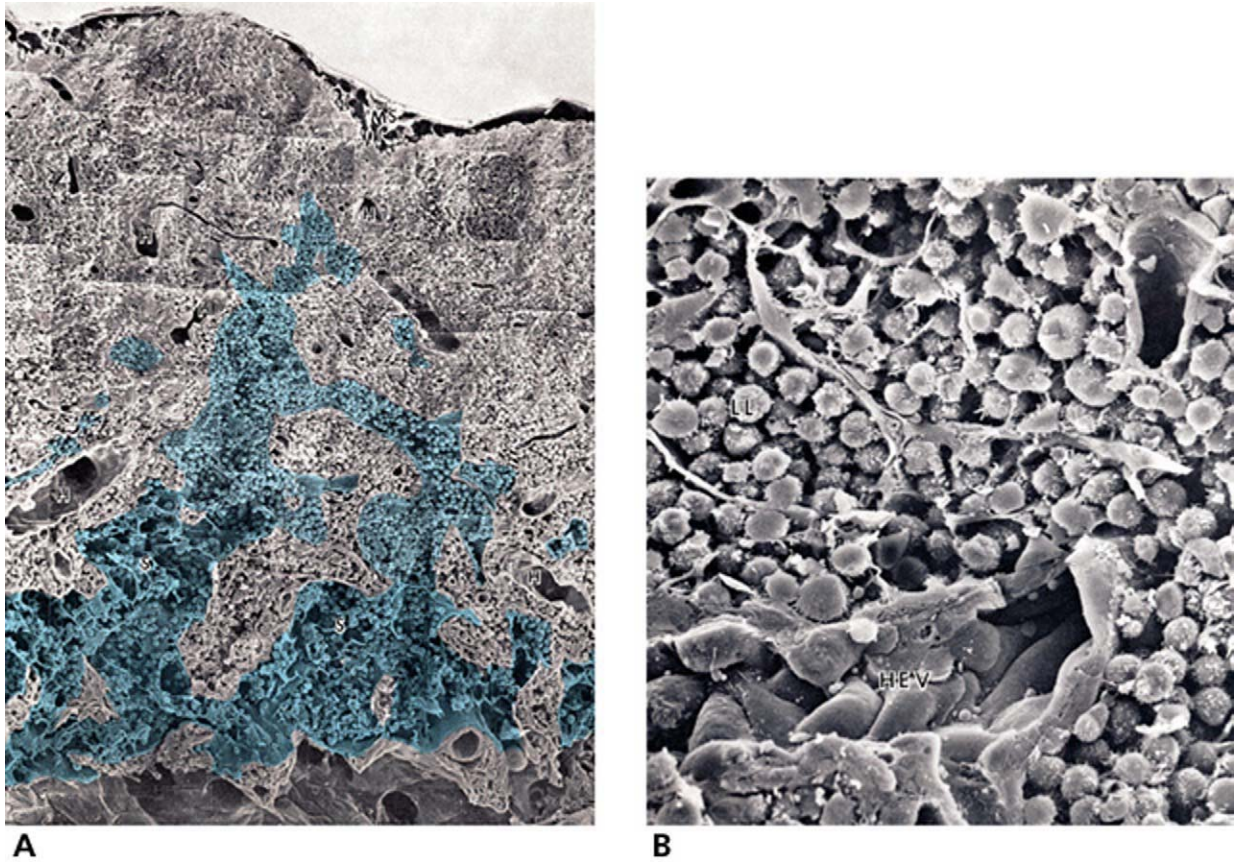


Plate 14 High endothelial venules in lymph node. (A) Rat mesenteric lymph node. Composite of about 200 scanning electron micrograph images. The capsule and the marginal sinus (MS) are seen at the top. The high endothelial venules (HEVs) are distributed mainly in the inner cortex and further in the interfollicular areas. The lymphatic labyrinths filled with lymphocytes are demonstrated in the inner cortex. S. medullary sinus. The lymphatic labyrinths and medullary sinuses are tinged in pale and dark blue, respectively. (B) Fractured portion of the inner cortex. An HEV is longitudinally opened at the lower left. The lymphatic labyrinth (L.L.) is shown near the HEV. (Kindly provided by Yechen He, Harbin Medical University, China, with permission.)

Plate 15

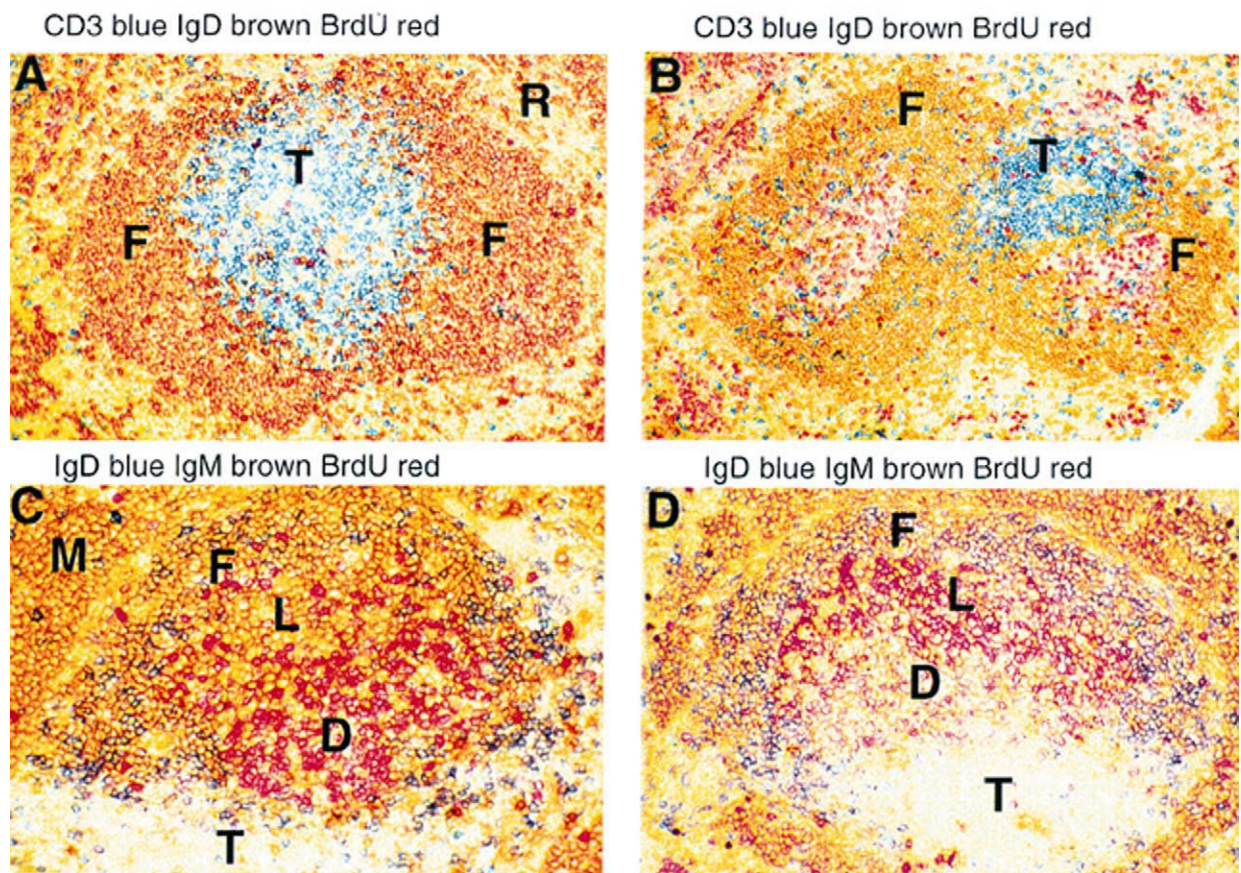


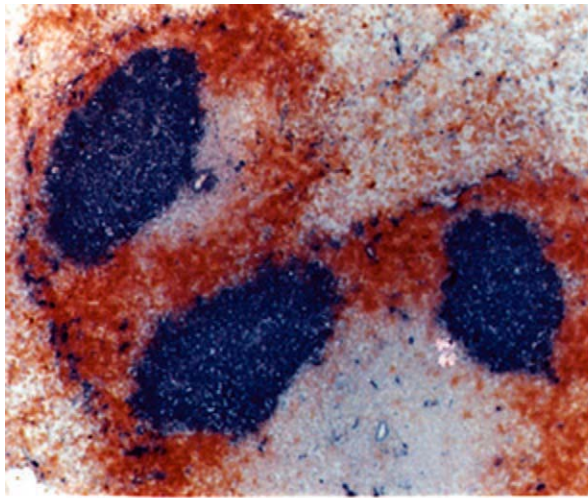
Plate 15 Germinal center. (a, b) Follicular T cell numbers rapidly increase during germinal center development. In a non-responding spleen (a) T cells in the white pulp are largely confined to the T zone (T) with only occasional T cells in the follicles (F); red pulp (R). (b) 7 days after primary immunization with antigen large numbers of T cells are found within the developing germinal center and surrounding follicular mantle – up to 10% of these have incorporated the thymidine analogue BrdU during a 2-hour pulse indicating that they are in cell cycle. (c, d) A pulse chase experiment where centroblasts in the dark zone (D) of a rat splenic germinal center are labeled during a 5-hour pulse with BrdU. In panel c the spleen is taken at the end of the pulse when almost all centroblasts are labeled but the majority of centrocytes in the light zone (L) are not labeled. The spleen in d was taken 7 hours later when centrocytes derived from the proliferating centroblasts are labeled. F - follicular mantle; M - marginal zone; T - T zone. (Kindly provided by Ian MacLennan, University of Birmingham Medical School, UK. Reproduced from MacLennan *et al.*, 1997, *Immunological Reviews* **156**, 53-66 with permission.)

Plate 16

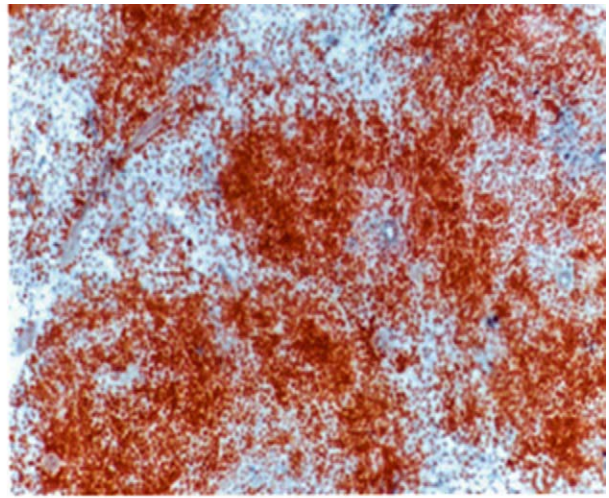


Plate 16 Delayed-type hypersensitivity. Brucellosis. Delayed-type hypersensitivity (DTH) reaction in the finger following needlestick injury. When vaccinating cattle, veterinarians are prone to needle stick injury. In those who have been sensitized by previous infection, albeit subclinical, this can be followed within 24-48h by a DTH reaction which ranges from the local reaction shown here, to a severe, generalized response which resembles many aspects of the infection itself. (Photograph courtesy K Hughes and the late J Forbes.)

Plate 17



Wild-Type



LT α -Deficient

Plate 17 Lymphotoxin knockout mice. Absence of germinal centers in mice lacking lymphotoxin α (LT $\alpha^{-/-}$ mice). Wild-type (left panel) and LT $\alpha^{-/-}$ (right panel) mice were immunized intra-peritoneally with 10^8 sheep red blood cells and 10 days later spleen sections were analyzed by staining with the germinal center marker peanut agglutinin (blue) and with anti-IgD (brown). (Kindly provided by David Chaplin, Washington University School of Medicine, USA. Reproduced from Matsumoto, M *et al.* 1997, *Immunological Reviews* **156**, 137-144 with permission.)

Plate 18



A



C



B

Plate 18 Cutaneous anaphylaxis. (A) (*above left*) Acute urticaria with marked erythema and whealing. (B) (*above*) Urticaria showing central clearing and an annular pattern. (C) (*left*) Symptomatic dermographism, marked linear whealing has occurred at sites of scratching. (Kindly provided by MHA Rustin and CH Ortey, The Royal Free Hospital, UK.)

Plate 19

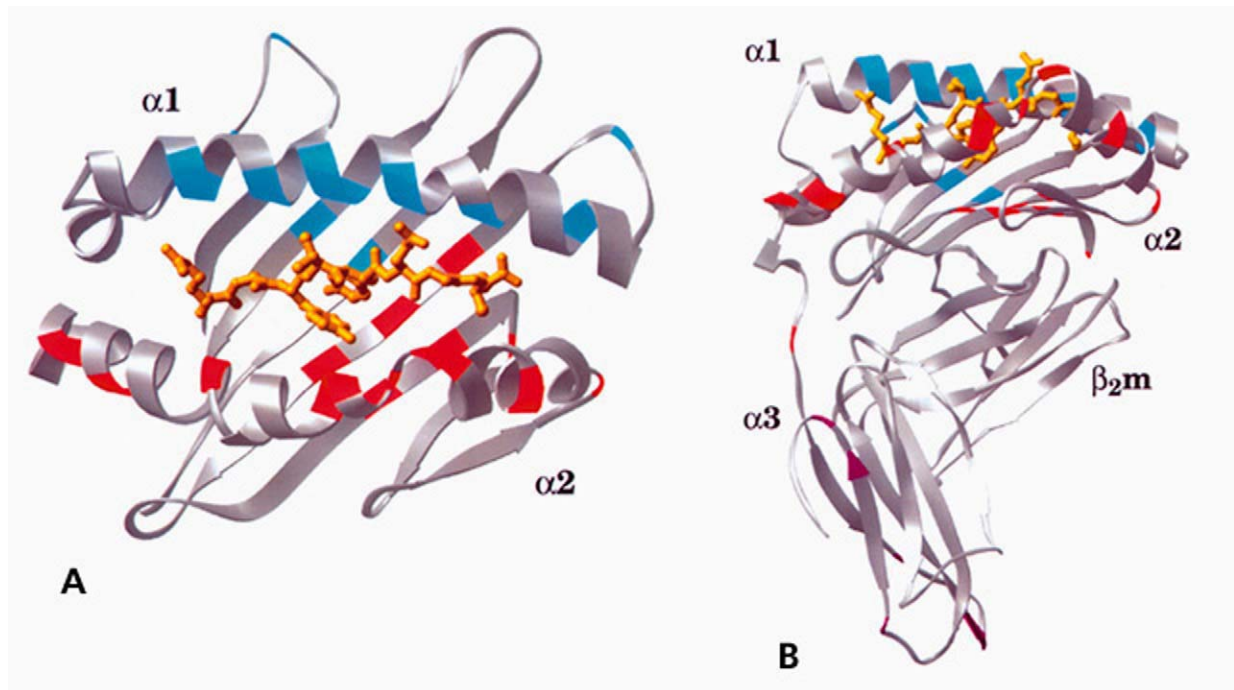


Plate 19 H-2K polymorphism. Ribbon diagrams of the H2-K^b molecule with the vesicular stomatitis virus 8mer (RGYVYQGL) bound. Polymorphic residues in the $\alpha 1$ domain (exon 2) are highlighted in blue, $\alpha 2$ (exon 3) in red, and $\alpha 3$ (exon 4) in purple. (A) T cell receptor view of the complex. (B) (*right*) side view of the molecule. (Kindly provided by JA Frelinger and EJ Collins, University of North Carolina, USA with permission.)

Plate 20

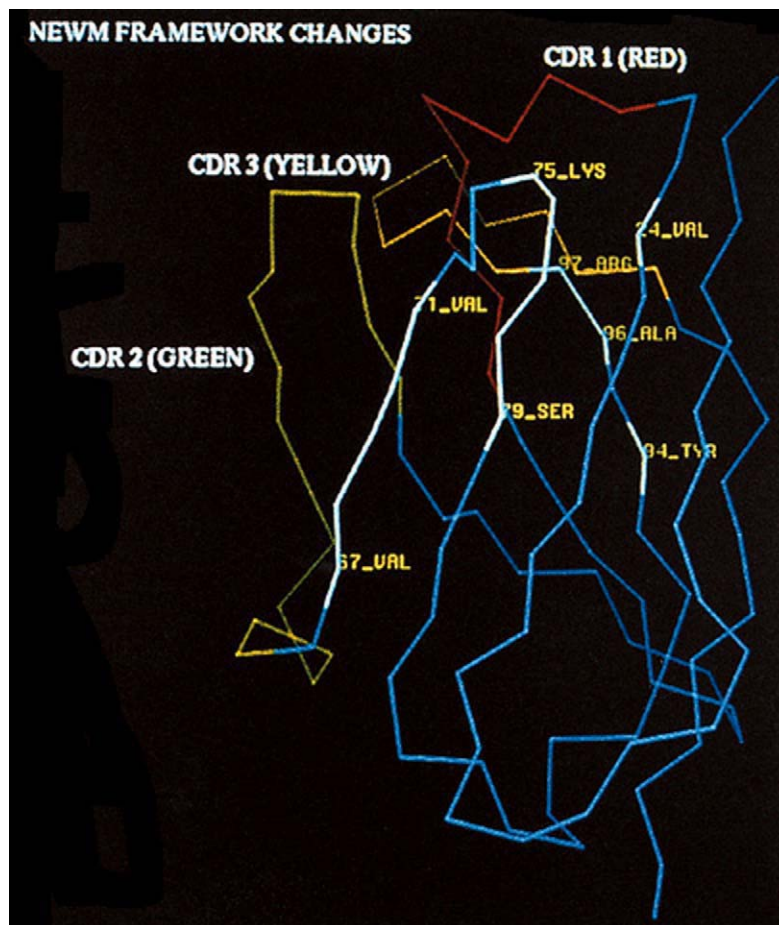


Plate 20 Antibody complementarity determining regions (CDRS). Computer graphic model of the structure of the V_H component of NEWM human antibody. The CDRs loop out from the scaffold structure of the framework regions (blue) to provide the antigen binding surface at one end of the molecule. The framework residues marked are those most commonly involved in maintaining the correct conformation of the CDR loops. (Reproduced with permission from Harris WJ and Cunningham CC (1995) *Antibody Therapeutics*; Austin TX: RG Landes.)

Plate 21

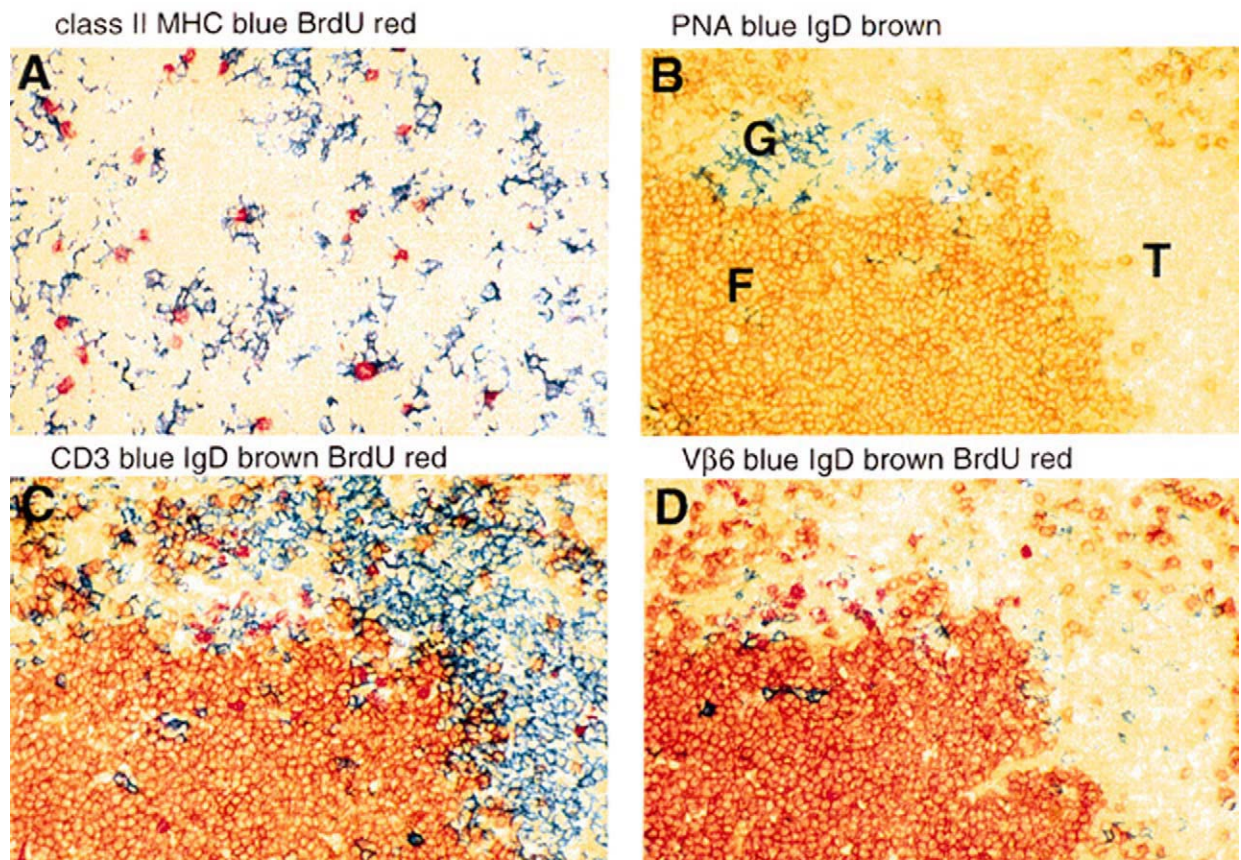


Plate 21 T cells in lymph node. (A) T cells proliferating during the priming process in association with interdigitating dendritic cells (IDC) in the T zone of a lymph node 3 days after immunization with hapten-protein antigen. The proliferating cells are stained red indicating that DNA in their nuclei contain the thymidine analogue BrdU, which was given as a 2 hr pulse before the node was taken. The IDC are identified by their expression of MHC class II molecules stained blue: staining on serial sections for the B-cell associated molecule B220 and CD4 indicates that the proliferating cells are T cells and the class II⁺ cells in contact with them are IDC. (B, C, D) Antigen-specific T cells are maintained long-term during a chronic follicular response in the popliteal lymph node draining the site of infection with murine mammary tumor virus MMTV (SW). Serial sections from the node taken 4 months after infection show a germinal center G stained in panel (B) with PNA (peanut agglutinin). T cells are shown in panel (C) and Vβ6 T cells in panel (D); these are present in the follicular mantle F, germinal center and T zone T. The superantigen-reactive T cells are selectively conserved in the responding node but are depleted from other lymphoid tissues. (Kindly provided by Ian MacLennan, University of Birmingham Medical School, UK. Reproduced from MacLennan I *et al.*, 1997, *Immunological Reviews* **156**, 53-66 with permission.)

Plate 22

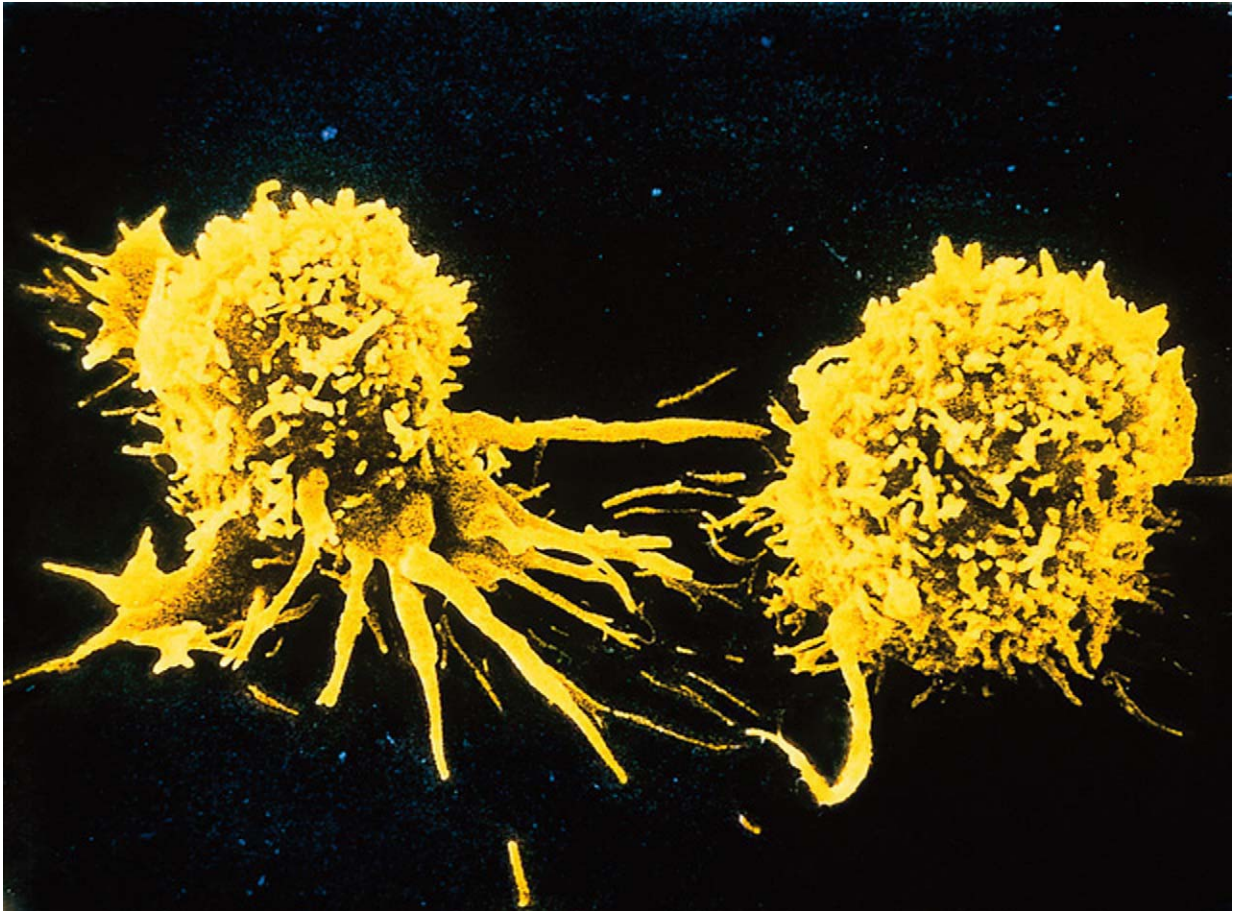


Plate 22 Cancer immunotherapy. False-color scanning electron micrograph of two lymphokine-activated killer (LAK) cells. In LAK immunotherapy, a patient's peripheral blood mononuclear cells are removed and cultured with interleukin-2 (IL-2) to allow LAK cells to develop. (With permission from Photo Science Library.)

Plate 23

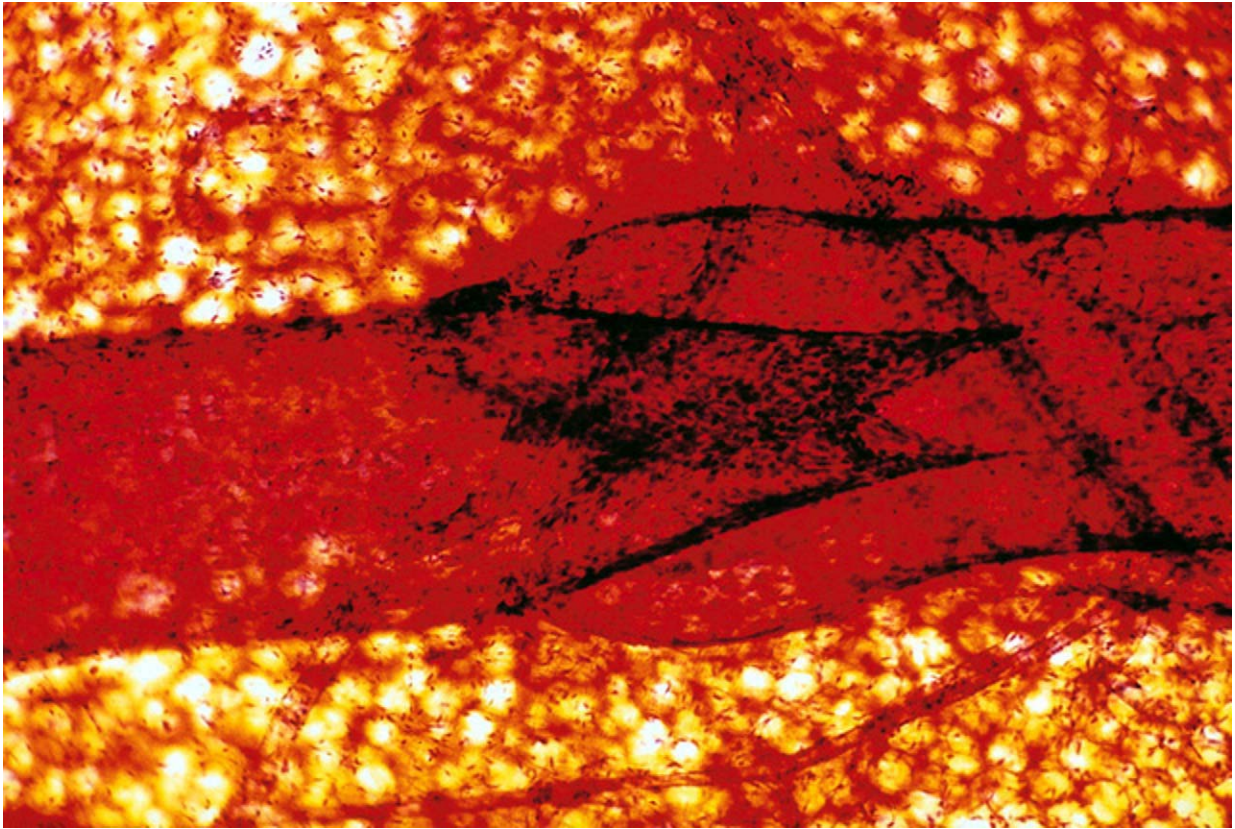


Plate 23 Lymphatic vessel. Light micrograph of a vessel in the human lymphatic system, showing a one-way valve. The vessel is red; the W-shaped valve (center) allows fluid to pass from right to left, but prevents backflow of lymph. (With permission from Photo Science Library.)

Plate 24

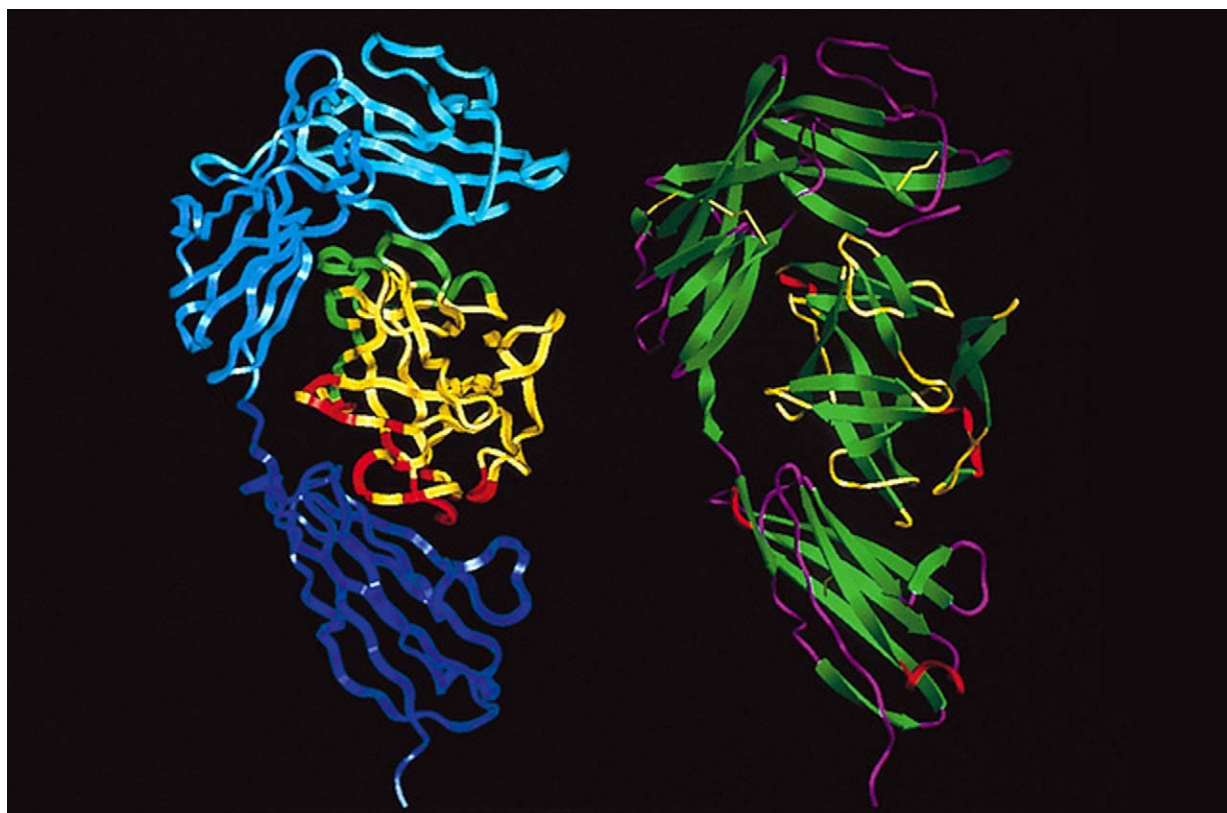


Plate 24 Interleukin-1 receptor (IL-1R). Soluble IL-1R (s-IL1R) complexed to IL-1 β . Left, Ribbon diagram showing domains 1, 2 and 3 of s-IL1R colored light, medium and dark blue, respectively. IL-1 β is yellow, with site A residues in green and site B residues in red. Right, Ribbon diagram with the β -strands shown as arrowed ribbons in green, α -helices in red, and the connecting loops in purple. The structure is oriented so that the carboxy terminus of s-IL1R and the cell membrane, if present, are at the bottom of the picture. (Kindly provided by Guy Vigers, Amgen Inc, Boulder, Colorado, USA. Reproduced from Vigers, G.P.A. *et al. Nature* 1997, **386**, 190-194 with permission.)

Plate 25

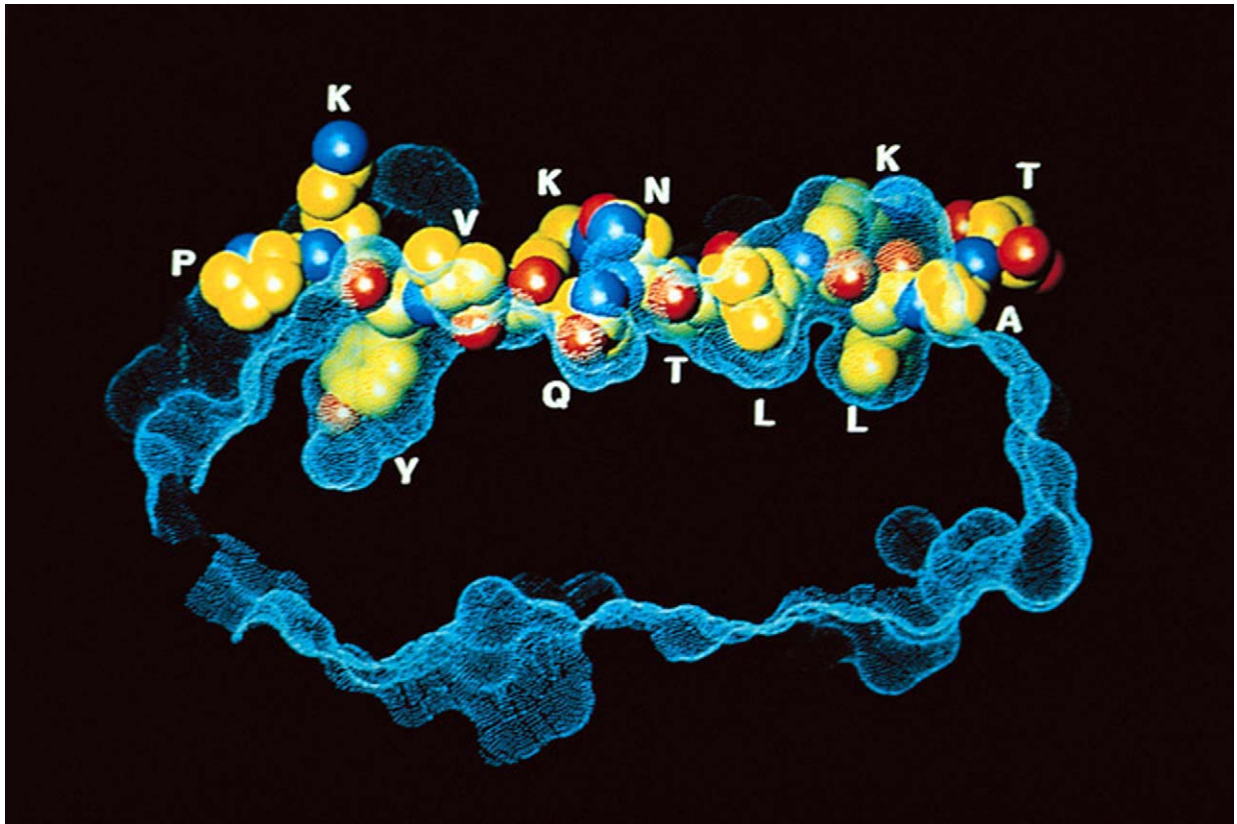


Plate 25 Peptide-MHC interaction. Pockets in the HLA-DR1 peptide binding site. Side view of the molecular surface of the HLA-DR1 peptide-binding site with She influenza hemagglutinin peptide 306-318. Peptide side-chains are labeled in one letter code. (Reproduced by copyright permission from Stern, LJ *et al* *Nature* 1994; **368**; 215-221.)

Plate 26

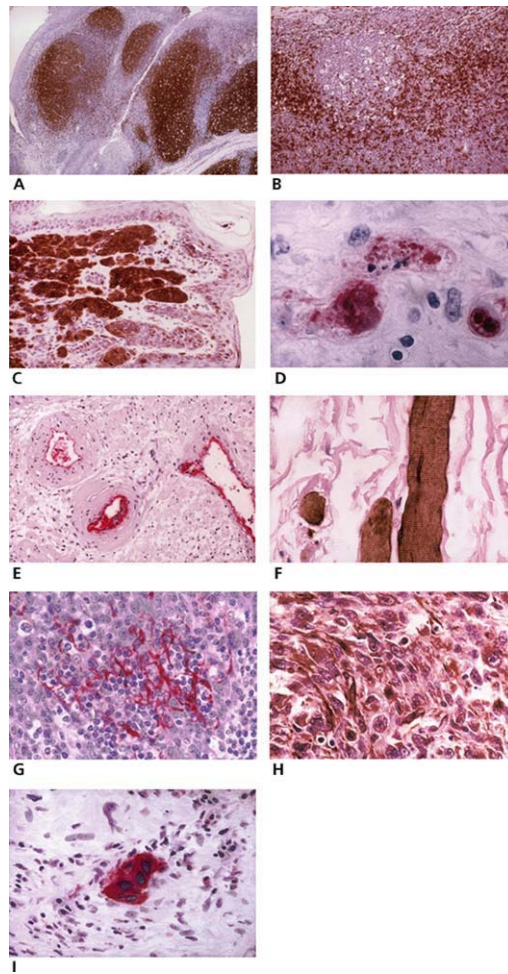


Plate 26 Immunocytochemistry. (A) DAB peroxidase-labeled anti-B lymphocyte antibody in human tonsil tissue. (B) DAB peroxidase-labeled anti-T lymphocyte antibody in human tonsil tissue. (C) Peroxidase-labeled anti-S100 antigen antibody in a skin sample. (D) AEC peroxidase-labeled anti-cytomegalovirus antibody in reproductive tract. (E) Antibody against factor VIII-vWB antigen labeled with AEC peroxidase. (F) DAB peroxidase labeled antidesmin in human muscle tissue. (G) Anti-EMA antigen in a case of cervical cancer. (H) Antibodies against vimentin in a human sarcoma tissue. (I) Anti-AE1/AE3 (cytokeratin) antibody labeled with AEC peroxidase in human tissue. (Kindly provided by CD MacKenzie, Environmental Pathobiology Laboratory, USA, with permission.)

Plate 27

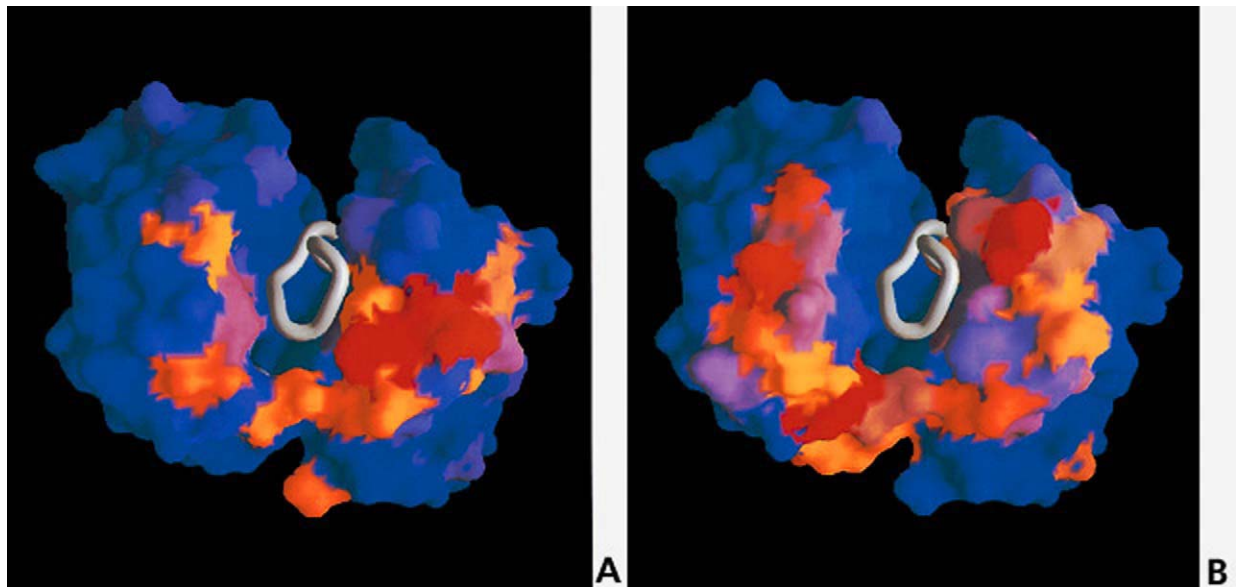


Plate 27 An 'antigen's eye view' of sequence diversity in human antibodies. Sequence diversity is plotted on a scale of blue (more conserved) to red (more diverse). The V_H domain is to the right and the V_L domain is to the left of each representation. (A) Germline diversity is focused at the center of the antigen binding site. (B) Somatic hypermutation spreads diversity to regions at the periphery of the binding site that are highly conserved in the germline V gene repertoire. Somatic hyper-mutation is therefore complementary to germline diversity. The CDRH3 region was not included in this analysis and is therefore shown in gray. The end of CDRL3 (also excluded) lies at the center of the binding site and is not visible in this representation. (Reproduced with permission from Tomlinson M *et al* (1996) *Journal of Molecular Biology* **256**: 813-817.)

Plate 28

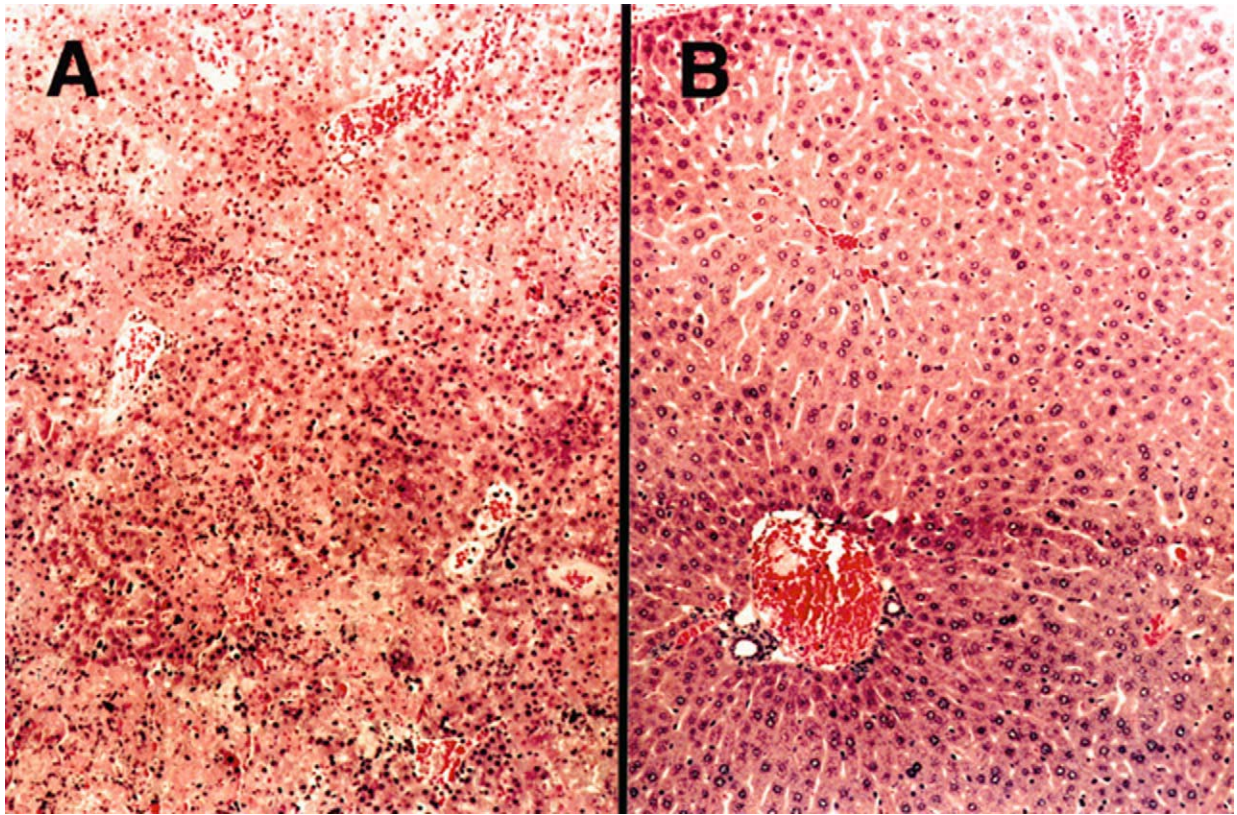


Plate 28 Stat 1 knockout mice. STAT1-deficient mice are extremely susceptible to viral infections. Transcriptional responses to interferons (IFNs) are mediated by tyrosine phosphorylation and nuclear translocation of transcription factors of the signal transducer and activator of transcription (STAT) family. Disruption of the gene for STAT1 in embryonic stem cells and in mice revealed that the STAT1 protein is required for all transcriptional responses to IFN (both type I and type II). STAT1-deficient mice grow and develop similar to wild-type animals, but show compromised innate immunity to viral disease. These animals fail to survive in the presence of otherwise innocuous pathogens, including mouse hepatitis virus. Histological examination of liver tissue from STAT1-deficient animals dying of disease showed multifocal hepatic necrosis with syncytial cell formation (A) and scant inflammatory response, consistent with viral hepatitis (caused by infection with mouse hepatitis virus). Analysis of hepatic tissue taken from wild-type siblings demonstrated no pathology (B) (Photograph courtesy of JE Durbin and DE Levy.)

Plate 29



Plate 29 *Staphylococcus aureus* septicemia. The bacteria may spread as emboli to peripheral vessels. (Kindly provided by Asa Ljungh, Lund University, Sweden.)

Plate 30

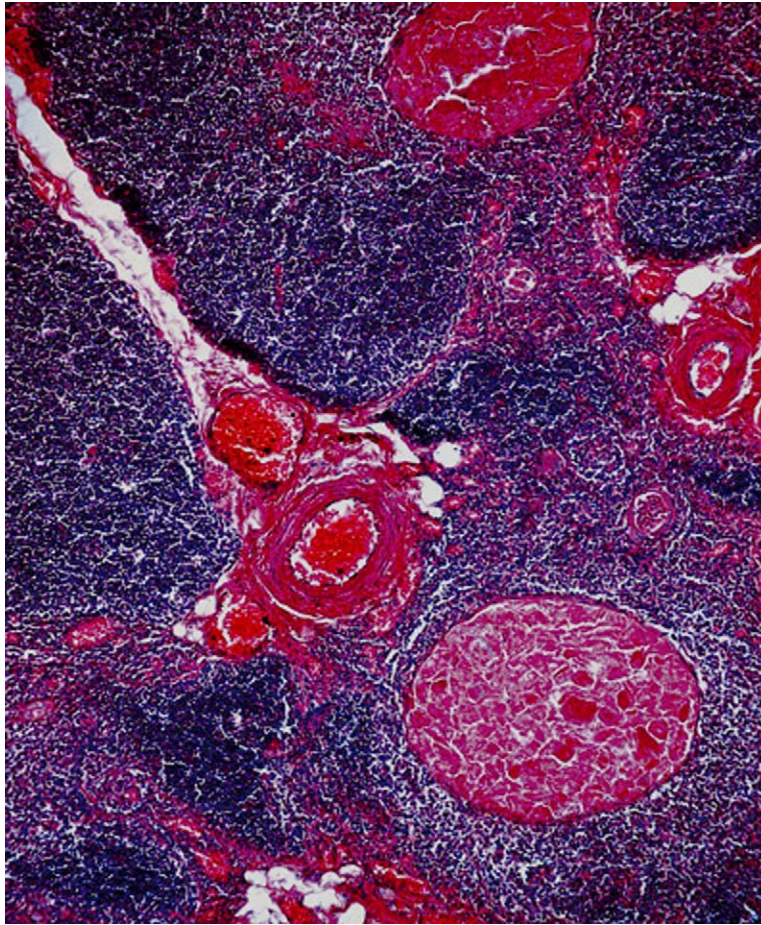
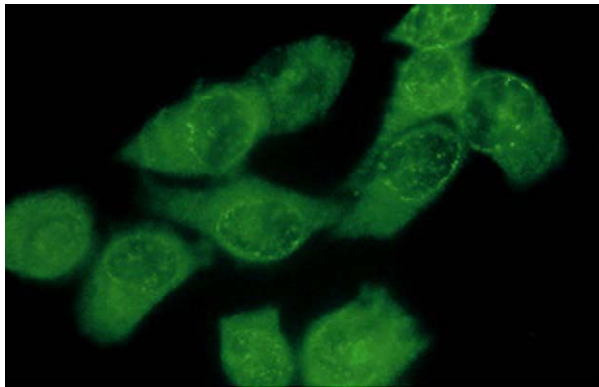
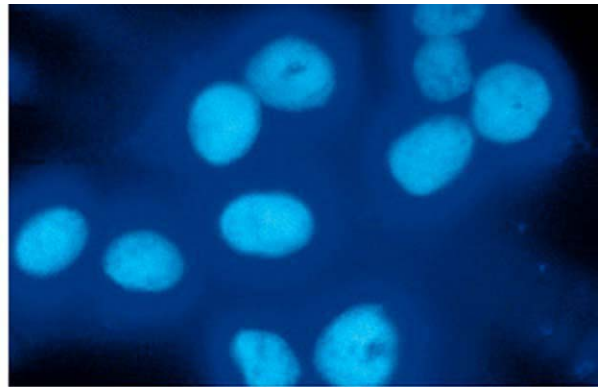


Plate 30 Human thymus. The thymus is divided into lobules separated by septa of connective tissue (white spaces) which may contain blood vessels (examples at center and far right). The body of the thymus is divided into an outer cortex (ink blue stain) and inner medulla; the medulla is less densely packed with lymphocytes. The pink, circular features within the medulla are called Hassal's corpuscles, cyst-like structures that may represent degenerated epithelial cells. (With permission from Photo Science Library.)

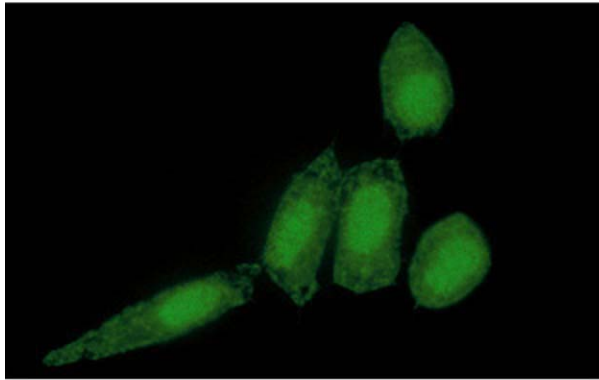
Plate 31



A



B



C



D

Plate 31 Heat shock protein. Immunofluorescence with monoclonal antibody W27 against heat shock protein (HSP) 70 (A, C) and the DNA-binding fluorescent molecule 4',6-diamidino-2-phenylindole (DAPI) (B, D). Staining of control (A, B) and heat shock activated (C, D) HeLa cells showing HSP 70 nuclear translocation in response to heat shock activation. (Kindly provided by Santa Cruz Biotechnology, Inc. USA, with permission.)

Plate 32

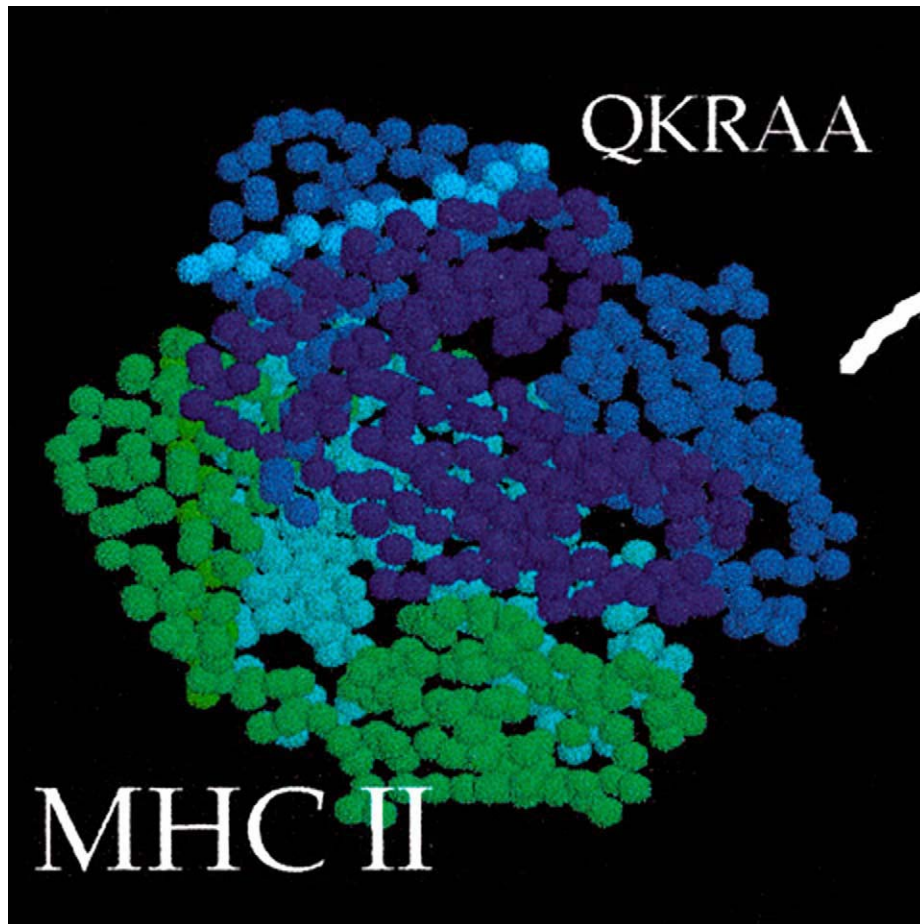


Plate 32 Rheumatoid arthritis. Three-dimensional representation of HLA class II molecule containing the antigenic processed peptide in the antigen-binding groove (light blue stretch, top of the figure). The five amino acid sequence QR/RRAA ('shared epitope') is common among all HLA alleles correlated to rheumatoid arthritis. (Kindly provided by A La Cava and S Albani, University of Carolina, USA, with permission.)

Plate 33

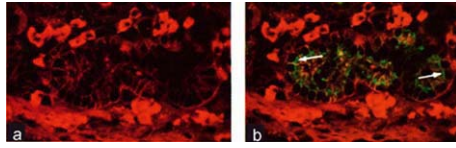


Plate 33 (A) Secretory component (the polymeric Ig receptor). Paired immunofluorescence staining for IgA and SC in tissue section from human nasal mucosa; the same field is shown after incubation with 'red' anti-IgA + 'green' anti-SC, (i) (red filtration) and (ii) (double exposure). IgA alone is present in immunocytes and throughout the stroma; SC alone is present in the Golgi zones (arrows) adjacent to nuclei of glandular acinar cells; both IgA and SC (mixed color) are present basolaterally on acinar cells and apically in their cytoplasm.

Plate 34

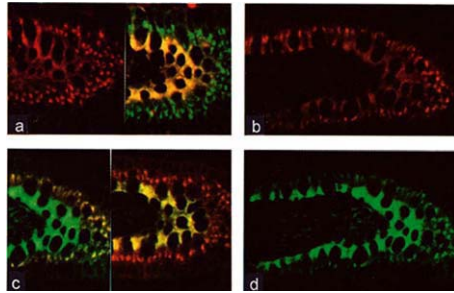


Plate 34 (B) Paired immunofluorescence staining for IgA and total SC, or for unoccupied and bound SC, in tissue sections of human colonic mucosa. (i) left part (red filtration) and right part (double exposure), comparable fields in adjacent sections of gland incubated with 'red' anti-SC and with 'red' anti-IgA + 'green' anti-SC, respectively. Prominent SC-containing granules are present in the Golgi zones, whereas the rest of the cytoplasm and the basolateral aspects of columnar epithelial cells are positive for both SC and IgA (mixed color). Goblet cells are devoid of both markers. (ii) (red filtration), (iii) (left part, double exposure), and (iv) (green filtration), same field in a section of gland incubated with 'red' anti-I determinant (specific for unoccupied SC) + 'green' anti-A determinant (accessible on free as well as bound SC). The two antigenic determinants show distinct differential distribution with a relative dominance of I in the Golgi zones, and of A in the remaining cytoplasm and weakly also basolaterally. Right part of (iii) (double exposure) shows a comparable field after prolonged exposure time for red (anti-I) emission. Note that neither of the two anti-SC reagents produced fluorescence of elements in the lamina propria.

Plate 35

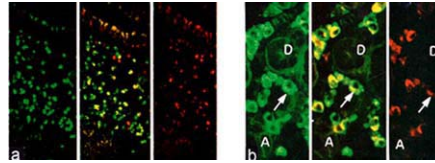


Plate 35 (C) (i) Paired immunofluorescence staining to demonstrate *in vitro* SC affinity to IgA immunocytes in section of human colonic mucosa preincubated with free SC followed by 'red' anti-SC + 'green' anti-IgA. Note in double exposure (center) that most IgA cells show varying degree of mixed color as evidence for binding of SC to cytoplasmic polymeric IgA (pIgA), whereas a few cells (probably pure monomer producers) lack SC-binding and are therefore only green. The purely red cells in the double exposure are SC-binding IgM immunocytes. The columnar epithelial cells of the glands at top and bottom contain innate SC and transport pIgA; they therefore show mixed cytoplasmic color with the exception of the Golgi zones which contain SC but no IgA. (ii) Similar SC-affinity test on section of human salivary gland. Double exposure (center) shows various tints of yellow in IgA immunocytes located between acini A and duct D on the figure; in several immunocytes the yellow color is restricted to areas close to the nucleus (arrow), suggesting accumulation of pIgA. One immunocyte (at the bottom) is purely green, suggesting production of only monomeric IgA. Acini and duct are faintly double-stained for unoccupied SC and translocated pIgA. (Kindly provided by P Brandtzaeg, F-E Johansen, P Krajčič and IB Natvig, University of Oslo, Norway, with permission.)

Plate 36

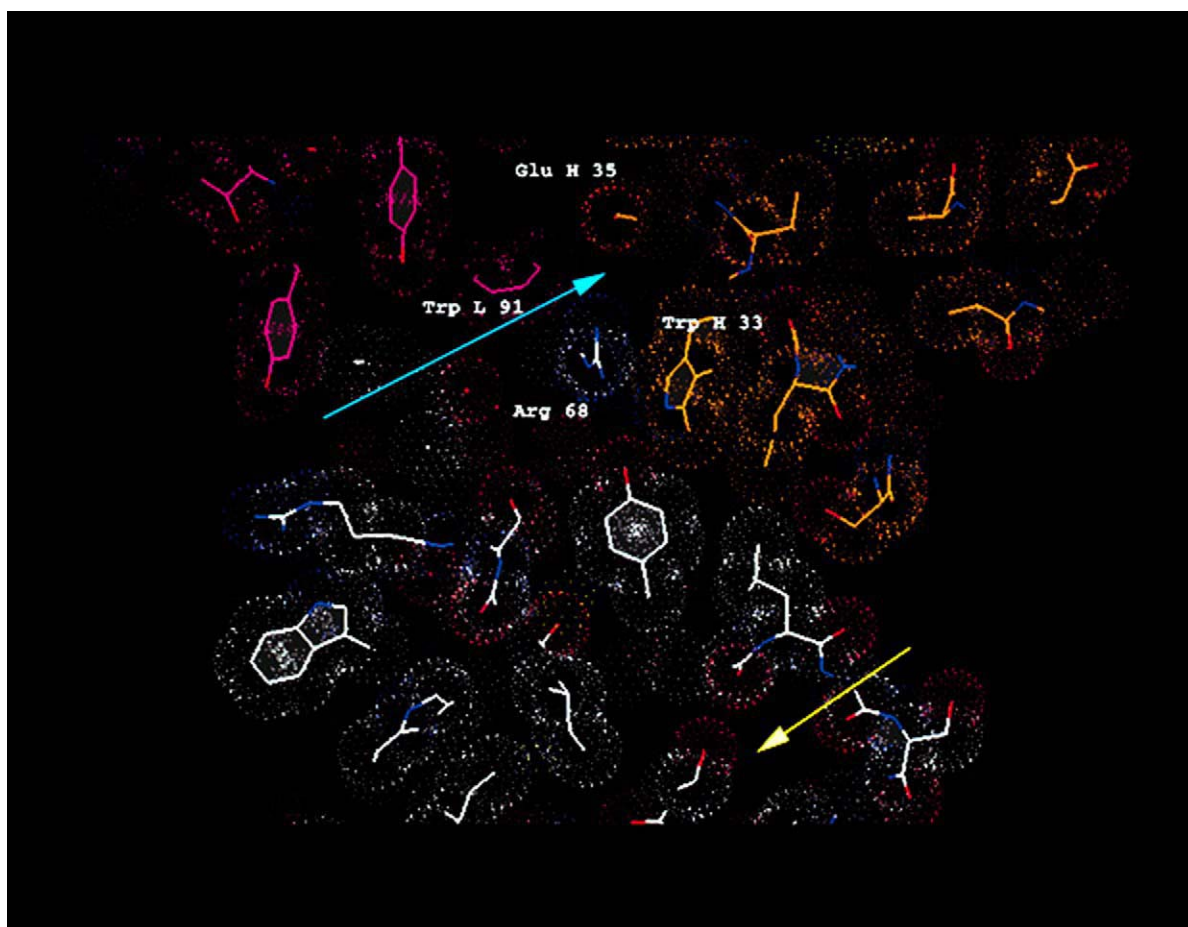


Plate 36 (A) Anti-lysozyme binding site Surface complementarity between the antigen, hen egg-white lysozyme and the antibody HyHEL-5 binding site. In this cross-reaction through the antigen-antibody complex, 5 Å thick, centers of atoms are connected by sticks and atomic volumes are shown as Conolly dot surfaces. Atom color-coding; oxygen, red; nitrogen, blue; sulfur, yellow; carbon in lysozyme, white; carbon in V_L domain, magenta; carbon in V_H domain, brown. The intermolecular interface, approximately traced by a line dividing the antigen atoms (lower part) from those of the antibody (upper part), consists of tightly packed regions, and occasional loosely packed regions, 'cavities'. The intermolecular cavities, for example the one delimited by lysozyme residue Arg 68 and the antibody residues L 91, H 33 and H 35 (blue arrow, on the top), are not any larger, however, than the occasional intramolecular cavities (white arrow, on the bottom). Crystallographic coordinates by Sheriff S *et al.* (1987), *Proceedings of the National Academy of Science of the USA* **84**: 8075-8079, with permission.

Plate 37

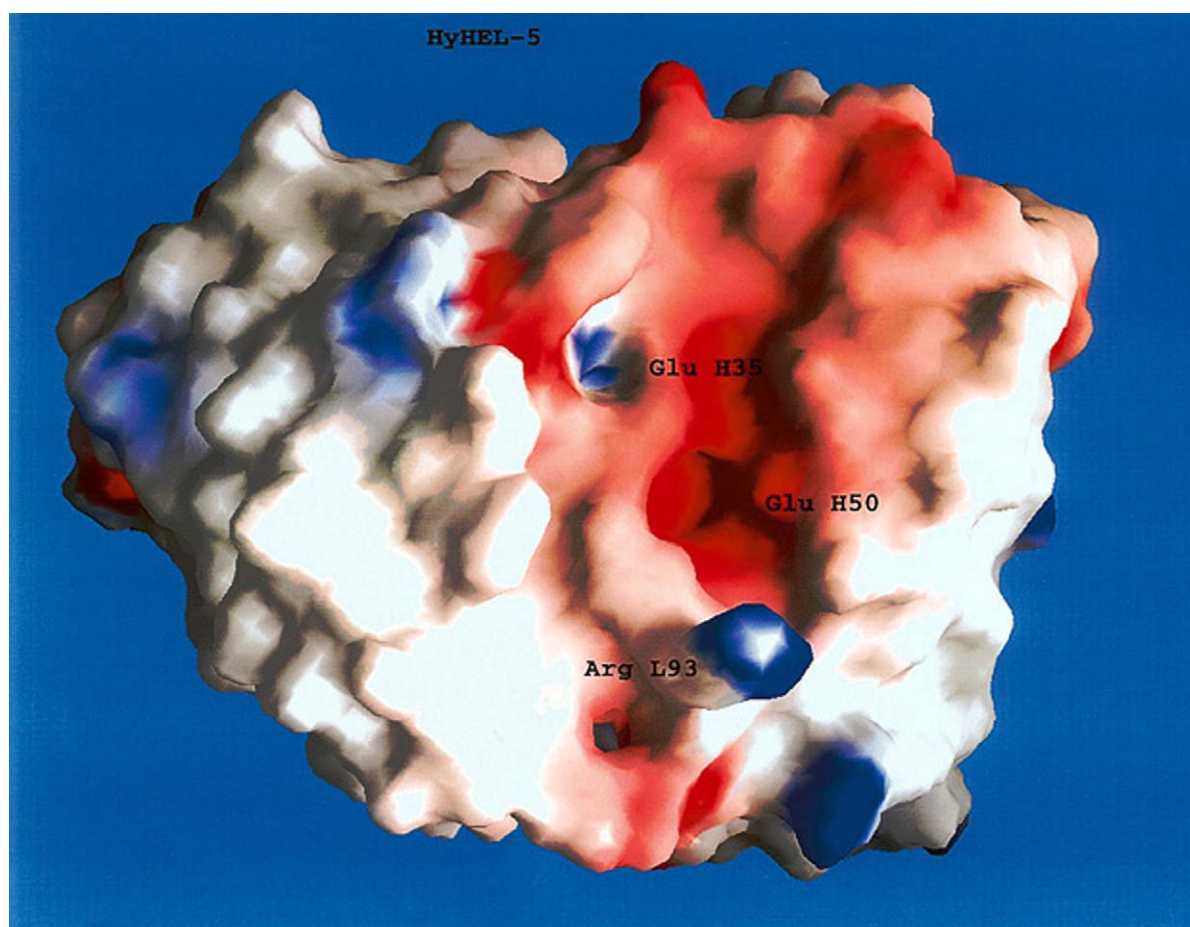


Plate 37 (B) Electrostatic fields at the surface of the HyHEL-5 antibody binding site. This monoclonal antibody is specific to lysozyme, a protein with an exceptionally high positive charge. Using the crystallographic coordinates of the HyHEL-5 V_H and V_L domains (Sheriff *et al*, (1987) *Proceedings of the National Academy of Science of the USA* **84**: 8075-8079, with permission.) molecular surface envelope was generated by the program GRASP (A Nicholls, Columbia University 1991) and was color-coded by the electrostatic field produced by all the formally charged residues in the molecule: red, negative potential; blue, positive potential; color intensity proportional to the strength of the field). At the center of the binding site there is a deep, electronegative cavity 'below' which is a protruding side-chain of the Arg residue L93, with a weaker, positive, electrostatic field. In the HyHEL-5-lysozyme complex, the central negatively charged cavity becomes occupied by the two positively charged lysozyme side-chains, Arg 45 and Arg 68. The positive field generated by the antibody residue Arg L93 gives rise to a dipole, close to the antibody surface, perhaps helping to preorient the antigen at its approach to the binding site.

Plate 38

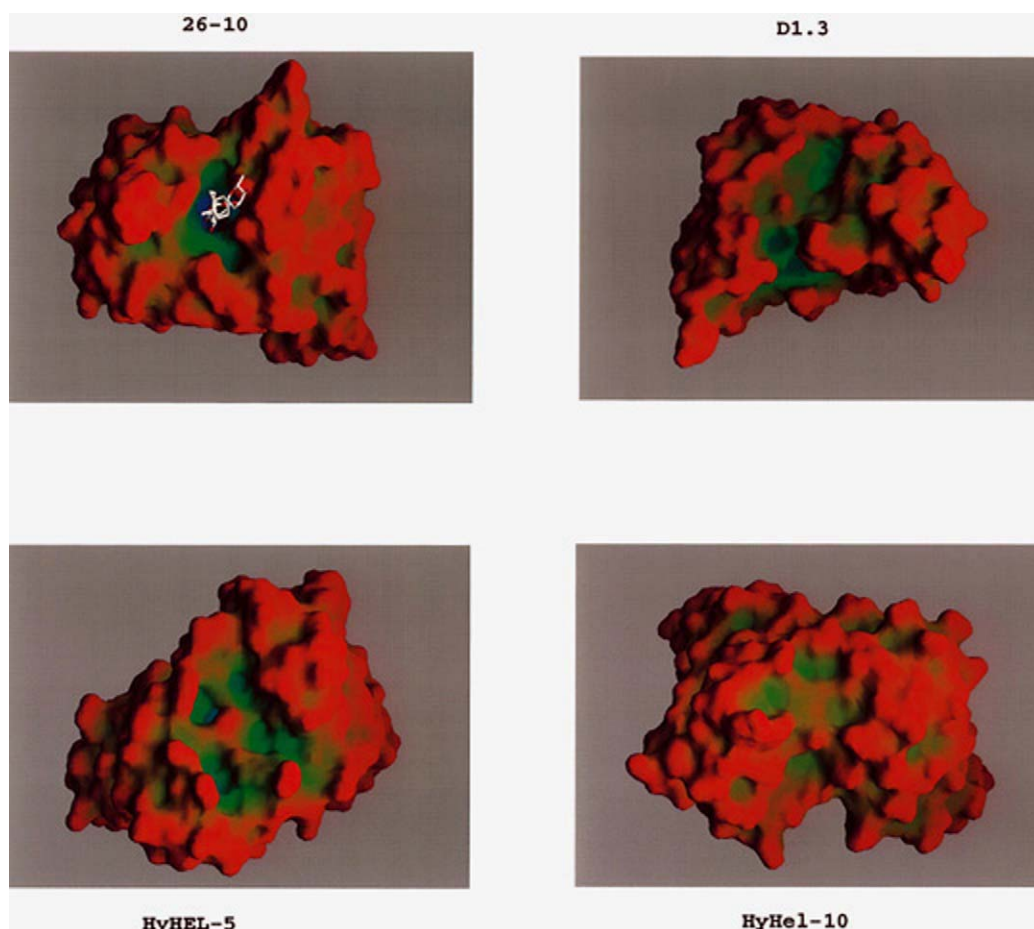


Plate 38 (C) Comparison of anti-digoxin and anti-lysozyme binding sites. In these 'elevation plots', points of the molecular surfaces, generated by the program GRASP, have been color-coded according to its distance normal to a three-dimensional envelope of the molecule. The envelope was defined, and constructed, as a smooth, curved plane connecting the 'highest' peaks of the molecular surface. The deepest protrusions on the surface of the molecule are shown in blue, the most elevated points of the surface are shown in red. The binding site of the 26-10 antibody directed against the hapten digoxin is a deep, narrow well (high 'global' curvature); the binding sites of the anti-lysozyme antibodies D1.3 (crystallographic coordinates by Bhat TN *et al.*, (1990) *Nature* **347**: 483-486, HyHEL-5 and HyHEL-10 (coordinates by Padlan EA *et al.*, (1989) *Proceedings of the National Academy of Science of the USA* **86**: 5938-5942) are all larger and more shallow (low 'global' curvature). The D1.3 binding site contains a smaller, deep 'local' hole in its surface (complementary to the lysozyme residue Gin 121), the HyHEL-5 binding site contains a similar but shallower hole (complementary to Arg '68,). The HyHEL-10 elevation plot shows virtually no local depressions. (Kindly provided by J Novotny and M Davis, Bristol-Myers Squibb Research Institute, USA with permission.)

Plate 39

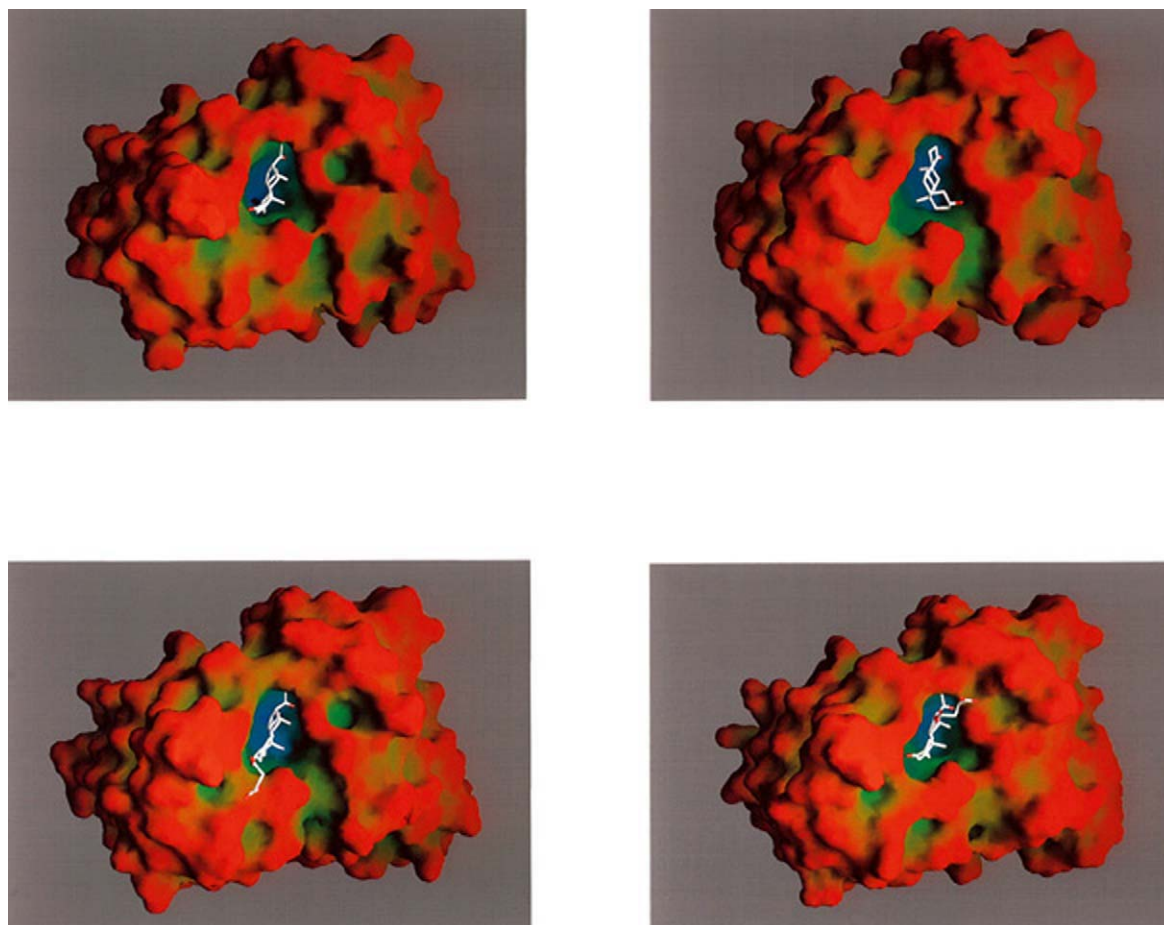


Plate 39 Antibody cross-reaction. DB3 antibody binding site in complex with four different cross-reacting steroid haptens. The elevation plots shown here were prepared as described in the legend to Plate 6 (C). Surface color-coding is from red (most elevated) to blue (the deepest). Crystallographic coordinates by Arevalo JH *et al* (1994). *Journal of Molecular Biology* **241**: 663-680. Upper left, DB3 with progesterone; upper right DB3 with 3-aetiocholanolone (5- β -androsterane-3, 17-dione); lower left, DB3 with 5- α -pregnane-3- β -hemisuccinate; lower right, DB3 with 3-progesterone-11- α -olsuccinate. Haptens are shown in stick representations; carbons are color-coded white, oxygens red. It can be seen that the overall shape of the binding site does not change on engaging the cross-reacting ligands; rather, the steroids orient themselves differently with respect to the binding site cavity. At the periphery of the binding site, however, changes to the shape of the surface can be seen, due to surface side-chain rearrangements. (Kindly provided by J Novotny and M Davis, Bristol-Myers Squibb Research Institute, USA with permission.)

Plate 40

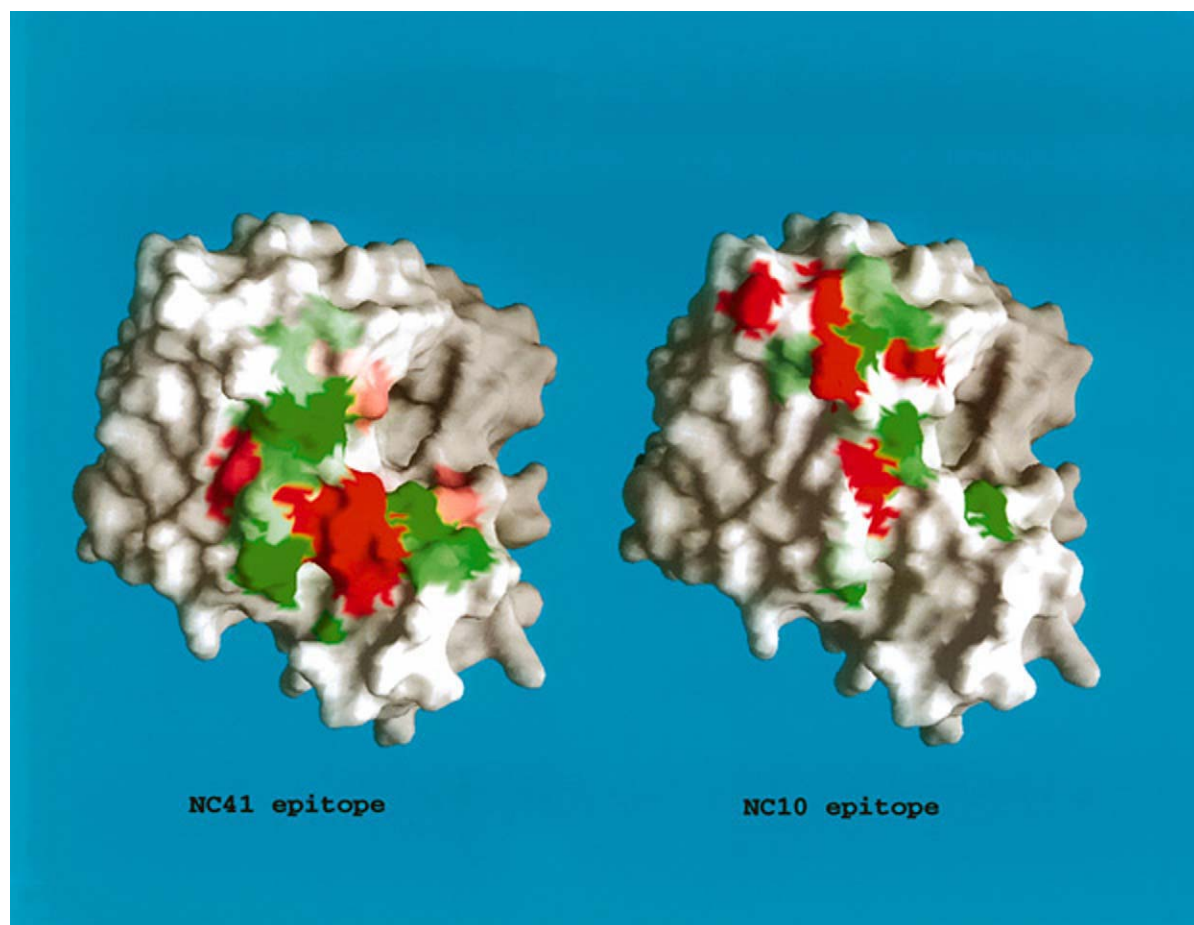


Plate 40 Epitopes on neuraminidase. The two energetic epitopes (NC41 and NC10) on the surface of the influenza N9 neuraminidase. The calculated 'energetic' epitopes (Tulip WR *et al.*, (1994) *Biochemistry* **343**: 7986-7994) were color-coded, antibody-antigen from green (residues with free energy contributions stabilizing the complex), through white ('neutral' residues), to red (residues whose contributions to the free energy of binding destabilize the complex). Although the two epitopes overlap by about 80%, their binding free energy attribution is strikingly different. (Kindly provided by J Novotny and M Davis, Bristol-Myers Squibb Research Institute, USA with permission.)

Plate 41

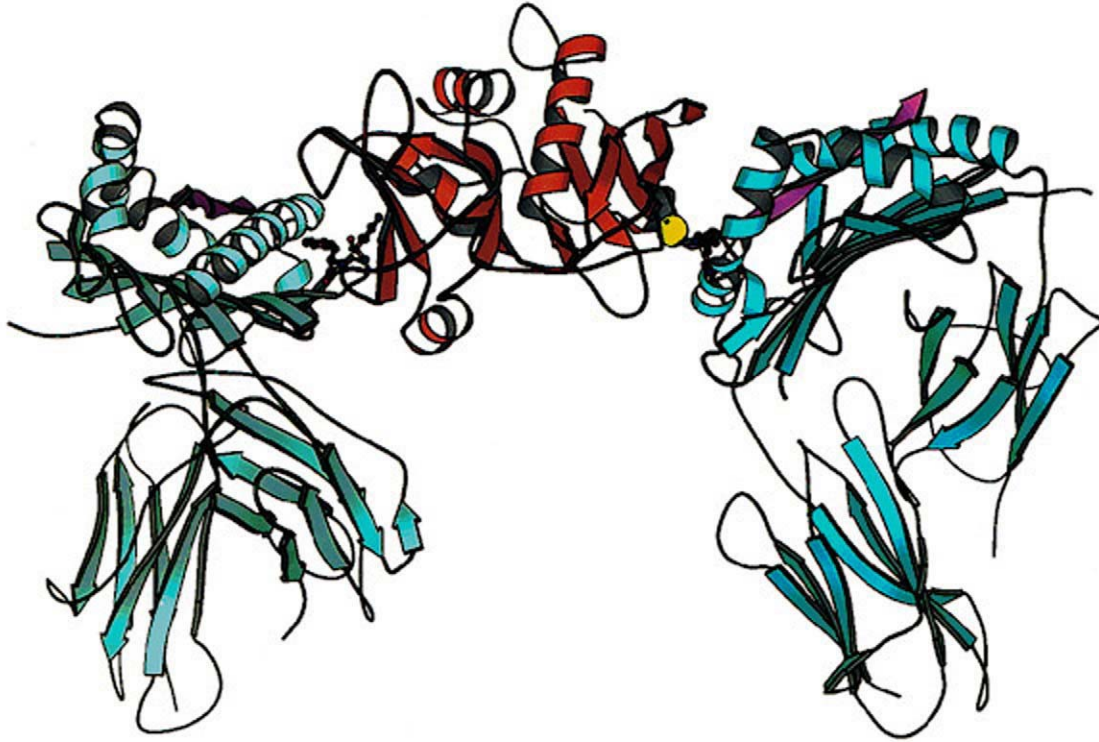


Plate 41 Superantigen. Molscript model of *Staphylococcus enterotoxin A* (SEA) interacting with two MHC class II molecules. SEA is colored red, MHC class II and bound peptide blue and purple, respectively. On the left-hand side MHC class II β -chain contacts the SEA coordinated zinc ion (yellow) through a histidine residue on the MHC class II β chain (His81, illustrated as ball-and-chain). The right-hand side shows the interaction between MHC class II α chain and SEA. This point of interaction is centered around a phenylalanine residue in SEA (Phe47, illustrated as ball-and-chain) and a lysine residue in MHC class II α chain (Lys39, illustrated as ball-and-chain). (Kindly provided by T Kalland, M Dohlsten, P Antonsson and Morten Sogaard with permission.)

Plate 42



Plate 42 Systematic Lupus erythematosus. The 'butterfly rash' seen in up to 40% of SLE patients. (Kindly provided by DA Isenberg, Bloomsbury Rheumatology Unit, UK with permission.)

A

ABO BLOOD GROUP SYSTEM

Anatole Lubenko and Marcela Contreras, North London Blood Transfusion Centre, Colindale Avenue, London, UK

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The ABO blood group system was discovered by Landsteiner in 1901. By mixing separated sera with suspensions of red cells obtained from the blood of different individuals, four patterns of agglutination could be obtained. The frequency of the four ABO groups varies in different populations: Amerindians are almost exclusively group O while Asians have a higher incidence of group B. In the UK the proportions of the four main blood groups are as follows: O (46.5%), A (42%), B (8.5%) and AB (3%). Agglutination of red cells arises because one individual's serum contains naturally occurring antibodies against ABO antigens that are missing from that individual's own red cells, but which are present on the cells of another; e.g. group A individuals have anti-B that agglutinates group B cells (see Table 1). Testing of red cells with selected potent anti-A, -B and -A,B reagents, while simultaneously testing the sera of the same subjects with reagent red cells (group A₁, A₂, B and O), provides the basis for ABO grouping (see Table 1).

The major subgroups of A are A₁ and A₂; the difference between them is partly quantitative and

partly qualitative. These subgroups can be distinguished using specific anti-A₁ reagents, and are only significant clinically if the serum of an A₂ individual reacts at 37°C, which may hence cause destruction of transfused group A₁ red cells. Quantitative variants of A (A_{int}, A_x, A_{end}, A₃, A_m, A_y, A_{cl}, etc.) and B (B₃, B_x, B_m, B_i, B_{ii}, B_{cl}, etc.) are also known, which are characterized by the absence of the appropriate ABO antibodies from their plasma; e.g. the cells of A_x individuals fail to react with anti-A from B subjects although they react with strong anti-A in group O subjects; such individuals do not have anti-A. A and B variants are rare and usually of little clinical significance.

Genetics

The genes controlling the expression of A or B antigens are autosomal codominant alleles (A and B respectively) that map to the 9q34 region of chromosome 9. Because the A and B antigens are sugars, the genes coding for ABO are glycosyltransferases. Group O individuals possess alleles at the ABO locus

Table 1 ABO grouping

ABO group	Agglutination of test cells with reagent				Agglutination by subject's plasma of control cells of group			
	Anti-A	Anti-A ₁ ^a	Anti-B	Anti-A,B	A ₁	A ₂	B	O
A ₁	+	+	0	+	0	0	+	0
A ₂	+	0	0	+	+/ ^{0b}	0	+	0
B	0	0	+	+	+	+	0	0
A ₁ B	+	+	+	+	0	0	0	0
A ₂ B	+	0	+	+	+/ ^{0b}	0	0	0
O	0	0	0	0	+	+	+	0

^aAnti-A₁ reagents can be: a lectin prepared from *Dolichos biflorus* seeds; sera of group B subjects absorbed with group A₂ red cells; or mouse monoclonal antibodies.

^bThe plasma of 1–8% of group A₂ and 22–35% of group A₂B individuals have weak naturally occurring anti-A₁.

on both homologous chromosomes that code for functionally inert proteins (see below), while A and B code for an *N*-acetylgalactosaminyl- and galactosyltransferase respectively. The appropriate alleles that encode the A¹, A² and B-transferases have been cloned and sequenced. The A and B genes code for 41 kDa proteins composed of 353 amino acids, which contain a 21 amino acid long leader peptide that is cleaved off to form the mature transferase. The two genes differ at seven nucleotide positions, which generate only four amino acid differences, found at positions 176, 235, 266 and 268 of the A- or B-transferase polypeptide. These correspond to Arg, Gly, Leu and Gly in the A-transferase, and to Gly, Ser, Met and Ala, respectively, in the B-transferase. The most critical differences are at positions 266 and 268 but the four are collectively responsible for the immunochemical differences between A and B and their associated transferases; substitution of one or more of these four key amino acids can critically affect ABO expression (see O² below).

Three types of O alleles have been described to date; the commonest has a nucleotide sequence similar to that of the A allele, but with a single base deletion that generates a change in reading frame (hence scrambling the amino acid sequence) at amino acid position 87. This deletion also produces a new in-frame stop codon that generates a truncated O allele polypeptide that is only 116 amino acids long, and which is enzymically inactive. An analogous O allele, with an incidence one-quarter of that of the former and representing an equivalent single base deletion in a B gene, has also been reported. The third type of O (sometimes called O²) arises from Arg→Gly and Gly→Arg substitutions at amino acids 176 and 268, respectively, of an A allele which abolishes the enzymatic activity of the resulting protein, thereby emphasizing the metabolic importance of these amino acids at the transferase active site.

The differences between the A¹ and A² alleles arise from a Pro→Leu mutation at amino acid position 156 coupled to a single base deletion near the C-terminus of an equivalent A¹-allele. This in turn results in a frame shift so that the original stop codon is no longer recognized, and a new stop codon is generated downstream, giving rise to a polypeptide for the A² enzyme that is 21 amino acids longer than the A¹ enzyme. The extra sequence thus generated, together with the Pro-Leu substitution in the A²-transferase, should account for the differences in kinetics and specificity between the two enzymes (see below). In addition to A¹ and A², the alleles encoding several examples of various other ABO subgroups have also been cloned: A₃, A_x, B₃, B(A) and *cis*-AB. All have so far proven to be heterogeneous at the

nucleotide level despite having been considered to characterize a single phenotypic subtype.

The A- and B-transferases synthesize A and B carbohydrate antigens by transfer of *N*-acetylgalactosamine (GalNAc) or galactose (Gal) moieties from UDP-GalNAc or UDP-Gal respectively to the C-3 carbon atom of a fucosylated terminal galactose residue present in a variety of oligosaccharide precursors with blood group H activity (see Figure 1). These precursors are the sugar component of glycolipids and glycoproteins. The transferases of the A₁ and A₂ alleles are known to have different pH optima, isoelectric points, thermal stabilities and metal ion cofactor requirements, which result in differences in their substrate transfer kinetics.

It is rarely possible to deduce a genotype from an ABO phenotype unless the groups of the family are available. For example, a group B individual could be B/B or B/O; an A₁ subject could be A¹/A¹, A¹/A² or even A¹/O. In contrast, group AB and group O individuals are A/B and O/O respectively, unless they are of the extremely rare *cis*-AB (with A and B antigens both derived from a rare allele at a single locus) or O 'Bombay' (see below) phenotypes.

Knowledge of the nucleotide sequences that distinguish the ABO alleles has enabled primers to be designed that can be used to determine ABO genotypes through the application of PCR. These include sequence specific primers that uniquely amplify A, B or O, as well as generic primers that amplify all ABO alleles; the products of the latter are distinguished by their sensitivity to restriction endonucleases that recognize the DNA sequences of specific ABO alleles.

ABO epitopes

ABO antigens are synthesized by glycosylation of oligosaccharides with H antigen activity. The H antigen is synthesized by a fucosyltransferase that is the product of the H gene on the long arm of chromosome 19. Carbohydrate chains carrying the A, B, and H antigens are present on 1) the short-chain oligosaccharides of simple glycolipids in plasma; 2) the heavily branched polysaccharides that form the polyglycosyl moieties of either soluble glycoproteins present in secretions or of polyglycosyl ceramides in the red cell membrane, and 3) the short O-linked and highly branched N-linked polysaccharides of integral membrane proteins. The immunodominant sugars of the A and B antigens are at the terminal (non-reducing end) of the various polysaccharide chains expressing A or B, and are invariably attached by an α1-3 linkage to a fucosylated galactose residue with H antigen activity (see Figure 1) such that the simplest A epitope is a trisaccharide with the structure:

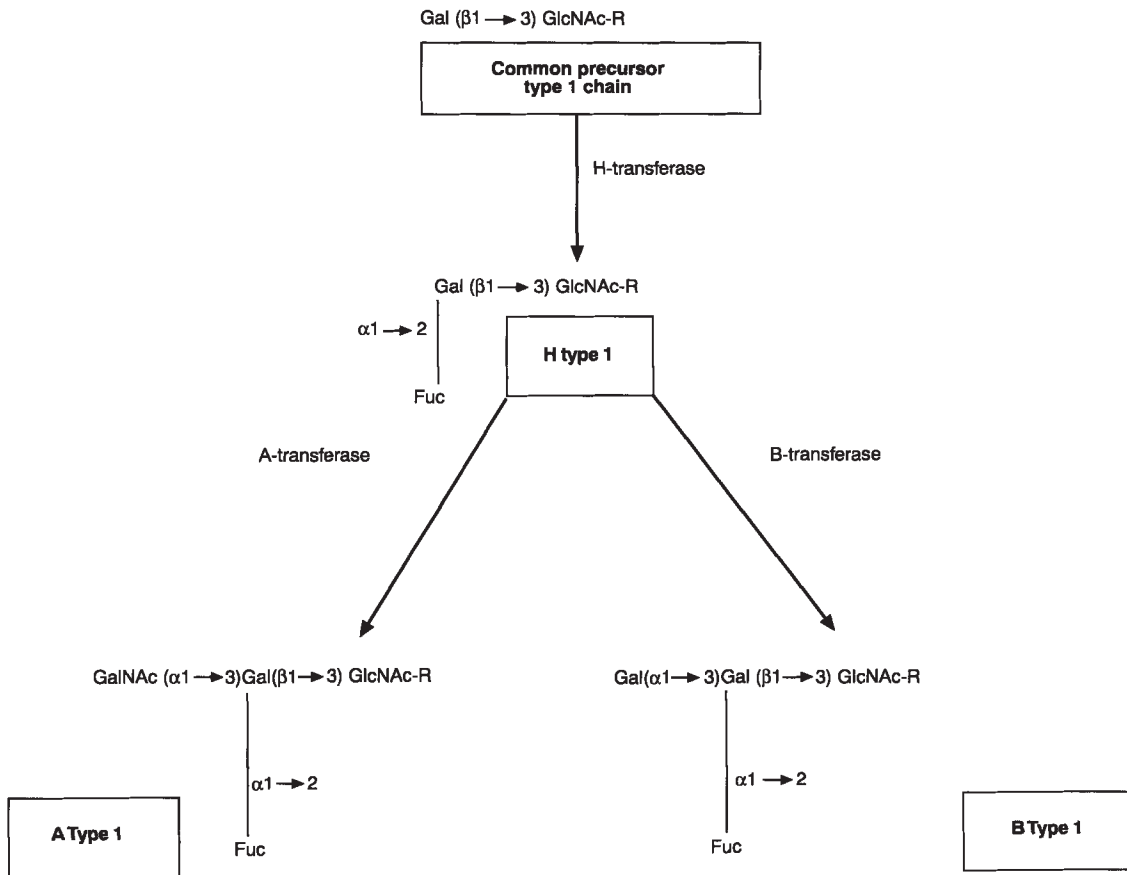
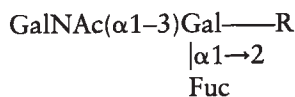


Figure 1 Biosynthetic pathways for the synthesis of A, B and H structures on type 1 precursor chains.



where R represents the rest of the polysaccharide chain. The B epitopes have Gal instead of GalNAc as the immunodominant sugar. The presence of the fucosyl residue, i.e. the H antigen, is essential for A and B expression: its absence, as in the rare 'Bombay' phenotype, leads to an inability of the A- and B-transferases to add their respective sugars to the Gal part of Gal—R, and hence A and B antigens are not expressed, even though the relevant transferases can be detected in cells and plasma and are functionally active in *in vitro* assays using fucosylated substrates from normal subjects as acceptors. Such 'Bombay' individuals lack an H gene, being homozygous for the silent allele h.

The terminal trisaccharides can be attached to R in at least six different ways: e.g. by either a 1–3 or 1–4 linkage to β GlcNAc (type 1 and type 2 A or B structures respectively), by a 1–3 link to α GalNAc (type 3), β GalNAc (type 4) or β Gal (type 5) or even a 1–4 link to β Glc (type 6). Of these, types 1 and 2 are the more abundant in the red cell membrane and the most important in red cell serology: integral red cell membrane proteins and glycolipids have almost exclusively type 2 linked sugars. Red cells may also contain glycolipids, passively adsorbed from plasma, that have exclusively type 1 chains. Secretions have a mixture of type 1 and type 2 epitopes. The existence of these various epitopes probably explains the heterogeneity in reactivity of different ABO antibodies with group A or B variants; seven reaction patterns for monoclonal anti-A and anti-B have been described.

In secretory tissues and other epithelia, ABH antigen expression is modulated by the genes of the 'secretor' (Se) locus which is not linked to the ABO locus; nonsecretor (se/se) individuals fail to produce type 1 and type 2 H in their secretions whereas red cell (type 2) H expression is unaffected.

Expression of ABO on red cells may be deliberately modified by treatment with glycosidases: an α -galactosidase extractable from green coffee beans can remove galactose from group B red cells and hence enzymically convert them to group O. Such 'ECO' (enzymically converted group O) red cells may survive normally when transfused to group O subjects. However, the clinical potential of such techniques is limited.

Biosynthesis and ontogeny

The A- and B-transferases and their respective antigens in adults are most abundant in intestinal and gastric mucosa, lungs and salivary glands. Significant levels are found in kidneys, bladder, urothelial cells, bone marrow and hematopoietic cells. The enzymes exist in the cytoplasm and bound to the membranes of the cells of the above tissues and in the membranes of red cells and platelets. The transferases are in free solution in the transtubular network and their relevant secretions, e.g. mucin droplets, plasma, ovarian cyst fluid, milk, saliva. Molecules glycosylated by the transferases include membrane enzymes, membrane structural proteins and receptors, as well as secreted proteins, e.g. IgA. The ABO transferases, but not others, can be lost in various cancers, e.g. carcinoma of the bladder and of the colon. Both N-linked and O-linked oligosaccharide moieties of glycoproteins with A or B activity are synthesized in the Golgi.

During ontogeny, ABH (and Lewis) activity is at its highest in the early embryo from the 5th week postfertilization; ABH antigens are found in large amounts on endothelial cells and most epithelial primordia, and in practically all early organs e.g. blood islands of the yolk sac, erythropoietic foci of the liver; digestive tube epithelia, pharyngeal pouches, the thymus, the pituitary, thyroid glands, trachea and bronchi, hepatic and pancreatic diverticula, the cloaca, urachos and allantois, mesonephros and the ducts of the metanephros. The CNS, liver, adrenal glands and secretory tubules show no ABH activity at this stage.

From the end of the 12–14th week of gestation, there is regression of ABH expression from epithelial cell walls, thyroid pituitary, and other glands and organs, to the adult vestigial pattern described above. The biological significance of this regression is unknown. The number of A and B sites on the red

cell is increased approximately fourfold in adults as compared with newborn infants, such that there are $0.25\text{--}0.37 \times 10^6$ A sites per red cell in the newborn and $0.81\text{--}1.2 \times 10^6$ in the adult, as compared with $0.2\text{--}0.32 \times 10^6$ B sites per red cell in the newborn and 0.75×10^6 for adults.

A- and B-like antigens have been detected in a variety of microorganisms and animal tissues. During phylogeny, ABH antigens are confined to the endodermal tissues of amphibia and reptiles, and are present on the epidermis of all mammals studied to date. While ABH is found on baboon vascular endothelium, only humans and the great apes have red cells expressing ABH. ABO are 'histocompatibility' antigens; it is simply the peculiarity of their original description that has led to ABH antigens being thought of as 'blood groups'.

ABO antibodies

The clinical importance of the ABO blood group system derives from the universality of its antibodies and their *in vivo* potency. The 'naturally occurring' antibodies of the majority of group A or B individuals are mainly IgM and produced in response to environmental ABO antigens, e.g. from microbes in the gut and respiratory tract. Such IgM antibodies, although displaying optimal activity in the cold, are reactive at 37°C and can activate the complement cascade up to the C9 stage, leading to the immediate intravascular lysis of transfused incompatible red cells *in vivo*. In the UK, roughly one in every three randomly selected, ungrouped blood donations would be incompatible with a given recipient; such incompatible transfusions can lead to renal failure, disseminated intravascular coagulation and even death. The majority of the signs and symptoms of severe ABO hemolytic transfusion reactions can be attributed to the generation of C3a and C5a fragments as a result of complement fixation, with the consequent release of vasoactive amines from mast cells and of cytokines such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor α (TNF α) from mononuclear cells.

Most, if not all, group O adults, and a small proportion of group A and B subjects, have naturally occurring, usually weak, IgG in addition to stronger IgM ABO antibodies. The IgG component can cross the placenta and bind to fetal red cells; however, lysis of fetal red cells is generally minimal and hemolytic disease of the newborn (HDN) caused by ABO antibodies is usually mild or inapparent in Western Europe and North America. The occurrence of HDN due to ABO antibodies cannot be predicted, but it only affects the offspring of group O mothers. The

lack of severity of most cases of ABO HDN is thought to be due to: 1) IgG ABO antibodies being predominantly IgG2, which is incapable of initiating complement-mediated hemolysis or destruction of antibody-coated red cells by the mononuclear phagocytic system (many sera have IgG1 ABO antibodies as well as an IgG2 component; a few sera have trace amounts of IgG3 and IgG4 antibodies); 2) substantial amounts of maternal IgG ABO antibodies binding to ABO sites on tissues other than red cells in the fetus; 3) soluble ABH antigens in plasma and body fluids of the fetus which neutralize IgG anti-A and anti-B and inhibit their binding; 4) the ABO epitopes of fetal red cells being present on unbranched oligosaccharides, which are thought to be unable to support the divalent IgG binding needed for complement activation by IgG1 or IgG3 antibodies; 5) fetal complement levels being too low to support efficient lysis of cord (or even adult) target red cells. However, in some parts of the world, ABO HDN is often more severe and this is attributed to environmental factors such as the stimulation of ABO antibodies by microbes and parasites.

Some individuals possess plasma IgA ABO antibodies irrespective of immunization; ABO antibodies of colostrum are often wholly IgA, although sometimes IgM antibodies can also be found.

Cord blood usually does not contain ABO antibodies although maternally derived IgG anti-A or -B can sometimes be detected. Newborn infants do not produce ABO antibodies until the 3rd–6th month of age (median titer = 4), reaching a maximal titer (approx. = 128) between the ages of 5 and 10 years. Anti-A seems to attain higher titers more rapidly than anti-B. Adult titers of anti-A range from 32 to 2048 (median = 256) and anti-B from 8 to 512 (median = 64). The vast majority of healthy adults

have readily detected ABO antibodies. Weakening of ABO antibodies can occur naturally in individuals aged over 50; a third of patients over 65 have ABO antibody titers of 4 or less. Occasional subjects may lack the appropriate ABO agglutinins, especially if hypogammaglobulinemic, or if their plasma IgM levels are low. Antibodies can be lost by exhaustive plasma exchange (used therapeutically in ABO incompatible bone marrow and organ transplantation) or by immunosuppression caused by therapy or by disease. IgM anti-A or -B are completely absent in individuals with the very rare Wiskott–Aldrich syndrome.

See also: Alloantigens; Blood transfusion reactions; Cold agglutinins; Complement, classical pathway; Embryonic antigens; Erythrocytes; Forssman antigen; Hemolytic disease of the newborn; Maternal antibodies; Natural antibodies; Rh antigens.

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ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

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In the early 1980s, reports appeared of a new immunodeficiency disease, which was later called the acquired immune deficiency syndrome or AIDS. Over the following decade and a half, the scale of AIDS has risen from a mere handful of cases in the USA to hundreds of thousands of cases affecting most countries worldwide. The human immunodeficiency viruses (HIV-1 and HIV-2), the causative retroviruses of AIDS, have already spread to 15–20 million individuals in less than three decades, and continue to spread rapidly in many communities, especially in the developing world. This epidemic of profound immunodeficiency, predominantly affecting cell-mediated immunity, has had a devastating and pervasive impact on many individuals and societies and has presented many challenges to immunology and to other clinical and scientific disciplines. Although an inadequate counterweight to its tragic personal and social impact, AIDS has served to focus and increase our knowledge of the immune system in health and disease as well as our understanding of individuals and of human society.

Epidemiology

Although the first cases of AIDS were seen in homosexual men in the USA, it soon became apparent that the epidemic of HIV and AIDS was occurring more widely in North America, Europe and Australasia, not only among homosexual men but also among intravenous drug users, recipients of blood and blood products, notably hemophiliacs, and to a lesser extent among heterosexual men and women, and among children of HIV-infected women. It also gradually emerged that a substantial epidemic was affecting several regions in the developing world, such as sub-Saharan Africa and the Caribbean, with a somewhat different pattern of infection, predominantly affecting heterosexual men and women and their children, as well as recipients of blood transfusions. South America showed a mixture of the two patterns, while other regions such as the Middle East and Asia initially showed a lower incidence of a type more similar to that seen in Africa. In the late 1980s, there was a rapid expansion of the epidemics in India and South-East Asia, predominantly through heterosexual spread and among injecting drug users.

These epidemics are almost entirely due to HIV-1, although there is regional variation in serotypes. Type B virus is found mainly in the USA and Europe, while other serotypes are seen in Africa and Asia; it has recently been suggested that type E, found in parts of Thailand, may have a greater capacity for heterosexual transmission. HIV-2, a related virus, has been seen in West Africa and remains largely restricted to that region. It appears to be less readily transmissible (though by the same routes) and is also less pathogenic, but causes the same spectrum of disease as HIV-1.

Such overall patterns of spread, together with detailed case and cluster studies, indicated that HIV was spread by three routes: sexual transmission, transmission by blood and from an infected mother to the fetus. Sexual transmission could occur through penetrative intercourse between homosexual men or between men and women. Regional differences in the type of sexual spread mainly reflect differences in prevalent sexual behavioural patterns and frequency of sexual partner change. Intercurrent sexually transmitted infections, especially those associated with genital ulceration, in either partner increase the risk of transmission but are clearly not essential. Blood-borne transmission occurred through transfusion of blood or blood products, but also, on a larger scale, between intravenous drug users who shared needles and syringes and other equipment; there is a small but definite risk of infection through inoculation injury. Vertical transmission occurs in part transplacentally, in part during delivery and in part through breastfeeding, although the proportions remain uncertain. Casual transmission has not been documented.

There has been considerable interest in the possibility that individuals exposed to HIV may develop protective immunity that prevents or clears initial infection or have features that prevent or reduce the risk of subsequent infection. For example, cohorts of female prostitutes in Africa and of homosexual men include individuals who appear to have been multiply exposed but remain uninfected. These show some HLA association and evidence of HIV-specific cytotoxic immune responses. The mechanism remains obscure. Recent observations on second receptors for

HIV, which are variably expressed among some populations, raise the possibility that lack of such receptors could be protective. Some infants appear to show evidence of transient infection as evidenced by polymerase chain reaction (PCR) or viral culture in the weeks after delivery but do not seroconvert or show any other signs of infection later. It has been proposed that they have cleared the infection, although the possibility of prolonged circulation of latently infected maternal cells has not been excluded.

Spectrum of HIV infection

A person infected by HIV will, within 3 weeks to 3 months, develop a detectable antibody response and will remain HIV antibody positive thereafter. Given that HIV establishes persistent infection, antibody positivity can be taken as evidence of current infection and has been widely used clinically and epidemiologically as a marker.

At the time of seroconversion, a significant minority of patients develop an acute transient glandular fever-like illness, with fever, malaise, rash, sore throat, lymphadenopathy, arthralgia and headaches (HIV seroconversion illness). In a few cases there is frank encephalopathy or aseptic meningitis. It is also now recognized that some patients develop acute, transient cellular immunodeficiency, manifested, for example, by oral and esophageal candidiasis. The symptoms and signs of acute HIV infection typically resolve after a few weeks. Patients with a severe and prolonged acute illness appear to progress to AIDS more rapidly, and those who develop any symptomatic seroconversion are more likely to progress than those who do not. After the resolution of the acute illness, patients will pass into a phase of chronic symptomless HIV infection.

Chronic symptomless HIV infection may take one of two forms – patients without any abnormal physical signs and those with persistent generalized lymphadenopathy. The lymph nodes are in most instances moderately enlarged in cervical, axillary and inguinal regions (although inguinal nodes may be enlarged to a similar degree in people without HIV infection) and some other peripheral sites, such as the epitrochlear nodes. They are sometimes slightly tender and may fluctuate in size with intercurrent illness, but are otherwise unremarkable. Patients may remain symptomless, with or without enlarged lymph nodes, for many years but over time a substantial proportion go on to develop disease. The risk for developing symptomatic disease is similar for patients with and without lymphadenopathy.

A few patients with otherwise symptomless

chronic infection and without immunodeficiency develop thrombocytopenic purpura. The pathogenesis of this is somewhat unclear, with evidence for platelet-specific antibodies and for immune complexes in different studies. The disorder is rarely associated with severe bleeding and seems to remit spontaneously after a few years in most cases. It does not presage progression.

With increasing years of infection, an increasing proportion of patients with chronic HIV infection will progress to develop evidence of immunodeficiency and other disease. This may initially be seen as constitutional illness (malaise, weight loss, diarrhea) or evidence of moderately severe immunodeficiency (oral candidiasis, oral hairy leukoplakia, multidermatomal shingles, salmonellosis). Patients may first develop such symptoms and later progress to AIDS, while others develop AIDS without any intervening symptomatic stage.

AIDS itself comprises two main categories: patients with major opportunist infections of cell-mediated type (e.g. *Pneumocystis carinii* pneumonia, cryptococcal meningitis, disseminated mycobacterial infection, cytomegalovirus retinitis), and those with opportunist tumors, Kaposi's sarcoma and B cell lymphoma. These will be considered in more detail below. It is worth noting that patients with Kaposi's sarcoma alone and some patients with lymphoma have less severe immunodeficiency than those with major opportunist infections.

In addition to the problems with cell-mediated opportunists, some patients, especially children, show significantly increased susceptibility to infections with capsulated bacteria of a type more typically associated with humoral immunodeficiency. This results from the dysglobulinemia seen in HIV infection, in which there is immunoglobulin G2 (IgG2) subclass deficiency and impairment of specific antibody development against antigens not previously encountered. These infections can occur with increased incidence in people with otherwise symptomless infection.

As well as its immunopathogenic effects, HIV can also cause chronic progressive disease of the nervous system, including HIV encephalopathy (or AIDS dementia complex), vacuolar myelopathy, peripheral and autonomic neuropathy, and occasionally an inflammatory myopathy. The central nervous system disorders typically occur after the onset of immunodeficiency disease, but go on to affect a high proportion to varying degrees.

A variety of other clinical disorders are seen, including: lymphocytic interstitial pneumonitis and an associated chronic lymphocytic parotitis, which are commonly seen in children but rarely in adults:

the appearance or increased severity of psoriasis and Reiter's syndrome and other seronegative arthropathies; HIV enteropathy with partial villous atrophy; and recrudescence of atopic disease in predisposed subjects.

Clinical features of AIDS

The profound and progressive immunodeficiency seen in AIDS is broadly characterized by infections with facultative intracellular and related pathogens and herpesviruses, and by opportunist tumors. While some of these pathogens only cause disease in immunodeficient subjects, others show altered and enhanced pathogenicity, causing different clinical disorders or more disseminated disease. The profile of these infections shows regional variation determined by locally prevalent pathogens and risk behaviour, which also affect the likelihood of infection by the relevant organisms, many of which are latent in the immunocompetent host.

Common and early infections in AIDS include *Pneumocystis carinii* pneumonia, disseminated tuberculosis, esophageal candidiasis, cryptococcal meningitis and ulcerative mucocutaneous herpes simplex infection. Other common infections seen, typically later in the course of the progressive decline in cell-mediated immunity, include cerebral toxoplasmosis, cytomegalovirus disease (presenting as retinitis, colitis, esophagitis and adenitis and, less often, pneumonitis, encephalitis and radiculitis), disseminated *Mycobacterium avium intracellulare* infection, histoplasmosis, gastrointestinal cryptosporidiosis, progressive multifocal leukoencephalopathy, visceral leishmaniasis, disseminated penicilliosis, nocardiosis and disseminated strongyloidiasis. Some of these organisms present predominantly in one organ system – lungs, gut, central nervous system, lymph nodes – but may also show varying degrees of dissemination.

Kaposi's sarcoma, which is most common among homosexual men and among heterosexually acquired cases of HIV in the developing world, is thought to result from infection with a newly recognized human herpesvirus, HHV-8. It often presents as cutaneous disease, later progressing in some patients to affect lymph nodes, lungs and gastrointestinal tract. Severe visceral disease more commonly presents early in patients in Africa. B cell lymphoma, of a variety of histological types, may present as isolated cerebral lymphoma or as systemic disease, with frequent extranodal involvement. Hodgkin's lymphoma, seminoma, squamous carcinoma of the anorectal region, cervical carcinoma and acute myeloid leukemia have also been observed somewhat more

frequently in HIV-infected subjects, although the association is less clear.

Natural history of HIV infection

The risk of development of disease in HIV-infected subjects has been assessed in large cohort studies. These have shown that in time the majority will develop AIDS (50% in 10 years; 65% in 14), with others progressing to less severe symptomatic HIV disease. It is now recognized that a small proportion of patients remain well and without evidence of immunodeficiency for 10 or more years. It is not clear whether they represent a discrete group or simply those with much slower progression rates. They are the subject of many studies attempting to define whether there are immune or other protective mechanisms.

Several factors affecting the likelihood of progression in HIV-infected subjects have been identified. Increased risk of disease and of immunological decline has been shown in patients with the HLA-A1, -B8, -DR3 haplotype in cohorts of hemophiliacs and homosexual men and HLA-B35 may also increase the risk of progression. In some ethnic groups HLA-DR5 is associated with increased risk of Kaposi's sarcoma. Patients with HLA-B27 appear to have a lower risk of progression.

Intercurrent infections may also affect progression: evidence of such an effect has been shown for sexually transmitted infections among homosexual men and for cytomegalovirus infection among hemophiliacs. It is plausible that other infections may have a similar effect, including systemic bacterial infections in injecting drug users using unsterile equipment, and possibly some of the infections that result from immunodeficiency, such as tuberculosis, listeriosis and pneumocystosis.

Age may also have some effect. This is most clearly seen in children infected in the neonatal period who show a much higher proportion of rapid progressions than adults. This is unlikely to reflect immunological immaturity and may result from immunological activation during the acquisition of an immunological repertoire. Children over 4 years of age show similar progression rates to adults. Adults over 50 years of age may also have slightly increased risk of progression.

The effect of pregnancy is controversial. Early studies that were carried out on women who had already given birth to a child with AIDS showed that subsequent pregnancies were associated with increased risk of progressive disease in the mother. More recent studies on women having their first HIV-positive pregnancy indicate no increased risk. It

is possible to reconcile these findings by concluding that having more than one HIV-positive pregnancy increases the risk to the mother.

Although formal epidemiological data are lacking, it is plausible that malnutrition, which itself induces cell-mediated immunodeficiency, may enhance the risk of progression to HIV disease. A higher rate of progression has been reported in a prospectively studied cohort in Uganda; several factors could have contributed to this, including different viral strains, different human genotypes, intercurrent parasitic and other infections and nutritional state. The use of immunosuppressive drugs such as prolonged high doses of corticosteroids may enhance risk.

The overall picture is one where increased risk of progression is associated with events that may activate the immune system, and thus active CD4 lymphocytes and macrophages which are latently infected with HIV, hence increasing virus replication, cell-to-cell spread and progressive immunological decline, as well as with events that suppress cell-mediated immune responses, thus compounding the factors leading to immunological deterioration.

Some strains of virus, whether the initially infecting virus or one that emerges through mutation within the infected host, may have greater pathogenic potential. One transfusion-associated cluster of HIV infections has been reported in which long-term nonprogression was a feature and where the HIV strain had a defective *nef* gene; this does not appear to be a generalizable feature but indicates the role of viral genotype, as do the lower rates of progression with HIV-2.

The role of protective immunity, whether humoral or cell mediated, is far from clear. Neither neutralizing antibody titer nor HIV-specific cytotoxicity, whether of antibody-dependent type or major histocompatibility complex (MHC)-restricted type, have any consistent relationship with progression. These responses are present throughout the spectrum of HIV infection and disease. It remains to be established whether or not innate resistance or susceptibility to HIV infection or disease play any part, or whether genetic factors serve rather to influence the ease with which, for example, immunological activation by intercurrent events is achieved, as is implied by the association with the HLA-A1, -B8, -DR3 phenotype. The study of long-term nonprogressors has attempted to elicit evidence of special features associated with nonprogression but has not yet shown any unique or general characteristic of host or virus. One problem with such studies is that of finding an appropriate comparator group, as their rapidly progressing peers may already have died and

current rapid progressors may have different initial host and viral characteristics.

Predictive markers for progression

Another objective of cohort analysis has been the identification of markers that can predict risk of future progression in HIV-infected subjects. The numbers of CD4 cells in blood have been widely used in this way, not least because they can reflect a significant component of the pathogenesis of disease. In grouped data over long-term follow-up, falling CD4 counts are strongly associated with progression. However, there is considerable variability in individual trends and, as many other factors can affect CD4 count, CD4 counts must be used with caution in individual patients. Serum markers of immunological activation such as β_2 -microglobulin, neopterin and interleukin 2 (IL-2) receptor are also associated with an increased risk of progression, as are raised IgA levels. The CD8 count may rise during the early years of HIV infection and then fall in the 2 years before progression, but the predictive value is unclear. CD8 derived cytokines may inhibit virus spread.

Virological markers are also of value. In early studies, HIV p24 antigenemia was strongly associated with progression in patients in temperate climates, although a significant proportion of AIDS patients do not develop antigenemia. Prior to the appearance of HIV p24 antigen, antibody levels against p24 fall, perhaps reflecting the complexing of p24 antigen and antibody following increased virus replication. Reduced levels of antibodies to p17 and to reverse transcriptase may show similar trends. These changes are not seen in patients in tropical environments, perhaps due to increased polyclonal B cell activation due to parasitic and other infections. It is evident that p24 antigen is an imperfect and crude index of virus replication.

Recently, it has become possible to measure viral load by quantitative PCR. Studies on a large prospective cohort of HIV-infected individuals have shown that viral load measures on initial samples provide a powerful predictive marker for subsequent progression. Such tests appear to be more discriminatory than CD4 count alone. Such findings are likely to provide a valuable means of assessing prognosis and suitability for antiretroviral therapy. They may help to identify those subjects with a high CD4 count who do poorly and those with a low CD4 counts who do well. Although the use of the two markers in conjunction has yet to be analyzed, this is likely to be a particularly useful combination for assessing future progression. Longitudinal analysis of both markers

may add further value. Earlier data showed that combining CD4 counts with serum neopterin, IgA, IL-2 receptor and p24 antigen could provide a better prediction of outcome than CD4 alone, although these are likely to be superseded by viral load measures, which probably correlate with activation markers.

Immunopathogenesis

HIV is a retrovirus, having an RNA gene that encodes for reverse transcriptase, which allows a DNA copy of the virus to be made. This DNA copy is then spliced into the gene of the infected cell, through the action of virally encoded endonuclease, leading to persistent and typically productive infection.

A major HIV gene product is the outer envelope glycoprotein gp120, which is expressed on the surface of the virus and of infected cells, and contains a region which binds with high affinity to the CD4 molecule on host cells. CD4 thus acts as the main virus receptor, enabling HIV gp120 to target cells of the immune system for infection and subsequent damage. Second receptors (CXCR4 and CCR5) for HIV on CD4 T lymphocytes and on antigen-presenting cells are a family of transmembrane proteins which are physiological receptors for chemokines. They appear to be critical to fusion events and cellular infectivity, and may also explain data showing that combinations of chemokines (RANTES, MIP-1 α and MIP-1 β) can inhibit cell-to-cell infection *in vitro*.

After the attachment of gp120 to CD4 and the second receptor on lymphocytes or macrophages, the virus membrane fuses with the membrane of the CD4-bearing cell, internalizing the virus genome and leading to cellular infection. This occurs both in initial infection and in the gradual process of cell-to-cell spread within the body that leads ultimately to disease. Infection of CD4 lymphocytes is central to the immunopathogenetic effects of HIV. Macrophages and related cells can also be infected by routes other than CD4 binding, e.g. Fc or C3b receptor binding. Such cells serve as an important reservoir of HIV infection and are implicated in the neuropathogenesis of HIV encephalopathy. Recent data have emphasized that most cell-to-cell infection occurs in lymphoid tissue, where extensive viral replication has been documented. As disease progresses, this results in significant structural damage to these key microenvironments.

The viral replication cycle proceeds at a rapid rate in infected patients, with durations of as little as 2.6 days, giving some 140 generations each year. This, combined with the error-prone transcription of retroviral genes, gives the opportunity for enormous

diversity of viruses within any individual. It is against this background that pathogenetically diverse strains, such as syncytium-inducing strains, and drug-resistant strains may emerge. In turn these affect the rate of immunological decline and responsiveness to therapy.

The most striking and most consistent effects of HIV in progressive disease are a reduction both in the number and in the function of CD4 lymphocytes. Infection of CD4 lymphocytes by HIV leads to their premature death or rapid elimination, so that few viable circulating CD4 cells show evidence of infection at any time, yet their numbers are progressively depleted. This may be due to virus cytopathic effects or to host-mediated cytotoxicity, or both. Virus-induced damage includes cell lysis and, more importantly, apoptosis and the formation of syncytia by fusion of infected as well as uninfected CD4 lymphocytes.

In addition to the progressive attrition of CD4 lymphocytes, various defects in the function of the CD4 cells that remain have been shown, even though most of them are uninfected at the time. These include failure of T cell responses to antigen, to T cell mitogens (phytohemagglutinin, concanavalin A) and to T cell-dependent B cell mitogen (pokeweed), whether measured by proliferation or cytokine/antibody production. Impaired cytokine release is seen for IL-2 and macrophage-activating cytokines such as interferon γ , as well as cytokines involved in regulating B cell responses. It seems that the failure of these mechanisms is largely responsible for the defects in killing of facultative intracellular pathogens, failure to control virally-induced tumors and herpesvirus infection and for some of the B cell defects seen in patients.

One common means whereby these effects on CD4 cell function could be mediated by HIV has been shown. HIV infection of lymphocytes *in vitro* or exposure of uninfected lymphocytes to gp120 causes activation of the inositol polyphosphate (InsP) signal transduction pathway; this causes a rise in resting levels of InsP₃ and InsP₄, and hence of intracellular calcium. This leads to a state of chronic cellular activation, rendering the cells less responsive to new signals. Similar changes are seen in lymphocytes taken from patients. These effects may be mediated by binding to CD4 and subsequent alterations in the intracellular signaling pathways involving tyrosine phosphorylation, starting with p56^{lck}. Similar changes in activation are seen in macrophages and it is possible that similar effects underlie the effect of HIV proteins on B cell function.

Depletion of CD4 lymphocytes and their functional impairment has knock-on effects on many

other cells, in addition to any direct effects that HIV has on them. Patients' macrophages show impaired microbicidal activity for intracellular pathogens in the presence of cytokines produced by autologous lymphocytes, although they retain responsiveness to exogenous interferon γ . Other macrophage functions, including antigen-presenting function and receptor expression, show changes that may result from loss of CD4 lymphocyte signals or from HIV infection. Dendritic cells, which may be infected by HIV, may be depleted and show marked dysfunction in antigen-presenting activity from an early stage of HIV infection. Natural killer (NK) cells and other cytotoxic cells show reduced recruitment and activity, in part due to reduced IL-2 and interferon γ production.

CD8 T lymphocytes are clearly altered in number with disease progression (see above) and show increased activation markers. Previously thought not to be infected, a recent report suggests that they may indeed be infected especially in patients with advanced disease, possibly as a result of infection of thymic precursors which coexpress CD4. This may contribute to the decline in their number in late disease and to their functional abnormalities. Furthermore, CD8 cells produce factors, which may include chemokines acting on the second receptor and/or other factors, that inhibit viral infection of CD4 cells. If these *in vitro* effects are operative *in vivo*, they may act to control or contain viral replication earlier in HIV infection. This is in addition to any role of CD8 cell-mediated specific cellular cytotoxicity, discussed above.

B cells show polyclonal activation in HIV-infected subjects, notably those with persistent generalized lymphadenopathy and Kaposi's sarcoma, with raised IgG1, IgG3, IgA, IgE and IgM levels. IgG2 and IgG4 levels may be low. Spontaneous immunoglobulin production is increased and responses to T cell-independent antigens are reduced. Enhanced B cell IgD production and raised serum IgD levels imply increased immaturity of B cells, which is also suggested by phenotypic alterations. In addition, responses to B cell mitogens and T cell-dependent mitogens and antigens are impaired, whether at a T cell level, antigen-presenting cell level, B cell level or a combination of any of these. Follicular dendritic cells in lymph nodes, which show evidence of HIV infection, show progressive destruction in patients with symptomatic HIV infection and AIDS. As a result of these various defects, patients show increased levels of antibodies to previously encountered antigens but are unable to develop new antibody responses.

The neuropathogenesis of HIV encephalopathy

and other nervous system disorders is more obscure. However, HIV encephalopathy also appears to result from both loss of cells and defective function of those that remain. The main cell type in the nervous system that harbours HIV is the macrophage, including perivascular macrophages and microglia, with little indication of infection of neuronal or glial elements. It is likely that HIV infection of such cells causes release of virus proteins or macrophage products, which in turn damage or alter the function of neighbouring nerve cells. gp120 can interfere with neuronal function in a similar way to that seen in lymphocytes, with activation of signal transduction pathways and raised intracellular calcium.

Treatment strategies in HIV disease

The AIDS pandemic has meant that large numbers of patients with severe immunodeficiency now require health care provision. Such patients need substantial specialist input from a wide variety of medical disciplines as well as a wider multidisciplinary team. Accessible and appropriate health care systems are being developed to cater for the various stages of HIV disease. Patients are increasingly involved in aspects of their care, notably in health maintenance (avoiding undernutrition, smoking, alcohol excess, and other cofactors). Specific therapies are of three types: treatment/prophylaxis of opportunist diseases, antiretroviral therapies and immunorestorative approaches.

Many of the opportunist infections are readily manageable, especially in the earlier stages of AIDS, with the use of specific antimicrobials and antitumor therapies. For many of the infections, maintenance therapy is required to avoid relapse. For some common infections, the use of antimicrobial prophylaxis offers considerable benefit, although there is often a trade-off of toxicity or drug hypersensitivity. Indeed drug hypersensitivity (type I and/or type III) to sulfonamides, antifungals and other drugs is notably more common in these immunodeficient patients. The increasingly effective use of antimicrobial treatment and prophylaxis has changed the clinical profile of disease, with deferral or elimination of some opportunistic events and decreased morbidity. However, it presents further challenges as more patients have more prolonged periods of severe immunodeficiency, leading to a greater proportion being affected by the less treatable infections and tumors, and new and more complex clinical events.

Antiretroviral therapy has developed rapidly in recent years. Zidovudine (AZT), a reverse transcriptase inhibitor and the first agent to show clinical efficacy, has had a significant impact when given as

monotherapy in AIDS and symptomatic HIV infection, reducing viral replication and hence reducing the rate at which disease progresses. This led to valuable increases in length and quality of life; improvement in HIV encephalopathy and reduction in its incidence are other notable benefits. Myelosuppression, gastrointestinal symptoms and a mitochondrial myopathy are seen, particularly at high doses, in patients with more advanced disease or where other drugs add to these problems. The benefit of zidovudine monotherapy seems on average to wane after some 2 years, whether due to its intrinsic limitations or the emergence of viral resistance. A logical approach to antiviral therapy would be to start treatment earlier, while the virus is active but when immune destruction is not so far advanced. With zidovudine alone, despite early encouragement, there is no general benefit in terms of clinical outcome. Zidovudine has, however, been shown to decrease vertical transmission if given to mothers during pregnancy and to the neonate.

Given the nature of the viral challenge, the use of combination therapies seems highly appropriate, with the objective of increasing efficacy and inhibiting the emergence of viral resistance, and possibly reducing toxicity. Initially this approach was focused on other nucleoside analogues, such as didanosine, zalcitabine, lamivudine and stavudine. Recent studies have shown the greater and more prolonged clinical efficacy of dual therapy with pairs of nucleoside analogs, confirming earlier studies showing their greater impact on CD4 count and on viral load. These therapies appear to offer some efficacy in earlier stages of infection, but the optimal timing is uncertain.

Two other major classes of antiretroviral agents have emerged – non-nucleoside reverse transcriptase inhibitors (NNRTI) (e.g. nevirapine, delavirdine, loviride) and protease inhibitors (e.g. saquinavir, ritonavir and indinavir). Triple combinations (e.g. two nucleosides and an NNRTI, or two nucleosides and a protease inhibitor) have an even more potent and durable impact on markers and, at least on early evidence, on clinical outcomes than two-drug regimens. Viral resistance appears to be more profoundly inhibited, which offers encouraging prospects for long-term efficacy. These more substantial effects raise the possibility of much earlier treatment, and some studies are exploring the effect of treating at or around the time of seroconversion.

Many issues still need to be resolved regarding antiretroviral therapy: optimal timing; optimal initial combinations; suitable means of monitoring patients on treatment; how these can inform decisions about if and when to change and, if so, what to change to;

and appropriate sequences of therapy. While some further approaches to antiviral treatment are being studied, the current portfolio of three classes seems likely to remain the core of treatment for some time, and there is a need to ensure that the armamentarium is deployed most effectively.

Among antiretroviral approaches that have been tried was the use of soluble CD4 or CD4 linked to an Fc molecule as a decoy for viral gp120. This looked promising *in vitro* and appeared not to interfere with cell function, but *in vivo* results were disappointing. This may be for a number of reasons, including the need to achieve very high levels to inhibit viral spread, the short plasma half-life of CD4 and the probability that much cell-to-cell transmission occurs directly, without a fluid phase.

Immunological approaches to therapy have been disappointing to date, although most were tried before effective antiretrovirals were available. Some are being re-explored in combination with antiretroviral therapy. Bone marrow transplantation has been generally disappointing, even when combined with antivirals. Several cytokines have been tried, including IL-2 and interferon γ , with some encouraging *in vitro* changes, and these are being tried with antiretrovirals. Immunostimulation has always been hard to achieve in reality and no clear agent of this type has been shown to be effective. One trial has shown that inosine pranobex (isoprinosine) may delay disease progression when given to asymptomatic patients, but other trials have not confirmed this; the mechanism of action is not clear.

Immunoglobulin therapy has proved helpful in patients, especially children, with antibody deficiency, by reducing problems with pyogenic infections. HIV antibody from asymptomatic subjects has been used as passive specific antibody therapy in a few studies but the results are not impressive and do not suggest that any effect is necessarily due to an antiretroviral mechanism; it is doubtful that passively acquired antibody will be any better than that produced actively in stemming viral replication and cell-to-cell transmission. Some have suggested that immunization with certain HIV antigens may be effective but such approaches may be subject to similar provisos; recent reports of therapeutic vaccines show no benefit with envelope glycoprotein or p24.

Vaccines

Extensive work is being done to develop vaccines against primary HIV infection. This work has utilised gp120 or other viral proteins and has employed a variety of viral and other vectors. Antibody and cytotoxic responses can be readily obtained, but

most work in animals shows that infection is not prevented or is restricted to a few strains. It remains unclear whether these problems reflect limitations of the immune responses obtained with such approaches or whether they reflect the fact that in most cases primary infection of target cells occurs at the mucosal level, where contact between virus and macrophage or lymphocyte is unlikely to be impeded by such antibody as reaches that site. Some studies have examined ways of producing more substantial mucosal immunity and this would seem a critical element in any vaccine approach. The prospect of an effective vaccine in the imminent future seems remote.

See also: Chemokines; Chimerism, hematopoietic; Dendritic cells; Human immunodeficiency viruses; Opportunistic infections; Retrovirus, infection and immunity.

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ACQUIRED IMMUNE RESPONSE

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The development of immunity to a particular pathogen occurs after initial exposure to the antigen. The immunity is due to the development of an acquired immune response to the pathogen. A baby is born virtually immunologically naive. Maternal antibodies that have crossed the placenta provide transient protection from infection until the newborn's own immune system has developed sufficiently to mount

an immune response strong enough and fast enough to protect against initial infection. After a primary immune response to a pathogen, an acquired immunity is developed such that any further infection by that pathogen is immediately controlled. The secondary or acquired immune response to the pathogen is more rapid and of greater magnitude and thus provides elevated protection against infection. From

birth throughout a person's life, the immune system is continually responding to new environmental challenges (e.g. bacteria and viruses), and developing immunity to those challenges. The study of the acquired immune response is the basis of the science of immunology.

Historical background

The phenomenon of acquired immunity was observed as early as 430 BC when the historian Thucydides in his description of the plague of Athens wrote: 'Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice – never, at least, fatally.' Thus even the early Greeks understood that individuals who had been exposed to a disease were provided protection from later susceptibility.

In the eighteenth century, the practice of active immunizations by inoculation with diseased material from smallpox victims (or later the inoculation of related or attenuated virus discovered by Jenner) was the impetus for modern studies of the acquired immune response. Today the acquired immune response is still being investigated on both the cellular and the molecular level.

Characteristics of the acquired immune response

The first time an antigen enters the body a wide spectrum of events occurs. The antigen is usually contained at the site of entry and in nearby lymph nodes by various phagocytic cells such as macrophages and dendritic cells. These cells process the antigen and present pathogen-derived peptides to CD4⁺ and CD8⁺ T cells in association with major histocompatibility complex (MHC)-encoded molecules. Antigen binding by surface immunoglobulin and cognate help by CD4⁺ T cells induce B cells to differentiate to antibody-secreting plasma cells. During a primary immune response there is a lag time of approximately 3–4 days before antigen-specific antibody is evident in serum, and the levels do not peak until approximately day 7. The antibodies produced are primarily immunoglobulin M (IgM), which are characteristically very heterogeneous in their affinity for the antigen. IgG antibodies do not appear in the serum until approximately day 7 and do not peak until day 14. Thus the primary humoral immune response is very slow and weak.

The acquired or secondary immune response occurs when the antigen is encountered by the

immune system any time after the primary response. This may occur several days or many years after the initial encounter. The acquired response is characterized by a more rapid and intense attack by the immune system. Increased levels of IgG antibodies appear in the serum within 24 h after infection. In the case of cell-mediated immunity, greater numbers of antigen-specific T cells rapidly respond. The ability of the immune system to 'remember' past exposure to antigens is called immunological memory and is one of the hallmarks of the immune system.

Mechanisms of acquired immunity

Development of high-affinity memory B cells

Towards the end of a primary response, higher affinity antibodies are found than were apparent early on. This phenomenon is called affinity maturation, and is found primarily with antibodies derived from B cells that have undergone isotype switching to IgG or IgA classes. During the primary response, many of the B cells differentiate to memory B cells. These memory B cells have been found to be extremely long-lived and, when activated, characteristically produce immunoglobulin that is similar to that found during the later stages of the primary response. Thus, when antigen is encountered by memory B cells, the cells are easily activated and able to produce a strong and rapid humoral immune response.

Memory B cells are highly effective antigen-presenting cells

The clonal expansion of antigen-specific memory B cells provides a large population of highly specific antigen-presenting cells. It has been shown by several groups that antigen-specific B cells are much more effective presenting cells than other known accessory cells such as macrophages and dendritic cells. Memory B cells take up antigen through their high-affinity surface immunoglobulin, and present high concentrations of antigen in association with MHC on their surface. Thus, antigen presentation to T cells, a critical early event in generating an immune response, is also more effective during a secondary immune response.

Clonal expansion of antigen-specific T cells

During the primary immune response, antigen-specific T cells are clonally expanded. It is believed that this expansion provides a further level of protection from reinfection. The mechanisms involved in the development and maintenance of T cell memory are

still unclear. After the initial T cell expansion, the antigen-specific T cells are downregulated through induction of programmed cell death and anergy. This stage is followed by the development of an antigen-specific memory T cell population. This population of T cells can remain with the individual for a lifetime, and can provide protection from further infections by the organism that expresses the antigen. There are two proposed mechanisms for how the body maintains these antigen-specific memory T cells. The first is that once these memory cells develop, they are extremely long-lived and can survive indefinitely to provide protection for the individual. The second hypothesis is that a small but significant amount of antigen persists in the individual. This antigen-persistence provides a continual level of stimulation for these memory T cells and is able to stimulate the production of new memory T cells.

Antigens that do not induce acquired immunity

Interestingly, not all antigens induce immunological memory. The best studied of these are T-independent (TI) antigens. TI antigens are capable of stimulating B cell proliferation and differentiation in the absence of cognate T cell help. Common TI antigens are polysaccharides and polymers, both of which are composed of highly repetitive subunits. The reason that these antigens are unable to effect an acquired immune response is still unclear. However, it is known that TI antigens tend to stimulate a subset of B cells, CD5⁺ cells, that may not be capable of differentiating into memory cells.

Medical indications of the acquired immune response

Clearly the most impressive medical indication of the

acquired immune response is vaccination. The simple and elegant theory of vaccination is that induction of a primary immune response by a nonpathogenic form of an antigen leads to immunity of the infectious form. Common types of vaccines are attenuated pathogen, related noninfectious antigen, and purified proteins from the pathogen. Examples of all of these vaccines have been used to induce a primary immune response that confers acquired immunity.

See also: Affinity maturation; Antigen-presenting cells; Antigens, T dependent and independent; Immune response; Immunoglobulin class switching; Maternal antibodies; Memory, immunological; Vaccines.

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ACUTE INFLAMMATORY REACTION

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The acute inflammatory reaction constitutes the first line of defense against infection as well as the initial stage in restoring injured tissue to normalcy. Acute inflammation is recognized by redness, swelling, heat and pain in the affected area. These symptoms stem from a multitude of events taking place within the inflamed site. The humoral arm of the immune response, including circulating antibodies and the complement system, is activated. Leukocytes which normally travel within the bloodstream recognize signs of tissue in distress, adhere to and traverse the

blood vessel wall and migrate toward the source of irritation. The permeability of small venules increases, leading to edema formation, and furthermore the blood flow through inflamed tissue is altered as a result of modification of arteriolar tone. Additionally, a multitude of cell types are activated to liberate various inflammatory mediators, including cytokines and arachidonic acid metabolites, which in turn can affect every aspect of inflammation mentioned above. The intricacy and complexity of the inflammatory reaction cannot be underestimated

and the detrimental impact of these events on normal function is often massive.

Cause of inflammation

While a classical immune response can elicit acute inflammation, both through antibody–antigen complex formation and through activation by cytokines secreted by T cell activated macrophages, the focus of this entry will be on effector cells such as neutrophils and endothelial cells. Signs of acute inflammation are seen in connection with infection by various pathogens, but tissue injury induced by physical, chemical or thermal means, without infection of foreign bodies *per se*, will also induce an inflammatory response. Additionally, a host of mediators, some of them listed in Table 1, will induce inflammation upon injection.

A particularly important clinical problem is the inflammatory response followed by tissue injury which is associated with ischemia/reperfusion situations such as myocardial infarction, stroke or transplantation of organs. Another example of an inflammatory response gone awry is the systemic inflammatory response syndrome (SIRS) sometimes seen in connection with systemic infection, extensive burns, ischemia, trauma or hemorrhagic shock. These conditions highlight the importance of understanding the inflammatory reaction for the purpose of adequate clinical intervention.

Table 1 Some inflammatory mediators and their actions *in vivo*

Mediator	Neutrophil accumulation	Edema formation ^a
Histamine		x
Bradykinin		x
Serotonin		x
Complement factor C3a		x
Complement factor C5a	x	x
PGD ₂ , PGE ₂ , PGI ₂		x ^b
LTC ₄ , LTD ₄ , LTE ₄		x
LTB ₄	x	
PAF	x	x
TNF α	x	
IL-1	x	
IL-8	x	
Substance P	x ^b	x

PG, prostaglandin; LT, leukotriene; PAF, platelet activating factor; TNF α , tumor necrosis factor α ; IL, interleukin.

^aIncludes mediators that induce edema through direct action on endothelium only.

^bIn some models only.

The neutrophil granulocyte

Mechanisms of neutrophil infiltration

The first leukocytes to infiltrate a site of inflammation are neutrophil granulocytes, which sometimes begin to accumulate within minutes of induction of inflammation. A host of adhesion molecules essential for neutrophil recruitment have been identified. During its journey through the vasculature, the neutrophil makes transient contact with the vascular wall of postcapillary venules, which gives the cell a rolling motion across the endothelium. This phenomenon is dependent on L-selectin (CD62L), present on the neutrophil surface, which recognizes and binds carbohydrates on the luminal surface of the vascular endothelium. Near an inflammatory site, however, the neutrophil becomes activated, the L-selectin is shed from the surface, and now the CD11b/CD18 adhesion complex, part of the integrin family, comes into play as the neutrophil adheres firmly to the vascular wall. Next, the neutrophil migrates out of the vessel through the junction between adjacent endothelial cells, and continues to move through the tissue toward the inflamed area, guided by inflammatory mediators which form a chemotactic gradient.

Other selectins, P- and E-selectin (CD62P and CD62E, respectively), can be found on the endothelial surface after stimulation with certain inflammatory mediators. P-selectin appears within minutes, whereas E-selectin is first found some hours after stimulation. Both P- and E-selectin can support neutrophil rolling along the endothelium together with neutrophil L-selectin.

An interesting exception to this scenario is seen in the lung. The low pressure in the pulmonary circulation combined with the narrow dimensions of lung capillaries leads to occasional trapping of neutrophils in these vessels, a phenomenon referred to as 'plugging'. When activated, neutrophils become stiffer as a result of intracellular actin polymerization, facilitating capillary trapping which may take place independently of the above-mentioned adhesion proteins. The cells may then migrate from the capillaries, which appear to be the main site for neutrophil extravasation in the lung. Plugging is also seen in some ischemia/reperfusion models, and has been suggested to account for the no-reflow phenomenon after severe ischemia, although this remains controversial.

Tissue injury by neutrophils

The neutrophil contains a formidable arsenal of proteinases, lysozyme and oxygen radical-forming enzymes in its granules, usually employed to

kill microorganisms ingested by the neutrophil. Occasionally, however, these injurious products may be released to the extracellular space and cause tissue damage, for example if the perceived foreign body is too large for ingestion. In addition, antiproteinases normally present in the interstitial fluids can be inactivated by neutrophil-derived oxygen radicals, thereby enhancing the damage inflicted by neutrophil proteinases such as elastase, gelatinase and collagenase. Experimental proof for neutrophil-inflicted tissue injury is found in models of immune complex-induced dysfunction of various organs such as the Arthus reaction and nephrotoxic nephritis, among others.

Vascular leakage

Tissue swelling is one of the most obvious signs of inflammation. It is generated by an increase in vascular permeability to plasma proteins and fluid. Under normal conditions the endothelial layer lining blood vessel walls is relatively nonpermeable to proteins and other molecules above 30 Å, and the small exchange between the intravascular and extravascular compartments occurs by transport through the endothelial cell bodies as well as through junctions between adjacent endothelial cells. During inflammation the endothelial cells in small venules retract from each other after cell junctions, in the form of cadherins, break apart. This results in large pore formation and massive exudation of plasma proteins. Many inflammatory mediators, including histamine, bradykinin, certain complement factors and serotonin, can act directly on the endothelial cells to cause retraction, although the exact underlying mechanism is yet to be described. This is partly due to the difficulty in studying these events in *in vitro* cell culture.

In some experimental models of inflammation the observed edema is dependent entirely on neutrophil infiltration. Many of the products liberated from activated neutrophils increase the permeability of vascular walls by causing endothelial damage. In some cases, however, neutrophils cause edema without apparent dependency on its proteases or oxygen radicals and in the absence of evidence of injury to the vessel wall. The migration through endothelial junctions by neutrophils could theoretically be enough to open a gap between endothelial cells. An alternative explanation is found in the multiple

polycationic proteins present in neutrophil granules, which can cause endothelial retraction through a poorly understood charge interaction with the endothelial surface. In a fulminant multifactorial inflammatory response, however, all of these mechanisms are likely to contribute to the observed edema.

Concluding remarks

The acute inflammatory reaction stems from a complex and interdependent set of events at the microvascular level which lead to the easily recognizable symptoms of inflammation: redness, swelling, heat and pain. Depending on the situation, it can constitute a necessary defense against a potentially threatening invasion of pathogens, or it can itself be a threat to the organism because of the destruction of tissue taking place in the process. A better understanding of the relative importance of the various mediators and cell types involved is crucial for successful therapeutic intervention in inflammatory disease.

See also: Anaphylatoxins; Arachidonic acid and the leukotrienes; Chemotaxis; Histamine; Mast cells; Neutrophils; Phagocytosis; Platelet-activating factor (PAF); Prostaglandins.

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ACUTE PHASE PROTEINS

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Acute phase proteins are plasma proteins, the synthesis and the circulating concentrations of which are adaptively regulated in response to most forms of inflammation, infection and tissue injury. The name arises from the fact that the first such protein, C-reactive protein (CRP), was originally discovered in serum of patients in the acute phase of pneumococcal pneumonia. However, it soon became apparent that increased levels of CRP and certain other plasma proteins occurred during active, tissue-damaging disease processes, whether acute or chronic. Nevertheless the term 'acute phase' has persisted and is in general use to describe a large group of disparate plasma proteins (Table 1) which share the property of increased production after injury and in disease states. A small number of proteins, including albumin, the single most abundant plasma protein, show the reverse pattern and are called 'negative acute phase proteins' because their levels fall during the acute phase response (Table 1).

Table 1 Plasma protein concentrations in the acute phase response

	Increased	Decreased
Proteinase inhibitors	α_1 -Antitrypsin α_1 -Antichymotrypsin	Inter α -antitrypsin
Coagulation proteins	Fibrinogen Prothrombin Factor VIII Plasminogen	
Complement proteins	C1s C2, B C3, C4, C5, C6 C9 C1 INH	Properdin (P)
Transport proteins	Haptoglobin hemopexin (Ceruloplasmin)	
Lipoproteins		High density lipoprotein Low-density lipoprotein
Miscellaneous	C-reactive protein Serum amyloid A protein α_1 -Acid glycoprotein Gc globulin Ceruloplasmin (Fibronectin)	Albumin Prealbumin (Transthyretin)

The acute phase proteins include members of the coagulation and complement systems, proteinase inhibitors and transport proteins, as well as several proteins of unknown function. The response as a whole, and many of the individual proteins, are conserved among endothermic animals and it therefore seems likely that it has an overall beneficial effect, possibly related to minimizing tissue injury, enhancing host resistance and promoting resolution of inflammation and repair. However, the whole, essentially nonspecific, phenomenon of the acute phase response is very complex and forms part of an even wider response to viable or nonviable noxious stimuli, involving fever, the immune system, the hematopoietic system, the stress-related endocrine system and protein, lipid and carbohydrate metabolism both locally and generally.

Synthesis of acute phase proteins

Most acute phase proteins are synthesized in the liver, although the genes for some are also expressed in cells and tissues elsewhere. Transcriptional control is the main mechanism for regulation of production but mRNA stability contributes in some cases. A large number of cytokines, including interleukin 1 (IL-1), IL-6, tumor necrosis factor α and various interferons, are capable of inducing increased, or in some cases decreased, production of various acute phase proteins *in vivo* and in cultured hepatocytes and liver cell lines *in vitro*. Glucocorticoids and steroid sex hormones can play an important permissive role and neural and neuroendocrine influences may be significant *in vivo*. Results obtained in different laboratories with different acute phase proteins, different cytokines and different cell lines or experimental systems have shown much variation. It has been difficult to reconcile all the findings and to identify the critical participation of particular mediators in control of particular reactants, especially because of the cascade effects by which some cytokines promote the production of others. Nevertheless it is striking that IL-6 knockout mice mount absolutely no acute phase response of serum amyloid A protein (SAA), serum amyloid P component (SAP) or complement component C3 following induction of sterile inflammation by casein or silver nitrate injection, whereas lipopolysaccharide (LPS) induces a definite, although subnormal, response.

Studies with transgenic mice bearing the human CRP gene, with transfected cells containing human SAA genes, and with hepatoma cell lines, have identified regulatory flanking regions of DNA which are targets for the action of nuclear factors responsive to IL-6 and IL-1.

Diversity of acute phase proteins

There is considerable diversity among acute phase proteins with respect to the concentrations attained, their structures, their behavior in different species and in different diseases. The concentration of a plasma protein depends on the balance between its secretion rate and its clearance rate. The availability of the protein for a particular function is the important factor in physiological and pathophysiological situations, rather than simply its serum concentration measured *ex vivo*. Increased availability can exist by virtue of increased production and yet be disguised by increased utilization or clearance so that plasma levels are normal or even decreased. Definition of acute phase proteins solely on the behavior of their plasma levels, while easy and convenient, is thus superficial and clearly misses important patterns of metabolic regulation of plasma proteins.

Among the acute phase proteins the increases vary between one- and twofold, for example, for some complement components, through up to five- to tenfold, for example for some clotting factors and proteinase inhibitors, and up to 1000-fold or more for CRP and SAA. CRP and SAA are trace constituents of normal plasma and are the most dramatic acute phase reactants increasing very rapidly to peak levels which may be up to 3000 times normal at about 48 h after an acute event. Persistently high levels may occur in chronic active disease processes, but with effective therapy or spontaneous resolution they fall to normal with a half-time as fast as 24–30 h. In contrast, all other acute phase proteins respond more slowly, taking days to reach their peak values, and also falling much more slowly, reflecting clearance and catabolic half-lives of days rather than hours. Furthermore, unlike most other acute phase proteins, there seems to be little effect of disease processes on the clearance or catabolism of CRP and SAA. The synthesis rate, which reflects the intensity of the pathology which induced their production, is thus the most important or even sole determinant of their plasma levels. For all these reasons these two acute phase proteins are the most useful for clinical purposes in humans.

Acute phase proteins are structurally extremely diverse. Most are glycoproteins produced predomi-

antly, though not exclusively, by hepatocytes. The extent and heterogeneity of glycosylation is often significantly increased during the acute phase response, apparently under the influence of the stimulatory cytokines. However, CRP and SAA are not glycosylated. SAA, unlike all the other acute phase proteins, is an apolipoprotein associated with high density lipoprotein, and unlike CRP, which shows no polymorphism, SAA is highly polymorphic. There are several sets of genes for SAA, including some which are predominantly expressed in tissues other than liver and the products of which are not significantly present in the plasma. There are also differences in the responses of different SAA genes to different acute phase stimuli. The importance of this diversity is not clear since the normal function of SAA, and its role in acute phase situations, are not known. However, SAA is the precursor of AA protein which forms the fibrils in reactive systemic, AA-type amyloidosis, a serious and usually fatal complication of chronic persistent infections and inflammatory disorders.

While the profile of acute phase plasma proteins is broadly similar across species there are nonetheless important differences. For example, SAP is a major acute phase reactant only in the mouse, and there are many other differences in normal levels and acute phase behavior of other members of the pentraxin family of proteins to which it belongs. While these differences may be important for the usefulness of particular proteins as markers in clinical or experimental situations, they may not reflect, as has been pointed out above, the underlying metabolic regulation. On the other hand, some species differences are clearly of physiological and pathophysiological importance. Thus, although rats have a gene for a homolog of SAA, the expression of which is regulated as an acute phase protein, the product does not appear as a plasma lipoprotein and rats never get AA amyloidosis. This contrasts with the behavior of SAA in all other mammals and birds which have been studied.

Finally, even within a species there is diversity in the acute phase response to different stimuli, to different disease processes and between different individuals. This has been most extensively studied in the case of CRP and SAA responses in humans, in whom there is a small number of serious inflammatory or tissue-damaging disorders characterized by absent or minimal acute phase production of these proteins. These exceptional disorders include systemic lupus erythematosus, dermatomyositis, Sjögren's disease, scleroderma, ulcerative colitis and leukemia, in all of which only a minority of patients have more than modest elevations of CRP or SAA concentration,

even in the face of extensive and active disease. Other acute phase proteins are produced, apparently normally, and if these patients acquire a significant intercurrent infection they then mount major acute phase responses of CRP and SAA. These phenomena are of considerable clinical value but the underlying mechanisms, whether of macrophage response, cytokine production or hepatocyte response are not known. In the great majority of other diseases in which CRP and SAA levels are greatly increased, the levels attained closely reflect the extent and activity of the pathological process in each individual, and serial monitoring provides valuable objective and quantitative information about the natural history of the disorder or its response to treatment. However, while there is usually a good general correlation between disease activity and CRP/SAA concentration there may nonetheless be considerable variation between the acute phase protein levels seen in different individuals with apparently comparable degrees of clinical activity. These variations are due partly to difficulties in clinical assessment of disease activity but mostly to genuine interindividual differences in the acute phase response.

Biological and clinical importance of acute phase proteins

Acute phase proteins and the response in general are stably conserved in evolution and are universal within each species. They are thus presumably of benefit to the organism undergoing infection, inflammation and/or tissue damage, although much remains to be learned of the properties and functions of many acute phase reactants. On the other hand, sustained, increased production of SAA is a necessary though not sufficient condition for the development of reactive, systemic AA amyloidosis, a grave complication of chronic infection or inflammation. Monitoring of the acute phase response, particularly by measurement of CRP in man, CRP or SAA in other species, and SAP in the mouse, is a powerful and

sensitive test for organic disease or the toxicity of an administered agent. It also provides the most accessible means for objective, quantitative assessment of disease extent and activity, and is valuable in the differential diagnosis of intercurrent infection especially in the immunocompromised host.

The application of new high sensitivity immunoassays for CRP in studies of atherosclerosis has lately revealed very important prognostic associations between previously undetected low grade acute phase responses and the progression and complications, especially of coronary heart disease. These findings open up a new area of considerable fundamental interest and clinical significance, relating mild chronic inflammation to the most common cause of death in the developed world.

See also: Acute inflammatory reaction; Amyloid; Clotting system; C-reactive protein; Cytokines; Plasma; Rheumatoid arthritis, human.

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ADENOVIRUS, INFECTION AND IMMUNITY

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Adenoviruses (Ad) are highly species-specific icosahedral DNA viruses. Multiple Ad serotypes cause a variety of clinical syndromes in humans including respiratory, genitourinary, gastrointestinal and conjunctival infections. Ads are relatively resistant to conventional antiviral chemotherapy, though a highly effective oral Ad vaccine has been used to prevent acute respiratory disease (ARD) in the military population. The human immune response to Ad infection is characterized by the development of serotype-specific neutralizing antibodies to Ad structural proteins which prevent reinfection with the same serotype. In addition, Ad genomes are characterized by four transcription groups that lead to the production of early (E) nonstructural proteins which exert important immunologic effects. The E3 region is not essential for viral replication and can be replaced with foreign DNA. Ad vectors for the delivery of foreign genes have been developed for immunization against non-Ad infections and for gene therapy. Achieving the long-term expression of foreign Ad-inserted genes and abrogating host immune responses that lead to rejection of delivered genes are active areas of research.

Adenoviruses were first isolated by Rowe and Hartley in 1953 when cytopathology was noted serendipitously in cultures of human tissue removed during tonsillectomies and adenoidectomies. Although the first Ad isolates were from patients without acute infectious symptoms, it was recognized subsequently that Ads were associated with a group of respiratory and ocular syndromes. Some of these diseases, such as conjunctivitis, had been known as distinct clinical entities for almost a century before the first isolation of Ads. The number of unique Ad serotypes proliferated and now there are at least 49 distinct types that are currently recognized by neutralization reactions. The Ads are classified into six subgroups (A–F) based on criteria which include the patterns of hemagglutination (HA) of a variety of species of red blood cells. Cross-hybridization of the Ad DNA genomes is greater than 50% for agents within a single sub-group. The discovery by Trentin in 1962, that Ad 12 was highly oncogenic in hamsters, began an era of excitement and uncertainty as attempts were made to link Ads and other DNA viruses with human tumors. All these efforts failed to reveal any reproducible association between

Ads and human malignancy. However, molecular biologic studies of the mechanism of Ad transformation have provided a paradigm for understanding virus-mediated transformation in general.

As a parallel development to the studies of Ad oncogenesis in model systems, investigators were exploring the use of Ad vaccines to prevent acute respiratory disease (ARD) in military recruits as they entered a new epidemiologic environment (see below). The first batches of inactivated Ad vaccine were contaminated with live simian virus-40 (SV40), an unknown virus at the time. The helper effect of SV40 on Ad growth in monkey cells was discovered during these studies.

Ads cause upper respiratory tract infections (URI) and pneumonia. Serotypes 8, 19 and 37 are associated with severe conjunctivitis. Ad infections of the urinary tract due to types 11 and 21 may produce hemorrhagic cystitis, especially in males. Ads 40 and 41, which induce gastroenteritis, are more difficult to grow in conventional tissue culture systems such as human embryonic kidney (HEK) and require special cells such as HEK 293 transformed by the Ad5 early region E1 for propagation. Other Ad serotypes cultivatable from enteric sources have not been reproducibly associated with diarrhea even though they may be persistently shed.

Characteristics of the organism and its antigens

The Ads of different serotypes have an icosahedral architecture, a double-stranded linear DNA, and more than 12 structural proteins, three of which are major components of the capsid (**Figure 1**). There is no virus envelope; 240 hexons, each a trimer of identical polypeptides, are the major subunits of the 252 capsomere shell with 12 different structures called pentons at each of the vertices. The penton is composed of five penton base and three fiber polypeptides which project out from the base in an antenna-like structure. These polypeptides are antigens for host immunologic responses to natural and vaccine-associated infection. The internal structural proteins are divided into those associated with the DNA in a core-like structure and others that bind to the inner surfaces of the hexon or penton capsomeres. None of the internal proteins appear to be involved in humoral or cell-mediated immunity.

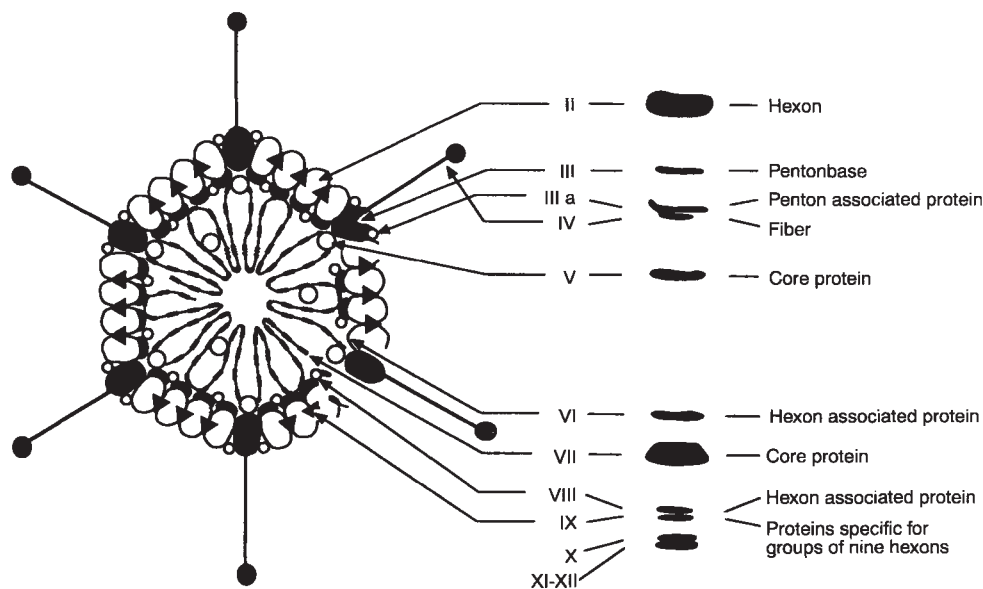


Figure 1 Schematic representation of an adenovirus. The mobilities and relative amounts of each protein after electrophoresis of the dissociated virus on a sodium dodecyl sulfate-containing polyacrylamide gel are shown on the right. The position of each polypeptide in the virion is designated (modified from Horwitz, M.S. (1990) *Adenoviruses and their Replication in Fields' Virology*, 2nd edition, pp. 1679–1721, Raven Press, edited by Fields, et al.

Several nonstructural proteins that are made early after infection have immunologic significance. These early proteins are the products of four transcription groups: the E1A, E1B, E3 and E4 genes have all been postulated to exert important immunologic effects. Both the E1A and E3 regions affect the expression of class I major histocompatibility complex (MHC)-encoded proteins on the cell surface. In model systems *in vivo* and *in vitro*, the E1A gene of Ad12 (which together with the E1B gene is responsible for transformation by the oncogenic Ads), has been shown to inhibit transcription of the class I MHC. This mechanism appears to be limited to the highly oncogenic Ads. In most of the other Ad serotypes that have been studied, a 19 kDa glycoprotein (E3-19 kDa) product of the E3 region has been demonstrated to bind tightly to the class I MHC heavy chain in the endoplasmic reticulum and prevent transport of this complex to the cell surface. Thus, the Ads have evolved two mechanisms to regulate cell surface MHC. The E3 region, which contains 10% of the Ad-coding sequence, includes other genes whose products may have immunologic significance. There is a 14.7 kDa protein and a heterodimer of 10.4/14.5 kDa that inhibit tumor necrosis factor (TNF) lysis of Ad-infected cells. The 14.7 kDa protein does not inhibit TNF by interacting with the ligand or its receptor: it exerts its effect intracellularly by inhibiting the cell death pathway. The details of this process are unknown, though 14.7 kDa inhibits TNF-induced release of arachidonic acid.

Four 14.7 kDa interacting intracellular proteins have been isolated recently; one of them, FIP-1, is a small GTPase with homology to members of the ras family. The 10.4 kDa protein also promotes the internalization of both the epidermal growth factor (EGF) and insulin receptors by a mechanism that is currently unknown. The biological importance of these proteins *in vivo* is under active investigation. For example, the entire cassette of E3 proteins has been expressed as a transgene in mice behind the rat insulin promoter. The E3-transgenic islets can evade the immune system in both allogeneic transplantation and autoimmune diabetes models. Recently, it has been shown that the Ad E3 that is overexpressed in rats by replication-defective Ad vectors used for gene therapy can suppress the humoral immune response to the virus. The E3 genes responsible for this effect and their mechanism of immunomodulation are under investigation.

Another nonstructural gene, VA-1, produces a transcript using RNA polymerase III. This transcript is not known to code for any protein, but the VA-1 can inhibit the action of interferon-induced phosphorylation and allow protein synthesis to continue in Ad-infected cells.

The Ad structural proteins – hexon, fiber and penton – are the antigenic determinants for both group- and type-specific antibodies. The fiber is the major attachment protein but entry is facilitated by the penton base protein. Adenovirus types 2 and 5 fibers bind through the terminal knob to a common recep-

tor shared with the coxsackie B viruses, but have also been reported to bind to class I MHC molecules. The penton base binds to both the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. The α determinant demonstrable on hexon is a cross-reacting group antigen found on most of the known human and animal Ads. The ϵ determinant of hexon and the γ determinant of fiber both give rise to type-specific neutralization (Nt) antibodies. Some of these determinants have now been mapped to specific nucleic acid sequences. Classically, type-specific antibodies have been detectable by hemagglutination inhibition (HAI), and Nt assays. Ad neutralization is serotype specific and generally demonstrable only with homologous antisera. However, some heterologous sera are capable of neutralizing virion at low pH (pH 5.5). Neutralizing antibodies recognizing the Ad hexon of serotypes 1–5 and the Ad fiber of types 1, 2 and 5 have been extensively studied in animal models. Antibodies against fiber can induce virion aggregation though virus attachment to host cells is facilitated by this mechanism. However, such aggregated Ads do not uncoat or enter the cytoplasm for eventual transport along microtubules to the nucleus. Although neutralization parallels aggregation, another mechanism for virus neutralization apparently occurs by antibody-mediated inhibition of viral entry from acidic vesicles into the cytoplasmic compartment. A similar mechanism has also been described for antibodies to penton base which also appear to inhibit by a mechanism independent of virus aggregation. Complement fixation or fluorescent antibody (FA) tests generally detect the presence of group-specific antibody, whereas HAI and Nt assays determine the serotype and the existence of serotype-specific immunity. In addition, type-specific monoclonal antibodies against Ads 40 and 41 can be used with FA, latex agglutination or ELISA assays to distinguish these enteric serotypes from others that are shed in the gastrointestinal tract as a component of Ad infection of the respiratory tract. Immunologic assays and other techniques such as nucleic acid hybridization, quantitative electron microscopy, direct Ad genomic DNA restriction endonuclease profiles of nucleic acid in stool specimens, and polymerase chain reaction (PCR) with selected serotype-specific primers are facilitating the diagnosis of Ad-related gastrointestinal and other clinical syndromes.

Immune responses of the host

Adenovirus infections are followed by serotype-specific immunity which is most likely mediated by serum-neutralizing antibodies. These antibodies protect against reinfection with the same serotype,

though asymptomatic viral shedding from tonsillar epithelium, lymphocytes and enteric epithelium may occur for many months in the presence of neutralizing antibodies. The role of cell-mediated immunity in recovery from acute infection and prevention of reinfection is not understood. Proteins encoded in the E1A or E1B regions are major targets for both cytotoxic T lymphocytes (CTLs) and for natural killer (NK) cell responses. CTLs have been induced in mice inoculated with human Ads.

Animal models of human Ad infection are imperfect because these viruses are highly species specific. In most species, human Ad infections result in abortive infections in which early viral genes are expressed. However, adenovirus infection of the cotton rat produces progeny virus and causes an inflammatory response in the lungs after intranasal infection. The effect of the E3 genes has been studied after intranasal infection of both cotton rats and mice with adenovirus E3-deletion mutants. Deletion of three anti-TNF genes (E3 14.7 kDa, 10.4 kDa and 14.5 kDa) resulted in worsening of pulmonary pathology as evidenced by the conversion of a mononuclear to a polymorphonuclear response in the peribronchial area of the cotton rat lung and the increase of alveolar inflammation in the mouse lung. The pulmonary response to deletion of gp19kDa was discrepant in that the inflammatory response increased in the cotton rat but was unchanged in the mouse.

Adenovirus infections are not as common as infections with members of the herpesvirus group in immunocompromised hosts. However, patients with various malignancies or following cytotoxic therapy have had more severe and more frequent episodes of Ad disease. Liver transplant patients in the pediatric age group have had fatal infection due to the ubiquitous Ad5 serotype. Patients with the acquired immune deficiency syndrome (AIDS) have had persistent infections with various Ad serotypes. Group B Ads 34 and 35 have been isolated chronically from the urine of many AIDS patients. Group D agents have been persistent in the stools of other AIDS patients studied. Both of these patient groups appear to be asymptomatic from their genitourinary or gastrointestinal tract Ad infections. Adult AIDS patients infected with Ads 34 and 35 do not make serum antibodies against these viruses though most of these patients had antibodies against Ad2, a serotype that they most likely acquired before HIV infection. The target of the major neutralizing antibodies against Ad35, the fiber gene, manifested changes from the prototype in many Ad35 isolates from AIDS patients. There appeared to be intermolecular recombination between genomes that were primarily Ad34 or 35 by restriction endonuclease analysis, but

that had the fibers characteristic of other group B Ads such as Ad 3, 7, 11, 16 and 21. Recombination between serotypes within the Ad subgroups is known to occur both in tissue culture and *in vivo*, though there was no evidence that the intermolecular changes, thought to occur by recombination, had arisen during chronic carriage of these Ads in any of the patients studied. This conclusion was reached by examining serial isolates over many months and finding that the serologic reactivity as well as the restriction endonuclease patterns of each Ad isolate had not changed during this period of observation. Thus, the frequency of these important antigenic changes is uncommon in each chronically infected individual.

Vaccines

Historically, the development of Ad vaccines provided new insights into important issues regarding the determination of the safety and efficacy of live viruses for disease prophylaxis. The development of an Ad vaccine became a major priority in the early 1960s after the realization that Ads were responsible for most of the ARDs in up to 80% of American military recruits (who often required hospitalization). Types 3, 4, 7, 14, and 21 Ads caused most of the disease in military recruits and trials of the first vaccines took place between 1958 and 1962. The initial vaccines consisted of inactivated whole-virus strains. However, besides not being completely effective, it was discovered that the seed stocks of vaccine virus were contaminated with fully viable SV40 as noted above. SV40 actually functioned as a helper virus that was essential for the replication of both Ad4 and Ad7. It was realized then that Ad grew in monkey kidney tissue culture only because many of these cells were contaminated *in vivo* by SV40 which produced a T antigen that complemented the Ad replication defect in cells of simian origin. Attempts to rid the Ad seed stocks of SV40 by the use of antibodies against the latter virus were responsible for promoting the intermolecular recombination of portions of the SV40 T antigen gene with the Ad genome. The resulting Ad-SV40 hybrid viruses were shown to be more oncogenic than Ad alone in various rodent species, though extensive studies have failed to show any oncogenic effect of these hybrid viruses in humans inadvertently inoculated with them during the early field trials of Ad vaccines. With increased knowledge of the biology of Ads, a live vaccine free of SV40 genes was formulated against Ads 4 and 7 for use in the military. Despite the anticipation that other respiratory

Ad strains would need to be included as vaccination programs proceeded, this has not been the case.

The adenovirus vaccine currently in use is an enteric coated tablet that protects the host from respiratory infection. It contains Ad 4 and 7 strains adapted for growth in human embryonic fibroblast (W1-38) tissue culture. Vaccine amounts of greater than 10^4 tissue culture infection dose₅₀ (TCID₅₀) in a single administration protect at least 95% of vaccinated recruits. Some individuals develop neutralizing antibodies without evidence of infection, although the vaccine infection rate is greater than 90% as measured by fecal excretion of vaccine Ad strains. In contrast to natural infection, Ad vaccine-induced neutralizing antibodies are not secretory IgA. Nonetheless, the vaccine is highly protective. It must be given within 2 h of the recruit's arrival at the training base because vaccine-mediated protection does not occur during the incubation period of wild-type Ad infection. The adenovirus vaccine strains are safe and are not oncogenic: since the introduction of the vaccine in 1965 it has been administered to over 10 million people.

Ad infections in children cause 5–10% of all pediatric respiratory infections. Since children are infected with other serotypes such as Ads 1, 2 and 5, it was anticipated that the adult vaccines would be reformulated to include these serotypes. However, Ad vaccines for civilians and children are not presently available. Ad vaccination of children has lagged behind the successful efforts to protect the military population because of some uncertainties about the initial safety of the vaccines, and the present enteric formulation which requires swallowing the vaccine tablet intact.

Adenoviruses as vectors for the delivery of foreign genes

Ads have been developed as vectors for the delivery of foreign antigens for immunization against other (non-Ad) infectious agents and for gene therapy. Ads are good vectors for the delivery of foreign DNA in that viral replication is not dependent upon host cell replication, they can be molecularly engineered to enter host cells and express foreign proteins without infectious viral progeny, and they are extremely safe immunogens that have already been administered to many people. For gene delivery, the E3 region, described above as a cluster of viral genes potentially controlling pathogenesis, has been physically or functionally deleted. Experience with Ad/SV40, described above, paved the way for these constructs by demonstrating that the Ad E3 region is not essential for viral replication. The E3 region can be

replaced with foreign DNA: insertion of the hepatitis B surface antigen (HBsAg) gene in place of the partially deleted Ad E3 results in the expression of HBsAg which elicited an antibody response in hamsters. Recently, many additional foreign genes have been cloned into Ad vectors. Foreign genes have also been placed and expressed in other Ad sites including positions downstream from the Ad major late promoter (MLP). The Ad MLP is a very active transcriptional start site which results in the expression of large amounts of foreign proteins. To elicit higher titers against a given antigen, duplicate cloning of the gene into several serotypes is performed, permitting booster immunizations without augmentation of a serotype-specific anti-Ad-neutralizing antibody response. Since there has been extensive use of the Ad4 and 7 serotypes in young adults in the military, these types are obvious choices for such recombinant vaccines. The efficacy of Ad constructs designed for immunization is largely dependent upon viral replication and expression of foreign proteins.

Ad constructs designed for gene therapy are replication defective. Foreign genes are inserted into deleted E1A and E1B regions. Growth of these vectors is dependent upon a cell line that is stably transfected with the E1A and E1B sequences. The viruses are infection competent and enter host cells where they efficiently transcribe inserted sequences without the production of significant progeny virus. Human trials have been attempted to test Ad constructs containing the cystic fibrosis gene, one of the numerous inserts placed into Ad vectors to date. Genes that can replace congenitally absent gene products and genes that can control cell growth or promote cell death of malignant cells have been engineered into Ads for therapeutic purposes. The p53 gene and the herpes simplex thymidine kinase gene (used with ganciclovir) are examples of this approach. Adenoviruses that have cell death genes such as Fas are also being used to control the overgrowth of vascular wall myocytes and endothelium after angioplasty. In many of these situations, there is a need for prolonged expression of the transgene or the requirement to re-administer the vector. However, the immune response to the initial injection of Ad, or even preexisting immunity from natural Ad infection, may preclude this possibility. A number of approaches to solve this problem have been undertaken in model systems, including short-term immunosuppression with FK506 or cyclosporine at the time of vector administration; oral or intrathymic tolerance with viral proteins; or using the immunosuppressive properties of overexpressed Ad E3. Inhibition of the antibody response to replication-deficient Ads containing overexpressed E3 genes has

allowed readministration of an Ad vector containing a functional bilirubin conjugating enzyme for correction of an enzymatic defect in rat liver. Though the expression of Ad-inserted genes is prolonged in some tissues, many problems remain to be solved to ensure the success of Ad vectors as delivery systems for gene therapy; achieving long-term expression of foreign Ad-inserted genes in the desired tissue(s) and circumventing host immune responses that ultimately lead to rejection of delivered genes are significant issues that must be resolved. This very active area of research is likely to lead to significant advances in the near future.

Therapy

Ads have remained rather resistant to antiviral chemotherapy. Although Ads code for their own viral DNA polymerase, this enzyme has not been hitherto exploited as a target for chemotherapy. The nucleoside analogs effective in the treatment of herpes simplex virus have not been effective in controlling Ad disease.

See also: Acquired immune deficiency syndrome (AIDS); Adjuvants; Eye infections; Vaccines; Viruses, immunity to.

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ADHESION MOLECULES

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In 1922, biologist Warren Lewis wrote, if 'cells (were) to lose their stickiness for one another and for the supporting extracellular white fibers, reticuli, etc., our bodies would at once disintegrate and flow off onto the ground. . . . The so-called cement, so commonly described as existing between various types of cells, may possibly be an adhesive substance'. Over 75 years later, we now know that this necessity for cells to adhere to their surroundings or to their cellular neighbors is reflected in the vast array of molecules identified that mediate cell adhesion. In the immune system, adhesion molecules are essential to many, if not all, aspects of leukocyte function, including normal recirculation of lymphocytes through lymphoid organs, recruitment of leukocytes into inflammatory sites, antigen-specific recognition, and wound healing. Defects in adhesion molecule expression can result in immunodeficiency, and the central role of adhesion molecules in the immune system suggests that adhesion can be used as a therapeutic strategy for intentional modulation of the immune response.

Five main structural families of adhesion molecules have been identified: integrins, immunoglobulin superfamily (IgSF) proteins, selectins, mucins and cadherins (Table 1). This discussion will focus on an introduction to the structure and function of these adhesion molecules, followed by emerging concepts of how the distinct functions of these many

adhesive proteins are integrated to mediate key immunological processes.

Integrins

The integrin family of adhesion molecules is composed of structurally related proteins that combine to form noncovalent $\alpha\beta$ heterodimers. Currently 16 α subunits and eight β subunits have been cloned, resulting in a minimum of 22 different combinations. The VLA (very late antigen) integrins, leukocyte integrins, and cytoadhesins represent integrin subfamilies based on the pairing of α subunits with the β_1 , β_2 or β_3 subunits, respectively. However, the identification of five other β subunits, together with the ability of certain α subunits to associate with more than one β subunit, blurs the distinctions between these integrin subfamilies. Integrin ligands include extracellular matrix (ECM) proteins, as well as cell surface proteins. Many of the integrin cell surface counter-receptors are members of the immunoglobulin (Ig) superfamily (see below). In addition, some bacteria and viruses use integrins to bind to host tissue. Ligand binding specificity is contributed by both α and β subunits. The ligand binding regions of integrins are invariably located at cation-binding sites, and consequently integrin-mediated adhesion is divalent cation dependent.

Table 1 Adhesion molecules in immunology

Receptor	Alternate name	Cell type	Extracellular matrix ligands	Cell surface/ other ligands
1. Integrins				
$\alpha_1\beta_1$	VLA-1	T _a , Mono, NK	COLL, LAM	
$\alpha_2\beta_1$	VLA-2	B, T, PI, Endo	COLL, LAM	Echovirus
$\alpha_3\beta_1$	VLA-3	B	COLL, LAM, FN, Epiligrin, Entactin/Nidogen	Invasin
$\alpha_4\beta_1$	VLA-4; LPAM-2	Thym, Mono, B, T, NK Eosin, Eryth _{imm}	FN, Thrombospondin	VCAM-1, Invasin
$\alpha_5\beta_1$	VLA-5; FnR	Mono, Leuko, T, PI, NK	FN, Thrombospondin	Invasin, L1
$\alpha_6\beta_1$	VLA-6	PI, Mono, T, Thym, NK	LAM	Invasin
$\alpha_4\beta_7$	LPAM-1, $\alpha_4\beta_p$	Some T, NK, Eosin, B	FN	VCAM-1, MAdCAM-1
$\alpha_E\beta_7$	HML-1	Intraepithelial lympho		E-Cadherin
$\alpha_L\beta_2$	LFA-1, CD11a/CD18	Lympho, Neutro, Mono, Macro, NK		ICAM-1, ICAM-2, ICAM-3
$\alpha_M\beta_2$	Mac-1, CD11b/CD18	Mono, Macro, Granulo, NK	FG	ICAM-1, ICAM-2, C3bi, Heparin, <i>B. pertussis</i> hemagglutinin, Haptoglobulin
$\alpha_X\beta_2$	p150/95, CD11c/CD18	Macro, Mono, Granulo, T _a , B _a , NK, Dendr	FG	C3bi, Endothelial ligand?, ICAM-1 (rabbit)
$\alpha_D\beta_2$		Some Macro and CD8 ⁻ T, Large granular lympho		ICAM-3, ICAM-1
$\alpha_V\beta_3$	VN-R	Endo, some B, Macro, Mono, PI	VN, FN, FG, COLL, VWF, Thrombospondin, Osteopontin	CD31, L1
$\alpha_{IIb}\beta_3$	gplIb/IIIa	PI, Megakar	VN, FG, VWF, FN, Thrombospondin	
2. Ig superfamily				
ICAM-1	CD54	Mono, Endo, *(B, T, Thym, Dendr, Endo, Keratino, epithel)		$\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$
ICAM-2	CD102	Endo, Mono, Dendr		$\alpha_L\beta_2$, $\alpha_M\beta_2$
ICAM-3	CD50	Leuko, Dendr		$\alpha_L\beta_2$, $\alpha_D\beta_2$
MAdCAM-1		Peyer's patch HEV, Mesenteric LN HEV, Mucosal lamina propria venules		$\alpha_4\beta_7$, L-selectin
VCAM-1	CD106	Endo _a , tissue Macro, Dendr		$\alpha_4\beta_1$, $\alpha_4\beta_7$
CD31	PECAM-1	PI, Endo, Mono, Granulo, some T		$\alpha_V\beta_3$, CD31
CD2	LFA-2, Leu-5	Thym, T, NK		LFA-3 (CD58), CD48, CD59
3. Selectins				
L-Selectin	CD62L, LECAM1 Leu-8, LAM-1	Most B and T, Granulo, NK Thym _{imm} , some mature Thym, Mono, bone marrow myeloid progen, eryth precursors, Eosin		GlyCAM-1, MAdCAM-1, CD34, Sgp200
E-Selectin	CD62E, ELAM-1, LECAM-2	endo _a		PSGL-1, CLA, ESL-1
P-Selectin	CD62P, GMP-140, PADGEM, LECAM-3	Endo _a , PI, Megakar		PSGL-1

Table 1 Continued

Receptor	Alternate name	Cell type	Extracellular matrix ligands	Cell surface/ other ligands
4. Mucins				
GlyCAM-1		PLN HEV, hemato progen		L-selectin
CD34		Wide intravascular expression		L-selectin
MAdCAM-1		See above (Ig superfamily)		$\alpha_4\beta_7$, L-selectin
5. Cadherins				
E-cadherin	Arc-1, L-CAM, Cell CAM 120/80, uvumorulin	Epithel		E-cadherin, $\alpha_E\beta_7$, internalin
6. Other adhesion molecules				
CD44	Hermes, Pgp-1, H-CAM, ECMR III	Wide expression	COLL, osteopontin	HA
VAP-1		HEV, PLN HEV, Synovium, tonsils		?
LVAP-2	CD73	HUVEC, B, CD8 ⁺ T, Lymphoid and nonlymphoid venules		?
uPAR		Mono, Neutro, T _a , Endo	VN	uPA

Ligands: FN, fibronectin; VN, vitronectin; COLL, collagen; HA, hyaluronic acid; LAM, laminin; FG, fibrinogen; VWF, von Willibrand factor. *Cell types:* Pl, platelet; T_a, activated T cell; Endo, endothelial cell; Mono, monocyte; Thym, thymocyte; PBL, peripheral blood leukocytes; NK, natural killer cells; Eosin, eosinophils; Eryth_{imm}, immature erythroid cells; Leuko, leukocytes; T_m, memory T cells; Epithel, epithelial cells; Lympho, lymphocytes; Neutro, neutrophils; Macro, macrophages; Granulo, granulocytes; Dendr, dendritic cells; Megakar, megakaryocytes; endo_a, activated endothelial cells; HEV, high endothelial venules; LN, lymph nodes; HUVEC, human umbilical vein endothelial cells; *, inducible expression.

The cytoplasmic domains of both the α and β subunits are critical to regulating the ability of integrins to mediate cell adhesion, as well as for transmitting information into the cell interior. Furthermore, recent studies have identified a number of intracellular proteins that associate with or are functionally coupled to integrins via these cytoplasmic tails. Additional complexity in the integrin subfamily is likely, given recent findings that variant forms of the β_1 and β_3 subunits exist that have different cytoplasmic tails as a result of alternative splicing.

Immunoglobulin (Ig) superfamily

Cell surface proteins of the Ig superfamily are characterized by the presence of a variable number of related 70–110 amino acid Ig-like domains originally described in the Ig variable and constant regions. Several IgSF members have been implicated in cell adhesion. CD2 has been shown to bind several distinct ligands, including the IgSF members CD58, CD48 and CD59. CD31, which can mediate homophilic adhesion, has also been implicated in binding to the $\alpha_v\beta_3$ -integrin. The integrin counter-receptors ICAM-1, ICAM-2, ICAM-3, VCAM-1 and MAdCAM-1 (Table 1) round out the list of IgSF adhesion molecules described to date. MAdCAM-1 is a unique IgSF member in that it contains mucin-like properties in addition to Ig domains (see below).

Selectins

Selectins represent a family of three structurally and functionally related adhesion molecules: E-selectin, P-selectin and L-selectin. Each selectin consists of an extracellular lectin-like domain, an EGF-like domain, and variable numbers of a consensus repeat bearing homology to complement proteins, followed by transmembrane and short intracellular sequences. The lectin-like domains are primarily responsible for Ca^{2+} -dependent interactions with fucosylated, mucin-like ligands. Functionally, the rapid association and dissociation rate constants that characterize selectin-mediated adhesion allow selectins to function as important initiators of leukocyte adhesion to endothelium under conditions of shear flow. A role for selectins as signal transducing receptors is also now becoming evident.

Mucins

Mucins are characterized by their extensive and dense array of carbohydrates. The carbohydrate linkages are primarily O-linked with sulfated core groups, termed sialyl Lewis x (sLe^x). The mucins characterized to date are primarily selectin counter-receptors. L-Selectin binds to at least four distinct mucins: GlyCAM-1, MAdCAM-1, CD34, and a poorly characterized protein called Sgp200. As men-

tioned previously, MAdCAM-1 has both Ig domains and a mucin domain, allowing it to bind to both an integrin ($\alpha_4\beta_7$) and a selectin (L-selectin). GlyCAM-1 is somewhat unique in that it lacks a transmembrane domain. PSGL-1 binds both E- and P-selectin, although additional ligands have been described for each of these selectins. The E-selectin ligand ESL-1 appears to require N-linked carbohydrate structures rather than the typical O-linked sialomucin structures. The CLA antigen also binds to E-selectin and is expressed on a subset of memory T cells that preferentially migrate to the skin. The protein backbones of mucins are also postulated to play a role in binding specificity.

Cadherins

Cadherins have been implicated in Ca^{2+} -dependent homotypic aggregation of nonlymphoid cells and play an important structural role in cell-cell junctions. Although there are several different cadherins, to date only E-cadherin has been implicated in regulating immunological function. Intraepithelial lymphocytes express the $\alpha_E\beta_7$ -integrin, which binds to E-cadherin expressed on epithelial cells. This adhesive interaction has been proposed to play a role in retaining $\alpha_E\beta_7$ -expressing T cells at specific tissue sites *in vivo*. E-Cadherin is also the cell surface receptor for the *Listeria monocytogenes* protein internalin.

Other adhesion molecules

Several molecules that are not easily categorized into these adhesion molecule families are listed in Table 1 and deserve mention here. VAP-1 is an adhesion molecule that is expressed on endothelium derived from peripheral lymph nodes, synovium and tonsils, and may play a role in tissue-specific migration of lymphocytes to synovial tissue. LVAP-2 is a putative endothelial adhesion molecule for lymphocytes and has been found on human umbilical vein endothelium (HUVEC), lymphoid and nonlymphoid venules, B cells, and CD8^+ T cells. The urokinase plasminogen activator receptor (uPAR) has been shown to bind vitronectin, as well as regulate the binding activity of β_1 - and β_2 -integrins (see below). CD44 is a widely distributed molecule that is related to cartilage link proteins and proteoglycan core protein. The CD44 transcript can undergo extensive alternative splicing, generating many different protein isoforms. Several CD44 ligands have been reported, the most notable being hyaluronic acid. Antibodies to CD44 can inhibit lymphocyte recirculation, and, like selectins, CD44 binding to hyaluronic acid can support

lymphocyte rolling under shear flow. However, the precise function of CD44 in the immune system remains elusive.

Roles of adhesion molecules in regulating immune function

As mentioned previously, the wide array of adhesion molecules and ligand possibilities provides for exquisite specificity as well as diversity in the tasks adhesion molecules perform in the immune system. Some salient points regarding the importance of these molecules in both normal immune regulation and with respect to the disease state are addressed below.

Adhesion molecules and lymphocyte recognition

The process by which an antigen-specific cytotoxic T lymphocyte (CTL) recognizes and kills a target cell has provided a valuable system for elucidating the function of adhesion molecules in the immune response. In the early 1980s, several monoclonal antibodies were identified that inhibited CTL killing. Subsequent analysis revealed that these antibodies recognize the adhesion molecules LFA-1, a β_2 -integrin, CD2 (LFA-2), and its cellular counter-receptor LFA-3 (CD58). Further analysis of CTL killing identified the first LFA-1 counter-receptor, ICAM-1. Antibodies against these molecules are effective in inhibiting CTL function, because they impair the physical contact between the CTL and the target cell that is required for T cell activation to occur. Thus, one function of adhesion molecules during T cell activation is to provide the adhesive force necessary to allow for effective binding of the antigen-specific T cell receptor with major histocompatibility complex (MHC)-peptide complexes on the antigen-presenting cell. In addition, adhesion molecules also provide intracellular signals during T cell activation that further facilitate T cell activation (see below).

Adhesion molecules and leukocyte recruitment and recirculation

Interaction of circulating blood cells with endothelial cells is essential for the recruitment of lymphocytes to sites of injury and inflammation and for normal recirculation of lymphocytes. The successful movement of leukocytes out of the bloodstream through the endothelium and into the underlying tissue requires the coordinated and sequential interaction of adhesion molecules on the leukocyte and the endothelium, as well as the correct temporal expression of activating signals provided by cytokines and chemokines. This process has been defined

generally as the multistep paradigm of leukocyte adhesion to endothelium. The process of adhesion under shear flow is typically initiated by low-affinity binding of leukocytes to activated endothelium, which results in a characteristic rolling or tumbling of leukocytes along the endothelial surface. Although selectins typically mediate rolling, α_4 -integrins can also mediate rolling in the absence of a contribution from selectins. Upon encounter with an appropriate stimulus, rolling can be converted rapidly into stable, shear-resistant attachment of leukocytes to the endothelial surface. This is accomplished by rapid agonist-induced activation of integrins such as $\alpha_4\beta_1$ (VLA-4), $\alpha_4\beta_7$, LFA-1, and Mac-1. A number of stimuli have been proposed to serve as the 'trigger' that activates integrins in this process, including chemokines immobilized on the endothelial surface by proteoglycans and, perhaps, intracellular signals generated by the selectins during rolling. Following tight adhesion to the endothelium, leukocytes migrate through the endothelium into the underlying tissue. Current evidence suggests a role for chemotactic factors, integrins and CD31 in this process. Thus, the successful extravasation of a leukocyte from the bloodstream into tissue requires the coordinated and sequential interaction of different adhesion molecules, each of which serves to carry out distinct phases of the process. Underlying this orchestrated utilization of adhesion molecules is the role of soluble factors, which serve to dictate the spectrum of adhesion molecules expressed on the endothelial surface, as well as to provide the appropriate signals and chemotactic gradients that initiate key steps in this cascade of adhesive events.

Regulation of adhesion molecule expression and function

The adhesive nature of cells is regulated at many levels. The level of expression of adhesion molecules on the cell surface is dependent in large part on the activation status of the cell. Activation of endothelial cells with inflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF α), results within hours in an increase in expression of E-selectin, ICAM-1 and VCAM-1. This cytokine-induced expression of these adhesion molecules is due to transcriptional upregulation of the genes encoding these molecules. P-selectin is also expressed on activated endothelium, as well as on platelets. However, P-selectin is stored in granules, ready to be upregulated within minutes in response to stimulation. Stimulation of neutrophils with agonists such as formyl-Met-Leu-Phe (fMLP) also results in the rapid upregulation of the Mac-1 integrin due to

fusion of intracellular granules containing Mac-1 molecules with the plasma membrane.

Adhesion molecules can also be rapidly lost from the cell surface. The most notable example is L-selectin, which is constitutively expressed on most peripheral blood leukocytes. However, leukocyte activation can result in rapid proteolytic cleavage (shedding) of L-selectin from the cell surface. Although the protease responsible for L-selectin shedding has not been identified, the cleavage of L-selectin from the cell surface has been demonstrated to be important for leukocyte interactions with endothelium. Other adhesion molecules, including CD44, E-selectin, ICAM-1 and VCAM-1, can also be found in soluble form. The functional significance of soluble adhesion molecules is not entirely clear, but changes in the level of expression of soluble adhesion molecules in various disease states have been noted.

Stable changes in the expression of adhesion molecules can also be observed during the process of lymphocyte differentiation. For example, when naive T cells are activated, some of the activated T cells differentiate into memory T cells. These memory T cells can be distinguished from naive T cells by differential expression of several adhesion molecules. Some adhesion molecules, such as the LFA-1 integrin, β_1 -integrins and CD44, show increased expression on memory T cells, while others, such as L-selectin, show reduced expression. These differences in adhesion molecule expression on naive and memory T cells are believed to be responsible in part for the differences in naive and memory T cell recirculation *in vivo*. For example, uniform expression of L-selectin on naive T cells is believed to be critical for the preferential migration of naive T cells into peripheral lymph nodes via adhesion and transmigration through peripheral lymph node high endothelial venules (HEV). Differentiation of thymocytes is also characterized by discrete changes in the expression of various adhesion molecules.

The functional activity of adhesion molecules can also be regulated by cellular activation. Stimulation of leukocytes and platelets results within seconds to minutes in rapid increases in integrin-mediated adhesion that do not require changes in levels of integrins on the cell surface. Such activation of integrins from a latent cell surface form is an essential element of the multistep paradigm of leukocyte interactions with endothelium. Furthermore, the ability of T cell receptor stimulation to activate integrins in such a manner implicates this mode of regulation in enhancing T cell contact with antigen presenting cells. T Cell receptor stimulation has also been reported to increase the adhesiveness of the CD2 molecule. The intracellular mechanisms by which activation rapidly

upregulates the adhesive nature of these molecules remain poorly defined.

Adhesion molecules may also be regulated by other molecules that physically associate with adhesion receptors and consequently alter adhesive activity. Integrin-mediated cell adhesion to fibronectin has been shown to be inhibited by expression of the uPAR, which physically associates with β_1 -integrins as well as the Mac-1 integrin. Studies of four transmembrane-containing (TM-4) proteins (CD9, CD63 and CD81/TAPA-1) have also demonstrated an association of these proteins on the cell surface with integrins, although the functional significance of this association for integrin function remains unclear. These observations suggest that additional membrane localized proteins may be identified in the future with similar roles of enhancing or inhibiting adhesion molecule function through physical association in the plasma membrane.

Signal transduction through adhesion molecules

While the term 'adhesion molecules' highlights the ability of these receptors to serve as the 'glue' that binds cells to each other and to ECM, it is now clear that engagement of adhesion molecules also results in the generation of intracellular signals that alter cell function. Evidence for adhesion molecule-mediated signal transduction is now plentiful. Analysis of integrin-mediated signaling, in particular, has been extensive and highlights the broad array of intracellular signals that adhesion molecules can transduce when appropriately stimulated. In the immune system, initial evidence for integrin-mediated signaling has been obtained by findings that interactions between integrin counter-receptors and ECM ligands can enhance T cell receptor-mediated activation of T cells. More direct evidence for integrin-mediated signaling has developed from biochemical studies of various signal transduction pathways using a large number of cell types. Integrin engagement by ligand or monoclonal antibodies has been shown to result in the activation of early signaling events, such as calcium influx, a rise in intracellular pH, and the activation of a number of intracellular kinases. These include tyrosine kinases, mitogen-activated protein kinases (MAPKs) and lipid kinases. Integrin stimulation also results in the tyrosine phosphorylation of a number of proteins, most notably an intracellular tyrosine kinase, focal adhesion kinase (pp125^{FAK}), the p130^{Cas} adapter protein, and several cytoskeletal proteins. In adherent cells, such as fibroblasts, many of these tyrosine phosphorylated proteins are associated functionally with integrins at focal adhesions,

the point of cell contact with the underlying ECM. A link between integrin-mediated tyrosine phosphorylation of pp125^{FAK} and activation of the Ras signaling pathway has been suggested. Certain transcriptional effects of integrin activation have been noted, including the induced expression of metalloproteinase and transcription factor genes. A critical role for integrins in cell survival has also been documented. However, the functional relationship between early integrin-mediated activation events, such as tyrosine phosphorylation, and later events, such as induction of gene transcription, remains poorly characterized.

Integrins are not the only adhesion molecules that transduce signals upon engagement. Stimulation of certain selectins (L- and E-selectin) and IgSF members (CD2, CD31) can upregulate β_1 - and β_2 -integrin activity. Thus, signals generated by one type of adhesion molecule can alter the functional activity of another. In the case of CD2, a role for CD2 as a signaling molecule was appreciated before any role for the molecule in adhesion was uncovered. Although not as extensively studied, it is now becoming clear that selectins, like integrins, also initiate intracellular signals upon ligand engagement. While typical markers of intracellular cascades, such as Ca²⁺ influx and tyrosine phosphorylation, have been noted following activation of many of these adhesion molecules, notable distinctions also appear to be emerging. Thus, there is likely to be considerable complexity and specificity in adhesion molecule signaling, reflecting the complex but specific nature of receptor-ligand interactions discussed previously and shown in Table 1.

Adhesion molecules and disease

The physiological relevance of adhesion molecule function is elegantly demonstrated by naturally occurring defects in the expression and/or function of adhesion molecules, which consequently leads to disease. The inherited bleeding disorder, Glanzmann's thrombasthenia, is caused by loss of expression and/or function of the $\alpha_{IIb}\beta_3$ -integrin on platelets. Consequently, platelet aggregation, which is necessary to prevent excessive loss of blood during injury, fails to occur. The importance of adhesive functions of the β_2 -integrins has been demonstrated by a syndrome termed leukocyte adhesion deficiency (LAD-1). Patients afflicted with this disease lack expression of the β_2 -integrin subunit, and the adherent function of their macrophages and granulocytes is essentially absent. As a result, affected individuals have severely reduced life expectancies and suffer from recurrent bacterial and fungal infections.

Recently, a syndrome, termed LAD-2, with a similar phenotype to that of LAD-1 has been characterized. LAD-2 results from a defect in fucose metabolism, which impairs the expression of the carbohydrate ligands recognized by selectins. The neutrophils of afflicted individuals fail to bind endothelial cells, further demonstrating the importance of adhesion molecules in normal physiological cell functions.

The *in vivo* relevance of adhesion molecules in the immune response has also been demonstrated experimentally in a variety of ways. Altered expression and/or function of adhesion molecules has been noted in various disease states. The functional relevance of these findings has been demonstrated by *in vivo* blocking studies using antibodies specific for adhesion molecules, soluble adhesion receptors, blocking peptides or carbohydrates, and antisense oligonucleotides. Thus, targeting of adhesion molecules with these reagents has been shown to be effective in many different animal models of human disease, including models of ischemia/reperfusion injury, acute inflammation, allograft rejection during organ transplantation and various chronic inflammatory disorders such as rheumatoid arthritis. In most of these studies, the primary focus has been on blocking or modulating leukocyte or lymphocyte attachment to endothelial cells. Modulation of adhesion molecule expression has also been shown to be effective in inhibiting tumor cell growth and metastasis.

The use of homologous recombination in embryonic stem cells to generate mice deficient in expression of various adhesion molecules has also been utilized to uncover the function of these molecules *in vivo*. Mice lacking expression of several individual integrin α and β subunits have been produced. For the most part, these integrin mutations have been either embryonic or neonatal lethal in phenotype, emphasizing the global importance of integrins in embryonic development. Mice lacking expression of signaling components linked to integrins, such as pp125^{FAK}, also fail to develop normally. Mice lacking expression of the β_7 -integrin subunit are viable, but exhibit defects in development of gut-associated lymphoid tissue, presumably due to the inability of T cells to migrate into this tissue site. L-selectin, E-selectin and P-selectin knockout mice have also been individually generated, confirming the importance of these molecules in leukocyte-endothelial interactions. E-selectin-deficient mice present with little change in phenotype from wild-type. However, mice genetically deficient for both E- and P-selectin show a synergistic reduction in neutrophil and lymphocyte adhesion to endothelium, confirming roles for both P- and E-selectin in this process.

Conclusions

Clearly, the variety of adhesion molecules described to date lends itself well to diverse mechanisms of immune regulation. New adhesion molecules are likely to be discovered, and the mechanisms of adhesion molecule function will continue to be further delineated. Given the breadth of this diversity, it will be crucial to begin a closer scrutiny of the structural and signaling requirements of these molecules. A more precise knowledge of these roles may lead to valuable tools that target adhesion molecules for therapeutic intervention in diseases whose etiology includes aberrations of the immune response.

See also: Carbohydrate antigens; CD2; CD44; Chemokines; Chemotaxis; Chemotaxis of lymphocytes; Chemotaxis of macrophages and monocytes; Chemotaxis of neutrophils; Diapedesis; Endothelium; Extracellular matrix; Fibronectin; High endothelial venules; Immune adherence; Immunodeficiency, animal models; Immunoglobulin gene superfamily; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Knockout, genetic; Lectins; Leukocyte adhesion deficiency; Lewis^x/sialyl-Lewis^x (CD15/CD15s); Lymphocyte function-associated antigen 1 (LFA-1); Lymphocyte function-associated antigen 3 (LFA-3); Lymphocyte trafficking; Proliferation, lymphocyte; Second signals for lymphocyte activation; Selectins (CD62-E/L/P); T lymphocyte activation; *Yersinia*, infection and immunity.

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ADJUVANT ARTHRITIS

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Adjuvant arthritis (AA) is a disease induced in rats by immunization with killed *Mycobacterium tuberculosis* (MT) organisms in oil, a substance known as complete Freund's adjuvant. The work of C.M. Pearson and his colleagues in the 1950s and 1960s helped establish AA as a standard model of chronic inflammation produced by immunological processes. Two opposing theories were put forth to explain the pathogenesis of AA: the first proposed that the joints were damaged as bystanders to an immune response directed to MT antigens disseminated to the sites of inflammation; the second proposed that AA was caused by an autoimmune attack against a cross-reactive self antigen. Despite the uncertainty of its pathogenesis, AA has served for many years as a reliable and convenient model for testing treatments for inflammation.

Induction of AA

AA appears about 2 weeks after immunization of rats to MT. The severity, duration and consequences of the arthritis seem to be influenced by various factors:

1. Genes; major histocompatibility complex (MHC) and other genes contribute to susceptibility. AA is most readily induced in rats and has been studied almost exclusively in this species. Mice, however, were reported to develop AA 2 months after administration of MT.
2. Gender; male rats are more susceptible than are female rats.
3. Microbial environment; germ-free rats and rats

maintained in very clean surroundings are much more susceptible to severe AA than are rats raised in contaminated quarters.

4. Amount of MT; the maximum clinical score of the arthritis is directly related to the amount of MT administered to the rats. About 0.2 mg of MT produces mild AA. Increasing the dose of MT causes more severe arthritis; a maximum clinical score is usually elicited by 1 mg of MT.
5. State of MT inoculum; finely pulverized MT is much more arthritogenic than is whole MT. Merely mixing the MT in oil is more arthritogenic than is emulsifying the MT in oil.
6. Site of immunization; intradermal inoculation is most arthritogenic, intramuscular is least and intraperitoneal is intermediate.

The severity of acute AA in rats peaks about 2 weeks after the disease first appears. The arthritis then slowly regresses and may disappear totally (for example in dirty female rats); but it may also persist for a year or more (for example in clean male rats). Protracted AA can lead to irreversible damage (ankylosis) to the joints of the extremities.

The rats that recover from AA acquire resistance to further attempts to induce AA. Resistance to AA can also be obtained by first administering MT under conditions that are not arthritogenic; for example, MT administered intramuscularly or without oil. Thus, the clinical expression of arthritis appears to result from many seemingly trivial factors impinging on the process of immunization to MT; the particulars of the immunization determine the nature of the response, which in turn determines the patho-

genic consequences. Such complexity implies regulation.

The hsp65 connection

In 1984 it became possible to study the complexities of AA using an etiologic agent of disease, a clone of T cells capable of adoptively transferring AA, called A2b. It was demonstrated that A2b recognized an MT antigen and an antigen in the proteoglycan moiety of joint cartilage. This supported the autoimmune theory: AA was caused by antigen mimicry between MT and a joint antigen.

The MT epitope recognized by clone A2b was later identified as the nine amino acids at positions 180–188 in the sequence of the 65 kDa heat shock protein (hsp65) molecule of MT. This molecule belongs to the hsp60 family and is closely homologous to the mammalian hsp60 molecules. The general importance of hsp65 to arthritis was suggested by the observation that T cells responsive to hsp65 could be detected in the synovial fluids of patients suffering from rheumatoid arthritis. Moreover, some patients with juvenile arthritis showed immunity to the human hsp60 molecule. Thus, immunity to hsp65/hsp60 could be associated with spontaneous forms of immunological arthritis in humans as well as with AA of rats.

Is T cell immunity to hsp65 the cause or only the result of AA? The answer awaits the resolution of four issues.

1. *Identity of the joint antigen.* The 180–188 peptide recognized by clone A2b is in a variable region of the hsp65 molecule of MT; despite an overall homology of 50%, the 180–186 amino acid sequence is not present in the known mammalian hsp60 molecules. Thus the epitope recognized by A2b in the joints is probably not on the endogenous hsp60 molecule but is likely to be present on another molecule. A very weak homology between the 180–188 peptide and a sequence in the link protein of cartilage proteoglycan exists. This is compatible with the earlier observation that clone A2b recognizes an element in cartilage proteoglycan. However, clone A2b does not proliferate in response to the link protein peptide, so some other cross-reactivity is probably responsible for AA. Cross-reactivity has been sought between hsp65 and chondrocyte and proteoglycan antigens, and between hsp65 and lactoferrin, but these studies have failed to shed any light on pathogenesis. AA seems to be distinct immunologically from the arthritis inducible by immunization to collagen type II, yet spleen cells from rats immunized to
2. *Induction of AA.* The fact that clone A2b caused arthritis led to the hypothesis that the target epitope of AA must be present in the hsp65 molecule of MT. This idea would be more tenable if active immunization to hsp65 were to actually induce AA. However, immunization with hsp65 has as yet failed to induce arthritis. Nevertheless, hsp65 must be involved in the process responsible for arthritis because administration of whole hsp65 or the 180–188 nonapeptide to rats induces resistance to AA.
3. *Specificity of hsp65 immunity.* The immune response to hsp65 is not exclusive to arthritis. It appears that inflammatory exudates outside of the joints, pleural effusions for example, are also rich in T cells responsive to hsp65. Moreover, immunization of some strains of mice with hsp65 or hsp60 has been found to induce diabetes mellitus. The epitope of hsp60 associated with diabetes differs from the 180–188 epitope associated with arthritis. Nevertheless, it is clear that hsp65 immunity is not limited to the inflammation of immunological arthritis. In fact, immunity to hsp65 is not necessarily associated with immunopathology; the T cells of healthy humans respond very well to hsp65 and even to self hsp 60. Moreover, hsp65 is a dominant antigen in the immune response to infection or vaccination with *Mycobacteria* or other bacteria.
4. *Regulation of AA.* AA is a manifestation of the inflammatory reaction, so it is not surprising that AA can be suppressed by various anti-inflammatory treatments, particularly treatments that inhibit proinflammatory cytokines, such as tumor necrosis factor α (TNF α), or that block the T-cell receptor. Interestingly, AA can be inhibited by T cells that recognize specific epitopes from the T-cell receptor of an anti-hsp65 T cell. In addition to regulatory T cells, it appears that antibodies may be important in the regulation of AA. AA can be inhibited by normal immunoglobulin (IVIg) and by immunoglobulins from strains of rats resistant to AA. Furthermore, AA is marked by changes in the glyco-

collagen type II could partially inhibit AA. Thus, the immunological connection between MT and AA, and between hsp65 and the joints, remains circumstantial.

sylation of antibodies of the IgG1 and IgG2b isotypes.

The future

Although AA has been a familiar laboratory model for decades, we are only now appreciating the charm of its complexity and the value of the secrets it can reveal about health, autoimmunity and the immunology of the host-parasite interaction. As we might have guessed from the constellation of elements affecting its induction, the development of AA is an outward expression of the subtleties of immune regulation. Health or disease is not the mere outcome of response or nonresponse. The immune system can respond to MT and to the hsp65/hsp60 molecules in many ways and it is the nature of this response that determines health or disease. 'The nature of this response' is a conceptual handle of the black box of immunological regulation. Present research is attempting to open the box to reveal the clockwork of the system.

See also: Adjuvants; Autoimmune disease, induced experimental models; Molecular mimicry; Rheumatoid arthritis, animal models; Stress proteins; T cell vaccination.

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ADJUVANTS

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The word 'adjuvant' is used in many ways in biology and medicine. Most commonly, it is used to refer to compounds that act in a nonspecific manner to augment specific immunity to an antigen. A series of experiments in 1925 by Ramon first indicated that compounds of a vaccine formulation other than antigen itself are important for a successful biological response. Ramon found that the addition of substances as diverse as metal salts, oil, tapioca and pyogenic bacteria to his vaccine preparations increased the antitoxic response to diphtheria and tetanus toxins. These simple but elegant experiments started a search, which is still ongoing, for vaccine adjuvants that are suitable for a wide range of uses.

Older vaccines contained antigen preparations that were frequently impure and poorly characterized. Retained contaminants resulted in toxicity and antigenic competition. In some cases, such as with killed bacterial vaccines, the contaminants themselves were adjuvants. Technical advances in biochemistry and molecular biology have led to the production of increasingly pure antigens in order to induce a specific immune response. Modern antigen preparations are usually very well characterized and are now often synthetic or recombinant constructs. These new preparations have reduced side-effects but in many cases are less immunogenic than older killed or subunit vaccines; therefore, in most cases, adjuvants are needed to increase their potency. Adjuvants are substances capable of stimulating an earlier, stronger or more prolonged response to an antigen. In some systems the antibody or the cell-mediated (CMI) immune response, as well as the antibody class or subclass and/or T helper subset (T_H1 and T_H2), can be selected for by manipulating the adjuvant and vaccine formulations.

There are several properties that an adjuvant should ideally possess to be effective, safe and practical for use in human vaccines. Foremost, it should accomplish its desired goal without toxicity. Additionally, however, it should be well characterized and stable even in the absence of refrigeration. Although there are numerous compounds presently utilized as adjuvants in animals, no compound fits all of these desired criteria for clinical use.

The development of vaccine adjuvants has been largely empirical. Antigen preparations have tra-

ditionally been administered with and without an adjuvant and the specific immune responses directed towards the antigen have been compared by assessing antibody and/or CMI utilizing assays current for the epoch of the experiment. Improved immunologic and biochemical techniques are permitting the construction of new, better characterized adjuvants and vaccine formulations. Nonetheless, a fundamental understanding of the mechanisms by which adjuvants stimulate immune responses is incomplete, slowing progress in the field and making classification of the numerous and diverse adjuvants that are currently known somewhat arbitrary.

Compounds with adjuvant activity (Table 1)

Aluminum compounds

Aluminum was used as an adjuvant as early as 1926 by Glenny. The positive results of these studies led to its use worldwide in animal and human vaccines in the following decades. Aluminum continues to be

Table 1 Commonly studied adjuvants, based on mechanism^a

<i>Depot based</i>
Aluminum
Degradable polymer microspheres
Emulsions
<i>Hydrophobic and surfactant</i>
Emulsions
Liposomes
ISCOMs
Saponin (QS21)
Nonionic block copolymers
<i>Bacterial derivatives</i>
Lipopolysaccharide and derivatives
Muramyl dipeptide and derivatives
Trehalose dimycolate
<i>Corynebacterium parvum</i>
Pertussis toxin
Cholera toxin
Heat-labile enterotoxin and derivatives
<i>Cytokines</i>
IL-1 and derived synthetic peptide
IL-2, IL-4, IL-6, IL-12, etc
IFN γ

^aThis list is not inclusive. Adjuvants may be listed in more than one category.

utilized today, primarily because it is generally regarded as safe.

Vaccines are prepared by adsorbing the antigen on to aluminum compounds such as aluminum hydroxide or phosphate, or by precipitating the antigen with alum $[\text{Al}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}]$. Several hypotheses have been proposed for its mechanism of action. However, most investigators believe that the primary mechanism by which aluminum acts is by the creation of a depot so that the antigen is relatively slowly released, prolonging the time in which it can interact with antigen-presenting cells and lymphocytes. Despite its wide acceptance for clinical use, there are some disadvantages in the use of aluminum as an adjuvant for human vaccines. Although clinical trials have confirmed that aluminum is safe, it augments antibody responses much more effectively than CMI. Furthermore, its adjuvanticity varies depending upon the antigen. Other problems are that vaccines containing aluminum cannot be frozen for storage or lyophilized, and therefore require refrigerated transport. Overall, aluminum compounds are adequate adjuvants if antibody is sufficient for protection and the antigen is a strong immunogen.

Hydrophobic compounds and surfactants

The empirical knowledge that hydrophobic substances tend to augment antibody production and CMI has been a framework for the development of several classes of adjuvants. Examples include emulsions containing water and oil, lipopolysaccharide (LPS), liposomes and saponin.

The use of emulsions began in the 1930s with the experiments of Freund, who mixed paraffin oil with solutions of killed mycobacteria. This compound, called Freund's complete adjuvant, is one of the most potent adjuvants known. It is, however, too toxic for use in humans. An oil emulsion without the mycobacteria, called Freund's incomplete adjuvant, is less toxic and was clinically utilized in the past for influenza and poliomyelitis vaccines. It is no longer used because of a low incidence of induced sterile abscesses, the question of carcinogenicity in mice, lot-to-lot variation, lack of long-term stability, and the fact that CMI is not substantially boosted.

The use of liposomes as adjuvants has been studied experimentally for 20 years. Adjuvant activity depends upon the charge, the number of multilamellar phospholipid layers, the composition and the method of preparation. Both antibody and CMI are augmented. Some liposome preparations induce a potent cytotoxic T lymphocyte (CTL) response. A physical association between antigen and liposome is necessary, suggesting that a depot effect and/or altered antigen presentation are important mechanisms.

Stability, long-term toxicity and lot-to-lot variation are potential problems.

Saponin is a compound that was initially purified from the bark of the tree, *Quillaja saponaria*. It has been studied for decades, but is too toxic for use in humans. Chemical extraction procedures led to the less toxic derivative, Quil A, which is utilized widely for veterinary vaccines, and subsequently to QS21. Complexes of cholesterol, Quil A and amphipathic antigen (called ISCOMs) provide an efficient means of inducing a potent antibody and CMI response, and may provide a formulation in which the toxicity of Quil A is diminished, while its efficacy, including its ability to induce CTL, is maintained. QS21 is less toxic than both parent compounds, and can stimulate a $\text{T}_\text{H}1$ response and antigen-specific CTL in addition to antibody responses. Its safety and efficacy are being evaluated in several clinical trials.

Adjuvants of bacterial origin

It is not surprising that animals have evolved an elegant immune response to bacterial products. Although many adjuvants of bacterial origin are too toxic in their natural form for use in humans, analogs of LPS and muramyl dipeptide have been developed for reduced toxicity.

Experiments in the 1960s and 1970s revealed that the adjuvant active moiety of LPS is a unique bacterial lipid called lipid A. Chemical manipulations such as phthalylation or succinylation of the LPS or removal of a phosphate group from lipid A to create monophosphoryl lipid A results in diminished toxicity of the compound, while retaining adjuvanticity.

Freund's complete adjuvant consists of an oil emulsion containing killed mycobacteria. Successive biochemical studies revealed that one of the most active components is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP). Over the last decade, numerous analogs of MDP have been synthesized and studied. Hydrophilic MDP analogs administered in saline induce mainly antibody responses, whereas CMI is induced if MDP is given together with antigen in oil emulsions or in liposomes. Similarly, if the MDP analog itself is made lipophilic by the addition of glycerol mycolate, CMI is induced. Nontoxic candidate MDP analogs for clinical use are murametide (NAcMur-L-Ala-D-Gln-OCH₃), murabutide (NAcMur-L-Ala-D-Gln- α -*n*-butyl ester) and muramyl tripeptide (MTP-PE). A combination of threonyl-MDP with a squalane and pluronic copolymer emulsion (Syntex adjuvant formulation, SAF) is a potent adjuvant in experimental models. MTP-PE and SAF are currently being

assessed as adjuvants for some human immunodeficiency virus (HIV) vaccine candidates.

The mucosal immune response is important for protection against many infections. Cholera toxin and *Escherichia coli* heat-labile enterotoxin (LT) are the most potent adjuvants described for the induction of mucosal immunity. Unfortunately, neither can be used in humans because of associated toxicity. Recently, however, a nontoxic derivative of LT has been developed which retains its adjuvanticity for mucosal immunization.

Cytokines

Interleukin 1 (IL-1), IL-2, IL-4, IL-6 and interferon γ (IFN γ) stimulate immune responses when injected together with antigens. A synthetic nonapeptide of IL-1 β has been identified that possesses immunostimulatory but not inflammatory activity. Other cytokines are attractive candidates for the development of vaccines that specifically stimulate certain arms of the immune system. For example, IL-12 is a potent stimulator of T_H1 responses and therefore may be helpful as a component of vaccines for some parasitic diseases, such as leishmaniasis, or for viral infections for which these T cell responses play an essential role.

Adjuvant and antigen combinations

Several strategies have been utilized to increase the immunogenicity of small or weak antigens. To increase its apparent molecular weight, an antigen can be coupled on to an inert support, coupled chemically or genetically to a larger 'carrier' molecule (such as tetanus toxoid), or coupled to itself or other antigens to create a univalent or polyvalent polymer. Peptides can be also directly synthesized on to a branching core structure to create defined branching molecules with multiple antigenic peptides (called MAPs). Polysaccharides that are T cell independent can be coupled to protein carriers to create protein-polysaccharide conjugates that confer T cell-dependent characteristics to the polysaccharide. Similarly, conjugation of the antigen directly to the adjuvant can increase its immunogenicity. Another approach to further augment the immune response is to utilize combinations of adjuvants, such as the use of LPS, MDP, saponin, or cytokines in liposomes or oil emulsions together with the antigen. Biodegradable delivery systems have also been developed that can be utilized for the controlled release of antigen and to target antigen to antigen-presenting cells. In particular, microspheres based on poly(lactic acid) and poly(lactic/glycolic acid) are being actively developed.

Living organisms as vector/adjuvants

An exciting recent approach is to engineer genetically a nonpathogenic but infectious living organism to deliver foreign protective antigen(s) to the immune system. The vaccine recipient is infected with the organism, which is utilized as a vector in order to induce an immune response to the antigen. The first live vector to be utilized was vaccinia virus; however, many other viral or bacterial vectors are now being developed to deliver protective antigens to the immune system. One excellent candidate for the construction of such an attenuated vaccine is BCG, which has already been extensively utilized worldwide in humans as a vaccine that has a very low incidence of serious side-effects. Attenuated *Salmonella* or *Shigella* strains that stimulate local immune responses after oral immunization are also being developed. Viruses, including adenovirus or poliovirus, have considerable potential for the ability to deliver foreign antigens to the immune system. Recombinant poxvirus vectors that are unable to replicate in mammalian species, such as fowlpoxvirus or canarypox, have also been used to elicit antibody and CTL responses against diverse pathogens in animals. A canarypox coding for the HIV-1 envelope proved to be safe and immunogenic in humans. Such live recombinant vaccines may be easier to prepare and administer than existing vaccines. Disadvantages include the risks inherent with all live vaccines and the fact that repeated use of the same vector for different antigens may decrease its efficacy.

Future prospects

Despite the large number of currently known substances that are capable of increasing specific immunity, only aluminum hydroxide is currently utilized routinely as an adjuvant in humans. The major obstacle to the use of the remaining agents is that the toxic:therapeutic ratio is too narrow. The documented dissociation of efficacy from severe toxicity with many adjuvants suggests that acceptable compounds can be developed in the future. With the growing use of sophisticated small antigenic fragments containing defined B and T cell epitopes, such adjuvants will become increasingly necessary. Since different subsets of the immune system may be needed in different settings, it is to be anticipated that multiple distinct adjuvants and vaccine preparations will be desirable. It is therefore appropriate that the adjuvants currently being utilized or studied are highly diverse, including minerals such as aluminum, bacterial products such as LPS and MDP, and living viral and bacterial vectors. Nevertheless, although progress is being made through

modifications of known adjuvants, a fundamental unifying hypothesis as to the necessary mechanisms needed for adjuvanticity is still lacking. As our understanding of the underlying physiology of the immune system broadens, the parameters that are important for the development of newer generations of adjuvants will hopefully become more evident.

See also: Bacterial cell walls; Carrier; Immunogen; Liposomes; Muramyl dipeptide; *Mycobacteria*, Infection and Immunity; Vaccination, methods of administration; Vaccines.

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ADRENAL AUTOIMMUNITY

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Adrenal autoimmunity is currently the most common cause of Addison's disease, a chronic disorder of the adrenal cortex characterized by atrophy of the adrenals. This results in deficient production of adrenocortical hormones together with increased secretion of anterior pituitary adrenocorticotrophic hormone (ACTH). The clinical picture of Addison's disease may comprise weakness, anorexia, nausea, vomiting and weight loss. Hypotension and mucocutaneous hyperpigmentation are usually present. Levels of serum potassium and urea nitrogen are elevated, whereas serum sodium may be decreased. Significant laboratory findings are low plasma levels of cortisol and elevations of ACTH. Stimulation with synthetic ACTH does not result in cortisol increases.

Idiopathic adrenal atrophy is probably an autoimmune disease, as suggested by the facts that the histopathology of the affected adrenals resembles that observed in tissues which are targets of other organ-specific autoimmune diseases, and that autoimmune responses to various adrenocortical antigens have been detected in patients with idiopathic Addison's disease.

Addison's disease can occur as an isolated (sporadic) disorder or in association with other conditions (autoimmune polyendocrinopathy syndrome type 1 or type 2) (see Table 1). Sporadic autoimmune

Addison's disease usually affects young females, who may outnumber male patients by a factor of 4. Contrary to the reported increased incidence of insulin-dependent diabetes mellitus (IDDM) and Hashimoto's thyroiditis, Addison's disease continues to be an extremely rare occurrence. In the United States, its incidence approximates 1 per 4500–6250 patients hospitalized for various disorders. In the London area the prevalence of Addison's disease has been estimated at 30 per million inhabitants and in Denmark it was reported to be 60 per million. In New Zealand there are approximately 4.5 new cases per million population per year.

Pathology and immunopathology

At autopsy, the adrenal glands of most patients with idiopathic Addison's disease are small, very thin and often are very difficult to recognize or may be missing altogether (substituted by adipose and fibrotic tissue). The most characteristic findings by light microscopy are: loss of adrenocortical cells, localized hypertrophy and hyperplasia of adrenocortical cells as well as chronic inflammatory infiltrates (Figure 1). A severe loss of adrenocortical cells is the most characteristic feature of adrenals from patients with autoimmune Addison's disease. Where cortical epi-

Table 1 Human diseases with adrenal autoimmunity

Characteristics	Adult Addisons disease	Autoimmune polyendocrinopathy type 1	Autoimmune polyendocrinopathy type 2
Clinical features	Idiopathic adrenal insufficiency	Idiopathic adrenal insufficiency + hypoparathyroidism + chronic mucocutaneous candidiasis + gonadal failure	Idiopathic adrenal insufficiency + insulin-dependent diabetes mellitus + autoimmune thyroiditis
Age of onset	All ages, but more commonly in adults	Most commonly in childhood	All ages, but more commonly in adults
Gender	Mostly females	Males and females	Mostly females
Prevalence	3–6 per 100 000	Unknown (very rare)	Possibly 50% of adult Addisons patients
Immunogenetics	HLA-DR3	No HLA-DR association	HLA-DR3 and/or DR4
Autoantibodies against	Steroid 21-hydroxylase P450	P450 side-chain cleavage enzyme Steroid 17 α -hydroxylase (?)	Steroid 21-hydroxylase P450
T cell responses to	Adrenal antigens (?)	?	?

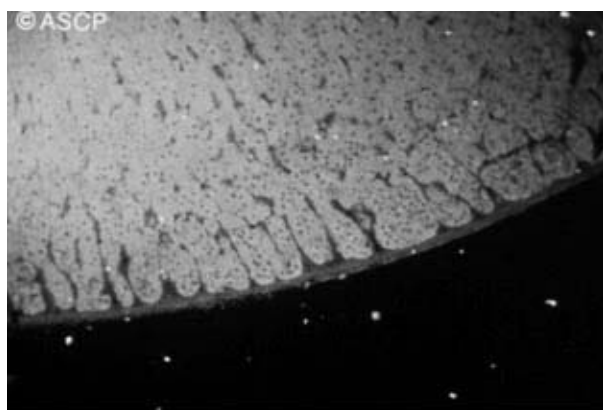
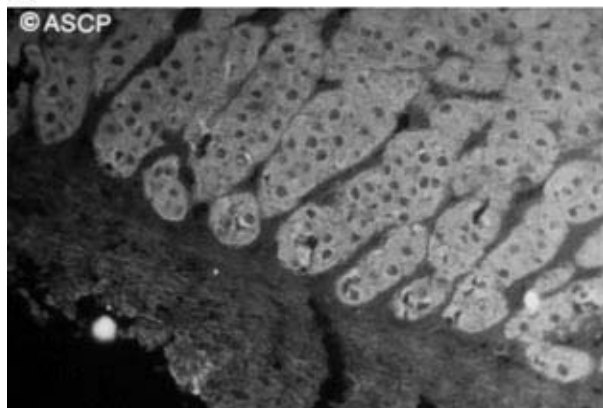
**(A)****(B)**

Figure 1 (A) Adrenal gland from patient with Addisons disease, showing atrophy of the cortex and infiltration with mononuclear cells. (B) Another area from same adrenal gland, photographed at higher magnification. (See also color Plate 6.)

thelium is present, it is not arranged in three different zones, but in strands or rows of irregular size, contour and direction. There is loss of cellular outline, swelling and vacuolar appearance. Some cells are extremely large, with granular or vacuolated cytoplasm, others are small. In addition, both hyperplasia and hypertrophy can be observed in adrenals from patients with idiopathic Addisons disease. Scattered islands of cells may be seen in the cortex as well as large well-circumscribed adenoma-like nodules, without typical zonal division of the parenchyma. These cells resemble those of the zona fasciculata, less often those of the zona glomerulosa. They may have a bizarre appearance and sometimes are huge, with deeply pigmented cytoplasm and large hyperchromatic nuclei. A chronic inflammatory infiltrate is usually present, with a prevalence of small lymphocytes. Plasma cells and macrophages are also present, but in lesser numbers. The adrenal medulla is usually intact or infiltrated by a few lymphoid cells. The lymphocytic infiltrate is usually diffuse, but in some areas lymphocytes may form aggregates of various dimensions. Germinal centers are seldom present.

Studies of adrenal immunopathology in patients with Addisons disease have been rather scarce. Direct immunofluorescence of the adrenals has shown dense deposits of immunoglobulin G (IgG) in some areas of the adrenal cortex, with specific fluorescence localized more intensely at the level of the cell membranes. To date, there are no reports on the composition of the adrenocortical lymphocytic infiltrates. However, immunohistopathology studies of micronodules of Cushing's disease have shown that the lymphocytic infiltrates, present around vessel

walls but also inside the nodules themselves, were composed mostly of T cells, usually CD4⁺ with a minority of CD8⁺. In addition, B cells were also present in the infiltrates. It is possible that a similar situation also occurs in the adrenals of patients with autoimmune Addison's disease. Class II major histocompatibility complex (MHC) expression by human adrenal cortical cells appears to be a physiological phenomenon. Approximately 10% of normal adrenocortical cells express MHC class II antigenic determinants. These antigens are mostly localized in the zona reticularis and occasionally may extend to the zona glomerulosa or fasciculata. In adrenal glands from Addison's disease patients, the majority of residual cortical cells show MHC class II antigens, with variation in extent from 50 to 100%. Staining is most intense at the periphery of residual nodules. Adrenal glands from patients with tuberculous Addison's disease have a similar pattern to normal glands, but show class II positivity in cortical cells immediately adjacent to foci of caseous necrosis.

Humoral and cellular autoimmune responses

Newly diagnosed patients with Addison's disease have a variety of circulating autoantibodies to adrenal antigens. Autoantibodies directed against organ-specific antigens localized in the cytoplasm of the adrenocortical cells are present in the circulation of approximately 70% of Addison's disease patients. These antibodies can be detected by indirect immunofluorescence, radioimmunoassay and ELISA, are usually of low titer, belong to the IgG class and react with one or more organ-specific antigens localized in the adrenal cortex. In most cases, immunofluorescence shows that all three layers of the cortex react with adrenal autoantibodies (Figure 2). However, different patterns of staining can be observed: sera from some patients stain all three zones of the cortex, whereas others react with the zona fasciculata and zona reticularis alone. A few sera react with the zona glomerulosa alone. The clinical significance of these findings is unknown, but suggests an immunologic heterogeneity of Addison's disease, with preferential involvement of different adrenal antigens. Complement fixing adrenal autoantibodies have also been observed by immunofluorescence. Autoantibodies to organ-specific surface antigens of adrenal cells are present in the sera of 86% of patients with Addison's disease who have circulating antibodies to adrenal cytoplasmic autoantigens. The adrenal-specific surface antigens appear by immunofluorescence as a continuous rim, but separate granules are visible on focusing over the plasma

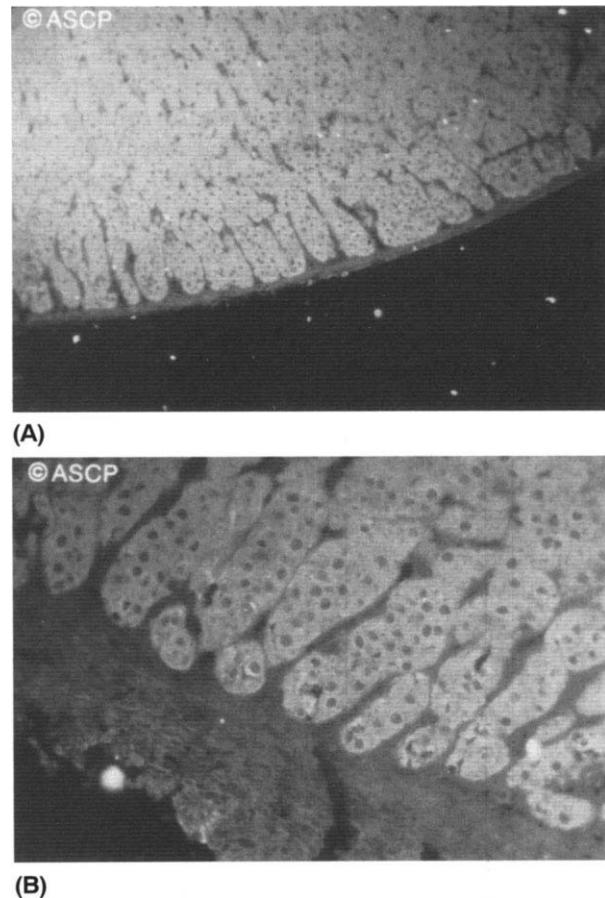


Figure 2 (A) Antibodies to adrenocortical antigens (indirect immunofluorescence with serum from a patient with Addison's disease on cryostat section of monkey adrenal). (B) Higher magnification of similar field. (With permission from American Society of Clinical Pathologists, Chicago, USA.)

membrane. Antibodies to steroid-producing cells of other tissues have also been detected in the circulation of patients with Addison's disease. These autoantibodies react with theca interna cells, interstitial cells and corpus luteum cells of the ovary as well as interstitial cells of the testis and placental trophoblasts.

Recent studies using molecular cloning and western blotting have shown that different steroidogenic enzymes of the cytochrome P450 family may be the target autoantigens of autoimmune Addison's disease. Purification of native human adrenal proteins, comparative western blotting using patients' sera and rabbit antibodies against recombinant 21-hydroxylase as well as antibody absorption studies, have demonstrated that steroid 21-hydroxylase P450, an enzyme involved in both the glucocorticoid-synthesizing pathway and the mineralocorticoid pathway, is the major target of adrenal autoantibodies found in patients with isolated Addison's dis-

ease or polyendocrine autoimmune syndrome type 2 (Table 1). On the other hand, patients with polyendocrine autoimmune syndrome type 1 (characterized by adrenal insufficiency, hypoparathyroidism and hypogonadism) have antibodies that react with P450 SCC (cholesterol side-chain cleavage) or, as reported by other authors, another enzyme, the microsomal steroid 17 α -hydroxylase (Table 1). It is still uncertain whether these autoantigens are expressed on the surface of adrenal cells or are otherwise accessible to adrenal antibodies *in vivo*. Finally, IgG preparations from patients with Addisons disease have been reported to cause a dose-dependent decrease of *in vitro* cortisol production and/or DNA synthesis induced by ACTH in guinea pig adrenal segments maintained in organ culture. The blocking effects occurred irrespective of the presence or absence of adrenal cytoplasmic autoantibodies and were attributed to the production of antibodies against the receptor for ACTH. This hypothesis has not been confirmed by recent studies that suggest an inhibitory effect of non-IgG components.

Studies of cellular immunity to adrenal antigens have been based on classical (but now rather outdated) methods to detect delayed hypersensitivity reactions and lymphokine production. In these procedures, peripheral blood lymphocytes from patients were incubated *in vitro* with the putative antigens, often used as a rather crude suspension. Thus, it has been shown that most patients with Addisons disease have cell-mediated immune reactions to adrenal antigens as determined by the leukocyte migration test. There was no correlation with onset of disease, its duration and the presence of adrenal antibodies. The antigen preparations that have been utilized in leukocyte migration studies were organ-specific, but not species-specific, since monkey and porcine adrenals were used with the same success as human adrenals. In some studies the antigenic activity was localized in the mitochondrial but not the microsomal fraction, whereas in other experiments leukocyte migration inhibition was observed after incubation with both components. More modern investigations of cellular immunity (e.g. with steroid 21-hydroxylase P450) are still lacking.

Immunogenetics and disease association

Adrenal autoimmunity may be a heterogeneous condition, as suggested by reports that Addisons disease is inherited in at least three different fashions: autosomal recessive, autosomal dominant with decreased penetrance and X-linked recessive. Inherited susceptibility to autoimmune Addisons disease is linked to

HLA-DR3 and/or DR4, except when the disease occurs as part of type 1 autoimmune polyglandular syndrome. A high prevalence of other autoimmune diseases (ovarian failure, Graves disease, Hashimoto's disease, hypothyroidism and insulin-dependent diabetes mellitus) is associated with Addisons disease (see Table 1).

Animal models of Addisons disease

Idiopathic Addisons disease occurs spontaneously in dogs and more rarely in cats. In the few cases that have undergone autopsy, atrophy, regeneration of adrenocortical cells and chronic inflammation of the adrenal cortex have been observed. The cause of adrenal insufficiency in dogs is probably of autoimmune, not tubercular, origin. A preferential occurrence in Chow-Chow dogs suggests a genetic predisposition. Adrenal autoimmunity with lesions of the chronic inflammatory type has been experimentally induced by immunization of monkeys, rabbits, guinea pigs, rats and mice with suspensions of adrenal cortex in complete Freund's adjuvant. Circulating autoantibodies to the adrenal cortex have been demonstrated in these models, whereas there is a paucity of reports on cellular autoimmunity. As for other experimental models, adrenalitis was induced in histocompatible recipients by adoptive transfer of lymph node cells from animals with adrenal autoimmunity. Modern immunogenetic studies are lacking, but there was a suggestion that some inbred strains may be more susceptible than others to the induction of experimental adrenalitis. More recently, adrenalitis and adrenal antibodies have been detected in BALB/c nu/nu mice reconstituted with thymic rudiments from young rats. The same mice also had thyroiditis, oophoritis or orchitis, i.e. were good models of autoimmune polyendocrinopathies.

Pathogenesis

Immunologically mediated lesions of the adrenal cortex may occur in a variety of ways. Adrenal parenchyma may be affected by a slow destructive process and is gradually replaced by connective tissue, with eventual loss of physiological function. The mechanisms involved may be cellular or humoral (autoantibody- and/or cytokine-mediated). The role of T lymphocyte-mediated cytotoxicity in Addisons disease is still unclear. Antibody-dependent cytotoxic destruction of adrenal cells might occur in several ways (phagocytosis or lysis of antibody-coated adrenal cells, antibody-dependent cellular cytotoxicity, immune complex-mediated reactions, inhibitory effects on steroid-producing enzymes). However,

babies born to mothers with autoimmune Addisons disease and circulating IgG antibodies to adrenal antigens develop none of the manifestations of the maternal disease during the neonatal period, despite the transplacental passage of adrenocortical antibodies. The role of cytokines and oxygen radicals in adrenal cortex damage is still relatively unexplored. Finally, the suggestion that antireceptor immunity may play a role in Addisons disease has not yet been confirmed.

Etiology

The cause(s) of autoimmune Addisons disease is(are) still unknown. Expression of MHC class II antigens, suggested as a key event in the initiation and/or maintenance of organ-specific autoimmunity, occurs in normal adrenals and, therefore, is unlikely to be the primary etiologic event of Addisons disease. Defects in HLA-DQB chain, found in IDDM, have not been reported in patients with Addisons disease. On the other hand, a deficiency of T suppressor cell activity was noted by some authors. To date, restricted T cell receptor (TCR) usage, alterations of the idiotypic-anti-idiotypic network and changes in the balance of the cytokine network have not been demonstrated in autoimmune Addisons disease.

Environmental agents (e.g. microorganisms and/or xenobiotics) might cause adrenal autoimmunity by

modifying autoantigens (so that they are no longer recognized as self), releasing tissue components normally absent from the circulation, or sharing antigens with tissues (molecular mimicry). In addition, they could inhibit T suppressor cells, stimulate T helper cells, B cells or macrophages, interfere with the idiotypic-anti-idiotypic network, alter tissue MHC expression and finally cause changes in the production of cytokines and lymphokines. However, none of these possibilities have yet been demonstrated in autoimmune Addisons disease.

See also: Autoantigens; Autoimmune diseases; Autoimmunity; Polyendocrine autoimmunity.

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AFFINITY

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The affinity (K_A) is a thermodynamic parameter that quantifies the strength of the association between two molecules in solution. For antibody-antigen complexes, K_A can be interpreted rigorously only for an equilibrium in solution involving a single species of antibody-combining sites and a single species of antigenic sites. Consequently, for polyclonal antibodies which contain a mixture of antibodies, no true affinity can be defined. The affinity of an antibody for an antigen depends on the structural complementarity of the binding site on the antibody (paratope) and the binding site on the antigen (epitope). Antibodies have a wide range of affinities that are generally between 10^5 (low affinity) and 10^{12} M^{-1} (high affinity). The overall stability of the complex between an antibody and an antigen is expressed by the term 'avidity' and is governed by

the affinity, the valency of the antibody and the antigen and the steric hindrance of the interacting components. Small changes in antigen structure can affect the strength of the antibody-antigen interaction. Similarly, changing the structure of the binding site of the antibody may alter the affinity of the antibody for the antigen. For instance, the progressive increase in affinity that occurs with time upon immunization (affinity maturation) arises from mutations in the complementarity-determining regions that build the paratope. Thus, antibodies that are different in their binding site structure are expressed and cells producing antibodies with higher affinities are stimulated preferentially to divide.

Definition and theoretical aspects

The antibody site to antigenic site association reaction at equilibrium can be written as:



with the concentrations of free antibody sites, free antigen sites and complex (saturated antibody or antigen sites) at equilibrium given as $[Ab]$, $[Ag]$ and $[x]$ respectively.

The affinity, K_A , is defined by the Law of Mass Action as:

$$K_A = [x]/[Ag].[Ab]$$

The concentration of antibody sites $[Ab]$ and the antigen sites $[Ag]$ at equilibrium are related to the total antibody sites $[Ab_t]$ and the total antigen sites $[Ag_t]$ by:

$$[Ab] = [Ab_t] - [x]$$

$$[Ag] = [Ag_t] - [x]$$

If $[Ag_t]$ is varied while $[Ab_t]$ is kept constant:

$$1/K_A = K_D = [Ag] ([Ab_t] - [x])/[x]$$

with K_D defined as the equilibrium dissociation constant.

Consequently

$$[x]/[Ab_t] = [Ag]/([Ag] + K_D)$$

Several linear plots have been proposed for the determination of K_D , the most commonly used of which are the Scatchard equation:

$$[x]/[Ab_t][Ag] = (1 - x/[Ab_t])/K_D$$

and the Klotz equation:

$$[Ab_t]/[x] = K_D/[Ag] + 1$$

If Ab_t and Ag_t are known, the experimental determination of K_D (or $K_A = 1/K_D$) requires precise measurement of only one of the three concentrations $[Ab]$, $[Ag]$ or $[x]$ (Figure 1).

K_A depicts an equilibrium property and therefore does not reflect the speed at which equilibrium is reached. Yet, K_A does depend on the association and dissociation rate constants. When binding is a simple one-step reaction, K_A is equal to k_{on}/k_{off} , where k_{on} and k_{off} are the association and dissociation rate con-

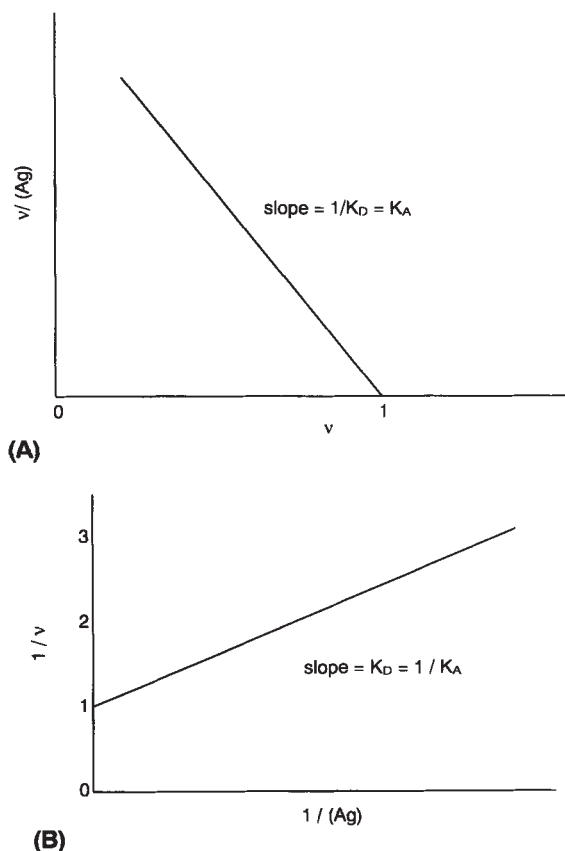


Figure 1 $(x)/(Ab_t)$ is usually referred to as v and corresponds to the fraction of bound antibody sites at equilibrium. (Ag) corresponds to the concentration of free antigenic site at equilibrium. (A) Scatchard plot; (B) Klotz plot.

stants. However, when a significant conformational change of either the monoclonal antibody or the antigen occurs upon binding, important deviations from that simple equation can be observed. For such cases, measuring only k_{on} and k_{off} would provide an erroneous estimate of the affinity.

The true affinity cannot be determined for antibody-antigen interaction in heterogeneous phase systems. No straightforward thermodynamic theory describes the equilibrium in heterogeneous phase systems, such as the binding of a monoclonal antibody to an antigen present on a cell surface or immobilized on an ELISA plate or a biosensor chip. K_A is defined in solution, with both the monoclonal antibody and the antigen diffusing and rotating freely in solution, while in the solid phase assay one partner is immobile. This can result in estimates of K_A by solid-phase assay being orders of magnitude away from the real affinity in solution. In such cases, the strength of monoclonal antibody-antigen interaction is best described by an appropriate operational parameter that is dependent of defined experimental conditions. Moreover it is now well established that immobil-

ization of either antibody or antigen often results in a partial denaturation of the protein, thus modifying its binding properties.

Methods for affinity measurement

As already pointed out, affinity is measured when the equilibrium between the two molecules has been achieved in solution. Hence, measuring K_A for monoclonal antibody and its antigen consists in mixing the monoclonal antibody (mAb) and the antigen at various initial concentrations, letting the equilibrium be established, measuring the concentrations of free and saturated sites at equilibrium and analyzing the binding curve. The experimental difficulty resides in distinguishing the free and bound state of either the monoclonal antibody or the antigen. Several methods can be used, such as equilibrium dialysis, radioimmunoassay (RIA) using precipitation with salts and other agents, filtration, fluorescence measurements and RIA- or ELISA-based methods. Equilibrium dialysis is the method of choice for hapten and dialyzable antigen but is not applicable for macromolecular antigens. Radioimmunoassays require radiolabeling of the antigen which may alter the binding properties of the antigen. Precipitation or filtration methods need the complex to be stable enough during the time required to achieve its separation so that the equilibrium is not disrupted. The use of fluorescence to determine K_A requires that either the monoclonal antibody or the antigen be fluorescent, and that a change in fluorescence should occur upon formation of the monoclonal antibody/antigen complex. The fluorescence signal used can be either intrinsic (e.g. tryptophan residues from the antibody and, possibly, from the protein antigens; some prosthetic groups such as pyridoxal-phosphate, NADH, NADPH, flavins, etc.) or result from prior fluorescent labeling of the antigen or the antibody with a fluorochrome. The fluorescent change observed upon association may be one of the following: excitation or emission wavelength shift, fluorescence quenching, fluorescence transfer, change in fluorescence polarization. As already underlined for radiochemical labeling, adding an extrinsic fluorescent label may affect the binding characteristics of the antigen or the antibody, either by steric hindrance or through a change in conformation, thus modifying the K_A . The sensitivity of current fluorimeters sets a higher limit of 10^8 – 10^9 M^{-1} on the K_A that can be determined. Some mAbs have much higher affinities for their Ag. Several methods such as indirect ELISA or surface plasmon resonance using immobilized antigen or monoclonal antibody have been developed to determine values of the affinity.

Such measurements yield real values of K_A only rarely. Friguet and colleagues have defined a procedure to measure by a competition ELISA method the real association equilibrium constant in solution. This method requires no labeling of either the antigen or the antibody, which ensures that the affinity is characteristic of native antibody and antigen. This method gives access to affinity constants as high as 10^{10} – 10^{11} M^{-1} and can be used for studies on protein–protein interactions that do not involve antibodies. Since this method has been frequently used for affinity determination, its principle is described in the next paragraph.

Affinity measurement in solution by competition ELISA

In the competition ELISA the monoclonal antibody at a fixed concentration and the antigen at varying concentrations are first incubated in solution until the equilibrium is reached. Then, the concentration of the free (i.e. not associated with antigen) monoclonal antibody at equilibrium is determined by a classical indirect ELISA using antigen-coated plates.

For correct determination of the free antibody concentration at equilibrium, several requirements must be fulfilled: 1) The absorbance obtained in the last step of the indirect ELISA, which reflects the free antibody concentration, must be proportional to the antibody concentration tested. 2) Only a small percentage of the free antibody molecules (i.e. less than 10%) must bind to the coated antigen, to prevent any significant disruption of the equilibrium in the liquid phase. This ensures that, during the ELISA, the equilibrium in solution is not significantly modified. By this means, the observed equilibrium constant corresponds to the real affinity. 3) Since the association constant K_A is generally dependent on the temperature, the temperature must be kept constant throughout.

To satisfy requirements (1) and (2), it is necessary in previous ELISA experiments to determine the total (i.e. initial) antibody concentration range that must be used, the concentration of the coated antigen and the optimal incubation time of the antibody solutions in the coated wells. The state (native or partially denatured upon coating) of the coated antigen and whether or not it is recognized by the mAb differently from the soluble antigen is not important as long as the coated antigen can specifically and quantitatively trap the free antibody.

An example of affinity determination by the competition ELISA method described above is given in Figure 2 using a computerized fitting program.

The multivalency of antibodies and antigens

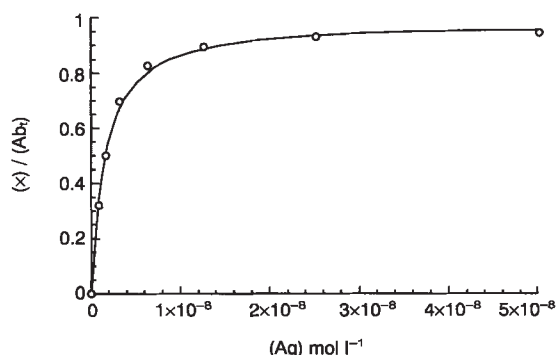


Figure 2 Saturation curve in solution of a monoclonal antibody by its antigen: $[X]/[Ab] = [Ag]/([Ag] + K_D)$. The value of K_D (1.5×10^{-9} M) was directly extracted with a computerized non-linear regression method.

sometimes complicates the determination of affinity. The distortion of the saturation curves (as compared with simple binding) caused by the multivalence of immunoglobulins in indirect competition ELISA was first analyzed by Stevens and has become the object of much attention. However, the simple data analysis initially proposed by Friguet and colleagues provides satisfactory results when divalent immunoglobulins are used, provided one extracts the affinity only from the part of the saturation curve obtained at high saturation of the monoclonal antibody by the antigen.

Why is affinity important?

The affinity is of considerable importance in understanding the biological activity of antibodies. For instance, when the immune system is challenged with antigen, the immune response and host defense is evaluated by the affinity of the antibodies. However, immune sera contain a mixture of antibodies with different affinities and the experimental conditions can considerably influence the apparent affinity. It is now well established that in the commonly used competition ELISA, the conditions must be carefully selected to minimize disruption of the liquid-phase equilibrium by the solid-phase antigen. In the case of a polyclonal serum-containing antibodies with widely different affinities, it is practically impossible to simultaneously fulfil this requirement for all the antibodies under a unique set of experimental conditions. Schematically, low-density coating of the antigen will trap essentially high-affinity antibodies, resulting in the determination of the apparent affinity of only these molecules, which will not be representa-

tive of the average affinity of the serum. In contrast, high-density coating of the antigen will trap low-affinity antibodies too, but the trapped fraction of high-affinity molecules will be large and their contribution to the average affinity will be seriously biased by the disruption of the equilibrium. The knowledge of antibody affinity and specificity for its antigen determines its reliability and usefulness as a reagent in a variety of immunochemical techniques such as histoimmunochemistry, immunochemical labeling of cells or cellular components, immunodetection by ELISA, RIA or western blotting. Most often, strict specificity and high affinity are a prerequisite for the accuracy of these techniques. However, it is sometimes useful to have antibodies of moderate affinity available as in the case of purification by immuno-adsorption which needs, as a final step, the immuno-complex to be dissociated.

Since the affinity of a monoclonal antibody for a protein depends on the structural complementarity of the binding sites on the two molecules, changes in affinity can reflect changes in protein structure occurring at the site recognized by the antibody. Thus, thoroughly characterized monoclonal antibodies have been used as probes to detect and analyze conformational changes within proteins.

See also: Affinity chromatography; Affinity maturation; Antibody-antigen intermolecular forces; Antigen-binding site; Clonal selection; Immunoassays; Somatic mutation; Surface plasmon resonance; Valency of antigens and antibodies.

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AFFINITY CHROMATOGRAPHY

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Affinity chromatography is the process by which the reversible biospecific interactions between a molecular species and its ligand are used in the isolation of that molecular species from a biological milieu. Conceptually the process is extremely simple. The ligand is attached to a solid support to form a specific adsorbent to which the biological system, usually in the form of a homogenate or extract containing the molecular species of interest, is exposed. Only the molecular species that shows appreciable affinity for the ligand will be retained or retarded; other materials which have no specificity for the insolubilized ligand will pass through the column. The molecular species that has become attached to the adsorbent must then be eluted without destroying its biological properties.

Unlike many of the other isolation techniques that depend upon differences in physical properties of the biological materials to effect a separation, the use of affinity chromatography takes advantage of functional properties of the system. Since almost all biological processes depend on specificity between different molecules, affinity chromatography has found a wide range of applications and new ones are always being developed. These include the use of antibiotics, antibodies, antigens, chromophores and dyes, coenzymes, drugs, hormones, enzymes, inhibitors, lectins, nucleic acids, nucleotides, polynucleotides, substrates and substrate analogs, sugars and polysaccharides, tRNA, and vitamins to isolate macromolecules, organelles, viruses and cells.

Successful application requires that the interactions between the biological molecules and their ligands have an affinity that is at least 10^5 , but not so strong that the binding cannot be reversed without

detriment to the biological materials of interest. Thus the preparation of appropriate ligand conjugates often requires a considerable amount of extra work, but, when successful, the results can be dramatic. The exquisite sensitivity of this system has allowed application to the separation of large protein assemblies involved in signal transduction, transcription complexes and cytoskeletal structure. The technique of affinity chromatography is one of the few procedures that will usually achieve a major purification in one or two steps, often producing an homogeneous sample for study. In addition the process is effective for extremely dilute solutions and allows extensive concentration of the biological sample. Such a dramatic example of the use of affinity chromatography is the successful isolation of 6.26 mg, a 12% yield of transcobalamin II from 1400 liters of human plasma.

Historical perspective

The earliest forms of affinity chromatography were actually developed by immunochemists. In 1951 Campbell, Luescher and Lerman developed antigen-cellulose columns for use in isolating specific antibodies, a separation that could not be achieved unless one could take advantage of the affinity of the antibody for its specific antigen. The conjugation of an antigen to a solid matrix was called an immuno-adsorbent and will be further described in the section on immuno-adsorbents. In 1968, Cuatrecasas, Wilchek and Anfinsen applied this concept to the isolation of enzymes and introduced the term affinity chromatography. Application of the technique exploded not only because the generality of the approach became clear, but also because a new solid

support based on agarose with appropriate chemistry for coupling many types of ligands was introduced. Since then this technique has had extensive application in all aspects of the biological sciences.

Recently, the use of affinity chromatography has been integrated with high-pressure liquid chromatography (HPLC) systems to combine the specificity of affinity chromatography with the speed and sensitivity of HPLC. This has been extended to include high-pressure immunoaffinity chromatography. One area that has been under-exploited is the use of specific antibodies to isolate biological materials such as growth factors that are produced at low concentrations in cell culture.

The procedure of affinity chromatography

Unlike the use of physical separation procedures, the use of affinity chromatography requires more extensive knowledge of the biological material and its binding to the ligand that will be used for isolation. The use of agarose has been the overwhelming choice for a solid support for attachment of ligands because of its hydrophilicity and thus low non-specific binding for most macromolecules, its good porosity and uniform spherical beads for preparing columns with suitable flow rates and good resolution, and its ability to be modified readily using cyanogen bromide to allow coupling of most important ligands either directly or via a linking molecule.

Ligand or ligand analogs must be selected that permit conjugation without significantly affecting the ligand's complementarity structure and thus detrimentally reducing its ability to bind the biological material. The attachment of the ligand may decrease or increase its affinity for the biological molecule depending on the nature of the linkage molecules and the conformational constraints induced by the coupling chemistry and the solid support, and these factors must be evaluated experimentally. For most isolations of macromolecules, the ligand-conjugated matrix is packed in a standard chromatography column, and the sample containing the biological molecules of interest is applied to the column. Biological materials that do not recognize determinants on the solid phase pass through, and the column is thoroughly washed with neutral buffers to remove all nonspecifically attached materials. Since the commonly used cyanogen bromide coupling chemistry introduces an isourea group at the linkage point which has a $pK_a = 10.4$, there are positive charges in the matrix, and these columns tend to have some weak ion exchange properties. Some of the commercially available supports have attached suitable func-

tional groups for coupling while avoiding the introduction of a charged moiety. However, if a protein such as albumin is used for linking the ligand, there is often increased weak binding between some of the impurities and the column. The amount of these weaker binding contaminants can be reduced by washing the column with higher ionic strength buffers, 1 or 2 M NaCl in borate or phosphate at pH 7–8, or 0.5 M carbonate buffer at pH 9.0 as long as they do not also elute any of the specific material.

Following removal of impurities and nonspecifically attached materials, conditions are changed to effect an elution of the desired biological molecules in their native state. Since the binding results from noncovalent interactions that include electrostatic, hydrophobic, and van der Waals forces, solvent changes must be made to reduce these interactions or compete with the ligand on the matrix to release the sample from the column without denaturing the macromolecule. The most commonly used method of elution is to reduce the pH to 2.8 with glycine-HCl buffer or 1 M propionic acid. If that fails to elute the sample or those conditions denature the protein, the use of alkaline conditions often yields better results. Probably the best alkaline buffer is 0.1 M diethylamine which has a pH of approximately 11. Many anti-idiotypic antibodies and some antibodies to protein determinants seem to prefer these elution conditions to the use of acid. In a number of cases, when a secondary antigen-antibody interaction (which usually is of the 'hydrophobic' variety) has set in, in addition to an increase in pH, a lowering of the surface tension of the medium should be effected at the same time by means of the admixture of a water-miscible organic solvent with strong hydrogen-acceptor properties, such as ethylene glycol, dimethylsulfoxide (DMSO, approximately 30–50%), or propanol (approximately 3–10%). Elution of various blood group antibodies from red cells can be effected at pH 9.5–10.0, in the presence of $\approx 47\%$ DMSO. Elution of DNA-specific antibodies from immobilized DNA (which is a preponderantly electrostatic system) can usually be effected by just a pH increase to 11 (for low-affinity antibodies), or 12 (for high-affinity antibodies), using glycine buffers. In the relatively rare cases where Ca^{2+} bridging is the main mechanism of antigen-antibody binding, elution is best done with Na_2EDTA , or other complexing agents. The protein should be maintained in the unfavorable conditions for as short a time as possible and should be kept cold during such procedures. The use of ligands can be very effective, but it is sometimes difficult to remove the ligand completely from the sample. The use of strong denaturants such as guanidine-HCl, urea and SDS should usually be

avoided since it is often difficult to recover the biological sample in a native state, although these reagents can be effective in regenerating the column for reuse. In those cases where the antigen and/or antibody do not become irreversibly denatured by some of the more robust dissociating agents, and those cases where an affinity column or surface has to be regenerated regardless of the fate of the eluate, the most efficient eluants are: 10 mM HCl (no salt or other additive), pH approximately 2; 50 mM NaOH (pH 10); or 50 mM NaOH together with 15–30% acetonitrile. These eluants have been found effective in regenerating antigen- or antibody-coated surfaces in the bioaffinity sensor apparatus.

Besides the use of affinity chromatography for specific interactions, there are many examples of using ligands to isolate a restricted family of biological molecules. Protein A and, more recently, Protein G can be used to isolate a large number of immunoglobulin subclasses from a variety of species and have found extensive use in the isolation of monoclonal antibodies. Hydrophobic columns have found application for membrane proteins; Cibacron Blue 3GA and related dyes have found restricted applications such as removal of albumin from serum. With more readily available molecular models of protein-binding sites, efforts have been made to design analogs of these textile dyes that would have higher specificity for the protein of interest. The use of metal chelate columns has permitted separations based on very subtle differences in amino acid residues, and sequence-specific DNA affinity chromatography allows the isolation of DNA-binding proteins important in regulating transcription and replication. Thus, affinity chromatography has made a major impact on the procedures used to isolate specific substances in all areas of biology and medicine.

See also: Antibody-antigen intermolecular forces;

Immunoabsorbents; Bacterial immunoglobulin-binding proteins; Protein separation techniques.

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AFFINITY LABELING

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Biological processes are catalyzed and regulated by the specific interactions between corresponding molecular species. The strength of interactions reflects the affinity between the molecules. The affinity is determined by the sum of the reciprocal interactions between the components in a given biological system. In biology, the proteins are the most prominent ligand-binding molecules. Whether we consider enzymes, immune proteins, receptors or transport proteins, the key step in their biological function is the specific recognition and binding of a ligand (substrate, antigen, hapten, hormone, etc.). A small, and in many cases well-defined, part of the total surface of the protein is presumed to be involved in this selective, high-affinity interaction with the specific ligand, and this part of the molecule is referred to as the binding site. Affinity labeling is one of the effective means of studying the binding site.

Affinity labeling and photoaffinity labeling

Affinity labeling is a technique for labeling the binding (or active) site of proteins by virtue of a ligand analog to which a chemically reactive or photoreactive group has been attached. The latter can form a covalent bond with a suitably oriented amino acid in the binding (active) site. Such a labeling reagent ($R-L$) would consist of 1) a biologically active substance (R) capable of forming a reversible complex with a given protein (P), and 2) a properly positioned, chemically or photochemically reactive leaving group (L) (**Figure 1**). Upon incubation, the affinity label interacts with its protein counterpart, resulting in the formation of an irreversible protein-ligand complex. This process is illustrated in **Figure 1** and described in the following formula:

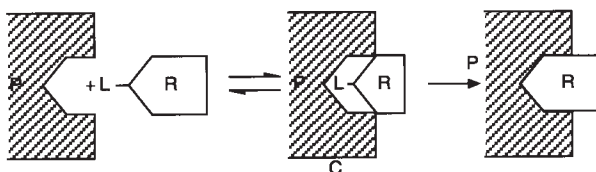
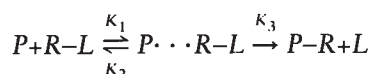


Figure 1 Affinity labeling.

The formation of the initial reversible complex ($P \cdots R-L$) increases the local concentration of the reagent at the active site relative to its concentration in solution. This ensures that the labeling reaction will take place at the binding site and not elsewhere. Following the formation of the reversible complex ($P \cdots R-L$), a functional group(s) on the protein adjacent to the active site reacts chemically with the affinity label, resulting in a covalent bond to form $P-R$. The affinity-labeled protein is either totally or partially inactivated by virtue of covalent bond formation.

The experimental criteria for a good affinity label are stipulated as follows: 1) its reaction with a given protein should result in a concomitant inactivation of the reversible binding activity; 2) it should not significantly label unrelated proteins; 3) specific ligands should protect against the affinity labeling; 4) it should facilitate the localization of the covalently bound ligand at the active site of the protein.

In planning an affinity labeling reagent, one should ensure that the reactive group (L) is relatively small and does not interfere significantly with the protein-ligand interaction. One should also consider the availability of radioactively labeled precursors and the facility of synthesis and stability. Finally, the labeled residue should remain stable to degradative techniques in order to permit its identification.

The first demonstration of affinity labeling by deliberate design was accomplished in 1961. Although affinity labeling was originally developed for investigations of purified enzymes, it has also added significantly to the study of the structure of active sites in other systems – for example, antibodies, ribosomes and membrane receptors. Affinity labeling of active transport carriers and hormone receptors has been used to identify and isolate these systems.

Affinity labeling of the antibody combining site

Antibodies and myeloma proteins against haptens comprise the most commonly used experimental system in which the determination of the antibody structure-function relationship has been attempted. Affinity labeling studies of antibodies were initiated by Singer, Wofsy and Metzger using diazonium

labeling reagents attached to a benzene arsonate hapten. Converse and Richards used diazoketones attached to dinitrophenyl (DNP) haptens as affinity labels, while Givol, Wilchek and coworkers used bromoacetyl derivatives attached to DNP haptens to label DNP-specific antibodies and MOPC 315 (a myeloma protein that binds DNP). A homologous series of reagents was prepared in which the bromoacetyl group was attached to the DNP hapten at progressively increasing distances from the haptenic moiety, thus allowing the labeling of residues in the combining site at various distances from the DNP ring. A photoaffinity label using aromatic azides was introduced by Porter's group. The photoaffinity reagent was introduced to overcome the limitations of the other reagents which can interact only with electrophilic reagents; the azide reacts with any amino acid residue. In addition, the design of this reagent made the chemical reactive group (L) part of the hapten.

Major results obtained

The results derived from affinity labeling studies of antibodies and myeloma proteins from different species that have different ligand specificities were surprising in their consistency.

1. Both light and heavy chains of the antibodies were labeled using affinity labeling reagents. With homogeneous myeloma proteins, it was possible to design reagents such that either the light or heavy chain could be labeled. Particularly interesting information was obtained with the homologous bromoacetyl derivatives of DNP ligands and the MOPC 315 protein. For example, it was shown that *N*-bromoacetyl-*N'*-DNP-ethylenediamine exclusively labeled tyrosine 34 on the light chain, whereas *N*^α bromoacetyl-*N*^ε-DNP-L-lysine labeled lysine 54 on the heavy chain. These experiments demonstrated that both chains contribute contact residues to the antibody-combining site.
2. Affinity labeling studies on antibodies derived from different species using five different reagents showed that the labeled residues were confined to three major stretches of the NH₂-terminal variable region, i.e. around positions 30, 55, 90 in either the light or heavy chain. These results clearly indicated that three restricted segments of both chains actually constitute the specific combining site. This was fully supported by independent evidence, e.g. the identification by statistical analysis of hypervariable segments (CDR) in the V region and by the isolation of the Fv fragment which provided final direct

proof that the combining site is entirely formed by the variable regions. X-ray crystallography of Fab-hapten complexes supported and extended this picture.

Affinity cross-linking

One outcome of affinity labeling using the homologous series of bromoacetyl derivatives, which labeled different residues of the light and heavy chains, was that it enabled the first demonstration of affinity cross-linking by a reagent which contained two bromoacetyl groups and a DNP moiety. This reagent cross-linked the heavy and light chains at the correct positions. Affinity cross-linking is today a major tool in biology and immunology for the identification, localization and isolation of receptors. This is usually accomplished by using a radioactive ligand that is allowed to bind to its receptor, after which a general cross-linking reagent is applied. Alternatively, a ligand to which a photoreactive group has been attached can be used; after binding to the target membrane, light is applied which establishes a covalent bond at the site of binding. Affinity cross-linking allows the identification of the labeled receptor and determination of its size by gel electrophoresis. Because it makes use of a cross-linker which is separate from the ligand, it can be applied to large complex ligands such as growth factors and other proteins. Examples of the use of affinity cross-linking in the immune system are interleukin-2 and its receptor, Fc and receptor, major histocompatibility complex (MHC) class II molecules and nominal antigen peptides on antigen-presenting cells, and photoreactive vinblastine and the 170 kDa protein from multidrug-resistant human cancer cells. Affinity cross-linking is mainly used to identify the molecular size and cellular localization of receptors. Its use, however, can be extended to study the ligand-binding site of the receptor, as has been shown for the epidermal growth factor receptor.

Immunoaffinity chromatography

Another outcome of the affinity labeling studies was the development of immunoaffinity chromatography. The localization of the modified residues in the antibody sequence requires the isolation of the labeled peptides. Givol and Wilchek introduced the immobilized antibody, with the same specificity as the labeled antibody, to 'fish out' the labeled peptides. Thus, peptides that were labeled with the DNP group could be isolated on a DNP-specific antibody column and peptides labeled with diazoarsonate were similarly isolated on an anti-phenylarsonate column. Immuno-

affinity chromatography on immobilized monoclonal and polyclonal antibody columns is the most useful approach today to isolate biologically active proteins prepared by recombinant technology and for purification of other proteins which have no known ligands. For example, a commercial preparation of human factor VIII utilizes an insoluble antibody column.

Conclusion

The most significant contributions of affinity labeling have been in the elucidation of structure–function relationships of the active site of a given biological system. For antibodies, affinity labeling provided structural information regarding the binding site before the X-ray structure was known. The techniques that have evolved from these studies are very important tools in modern biology and will continue to be so in the future. It is hoped that refined information regarding binding sites in various complex biological systems will revive the initial impetus which led to the development of affinity labeling as a method: namely, the potential for designing better and more effective drugs. Such drugs may inactivate receptors or intracellular enzymes via affinity labeling.

See also: Affinity chromatography; Antigen-binding site; Hapten.

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AFFINITY MATURATION

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The changes of antibody quality in the course of the antibody response is a classical immunological phenomenon. One such change is the gradual increase of antibody affinity for the immunizing antigen with time, called affinity maturation. Affinity maturation is typical of, and regularly observed in, T cell-dependent responses and is a key feature of immunological memory in B cells. Upon secondary immunization with a T cell-dependent antigen an anamnestic antibody response is produced, consisting of antibodies with a higher affinity than that of primary response antibodies.

Burnet's clonal selection theory provided a theor-

etical framework for affinity maturation, as shown in **Figure 1**. The B cells in the peripheral B cell pool express a clonally distributed, diverse antibody repertoire. This repertoire includes antibody specificities against any given antigen or antigenic determinant, but, in general, high-affinity antibodies should be less frequent than antibodies of average or low affinity. The clonal selection theory postulates that antigen selectively expands B cells expressing antibodies on their surface to which it can bind. With decreasing antigen concentration in the course of the response this mechanism results in the preferential expansion of B cells expressing high-affinity antibodies, as depicted in the upper part of **Figure 1**. We have

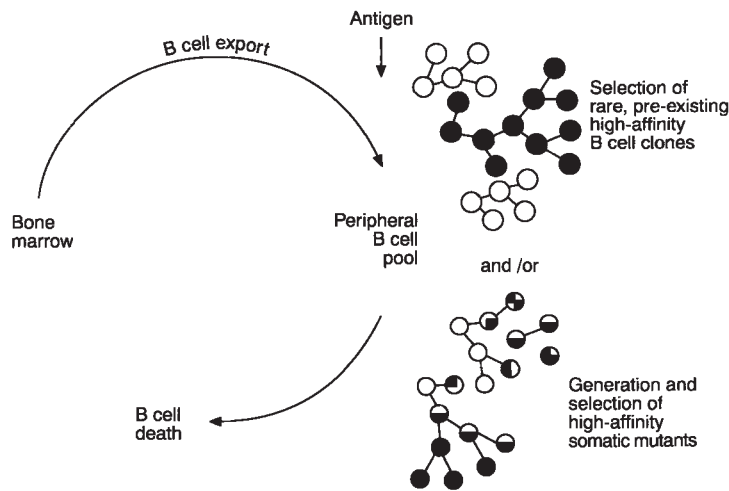


Figure 1 Models of affinity maturation. (Taken, with modification, from Rajewsky K (1989) *Progress in Immunology* 7: 400.)

learned in the meantime that the process of B cell triggering is more complex: the B cell captures antigen by its receptor antibody from antigen-presenting (follicular dendritic) cells, processes it intracellularly and exposes fragments of the antigen on its surface, in the groove of major histocompatibility complex (MHC) antigens of class II. Subsequently, T helper cells recognize the antigen-loaded MHC molecules and signal the B cell into its pathway of proliferation and differentiation. This multistage process, together with the variability of antigenic valency, makes the relation of antibody affinity to cellular selection complicated (indeed selection seems sometimes driven by the on-rate of antigen binding rather than affinity), but does not change the picture in principle.

The model depicted in the upper part of **Figure 1** is in line with experimental evidence that the B cell clones dominating secondary and hyperimmune responses to a given antigen often differ strikingly from those dominating the primary response in terms of antibody variable (V) region gene usage. However, it has become clear that the central mechanism of affinity maturation is not the selection of rare, pre-existing high-affinity antibodies, but a process of somatic evolution, in which somatic antibody mutants are generated at high rate as a consequence of antigenic contact and high-affinity mutants subsequently selected by antigen (**Figure 1**, lower part). It is often forgotten that Burnet considered this possibility already in his clonal selection theory: 'The . . . postulate, that active sites on cell surface or globulin molecules can be modified to a wider reactivity by somatic mutation, provides the chief agent to allow change in antibody character as immunization proceeds. If the primary postulates of the clonal selection theory are accepted, such a result of somatic mutation is as much in order as any other'. The tools

of molecular biology allowed this possibility to be studied directly, by isolating and sequencing antibody V genes expressed by B cells participating in model immune responses. Initially, the V genes were identified in hybridoma cells selected from various stages of the immune response. They can now be isolated from the overall B cell population or even single B cells by specific gene amplification, using the polymerase chain reaction. These experiments demonstrated that, within a short time after priming with a T cell-dependent antigen, the initial repertoire of germ line-encoded antibodies is completely changed into a repertoire of somatic antibody mutants as they are exclusively seen in secondary and hyperimmune responses. This is achieved by a mechanism introducing somatic point mutations into rearranged antibody genes at an exceedingly high rate, estimated to be in the range of 1×10^{-3} per base pair per generation. The mutations are introduced in a stepwise manner in the course of cellular proliferation and mutants expressing high affinity for the antigen are selected out in a highly efficient manner, preventing the accumulation of mutants expressing useless, potentially harmful (autoaggressive) specificities. Genetic engineering experiments have directly demonstrated that certain key mutations, often repeatedly seen in the repertoire of antibody mutants as it arises after immunization with a given antigen, are directly responsible for an increase of affinity for the antigen. However, because of the high rate of somatic mutation the antibodies also harbor a multitude of mutations which are not affinity selected (e.g. 'silent' exchanges). Affinity maturation through somatic hypermutation occurs in a particular B cell differentiation pathway, namely the proliferation of B cells and their maturation into memory or plasma cells in germinal centers. In this pathway the

hypermutation mechanism (whose molecular basis remains to be elucidated) is turned on through an unknown (T cell-derived?) signal and is later turned off to allow the stable expression of the affinity-matured secondary antibody repertoire.

Taken together, affinity maturation of antibodies is mainly based on a process of somatic evolution in which somatic antibody mutants are generated at high rate upon T cell-dependent immunization, and high-affinity mutants selected by antigen. This allows the antibody system to rapidly adapt to any given immune stimulus – the key requirement of acquired immunity. Only in this way will the system be able to deal efficiently with microbial infection, in the course of which mutants of the infecting agent often accumulate rapidly, threatening to circumvent immune protection.

See also: Affinity; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; B lymphocyte repertoire; Clonal selection; Diversity, generation of; Germinal center; Immunoglobulin genes; Maturation of immune responses; Memory, immunological; Somatic mutation.

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AFRICAN SWINE FEVER

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African swine fever (ASF) is a highly contagious acute hemorrhagic disease of domestic pigs caused by a large icosahedral DNA virus which produces a wide range of clinical signs. Protective immunity can be stimulated *in vivo* but classical neutralizing antibodies have not been demonstrated. The complete genome has been sequenced and some proteins that could enable the virus to evade the host immune response have been identified. There is no vaccine.

Characteristics of the organism and its antigens

The virus persists in the southern half of Africa, either in wildlife reservoirs – wart hogs and soft ticks (*Ornithodoros moubata*) – or as an enzootic disease of domestic pigs. After more than 30 years as an enzootic disease in domestic pigs in southern Europe it has been eradicated from Portugal (1993) and

Spain (1995). Because it has icosahedral symmetry and replicates in the cytoplasm of infected cells, ASF virus was originally classified as an iridovirus but the genome structure and replication strategy differ substantially from the Iridoviridae and are more like the Poxviridae. The virus is currently classified as the only member of a family called African swine fever-like viruses. The entire genome of a tissue culture-adapted Spanish isolate has been sequenced; it is 170 101 nucleotides long and contains 151 open reading frames which include five multigene families of unknown function.

The only cells in the pig in which ASF virus is known to replicate are those of the mononuclear phagocytic system in which virus produces hemadsorption (HAD) of pig erythrocytes to a CD2 homolog expressed on the cell surface (Figure 1).

Infection occurs by receptor-mediated endocytosis via saturable binding sites; it has been shown that

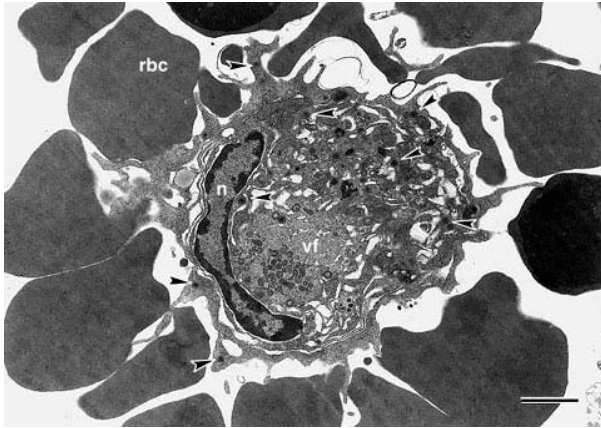


Figure 1 Hemadsorption to an alveolar macrophage infected with the Malawi isolate of African swine fever virus. The red blood cells (rbc) adhere to the surface of the infected cells producing a classical rosette. The viral structures include the perinuclear virus factory (vf) containing virus particles at various stages of assembly and cytoplasmic virus (arrow heads) prior to leaving the cell by budding. Cell nucleus (n). The bar represents 5 μ m. (Provided by S.M. Brookes).

one receptor-binding protein of the virus is a low molecular weight protein (12 kDa) expressed as a homodimer (17 kDa).

Virus is released from infected cells by budding and acquires an external lipoprotein envelope containing glycosylated components; extracellular virus does not contain glycoproteins. The virus contains over 50 structural proteins, the most highly conserved of which are good immunogens and are used as antigens for serological tests to detect virus. There is no clear identification of virus strains by traditional serological methods but some differentiation is possible by monoclonal antibodies and antibodies that inhibit HAD. There is evidence that field isolates contain viruses with variable polypeptides, that these may also vary on passage in pig macrophages and that there is a complex variety of virus serotypes. Different and distinct genotypes have been identified by restriction enzyme analysis.

Immune response of the host

Circulating antibodies in infected pigs can be detected by radioimmunoassay or ELISA 3–4 days after infection and they persist for life. Neutralizing antibodies have never been demonstrated. After recovery from infection with less virulent isolates, pigs are persistently infected for at least 6 months and are resistant to challenge with homologous virus, although the challenge virus may undergo limited replication without producing clinical disease; they are usually fully susceptible to infection with different virus genotypes.

Pigs given passive antibody or piglets given colostrum antibody have reduced levels of virus in the blood and tissues when challenged with virulent virus and show little or no clinical signs of disease. Studies *in vitro* have also shown that antibodies can reduce virus yield from infected pig macrophages in the absence of complement but the transfer of immune lymphocytes has no effect on the course of disease.

Antibodies which mediate lysis of ASF virus infected pig kidney cells (IBRS2) *in vitro*, by either antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-mediated lysis, are present in serum 12–14 days after infection but are not able to lyse infected pig macrophages.

Because the humoral immune response does not appear to mediate the immunity of pigs which recover from infection, the cell-mediated immune responses have been investigated to determine what immune mechanisms may be important, and the following observations have been made.

Natural cytotoxic cells are also unable to lyse infected macrophages but may be important as producers of interferon γ (IFN γ). Some isolates of ASF virus can induce IFN γ production, and recombinant bovine IFN α and porcine IFN γ reduce infectious virus production by pig alveolar macrophages and monocytes *in vitro*.

Stimulation of peripheral blood lymphocytes from uninfected pigs with recombinant porcine interleukin 2 (IL-2) will induce a lymphokine-activated killer (LAK) cell which will lyse infected pig macrophages. Lymphocytes from recovered animals stimulated with ASF virus *in vitro* will produce IL-2, which indicates that a lymphokine-activated cytotoxic lymphocyte may be involved. ASF virus-specific cytotoxic T lymphocytes obtained from infected inbred pigs with defined SLA haplotypes are major histocompatibility complex (MHC) class I restricted.

Evasive strategies by the organism

The interaction of ASF virus with the host cells and its immune system and the processes involved in producing disease are still poorly understood; however, the complete genome sequence has indicated that some novel strategies may be employed to avoid the immune response, by direct and indirect effects of the virus on the cells of the immune system, in the absence of neutralizing antibody.

ASF virus replication in pig macrophages, and the release of immune mediators, may be important in aiding the virus to evade the immune system, in addition to being possible factors in the pathogenesis of the disease. Infection of pig macrophages does not

alter the expression of Fc receptors nor their ability to mediate ADCC of antibody-coated bovine erythrocytes but does modulate antibody-mediated phagocytosis and chemotaxis. A protein isolated from infected cells has been shown to have a strong immunosuppressive effect *in vitro*. Infection of these cells will cause the downregulation of the pro-inflammatory cytokines IL-8, IFN α and tumor necrosis factor α (TNF α) but not of TGF β .

The ASF virus genome also encodes for a Bcl-2 homolog which may aid the survival of infected cells and maintain persistence. In contrast, lymphocytes in infected tissues have been found to be apoptotic. Thus ASF virus may downregulate inflammation, increase the survival of infected cells and cause apoptosis of surrounding effector cells.

Although virus-specific delayed-type hypersensitivity, antibody production and lymphoproliferation are readily demonstrable, there are reports that the more virulent isolates can interfere with the immune response to foot-and-mouth disease virus in pigs inoculated with this virus 3 days after infection with ASF virus.

Vaccines

There are no commercial vaccines available for ASF. All attempts to produce suitable vaccines from either inactivated virus or viruses attenuated by passage in culture have been unsuccessful. There are reports in which a limited number of pigs were protected from death, but not clinical disease, after inoculation with

infected spleen treated with a nonionic detergent in Freund's complete adjuvant.

See also: Iridovirus, infection and immunity; Porcine immune system; Poxvirus, infection and immunity; Viruses, immunity to.

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AGGLUTINATION

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A variety of particles bearing either natural or conjugated epitopes on their surfaces are specifically agglutinated by polyclonal or monoclonal antibodies to these epitopes. These particles can be inert but bearing these epitopes on their surfaces. They can also be large viruses, eukaryotic cells, or a variety of other cells, including bacteria, yeasts, fungi, protozoa and parasites. Inasmuch as very low concentrations of these antibodies can specifically agglutinate these cells, this reaction has served as a very sensitive and specific assay for the presence of specific antibodies in the serum or body fluids, e.g. antibodies to the bacillus of typhoid fever. The reaction can also be

employed for the detection and measurement of antigen in the serum or body fluids. Agglutination is rarely used in research and for diagnosis because it has not been possible to easily and precisely quantitate this reaction. Therefore, it has been replaced by more quantifiable assays for antibodies, including the ELISA (enzyme-linked immunosorbent assay), various radioimmunoassays and other types of immunoassays. More recently, however, methods which facilitate, speed up and make more precise the reading of agglutination reactions may eventually result in the more frequent use of this very sensitive reaction for both research and diagnosis.

History

Specific bacterial agglutination was first observed about 100 years ago by Charrin and Roger. *Pseudomonas pyocyanea* grown in the serum of an immunized animal yielded a clear supernate with clumps of microbes at the bottom of the culture tube, whereas organisms cultured in normal serum were uniformly distributed throughout the tube. Bordet was the first to suggest that specific agglutination could be used for the diagnosis of infectious diseases. Widal and Grunbaum in 1896 applied this reaction to the diagnosis of typhoid fever. Bordet and Gay made the first fundamental analysis of the mechanisms of this reaction.

Landsteiner and Ehrlich, and Morgenroth in 1890 independently observed that normal human sera agglutinated the erythrocytes of other individuals. They also found that the injection of human red cells induced the formation of antibodies which by agglutination could distinguish the blood cells of different individuals within the species. These observations formed the basis of blood typing for transfusion.

In 1951 Boyden reported that proteins are observed on tannic acid-treated erythrocytes and that these cells are specifically agglutinated by antiprotein sera. Battisto and Chase, in 1963, extended this passive hemagglutination method to the detection of antibodies to haptens by the absorption of soluble hapten-protein conjugates to tanned erythrocytes.

Coombs and colleagues detected 'incomplete' Rh antibodies by the use of a second antiglobulin reaction. Pickles showed in 1946, that these 'incomplete' antibodies specifically agglutinated enzyme-treated erythrocytes. Bozicevich and coworkers in 1951 adsorbed various antigens to bentonite particles and observed that these particles were then flocculated by specific antisera. In 1956 Singer and Plots demonstrated that human gammaglobulin-coated polystyrene latex particles could be used for the detection of rheumatoid factors in human sera.

Boyd and Renkonen independently reported in 1946 and 1947, respectively, that different seed extracts contained substances which selectively agglutinated the erythrocytes of various species through binding to particular polysaccharides on the cell surface. These plant proteins are called lectins.

Definitions and classification of agglutination reactions

Agglutination is defined as the formation of clumps of cells or inert particles by specific antibodies to surface antigenic components (direct agglutination) or

to antigenic components adsorbed or chemically coupled to red cells or inert particles (passive hemagglutination and passive agglutination, respectively). Erythrocytes are also agglutinated by nonantibody substances such as plant proteins, viruses, salts of heavy metals, inorganic colloidal acids and bases, and basic proteins (protamines, histones). Agglutination inhibition or hemagglutination inhibition refers to the inhibition of these reactions by soluble antigen which reacts with the combining sites of the antibodies and thereby prevents their binding to and agglutination of the particles.

Mechanisms of specific agglutination

Agglutination occurs in two stages: first, specific combination of antibody with antigen on the cell surface; second, formation of visible aggregates of the particles. According to Bordet, the antibody sensitized the cells to subsequent nonspecific agglutination by electrolytes but the alternative 'lattice' theory suggested that the cells were agglutinated through their specific linkage by bivalent or multivalent antibodies. The bulk of the evidence favors the 'lattice' theory, but under some conditions the second stage may be nonspecific so that cells of different antigenic specificities are coagglutinated in the presence of antibody to only one of the specificities. Usually, however, the second stage is also specific.

Agglutination may be influenced by many factors, including ionic strength (0.15 M sodium chloride is optimal), temperature (from 0 to 30°C agglutination is increased), and viscosity of the medium. Hemagglutination cannot be achieved with certain immunoglobulin G (IgG) class anti-protein or anti-blood group antibodies. Erythrocytes suspended in isotonic saline on account of mutual repulsion by negative zeta potential and intercellular attraction induced by van der Waals forces cannot approach each other more closely than 5–10 nm. The two valences of single IgG class antibodies are at most about 12 nm apart; therefore, they cannot bring the erythrocytes close enough together to agglutinate them. On the other hand the two diametrically opposed valences of the potentially decavalent IgM antibodies can easily bridge a distance of about 300 nm. Hemagglutination by IgG antibodies can be achieved by extending the IgG antibodies with anti-IgG antibodies or through the treatment of erythrocytes with enzymes which permits a much closer approach of the erythrocytes.

It seems paradoxical that agglutination often does not occur in the presence of the highest antibody concentrations. This is called the prezone or prozone phenomenon. This phenomenon may be due to the

coating of the antigenic particles with such large numbers of antibody molecules that the number of cross-particle linked antigen-antibody complexes is decreased. The presence of nonagglutinating or blocking antibodies or other nonspecific factors which inhibit agglutination has not been excluded.

Applications of agglutination and agglutination inhibition reactions

Because of the ease of their performance and high sensitivity, specific agglutination reactions have been employed for: 1) the serological diagnosis of infectious diseases through the detection of a rising titer (concentration) of serum antibody; 2) screening for specific tissue-directed antibodies in connective tissue and autoimmune diseases; 3) detection of the presence of antigenic determinants on cells (mixed agglutination reaction); 4) blood typing and cross-matching procedures for blood transfusion, including the use of antiglobulin as a second antibody to directly cross-link incomplete agglutinins bound to Rh and ABO blood cell antigens; 5) diagnosis of viral infection: influenza, rubella and other viruses that directly agglutinate erythrocytes. The inhibition of viral hemagglutination by specific antibody to the virus is a particularly useful and sensitive test for titers of antiviral antibody.

A number of lectins are employed in blood banks for the hemagglutination of erythrocytes through binding to A, B and H blood group substances, thus providing an alternative method of identification of blood group substances.

Quantitation

The agglutination method has fallen somewhat into disuse for research and, to some extent, even for diagnosis because it is not possible to precisely quantitate the reaction. Concentrations of agglutinating antibody are assayed by preparing a series of doubling dilutions of the test serum, adding cells to each dilution, incubating the cell suspension, and then observing the highest dilution of the serum which produces a visual agglutination pattern at the bottom of the tube. Thus, failure to observe the presence or absence of agglutination in one tube or one doubling dilution represents a 100% error in determining the quantity of antibody. Therefore, agglutination has been replaced by more quantifiable assays, including the ELISA, various radioimmunoassays and other types of immunoassays. However, agglutination can be quantitated. The actual amount of antibody nitrogen or protein bound by cells can be determined, but is too complicated and time-consuming to be a rou-

tine assay. Modern technology has provided a number of methods for the quantitation of agglutination through measurements of turbidity, light scattering or the mean diffusion constants of the particulate suspension.

Automated reading and processing of agglutination results

Microplate agglutination techniques are commonly used for the quantitative or qualitative isotypic analysis of specific antibodies, but require optical reading by the researcher and, therefore, can be quite subjective. Aubert and colleagues have used an automatic reader scanning each of the wells of a standard microtiter plate. The advantages of the automatic reader were additionally maximized by coupling it to a dedicated computer running customized software designed to process data coming on-line from the spectrophotometer. This approach was applied to the diagnosis of human toxoplasmosis and candidiasis. Suspensions of *Toxoplasma gondii* tachyzoites or of sensitized erythrocytes were used for the determination of IgG antibodies or the quantitation of IgM-, IgA- or IgE-specific isotypes.

Rapid agglutination testing in an ultrasonic standing wave

The time required to perform diagnostic agglutination tests can be significantly reduced by applying an ultrasonic standing wave field to a droplet of reagents in a capillary tube. Avian erythrocytes, bacteria and latex particles were agglutinated in 15 s, 5 min and 1 min, respectively with no loss in sensitivity or specificity. Moreover, a series of five droplets in a single capillary can be tested in less than 4 min by drawing the capillary along the axis of the ultrasonic field of a ring transducer.

Agglutination as a protective mechanism in infections

Specific agglutination of bacteria by antibody *in vivo* is observed only with rather enormous numbers of bacteria. Therefore, this serologic reaction is presumably not very important in natural infections of the bloodstream where enormous numbers of bacteria are unusual. In a local lesion, however, where the number of bacteria may be high, it is possible that agglutination of bacteria by antibody may aid in localizing the infection as well as keeping the growing bacteria in a clump rather than disseminating.

See also: **Alloantigens; Antigens; Antiglobulin**

(Coombs') test; Autoantibodies, tests for; Bacterial cell walls; Erythrocytes; Lectins; Phytohemagglutinin (PHA); Valency of antigens and antibodies; Viruses, immunity to.

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AGING AND THE IMMUNE SYSTEM

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Lifespan and hence the rate of aging is a component of the evolution of different species. The magnitude of lifespan affects programs for sexual maturation and subsequent decline of reproductive activity. Body size and metabolic activity are in turn correlated with capacity for repair; this has been demonstrated for excision repair after DNA damage induced by ultraviolet light. Since the capacity for repair is limited, damage will ultimately result in age-related decline of synthetic capacity. While the quantity of products and the propensity for cell division generally diminish as a function of advancing age, amino acid sequences of proteins and nucleotide sequences of DNA do not change with age.

Having referred to factors which may determine differences in age-related progression of different species, we shall turn to differences in aging which occur in different individuals of the same species. It is reasonable to assume that some or many of these result from the presence of multiallelic genes with products which differ in their capacity to overcome wear and tear. This is certainly the case for individual differences in onset of diseases caused by inborn errors of metabolism. The age of the onset of several genetic diseases is related to the functional competence of the molecule which is defective in a

given disease. The absence of a particular molecule crucial for a given function, or the synthesis of an incompetent molecule, results in an early onset of the disease; the disease onset may be delayed if the molecule retains some functional capacity (e.g. Duchenne Becker muscular dystrophy, Sandhoff disease).

Aging of the immune system involves changes in the capacity for self-renewal, in the relative preponderance of cell populations and in the quantity of immunoglobulin molecules of different isotypes and of different interleukins. We have relatively little definitive information on the exact nature of changes in the capacity for self-renewal of stem cells and of precursor cells. There appears to be an age-related decrease in the number of cells which synthesize terminal deoxynucleotidyl transferase (TdT) and are precursors of both T and B cells. However, it remains possible that the observed decrease does not depend on changes in the precursor cells themselves but is due to regulator cells: feeder cells from old mice cannot support the *in vitro* growth of TdT⁺ cells, while feeder layers from young animals can do so. In short it is possible that self-renewal decreases with age as a function of regulator cells rather than of precursor cells themselves.

Age-related changes in the quantity of molecules

may be controlled by feedback mechanisms. There is considerable polymorphism in the quantity of molecules produced in young individuals of different genetic backgrounds. The quantity produced by the youthful organism is in many cases directly correlated to the extent of subsequent decrease; this correlation has been referred to as 'economic correction'.

In the immune system the most striking age-related change occurs in the thymus, in which some cell populations decrease and others show a compensating increase up to late middle age when the total number of self-renewing thymus cells becomes drastically reduced. These central cellular changes cause marked age-related changes in various subpopulations of T cells, and particularly in different classes of suppressor and of helper cells.

Individual differences, i.e. the polymorphism of age-related changes, will be exemplified in terms of two classes of suppressor capacity, one affecting the humoral, the other cell-mediated immunity.

Suppressor capacity in the thymus, affecting humoral response, can be measured by reconstitution experiments, in which lethally irradiated mice are given lymphocytes of young donors, mixed with thymus cells from donors of different ages; the reconstituted mice are then immunized, and antibody is measured. In some strains of mice, thymus cells from young donors reduce the humoral response of the lymphocytes, while thymus cells from old donors increase it.

Changes in suppressor capacity for cell-mediated immunity can be measured by the ability of thymus cells to prevent lymphocytes to be sensitized by targets and to kill them.

Within the same strain the potency of the two classes of suppressor capacity, i.e. suppressor capacity for humoral immunity and suppressor capacity for cell-mediated immunity can change in opposite directions. The independence of aging of the two classes can also be seen when progression of age-related changes are modified by nutritional, hormonal or pharmacological interventions; these can affect aging of one class without affecting aging of the other. In general, it seems that age-related changes of the two classes vary as if they were controlled by two different genes.

We shall now turn to changes in T helper cells, in terms of interleukins. In mice of different strains, interleukin 1 (IL-1) and IL-2 decrease with age, though to different extents. In striking contrast, IL-3, IL-4, IL-6 and IL-10 increase up to a fairly advanced age. These contrasting changes in the direction of age-related alterations of different interleukins may be attributable to relative changes in the proportion of two sets of T helper cells, T_H1 and

T_H2 cells, the latter include memory cells. Thus, changes of interleukins may result from a lifelong accumulation of encounters with foreign macromolecules, and so reflect immunological experiences.

In summary, considerable age-related changes in suppressor cells occur to an extent which shows individual variation; different types of suppressor cells can change in opposite directions in the same individuals. Changes in helper cells are attributable to changes in the relative proportion of subpopulations and manifest themselves in age-related changes in the quantity of different interleukins. In some strains of inbred mice these changes can result in a decrease in humoral response. In other strains of mice a marked decrease in suppressor cells for the humoral response can result in autoimmune disease.

In the heterogeneous human population, there is an age-related increase in the proportion of individuals who show a decrease in the capacity to make a potent immune response and a greater tendency to make autoimmune responses. From the foregoing discussions on age-related changes in different strains of inbred mice, it is clear that both changes observed in human populations can be interpreted as resulting from the heterogeneity of the population i.e. from individual differences in genetic background; some individuals have a tendency to the changes which we have observed in the age-related patterns of certain inbred strains of mice. In this context, it is intriguing that certain interleukins seem to increase in each of the five inbred strains examined; the question arises whether these changes might be advantageous.

In fact, this is not necessarily the case. Immunity to leishmania can be reduced via IL-3 and IL-4, which impair the capacity of macrophages to kill intracellular organisms. IL-4 can directly inhibit the induction of cytotoxic T lymphocytes (CTL) and lymphokine activated killer (LAK) cells against virus-infected cells and tumors. It is possible, therefore, that increases in interleukins may reduce the ability of the immune system to deal effectively with some infections. However, this is not necessarily applicable to all infections, indeed some changes of old age can have adaptive advantages. This appears to be the case in age-related decreases of factors which affect growth of certain tumors. In one situation, age-related decrease in the growth of some tumors is attributed to age-related decrease in the production of angiogenic factors by T cells of aged animals.

In several strains of mice, NK cells have been found to increase to middle age and then to decrease. Similar observations have been made with respect to LAK cells, which show polymorphism in the quantity of these cells in young individuals and subsequent decreases that are proportional to the magnitude of

the youthful level, i.e. 'economic correction'. There is a diversity of findings concerning the aging of human NK cells. One group reports an age-related decrease; others find no change; yet others find an increase of NK function with aging, most often correlated with an increased number of NK cells. Thus it might appear as if NK cells of humans and mice age in a different manner. In fact, this difference may be due to the difficulty of analyzing the relation between a given parameter in old, middle-aged and young human populations, since individuals with certain genetic traits are eliminated before old and middle age, but are present in the young population.

Some age-related changes of B cells are a manifestation of change in T cells and the accumulation of memory. However, there is some evidence that B cells from old mice are specifically defective in their ability to progress from the G₀ phase into the S phase of the cell cycle.

Having examined aging in T, NK and B cells, it remains to summarize the reason for the increase in autoimmunity in aging human populations. In part this may be attributable to the loss of suppressor capacity as has been found in some strains of inbred mice. In part it may be due to accumulation of denatured proteins which is the consequence of the age-related decrease in proteolytic enzymes. These denatured proteins may cause a breakdown of the tolerance which exists for intact autologous molecules.

Age-related changes are clearly compartmentalized in terms of different types of T suppressor cells and in terms of different subsets of helper cells. There is also a difference in age-related changes in the mucosal system and in the peripheral immune system; the former appears to be much less subject to age-related changes than the latter. The quantity of NK cells in mice goes through a peak in middle age and then declines moderately. In humans, this decline has not been observed and selected groups of aged individuals appear to have high levels of NK activity. There may be a decline in the self-renewal capacity of some stem cells but it is not clearly established whether or not this is in fact the outcome of the effect of controlling cells which prevent proliferation.

See also: Effector lymphocytes; Sex hormones and immunity; Suppressor T lymphocytes; Thymus.

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ALLELIC EXCLUSION

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In agreement with the proposal of the 'clonal selection theory' by Burnet in 1959, Nossal and Mäkelä could show in 1961 that indeed each B cell produced many copies of only one type of antibody. Thus each B cell is restricted to one specific antibody. With the discovery of allotypes another restriction of B cells was shown. Allotypes are serological markers on heavy or light chain of immunoglobulin (Ig) which differ among genetically different strains of one species. Anti-allotypic antisera can be produced by immunizing strain A₁ with strain A₂ Ig. Using anti-allotypic sera, Pernis, Chiappino, Kelus and Gell, as well as Cebra, Colberg and Dray, showed in 1965 that in an (A₁ × A₂) F₁ animal B cells express either A₁ or A₂ but not both allotypes on their cell surface. Hence, B cells are restricted to express Ig chain of only one of their two alleles, a phenomenon which is called 'allelic exclusion'.

The allelic exclusion control ensures that each B cell expresses only one type of binding site because an antibody binding site is determined by the combination of a particular heavy and light chain. Figure 1A shows that a B cell with a defined binding specificity can be efficiently selected and activated by the corresponding antigen. If expression of the Ig alleles was not regulated each B cell would express several combinations of the two different heavy and light chains and thus express a variety of antigen

specificities (Figure 1B). Such unregulated B cells would not be able to mediate a specific immune response.

Allelic exclusion in the context of V gene assembly

Most cells of the vertebrate species have a diploid chromosomal organization and express both alleles of an active gene. The Ig genes are an exception to this rule because only one of their alleles is expressed as a functional product. This phenomenon is connected with another special feature of the Ig genes; namely, that the gene for the variable part of an Ig chain (V gene) is not encoded in the germ line but assembled from different gene segments during B cell development. The genes for the variable region of the heavy (H) and light (L) chain are assembled out of three (V_H, D and J_H) or two (V_L, J_L) germ-line gene segments, respectively. This assembly is mediated by two recombinase proteins, RAG-1 and RAG-2 which are only expressed in cells of the lymphoid lineage. The V gene segments are found in multiple different copies at the IgH and the two IgL gene loci (Igκ and Igλ). V Gene rearrangements occur in the developing B cell in an ordered fashion first at the IgH locus and only later at the Igκ locus. Before the segments are assembled into either a V_HDJ_H or a V_LJ_L complex

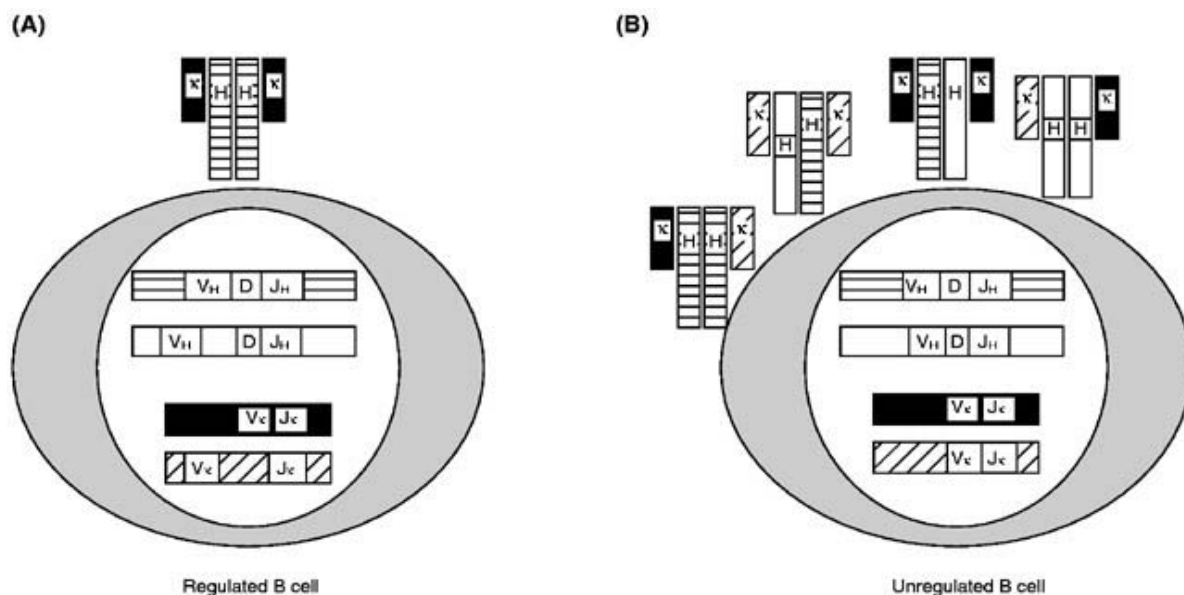


Figure 1 Allelic exclusion ensures expression of a single specificity of antigen-binding site on the surface of the B cell.

their ends are frequently modified by the removal and/or addition of nucleotides. These modifications increase the variability of the expressed V genes. However, they may also result into a nonfunctional V gene in which the V_H or V_L segments are not joined in the correct reading frame to the DJ_H complexes or to the J_L segments (out of frame join). When the first sequences of both IgH and Ig κ alleles of B cells became available it was noticed that in most cases only one allele carried a functional (in frame) V_HDJ_H or $V_\kappa J_\kappa$ complex. At the Ig κ locus the second allele either did not carry any V gene rearrangements (germ-line configuration) or had assembled a nonfunctional $V_\kappa J_\kappa$ complex. At the IgH locus the second allele carried either a DJ_H or a nonfunctional V_HDJ_H complex. In a first attempt to explain these data it was proposed that allelic exclusion was simply due to a high frequency of nonfunctional V gene rearrangements (stochastic model). This model assumed that a V gene rearrangement occurs only once per allele and that due to the low frequency of a productive V gene rearrangement most developing B cells have either no or only one functional V gene. The latter population would then

be selected by the immune system. However, in studies of Abelson murine leukemia virus-transformed pre-B cell lines it became clear that the V gene assembly is a very dynamic process which can occur repeatedly on one allele. This is due to the fact that V gene segments are present in multiple different copies at each Ig locus. Thus a nonfunctional $V_\kappa J_\kappa$ complex can be deleted and replaced by a functional $V_\kappa J_\kappa$ complex on the same allele simply by joining an upstream V_κ to a downstream J_κ segment. Similarly a nonfunctional V_HDJ_H allele can become productive by performing a V_H to V_HDJ_H join. Further studies with pre-B cell lines also showed that the frequency of a functional V gene rearrangement was not very low. In a developing pre-B cell line roughly 30% of the analyzed V gene rearrangements are productive. However, in this analysis no cell line was found with a productive V gene on both alleles.

These data support the regulated model of allelic exclusion as proposed by Alt and Baltimore in 1982. According to this model the products of a functional V gene rearrangement exercise a feedback control preventing any further V gene assembly at a particular Ig locus. Thus in pre-B cells V_H gene rearrange-

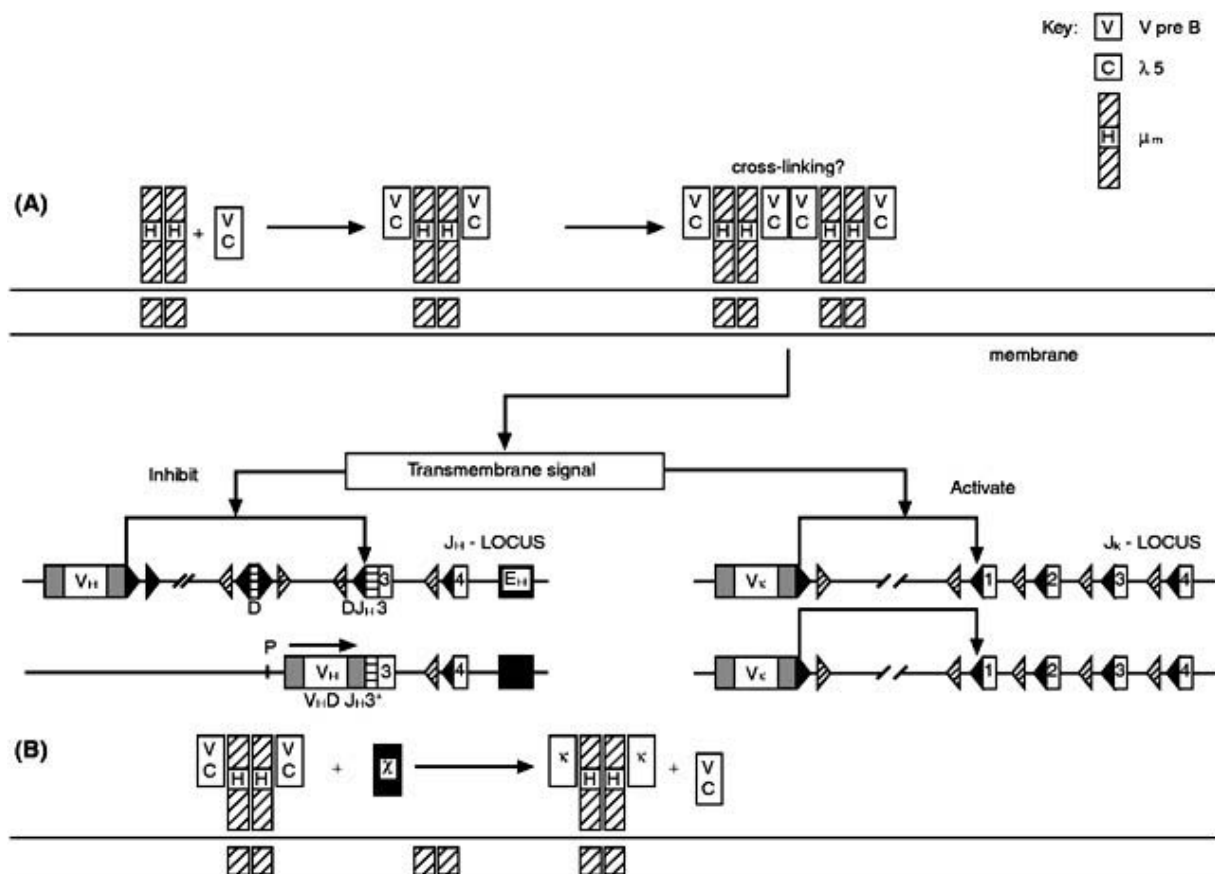


Figure 2 Transmembrane signals regulate antigen-receptor gene rearrangement.

ment occurs on one or both IgH alleles until a productive V_HDJ_H complex is formed and a μ chain is produced. The μ chain then generates a signal preventing any further V_H gene assembly on both IgH alleles. Specifically it is the membrane-bound form of the μ heavy chain which exerts this feed back control, preventing any further V_H gene assembly on the two IgH alleles. In the endoplasmic reticulum of the pre-B cell the μ heavy chain binds to the surrogate light chain proteins VpreB and $\lambda 5$ as well as to the Ig α /Ig β heterodimer, the signaling component of the B cell antigen receptor. Together these molecules compose the pre-B cell receptor which at the cell surface initiates the signal for the expansion of the pre B cell clone and the inhibition of further V_H to DJ_H rearrangement (Figure 2A). Pre-B and pro-B cells in mutant mice lacking μ heavy chain, $\lambda 5$ or Ig β expression do not show allelic exclusion of the IgH alleles, thus demonstrating the importance of the pre-B cell receptor in this control. How signals from the pre-B cell receptor stops further V_H gene rearrangement is not known at present; presumably the V_H segments are the target of this control as the J_H-C_H locus is already transcriptional active during pre-B cell development. During the expansion of the pre-B cell clone the RAG genes are switched off, but are re-expressed later on so that V_L to J_L rearrangements can occur. These rearrangements continue until a light chain is produced which can bind to the pre-existing μ heavy chain (Figure 2B). The subsequent expression of a B cell antigen receptor on the cell surface results in termination of further V_L to J_L rearrangements, thus establishing allelic exclusion of the IgL loci. Allelic exclusion occurs not only at the IgH and IgL loci but also at the TCR β chain locus. Similar to that which has been described above for the pre-B cells, the control of the TCR β chain locus is mediated via a pre-T cell receptor consisting of the CD3 complex, the TCR β chain, and the surrogate

TCR α chain pre-T α . In contrast to the IgL loci the TCR α chain locus does not show a tight allelic exclusion control; why this is so remains to be established.

See also: B lymphocyte differentiation; Clonal selection; Immunoglobulin, cell surface; Immunoglobulin genes.

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ALLERGENS

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The modern history of 'allergens' dates back to 1873, when Charles Blackley showed that wind-borne pollen grains caused the symptoms of June hayfever and that aqueous pollen extracts caused immediate wheal and flare reactions when scratched into the skin of a hayfever sufferer (himself). At the turn of the century, the causative agents of this

'supersensitivity' reaction were defined as allergens (Von Pirquet) and this form of immunity was shown to be mediated by homocytotropic (or 'reaginic') antibody (Prausnitz and Kustner). These historic developments are summarized in Table 1. By the 1920s, it was known that inhalation of allergens derived from pollens, house dust and animals, or

Table 1 A brief synopsis of the history of allergens

1873	Blackley	Showed that windborne pollen grains caused hayfever and that pollen extracts caused wheal and flare reactions when 'scratched' into the skin
1906	Von Pirquet	Defined 'allergy' as supersensitivity to foreign antigens or 'allergens'
1911	Noon	Used desensitizing injections of pollen extracts ('pollen toxin') to treat hayfever
1916	Cooke	Demonstrated familial inheritance of hayfever/asthma, i.e. a genetic trait
1921	Prausnitz and Kustner	Passive transfer of immediate skin test reactivity using serum from an allergic patient (the P-K test)
1940	Loveless	Production of 'blocking antibodies' during immunotherapy
1960s	Osler and Lichtenstein	Development of histamine release assay (1963)
	Voorhorst and Spijksma	Discovery of house dust mites (1964)
	King, Norman and Marsh	First allergens purified from ragweed (antigen E) and rye (Rye 1) (1964–1965)
	Ishizaka and Ishizaka	Discovery of IgE (1967)
	Norman and Lichtenstein	Controlled trials of ragweed immunotherapy (1966–1970)
1980	Chapman and Platts-Mills	Purification of dust mite allergen, Der p 1
1988	Chua and Thomas	First allergen to be cloned – Der p 1

ingestion of foods, was associated with the clinical symptoms of hayfever, asthma, atopic dermatitis or food allergy, and that these conditions affected 10–20% of the population. This was a 'golden era' of research on allergic disease and, indeed, the first edition of the *Journal of Immunology* contained an article by Robert Cooke, some 100 pages long, on the genetics of asthma, showing that the disease had strong hereditary links. The second golden era was in the 1960s, when the first allergens were purified from ragweed and rye grass pollen, histamine release assays were developed, and the landmark studies of the Ishizakas established that immediate hypersensitivity reactions were mediated by a new class of antibody, termed immunoglobulin E (IgE).

Inhalation of environmental allergens is the most common cause of IgE responses in humans and this feature distinguishes this class of antigens from other macromolecules or infectious agents which, with the exception of helminth parasites, do not usually cause IgE responses. There has been tremendous recent progress in research on allergens. Most important allergens from pollens, dust mite, animal danders, insects and foods (e.g. peanut, shrimp) have now been cloned and expressed as recombinant proteins. Amino acid sequence data derived from these studies have been used to establish the structure and biologic function of allergens, to identify the antigenic sites that are involved in IgE responses, and to investigate cellular mechanisms of inflammatory responses in asthma and atopic dermatitis. Recombinant allergens are being introduced for the diagnosis of allergic diseases and for developing therapeutic products. Allergens are one of the most well-defined groups of biomedically important proteins and we are now entering a new era in which the molecular basis of

the immune response to these proteins is becoming established.

Nomenclature and general properties

Atopic individuals become sensitized to allergens through exposure by inhalation (e.g. pollen grains, mite feces, animal danders, cockroach secretions, and fungal spores), by ingestion (e.g. milk, egg, peanut, fish), and by injection (e.g. insect venoms and parenterally administered drugs, such as penicillin). In general use, the term allergen can refer to these sources, to aqueous extracts prepared from the source materials, or to highly purified or cloned allergen proteins. The systematic nomenclature for purified allergens developed by the World Health Organization and International Union of Immunological Societies was revised in 1994. The nomenclature uses the taxonomic name of the source material and an Arabic numeral to describe the allergen, in the chronologic order of identification and purification. In the abbreviated form, the first three letters are used for the genus, followed by a single letter for the species. For example, ragweed antigen E (the first pollen to be purified) is *Ambrosia artemisiifolia* allergen 1, or Amb a 1. To be included in this nomenclature, newly described allergens have to have clearly defined molecular properties and to have demonstrable allergenic importance according to criteria established by the IUIS subcommittee on allergen nomenclature (Table 2). This committee reviews all proposals for inclusion of allergens in the nomenclature. The aim is to avoid redundancy and to ensure consistent use of allergen names in scientific and medical journals.

In most cases, multiple allergic proteins have been

purified from a given source. Thus more than ten different allergens have now been isolated or cloned from house dust mites or grass pollens. Usually, 80–90% of patients make IgE antibody responses to one or two proteins, e.g. mite allergens from *Dermatophagoides pteronyssinus*, Der p 1 and Der p 2, and these 'major' allergens are often used for immunologic and clinical studies. The prevalence of IgE antibodies to other 'minor' allergens is lower (<40%) and there is also evidence that a greater proportion of IgE is directed against the major allergens.

Allergens possess several characteristic features that appear to be important for immediate hypersensitivity reactions. They are usually low molecular weight proteins or glycoproteins (5–50 kDa) that are readily soluble and elute rapidly (within 1 min) from the allergen source, e.g. pollen grains or mite feces. These properties facilitate rapid penetration at mucosal surfaces, cross-linking of IgE antibodies on the mast cell surface, and mediator release to give immediate symptoms. Some allergens, such as penicillin and low molecular weight chemicals, act as haptens and require binding to a protein carrier (usually albumin) in order to elicit IgE responses. Other characteristic features of inhaled allergens are that allergic individuals become sensitized following exposure to extremely low doses of inhaled allergen, typically <1 µg of a single mite or pollen allergen per year, with no apparent adjuvant, on airborne particles of ~5–40 µm diameter. This form of

immunization appears to prime preferentially for IgE antibody production and may be important in generating T cell (T_H2) responses with cytokine profiles that are required for IgE production.

Molecular biology

Cloning and sequencing

By the mid-1980s a large number of allergens had been purified using biochemical techniques, but in general the yields were poor and, apart from physicochemical properties, the molecular structures of these proteins were poorly defined. With the advent of molecular cloning techniques, the primary structures of many allergens were rapidly established and, by screening cDNA expression libraries with IgE antibodies, new allergens were identified and sequenced. More than 100 allergen sequences are now listed in protein databanks (GENBANK) and a partial listing of major allergens is shown in Table 3.

The aim of cloning and sequencing is to obtain the primary structure of the allergen and to express the allergen in recombinant form. For many major allergens, N-terminal or internal amino acid sequences, and monospecific antibodies, were available to confirm the identity of cDNA clones. In some cases, e.g. cat allergen, Fel d 1 or *Aspergillus* allergen, Asp f 1, the nucleotide sequences were obtained by polymerase chain reaction (PCR) using primers derived from amino acid sequence. However, the usual approach has been to isolate mRNA from allergen source material (e.g. pollen grains, mite or cockroach bodies, fungal spores) and to use reverse transcriptase to generate double-stranded cDNA, which is then packaged into λgt11 or similar expression vectors. The cDNA libraries are screened with pooled IgE antibodies from 6–8 allergic patients, or with high titer monospecific polyclonal antibodies, to identify cDNA clones expressing allergen. Increasingly, allergens are being identified solely based on screening cDNA libraries and this approach has been very successful for allergens which were previously ill defined, e.g. fungi (*Alternaria*, *Aspergillus*, *Trichophyton*); foods (peanut, shrimp); and insects (cockroaches, fire ants, hornets).

Protein families and biologic function

Phylogenetically related species usually produce allergens that share significant amino acid sequence homology. For example, the group 1 allergens from *D. pteronyssinus* (Der p 1) and *D. farinae* (Der f 1) show 80% homology and the group 2 allergens from these mites show 88% homology. Allergens showing

Table 2 Allergens: criteria for inclusion in the WHO/IUIS nomenclature

1. The molecular and structural properties should be clearly and unambiguously defined, including:
 - Purification of the allergen protein to homogeneity
 - Determination of molecular weight, pI and carbohydrate composition
 - Determination of nucleotide and/or amino acid sequence
 - Production of monospecific or monoclonal antibodies to the allergen
2. The importance of the allergen in causing IgE responses should be defined by:
 - Comparing the prevalence of serum IgE antibodies in large population(s) of allergic patients. Ideally, at least 50 or more patients should be tested
 - Demonstrating biologic activity, e.g. by skin testing or histamine release assay
 - Investigating whether depletion of the allergen from an allergic extract (e.g. by immunoabsorption) reduces IgE binding activity
 - Demonstrating, where possible, that recombinant allergens have comparable IgE antibody-binding activity to the natural allergen

Table 3 Molecular properties of common allergens

Source	Allergen	Mol. wt (kDa)	Homology/function
Inhalants			
Indoor			
House dust mite (<i>Dermatophagoides pteronyssinus</i>)	Der p 1	25	Cysteine protease ^b
	Der p 2	14	Epididymal protein
	Der p 3	30	Serine protease
	Der p 5	14	Unknown
Cat (<i>Felis domesticus</i>)	Fel d 1	36	(Uteroglobulin)
Dog (<i>Canis familiaris</i>)	Can f 1	25	Calycin
Mouse (<i>Mus musculus</i>)	Mus m 1	21	Pheromone binding protein (calycin) ^b
Rat (<i>Rattus norvegicus</i>)	Rat n 1	21	Pheromone binding protein (calycin) ^b
Cockroach (<i>Blattella germanica</i>)	Bla g 2	36	Aspartic protease
	Bla g 4	21	Calycin
Outdoor			
Pollens – grasses:			
Rye (<i>Lolium perenne</i>)	Lol p 1	28	Unknown
Timothy (<i>Phleum pratense</i>)	Phl p 5	32	Unknown
Bermuda (<i>Cynodon dactylon</i>)	Cyn d 1	32	Unknown
Weeds			
Ragweed (<i>Artemisia artemisifolia</i>)	Amb a 1	38 ^a	Pectate lyase
	Amb a 5	5	Neurophysins ^b
Trees			
Birch (<i>Betula verucosa</i>)	Bet v 1	17	Pathogenesis-related protein
	Bet v 2	14	Profilin
Foods			
Milk	β-Lactoglobulin	36 ^a	Retinol-binding protein (calycin) ^b
Egg	Ovomucoid	29	Trypsin inhibitor
Codfish (<i>Gadus callarias</i>)	Gad c 1	12	Ca ²⁺ -binding protein (muscle parvalbumin)
Peanut (<i>Arachis hypogea</i>)	Ara h 1	63	Vicilin (seed-storage protein)
Venoms			
Bee (<i>Apis mellifera</i>)	Api m 1	19.5	Phospholipase A ₂
Wasp (<i>Polestes annularis</i>)	Pol a 5	23	Mammalian testis proteins
Hornet (<i>Vespa crabro</i>)	Ves c 5	23	Mammalian testis proteins
Fire ant (<i>Solenopsis invicta</i>)	Sol i 2	13	Unknown
Fungi			
<i>Aspergillus fumigatus</i>	Asp f 1	18	Cytotoxin (mitogillin)
<i>Alternaria alternata</i>	Alt a 1	29 ^a	Unknown
Latex			
<i>Hevea brasiliensis</i>	Hev b 1	58	Elongation factor
	Hev b 5	16	Unknown – homologous to kiwi fruit Protein of unknown function

^aMost allergens have a single polypeptide chain; dimers are indicated.

^bAllergens of known three-dimensional structure are also indicated.

a high degree of structural homology are also found in grasses and trees (group 1, group 5, etc.). It is quite common for a single allergen to show sequence polymorphisms, and variants with limited amino acid substitutions are termed *isoforms*. In some cases, e.g. birch pollen allergen, Bet v 1, the isoforms may have altered IgE antibody-binding properties.

Sequence similarity searches have enabled the protein families to which allergens belong to be ascertained and their biologic function to be identified. The major tree pollen allergens (e.g. Bet v 1) are related to pathogenesis resistance proteins; many

mite allergens are proteolytic enzymes (cysteine and serine proteases, trypsin and chymotrypsin); and muscle proteins such as tropomyosin and troponin have been identified as allergens in mites, insects and shellfish (Table 3).

Recently, a new family of proteins has been identified that causes IgE antibody responses – ligand-binding proteins or calycins. These proteins bind small hydrophobic molecules such as pigments and pheromones. Calycins were first identified as a result of cloning cockroach allergens and the family also includes the major rodent urinary proteins, milk

allergen β -lactoglobulin, and dog, bovine and equine allergens. It has been postulated that because many allergens, particularly from dust mites, are proteolytic enzymes, that this facilitates penetration through mucosal surfaces and induction of IgE antibody responses. There is also evidence that Der p 1 may upregulate IgE synthesis through its ability to cleave CD23, the low affinity receptor for IgE. However, several clinically important allergens have no enzymatic activity and there are other allergens which show no sequence homology to known proteins and whose function is as yet unknown (e.g. mite group 5 allergens) (Table 3).

Expression and biologic activity of recombinant allergens

Traditional methods of allergy diagnosis and treatment have relied on the use of heterogeneous allergen extracts. The problems with these extracts are that the absolute potency is difficult to define and some patients give adverse reactions when the extracts are injected (during immunotherapy), which can be life threatening. While there has been significant progress in the standardization of allergen extracts; the use of purified recombinant allergens expressed in bacteria, yeast or insect cells offers the prospect of using defined proteins for both diagnosis and treatment. An increasing number of recombinant allergens have been produced and it is possible to investigate whether cocktails of 3–4 allergens would be suitable for clinical purposes. Typical vectors in which allergens have been expressed include pGEX and pET (in *Escherichia coli*); pSAY-1 and *Pichia pastoris* (yeast), and the baculovirus system, giving yields of 1–10 mg l⁻¹. The allergens can be obtained with a high degree of purity and, in many cases, have excellent immunoreactivity on skin testing and in serum IgE antibody assays. Several recombinant allergens from mite (Der p 2, Blo t 5, Der p 5), aspergillus (Asp f 1), cockroach (Bla g 4, Bla g 5) and grasses (Phl p 1, Phl p 5) have been shown to have good biologic activity on skin testing, and elicit positive wheal and flare reactions using picogram doses of protein.

Molecular modeling and tertiary structure

Recombinant allergens are essential for providing sufficient quantities of highly purified protein for structural studies. It is also possible to manipulate allergen sequences and to produce partial constructs for investigation of functional or immunologic activity. Using site-directed mutagenesis, it has been possible to generate site-specific variants of Der p 2 that have reduced binding for IgE antibodies. These experiments allow B cell epitopes to

be localized and can also be applied to investigate functional domains of the allergen, for example, the active sites of enzyme activity or the ligand-binding regions.

The homology between allergens and other protein families can be used to generate molecular models of the tertiary allergen structure. Two recent examples involve mite allergen Der p 1, whose structure has been modeled on the cysteine protease, papain, and cockroach allergen, Bla g 4, which has been modeled on other calycin structures for which X-ray crystal coordinates were known (Figure 1). The X-ray crystal structures of several allergens have been determined, including mouse and rat urinary proteins (Mus m 1 and Rat n 1), ragweed Amb a 5, and chironomid hemoglobin.

Immune response

Allergic individuals make IgG, IgA and IgE antibodies to allergens in both serum and nasal secretions. It is not uncommon to find asymptomatic individuals with positive skin tests and allergen-specific serum IgE antibody; however, most 'nonallergic' individuals do not make IgE antibody responses. The levels of IgG antibody in nonallergic individuals are usually lower than in allergic

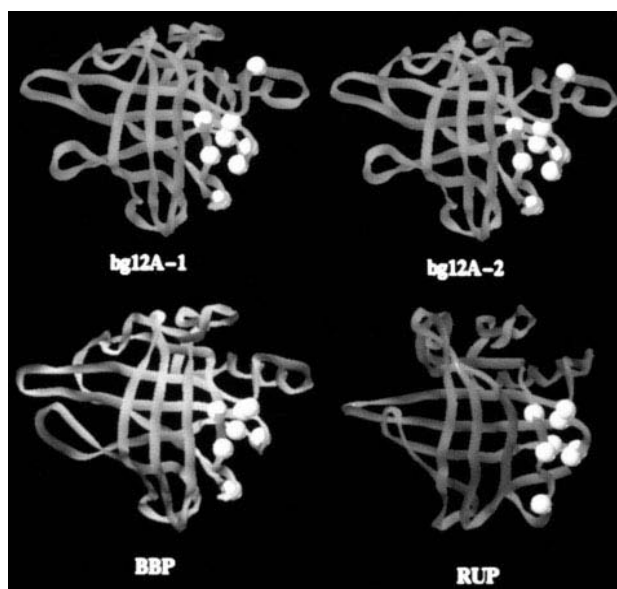


Figure 1 Molecular modeling of the cockroach calycin allergen, Bla g 4. The α backbone structures for two models of Bla g 4 (designated bg12A-1 and bg12A-2) were modeled on the X-ray crystal coordinates for butterfly bilin-binding protein (BBP) and are compared with rat urinary protein allergen (RUP), for which the X-ray crystal structure has also been determined. The conserved amino acid residue spheres form motifs which define the ligand-binding proteins, or calycins. (See also color Plate 7.)

patients and there is also evidence to suggest that the affinity for these IgG antibodies is lower. In hayfever patients, IgE antibodies to pollen allergens can account for a significant proportion (>20%) of the total IgE. IgE antibody responses to food allergens develop within the first few months of life, whereas antibody responses to inhaled allergens develop over the first 2–3 years of infancy. IgG antibody levels rise (up to 50-fold) in patients being treated by immunotherapy using aqueous allergen extracts. A rise in IgG antibody correlates with clinical efficacy in patients with insect venom allergy, but is not significantly associated with clinical improvement with inhaled allergens.

Recent work has focused on the role of T cells in regulating allergen-specific IgE responses. Proliferative T cell responses can be demonstrated *in vitro* using peripheral blood from allergic patients, and T cell clones specific for mite, pollen and cat allergens have been obtained. Assessing T cell responses to allergens is difficult because many patients, even with high antibody levels, make weak or inconsistent T cell responses. For these reasons, studies have often focused on small numbers of highly selected patients and there are few large population studies. T cell clones derived from allergic individuals have a predominantly T_H2 phenotype and secrete interleukin 2 (IL-2), IL-4 and IL-5. T cell clones have also been obtained from nonallergic individuals and these are T_H1 cells secreting interferon γ (IFN γ). Epitope mapping studies with Der p 1, Der p 2, Fel d 1 and Bet v 1, and other allergens, suggest that most allergens have multiple T cell epitopes, which are not HLA restricted, i.e. 'promiscuous' epitopes. In some cases immunodominant T cell sites have been identified, e.g. two 27 amino acid peptides on Fel d 1, chain 1.

There is also good evidence that allergen-specific T cells have inflammatory effects *in vivo*. Patch tests with purified mite allergens result in the recruitment of basophils, eosinophils and mononuclear cells into the skin of atopic dermatitis and the production of eczematous lesions at 48 h. The frequency of allergen-reactive T cells in the skin of these lesions is higher than in the peripheral blood. Allergen challenge in the lung also results in recruitment of inflammatory cells and is thought to be in part T cell mediated.

Now that the amino acid sequences of many allergens are known, strategies are being developed for new forms of allergen immunotherapy involving T cell epitopes and specifically designed molecules which no longer bind IgE. The key advan-

tage of these approaches is that the peptides can be synthesized, and any alterations in allergen structure can be clearly defined. One approach has been to immunize cat allergic patients with two immunodominant T cell epitopes of Fel d 1, with the aim of occupying the T cell receptor binding site without delivering the costimulatory signals that are necessary for IgE production. The cat vaccine is now undergoing clinical trials and early results show clinical improvements in treated patients, particularly at high peptide doses. Another approach is to design allergen vaccines containing a cocktail of recombinant allergens or variants which have been modified by mutagenesis so that they have markedly reduced binding to IgE. The rationale here is to deliver a broad spectrum of T cell epitopes and to reduce the possibility for IgE mediated side-effects.

Clinical significance

Allergic diseases such as hayfever, asthma and atopic dermatitis affect 10–20% of the population. Asthma is the most common chronic disease of children in Western countries and the prevalence of the disease has been increasing. Over the past 5–10 years, the clinical significance of allergens in causing asthma has become firmly established and there is now overwhelming evidence that sensitization to allergens is the single most important risk factor for the development of asthma. Asthma management has relied heavily on the use of bronchodilator and anti-inflammatory drugs (inhaled or oral steroids). In addition, greater emphasis on the role of indoor allergens in causing the disease has focused attention on the need to reduce allergen exposure in houses. Measuring allergen exposure in houses using monoclonal antibody based ELISA tests has established levels at which patients become sensitized (typically exposures of 1–2 μg allergen per gram dust) and at which they are likely to develop asthma exacerbations (>10 $\mu\text{g g}^{-1}$). Allergen avoidance studies carried out at high altitude or in hospital rooms have shown that reducing allergen exposure to <1 $\mu\text{g g}^{-1}$ can reduce bronchial hyperreactivity and improve asthma symptoms. Studies are now being designed to assess whether allergen avoidance protocols can be developed for routine use and whether early allergen avoidance procedures (in the first 3 years of life) will reduce the prevalence of asthma.

See also: Antigens; Asthma; Atopic allergy; Enzyme-linked immunosorbent assay (ELISA); Epitopes; Food allergy; Hypersensitivity reactions; Immunoas-

says; Monoclonal antibodies (mAbs); T cell vaccination.

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ALLOANTIGENS

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Molecules expressed on biologically active cells include structural components, enzymes, receptors, transport system molecules, viral proteins and accessory molecules. Many of them carry unique determinants that can elicit xenogeneic and/or allogeneic immune responses. Alloantigens play crucial roles in the body's defense mechanisms and are of importance in the clinic with respect to blood transfusion and tissue or organ transplantation. Recent advances in serological and molecular biological techniques and in X-ray crystallographic structural analysis have increased our ability to identify and characterize new cell surface antigens and to understand their structure and function.

Alloantigenic polymorphism can arise in several ways: first, sequence variation in structural genes (e.g. major histocompatibility complex (MHC) antigens, Rh blood group antigens); second, variation in the activity of enzymes involved in the synthesis of antigenic epitopes (e.g. ABH or Lewis blood group carbohydrate determinants); third, sequence variation in regulatory elements controlling the expression of structural genes (e.g. expressed versus null alleles as for H2-T antigens in the mouse).

Alloantigenic responses also can occur in several ways: 1) alloimmunization; 2) natural immunization (e.g. naturally occurring antibodies against ABH antigens); and 3) the maternal immune response against fetal antigens during pregnancy (e.g. antibodies against human MHC antigens). Some major alloantigenic systems are described in the following section and summarized in Table 1.

Major alloantigenic systems

Blood group antigens

Hundreds of blood group antigens have been identified by red blood cell serology. The well-known major blood group alloantigenic systems (ABO(H), Rh) are very important from a clinical standpoint. The minor blood group systems (e.g. Lewis, MNS, Lutheran, Kell, Duffy, Kidd, P, I) also are clinically significant, but less so. The Rh system is one of the most polymorphic antigen systems known, as it comprises at least 50 antigens, all of which are encoded by alleles at either of two loci, D or CcEe; the D antigens are the most immunogenic of all the red blood cell antigens. The MNS system is also highly

Table 1 Major alloantigenic systems with their basic functions and clinical significance1. *Blood group antigens*

Major systems: ABO(H), Rh

Minor systems: Lewis, MNS, Lutheran, Kell, Duffy, Kidd

Functional and clinical significance: blood transfusion, tissue or organ transplantation, hemolytic diseases and inflammatory responses2. *Histocompatibility antigens*

A. Major Histocompatibility antigens (MHC)

(a) Classical

Class I: Human HLA-A, B, C

Mouse H2-K, D, L

Rat RT1.A, F, E

Class II: Human HLA-DP, DQ, DR

Mouse H2-A, E

Rat RT1.H, B, D

Functional and clinical significance: presentation of antigenic peptides to T cells; association with susceptibility to a number of diseases; tissue and organ transplantation

(b) Nonclassical:

Class I: Human HLA-E, F, G, H, J, K, L; MIC (MHC class I chain related)

Mouse H2-Q, T, M

Rat RT1.C, K, G, O, N, M

Class II: Human HLA-DMA, DMB

Mouse Ma, Mb₁, Mb₂*Functional and clinical significance:* presentation of specific antigenic peptides to T cells; DMA/DMB act as accessory molecules in antigen presentation by class II antigens.

(c) MHC class III antigens

Human: C2, factor B (Bf), C4A and C4B (complement proteins); TNF α , TNF β (tumor necrosis factors); steroid 21-hydroxylase (CYP21); major 70 kDa stress protein Hsp70; and TAP (transporter of antigenic peptides)Mouse C2, C4, Bf, Slp (sex-limited protein), 21-hydroxylase (21-OH), Hsp70 and TNF α , TNF β Rat C2, C4, Bf, Hsp70, Cyp21, TAP, TNF α *Functional and clinical significance:* complement functions; transport of antigenic peptides; chaperones in antigen processing and presentation

(d) MHC-like antigens encoded by genes residing outside the MHC

Class I-like

Human, mouse, rat: FcRn

Human, mouse, rat: CD1

Human: Zinc α_2 -glycoprotein (ZAG)*Functional and clinical significance:* FcRn binds IgG from ingested milk from mother and transfers it across intestinal cells to the bloodstream; CD1 predominantly presents nonpeptide lipid and glycolipid antigens to T cells; and ZAG has no known physiological function

B. Minor histocompatibility antigens

(a) Sex-specific: H-Y (mouse, rat, human)

(b) Many tissue-specific antigens in all species

(c) Mls endogenous superantigens

Mouse: Mls-1^a, Mls-2^a, Mls-3^a*Functional and clinical significance:* slow tissue rejection, mixed lymphocyte stimulation

3. Differentiation antigens (CD)

In excess of 100 clusters have been identified in humans

Functional and clinical significance: Involved in cellular interactions.

complex, comprising two closely linked homologous genes GYPA and GYPB, that encode for at least 38 antigens. Recent studies at the molecular level indicate that a variety of genetic mechanisms gives rise to this complexity. So far, Rh antigens have been detected only on red blood cells, but the ABO(H), Lewis, P and I antigens are widely distributed throughout the body. Recent data suggest functional roles for blood group antigens unrelated to erythrocytes: cell adhesion, association with diseases, tumor

markers and receptors for parasites, bacteria and viruses.

Major histocompatibility complex antigens

There is extensive allelism of genes within the MHC; their numbers vary from a few in the miniature swine to hundreds in the African pigmy mouse. The MHC-encoded classical class I (class Ia) and class II molecules bind peptides derived from endogenous or exogenous self proteins as well as nonself proteins

from viruses, bacteria, multicellular parasite and many types of tumors. They present these peptides to mature CD8⁺ and CD4⁺ subsets of $\alpha\beta$ T cells, respectively, for subsequent generation of specific cytotoxic/suppressor or helper/inducer T cells. Within a population, the classical MHC molecules are highly polymorphic: for example, in the human, at least 20 HLA-A, 45 HLA-B and 14 HLA-C class I antigens are known to date. A similar degree of polymorphism exists for class II antigens.

Besides the classical MHC genes, there are a large number of MHC genes with limited polymorphism and restricted tissue distribution of their products. The nonclassical antigens (class Ib) RT1.K and HLA-G are the only MHC antigens expressed, respectively, in rat and human placental trophoblasts. In the mouse, the Qa-2 molecules are restriction elements for a wide variety of nonameric peptides with restrictive binding motif. For example, Qa-1 binds peptides derived from heat shock proteins. Some H2-T antigens present peptides to $\gamma\delta$ T cells, and the H2-M3 antigen presents peptides from intracellular pathogens such as *Listeria monocytogenes* and from allelic variants of a mitochondrial enzyme specifically containing N-formylated methionine, a unique structural component of prokaryotic mitochondrial proteins.

Recently, a second MHC class I-related oligomorphic gene family, MIC, has been detected in the human (near HLA-B), goat, pig, dog and hamster. This family appears to have evolved independently from other class I genes, since its gene sequences are quite divergent. No functions are known but their structure suggests antigen presenting capability.

There are also class I-like molecules encoded by genes residing outside the MHC and on different chromosomes. 1) Neonatal Fc receptor (FcRn) first detected in rats and more recently in mice and humans, is involved in the acquisition of passive humoral immunity in young mammals during the first few weeks after birth. FcRn binds immunoglobulin G (IgG) from ingested mother's milk and transports it through gut epithelial cells into the bloodstream by transcytosis. 2) CD1 is a small multigene family, so far detected in several mammals including the mouse, rat and human. These antigens do not show significant polymorphism. CD1 proteins present nonpeptide lipid (mycolic acid) or glycolipid antigens from intracellular mycobacteria such as *Mycobacterium leprae* and *M. tuberculosis* to CD8⁺ $\alpha\beta$ T, CD4⁺CD8⁺ $\alpha\beta$ T or $\gamma\delta$ T cells or T cells within epithelial sheets. 3) Zinc α_2 -glycoprotein, a zinc-precipitable protein of human plasma, is another oligomorphic MHC-unlinked

class I protein. No physiological role for this protein is known.

Unlike the nonclassical class I MHC genes, only a few nonclassical class II MHC genes have been reported. Human HLA-DMA and HLA-DMB and the murine homologs Ma, Mb₁ and Mb₂ are examples. The human genes encode a heterodimer, DMA/DMB, which is not a cell surface molecule but is associated with class II antigens in the endosomal compartment. There is evidence that it helps peptide presentation by conventional class II molecules.

Minor histocompatibility antigens

A large number of minor histocompatibility antigens which contribute to the slow rejection of grafts has been detected. For example, the sex-specific antigen H-Y in males may cause rejection of male organs or bone marrow grafts by MHC-identical female recipients. The epitope of the H-Y antigen has been mapped to an 11-residue peptide located near one end of the protein encoded by the conserved Y-chromosome gene SMCY, which is homologous to the SMCX gene on the X chromosome. Identical results have been reported for the mouse H-Y antigen.

Mls and superantigens

Mls, or minor lymphocyte-stimulating, antigens represent another class of alloantigens that are recognized by T cells *in vitro*. They are encoded by endogenous mouse mammary tumor virus genes (Mls-1^a, Mls-2^a and Mls-3^a) and they elicit a strong primary mixed lymphocyte reaction (MLR) between cells derived from mice sharing the same MHC haplotype. Subsequently, similar antigens, known as superantigens (SAGs), have been found which include exogenous products of gram-positive bacteria (toxins), retroviruses and mycoplasma. These antigens are presented to CD4⁺ T cells by class II molecules in association not with the peptide-binding groove but with an external face.

Immunoglobulin allotypes

Allotypic variants of immunoglobulin light chains and heavy chains exist in different species, and they arise due to allelic variation in the constant region genes. In humans, for example, the IgE isotype has two alleles, and the IgG3 isotype has as many as 14 alleles. Allotypes are useful in population studies, paternity testing and forensic science. Variation in the expression of certain allotypes may be related to disease susceptibilities. For example, the IgG2 isotype is associated with immune responses to certain

bacteria, and lack of the Km1 or G2m(n) allotypes increases the risk of *Haemophilus influenzae* and *Meningococcus C* infections. Disease susceptibility due to interactive effects between Gm allotypes and HLA antigens has also been reported

Differentiation antigens

Leukocyte differentiation antigens provide markers for different lymphoid cell types, define cell lineages and mediate crucial leukocyte-specific functions. From serological, biochemical, structural and functional homologies, many of the antigens have been grouped into clusters of differentiation (CD), and some CD genes are polymorphic or oligomorphic, while others are monomorphic.

The CD antigen clusters are involved in numerous functions. Some examples are given below.

Accessory functions:

1. Interaction with MHC antigens leading to T cell activation: CD3 associates with $\alpha\beta$ TCR on CD4⁺ and CD8⁺ T cells, and the complex binds to MHC antigens along with antigenic peptides.
2. Activation of T cells requires a second signal that is delivered by the interaction of CD80(B-7)/CD28 and B cells via CD40/CD40-ligand molecules.
3. Signal transduction: Thy 1 (CDw90) is a signal transduction molecule that plays a role in T cell activation through the TCR/CD3 complex. CD19 and CD21 are involved in signal transduction in B cell activation.
4. Cell adhesion (integrins and their ligands): involves cell-cell or cell-tissue interactions during microbial infections, inflammation, tumor formation, metastasis and neural development. For example, the antigen-presenting cells (APC) and CD4⁺ T cells are brought into close contact by interaction between LFA-1(CD11a/CD18) antigen on the T cells with its ligands ICAM-1 and ICAM-2 on the APC and also by interaction between LFA-3 (CD58) and its counterpart CD2 on the T cells.

Markers for different cell types: T cells (CD3), MHC class II-restricted T cells (CD4), MHC class I-restricted T cells (CD8), activated T and B cells (CD95), B cell lineage (CD19, CD20) and granulocytes (CD66).

Receptors for soluble molecules: Interleukins (CD25, CD121 through CDw130); Fc portion of IgG (CD16: Fc γ RIII; CD32: Fc γ RII; CD64: Fc γ RI) or IgE (CD23) and complement C3d (CD21).

Viral receptors: Human immunodeficiency virus (CD4); and Epstein-Barr virus (CD21 on B cells).

Enzymatic activity: CD10, CD13, CD26 (dipeptidylpeptidase IV); CD45 (phosphotyrosine phosphatase) and CD73 (ecto-5'nucleotidase).

Ion channels: CD20 forms ion channels and functions during receptor-mediated events.

Disease associations and clinical use: A few examples: defective CD43 expression on T cells with X-chromosome-linked Wiskott-Aldrich syndrome; some minor GPI (glycosyl-phosphatidylinositol)-anchored antigens (e.g. CD55, CD58, CD59) are deficient on the red blood cells from patients with paroxysmal nocturnal hemoglobinuria (PNH). Anti-CD19 antibodies are used to treat leukemias and lymphoma and anti-CD3 antibodies to treat renal, hepatic and cardiac allograft rejection.

Biochemistry and molecular biology of alloantigens

The majority of alloantigens are glycoproteins (e.g. MHC, MNS antigens), and the others are sialoglycoproteins (e.g. CD43), oligosaccharides (ABO(H), Secretor, Lewis, Li and P), sialo-oligosaccharides (sialyl-Lewis^x or sialyl-Lewis^a) or proteins (Rh). Most cell surface protein antigens are anchored through transmembrane hydrophobic interactions, but there is an increasing number of structurally diverse cell surface antigens that are anchored by a covalent linkage through GPI: Thy-1 (CDw90), Ly-6, Qa-2, RT6, CD14, CD55, CD59, CD73. Some antigens (CD58 (LFA-3) and probably CD55) occur in two forms: one has a GPI anchor, and the other has a transmembrane anchor. The GPI-anchored proteins are active in transmembrane signaling; for example, the GPI anchor of Qa-2 is essential for T cell activation via this molecule. In addition, a large number of alloantigens belong to the immunoglobulin supergene family: for example, membrane-proximal domains of MHC polypeptides, CD1, CD2, CD3, CD4, CD7, CD8, Ly-6, Qa-2, RT6, CD19, CD22, CD56, CD58(LFA-3), CD59 and CDw90 (Thy 1).

Molecular cloning and sequencing of many allo-antigen genes have revealed the basis of their polymorphism and provided some insight into their function, either directly or by analogy with sequences of previously known genes. Many of the blood group antigens have been expressed by transfection of cDNA into nonerythroid or nonhuman mammalian cells in order to study their functions. Also, recent

X-ray crystallography of a number of peptide-MHC antigen complexes and protein modeling have yielded detailed information about the structure-function relationships of the MHC antigens.

See also: ABO blood group system; Allotypes, immunoglobulin; Antigens, cell surface; H2 class I; H2 class II; H2 class III; HLA class I; HLA class II; HLA class III region; Antibodies, detection of; Minor transplantation (histocompatibility) antigens; Rh antigens; Superantigens; Thy-1.

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ALLOTYPES, IMMUNOGLOBULIN

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Allelism is the existence of two or more variants of a given gene in an outbred population. Allelism within the immune system was first recognized for genes encoding immunoglobulin (Ig) molecules, through the antigenic properties of their protein products, i.e. antibody molecules. The extent of allelism at the Ig loci has resulted in the application of Ig phenotyping to paternity testing, forensic science, bone marrow transplantation, etc. Allelism and allotypy may be evident in its most diverse form within the major histocompatibility loci, where the phenotype of an individual may constitute immunological self-identity.

Nomenclature

In 1976 the World Health Organization (WHO) sponsored an expert committee meeting at which the nomenclature for human immunoglobulin allotypes

was systematized and a logical numerical system was proposed to replace the apparently illogical alphameric system that had evolved. However, many expert laboratories continue to use the alphameric system since reference can be made to original publications in which the allotypes were defined. Both systems may therefore be encountered in current literature. The allotype of an Ig molecule is recognized by the expression of a unique epitope (allotope) and is designated by a capital letter representing the heavy-chain isotype or light-chain type with a small m standing for marker, viz: Gm, Am, Em, Km. Further, where appropriate the subclass of the protein is shown by the inclusion of the appropriate numeral. Thus, G1m(f) refers to genetic marker or allotope f expressed on molecules of the IgG1 subclass; G2m(n) to allotope n expressed on molecules of subclass IgG2, etc. The human Ig allotypes currently recognized are summarized in Table 1 and those of mouse

Table 1 Human immunoglobulin allotypes

Isotype/type	Heavy chains						Light chain
	IgG1	IgG2	IgG3	IgG4	IgA	IgE	κ
Allotypes	G1m	G2m	G3m	G4m	A2m	Em	Km(Inv)
	a(1)	n(23)	g1(21),g5(28)	4a(1)	1	1	1
	x(2)		b0(11),b1(5)	4b(1)	2		2
	f(3)		b3(13),b4(14)				3
	z(17)		b5(10),s(15)				
			t(16),c3(6)				
			c5(24),u(26)				
			v(27)				

NB: Numeric notation given within brackets.

and rabbit in Table 2. The genes encoding the allotypic products are identified using the italicized form of the epitope nomenclature and the alphameric or numeric identity as a superscript, e.g. *G1m^a*, *Km¹*, etc.

Serological reagents that identify the products of some allelic genes may not be available, presumably because the resultant protein structure is not immunogenic, in humans. An example is IgG2 proteins for which reagents are available that identify a G2m(n) allotype and allow IgG2 paraproteins to be

designated G2m(n⁺) or G2m(n⁻); a reagent is not available that specifically recognizes G2m(n⁻) molecules.

Some reagents define isoallotypic epitopes; these epitopes are expressed on the product of a gene that is allelic for a given Ig subclass but are also expressed on the products of the other subclass genes. An example is a reagent recognizing the nG1m(a) epitope; IgG1 proteins that are the product of the *G1m^a* gene express the G1m(a) allotope whilst proteins that are products of the allelic gene express the non-G1m(a) or nG1m(a) epitope. However, proteins of the IgG2 and IgG3 isotype also express the nG1m(a) epitope. Such reagents allow the typing of homogeneous proteins, but cannot be applied to polyclonal IgG or whole serum analysis.

Table 2 Mouse and rabbit immunoglobulin allotypes

Isotype/type	Allotypes
<i>Mouse</i>	
κ	None identified
λ	None identified
<i>Heavy</i>	
G1	4 ^{a,c,d,e,f,g,h} , 4 ^b
G2a	1 ^a , 1 ^b , 1 ^c , 1 ^d , 1 ^e , 1 ^f , 1 ^g , 1 ^h
G2b	3 ^{a,c,h} , 3 ^b , 3 ^d , 3 ^e , 3 ^f , 3 ^g
G3	None identified
A	2 ^{a,h} , 2 ^b , 2 ^{c,g} , 2 ^{d,e} , 2 ^f
D	5 ^a , 5 ^b
M	6 ^a , 6 ^b
<i>Rabbit</i>	
κ	b4, b5, b6, b9
λ	c7, c21
<i>Heavy</i>	
V _H	a1, a2, a3 x32, x- y33, y- n81, n82
C _{μ}	f71, f72, f73
C _{ϵ}	g74, g75
C _{γ}	d11, d12 e14, e15

Haplotypes

All the allotypes presented in Table 1 are expressed on immunoglobulin constant regions. Since the genes encoding the constant region of the heavy chains are closely linked within the IgG gene locus they are inherited together as haplotypes with a very low frequency of cross-overs. However, cross-over events have occurred during evolution, resulting in present populations expressing characteristic haplotypes, hence the usefulness of the allotype system in population studies. Thus in northern Europe (The Netherlands) the haplotype *G1m^f*; *G2mⁿ*; *G3m^b* is present with a frequency of 0.45 and virtually all individuals are homozygous for the *A2m¹* allele, however, amongst Nigerian Africans the *G1m^a*; *G2mⁿ*; *G3m^b* haplotype occurs with a frequency of 0.678 (the *G1m^f*; *G2mⁿ*; *G3m^b* haplotype not being encountered) and the *A2m²* allele with a frequency of 0.826.

The structural difference between alleles is frequently restricted to a single base change within a codon resulting in a conservative amino acid inter-

change; examples are lysine or arginine at residue 214 in IgG1 proteins correlating with the expression of G1m(z) and G1m(f) allotypes respectively and leucine or valine within κ light chains with the expression of the Km1,2 or Km3 allotypes. Similar minor structural differences are observed between allotypic variants of Ig constant region in the mouse, rat and rabbit.

Applications

The value of allotypes in population studies, paternity testing and forensic science will be apparent from the heterogeneity of haplotypes present within and between population groups (Table 3). The value in forensic science is emphasized by the sensitivity of assay systems and the stable expression of allotypes by immunoglobulin present in aged, dried blood samples. A more recent application is in bone marrow transplantation where allotype disparities between the donor and recipient allow monitoring of the success of the transplantation protocol. Postoperative expression of donor allotypes, not expressed by the recipient B cells, is indicative of successful grafting. Allotype matching of human (humanized) monoclonal antibodies to recipients of *in vivo* immunotherapy may be important in minimizing immunogenicity and anti-idiotypic responses.

Disease associations

Immunoglobulin levels may also be associated with allotype and several studies have established that individuals homozygous for G2m(n) have higher IgG2 subclass levels than those homozygous for G2m(n⁻), heterozygotes having intermediate levels. Since protective immune responses to certain bacterial species are restricted to the IgG2 isotype, these

findings may have implications for disease susceptibility. Such an effect has been reported for post-immunization levels of antibody to *Haemophilus influenzae* and *Meningococcus C* polysaccharides with individuals lacking the Km1 and G2m(n) allotype having an increased risk of infection. A more established finding is an interactive effect between Gm allotypes and HLA antigens on disease susceptibility. There is some evidence that antibody molecules of the same isotype but differing allotype may differ in their potential to activate effector mechanisms.

Detection

Although allotypes may be detected by a relatively simple hemagglutination system, it does employ a range of reagents that are only available from specialist hematology institutions such as national blood banking centers. Anti-allotype reagents are generally alloantibodies produced by apparently healthy blood donors and are induced to paternal allotype in multiparous women or to nonself allotypes in individuals receiving transfusions or undergoing deliberate immunization. The sample under review is assayed for inhibition of agglutination of human red blood cells coated with anti-Rh antibody of the appropriate allotype. Limited availability of these reagents has resulted in the development of some polyclonal reagents in heterologous species, but this is a generally difficult and unsatisfactory procedure.

The development of monoclonal antibodies has made assay systems widely available and allow new studies to confirm and extend the influence of Ig allotype on immune responsiveness. Generally, these reagents show preferential reactivity with the target allotope, rather than exclusive specificity. They must be used with care in assay protocols defined by the originating laboratory.

See also: Allelic exclusion; Antibodies, antigenicity of; Idiotypic; Immunoglobulin genes; Immunoglobulin structure; Isotype.

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Table 3 Gm haplotypes characteristic for a Race

	Whites	Blacks	Oriental
G1m;G2m;G3m			
f ; n ; b	+	–	–
f ; .. ; b	+	–	–
za ; .. ; g	+	–	+
zax ; .. ; g	+	–	+
za ; .. ; b	–	+	–
za ; .. ;c3c5	–	+	–
za ; .. ; c3	–	+	–
za ; .. ; s	–	+	–
za ; .. ; st	–	–	+
af ; n ; b	–	–	+

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AMEBIASIS

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The World Health Organization definition of amebiasis describes it as 'infection with *Entamoeba histolytica*, with or without clinical symptoms'. It has long been known that at least 90% of patients with amebiasis will be asymptomatic cyst passers but only recently appreciated that these cases are caused by a separate species of ameba, now usually referred to as *Entamoeba dispar*; it is generally felt that about 450 million people carry this organism without either pathology or an immune response. At least 50 million new cases of infection with *E. histolytica sensu stricto* are thought to occur each year; the proportion of those infected who show clinical symptoms at any one time is hotly debated but everyone agrees that all are at risk. The remainder of this entry will deal exclusively with *E. histolytica* in its new sense – previously described as the 'pathogenic zymodeme'. Methods of distinguishing it from *E. dispar* (they are morphologically identical) are only slowly coming into use and this must be borne in mind when reading any but the most recent literature.

Characteristics of the organism and its antigens

E. histolytica is a protozoan parasite that lives in the large bowel (probably mostly adhering to the mucosa) as an ameboid and phagocytic trophozoite dividing by binary fission. Responding to unknown stimuli, trophozoites may develop into resistant cysts which are shed in the feces and, by being ingested orally, are responsible for parasite transmission. Pathology occurs when trophozoites penetrate the mucosa causing ulceration and bloody diarrhea (amebic dysentery, AD); less frequently dissemination to the liver takes place with, ultimately, extensive tissue destruction (hepatic amebiasis or amebic liver abscess, ALA). ALA is about seven times less

common than AD, and dissemination to other sites (brain, skin, etc.) is rarer still, but together these conditions cause much morbidity and 40–100 000 deaths a year, mainly in developing countries. Dissemination is aided by a formidable array of cytotoxic and cytolytic activities, most of which seem to have homologs in *E. dispar*; the specific factors which account for the invasive potential of *E. histolytica* have not yet been identified.

Western blots probed with patients' sera reveal a large number of immunogenic molecules, of which the best characterized are: the galactose/N-acetyl-D-galactosamine inhibitable lectin (GIL), a 260 kDa heterodimeric molecule involved in binding to and killing enterocytes; SREHP, a phosphorylated and glycosylated serine-rich membrane protein; a 29 kDa cysteine-rich protein (CRP) which may be involved in protection against oxygen toxicity; a 125 kDa variable surface antigen; additional 112 and 220 kDa molecules also capable of mediating adhesion; and a number of proteases.

Immune responses of the host

Antibody

Using sensitive tests, specific serum antibody may be detected in virtually all persons infected with *E. histolytica*, whether they have symptoms or not, although levels are significantly higher in those with ALA. Principally immunoglobulin G (IgG), and to a lesser extent IgA, are represented; IgM declines fairly rapidly while specific IgG remains elevated for months or years, reducing the diagnostic value of serology in endemic areas. In one study levels of specific IgG4 >> IgG2 > IgG3 > IgG1 were found. Coproantibodies have been detected in the feces of AD patients, and specific secretory IgA in the saliva and colostrum of those with ALA. Autoantibodies

(directed against liver, colon, neutrophils and IgG) and circulating immune complexes have been described in patients but there is no real evidence for immunopathology in human amebiasis.

Cell-mediated responses

On histological examination neither the ulcers of AD nor the hepatic abscesses of ALA show much evidence of cellular infiltration and granuloma formation; one exception to this is the ameboma, a rarely found mass of granulation tissue in the colonic lumen. In small animal models intense inflammatory changes occur early in the development of lesions but whether this occurs in human amebiasis is not known. After recovery, most ALA patients are skin-test positive but appear to be specifically unresponsive to *E. histolytica* antigens earlier on.

Most studies have shown that normal human leukocytes of all sorts are harmless to and are killed by *E. histolytica* trophozoites. ALA patients' CD8⁺ cells became cytotoxic after incubation with soluble amebic antigen, phytohemagglutinin or GIL. More importantly, antigenic stimulation results in the production of cytokines (interferon γ (IFN γ) seems to be the most important) which activate macrophages to kill trophozoites. *E. histolytica* is powerfully chemotactic for neutrophils, which are rapidly killed by a contact-mediated, GIL-dependent process; lytic enzymes liberated from these dead cells may be responsible for much of the tissue destruction seen in amebiasis. IFN γ and tumor necrosis factor α (TNF α)-activated neutrophils can kill trophozoites and N-formylmethionine leucyl phenylalanine (fMLP)-activated eosinophils can do so even more effectively, although, again, normal cells are killed by contact with *E. histolytica*.

Role of complement

It seems inevitable that *E. histolytica* must reach the liver via the portal circulation and therefore obvious that it must survive the journey. Reed and her colleagues showed that while all *E. dispar* isolates (and old, laboratory-adapted *E. histolytica* isolates in axenic culture) were lysed, freshly isolated strains of *E. histolytica* in xenic culture were apparently largely resistant to complement activated by the alternative pathway. This fact alone could have been enough to explain the noninvasiveness of *E. dispar*. At least two resistance mechanisms have been postulated – cleavage of C3 by the parasite's own cysteine protease and one involving yet another function for GIL – the molecule shares sequence similarities and antigenic cross-

reactivity with the complement regulatory protein CD59 and inhibits *E. histolytica* lysis mediated by C5b-9. However, Hamelmann and coworkers have obtained quite different results – non-pathogenic isolates (i.e. *E. dispar*) were nearly unaffected; freshly isolated pathogenic strains (i.e. *E. histolytica*) were susceptible to lysis and became more so on long-term cultivation, with or without bacteria. Complement resistance could only be acquired by hamster liver passage or by growth in media containing increasing concentrations of normal human serum. The results of the two groups are incompatible and the differences need to be resolved before the role of complement in amebiasis can be assessed.

Animal models

There are currently no realistic small animal models of human amebiasis; in all cases trophozoites must be injected directly into cecum or liver. Mice, with the exception of SCID animals, are almost completely resistant and the large literature is concerned with the *in vitro* effects of murine macrophages. These cells (like their human counterparts) can be activated for killing by IFN γ and TNF α ; the process appears to be nitric oxide mediated and TNF α increases the expression of nitric oxide synthase. The preferred model of ALA involves intrahepatic challenge of gerbils or hamsters (see below, Vaccines) where treatments which suppress cell-mediated immunity seem to enhance abscess formation.

Evasion strategies by the organism

These obviously exist, since amebic liver abscesses can enlarge relentlessly in the presence of high levels of circulating antibody, but their nature is not fully understood. Surrounded by necrotic tissue the parasite may not be exposed to the full concentration of serum antibody, and that which does bind is capped and shed very rapidly. Host cell-mediated responses are probably delayed and blunted by a number of mechanisms: antigen processing cells and effector cells are often killed on contact with the parasite; a monocyte (but not polymorph) locomotion inhibitory factor is produced which will minimize infiltration; and patient serum contains a factor that suppresses the production of activating cytokines by T cells – this may be the same as the parasite T cell mitogen which has recently been identified with GIL. Animal experiments have shown that macrophages from gerbil liver abscesses are deficient in the generation of reactive oxygen species, unresponsive to lymphokines and not cytotoxic (cells treated with *E. histolytica* extract *in vitro* showed much the same defects) while parasite proteins suppress the IFN γ -

induced expression of major histocompatibility complex (MHC) class II molecules by murine macrophages. All this suggests that host immune responses need to be suppressed to permit long-term parasite survival and so it is surprising that, while the severity of amebiasis is markedly increased in pregnancy or by corticosteroid treatment, there is virtually no evidence for such a phenomenon in patients with the acquired immune deficiency syndrome.

Vaccines

Vaccine developers always like to see solid immunity following the natural infection, but only one study has produced evidence of this for ALA and there is none from AD patients. Considerable success has been achieved in protecting small animals against challenge, but the artificiality of the models (see above) should be remembered when assessing how close we are to a human vaccine. Gerbils have been protected against intrahepatic challenge by CRP, by GIL (as have rats) and a recombinant fragment thereof, and by recombinant SREHP; and SCID mice by passively transferred anti-SREHP. The same antigen administered orally with cholera toxin has produced high levels of sIgA in mice and primates (but these animals cannot be realistically challenged) and the SREHP gene has been expressed in a *Salmonella typhimurium* vaccine strain. Progress is being made but there is still a long way to go.

See also: Cell-mediated immunity; Gastrointestinal tract infections; Parasites, immunity to.

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AMPHIBIAN IMMUNE SYSTEM

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Living amphibians are the descendants of the first tetrapod vertebrates that diverged from their lungfish-like ancestors in the Devonian period, about 450 million years ago. Urodela (newts, salamanders), Anura (toads, frogs) and Gymnophiona (limbless coecilians) are the three orders of the Lissamphibia infraclass and they actually represent about 4000

species. From their key position in vertebrate evolution, amphibians are important models for the developmental and comparative analysis of the immune system. Two species are currently studied: the New World urodele *Ambystoma mexicanum* (axolotl, a neotenic species) and the African anuran *Xenopus laevis* (South African toad, or clawed toad).

Lymphoid organs and lymphocytes

The amphibian thymus anlagen arise from the dorsal epithelium of the second (anuran), or third, fourth and fifth (urodeles) pharyngeal pouches. In *Xenopus*, the thymus buds are rapidly colonized by hematopoietic precursors, and by day 6–8 the cortex–medulla architecture becomes visible. The larval thymus involutes during metamorphosis, but efficient regeneration occurs in the metamorphosed froglet. The thymus then regresses at the time of sexual maturity. In axolotl the thymic anlagen appear at the time of hatching, 3 weeks after fertilization. The thymus buds are invaded by hematopoietic precursors 12 days after hatching, and the thymus develops slowly up to sexual maturity (about 12 months), and then regresses. The urodele thymus shows no cortex–medulla differentiation. The axolotl and *Xenopus* thymus contains most of the cell types that constitute the stroma of mammalian thymus, such as macrophages, nurse-like cells, cortical large dendritic cells and different types of epithelial cells.

The spleen appears about 12–14 days following fertilization in *Xenopus* and the mature spleen has a clear-cut red and white pulp, but no structures reminiscent of the avian and mammalian germinal centers. The axolotl spleen anlagen appear at the time of hatching (about 3 weeks after fertilization). Although some red/white pulp-like organization can be seen macroscopically, there is no follicular-like structure and no germinal centers. In urodeles and anurans, the hematopoietic peripheral layer of the liver supports B cell lymphopoiesis and granulocytopoiesis.

Immunoglobulin M (IgM)-producing cells can be observed along the digestive tract in axolotl, and IgM-, IgX- but no IgY-producing cells are present in the digestive tract of *Xenopus*. The anuran kidneys contain B lymphocytes, and can retain antigens in the intertubular tissue. Amphibians possess B cells and T-like cells in the blood and in *Xenopus* these cells can collaborate *in vitro* in a major histocompatibility complex (MHC)-restricted manner for the production of antibodies to T cell dependent antigens.

B Cells can be recognized in axolotl and *Xenopus* by monoclonal antibodies (mAbs) specific for the different heavy (H) and light (L) Ig chain isotypes. More difficult was the production of mAbs specific for T cells. In the axolotl, mAb 34.38.6 labels all thymocytes, 60–63% of sIg⁺ splenic lymphocytes, hematopoietic stem cells, granulocytes and macrophages of normal animals, but only 9% of splenic lymphocytes from thymectomized animals. A polyclonal antibody (L12) coprecipitates several (38, 43 and 22 kDa)

covalently linked molecules that form a multimeric complex on the axolotl T cell surface.

A membrane glycoprotein (120 kDa), which may be a CD8 equivalent, has been identified on *Xenopus* J strain T cells using mAbs XT-1 and AM22 which recognize *Xenopus* cortical thymocytes and a subpopulation of peripheral T cells. mAb AM15 recognizes a 18 kDa protein on a subpopulation of *Xenopus* T cells. Two mAbs (anti-CTX: 1S9-2 and X71) stain a subpopulation (65–80%) of *Xenopus* cortical thymocytes which are also labeled by mAb AM22 (anti-CD8-like), and thus could be the *Xenopus* equivalent of the avian and mammalian cortical DP thymocytes.

In tadpoles and adult *Xenopus*, the use of classical mitogenic agents, such as lipopolysaccharide (LPS), phytohemagglutinin (PHA), concanavalin A (Con A) and purified protein derivative (PPD), stimulate *in vitro* proliferation of B cells and T cells with the same responses that are seen in mammals. The PHA and mixed leukocyte responses are sensitive to thymectomy. Furthermore, a T cell growth factor similar to interleukin 2 (IL-2) is produced by stimulated T lymphocytes.

The axolotl has a population of B lymphocytes that proliferate in the presence of LPS at all steps of ontogenesis. The splenic T cells from adult, but not from young axolotls, proliferate significantly in response to PHA and Con A. Axolotl lymphocytes are also stimulated *in vitro* by the enterotoxins A and B from *Staphylococcus aureus*.

Lymphocyte antigen-specific receptors

T cell receptors (TCRs)

No antibodies have yet been produced against TCR-equivalent molecules in amphibians and the present knowledge regarding these molecules comes from recombinant DNA studies. Genes encoding the α and β chains of the axolotl TCR have been cloned. Five V α and 14 J α segments have been identified, and the structure of the CDR α 3 loops is in good agreement with that of mammals. The C α domain has the typical structure of mammalian and avian C α s, including the charged residues in the transmembrane segment that are thought to interact with other proteins in the membrane.

Fourteen V β families have been identified in the axolotl and several V β are sequence-related to the human V β 13 and V β 20 segments and to their murine V β 8 and V β 14 homologs. Four C β isotypes have been detected, C β 1, C β 2 and C β 3 show a high degree of identity, but the C β 4 is divergent (43–48% identity with C β 1–3). The transmembrane axolotl C β domain maintains the lysine residue which is

thought to be involved in the charged interaction between the TCR $\alpha\beta$ heterodimer and the CD3 complex. Specific J β segments are associated with each C β isotype and 4 D β -like sequences have been identified, suggesting that the axolotl TCR β locus may be organized into at least four independent (D β -J β -C β) clusters that use the same collection of V β segments.

The V β segments in *Xenopus* are organized into nine independent families, each containing one, or a few, members. Eight J β segments and two D β core segments have been detected, with probably a single C β .

B cell receptors (BCRs)

Two high molecular weight (IgM and IgX) and one low molecular weight (IgY) Ig classes have been characterized in *Xenopus*. The three heavy (H) chain isotypes can associate with 25, 27 and 29 kDa light (L) chains but almost no C ν (IgY) chain is associated with the 27 kDa L chains.

The genes encoding *Xenopus* H μ , H ν and H χ chains and the three L chain (ρ , σ and type III- λ -like) isotypes have been recently cloned. The C μ region is well related to other vertebrate C μ , and the C ν region of the IgY molecule is structurally related to avian C ν and mammalian C ϵ . The C χ region is not clearly phylogenetically related to any other vertebrate isotype. Switch regions are found 5' to the C ν and C χ genes and a DNA switch recombination event leads to the expression of C χ . Eleven V H families, about 15 D H segments and 9 J H segments have been detected in the *Xenopus* IgH locus. The V, D and J elements can rearrange randomly, and junctional diversity arises at the V-D and D-J junctions, with the possible occurrence of random nucleotide deletion (nibbling) and template (P) or non-template (N) nucleotide addition.

The *Xenopus* L ρ chains are homologous to the mammalian L κ chains. One C ρ and five J ρ elements that rearrange randomly with several members of a single V ρ family have been detected. The L λ (also called type III) chains are built from at least six V λ families, two J λ and two C λ segments; this is the most diverse *Xenopus* L chain isotype. The L σ chains consist of two (C σ 1 and C σ 2) isotypes and two (V σ 1 and V σ 2) families that rearrange with their own set of J σ 1 and J σ 2 elements.

High molecular weight (IgM) and low molecular weight (IgY) Ig classes are present in the axolotl. No B cells double stain with anti- μ and anti- ν mAbs, suggesting that B μ and B ν cells might represent independent B cell lineages. The axolotl C μ and C ν are closely related to their *Xenopus* counterparts and additional cysteines in C ν 1 and C ν 2 domains are consistent with an additional intradomain S—S

bond similar to the avian C ν and the human C ϵ . Eleven V H families, six J H and two D H segments have been identified and the same collection of V H segments is used by IgM and IgY molecules. However, it is not known whether, as found in *Xenopus*, an IgM to IgY switch occurs in individual B cells.

Major histocompatibility complex (MHC)

The *Xenopus* MHC is a single genetic region encoding classical class Ia and class II molecules. A distinct chromosome harbors nine subfamilies of nonclassical class Ib genes. *Xenopus* has a single classical class Ia locus, and a single polymorphic class Ia molecule per MHC haplotype is expressed in all *Xenopus* species, regardless of the ploidy. They display three class II β genes, with a pattern of nucleotide substitutions in the β 1 distal domain essentially similar to that found in functional mammalian class II genes.

Classical class Ia genes have also been characterized in the axolotl and show a strong structural homology with *Xenopus* class Ia genes. However, axolotls display 10–12 class Ia genes detected by Southern blot. Class II genes are not yet characterized.

The tissue distribution of MHC class I and class II molecules in the adult amphibian is somewhat similar to that seen in mammals: class Ia are ubiquitously expressed in all tissues except brain and gonads and class II are expressed by B lymphocytes and T lymphocytes. Class I molecules are not expressed in *Xenopus* before metamorphosis, but can be detected as soon as hatching in the axolotl.

The *Xenopus* MHC genetic region encoding class I and class II molecules directs rapid graft rejection, mixed lymphocyte reaction (MLR) and T-B collaboration. T cells interact with antigen-presenting cells similarly to mammals, and the *in vitro* secondary proliferative response is MHC restricted.

Immunobiology of T and B cells

Thymectomy in *Xenopus* either decreases or abolishes allograft rejection, MLR, PHA responsiveness, IgY antibody synthesis and antibody responses to thymus-dependent antigens. It does not abolish *in vivo* or *in vitro* responses to thymus-independent antigens or B cell polyclonal activation (LPS). *In vitro* assays using purified T and B cells from carrier- or hapten-primed *Xenopus* of various MHC types indicate that T cell help is involved in the differentiation of thymus-dependent antigen-primed B cells and that T-B collaboration is MHC restricted. IgM is produced first following antigenic stimulation, and

then is produced in conjunction with IgY. A second injection generates a significantly stronger ($\times 10$ – 100), mainly IgY, secondary response. Although somatic mutation occurs in *Xenopus* V_H segments at almost the same frequency found in mammals, these mutations may not be properly selected, perhaps because of the absence of germinal centers in the lymphoid organs.

Larval thymectomy suppresses allograft rejection in urodeles, but does not abolish *in vivo* responses to certain thymus-dependent antigens, such as sheep or horse erythrocytes. Larval and adult thymectomy, low dose (50–150 rads; 0.5–1.5 Gy) irradiation and hydrocortisone treatment enhance specific antibody synthesis against erythrocyte antigens. These observations suggest that T cell help is impaired in urodeles, but that some kind of T cell-dependent suppression acts on antibody production. Urodeles can be immunized against particulate but not against soluble antigens, and in normal and thymectomized axolotls IgM is the single antigen-sensitive Ig class, specific IgY is not produced. The kinetics of the antibody response is slow and there is no occurrence of a typical secondary response following hyperimmunization. Thus, although the *Xenopus* and axolotl IgY molecules are clearly homologous at the molecular level, their respective physiological functions are different: IgY are IgG-like (thymus dependent, sensitive to thymectomy) in anurans, but IgA-like in axolotl, at least in the first 7–8 months of development; most IgY are found associated with the digestive epithelium and are secreted into the gut lumen following transepithelial transport in association with secretory component-like molecules.

Ontogeny of the immune response

Xenopus tadpoles develop allorecognition (graft rejection, MLR) from about 12 days after fertilization, but the capacity to reject or to tolerate grafts depends on genetic background (minor non-MHC, or MHC differences) and on the balance of several parameters, such as the size of the grafts, age of tadpoles and breeding conditions. Metamorphosis is a crucial period when a high frequency of allogeneic grafts can be tolerated and this seems to be an active phenomenon, as thymectomy before metamorphosis decreases the level of tolerance. MLR and the splenic response to T cell mitogens are depressed at metamorphosis, but recover in the young froglet. This might be correlated to the transient decline in the number of lymphocytes at the time of metamorphosis, and could be a means by which the metamorphosing tadpole is able to remain unreactive to the numerous new self antigens that appear during

the metamorphosis climax, and an opportunity for the young froglet to build a new repertoire of self tolerant T cells.

The anti-DNP antibody spectrotypes are different and less heterogeneous in *Xenopus* tadpoles compared with adults, suggesting that pre- and post-metamorphosed *Xenopus* express different antibody repertoires. B cells can first be detected in the liver at 3–4 days; a second wave of B cell production possibly arises after metamorphosis, accounting for the detection of two successive antibody repertoires. Sequential rearrangement of V_H genes occurs from day 5 in developing tadpoles, all the J_H and D_H elements are randomly expressed at early larval stages, and the production of specific antibodies arises at about 10 days. Most antibodies are IgM; switching to IgY seems not to be efficient before metamorphosis, although Igs of the three classes are present in tadpole serum.

Urodele development is much slower than anuran, and lymphoid organs begin to develop at the time of hatching, 2–3 weeks after fertilization. *Ambystoma tigrinum* (tiger salamander) larvae begin to reject allografts at about 40 days following fertilization and seem to be fully competent for rejection at 100 days. Thymectomy efficiently suppresses allograft rejection when performed at 50–60 days. Clusters of IgM- and IgY-producing B cells are first seen in the spleen of 70-day-old axolotls, 30 days after differentiation of the spleen anlagen. IgM is first detected in the serum at 90 days (4% of the adult value) and its concentration linearly increases until 13 months. IgY is first detected at 7 months (5% of the adult value) and its concentration increases rapidly until 11 months. However, abundant secretory IgY molecules are present in the stomach and intestinal mucosa of young axolotls from day 50, and until the 7th month. IgY then progressively disappears from the gut but becomes detectable in the serum.

Tumors of the immune system

Several spontaneous thymus lymphoid tumors have been detected in the *Xenopus* colony by Louis Du Pasquier at the Basel Institute for Immunology. The first one (MAR1) was from a 4-year-old *Xenopus* of the ff strain and was transplantable in isogenic ff tadpoles and froglets, but not in fully adult animals. *In vitro* cell lines were derived from MAR1 (e.g. B3B7). Three other tumors were found, one from the ff strain (ff-2), and two from the LG15 strain (LG15/0 and LG15/40). All these tumors are sIg⁺, and MHC class I molecules are not expressed on the surface of B3B7, 15/0 and 15/40, but are present (with β_2 -microglobulin) on the surface of ff-2

cells. All the lines are labeled by mAbs X21.2 (thymocytes), AM22 (CD8 equivalent), and B3B7, ff-2 and 15/40 express large quantities of the CTX antigen which is thought to be a marker of the cortical DP lymphocytes. In the B3B7 line, the two alleles of the IgH locus are rearranged in frame (but are not transcribed), the two alleles of the IgL ρ locus seem to be deleted, and the two alleles of the IgL σ locus are rearranged – one is out of frame and the second uses a V σ pseudogene. These *Xenopus* lymphoid cell lines resemble some rare types of mammalian leukaemia and represent very useful tools for the analysis of lymphoid cell differentiation and Ig and TCR gene expression.

See also: Immunoglobulin, evolution of; MHC, evolution of; Phylogeny of the immune response; T cell receptor, evolution of.

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AMYLOID

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Although a waxy tissue deposit was observed in pathologic laboratories in the early eighteenth century, it was Rudolph Virchow in 1854 who called these 'lardaceous deposits' of the liver and spleen amyloid, when he found them to stain with iodine and sulfuric acid. This belief that the material was made up of carbohydrates (i.e. cellulose) was challenged, and the proteinaceous nature of the substance was generally accepted by the turn of the century. Most amyloid was thought to be related to chronic infections (tuberculosis, osteomyelitis) and subsequently to chronic inflammation (rheumatoid arthritis) and the term secondary or reactive amyloid has persisted in the clinical literature. In 1886, it was noted that amyloid could appear *de novo* and the then unusual clinical form named primary or idiopathic amyloid was discovered. The association of amyloid with aging, especially in the brain and heart, was long known, but regarded as incidental epiphenomena until recent years. Finally in 1952, in Portugal, a hereditary amyloid polyneuropathy was described, and has led to a growing literature of multiple kinships of hereditary amyloid.

Definition

Amyloidosis is now regarded as a generalized deposit of the protein amyloid extracellularly throughout parenchymal tissue (liver, spleen, kidney, adrenal), blood vessels, and heart. Virtually any tissue or organ may be involved, and in some cases the deposition is localized to a single area. The amyloid itself is an eosinophilic hyalin material, which after Congo red staining demonstrates green birefringence on polarization microscopy. Electron microscopy has shown amyloid to have a fibrillar ultrastructure and by X-ray diffraction it has a classical cross- β (β pleated sheet) appearance. It has generally been classified clinically as 1) Secondary (reactive) amyloidosis; 2) primary (idiopathic) amyloidosis; 3) amyloid associated with multiple myeloma (now known to have the same chemical characteristics as primary amyloid); 4) hereditary or familial amyloidosis; 5) amyloid associated with endocrine organs; 6) amyloid associated with aging (brain or heart); 7) amyloid associated with chronic hemodialysis; and finally 8) localized amyloid (Table 1). In the past few years,

Table 1 Simplified classification of amyloid

Clinical aspects	Protein or variant or precursor	Amyloid nomenclature
1. Secondary (reactive) Familial Mediterranean fever	apoSAA (precursor)	AA
2. Primary (idiopathic) Multiple myeloma associated	κ or λ chains	AL
3. Familial amyloid polyneuropathy	Transthyretin variant prototype Met 30 (multiple mutations)	ATTR
	Apolipoprotein A variant Arg 26 (multiple mutations)	AApoAI
	Gelsolin Asn 15	AGel
Familial amyloid cardiopathy	Transthyretin Met 111	ATTR
Senile cardiomyopathy	Transthyretin Ile 122	ATTR
4. Alzheimer disease	β protein from amyloid β protein precursor	A β
Down syndrome		
Dutch hereditary cerebral hemorrhage with amyloid		
5. Chronic hemodialysis	β_2 microglobulin	AB ₂ M
6. Adult onset (type II) diabetes mellitus	Islet amyloid polypeptide (IAPP) (amylin)	AIAPP
7. Medullary carcinoma of thyroid	Procalcitonin	ACalc
8. Icelandic hereditary cerebral hemorrhage with amyloid	Cystatin C (trace γ) Gln 68	ACyst
9. Isolated amyloid of the heart (atrium)	Atrial natriuretic factor	AANF
10. Creutzfeldt-Jacob disease	Prion protein (scrapie protein)	AP γ P

it has been demonstrated that while all amyloid has common physical characteristics, biochemically there are multiple proteins, unique for each clinical form of amyloid listed above, and that a variety of proteins with specific point mutations are found in the hereditary dominant forms of the disorder.

Pathogenesis

AA

Secondary amyloid consists of the protein AA (amyloid A) an 8.5 kDa protein that is heterogeneous (at the amino terminal end). It is putatively derived by proteolysis from the normal circulating protein apoSAA (serum amyloid A related protein) which has a molecular weight of 12 kDa. apoSAA is an apolipoprotein associated with high density lipoprotein (HDL) especially HDL₃ and acts as a very sensitive acute phase protein. SAA is polymorphic in mice (four genes and two pseudogenes have been identified) and in man (three genes have been identified). In man SAA₁ isotype appears to be the major contributor to AA while in mice SAA₂ isotype is the sole contributor.

AL

Idiopathic (primary amyloid) and that associated with multiple myeloma consists of whole or partially degraded immunoglobulin light chains (i.e. whole or part of the V_L domain, with a molecular weight of 8–23 kDa). These fragments derived from the amino terminal region are more predominantly λ than κ chains. It has been deduced that some λ structures make the light chain fragments more likely to polymerize as amyloid. Indeed, λ VI was first discovered as an amyloid protein. The identical fragment that constitutes the amyloid deposit may be found in the serum or urine as a monoclonal spike. Cases of heavy chain amyloid have been described.

ATTR

In the prototypic Portuguese (autosomal dominant) familial amyloid polyneuropathy, transthyretin (prealbumin) variants have been found to constitute the amyloid. This molecule circulates as a 55 kDa tetramer composed of 4 identical 127 amino acid monomers. This molecule is a carrier of thyroxine and of retinol binding protein. It also acts as a negative acute phase reactant. It is determined by one gene located on chromosome 18. The most common abnormality is the substitution of methionine for valine at position 30 in families of Portuguese, Swedish,

Japanese, Italian, and Greek origin. Substitutions in the families with other phenotypic expression occur at over 50 other positions. Several different mutations may be present at one position (i.e. 30, 33, 35, 45, 47, 50, 58, 84). Hereditary amyloid composed of variant apolipoprotein A1, of the proteins gelsolin, fibrinogen A α , lysozyme and of β protein have also been described, each in a different clinical syndrome.

AP

Other substances are associated with amyloid. Amyloid P component (AP) has been identified in all forms of amyloid as a molecule independent of the amyloid fibril but nonetheless intimately bound to it. AP is a glycoprotein of molecular weight 22 kDa in its monomeric form. It circulates as SAP (serum amyloid P component), a pentraxin composed of two pentameric units. SAP acts as an acute phase reactant in mice, but is clearly a constitutive protein in humans. Its role in amyloidogenesis is unclear. Do its ligand binding properties make it a focus for fibril deposition or is it an epiphenomenon? Glycosaminoglycans are also intimately associated with amyloid deposits.

AEF

Amyloid enhancing factor (AEF) has been extensively studied in animals and dramatically shortens the induction time for experimental AA deposition in mice. It appears to be a monokine produced by macrophages when stimulated by inflammation. Whether it plays a more pervasive role is yet to be determined.

AB2M

Other forms of amyloid exist. In chronic long-term hemodialysis, patients often develop a carpal tunnel syndrome, arthritis and cystic bone lesions, all due to amyloid. The protein isolated has been β 2 microglobulin, a single chain low molecular weight (11.8 kDa) polypeptide that is continually shed from all membranes. It does not pass through most dialysis membranes and in chronic hemodialysis its concentration may rise 50-fold. The specific local factors influencing its precipitation as amyloid are not yet known.

A β

It has long been known that the senile plaques of Alzheimer disease stain positively for amyloid. The amyloid fibrils of the senile plaque and cerebral amyloid angiopathy from both Alzheimer disease and Down syndrome have been isolated and identified as

β protein (also known as A4 protein), a 4.2 kDa protein (39 amino acid segment isolated from vessels; 43 amino acid segment from plaques). The 28 N-terminal amino acids are almost identical in the senile plaques and vessel. Beta protein is derived from a 695 amino acid glycoprotein of 79 kDa (β amyloid protein precursor A β PP) that crosses and constitutes part of the cell membrane. The precise cause of the cleavage of the A β PP is not known, but aberrant degradation involving serine protease inhibitors such as α 1 antichymotrypsin (ACT) which is regularly found in plaques has been suggested.

See also: Acute phase proteins; Bence Jones proteins; β_2 -microglobulin; Lymphoma.

ANAPHYLATOXINS

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The term anaphylatoxin stems from the observation, originally made by Friedberger in 1910, that injection of complement-activated serum in small experimental animals, such as guinea pigs, results in an anaphylactoid reaction similar to the symptoms of an acute hypersensitivity shock which occurs after exposure of sensitized animals to the antigen. This acute, potentially lethal, reaction was found to be due to small bioactive fragments, termed anaphylatoxins to describe the functional behavior of these humoral factors. Some of the effects of systemic administration of anaphylatoxins are due to the release of bioactive cell-derived mediators as in hypersensitivity reactions, but the lethal effect is rather related to the potent spasmogenic activity of the anaphylatoxins causing respiratory failure due to acute bronchiolar constriction. It is unlikely that in humans anaphylatoxins have such dramatic effects, although they certainly contribute to the pathogenesis of, for example, septic shock and the acute respiratory distress syndrome (ARDS) in synergy with other proinflammatory factors, such as lipopolysaccharide (LPS) and tumor necrosis factor (TNF). Indeed, high levels of C3a can be detected *in vivo* under these clinical conditions.

Mainly based on their activity on the isolated guinea pig ileum, the anaphylatoxins C3a, C4a and C5a have been isolated from complement-activated serum and their amino acid sequence was determined many years ago. A large number of more recent func-

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tional studies, and the characterization of anaphylatoxin receptors, however, clearly established that the anaphylatoxins are typical chemotactic cell agonists. They act in a very similar fashion to other chemotactic agonists, such as the bacterial leader sequence analogue N-formyl-met-leu-phe, the cell-derived lipid mediators leukotriene B₄ and platelet-activating factor, and the very large group of cell-derived chemotactic cytokines, the chemokines. All these agonists act on cells mainly of hematopoietic origin, by interaction with specific, pertussis toxin-sensitive, G_i-coupled seven-transmembrane (serpentine) receptors, and they activate the cells by utilizing very similar signal transduction pathways leading to similar cellular functions. Based on sequence homologies and the similarity in function, the receptors for all chemotactic agonists, including the receptors for anaphylatoxins, constitute a subfamily of the rhodopsin receptor superfamily. It is important to note that, despite this redundancy, the anaphylatoxins are the only well-characterized endogenous humoral inflammatory mediators among this large group of chemotactic leukocyte agonists.

Generation of anaphylatoxins

The anaphylatoxins C3a, C4a and C5a are formed by specific cleavage of a single Arg–X bond in the α chains of C3, C4 and C5, respectively, through the corresponding convertase. The C3, C4 and C5 con-

vertases are formed during complement activation by the classical pathway, the lectin pathway and (C3 and C5 convertases only) the alternative pathway. Thus, by the generation of bioactive activation products, the complement cascade connects recognition systems of the innate and adaptive immune system with effector systems for host defense. A major consequence of the anaphylatoxins generated locally due to tissue injury, infection or immunoglobulin-antigen complexes is to promote an inflammatory reaction. This inflammatory response is primarily due to the capacity of the anaphylatoxins to induce the release of cell-derived vasoactive mediators and to directly attract and activate inflammatory effector cells. C3a and C5a may also be generated without complement activation by certain proteases of the host or from pathogens (e.g. mast cell tryptase and a cysteine proteinase from *Bacteroides gingivalis*, cleaving C3 and C5, respectively).

The C-terminal arginine of the anaphylatoxins is rapidly cleaved by serum carboxypeptidase N, resulting in peptides with negligible (C3a_{desarg}, C4a_{desarg}) or altered (C5a_{desarg}) bioactivity. Thus, the radius and duration of action of C3a is rather short, while the major stable soluble bioactive product of complement activation is C5a_{desarg}. C5a_{desarg} retains sufficient affinity for the C5a receptor to be cleared from the circulation or from inflammatory exudates by receptor-mediated endocytosis. C3a_{desarg} and C4a_{desarg}, however, can be detected in bodily fluids, and assay systems to monitor the levels of these anaphylatoxins have been developed as an efficient and sensitive way to assess complement activation *in vivo*. Because of the central role of C3 in all the complement pathways, C3a is a general parameter of complement activation, while C4a has been regarded as a specific marker for the activation of the classical pathway by immune complexes. The recent discovery of the lectin pathway, which is initiated by recognition of certain carbohydrates on pathogens and which is homologous to the classical pathway, makes this assumption and the clinical utility of C4a measurements less certain. The highest levels of anaphylatoxins are found in acute states such as ARDS, multisystem organ failure and sepsis, where anaphylatoxins may actually contribute to a potentially lethal outcome. However, more subtle changes in anaphylatoxins (C3a or C4a) have been described in a number of clinical patient studies in a variety of (primarily inflammatory) disorders. Complement is also activated by contact of plasma with artificial surfaces *in vivo* and well as *in vitro*. Thus, complement activation *ex vivo* must be carefully monitored when collecting blood samples. Furthermore, significant complement activation occurs during

extracorporeal blood treatment by cardiopulmonary bypass surgery and dialysis, probably contributing to side-effects and complications of these procedures. Progress in decreasing complement activation by artificial surfaces has allowed an increase in the biocompatibility of such membranes.

Structure of anaphylatoxins

The complement fragments C3a, C4a and C5a released during complement activation are, in humans, 77–74 amino acid residues in length, and share, as their parent proteins C3, C4 and C5, significant homology in their primary sequence. They all have a core with three disulfide bridges. More importantly, crystallographic data of C3a and nuclear magnetic resonance (NMR) studies of C3a and C5a in solution showed that C3a and C5a have very similar secondary and tertiary structures. The first 63 residues of C5a in solution form an antiparallel bundle of four helices similar to the coiled-coil arrangement of helices in many other four-helix proteins, while the C-terminus is disordered. The helices I, II, and IV are quite regular and are compatible with an α -helical pitch. The N-terminal helix is amphiphilic and its hydrophobic residues are interdigitated with hydrophobic residues on helices II and IV. The structure of C5a seems to differ somewhat from the crystal structure of C3a, which shows a disordered N-terminus and a helical C-terminus, but NMR investigations of C3a in solution indicate that the terminal regions of both anaphylatoxins are similar.

Thus, based on similarities of bioactivity, of their receptors as well as of their structure, the anaphylatoxins must be regarded as a family of closely related molecules. Anaphylatoxins of other species are most likely very similar in structure as well. Furthermore, the bioactivity of C3a and C5a is not species restricted, as is the case for many cytokines. Human C5a has, however, some notable peculiarities. First, there is a N-terminal odd cysteine at position 27 in the loop connecting helix II with helix III, facing towards the solvent. It can be attached to molecules such as glutathione without effect on bioactivity, and it may covalently link to other proteins with a free accessible -SH group under an oxidative environment. Indeed, yet poorly defined high molecular weight chemotactic factors have been described in serum. Second, a bulky sugar moiety is attached in human C5a near the C-terminus which is of major importance in receptor activation. This glycosylation may be responsible in part for the fact that natural human C5a_{desarg} is a potent chemoattractant but a very ineffective cell activator and spasmogen.

C3a and C4a receptors

It is still unclear whether C3a and C4a mediate their effects by common or separate receptors. Based on their activity and cross-desensitization on the guinea pig ileum contraction assay, C4a has been regarded as a weak C3a receptor agonist. This assumption requires confirmation on cells transfected with the C3a receptor. Other less conclusive data on guinea pig macrophages indicate that there may be separate receptors.

The C3a receptor could not be identified until recently, although C3a was isolated decades ago. Despite this lack of a defined cellular model, extensive investigations, mainly by T Hugli and coworkers in the heterologous guinea pig system, established the structure-function relationships of C3a analog peptides and identified the C-terminal pentapeptide as a full C3a receptor agonist. Further studies revealed that the C-terminal 21-residue fragment is nearly as potent as the complete anaphylatoxin, and, by attaching several hydrophobic residues to the N-terminus of a synthetic peptide based on the 12–13 residue C-terminal fragment of C3a, superagonists of even greater potency than the natural factor were obtained (Figure 1). Thus, the interaction of C3a with its receptor is clearly less complex than that of C5a and probably most other protein ligands of seven-transmembrane receptors.

The human C3a receptor has recently been cloned by two independent groups. It is a typical seven-transmembrane receptor of the chemotactic factor subfamily. As for other chemotaxin receptors, activation of the C3a receptor in transfected cells promotes a transient elevation of cytosolic calcium concentration by a pertussis sensitive G_i -coupled activation of phospholipase C. In contrast to other G protein-coupled receptors, the C3a receptor carries an unusually large second extracellular loop of over 160 amino acids of unknown function. Neglecting this loop, the C3a receptor is most closely related to the C5a and fMLP receptor, with 37% and 34% homology, respectively. The C3a receptor mRNA is widely expressed in peripheral blood lymphocytes, different lymphoid tissues as well as in different solid organs. The identification of the receptor for C3a will now permit better characterization of the role of this anaphylatoxin in homeostasis and host defense, and will aid in the development of specific antagonists.

C5a receptors

The C5a receptor was cloned independently by two groups several years ago, and was shown to be a G

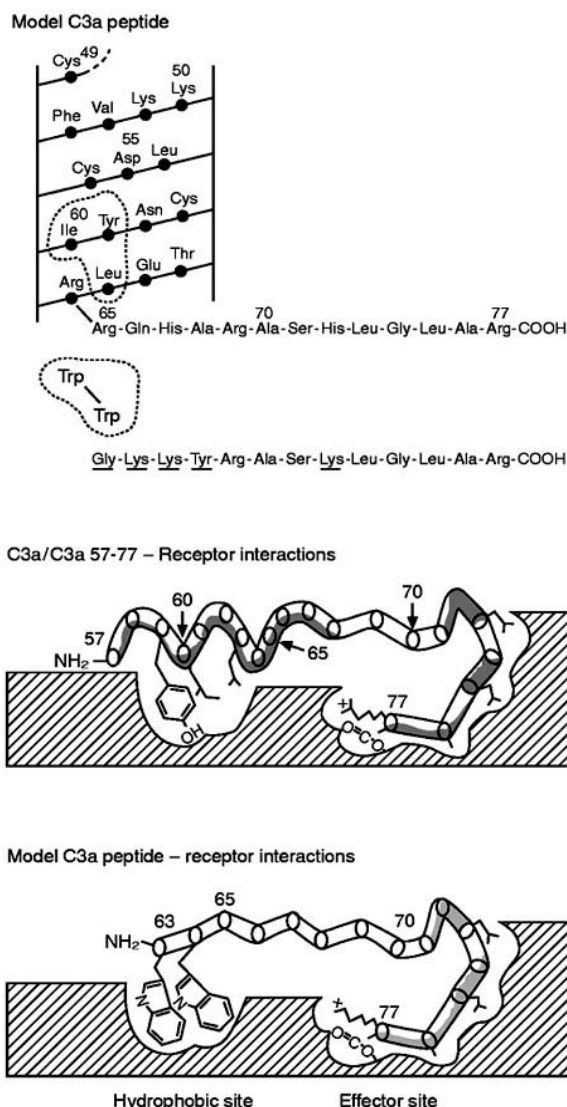


Figure 1 (Upper) The helical region of the C-terminal end of C3a is shown with the hydrophobic patch outlined (dashed line) including residues Tyr59, Ile60 and Leu63. This patch is believed to interact with a hydrophobic sub-binding site of the C3a receptor. The superpotent synthetic analog of C3a has two tryptophan residues substituted for the hydrophobic patch on the natural factor. The residues that are underlined are nonessential replacements that improve solubility of the analog. (Lower) The schematic model illustrates how the natural factor C3a and the synthetic analog C3a 57–77 interacts with the receptor. The hypothesis is that the model Trp-containing superpotent peptide mimics C3a by interacting with the receptor in a similar manner. Interaction at the hydrophobic sub-binding site cooperatively enhances binding of the essential Leu-Gly-Leu-Ala-Arg effector site. If binding of Trp-Trp at the hydrophobic site is greater than the Tyr-Ile-Leu patch, it could explain the superpotency of the analog peptide.

protein coupled seven-transmembrane receptor of 350 residues in length with high (34%) homology to the fMLP receptor. This has allowed a detailed molecular characterization of ligand–receptor interaction using monoclonal antibodies and mutants of both C5a and its receptor. Furthermore, based on weak binding of the C-terminal octapeptide of C5a to the C5a receptor, several peptide analogs with reasonable affinity and agonistic, partial agonistic or antagonistic properties were developed. These studies led to a two-binding model of ligand–receptor interaction, as first proposed by MS Springer (Figure 2). One involves the core of C5a (with participation of Lys19 and Lys20) interacting with the extracellular N-terminus (in particular with several aspartic acid residues in this region), presumably resulting in a conformational change of C5a, which allows its C-

terminus to interact with a second, ill-defined, binding site in a transmembrane segment. Using synthetic C5a hexapeptide analogs and mutant receptors, an interaction of the peptide C-terminal arginine with Arg206 in the fifth transmembrane helix of the receptor has been proposed as a mechanism to activate this second binding site. It should be noted, however, that C5a_{desarg}, which has no C-terminal arginine, is a potent chemoattractant, but is rather ineffective in activating many other cell functions, indicating that different types of ligand–receptor interactions exist which lead to distinct signaling events.

Bioactivity of anaphylatoxins

C3a

While most of the earlier investigations on C3a bioactivity dealt with its spasmogenic and vasoactive properties *in vivo* and in tissues of experimental animals, more recent studies revealed that the effect of C3a is not so much different from that of other chemotactic agonists. However, the target cell profile of C3a is more restricted than that of C5a. C3a induces calcium transients in all myeloid cells indicating wide expression of C3a receptors on leukocytes, although C3a has negligible effects on the function of monocytes and neutrophils. C3a is a chemoattractant and potent inducer of exocytosis for eosinophils. The efficacy of C3a in inducing the release of cytotoxic products is similar to that of C5a and clearly higher than that of chemokines, such as eotaxin. C3a also induces mediator release in cytokine-primed basophils and in mast cells from the skin but not from the lung, while the effects on mast cells from other sites (e.g. intestine, heart, uterus) are largely unknown. These data indicate that C3a may be important in allergic inflammation and other conditions involving mast cells, eosinophils and basophils. C3a_{desarg} cannot activate the C3a receptor. High levels (around 10 μM) of C3a_{desarg} seem to directly activate G proteins similar to other cationic peptides, such as mastoparan. The biological meaning of this effect is uncertain, although such high levels can theoretically be achieved *in vivo*.

C5a

C5a is a classical and most potent and effective chemoattractant and activator of effector functions of different leukocyte types, and, among the different chemotaxins, the effects are most similar to that of fMLP. Unlike chemokines, which have a more restricted target cell profile, C5a is rather pleiotropic and acts efficiently on neutrophils, monocytes, macrophages, eosinophils, basophils, dendritic cells

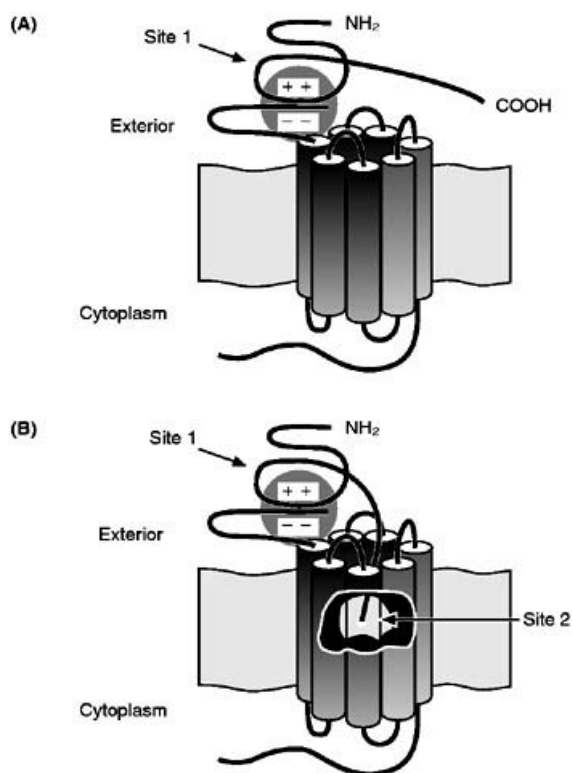


Figure 2 Model for the binding of C5a to its receptor. As shown in (B), the interaction takes place at distinct sites. The first, designated site 1, is between the N-terminus, and possibly the second extracellular loop of the receptor, and the core of C5a. Specifically, primary interaction is thought to be between several of the aspartic acid residues in the N-terminus and Arg40 and possibly Arg37 and His15 of C5a. The second interaction site, designated site 2, is between the C-terminus of C5a and the interhelical region of the receptor. The primary interaction involves Arg74 and Lys68 of C5a. The initial site of productive contact is believed to take place at site 1, as depicted in (A). The contact at site 1 effectively raises the local concentration of C5a and thereby promotes the more difficult interaction at site 2. (Reproduced from Siciliano *et al* (1994).)

and possibly some lymphocyte subpopulations. Neutrophil responses to C5a have been most extensively studied and shown to include the induction of calcium transients, shape change, cell polarization and chemotaxis, upregulation of receptors, integrin activation and, at higher concentrations, exocytosis, oxygen radical production, induction of a hyperadhesive state and enhancement of phagocytic and cytotoxic functions. Furthermore, after priming with the appropriate cytokine – granulocyte-macrophage colony-stimulating factor (GM-CSF) for neutrophils and monocytes, interleukin 3 (IL-3), IL-5 and GM-CSF for basophils and eosinophils – C5a promotes the synthesis of large amounts of the lipid mediators leukotriene B₄ (LTB₄), platelet-activating factor (PAF), and LTC₄. Together with fMLP, C5a induces the broadest range of biological responses in effector cells, in contrast to most other chemotactic agonists which more preferentially activate either chemotaxis or cell activation, indicating important, yet ill-defined, differences in receptor signaling. Interestingly, C5a_{desarg} is a poor activator of most leukocytes, but retains potent chemotactic activity, thus becoming more similar to other chemotaxins such as IL-8 and LTB₄. The bioactivity of C5a_{desarg} also depends on the cell type: it is still chemotactic for a broad range of leukocytes, yet stimulates efficient exocytosis only in basophils, but not in mast cells or mast cell lines. The reason for this complexity is unknown.

Cytokine expression and immunoregulation

More recent studies have shown that C5a in synergy with LPS promotes expression of IL-1 and IL-6 in monocytes. Combined stimulation of basophils with IL-3 and C5a induces the selective expression of IL-4 and IL-13, which are key immunoregulatory cytokines for the induction of a T_H2-type immune response and for the synthesis of immunoglobulin E (IgE). C5a is unique in this respect, as other chemotactic agonists, such as chemokines which are also effective triggers of chemotaxis and/or exocytosis, are unable to induce cytokine expression. Thus, C5a is not just an inflammatory mediator but may also have indirect immunoregulatory properties, and promote humoral and T_H2 type immune responses. These observations also emphasize the importance of studying the effects of anaphylatoxins within the network of other factors.

Activities outside the immune system

C5a also acts on liver cells and induces the expression of acute phase proteins. Furthermore, the mRNA for the receptors for C5a and C3a are widely expressed in a large number of tissues and organs, indicating that anaphylatoxins may have other, largely unexplored, functions outside the immune system. Particularly abundant transcripts for the C5a receptor are found in lung, spleen, heart, placenta, spinal cord and throughout the brain, and for the C3a receptor in lung, spleen, ovary, placenta, small intestine and less ubiquitously in the brain.

See also: Acute inflammatory reaction; Arachidonic acid and the leukotrienes; Complement, alternative pathway; Chemokines; Chemotaxis; Complement, classical pathway; Complement, membrane attack pathway; Complement receptors; Cutaneous anaphylaxis; Platelet-activating factor (PAF).

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ANEMIA, AUTOIMMUNE HEMOLYTIC IN ANIMAL MODELS

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Autoimmune hemolytic anemia (AIHA) is a classic example of an antibody-mediated autoimmune disease. It is well documented how autoantibody-coated red blood cells (RBCs) can be destroyed by splenic macrophages, but by contrast, the mechanisms that initiate the production of RBC autoantibodies remain unclear. The New Zealand Black (NZB) strain of mice spontaneously develops AIHA. RBC-bound immunoglobulin G (IgG) autoantibodies can be detected by Coombs' test from 3 months of age and the mice develop signs of anemia 2–3 months later. For two decades after these mice were first bred in 1959, a flood of papers appeared documenting the specificity of the autoantibodies and attempting to pinpoint lesions in particular cells which could result in the generation of RBC autoantibodies. Many of these results are now open to re-evaluation in the light of recent studies.

Antigen specificity of RBC autoantibodies from NZB mice

Historically, four distinct RBC autoantigens were described in NZB mice, namely X, HB, I and HOL. The IgG response to the antigen designated X appears to be of primary pathogenic importance, since only antibodies of this specificity are exclusive to the NZB mouse and are capable of binding untreated murine RBCs, i.e. to determinants normally exposed on the RBC surface. By contrast, IgM autoantibodies reactive with the antigens HB and I, and IgG antibodies specific for HOL are also found in strains of healthy mice, and recognize cryptic epitopes on the RBC membrane. Recent work has confirmed that antibodies eluted from the RBCs of old, anemic NZB mice are pathogenic when injected into younger animals with prepatent disease. Moreover,

as judged by immunoprecipitation, the target of these antibodies is band 3, the RBC anion channel protein. These results would suggest therefore that X is band 3.

One approach to understanding the pathogenesis of AIHA in NZB mice is to prepare and characterize hybridoma antibodies derived from spleen cells of anemic animals. A number of such monoclonal antibodies that react with intact murine RBCs have been generated and their properties examined in detail. For example, analysis of the variable region gene usage of NZB-derived RBC-specific monoclonal antibodies reveals evidence of somatic mutation, consistent with an antigen-driven response. Some of the monoclonal antibodies are pathogenic *in vivo*. Injection of pathogenic IgG monoclonal antibodies into mice induces hemolysis by Fc-dependent erythrophagocytosis, whereas massive accumulations of hemagglutinated RBCs are found in the recipients of pathogenic IgM monoclonals. It should be noted that hemagglutinated RBCs are not prominent on histological examination of spleens from anemic NZB mice.

The erythrocyte autoantigens recognized by a panel of eight hybridoma RBC autoantibodies were recently characterized. Two of the three pathogenic IgG monoclonal antibodies immunoprecipitated band 3. A third nonpathogenic IgG monoclonal also precipitated band 3 but only at high concentrations. This monoclonal was the only one to react with band 3 by immunoblotting and it alone of the IgG monoclonals reacts with RBCs from other species. None of the four IgM monoclonal antibodies precipitated any labeled RBC components. However, by immunoblotting, the two pathogenic IgM monoclonals bound to a doublet which corresponds in electrophoretic mobility with band 4.1 from the

internal RBC membrane skeleton. An explanation of how these antibodies bound intact RBCs may come from the finding that they also bound to histones, suggesting that they are polyreactive and recognize surface autoantigens such as phospholipids. Thus, the demonstration that two pathogenic monoclonal autoantibodies recognize band 3 confirms that this protein bears important autoantigenic epitopes but also reveals that some pathogenic monoclonal antibodies do not share this characteristic. It could be argued that the latter monoclonal antibodies represent minor components of the total RBC autoantibody population or that antibodies to multiple RBC antigens, in addition to Band 3, contribute to hemolysis in NZB mice.

Transgenic mice

Honjo and his colleagues generated transgenic mice expressing one of the pathogenic IgM monoclonal antibodies derived from NZB mice. In these mice conventional (B-2) B cells were deleted, presumably after contact with RBCs, but normal numbers of CD5 B-1 cells were present in the peritoneal cavity and lamina propria. About 50% of these mice developed AIHA in a conventional environment but not if maintained in isolators. Oral administration of LPS induced AIHA in asymptomatic anti-RBC transgenic mice but not in transgenic mice bearing the *xid* (B-1 cell deficient) mutation. Since intraperitoneal injection of RBC into the transgenic mice induced apoptotic death of peritoneal B-1 cells and prevented AIHA developing, it appears that RBC-reactive B-1 cells survive in the RBC-free regions of the peritoneal cavity and gut and are stimulated by microorganisms to produce RBC autoantibodies. Are these findings relevant to NZB disease? It has been known for some years that the introduction of the *xid* mutation into NZB mice reduces the production of RBC autoantibodies and the development of anemia. The therapeutic effect of the *xid* gene could result not only from the lack of B-1 cells but also from defects introduced into conventional B cells. However, NZB mice injected intraperitoneally with distilled water weekly from birth to 12 months of age have markedly reduced splenic B-1 cells, increased hematocrit values and reduced levels of RBC-bound IgG and IgM. These results suggest that B-1 cells contribute directly to the development of NZB disease by secreting IgM RBC autoantibodies and that some IgG RBC autoantibody-secreting cells are derived, directly or indirectly, from B-1 cells.

Ontogenesis of RBC autoantibody response

Early work showed that neonatal thymectomy of NZB mice failed to prevent the generation of RBC autoantibody, although thymectomy immediately after birth appeared to delay the onset of autoantibody production. It was also established that RBC autoantibody production can be transferred to irradiated non-NZB H-2^d recipients with bone marrow cells. Since the anti-RBC plaque-forming response still occurred in chronically T-depleted recipients, it was argued that an intrinsic defect was present in NZB B cells and implied that the RBC autoantibody response was T-independent. Recent work calls these interpretations into question. First, SCID mice repopulated with NZB pre-B cells fail to develop RBC autoantibodies despite that fact that high levels of IgM anti-DNA and low levels of IgG anti-DNA are generated. Secondly, chronic treatment of NZB mice with monoclonal anti-CD4 prevents or considerably delays the generation of RBC autoantibodies. Interestingly, anemia still occurs in anti-CD4-treated NZB mice, possibly because they developed an ulcerative colitis. Finally, Gershwin and his colleagues have generated CD4 and CD8 gene-deleted NZB mice. Again the incidence and titers of RBC autoantibodies (particularly of the IgM isotype) were significantly reduced in NZB.CD4^{-/-} mice as compared with wild-type NZBs. By contrast, no significant change in RBC autoantibody responses was observed in NZB.CD8^{-/-} mice. Thus CD4⁺ T cells clearly contribute to the development of NZB AIHA.

Specificity and characterization of T cells

Since the generation of pathogenic anti-RBC autoantibodies is CD4 T lymphocyte dependent, it would be predicted that NZB mice harbor helper T cells responsive to RBC autoantigens. Splenic T cells from NZB mice, but not other H-2^d strains such as BALB/c or DBA2 mice, do indeed proliferate *in vitro* in response to band 3. The majority of the responding cells express CD4 and the response is major histocompatibility complex class II restricted. Thus the T cells which help the production of the band 3 reactive autoantibodies may also be band 3 specific. The epitopes which stimulate band 3-reactive T cells have been mapped. Splenic T cells from old NZB mice respond to some 20 15mer peptides corresponding to the sequence of band 3. However, the response to one peptide is dominant in terms of magnitude and the appearance of the response with age. Splenic T cells from NZB mice as

young as 3 weeks old respond to this peptide but generally not to others, suggesting that the response is initially focused on the dominant peptide and later spreads to other band 3 epitopes. So far attempts to demonstrate that band 3-reactive T cells are present and primed in the thymus have failed. Thus band 3-reactive T cells may be primed in the periphery. Nevertheless, the observation of abnormalities in the structure of the NZB thymus suggests that events in the thymus should not be neglected.

CD4⁺ T cells can be classified into two types, T_H1 and T_H2, depending on the cytokines they produce. For example, T_H1 cells typically secrete interferon γ (IFN γ) and T_H2 cells interleukin 4 (IL-4). The type of cytokine secreted by helper T cells affects the isotype of antibody produced, and in mice T_H1-dominated responses are associated with IgG2a antibodies and T_H2 with IgG1. Whilst there is now a consensus that pathology of cell-mediated autoimmune diseases is linked to the stimulation of T_H1 autoreactive cells, it might be expected and indeed it has been suggested that T_H2 responses may be important in initiating and driving antibody-mediated autoimmune disorders such as AIHA. However, NZB T cells stimulated with band 3 produced high levels of IFN γ , but little or no IL-4, IL-5 or IL-10. Similar patterns were produced by NZB T cells responding to a spectrin preparation from the RBC membrane skeleton, or to mycobacterial heat shock protein (hsp) 65 following immunization of mice with hsp65 in incomplete adjuvant. By contrast, T cells from CBA mice similarly immunized with hsp65 produced high levels of IL-4 and IL-5 in response to hsp65.

Examination of the isotype of the RBC-bound immunoglobulins in NZB mice revealed that IgG2a autoantibodies were the first to be detected in most mice and that later in the disease, IgG3 autoantibodies were often prominent. Consequently, contrary to expectation, the development of RBC autoantibodies in NZB mice is associated with T_H1 cytokine-dominated responses. How T_H1 cytokines effect NZB disease remains to be determined, although it has been suggested that T_H1 cytokines can alter antigen processing so that previously cryptic epitopes become presented and are thus responsible for epitope spreading.

See also: Anemia, autoimmune hemolytic in human; Antiglobulin (Coombs') test; Autoantigens; Autoimmune diseases; Suppressor T lymphocytes; Tolerance, peripheral.

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ANEMIA, AUTOIMMUNE HEMOLYTIC IN HUMAN

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The autoimmune hemolytic anemias represent a group of disorders in which individuals produce antibodies directed toward one or more of their own erythrocyte membrane antigens. This in turn leads to destruction of the erythrocytes. An effective manner in which to approach autoimmune hemolytic anemia is to analyze which class of antibody is responsible for the hemolysis. In general, there are two major classes of antierythrocyte antibodies which produce hemolysis in humans: immunoglobulin G (IgG) and IgM. The pattern of red blood cell clearance, the site of organ sequestration, the response to therapy, and the prognosis all relate to the class of antierythrocyte antibody involved.

IgM-induced immune hemolytic anemia

Autoimmune hemolytic anemia caused by IgM antibody in humans is generally restricted to the clinical entity of cold hemagglutinin disease, in which the antibodies only bind significantly to the erythrocytes at temperatures below 37°C. The most common form of chronic cold hemagglutinin disease is the primary or idiopathic form caused by an IgM antibody. This is a clonal disorder and is associated with the presence of a monoclonal IgM antibody which usually has a high cold agglutinin titer. The IgM antibody is usually directed against the I antigen or related antigens on the human erythrocyte membrane. As with all IgM antibodies agglutinating activity is particularly efficient because of the multiple antigen combining sites on the IgM molecule. The cold agglutinin titer represents the least dilution of patient plasma or serum capable of agglutinating human red blood cells in the cold. In most patients with hemolysis the cold agglutinin titer is greater than 1:1000.

Secondary cold hemagglutinin disease is most commonly associated with an underlying mycoplasma infection, particularly *Mycoplasma pneumoniae*. It may also occur with other infections, such as infectious mononucleosis or cytomegalovirus. Cold hemagglutinin disease (IgM-induced immune hemolytic anemia) can also be seen with an underlying immunoproliferative disorder, such as chronic lymphocytic leukemia, non-Hodgkin's lymphoma, or systemic lupus erythematosus. The disease may be chronic and the IgM protein may be of restricted het-

erogeneity (oligoclonal) or even be monoclonal. When this is the case, one should suspect an underlying malignant immunoproliferative disorder and the prognosis is adversely affected.

As in all patients with autoimmune hemolytic anemia, erythrocyte survival is generally proportional to the amount of antibody on the erythrocyte surface. In cold hemagglutinin disease the extent of hemolysis is a function of the titer of the antibody (cold agglutinin titer), the thermal amplitude of the IgM antibody (the highest temperature at which the antibody is active), and the level of the circulating control proteins of the C3b inactivator system. Hemolysis is complement-dependent and the IgM-coated erythrocytes become coated with C3b by classical complement pathway activation. These C3b-coated erythrocytes are cleared primarily by the macrophage C3b receptors in the liver. Two uncommon variants of cold hemagglutinin disease, low IgM titer cold hemagglutinin disease and cold hemagglutinin disease mediated by IgG antibody are more responsive to therapy.

IgG-induced immune hemolytic anemia

In IgG-induced immune hemolytic anemia, the antibodies are of the IgG class and the antigen to which the antibody is directed is usually an Rh erythrocyte antigen. The antibody has its maximal activity at 37°C, and, thus, this entity has been termed warm antibody-induced hemolytic anemia. IgG-induced immune hemolytic anemia may occur without an apparent underlying disease (idiopathic type); however, it may also occur with an underlying immunoproliferative disorder, such as chronic lymphocytic leukemia, non-Hodgkins lymphoma or systemic lupus erythematosus. The signs and symptoms are those of anemia in general. The diagnosis is established by directly examining the erythrocyte surface for the presence of IgG and C3b.

In addition to C3b receptors, macrophages within the reticuloendothelial system have receptors for the Fc fragment of IgG, called Fcγ receptors. The macrophage Fcγ receptors can detect IgG-coated erythrocytes, bind them, make them spherical, or phagocytose them in the absence of C3b. However, once C3b is placed on the erythrocyte surface, through com-

plement activation, erythrocyte clearance is further accelerated. Thus, IgG-coated erythrocytes are progressively cleared from the circulation by macrophages possessing Fc γ receptors. Hemolysis is almost always extravascular and these IgG-coated cells are cleared predominantly in the spleen. Complement activation accelerates the clearance of IgG-coated cells.

Drug-induced immune hemolytic anemia

Drug-induced immune hemolytic anemia can be divided into four major pathophysiologic groups. Clinical signs and symptoms are identical to those of the other autoimmune hemolytic anemias. The diagnosis is established primarily by history.

Hapten type

This type of drug-induced immune hemolysis classically develops in patients exposed to high doses of penicillin. A portion of the penicillin molecule or its active metabolites combines with the erythrocyte surface, acting as a hapten. This induces an antibody response directed against the penicillin-coated erythrocyte membrane. This is usually an IgG response, and complement activation is common. The patient's erythrocytes become coated with IgG and often with C3. Patients rarely develop this syndrome unless they have received 10–20 million units of penicillin a day. Diagnosis can be established by incubating the patient's serum with donor erythrocytes preincubated with penicillin. The deposition of IgG antibody will occur only in the presence of penicillin and can be detected with the Coombs test.

Quinidine type

This type of autoimmune hemolytic anemia most commonly occurs with the use of quinidine or its derivatives. Commonly called an innocent bystander reaction, it is believed to be due to an antibody directed against quinidine bound to a plasma protein or the red blood cell membrane acting as a hapten. This interaction results in activation of the classic complement pathway and deposition of C3 on the erythrocyte surface. With quinidine it is commonly caused by IgM antiquinidine antibody. The diagnosis can be established *in vitro* by examining for complement deposition on donor erythrocytes by patient serum which occurs only in the presence of the drug, e.g. quinidine.

α -Methyldopa type

α -Methyldopa and its derivatives produce a clinical syndrome virtually identical to IgG-induced immune

hemolytic anemia. The mechanism of the IgG antibody formation is poorly understood. Many patients, up to 25%, exposed to α -methyldopa, develop a positive Coombs' test for IgG. These IgG antibodies have specificity for Rh antigens. Most patients do not develop sufficient IgG coating for hemolysis; however, hemolysis is observed in approximately 0.8% of patients exposed to α -methyldopa. Diagnosis can be made by examining the patient's red blood cells and plasma. *In vitro* it is not necessary to have the drug present for the patient's plasma to deposit IgG antibody on donor erythrocytes. A similar syndrome has been reported with mefenamic acid.

Nonspecific coating

Nonspecific coating of the erythrocyte surface has been observed with the antibiotic cephalothin, in which cephalothin becomes bound to the erythrocyte membrane and causes the red blood cells to be coated by many plasma proteins. The Coombs test is positive, but hemolytic anemia is rare. Cephalosporins, however, can cause hemolytic anemia by acting as a hapten by a mechanism similar to that of penicillin.

In all these situations, the patients respond to withdrawal of the offending drug. If necessary, a brief course of glucocorticoid therapy can be effectively administered.

Therapeutic measures

In many patients with IgG- or IgM-induced immune hemolytic anemia, no therapeutic intervention is necessary, since the hemolysis may be mild. If an underlying disease is present, control of this disease often brings the hemolytic anemia also under control. However, if the patient is having significant anemia secondary to hemolysis, therapeutic intervention is in order.

Glucocorticoids

Patients with IgG-induced immune hemolytic anemia usually respond to glucocorticoid therapy in dosages equivalent to 40–120 mg of prednisone a day. Glucocorticoids work in IgG-induced hemolytic anemia by three primary mechanisms. First, they decrease the production of the abnormal IgG antibody. This is the most common effect and produces a gradual increase in hemoglobin within 2–6 weeks. Second, glucocorticoids have in several cases been demonstrated to cause the elution of IgG antibody from the erythrocyte surface, improving the red blood cell survival. This is probably an uncommon effect of therapy.

Third, glucocorticoids have been shown *in vitro* and *in vivo* to interfere with the macrophage Fc γ and to a lesser extent C3b receptors responsible for the erythrocyte destruction in this disease. This effect may be rapid and is probably responsible for the rise in hemoglobin which occurs in some patients within 1–4 days of therapy. This action of glucocorticoids causes an improvement in erythrocyte survival despite the continued presence of IgG and C3b on the erythrocyte surface.

Once a therapeutic response is achieved, tapering of glucocorticoids should be initiated. This may take several months. Alternate-day glucocorticoid therapy may be utilized during this time, or until the patient's hematologic picture stabilizes.

Since glucocorticoids may improve erythrocyte survival by interfering with macrophage detection of IgG-coated erythrocytes, the patient's Coombs test may remain positive in the face of an improved erythrocyte survival. Thus, some patients may continue to improve hematologically despite a persistently positive Coombs' test.

Approximately 80% of patients have an initial response to high-dose glucocorticoids. Nevertheless, only 20–30% have a sustained response following discontinuation of therapy. Several patients maintain control of their hemolytic process on low- or medium-dose glucocorticoid therapy. For the patients who are steroid dependent, the initial and long-term side-effects of steroids must be considered. These include gastritis, peptic ulcer disease, emotional lability, exacerbation of diabetes and hypertension, electrolyte imbalance, increased appetite and weight gain, moon-like faces, osteoporosis, myopathy and increased susceptibility to infection. The severity of these side-effects relates to both dosage and duration of therapy. Splenectomy is usually recommended for patients who are unresponsive to steroids or who require more than 10–20 mg per day or 20–25 mg every other day for maintenance. Each patient requires individual evaluation of underlying disease, surgical risk, extent of anemia and steroid intolerance. In some patients the presence of a mild hemolytic anemia may be preferable to splenectomy or other treatment options. The initial goal of therapy is to return the patient to normal hematologic values with nontoxic levels of glucocorticoid therapy. However, in some patients, a secondary goal of achieving improvement in hemolysis to a clinically asymptomatic state with minimum glucocorticoid side-effects is more realistic. Alternate-day glucocorticoids are worthy of consideration in many patients. Their efficacy is not generally appreciated.

Glucocorticoids are not usually effective in cold hemagglutinin disease. This is probably due to the

fact that these patients generally have large amounts of IgM antierythrocyte antibody and large amounts of C3b on the erythrocyte surface. In addition, some of the hemolysis may be intravascular. A few patients with a low titer cold hemagglutinin disease syndrome, in which the IgM antierythrocyte antibody is active at temperatures approaching 37°C, and patients with cold hemagglutinin disease mediated by IgG antibodies, do respond to glucocorticoid therapy. Patients with cold hemagglutinin disease respond best to the avoidance of cold and control of their underlying disease. Fortunately, in many patients hemolytic anemia is mild.

Splenectomy

The reticuloendothelial system of the spleen with its resident macrophages is the major site for sequestration of IgG-coated blood cells. This appears to be due to the unique circulatory pathways in the spleen whereby hemoconcentration occurs in the splenic cords. This results in intimate contact between macrophages (with their membrane Fc γ receptors) and IgG-coated blood cells in the presence of a minimal amount of plasma IgG; plasma IgG competitively inhibits the macrophage Fc γ receptor binding of IgG-coated cells. Thus, the spleen is usually the major site of red cell sequestration in autoimmune hemolytic anemia, with the liver accounting for a variable degree of sequestration.

Removal of the major site of red cell destruction is an effective therapeutic strategy in IgG-induced immune hemolytic anemia. The response rate to splenectomy is approximately 50–70%; however, the vast majority of the responses are partial remissions. The partial remissions are often quite helpful in that they result in a lessening of the hemolytic rate, with a rise in the hemoglobin value, and/or allow for a reduction in the amount of glucocorticoid needed to control the hemolytic anemia. Patients who are unresponsive to glucocorticoids, require moderate to high maintenance doses of glucocorticoids, or have developed glucocorticoid intolerance are generally candidates for splenectomy. ⁵¹Cr-labeled red cell kinetic studies are probably not beneficial, since the procedure is time consuming, expensive and not a reliable indicator of response to splenectomy.

Splenectomy is effective in IgG-induced hemolytic anemia because the cells are cleared primarily in the spleen. In addition, it has been shown that splenectomy can decrease the production of the IgG-antierythrocyte antibody, as the spleen contains a large B cell pool. Those patients in whom splenectomy fails are probably those with very high concentrations of IgG on the erythrocyte surface; in such

patients the liver plays a more prominent role in clearance.

Splenectomy is not effective in patients with traditional cold hemagglutinin disease because IgM-coated erythrocytes are cleared predominantly in the liver, not in the spleen. An occasional case in which a patient with an apparent IgM-induced hemolytic anemia responded to splenectomy has been reported. This may be due to decreased production of IgM antibody by the spleen in these patients. Patients with the uncommon IgG-induced cold hemagglutinin disease syndrome do, however, respond to splenectomy.

The side-effects of splenectomy vary greatly from institution to institution. We have generally attempted to identify one or two surgical colleagues who carry out most of the splenectomies on our patient population. This experience enhances both the surgical procedures (speed and safety) and the postoperative follow-up. Postoperative thromboses and infection both above and below the diaphragm can occur. The risk of morbidity and mortality is greater in older patients, in those with unrelated underlying disease (benign or malignant immunoproliferative disorder), in those with unrelated medical problems, and, to some extent, is influenced by the degree of glucocorticoid side-effects. Thus, a thoughtful analysis of the benefit-versus-risk factors needs to be undertaken for each patient. A final consideration is that adults who undergo splenectomy may, in rare instances, have a propensity to life-threatening infection, particularly with *Pneumococcus*. For this reason it is generally recommended to immunize patients with Pneumovax some weeks prior to elective splenectomy in an effort to reduce this long-term complication.

Immunosuppressive agents

Several chemotherapeutic agents with known immunosuppressive effects have been used to treat immune hemolytic anemia. The drugs most commonly used include the thiopurines (6-mercaptopurine, azathioprine and thioguanine) and alkylating agents (cyclophosphamide and chlorambucil). Immunosuppressive therapy may be effective for treating patients with IgG-induced immune hemolytic anemia when patients are refractory to steroids or splenectomy. Immunosuppressive agents work by decreasing the production of antibody, and therefore it generally takes at least 2 weeks before any therapeutic result is observed.

Patients are selected for immunosuppressive therapy because they have a clinically unacceptable degree of hemolytic anemia resistant to glucocort-

icoid and splenectomy treatment. Alternatively, they may be intolerant of, or resistant to, glucocorticoids and poor surgical risks for splenectomy. Clinical benefit has been noted in about 50% of patients. A reasonable trial of this type of agent is about 3–4 months, and if no beneficial effect is noted, therapy is discontinued. If clinical benefit occurs, one can maintain the dosage level for a total of 6 months and then taper over several months. During therapy, patients are instructed to maintain a high fluid intake to reduce the incidence of chemical cystitis seen with cyclophosphamide and the need to have weekly blood counts to monitor bone marrow suppression, which can be seen with any of these immunosuppressive drugs. Dosage should be adjusted to maintain the leukocyte count >2000 , granulocyte count >1000 and platelet count $>50\,000$ – $100\,000\text{ mm}^{-3}$.

Cyclophosphamide in this dosage range is usually well tolerated but a variety of side-effects may occur, including bone marrow suppression (primarily leukopenia), hemorrhagic cystitis, nausea, partial alopecia, amenorrhea and impaired spermatogenesis. The use of alkylating agents may also have long-term potential for increasing the incidence of malignancy, particularly acute leukemia. These side-effects require that the clinical indications for immunosuppressive therapy be strong and that patient exposure to the drug be limited.

Aside from supportive measures, immunosuppressive therapy has been the major therapy in traditional high titer IgM induced cold hemagglutinin disease. Alkylating agents (cyclophosphamide or chlorambucil) have been the primary drugs used and appear to have a beneficial effect in up to 50–60% of cases. Unresponsiveness is probably due to the fact that very little antibody is required for significant hemolysis to occur.

Miscellaneous therapy

Plasmapheresis has been used in patients with severe IgG-induced immune hemolytic anemia but has met with limited success, possibly because more than half of the IgG is extravascular and the plasma contains only small amounts of the specific antibody (most of the antibody being on the red blood cell surface). However, plasmapheresis has been effective in IgM-induced hemolytic anemia. This is only of short-term benefit, but has reduced the level of cold agglutinins by virtue of the fact that IgM is a high molecular weight molecule that remains predominantly within the intravascular space.

Other measures that have been used effectively are vincristine and vinblastine infusions, gamma globulin infusion and hormonal therapy. Gamma globulin

infusion is effective in IgG-induced immune hemolytic anemia, probably by primarily inhibiting the clearance of the IgG-coated cells. In hormonal therapy the synthetic androgen danazol has been effective in several patients. Because of the limited side-effects (limited masculinizing effects, mild weight gain), danazol or a similar agent may become an attractive alternative to glucocorticoid therapy in some patients with IgG-induced immune hemolytic anemia. However, danazol does not appear effective in IgM-induced hemolytic anemia. The data suggest that one mechanism of danazol's effect is through modulation (inhibition) of macrophage Fc γ receptor expression. Hormonal influence of macrophage Fc γ receptor expression probably explains, at least in part, the increased clinical activity of IgG-induced immune hemolytic anemia during pregnancy. During pregnancy, blood estrogen levels increase to an extent where they enhance splenic macrophage Fc γ receptor expression and thereby accelerate the clearance of IgG-coated cells.

Supportive transfusion therapy

The majority of patients with autoimmune hemolytic anemia do not require transfusion therapy because the anemia has occurred gradually and there has been physiologic compensation. However, occasionally patients experience acute and/or severe anemia and require transfusions for support until other treatment modalities reduce the hemolysis. Transfusion therapy is complicated by the fact that the blood bank may be unable to find any 'compatible' blood. This is because the autoantibody is directed to a product of the Rh locus which is present on the erythrocytes of essentially all potential donors, regardless of Rh subtype. The usual recommendation is for the blood bank to identify the most compatible units of blood of the patient's own major blood group and Rh types. Using this approach, it is unlikely that the donor blood will have a dramatically shortened red blood cell survival.

The indication for transfusion therapy is prevention of any serious complications due to the anemia. These include angina, congestive heart failure and central nervous system symptoms of hypoxia (for example, syncope, lightheadedness, impairment of mental acuity). In addition, if the patient is experiencing blood loss, transfusion is required. The slow infusion of 1–2 units of packed red cells usually improves the clinical status of the patient. Elderly patients especially need to be monitored for circulatory overload and for transfusion reactions. The latter are unlikely to be serious since hemolysis is usually

extravascular. In the rare patient with little or no response to steroids and a continuing need for transfusions because of serious side-effects of anemia over the first 4–6 days of therapy, an early decision for splenectomy may be necessary.

In cold agglutinin disease it is important to pre-warm all intravenous infusions, including whole blood, to 37°C because a decrease in temperature locally in a vein can enhance the binding of the IgM antibody to red cells and accelerate the hemolytic process. In addition, erythrocyte agglutination in a small blood vessel can result in vascular compromise and ischemia to an extremity.

See also: Anemia, autoimmune hemolytic in animal models; Anemia, drug induced immune hemolytic; Autoimmune diseases; Autoimmunity; Erythrocytes; Glucocorticoids; Immunosuppression; *Mycoplasma*, infection and immunity; Plasmapheresis; Rh antigens; Spleen.

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ANEMIA, DRUG-INDUCED IMMUNE HEMOLYTIC

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Acquired hemolytic anemias may develop as the result of immunological reactions consequent to the administration of certain drugs. Clinically, they often closely mimic autoimmune hemolytic anemia of 'idiopathic' origin and for this reason a careful enquiry into the taking of drugs is a necessary part of the investigation of any patient suspected of having an immune hemolytic anemia.

It is common experience that a particular drug may cause hemolysis in one patient, thrombocytopenia in another, neutropenia in yet another, and sometimes combinations of these in the one patient. The drug-induced antibodies responsible for these immune cytopenias are cell specific. We are still at a loss to know why a particular drug binds to a particular cell in a particular patient.

Mechanisms of drug-induced immune cell destruction

The basic mechanisms are widely accepted, although some essential details remain hypothetical.

In a unifying concept, the primary immune response is initiated by an interaction between the drug or its metabolites and a component of the blood cell membrane to create a neoantigen (Figure 1). The target orientation of the antibodies covers a spectrum from drug-dependent antibodies, which bind to both the drug and the cell membrane, but not to either separately, to drug-independent autoantibodies for which the greater part of the neoantigen is sufficiently similar to the normal cell membrane to allow binding without the drug being present. Both types of antibody may be present at the same time in some patients.

Drug-dependent immune hemolytic anemias

In these cases the drug is required in the *in vitro* sys-

tem for the antibodies to be detected. The red cells may be damaged in two ways.

1. *Complement-mediated lysis.* A typical history is for hemolysis, which may be severe and intravascular, to follow the readministration of a drug with which the patient has previously been treated, and for the hemolysis to subside when the offending drug has been identified and with-

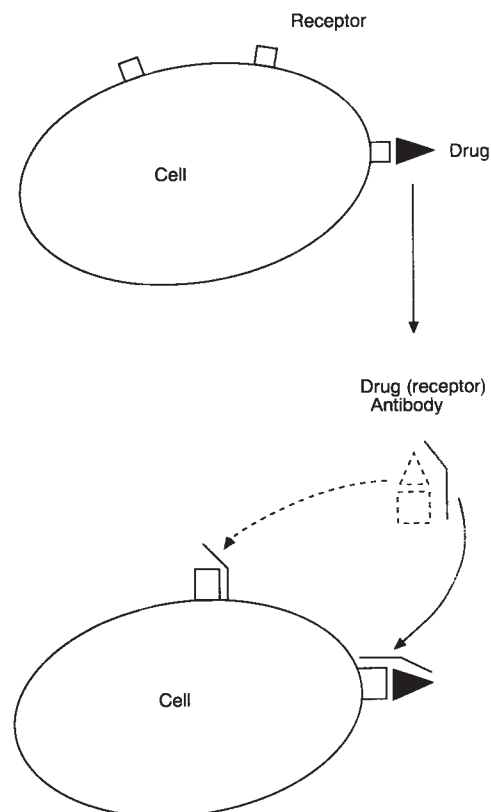


Figure 1 Mechanisms of drug-induced immune cytopenia. Drug-induced antibodies may react with the drug-receptor complex (drug-dependent antibody) or with the drug receptor alone (drug-independent autoantibody). Both types of antibody may be present at the same time.

drawn. The direct antiglobulin test (DAT) is likely to become strongly positive during the hemolytic phase, the patient's red cells being agglutinated by antibodies to complement and sometimes by anti-immunoglobulin G (IgG).

Drugs that have been shown to cause hemolysis by this mechanism include quinine, quinidine and rifampicin, as well as chlorpropamide, hydrochlorothiazide, nomifensine, phenacetin, salicylazosulfapyridine, the sodium salt of *p*-aminosalicylic acid and stibophen.

2. *Extravascular hemolysis.* This is brought about by IgG antibodies that usually do not activate complement, or if they do, not beyond C3. The direct antiglobulin test will be positive with anti-IgG, and sometimes also with antibodies to complement.

The hemolytic anemia associated with prolonged high-dose penicillin therapy is caused by this mechanism, and other penicillin derivatives, as well as cephalosporins and tetracycline, may cause hemolysis in a similar fashion. Hemolysis ceases when the offending drug has been identified and withdrawn.

Drug-independent autoimmune hemolytic anemias

In these cases the antibody reacts with the red cell in the absence of the drug. The anti-red cell autoantibodies seem to be serologically identical to those of 'idiopathic' warm-type autoimmune hemolytic anemia. The great majority of cases have followed the use of the anti-hypertension drug α -methyldopa (Aldomet). The red cells are coated with IgG and the serum contains autoantibodies which characteristically have Rh specificity.

In patients receiving α -methyldopa the autoantibody is often present (15–20%), but hemolysis is not common (<1%). Studies of reticuloendothelial clearance in patients receiving α -methyldopa suggest that the drug itself may blockade the reticuloendothelial

system in most patients, effectively blocking cell destruction by the autoantibody.

Other drugs that have been reported to act in a similar fashion to α -methyldopa include chlor-diazepoxide (Librium), mefenamic acid (Ponstan), flufenamic acid and indomethacin.

Serological investigation

Typical serological features of the different types of drug-induced hemolytic anemia of immunological origin are summarized in Table 1. The drug may be added directly to the assay system (and included in the wash solution) or the cells may be pretreated with the drug (e.g. penicillin). For some drugs a metabolite and not the native drug is the appropriate antigen for testing; in these cases an *ex vivo* drug antigen may be used, from plasma or urine collected from volunteers or other patients after ingestion of therapeutic doses of the drug.

Review of the table reminds us that drug-induced antibodies may react as autoantibodies, so that positive reactions of serum and eluate with normal red cells do not exclude a drug as an etiologic agent.

Idiosyncrasy of drug-induced cytopenias

As already indicated, the antibody may recognize not only the drug but also a specific cell membrane component. Antibodies from patients with drug-induced thrombocytopenia or neutropenia show restriction, in that they react with variable proportions of normal donor cells exposed to the drug (10–80% in one study). This is not related to HLA or known cell-specific antigens, and family studies suggest that an unidentified polymorphic membrane determinant may be involved as a cell receptor/carrier. Another feature of drug-induced antibodies is their extreme heterogeneity from patient to patient and their individual specificity, reflecting a unique immune response.

See also: Anemia, autoimmune hemolytic in human;

Table 1 Serological features of the different types of drug-induced hemolytic anemia of immunological origin

Mechanism	Prototype drug	DAT	IAT No drug	IAT	
				Serum + drug	Eluate + drug
Drug-dependent antibody					
C activation	Quin(id)ine	C ^a	Negative	C ^a	Negative
No C activation	Penicillin	IgG	Negative	IgG	IgG
Autoantibody	α -Methyldopa	IgG	Positive (IgG)		

C, complement; ^a Occasionally also IgG. DAT, direct antiglobulin test; IAT, indirect antiglobulin test. Reproduced from Waters (1995) with permission of Churchill Livingstone.

Carrier; Drugs, allergy to; Erythrocytes; Hapten; Idiopathic thrombocytopenic purpura.

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ANEMIA, PERNICIOUS

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Pernicious anemia is an anemia which occurs in the terminal stages of the chronic destructive autoimmune disease, autoimmune gastritis. The anemia results from the death of parietal cells in the body of the stomach due to an immunologic reaction and in the absence of gastric parietal cells, a blood-sustaining vitamin B₁₂ is no longer transported to its site of absorption.

Thomas Addison is acknowledged as the physician who, in 1849, described the clinical features of the anemia. The term 'pernicious' emphasized the inevitable progress and fatal outcome of the anemia and this description given by Biermer in 1972 has endured despite the knowledge that it is appropriate only to the terminal phase of the disease. The disease is, in fact, autoimmune gastritis for which histologic evidence was documented by Fenwick in 1870. However the importance of the link between the anemia and gastritis was not recognized until many years later.

Treatment played a major role in the eventual understanding of the development of pernicious anemia. The earliest studies were directed towards halting the progress of the anemia, and the prevention of death from the disease. Meals of crude extracts of hog stomach or cooked liver produced a reticulocyte response, indicating that new erythrocytes were made in response to ingestion of such meals. The reason for this became clear when Castle, in the 1950s, showed that the response was dependent on the pres-

ence in food of an extrinsic factor which reacted with intrinsic factor secreted into gastric juice. However, despite intensive treatment, relapses occurred and, in some instances, the anemia became refractory to treatment. The refractory state appeared to be due to the development of an inhibitory factor in the serum because serum mixed with gastric juice inhibited the effectiveness of the gastric juice. Rabbits injected with gastric juice produced antibodies which showed the same inhibitory effect. The essential factor, extrinsic factor, in food was shown to be vitamin B₁₂ and the inhibitory factor in gastric juice (and serum) was autoantibody which reacted with the receptor for vitamin B₁₂ on intrinsic factor. Binding of vitamin B₁₂ to intrinsic factor was inhibited and the inhibition of this normal physiologic process prevented the transportation of vitamin B₁₂ to its site of absorption. Blood cells deprived of their essential building block, vitamin B₁₂, failed to mature and the outcome was the development of a pernicious and progressively fatal anemia.

Clinical and hematologic features of pernicious anemia

The autoimmune processes that finally result in vitamin B₁₂ deficiency may be operative for many years, perhaps 20 years or more, before the gastric parietal cells are functionally exhausted. The patient is usually a middle to older-aged woman of Northern

European origin. She is pale, mentally depressed, physically tired, has abdominal discomfort and may show signs of neurologic degeneration if vitamin B₁₂ deficiency has been prolonged and severe. A film of blood or aspirate of bone marrow will show features of megaloblastic anemia. Her gastric juice is greatly reduced in volume and lacks acid. Her levels of vitamin B₁₂ and pepsinogen 1 are low and there is an abnormally high level of serum gastrin.

These diagnostic criteria for anemia are 'insensitive' as disease markers for autoimmune gastritis because they are not evident until late in disease and therefore identify only a small proportion of patients. The earliest marker indicating the presence of chronic autoimmune gastritis is the gastric parietal cell autoantibody (GPCA), and since it is detectable early in the disease and persists throughout the evolution of the disease, it assumes considerable importance as a marker of the autoimmune process that may terminate in pernicious anemia. GPCA (Figure 1) is detected by immunofluorescence using frozen sections of parietal cell-rich areas of human or rodent stomach, preferable murine rather than rat because human serum may react nonspecifically with rat parietal cells. Over 90% of patients with autoimmune gastritis have GPCA.

Immunology

The gastric autoantigens

The two autoantigens targeted by autoantibodies are components unique to the gastric parietal cell in the

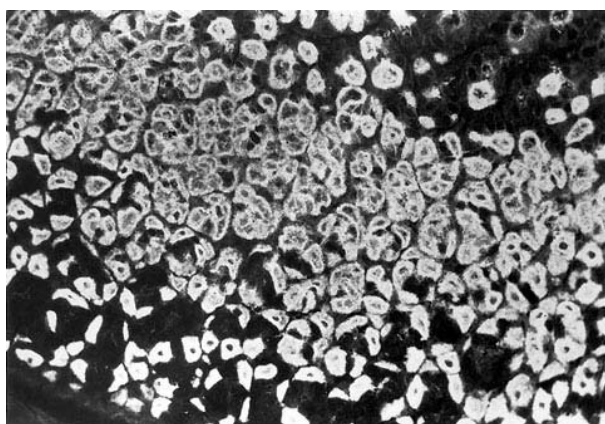


Figure 1 The gastric parietal cell antibody detected by immunofluorescence and illustrated here on murine gastric mucosa reacts with the cytoplasm of gastric parietal cells. Characteristic of the immunofluorescence reaction is the denser staining of the newly formed cells at the base of the mucosa (bottom left) and lighter staining of the mature cells towards the top of the mucosa. Experimental evidence suggests that the autoantigen in the parietal cell is the enzyme H⁺,K⁺-ATPase which is involved in production of acid secreted into the gastric juice.

body of the stomach. The autoantigen with which GPCA reacts is the enzyme H⁺,K⁺-adenosine triphosphatase (H⁺,K⁺-ATPase) which is cell-specific but not species-specific, indicating the importance of the physiologic role of the molecule in the gastric parietal cell. The second autoantigen is a secreted product of the gastric parietal cell, gastric intrinsic factor.

H⁺,K⁺-ATPase Gastric H⁺,K⁺-ATPase is the enzyme responsible for the acidification of gastric juice and is otherwise known as the proton pump. It belongs to a family of ion-motive-P-type ATPases which include Na⁺,K⁺-ATPase and Ca²⁺-ATPase which have highly conserved catalytic α subunits that are phosphorylated during their reaction cycles. Only H⁺,K⁺-ATPase and Na⁺,K⁺-ATPase have a β subunit which includes a heavily glycosylated 35 kDa core protein. The gastric enzyme, which is heterodimeric, is located on intracellular and apical membranes of the gastric parietal cells. Autoantigenic determinants of H⁺,K⁺-ATPase are located on both the catalytic 100 kDa (α) and the glycosylated 60–90 kDa (β) subunits.

Gastric intrinsic factor Gastric intrinsic factor, the other major autoantigen in autoimmune gastritis, is a glycoprotein with a molecular weight of 44 kDa. Each molecule has the capacity to bind to one molecule of vitamin B₁₂ and of its two autoantigenic determinants, one is at the binding site for vitamin B₁₂ and the other is on a part of the molecule remote from this site.

The gastric autoantibodies

The major autoantibodies implicated in autoimmune gastritis are GPCA and the two antibodies that react with gastric intrinsic factor. Since H⁺,K⁺-ATPase is unique to the gastric parietal cell and is responsible for acid production, the inhibitory effect of the immune response on anti-H⁺,K⁺-ATPase accounts for hypochlorhydria. The scenario for the effect of autoantibodies is as follows.

Upon reaching the stomach after ingestion, vitamin B₁₂ in the acid medium of gastric juice is freed from protein to bind to intrinsic factor. When acid secretion is inhibited by anti-H⁺,K⁺-ATPase the amount of vitamin B₁₂ free to bind to intrinsic factor is greatly reduced and if the vitamin B₁₂ site on intrinsic factor is blocked by antibody little, if any, will bind to intrinsic factor. The result is malabsorption of vitamin B₁₂ and as the autoimmune gastritis progresses the stores of vitamin B₁₂ dwindle to levels that can no longer sustain blood and nervous system metabolism. The patient becomes anemic and neurologic complications follow (Figure 2).

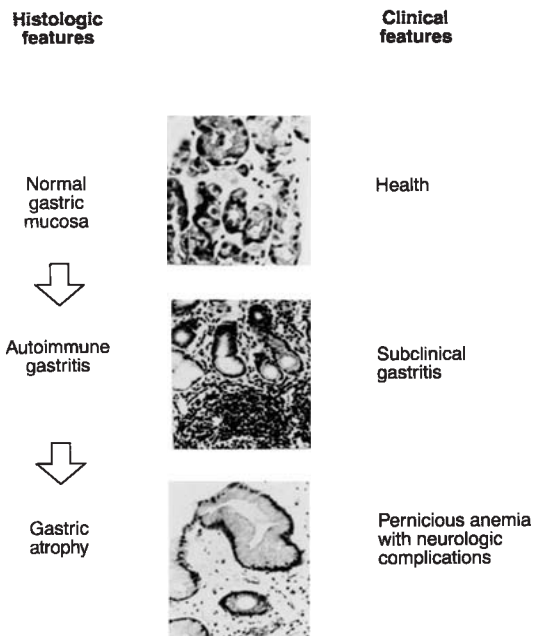


Figure 2 The end-stage of autoimmune gastritis is gastric atrophy. This results in malabsorption of vitamin B₁₂ and pernicious anemia with neurologic complications when vitamin B₁₂ deficiency is severe. The histologic appearance of autoimmune gastritis is that of chronic inflammation.

In addition to autoantibodies to gastric antigens, patients with pernicious anemia have a substantially higher than normal frequency of antibodies to components of cells which are targets in diseases associated with autoimmune gastritis (Table 1). These autoantibodies include those reactive with thyroid peroxidase, thyroglobulin, pancreatic islet cells, adrenal cortical cells and ovarian cells and indicate the patient either has or is predisposed to the associated disease in which these antibodies occur.

Cell-mediated immunity to gastric antigens

The contribution of cell-mediated immunity to the gastric lesion in the body of the stomach is poorly understood. Increased numbers of T lymphocytes of the CD4 and CD8 lineages as well as a greatly increased number of B cells have been observed in

the gastric lesion. It is presumed that T cells provide help required for the production of the gastric autoantibodies because these antibodies are predominantly of the immunoglobulin G (IgG) isotype, and that they also participate in the destruction of the gastric cells. One elegant study by electron microscopy showed T lymphocytes lined up against the membranes of gastric parietal cells and chief cells as if poised to destroy these cells.

Both gastric parietal cells and chief cells become greatly reduced in numbers as the inflammation progresses. Why there is accompanying loss of chief cells is, as yet, an enigma because the autoimmune attack is not thought to be directed at chief cells. Levels of serum gastrin are high probably because gastrin production by antral cells is under the control of gastric acidity and when acid is insufficient due to the absence of the parietal cells an essential feedback mechanism fails and levels of gastrin rise.

Gastritis

The autoimmune reaction is confined to the body of the stomach. The end-result is gastric atrophy but the rate at which the lesion progresses to this stage cannot be predicted in any individual patient. Some patients may maintain adequate vitamin B₁₂ absorption for 20 years or more while others progress rapidly to atrophy.

The recognition of the 'pernicious anemia type' of gastritis was first made by Strickland and Mackay in 1973 who proposed a classification based on differences in the histologic findings in the body and antrum of the stomach. Type A gastritis was the 'pernicious anemia type' in which the immune reaction progressed until the glandular destruction was total and the mucosa atrophic. It was confined to the body of the stomach, and was associated with GPCA and high levels of serum gastrin. Type B gastritis was the 'simple' gastritis now mainly attributed to infection with *Helicobacter pylori* in which glandular destruction did not progress to total atrophy of the glands of both body and antrum, and was not associated

Table 1 Autoantibodies to nongastric components in pernicious anemia

Autoantibodies to:	Frequency	Disease association
Thyroid cells	30%	Hashimoto's thyroiditis
Thyroid peroxidase		Graves' disease
Thyroid hormone receptor		Atrophic hypothyroidism
Thyroglobulin		
Pancreatic islet cells	5%	Insulin-dependent diabetes mellitus
Adrenal cortical cells	5%	Primary Addison disease of the adrenal gland
Ovarian cells	Rare	Primary ovarian failure associated with Addison's disease
Neuromuscular junction	Rare	Lambert-Eaton syndrome

with GPCA or abnormally high levels of gastrin. Regeneration of gastric parietal cells, improved gastric function, and hematologic remission occurred after treatment with immunomodulating drugs such as the corticosteroid drugs and azathioprine, further suggesting that type A gastritis was immunologically mediated.

Animal models of autoimmune gastritis

Spontaneously occurring autoimmune gastritis

There is no animal model in which the primary disease is autoimmune gastritis although atypical forms of autoimmune gastritis are present in a proportion of animals whose primary autoimmune disease is one of the diseases associated with autoimmune gastritis. For example BB/W rats whose primary disease is insulin-dependent diabetes mellitus may have gastric parietal cell antibodies and histologic evidence of mild to moderate gastritis. However they have no reduction in acid secretion or fall in level of vitamin B₁₂. Another example, the obese strain chicken model of Hashimoto's thyroiditis may also have gastric parietal cell antibodies but the chicken provides a poor model of the human gastritis.

Experimentally induced autoimmune gastritis

Attempts to develop models of autoimmune gastritis in rhesus monkeys, dogs and rats by injecting gastric mucosa with immunologic adjuvants have met with only partial success and when inflammatory gastritis is induced it does not persist and progress like the human disease. The model that best mirrors the human disease is that which develops in BALB/c mice following four injections of murine H⁺,K⁺-ATPase emulsified in Freund's complete adjuvant. Although the lesion is reversible after cessation of immunization, the response to immunization is a florid gastritis with destruction of the parietal and chief cells and development of GPCA. An alternative model in the BALB/c mouse is the gastritis which follows thymectomy performed within 2–4 days of birth of the BALB/c mouse or thymectomy and a single dose of cyclophosphamide in the adult mouse. Two criteria are important in the development of these thymectomized models. One is the timing of thymectomy in the neonate which must be performed within 2–4 days of birth and the second is the need for combined treatment in the adult because neither thymectomy alone nor cyclophosphamide alone will result in gastritis.

Analysis of the cells in the early gastric lesion in the BALB/c models shows B cells, CD4 T cells and macrophages predominate. Transfer of splenic lym-

phocytes from affected animals to immunocompromised murine hosts results in autoimmune gastritis in the hosts, the cell responsible for the transfer being the CD4 T cell and its target the β subunit of gastric H⁺,K⁺-ATPase.

Inheritance

Three pieces of evidence converge to suggest that there is a genetic predisposition to pernicious anemia: the high prevalence of pernicious anemia in some racial groups, the high frequency of pernicious anemia in families within those racial groups, and the association of particular genetic markers among patients with pernicious anemia.

Pernicious anemia is predominantly a disease of people of Northern European origin. It is rare among Southern Europeans, Asians, Latin-Americans, and African and American Blacks and when it occurs in African and American Blacks it does so at an earlier age than it does in White Europeans and there is a higher frequency of antibodies to gastric intrinsic factor.

Multiple cases of pernicious anemia have been reported in families, often for several generations, and this frequency may be as much as 20 times greater than in the normal population. There is a raised but not absolute concordance of pernicious anemia in monozygotic twins and there are intrafamilial aggregations of the autoimmune diseases associated with pernicious anemia, namely the thyroid autoimmune diseases, insulin-dependent diabetes mellitus and primary Addison's disease of the adrenal gland which may be expressed as frank or subclinical disease marked by the presence of autoantibodies specific to these diseases. The prevalence of gastric parietal cell antibodies in first-degree relatives of patients with pernicious anemia is 20% which is three times higher than that found in normal controls and the frequency of autoantibodies to thyroid, pancreatic islet cells and adrenal cortical cells is also higher among relatives.

The higher frequency of gastric autoantibodies among relatives of patients with pernicious anemia is also in keeping with the high frequency of type A gastritis among relatives. Studies by Varis and colleagues on a large population of patients with pernicious anemia and their relatives in Finland showed that the prevalence of autoimmune gastritis was 13% higher in relatives than in controls. This familial aggregation of autoimmune gastritis was attributed to a genetic effect rather than an environmental factor. A delay in the onset of gastritis in males suggested that 'maleness' may have a protective effect.

Blood group A and blue eyes are phenotypes

which are common among Northern Europeans with pernicious anemia. Female gender on this phenotypic background potentiates predisposition to the autoimmune gastritis. Various HLA antigens have been associated with pernicious anemia although these associations are generally weak and have not been corroborated in all studies. Evidence for genetic polymorphism has been observed: HLA-B7, -B12, DR2 and DR2/4 heterozygosity has been shown to be increased in frequency in patients with pernicious anemia alone, and HLA-B8, -B18, -Bw15, DR3 and DR3/4 heterozygosity has been demonstrated in patients with pernicious anemia with an associated autoimmune endocrine disease. HLA-B12 has associated with severe vitamin B₁₂ malabsorption and neuromyelopathy, and an increased frequency of Dw2 has been reported in patients with antibodies to gastric intrinsic factor.

Treatment

The standard treatment is administration of vitamin B₁₂ by regular injection to replace that no longer absorbed from food. Being an immune-mediated disease, autoimmune gastritis is also ameliorated by immunomodulating drugs. However serious side-effects may result from treatment with such drugs and this is not the treatment of choice. If untreated, the outcome of the gastritis and vitamin B₁₂ malabsorption is a fatal anemia with neurologic complications.

Conclusion

Pernicious anemia is the result of functional failure

of the gastric parietal cell, and the gastritis associated with it fulfils the criteria for an autoimmune disease. Like most human autoimmune diseases, autoimmune gastritis predominates in women. It is associated with autoantibodies which react with the highly conserved and functionally important molecules, gastric H⁺,K⁺-ATPase and gastric intrinsic factor. There is a genetic predisposition to its development and this is potentiated in the female. It is associated with other well-defined organ-specific autoimmune diseases affecting endocrine glands. How these data are related to the cause, potentiation and maintenance of the gastritis remains unresolved.

See also: Autoantibodies, tests for; Autoantigens; Autoimmune diseases; Autoimmunity.

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ANERGY, B CELL

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Generation of immunological diversity and the need for tolerance

The adaptive immune system, comprising B and T lymphocytes, functions by the clonal response of those lymphocytes to foreign antigen. Each lymphocyte recognizes a single antigen, and the existence of a particular specificity among a population of lymphocytes does not depend on the prior exposure of the animal to that antigen. That is, the introduction of an antigen does not induce the appearance of lymphocytes specific for that antigen; the cells already

exist. What antigen does is to expand selectively those lymphocyte clones which recognize the antigen. This is the basis of the clonal selection theory formulated by Burnet. Defense against a potentially infinite number of foreign antigens requires the immune system to be able to generate an infinite number of specificities, each borne on a unique lymphocyte clone. The immune system can achieve a spectacular level of diversity among the antigen receptors of B and T lymphocytes by using the random association of gene segments to create the final receptor gene products during lymphocyte develop-

ment. Because the selection of individual gene segments is random, the range of specificities embodied in the final receptor molecules is also random. Some lymphocytes will develop expressing receptors which recognize self antigens, while others will be specific for as yet unencountered foreign antigens. The problem for the immune system is how to prevent the self reactive lymphocytes from responding if and when they encounter their cognate self antigen, while allowing the remainder to survive. The means by which the body silences self reactive lymphocytes is called tolerance. Once the requirement for immunological tolerance had been recognized, it was proposed that its imposition was restricted to the early or neonatal stage of life. This model was subsequently modified by Lederberg to one in which each lymphocyte went through a tolerance-sensitive stage during its development. Encountering antigen during this phase resulted in the lymphocyte being permanently disabled. If, however, an antigen was encountered after this developmental stage, then the lymphocyte would be activated and initiate an immune response. Thus while some lymphocytes specific for a foreign antigen may be in a tolerance-sensitive state when they first encounter antigen, other lymphocytes with the same specificity which developed prior to the introduction of the antigen and which are therefore not tolerant, would respond. Self antigens, on the other hand, would always be present and thus able to tolerize each developing lymphocyte.

Early models of tolerance induction invariably invoked clonal deletion as the means by which self reactive cells were rendered tolerant. That is, self reactive lymphocytes were killed by interaction with self antigen. Numerous experiments dating from the 1970s, however, suggested that not all self reactive B cells were deleted. After Naor and Sulitzeanu described the enumeration of antigen-specific lymphocytes by their ability to bind radioactively labeled antigen, others used the same technique to determine whether antigen-specific B cells persisted in tolerant animals. In some instances a reduction in the number of such B cells was observed, while in others no change was seen, suggesting that B cell tolerance was not obligatorily associated with deletion. Using this technique it was possible to demonstrate in apparently healthy animals the presence of lymphocytes which could bind self antigens such as thyroglobulin. Yet other groups showed that polyclonal B cell activators such as lipopolysaccharide could induce the production of autoantibodies from animals with no overt signs of autoimmunity. The overall effect of these and other experiments was to call into question

the exclusive role of clonal deletion in establishing and maintaining immunological tolerance.

Clonal anergy

Experiments pioneered by Nossal and Pike in the 1980s led to the enunciation of clonal anergy as an alternative mode of B cell tolerance induction. In the original experiments these workers reported that while both high and low doses of antigen could induce tolerance, only the high doses resulted in deletion. Low doses of antigen tolerized the animals such that they were subsequently unable to respond to immunogenic forms of the same antigen, despite the persistence of antigen-specific B cells. Data supporting clonal anergy as a means of tolerizing T cells subsequently emerged. Progress in resolving the relative importance of clonal deletion and anergy in maintaining immunological tolerance was hindered by the difficulty in detecting and enumerating antigen-specific lymphocytes in normal animals and in quantifying changes to this population upon the induction of tolerance. Subsequent advances in this field have depended on the use of immunoglobulin transgenic animals.

Transgenic models of B cell tolerance

Immunoglobulin transgenic mice are derived by the introduction of genes encoding functional immunoglobulin heavy and light chains into a zygote. Although every cell in the resultant transgenic mouse carries the introduced genes, expression will only occur in those cells containing the appropriate transcription elements. In the case of immunoglobulin genes, this means B cells and occasionally T cells. Expression of the transgenic immunoglobulin chains in early B cell precursors inhibits the rearrangement of the endogenous immunoglobulin genes and therefore results in an animal in which essentially every B cell develops with the same antigen specificity. If the antigen for which the immunoglobulin transgenes are specific is present in the same animal, then all the B cells will be autoreactive. A number of model systems have been developed employing this approach. In various forms the transgenic B cells are specific for DNA, for major histocompatibility complex (MHC) class I antigens, for mouse red blood cells and, in a particularly flexible model, the B cells are specific for hen-egg lysozyme (HEL). While HEL is obviously not a mouse self antigen, mice transgenic for HEL have been made and, when crossed with the immunoglobulin transgenic mice, create a situation of self reactivity. An advantage of this system has been that the form in which the HEL is expressed

can be altered by changing aspects of the transgene, thereby allowing different forms of B cell tolerance to be studied. Using these transgenic models, it was quickly revealed that if the self antigen was a membrane-bound protein expressed on cells present in the bone marrow, blood or peripheral lymphoid organs, then self reactive B cells were deleted. This deletion was rapid, efficient and required a surprisingly low affinity for the antigen. In situations in which the self antigen was not membrane bound, but rather existed as a serum protein to which the B cells were chronically exposed, then the self reactive B cells were not rapidly deleted, but rather rendered anergic. Analysis of transgenic self reactive systems that promote B cell anergy rather than deletion has revealed some of the cellular and molecular changes that characterize this state of immunological unresponsiveness.

Anergic B cells

The biology of anergic B cells differs from that of nonself reactive B cells in several respects, including the level of expression and functional capability of surface immunoglobulin, activation of helper T cells, lifespan and migration within the lymphoid system. Mature B cells ordinarily express both immunoglobulin M (IgM) and IgD on the cell surface at relatively uniform levels. Anergic B cells express IgD at levels equal to that of nonself reactive (or normal) B cells, but they downregulate IgM by between 5- and 50-fold. The downregulation of IgM is associated with an inhibition in signaling such that phosphorylation of critical signal transduction molecules associated with surface immunoglobulin is diminished. Molecules such as the syk tyrosine kinase and the immunoglobulin coreceptors $Ig\alpha$ and $Ig\beta$ are underphosphorylated in anergic B cells following receptor stimulation. A further consequence of this inhibition in signaling is the fact that receptor stimulation of anergic B cells does not result in the release of intracellular calcium, a critical component in the pathway of B cell activation.

While the changes which are intrinsic to the B cell clearly inhibit the ability of the anergic B cell to respond to subsequent exposure to its cognate antigen, such a state of unresponsiveness can be reversed by signaling through CD40. In certain situations in which anergic B cells are exposed to either CD40 ligand or antibodies specific for CD40 in the presence of the cytokine interleukin 4 (IL-4), the B cells proliferate and differentiate into antibody-secreting plasma cells. This experimental result, however, does not extend to the anergic B cell being able to solicit help from antigen-specific T cells. B cells encountering their cognate antigen in a form which cross-

links surface immunoglobulin will not only process and present the antigen to T cells on MHC class II, but will also upregulate the costimulatory B7 molecules. These molecules are critical for the activation of helper T cells which induces expression of CD40 ligand on T cells, which in turn is critical for the final differentiation of the B cell into an antibody secreting cell. Anergic B cells fail to significantly upregulate B7-2 in response to receptor cross-linking, meaning that they are unable to activate T cell help. This further diminishes the possibility of anergic B cells responding to self antigen.

Self reactive B cells, however, never constitute the entire output of the lymphoid system – they exist among cells which show no self reactivity. In this respect the transgenic models described above, while useful, are somewhat less than ideal. More realistic situations have been developed in which only a fraction of the B cells are specific for a self antigen and their behaviour in relation to their nonself reactive peers can be assessed. Such studies have been conducted by two groups, one being that of Cyster and Goodnow and the second Fulcher and Basten. These experiments have revealed that anergic B cells show a distinct pattern of recirculation in the lymphoid organs relative to nonself reactive B cells. Normally, mature B cells leave the blood, migrate through the T cell areas of the lymphoid organs and enter the B cell follicles. From here they enter the lymphatic circulation which eventually returns them to the blood. Self reactive B cells in the presence of their cognate self antigen enter the secondary lymphoid organs but are unable to enter the B cell follicles. Instead they accumulate at the border of the B and T cell areas of the spleen where they die within a few days in the absence of T cell help. If T cell help is available then the B cells become activated and give rise to structures characteristic of an immune response, namely antibody-forming cells and germinal centers. Thus tolerance of the immune system is a cooperative event, requiring both tolerance in the T and B cell compartments. The failure of self reactive B cells to enter the follicles has been the subject of two interpretations. In one case, Cyster and coworkers suggest that self reactive B cells are unable to compete effectively for a homing signal emanating from the follicle and therefore are excluded. In order for B cells to have a long life they need to have access to survival factors in the follicle, and thus excluded self reactive B cells die quite rapidly. Fulcher and Basten, on the other hand, suggest that localization of the cells in the T cell area of the spleen is a consequence of receptor stimulation and is indicative of the B cells searching for T cell help. If that help is not forthcoming, as would normally be the case for

a self antigen, then the B cells rapidly die. Furthermore, this group argues that the critical factor determining whether an individual B cell is able to enter the follicles or not is the level of receptor engagement. If the concentration of self antigen is low, then the B cell is able to ignore the signal and behave like a nonself reactive cell. Once the concentration is above some threshold level, however, then the lymphocyte changes its trafficking pattern and has a reduced lifespan in the absence of T cell help.

Breaking anergy

B cells made anergic by chronic exposure to a self antigen are able to be activated in some instances. As indicated above, stimulation of anergic B cells through the CD40 molecule in the presence of IL-4 is able to induce proliferation and differentiation of the B cell. Similarly, extensive cross-linking of surface immunoglobulin on the B cell is able to activate the cell. This can be achieved, for example, when a B cell chronically exposed to soluble monomeric self antigen encounters the same antigen in a highly multimerized form such as a cell protein. In this case, the B cell will proliferate and differentiate into an antibody-forming cell in the presence of the appropriate cofactors. This reversal of anergy is dependent on the strength of the receptor signal. In the presence of soluble self antigen, anergic B cells are killed by antigen specific nontolerant helper T cells. This death is dependent on anergic B cells expressing the apoptosis-inducing molecule Fas and the helper T cells expressing its ligand. This lethal embrace can be transformed into a stimulatory one if the antigen receptor stimulation given to the B cell is sufficiently strong.

Why is anergy reversible?

Clonal anergy, defined as the presence of antigen-specific B cells in an unresponsive state, can be achieved by a number of mechanisms. Some involve modulation of the signaling of the B cell itself, while others alter the ability of these B cells to solicit and respond to T cell help. Still other mechanisms alter the location of anergic B cells in the lymphoid system and their lifespan. Why are so many tolerance mech-

anisms employed when it would be simpler to delete any lymphocyte which showed traces of self reactivity? Several explanations have been put forward to answer this question, most cogently by Goodnow. First, not all self antigens will be present in bone marrow when the B cells are developing and sensitive to tolerance induction. Second, if all B cells which show any degree of binding to self antigens were to be deleted, it is questionable that the lymphoid system could generate a sufficient number of cells to counter the infectious agents animals are exposed to. That is, anergy may be a means by which the immune system is able to silence potentially harmful B cell clones and yet have B cells survive long enough to be exported to the peripheral lymphoid organs. In these locations it is possible that the anergic B cells may encounter a foreign antigen to which they have a higher affinity than that with which they recognize the self antigen. If this were the case, then the anergic B cell could be activated and participate in a protective immune response. The anergic B cells would quickly die, however, if no such foreign antigen existed at the time it reached the periphery. This may also account for the reversibility of B cell anergy.

See also: **Anergy, T cell; Antigen presentation via MHC class II molecules; B lymphocyte activation; B lymphocyte repertoire; CD40 and its ligand; B7 (CD80 & CD86); Helper T lymphocytes; Clonal deletion; Clonal selection; Diversity, generation of; Effector lymphocytes; Fas (CD95) and fas ligand; Interleukin 4; Second signals for lymphocyte activation; Tolerance, peripheral.**

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ANERGY, T CELL

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In 1908 C. Von Pirquet first used the term anergy (from the Greek meaning without work or not working) to describe the loss of response to a tuberculin skin test in patients infected with the measles virus. Since then this type of phenomenon has been described for delayed-type hypersensitivity responses to a variety of antigens during the course of a number of different infectious diseases. It appears to be a nonspecific suppression of the T cell response which reverses when the infectious agent is cleared. In 1980 Nossal and Pike borrowed the term and used it to describe the state of antigen-binding B cells isolated from an adult mouse that had been made unresponsive by injection of a tolerogenic dose of antigen. The B cells failed to differentiate into immunoglobulin secreting cells when restimulated *in vitro* with either antigen or the mitogen lipopolysaccharide. They called this form of unresponsiveness 'clonal anergy', to denote that the unresponsiveness was antigen specific. The tolerogenic regimen used in these experiments was derived from the earlier work of Dresser, who demonstrated that intravenous injection of deaggregated proteins in the absence of an adjuvant would induce a subsequent state of unresponsiveness to that antigen. In contrast, if the same preparation of antigen was given with Freund's complete adjuvant as a costimulator, it was immunogenic. This work eventually gave rise to the concept that antigen stimulation of a lymphocyte in the absence of costimulation was tolerogenic. When a similar phenomenon was discovered for cloned T cells in tissue culture, the term clonal anergy was extended to this population as well. In this case the unresponsiveness was a failure of the T cells to proliferate on rechallenge with antigen presented by professional antigen-presenting cells (APCs). It is this last category of the use of the term clonal anergy to which this entry is confined.

The induction of T cell clonal anergy in the mouse was first achieved by stimulating cultured lymphocyte clones with peptide antigens and chemically fixed APCs. Induction of this same state with purified major histocompatibility complex (MHC) class II molecules in planar membranes made it clear that T cell receptor (TCR) occupancy by peptide – MHC complexes was all that was necessary for anergy to occur. Other forms of pure TCR engagement, such as concanavalin A crosslinking in the absence of

APCs or plate-bound anti-CD3 monoclonal antibody, also induced clonal anergy, although significant amounts of cell death were observed as well. Anergy induction was prevented by addition of APCs, giving support to the paradigm that signal 1 (the antigen-specific signal) alone was inhibitory, whereas signal 1 plus signal 2 (costimulation) was stimulatory. The nature of the costimulatory signal was subsequently shown to be engagement of the T cell CD28 molecule by its ligands CD80 and CD86 on the APC. The requirement for APC activation in order to express CD80/86 explains why the original chemical fixation experiments prevented costimulation.

Anergic T cell clones have a very distinct pattern of partial inactivation. Proliferation is prevented on restimulation by a number of mechanisms. First and foremost, interleukin 2 (IL-2) production is inhibited by 20–50 fold. This block is at the level of transcription and appears to involve interference with signal transduction through the p21^{ras}/MAP kinase pathway. The latter prevents activation of the transcription factor AP-1 which is required for IL-2 gene transcription. Overinduction of the IL-2 transcriptional repressor, Nil-2a, has also been reported in anergic T cells. Production of other cytokines is inhibited, but to varying degrees. IL-3 and tumor necrosis factor α production are inhibited about tenfold. In contrast, IL-4 and interferon γ production are only reduced slightly. Interestingly, in those clones capable of using IL-4 as a growth factor, anergy induction blocks only the ability of the cells to respond to IL-4. Similarly the augmentation of TCR-induced proliferation by IL-12 is inhibited. Finally, expression of CD40 ligand, which is required for T cell mediated, B cell proliferation, is inhibited. The overall pattern suggests that anergy is a growth arrest state designed to reduce proliferation in the T cell immune response.

Costimulation given within 2 hours of TCR occupancy blocks the induction of anergy. This is due to an increase in IL-2 production mediated by a CD28-induced augmentation of IL-2 mRNA stability and an increase in the activity of Jun N-terminal kinase (JNK), which enhances IL-2 gene transcription. The IL-2 produced inhibits anergy by two mechanisms. One is by activation of Janus kinase (JAK)3 via stimulation through the common γ chain of the IL-2 receptor. The other is by driving the cell into

cycle. The division process antagonizes and even reverses the block in the Ras/MAP kinase pathway by an as yet unknown mechanism.

Anergy can also be induced in murine T cell clones by stimulating them with altered peptide ligands. In this case costimulation is normal, but signal transduction through the TCR is suboptimal. This partial signaling possibly results in the production of the inhibitor of the Ras/MAP kinase pathway, but is not sufficient to produce IL-2. As a consequence, the small production of inhibitor is unopposed and the cell becomes anergic. Human T cell clones can also be induced into an unresponsive state by exposure to high concentrations of normal peptide ligands. In this case too, the presence or absence of costimulation makes no difference. A biochemical analysis, however, suggests that the unresponsiveness is mediated by a different mechanism. Here signaling through the calcium/calcineurin pathway is impaired, while the MAP kinase pathway is normal. A similar biochemical block can be observed in mouse cells, but only after they are stimulated through the IL-2 receptor. The murine block, however, is transient, and the cells return to their normal responsiveness in 5–7 days. In order to distinguish the state of unresponsiveness in human clones stimulated with high concentrations of peptide from anergy induced in human and mouse clones in the absence of costimulation, it has been proposed that the latter be called Ras-blocked anergy and the former calcium-blocked anergy.

The *in vitro* demonstration of T cell anergy led to a major effort to identify this state *in vivo*. Two systems have been described which look as though they represent this phenomenon. The first is induced with superantigens such as staphylococcal enterotoxin B (SEB) and minor lymphocyte stimulating (Mls) antigen. In this case subsets of T cells expressing particular V_{β} receptors expand in response to the superantigen stimulus. Several days later many of these cells die, but a small cohort remains and these residual cells are often unresponsive to restimulation with either the superantigen or an antibody against the V_{β} receptor. An analysis of the biochemical events taking place in this population during restimulation revealed a profile similar to that seen in anergic T cell clones. Interestingly the cells *in vivo* showed a block in IL-2 receptor signaling as well as one in IL-2 production. Furthermore, multiple injections of the superantigen seemed to produce a more profound state of anergy in which signaling through the Ras/MAP kinase pathway was blocked to the point of impairing activation of the NF κ B and NF-AT transcription factors in addition to AP-1. A recent report has suggested that this deeper state of

anergy can also be achieved *in vitro* if the T cell clones are exposed to IL-10 during their stimulation through the T cell receptor. The molecular mechanism for this IL-10 enhancement is not known.

The second *in vivo* system in which anergy has been identified involves TCR transgenic mice. CD4 cells from such animals have been transferred to normal recipients and challenged with antigen intraperitoneally in incomplete Freund's adjuvant. Cells recovered from the lymph nodes failed to proliferate *in vitro* when rechallenged with antigen. In a second model, H-Y specific, TCR transgenic, CD8 cells, when transferred into male nude mice, responded in an analogous way to that seen for normal T cells stimulated with superantigens. After an initial expansion and death phase the remaining cells were unresponsive to restimulation with either the antigen or an anti-TCR antibody. To date, the biochemical state of the unresponsive cells has not been examined in either transgenic model. Hence at this time one cannot be certain that the state is the same as that observed in T cell anergy studied *in vitro*.

Given the existence of anergic T cells *in vivo*, one can now ask what their biological function is. No consensus exists on this point. It was originally thought that anergy played some role in self tolerance. Naive T cells make only IL-2 following T cell activation and it was postulated that anergy induction would block this response, thus preventing the T cell from expanding and differentiating into an effector cell. At this point, however, there is no definitive evidence that naive T cells *in vivo* are turned off when given signal 1 in the absence of signal 2. Some experiments suggest that instead the cells may differentiate into T_H2 cells. Anergy and T_H2 differentiation can be distinguished by their responsiveness to IL-4. Anergic T cells cannot proliferate in response to IL-4, whereas T_H2 cells can. So far no investigation has clearly made this distinction. Another possible scenario for anergic T cells *in vivo* is that they are on their way to die. The biochemical events seen inside the T cell during and after anergy induction are not those associated with apoptosis; however, Fas ligand is upregulated *in vitro* and could be used in a subsequent encounter with antigen to mediate cell death. On the other hand, transfer of CD8 TCR transgenic cells into female nude recipients suggests that the cells do not die and that they can actually spontaneously come out of the anergic state in the absence of antigen. Anergy can also be reversed *in vitro* by stimulating the T cell clones with IL-2. Whether this reversibility would provide a useful mechanism for transiently recruiting autoreactive clones for use in an ongoing immune response to foreign antigens remains an intriguing possibility.

See also: Anergy, B cell; Delayed-type hypersensitivity; Second signals for lymphocyte activation; Superantigens; T lymphocyte activation.

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ANNEXINS (LIPOCORTINS)

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The annexin (lipocortin) family of proteins possess a highly conserved core region, consisting of four or eight repeats of approximately 70 amino acid residues, and a highly variable N-terminal region. The core region mediates Ca²⁺-dependent binding to phospholipid membranes and forms a Ca²⁺ channel-like structure. The physical and structural properties of annexin proteins suggest that they regulate multiple aspects of cell membrane function, including involvement in membrane trafficking, signal transduction and cell–matrix interactions. Some of their actions are akin to those of glucocorticoids, including anti-inflammatory, anti-edema and immunosuppressive effects.

Historical background

Glucocorticoids are widely used as one of the most efficacious medicines against various inflammatory and immunological diseases such as rheumatoid arthritis, systemic lupus erythematosus and bronchial asthma. Their anti-inflammatory action is mediated by inhibition of arachidonic acid release, a precursor of prostaglandins (PGs), hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs). Phospholipase A₂ (PLA₂) and alkylphospholipids, a precursor of platelet-activating factor (PAF), in biomembranes have been considered as the key enzyme and the major source of arachidonic acid, respectively. Anti-inflammatory steroids also inhibit the forma-

tion of both PAF and its inactive lyso-form (deacylated molecule). Since most of the anti-inflammatory actions of glucocorticoids are inhibited *in vivo* and *in vitro* by inhibitors of protein synthesis as well as mRNA synthesis, it was proposed that the anti-inflammatory action of glucocorticoids is partly, if not exclusively, mediated through the induced synthesis of PLA₂ inhibitory protein(s).

Between 1980 and 1984, attempts were made by several groups to isolate a protein 1) whose synthesis is induced by glucocorticoids, 2) that inhibits the activity of PLA₂ *in vitro* and *in vivo*, and 3) that displays an anti-inflammatory action like glucocorticoids. Several different proteins that inhibit PLA₂ *in vitro* were identified and partially purified. These included 'lipomodulin' from rabbit neutrophils (molecular weight = 40 kDa), 'renocortin' from rat kidney cells (molecular weight = 30 kDa) and 'macrocortin' from rat macrophages (molecular weight = 15 kDa). Among these proteins, lipomodulin was best characterized: a possibly glycosylated protein with an apparent molecular weight of 40 kDa and a pI value of 9.0. This protein can bind 2 mol of Ca²⁺ mol⁻¹ protein, and the concentration of Ca²⁺ required for half maximal binding is approximately 1 μM. It was proposed that this protein suppresses PLA₂ activity by forming a complex with PLA₂ at the Ca²⁺-binding site(s). Furthermore, various protein kinases including protein kinase C and tyrosine

protein kinase, can phosphorylate lipomodulin to modify its function and structure as detected by the inhibitory activity against PLA₂ and the susceptibility to proteases.

Despite differences in their chemical properties, all proteins isolated as second messengers of glucocorticoid anti-inflammatory action have similar, if not identical, biological properties: 1) the synthesis of these proteins is induced by glucocorticoids, 2) preparations of these proteins, regardless of their purity, inhibit porcine pancreas and/or snake venom PLA₂ *in vitro*, 3) these protein preparations mimic some actions of glucocorticoids, especially with regard to arachidonic acid release and production of lipid mediators such as prostaglandins, and 4) some monoclonal antibodies raised in one laboratory cross-react with the proteins prepared by the other laboratories. Based upon these findings, it was proposed in 1984 that a family of PLA₂ inhibitory proteins whose syntheses are induced by glucocorticoids be named as *lipo(modulin, macro- or reno-)cortins* (lipocortins). In 1986, the Biogen group purified two proteins from human placenta whose properties are almost identical to those of lipomodulin. They isolated cDNA clones encoding these proteins, and described their chemical natures as follows: 1) their molecular weights are around 36 kDa, 2) they have multiple phosphorylation sites including one for tyrosine protein kinase and a potential glycosylation site, 3) they have four repeats which can bind acidic phospholipids and Ca²⁺, and 4) they inhibit PLA₂ *in vitro*. Interestingly, these cDNA clones were almost identical to the simultaneously discovered cDNAs that encode two of the major substrate proteins (calpactins) for the EGF receptor (tyrosine) kinase.

Diversity in the annexin family

Two protein inhibitors of PLA₂, namely annexin I and II, have the same characteristic structural organization: following unique N-terminal sequences of about 40 amino acid residues, they both are composed of four repeated sequence motifs of 70–80 amino acids, in which a conserved 17 amino acid consensus (Lys-Gly-X-Gly-Thr-Asp-Gln-X-X-Leu-Ile-X-Ile-Leu-Ala-X-Arg) is thought to be important for Ca²⁺-dependent binding to phospholipids. These features have now been identified in sequences of 18 related proteins (Table 1), although annexin VI has 8 repeats instead of the 4 in other annexins. Due to this conserved structure in the C-terminal domain, annexins have a high degree of identity with each other, and maintain around 40–60% amino acid sequence homology. This family of proteins is ubiquitously distributed, being present in lower organisms such as *Drosophila* and plants. They are referred to as annexins since their main property is to 'annex' (bind to) cellular membrane phospholipids in a Ca²⁺-dependent manner. Distinct from the 'EF-hand calcium-binding proteins' such as calmodulin, their intrinsic affinity for Ca²⁺ is generally low (around 0.1–1 mM), and the number of Ca²⁺-binding sites is between 2 and 4. However, the presence of phospholipids increases their affinity for Ca²⁺ up to 100-fold, while Ca²⁺ facilitates their binding to acidic phospholipids (phosphatidic acid > phosphatidylserine > phosphatidylinositol). Although some annexins such as annexin I and II can bind to neutral phospholipids such as phosphatidylethanolamine, their binding to phosphatidylcholine and sphingomyelin, choline lipids, is not detectable. In

Table 1 The annexin (lipocortin) family

	<i>Previous terminology</i>	<i>Biological functions</i>
Annexin I	Calpactin II, Chromobindin 9, GIF, p35	EGF receptor kinase substrate
Annexin II	Calpactin I, Chromobindin 8, p36, PAP-IV, Protein I	Src-kinase substrate
Annexin III	Calcimedlin 35- α , Calphobindin III, PAP-III	
Annexin IV	Calcimedlin 35- β , Calelectrin 32.5K, Chromobindin 4, Endonexin I, PAP-II, PP4-X, Protein II	F-actin-binding protein
Annexin V	Anchorin CII, Calelectrin 35K, Calphobindin I, Calcimedlin 35- γ , Endonexin II, IBC, VAC- α , PAP-I, PP4	Anticoagulant protein
Annexin VI	Calcimedlin 67K, Calelectrin 67K, Calphobindin II, Chromobindin 20, p68, p70, 73K, Protein III, Synhibin	
Annexin VII	Synexin	
Annexin VIII	Vascular anticoagulant (VAC- β)	
Annexin IX	<i>Drosophila melanogaster</i> annexin	
Annexin X	<i>Drosophila melanogaster</i> annexin	
Annexin XI	Calcyclin-associated annexin (CAP)-50	
Annexin XII	<i>Hydra vulgaris</i> annexin	
Annexin XIII	Intestine-specific annexin (ISA)	

contrast to the conserved C-terminal core, each annexin has a unique and extremely variable sequence in the N-terminal domain with the length varying from a few amino acids to 160 amino acids. This domain contains various sites for phosphorylation, glycosylation, acylation and other post-translational modifications. A dimer of annexin II forms a heterotetrameric structure via its N-terminus with a dimer of the p11 protein, a complex which can bind to phosphatidylserine at nanomolar Ca^{2+} concentrations. Therefore, this domain is considered as the regulatory region of the protein for various biological functions.

Results of genomic analysis suggest that annexin genes may be derived from a common ancestor gene, and that the evolution and diversification of the family arose by two consecutive duplications of a DNA sequence encoding a protein resembling a single repeat. The structure of all the annexin genes is well conserved. The 5'-flanking region of annexin genes contains sequences for the binding sites of several transcriptional factors, indicating transcriptional regulation by mitogenic stimulation, steroids and metals, while the 5'-flanking region demonstrates *alu*-like repeats. The presence of potential splice sites and Z-DNA structure in the C-terminal core region produces different isoforms of annexins.

Mechanism of action of annexins

In contrast to the initial proposed mechanism of phospholipase A_2 inhibition whereby annexin I (lipomodulin) was thought to complex with PLA_2 via Ca^{2+} -binding sites, the currently accepted mechanism proposes that annexins bind phospholipids in a Ca^{2+} -dependent manner, thus depleting phospholipid substrates for phospholipase A_2 . Since a certain type of PLA_2 (type II) is released in inflammatory loci, the *in vivo* anti-inflammatory action of annexins is also explained by a hypothetical mechanism in which annexins protect membranes from attack by PLA_2 by binding to membrane phospholipids. Since annexins bind acidic phospholipids such as phosphatidic acid, phosphatidylinositol and phosphatidylserine but not phosphatidylethanolamine or phosphatidylcholine, this interpretation does not explain their specificity for PLA_2 . Lipases such as phospholipase C and phospholipase D, the enzymes that hydrolyze these phospholipids are also potentially inhibited by the same mechanism. Annexins are now found to form complex homo- and heterodimers or tetramers, with annexins or other proteins. The annexin-binding proteins are annexins, collagens, S-100, apolipoproteins, helicase, DNA methyltransferase, protein kinase C, glycerol-3-phos-

phate dehydrogenase, malate dehydrogenase, calyculin, F-actins and PLA_2 . Despite the findings that ratios of annexin I to phospholipid substrate (phosphatidylserine) are an important determinant factor of the *in vitro* inhibition of PLA_2 , the possibility of complex formation is not ruled out. Such protein-protein interaction, whether it be a specific interaction or nonspecific electrostatic interaction, alters the function and stability of these binding proteins. In this sense, annexins have some properties similar to those of 'house-keeping' proteins.

Crystallographic and molecular modeling studies demonstrate that annexins have a calcium channel-like structure. In fact, annexin V and VII are shown to function as voltage-dependent and GTP-sensitive Ca^{2+} channels, respectively. GTP-binding activity has been implicated by a homology between the amino acids 78–236 (the core region) of annexin I and the amino acids 22–171 of *c-K-ras* 2a, a mammalian *ras-onc* gene product which has GTPase activity. It is noteworthy that maize annexin has ATPase activity. It is also speculated that annexins which bind to mitochondrial dehydrogenases such as isocitrate dehydrogenase and malate dehydrogenase may act as Ca^{2+} sensors, since these enzymes are known to be activated by Ca^{2+} without apparent Ca^{2+} -binding sites. Therefore, annexins are distinct from EF-hand and Ca^{2+} -binding proteins such as calmodulin, whose major functions are Ca^{2+} buffering inside cells.

Biological actions and possible physiological roles

Initial efforts have been made to identify and characterize the annexins, particularly annexin I and II, as possible second messengers in response to anti-inflammatory steroids. The observations to date have shown that these recombinant annexins are able to mimic a part, if not all, of the anti-inflammatory action of glucocorticoids, while antibodies against annexins diminish such actions (Table 2). Even some of their fragments containing the N-terminus exhibit similar but weak anti-inflammatory actions. The inflammatory reactions inhibited by annexins and their fragments include not only the release of arachidonate and its metabolites and the formation of PAF but also neutrophil chemotaxis and release of lysozymes. The cytotoxic reaction by cytotoxic T cells and NK cells is also inhibited by both annexins and glucocorticoids. Although the annexin I and II genes contain GRE sequences in their 5'-flanking regions, thus implicating transcriptional regulation by glucocorticoids, induction of the synthesis of annexins by the steroids remains to be determined. Such induc-

Table 2 Biological action of annexins

1.	Inflammatory responses
a)	Inhibition of <i>in vivo</i> inflammation Carrageenan-induced paw edema and pleurisy
b)	Inhibition of <i>in vitro</i> inflammatory response Inhibition of neutrophil chemotaxis Slight inhibition of O_2^- production Inhibition of lysozyme release Inhibition of phagocytosis Inhibition of arachidonic acid liberation Inhibition of PAF formation
2.	Immune system
a)	<i>In vivo</i> Tumor-induced immunosuppression Modulation of <i>in vivo</i> pyrogenic action by cytokines Suppression of IgE synthesis
b)	<i>In vitro</i> Inhibition of cytotoxic reaction of cytotoxic T cells and NK cells Inhibition of <i>in vitro</i> cytotoxic T cell generation Inhibition of IL-1 production Inhibition of IL-1 action Inhibition of IL-2 production Inhibition of T cell proliferation Induction of maturation of suppressor T cells Induction of memory cells Inhibition of IgE synthesis Change from necrosis to apoptosis of H_2O_2 -treated thymocytes
3.	Cell proliferation and differentiation
	Differentiation of leukemia cell lines Differentiation of macrophage cell lines Differentiation of neuronal cell lines Differentiation of glial cell lines Differentiation of fibroblast cell lines Differentiation of epidermal cell lines Differentiation of epithelial cell lines
4.	Secretion
a)	Inhibition of ACTH, TSH, prolactin and other peptide hormones from pituitary cells
b)	Inhibition of catecholamines from chromaffin cells
5.	Other biological actions
a)	Inhibition of <i>in vitro</i> coagulation
b)	Inhibition of <i>in vivo</i> brain edema
c)	<i>In vitro</i> mucous production of tracheas
d)	<i>In vitro</i> induction of β -adrenoceptors and/or inhibition of β -adrenoceptor downregulation
e)	Inhibition of <i>in vivo</i> LPS induction of nitric oxide (NO) synthetase
f)	Enhancement of DNA replication in the oocyte cell free system and in mitochondria
g)	Osteoclast formation and bone reabsorption

tion has been often demonstrated in intact animals and organs, while cultured cells mostly fail to respond to glucocorticoids at either mRNA or protein levels. Since a whole family of annexin proteins which maintain a highly conserved structure in the C-terminal core with each other, occupy approximately 2% of the total cellular proteins, and since their synthesis is dependent on the cell cycle, changes in their mRNA and/or protein levels might be often

underestimated. Alternatively, an unknown mechanism beside the genomic effect of glucocorticoids might be involved in induction of the synthesis of annexin I and II.

Most annexins are localized intracellularly, particularly in the cytosol. However, various stimuli including increased intracellular Ca^{2+} are known to translocate annexins to plasma membranes and other subcellular vesicles including chromaffin granules. Glycosylation and acylation of annexins is thought to be important for such insertion and/or attachment to membranes. Some annexins are known to be extracellularly released. Autoantibodies against annexins in patients with rheumatoid arthritis and systemic lupus erythematosus (SLE) neutralize these annexins, thereby augmenting inflammatory responses in these diseases. Similar observations have been extended to inflammatory diseases such as asthma, psoriasis, and ulcerative colitis. Among these diseases, SLE may involve dysfunction of suppressor T cells in the immune system. During immunoglobulin E (IgE) synthesis *in vitro* annexin I enhances suppressor function by inhibiting the glycosylation of IgE-binding factor.

Similarly, annexins regulate the proliferation-differentiation of suppressor T cells, including cytotoxic T cells. This leads to the suggestion that the immunosuppression of animals bearing tumors can be attributed to monocytes/macrophages in which the synthesis of annexin I is stimulated. Because of the chemical nature of annexin I and II as substrates for tyrosine kinases, including EGF receptor kinase and Src kinases, annexins are associated with the proliferation-differentiation process of many cells other than suppressor T cells (Table 2). Effects of annexins, especially annexin I and II, are similar, if not identical, to those of glucocorticoids. However, the mechanism of action of exogenous annexins cannot be explained by their binding to plasma membrane acidic phospholipids, which are mainly located on the intracellular side of the membranes. In fact, the binding of annexin V is currently used to detect apoptotic cells which have an inverted membrane phospholipid distribution. This suggests that the existence of specific receptors for annexins mediate their actions. Alternatively, annexins may be inserted into membranes to interact with specific enzymes and/or proteins.

Annexin VII has been identified as synexin, a protein which modulates presynaptic functions including the release of neurotransmitters. Similarly, other annexins are reported to participate in exocytosis and endocytosis in a variety of cell functions such as catecholamine release from adrenal chromaffin cells and ACTH secretion from pituitary cells. Such

activity is attributed to the binding of annexins to subcellular vesicle membranes and Ca^{2+} channel and/or sensor action, although the possibility of an interaction between annexins and actin-like cytoskeletal elements, thus changing their bundling, cannot be neglected.

The presence of annexins in nuclei and mitochondria has also been reported. Although annexin II can regulate the replication of DNA in cell-free systems, the detailed mechanism(s) remains to be solved. Since annexin I and II and annexin I-IV have phosphorylation sites for tyrosine protein kinases and protein kinase C (enzymes that regulate the proliferation-differentiation and/or cell cycle of cells) respectively, many reports describe their involvement in the proliferation-differentiation processes of various cells. Such phosphorylation of annexins alters their affinities for Ca^{2+} and phospholipids, and their interactions with other enzymes and/or proteins. A recent report describing the involvement of annexin I in the apoptosis of H_2O_2 -treated thymocytes may also substantiate this contention, because apoptosis, a type of cell death distinct from necrosis, is explained by an abortive cell activation mechanism.

Although the structure and chemical and physical nature of annexins have been extensively studied, the mechanism of most of their biological and physiological actions remain unclear. Considering the unique distribution of certain annexins in specific cell types, it is likely that additional physiological and/or biological roles and mechanisms of actions of the

annexin family of proteins will be explored in the near future.

See also: Acute inflammatory reaction; Arachidonic acid and the leukotrienes; Autoimmune diseases; Glucocorticoids; Hypersensitivity reactions; Platelet-activating factor (PAF); Proliferation, lymphocyte; Prostaglandins.

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ANTI-GLOBULIN (COOMBS') TEST

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The anti-globulin test was developed to detect human blood group immunoglobulin G (IgG) antibodies by agglutination. The negative charge on the surface of red cells prevents them from coming closer than within 20 nm of each other, thus IgG antibodies are unable to cross-link red cells as the Fab arms, even when fully extended, are unable to span this distance. On the other hand, the Fc portion of cell-bound antibody projects outwards sufficiently far to allow an anti-IgG antibody to cross-link between IgG molecules on one cell and those bound to neighboring red cells, thus bringing about agglutination (Figure 1). The development of the test occurred following the discovery of the Rh blood group system in the early 1940s. It was soon realized that there

were two types of antibody involved in this system – those that would directly agglutinate red cells in saline (retrospectively identified as IgM antibodies), and the so-called 'incomplete' or 'blocking' antibodies (now identified as IgG) which would not directly agglutinate but would block the action of the direct agglutinators.

R.R.A. Coombs, A.E. Mourant and R.R. Race, working in Cambridge in the early 1940s, were investigating the various specificities of the antibodies that appeared to be associated with the Rh system. As reported in their publication of 1945, the seminal idea was that red cells sensitized with nonagglutinating anti-Rh antibody 'had absorbed antibody globulin at some points on their surface and that an

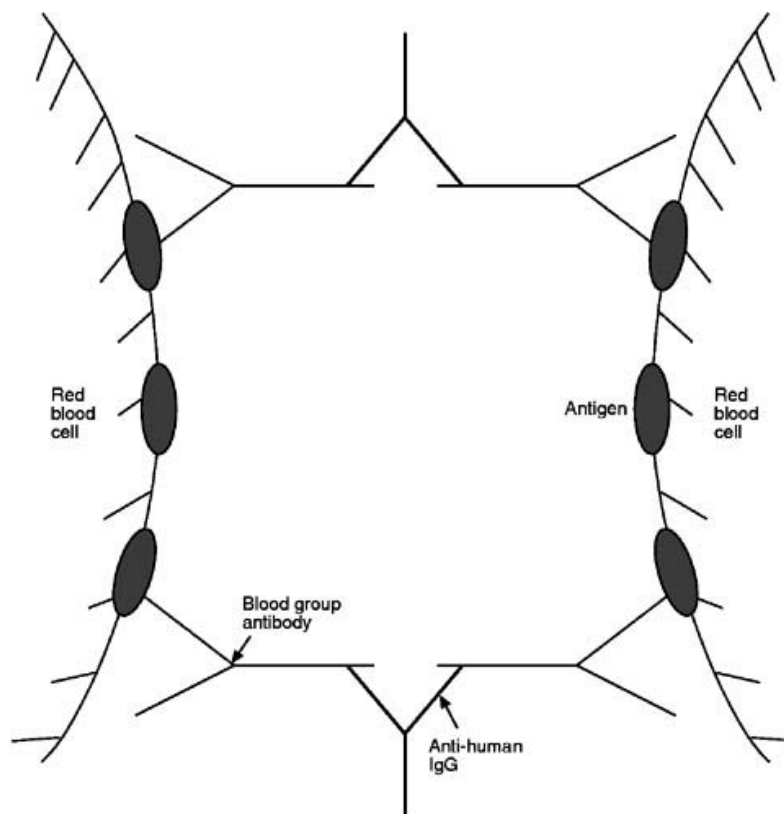


Figure 1 Cross-linking of cell bound IgG.

anti-human globulin serum might be expected to react with this in some observable way'. The principle of the test was soon established. In a later publication, they indicated that the test could probably be used to detect any nonagglutinating antibody present in serum (the indirect Coombs' test) by initially absorbing the antibody on to red cells; they also showed that it could also be used to detect antibody on the surface of fetal red cells in hemolytic disease of the newborn (direct Coombs' test).

It is interesting to note that the use of an anti-globulin serum in this way was first described by the Italian physiologist, Moreschi, in 1908. Moreschi showed that if rabbit red cells were reacted with a goat anti-rabbit red cell antibody at too low a concentration to bring about agglutination, they could subsequently be agglutinated by the addition of a goat serum-specific antibody. At that time, human nonagglutinating antibodies were unknown and hence the significance of the reagent was not appreciated and its description was forgotten.

The test is widely used in two areas in clinical immunohematology. First, it is of major importance in blood transfusion in cross-matching for determining compatibility between donor and recipient. Donor red cells are added to the recipient's serum and any nonagglutinating antibody which binds to

the cells can be detected by the addition of the anti-globulin reagent. Second, it is used in the diagnosis of acquired hemolytic anemias for demonstrating the presence of antibodies bound to red cells *in vivo*.

Antiglobulin reagents can be made specific for IgM, IgA and the four subclasses of IgG. The reagents used in cross-matching also contain antibodies specific for the complement component C3, because some antibodies are best detected by testing for the bound complement which is deposited as the result of the antigen-antibody reaction.

See also: Antibodies, detection of; Blood transfusion reactions; Gammaglobulin; Hemolytic disease of the newborn; Maternal antibodies; Rh antigens; Transfusion.

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ANTI-INFLAMMATORY (NONSTEROIDAL) DRUGS

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed drugs and are also widely used in over-the-counter preparations. The first NSAID, aspirin, was first commercialized at the end of the nineteenth century. Since that time, dozens of more potent NSAIDs have been marketed. NSAIDs are used primarily for their anti-inflammatory, analgesic and antipyretic effects, although more recently aspirin has been used increasingly for prevention of myocardial infarction and stroke. While widely used, these drugs are not entirely safe, with well-documented toxicity in the gastrointestinal tract and kidney. In this entry, the mechanisms of action of NSAIDs are reviewed, as are the major adverse effects associated with their use.

NSAIDs and prostaglandin synthesis

NSAIDs fall into a number of different chemical classes, but despite structural heterogeneity, they share activity, toxicity and most probably, mechanism of action (Table 1). While some NSAIDs may have additional activities which contribute to their anti-inflammatory action, it is the ability of all NSAIDs to inhibit the enzyme cyclooxygenase (COX) that is the primary mechanism through which these drugs reduce pain, inflammation and fever. Cyclooxygenase catalyzes the conversion of arachidonic acid to prostaglandins G and H. Subsequent

enzymatic and nonenzymatic reactions result in the conversion of these highly unstable prostaglandins to more stable and physiologically important prostanoids, including prostaglandin E₂, prostacyclin (PGI₂) and thromboxane A₂. Arachidonic acid is a 20 carbon, unsaturated fatty acid found in membrane phospholipids. It can be cleaved from these phospholipids through the actions of phospholipase A₂ or phospholipase C, whereupon it can react with cyclooxygenase to form prostanoids. One of the mechanisms through which glucocorticoids reduce inflammation is by inhibiting this phospholipase-mediated liberation of arachidonic acid from membrane phospholipids. Prostaglandins contribute to the inflammatory process primarily by causing relaxation of vascular smooth muscle around arterioles, thereby increasing blood flow. While prostaglandins themselves cause only minor changes in the permeability of blood vessels, the increase in blood flow they produce results in a synergistic increase in plasma exudation (edema formation) caused by other mediators, such as histamine and bradykinin. In doing so, prostaglandins contribute to three of the cardinal signs of inflammation, namely, redness, swelling and heat.

Prostaglandins also contribute to the pain associated with inflammation. Prostaglandins themselves are not particularly potent at inducing a pain response when applied, for example, to the base of a blister in human skin. However, they can produce a state of prolonged hyperalgesia and increase the sensitivity of peripheral pain receptors to chemical and mechanical stimuli. For example, the presence of prostaglandins at a site of inflammation or injury will greatly increase the sensitivity of pain receptors to mediators such as bradykinin and histamine. By inhibiting prostaglandin synthesis, NSAIDs remove this hyperalgesic component of an inflammatory reaction. NSAIDs exert analgesic effects both peripherally and centrally, although it is the peripheral action that predominates.

Prostaglandins also play a key role in the generation of fever. There is strong evidence that prostaglandins are produced in the hypothalamus in response to interleukin 1 (IL-1). These prostaglandins mediate the resetting of temperature-regulating systems in the hypothalamus. Blockade of

Table 1 Chemical classes of NSAIDs and examples

<i>Chemical class</i>	<i>Examples</i>
Salicylic acid derivatives	Aspirin Diflunisal
Propionic acids	Ibuprofen Naproxen Flurbiprofen Ketoprofen
Indole acetic acids	Indomethacin Etodolac Sulindac
Enolic acids	Piroxicam Meloxicam
Heteroaryl acetic acids	Diclofenac Ketorolac
Fenamates	Mefenamic acid

prostaglandin synthesis by NSAIDs results in a blockade of this effect of IL-1 and other pyrogenic substances. Interestingly, acetaminophen (paracetamol) shares this ability to inhibit prostaglandin synthesis in the hypothalamus and therefore reduce fever, but this drug is a poor inhibitor of prostaglandin synthesis in the periphery, so is not an effective anti-inflammatory drug.

Interaction of NSAIDs with cyclooxygenase

The inhibition of COX activity by NSAIDs occurs in two main ways. Most NSAIDs inhibit this enzyme by binding in a reversible manner to a site within the channel through which arachidonic acid must insert to be metabolized (Figure 1). In contrast, aspirin produces an irreversible alteration in the conformation of the active site such that arachidonic acid can no longer gain access. Aspirin produces this effect by acetylating a serine residue in COX. Thus, the recovery of prostanoid synthesis after administration of aspirin is a function of the rate of turnover of COX in each tissue. The irreversible blockade of COX by aspirin accounts for the utility of this drug in the prophylaxis of stroke and myocardial infarction. Due to their reversible binding to COX, most NSAIDs are competitive inhibitors of COX. When plasma levels of the NSAID decline below a critical level, COX activity is restored. On the other hand, COX activity is only restored following aspirin administration through the synthesis of new enzyme. Platelet aggregation is regulated by two key prostanoids. Thromboxane produced by the platelet stimulates aggregation, while prostacyclin produced by the vascular endothelium inhibits platelet aggregation. Following aspirin administration, endothelial prostacyclin synthesis is inhibited until such time as new COX is synthesized (the plasma half-life of aspirin is only ~15 min). However, platelets lack the nuclear machinery to synthesize new COX, so aspirin effectively blocks their ability to synthesize thromboxane permanently. Thromboxane synthesis only returns with the appearance of new platelets in the circulation. Thus, giving small doses of aspirin daily leads to marked suppression of thromboxane synthesis with little or no effect on endothelial prostacyclin synthesis, thereby producing a net antithrombotic effect.

In recent years, it has become clear that COX exists in at least two distinct forms. COX-1 is constitutively expressed in many tissues, including the stomach, kidney and platelet, and is believed to produce the prostaglandins that mediate important physiological functions in these tissues (Figure 2). On the

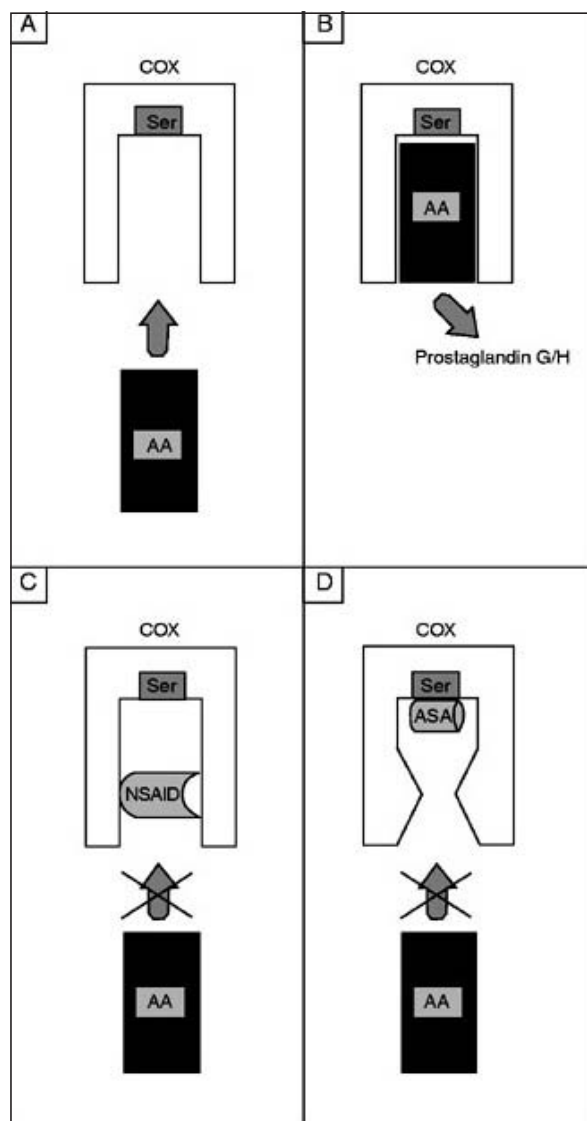


Figure 1 Cyclooxygenase metabolizes arachidonic acid to prostaglandins G and H, which are in turn converted to a number of bioactive prostanoids (PGE_2 , PGI_2 , TXA_2). Arachidonic acid inserts into a channel in COX (panel A) and can then undergo conversion to $\text{PGG}_2/\text{PGH}_2$ (panel B). Most NSAIDs bind to a site within the channel through which arachidonic acid must insert to be metabolized, thereby blocking the activity of COX (panel C). Aspirin acetylates a serine residue in COX resulting in a conformational change in the active site, such that arachidonic acid can not be converted to $\text{PGG}_2/\text{PGH}_2$ (panel D).

other hand, COX-2 is not constitutively expressed in many tissues, but can be induced in many tissues by a number of cytokines and mitogens. NSAIDs currently on the market are nonselective, inhibiting both isoforms of COX. As mentioned above, aspirin is able to produce its inhibitory effects on both COX-1 and COX-2 through acetylation of a serine residue, although the position of this residue differs slightly between the two COX isoforms. COX-2 has also

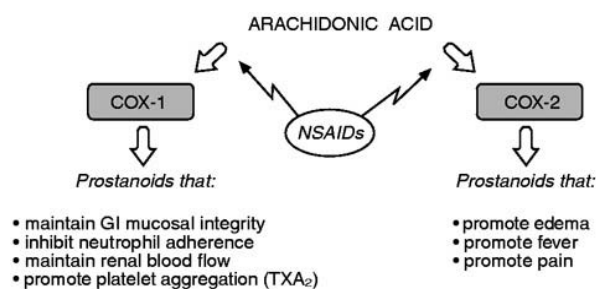


Figure 2 Arachidonic acid is metabolized by the enzyme cyclooxygenase (COX) to form prostaglandins. It has been suggested that the COX-1 isoform is responsible for producing prostaglandins important to the function of the gastrointestinal (GI) tract, kidney and platelet, while COX-2 is expressed at sites of inflammation and produces the prostaglandins that contribute to the associated pain, swelling and redness. Currently marketed NSAIDs show little selectivity for COX-1 versus COX-2, perhaps explaining their detrimental effects on the gut and kidney when used at doses required to reduce inflammation.

been shown to be the predominant form of COX expressed at sites of inflammation. This has given rise to the hypothesis that NSAIDs reduce inflammation through their inhibitory effects on COX-2, while producing their toxicity in the gastrointestinal tract and kidney through inhibitory effects on COX-1. If this is true, highly selective inhibitors of COX-2 should produce the beneficial effects of NSAIDs without the toxicity they exhibit in some tissues. This hypothesis is supported by considerable data, although there is evidence of prostaglandins derived from COX-2 performing important physiological functions in some circumstances, such as in situations in which the gastrointestinal tract is inflamed (see below).

Other mechanisms of action of NSAIDs

While there is very strong evidence supporting the hypothesis that NSAIDs produce their anti-inflammatory, analgesic, antipyretic and antithrombotic effects through inhibition of prostanoid synthesis, there is also evidence for other mechanisms of action. These other mechanisms of action may explain the differences in activity from one NSAID to another. For example, NSAIDs such as indomethacin have been shown to inhibit other enzymes, including phospholipase A₂, diacylglycerol kinase and diacylglycerol lipase. These effects are observed at concentrations achieved by therapeutic doses of these drugs, and may explain the observation that some drugs, including indomethacin, are more potent as anti-inflammatory agents than they should be based solely on their potency as inhibitors of COX.

Many NSAIDs have also been shown to inhibit the motility of neutrophils and monocytes, which could

explain their ability to reduce the number of inflammatory cells emigrating to sites of injury. Some NSAIDs have also been shown to suppress the function of helper T cells and B cells. However, at least in some cases, these effects are seen only with supra-therapeutic concentrations of the drugs.

There may also be COX-related effects of some NSAIDs that contribute to their beneficial effects, but are not due to inhibition of the activity of this enzyme. For many years, researchers have been puzzled by the observation that salicylic acid, the metabolite of aspirin, could reduce inflammation without having significant effects on COX activity. It appears that one of the mechanisms through which salicylate acid produces this effect is by inhibiting the induction of COX-2 at sites of inflammation, possibly by blocking the activity of the transcription factor NF κ B (which regulates the expression not only of messenger RNA for COX-2, but also of a number of other proinflammatory mediators).

Proinflammatory effects of NSAIDs

Ironically, while prostaglandins are typically described as mediators of inflammation, these substances exert a wide range of anti-inflammatory effects (Figure 3). For example, prostaglandins are potent inhibitors of the release of tumor necrosis factor α (TNF α) and IL-1 from macrophages, and potent inhibitors of TNF α and platelet-activating factor from mast cells. Prostaglandins also suppress the release of a number of proinflammatory mediators from platelets. On the other hand, NSAIDs, by suppressing endogenous prostaglandin synthesis, can increase the release of a number of proinflammatory mediators from various cells, and can therefore enhance an inflammatory reaction (while simultaneously reducing edema and pain). Prostaglandins have also been shown to be potent inhibitors of adhesion molecule expression on the vascular endothelium (e.g. ICAM-1) and on leukocytes (e.g. CD11/CD18), thereby reducing the ability of leukocytes to extravasate. Conversely, NSAIDs have been shown to increase adhesion molecule expression in some experimental models, and to cause a corresponding increase in leukocyte adherence to the vascular endothelium. Actions such as these might explain the observation that NSAIDs can actually lead to increased tissue injury in some forms of inflammation, as well as some of the adverse reactions to NSAIDs in settings of inflammation (e.g. inhibition of the healing of peptic ulcers). Removal of the immunomodulatory effects of endogenous prostaglandins through NSAID administration may

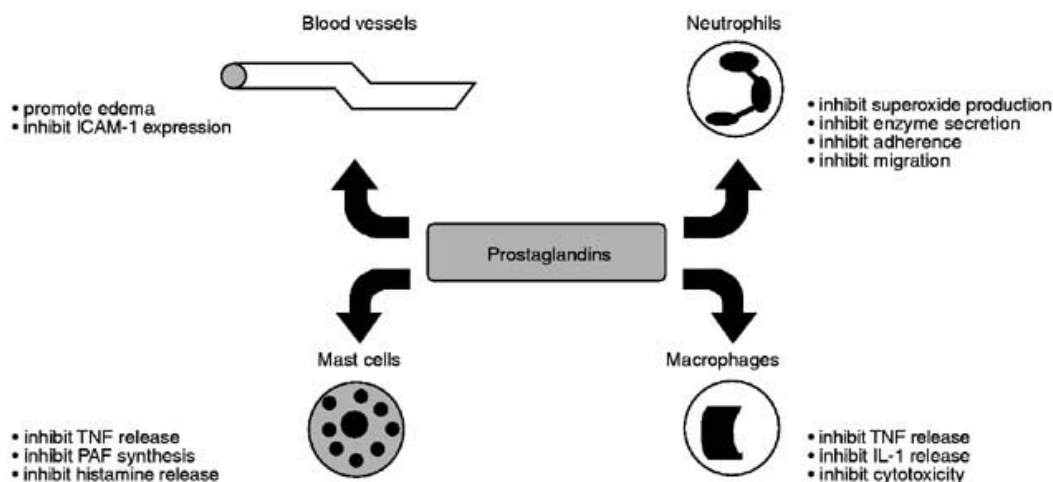


Figure 3 Prostaglandins exert both pro- and anti-inflammatory effects. Prostaglandins, through vasodilation, increase edema formation. However, prostaglandins can reduce inflammatory reactions through inhibition of adhesion molecule expression on the vascular endothelium, and inhibition of inflammatory mediator release from macrophages, mast cells and platelets. Prostaglandins can also reduce tissue injury through inhibition of oxygen-derived free radical production by leukocytes and monocytes.

also contribute to the hypersensitivity reactions to these drugs (see below).

Adverse effects of NSAIDs

Like the beneficial effects of NSAIDs, the adverse effects of these drugs can be largely attributed to their effects on prostaglandin synthesis. The most common detrimental effects of NSAIDs are ulceration in the gastrointestinal tract, renal damage, delayed labor and hypersensitivity reactions (often manifest as asthma). NSAIDs can cause gastric and duodenal ulceration and can exacerbate or delay the healing of pre-existing ulcers. Inhibition of prostaglandin synthesis in the upper gastrointestinal tract is central to the pathogenesis of this ulceration. On the other hand, small intestinal damage caused by NSAIDs may be more a consequence of their topical irritant properties and enterohepatic recirculation than to inhibition of prostaglandin synthesis. Renal injury by NSAIDs, which includes papillary necrosis and chronic interstitial nephritis, is also believed to be, at least in part, attributed to inhibition of prostaglandin synthesis. Prostaglandins play an important role in autoregulation of renal blood flow, with a particularly important role being played in individuals with renal and/or hepatic insufficiency. In the case of delayed labor, once again it is the suppression of prostaglandin synthesis that causes alterations in uterine contraction; however, there is evidence that the prostaglandins in this case are derived largely from COX-2, which is induced in labor. Similarly, in settings of gastrointestinal inflammation, such as in inflammatory bowel disease and peptic ulcer disease, the exacerbation of inflammation and delayed

healing of ulcers may be due to inhibitory effects of NSAIDs on COX-2. Because of this, the notion that selective COX-2 inhibitors will be devoid of toxicity, particularly in the gastrointestinal tract, may have to be reconsidered in cases where individuals have a pre-existing inflammatory disease in those tissues in which COX-2-derived prostaglandins appear to play an important immunomodulatory and anti-inflammatory role.

Hypersensitivity reactions to NSAIDs are not common in children, but may occur in up to a quarter of middle-age patients. The reaction, which can range in severity from rhinitis and bronchial asthma to hypotensive shock, can be life-threatening and can occur following ingestion of small doses of these drugs. While often regarded as occurring only with aspirin, hypersensitivity reactions can occur with any NSAID. The mechanism responsible for this reaction has not been firmly established, but does not appear to be immunological in nature. However, as it can occur with NSAIDs with diverse chemical structures, the reaction may be related to suppression of COX. There is evidence that administration of NSAIDs to susceptible individuals results in a profound increase in the production of peptido-leukotrienes, which are potent proinflammatory and bronchoconstrictive agents. These substances are also derived from arachidonic acid, so the increase in leukotriene synthesis may be a consequence of 'shunting' of substrate to that pathway when COX is inhibited, or due to the removal of the inhibitory effects prostaglandins can exert on leukotriene synthesis.

See also: Arachidonic acid and the leukotrienes; Fatty acids (dietary) and the immune system; Glucocort-

icoids; Prostaglandins; Rheumatoid arthritis, animal models; Rheumatoid arthritis, human.

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ANTI-LYMPHOCYTE SERUM

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Anti-lymphocyte serum (ALS) is a serum which contains antibodies against lymphocytes, and is usually prepared in horse, goat or rabbit by immunization with human lymphoid cells. ALS may be obtained by immunization with, for example, lymph node cells, T cell lines, B cell lymphoblasts, thoracic duct lymphocytes or thymocytes. In the latter case the produced serum is referred to as antithymocyte serum (ATS). Most often, the immunoglobulin (Ig) fractions of ALS or ATS were isolated and used as anti-lymphocyte globulin (ALG) or antithymocyte globulin (ATG). Removal of cross-reacting antibodies (such as to erythrocytes or platelets) reduces the side-effects. Some frequently used preparations are ATGAM (equine ATG), Lymfoser, Minnesota anti-lymphoblast globulin (MALG), and different RATG (rabbit ATG) preparations, such as ATG Fresenius, Nashville antithymocyte serum (nATS), and thymoglobulin. In this text, all these different preparations are referred to as ALS. With modern techniques, highly specific monoclonal antibodies (mAbs) against different cell surface molecules have been prepared, and are used in a similar fashion as ALS. Both with polyclonal ALS and mAbs, the effects may be counteracted by sensitization against the xeno-geneic proteins.

ALS as an immunosuppressive agent

ALS is used, often in combination with other immunosuppressive agents, to avoid allograft rejection. In a quadruple therapy protocol it is used with azathioprine, steroids and cyclosporine. ALS is used both as a preventive immunosuppressant, for initial

treatment of rejections, and to treat steroid-resistant rejection. The mechanism of action is not fully clarified, but depletion of circulating lymphocytes does occur. This may result from either cytotoxic mechanisms or opsonization. The widest experience with ALS is from kidney transplantation, but ALS has also been used in connection with transplantation of small bowel, heart, bone marrow, liver, pancreas (or islet cells), cornea and skin.

ALS in bone marrow disease and bone marrow transplantation

ALS is widely used in the treatment of bone marrow disorders, particularly severe aplastic anemia. When bone marrow transplantation is not possible, ATG represents the second-best therapeutic alternative in aplastic anemia. It has also been used together with bone marrow transplantation and with good results.

The graft-versus-host reaction is a persistent problem in bone marrow transplantation. The removal of mature T cells and T cell precursors with different lymphocyte-specific antibodies has been a way of preventing it. There are, however, studies that claim that the removal of T cells from bone marrow before transplantation does not improve survival rates, and in fact can cause a higher frequency of graft failure.

An antigen on T lymphocytes which is a very good target for cell lysis is the CDw52 antigen. mAbs to this antigen, prepared in the rat (CAMPATH-1), have been used successfully to deplete donor bone marrow of T lymphocytes. The variants CAMPATH-1G (IgG2b) and CAMPATH-1M (IgM) exist, as well as the humanized CAMPATH-1H (human IgG1).

Therapeutic use of ALS in other diseases

Other cases in which ALS has been reported of value include autoimmune disease such as rheumatoid arthritis and multiple sclerosis, autoimmune thrombocytopenic purpura, Omenn's syndrome, Wegener's granulomatosis, pure red cell aplasia and familial hemophagocytic lymphohistiocytosis. The use of ALS in these conditions is not widespread, however, possibly because of problems related to repeated treatment, such as reduced effect, or anaphylactic reactions. In mice, ALS (or anti-CD4 mAbs) prevents the development of spontaneous systemic lupus (NZB strain) and diabetes (NOD strain). Experimentally induced diabetes (BALB/c strain), arthritis and myasthenia gravis are also prevented this way. In addition, ALS or more specific antibodies may prove to be useful in leukemia. A monovalent, CD3-specific mAb has been shown to deplete CD3⁺ tumor cells effectively in a patient with T cell leukemia. CAMPATH-1 is very effective in the treatment of lymphoid malignancy and lymphoproliferative diseases. This mAb has also been tried in the treatment of rheumatoid arthritis and multiple sclerosis.

Monoclonal anti-lymphocyte antibodies

Although the polyspecific ALS (shown to contain antibodies to a great number of T lymphocyte surface antigens) is still very useful, with the advent of monoclonal antibodies to various surface molecules on lymphocytes, more specific tools are available. Antibodies specific for epitopes on molecules such as CD2, CD3/TCR components, CD4, CD6, CD8, IL-2R (CD25), LFA-1, and HLA class II have been tried. OKT3 (anti-CD3), which is by far the most commonly used mAb to lymphocytes, has been used for immunosuppression in clinical practice for about 15 years. It has a half-life after injection of 18 h. As with ALS, such mAbs will be used for depletion of lymphocyte populations, but may also be used to modulate the function of lymphocytes by way of interacting with the respective surface molecules. Many studies have compared the therapeutic use of OKT3 and ALS, and in many cases they were found to be equivalent. OKT3 has been effective in cases where graft rejection was both steroid and ALS resistant. Many factors influence the effectiveness of lymphocyte-specific mAbs, such as the properties of preparation itself, the pharmacokinetics, the rate of production of the target molecules, the presence of blocking antibodies, etc. Advantages with mAbs are that they are uniform in immunoglobulin subclass and have a better defined specificity and more repro-

ducible effects. A problem is the production of anti-antibodies which counteract the effect of the administered mAb (Figure 1). It is a smaller problem when using polyclonal ALS, and is to some extent prevented by the use of concomitant immunosuppression. The problem with anti-antibodies may be circumvented by changing to a mAb against a different epitope or by using mAbs with a human type of molecular structure. In an experimental system, pretreatment with ALS was found to suppress formation of neutralizing anti-antibody to a mAb administered later. Only mAbs against CD4 seem to avoid stimulating the production of anti-antibodies by the recipient. This may reflect the unique ability of CD4-specific mAbs to induce tolerance. A humanized CD4-specific mAb has been used in clinical trials on patients with autoimmune disease. The effect of mAbs may be modified by removing the Fc fragment, which is of importance for the elimination of the target cells by phagocytes. Thus, CD3-specific mAbs which lack the Fc part cause immune activation rather than immunosuppression. Antibodies may be raised against the antigen-binding epitope of the T cell receptor. This will probably become of value in the treatment of autoimmune disease. In the comparison between ordinary, polyclonal ALS and mAbs, contradictory opinions have been published regarding the effectiveness and severity of side-effects.

Side-effects

ALS is administered via a central nervous catheter to avoid irritation following intramuscular or ordinary intravenous injection. Many side-effects seen after administration of polyclonal or monoclonal antibodies against lymphocytes are probably due to antibodies cross-reacting with other tissues. A common problem is serum sickness (fever, malaise, dyspnea, hypotension, edema, cutaneous eruptions, lymphadenopathy, arthralgia and headache). Histologically there may be signs of vasculitis. Many of these symptoms seem to be associated with occurrence of immune complexes. Monoclonal antibodies also have side-effects, even in their humanized forms. The first few injections of anti-CD3 often cause fever, gastrointestinal and respiratory disturbances, headache and anorexia, symptoms sometimes referred to as 'the first-dose syndrome'. The side-effects caused by both ALS and anti-CD3 are thought to be mediated by release of tumor necrosis factor (TNF), interferon γ (IFN γ) and possibly interleukin 2 (IL-2). Transient renal dysfunction attributed to cytokine-induced renal capillary leakage, has also been reported. Other T cell-specific mAbs do not cause

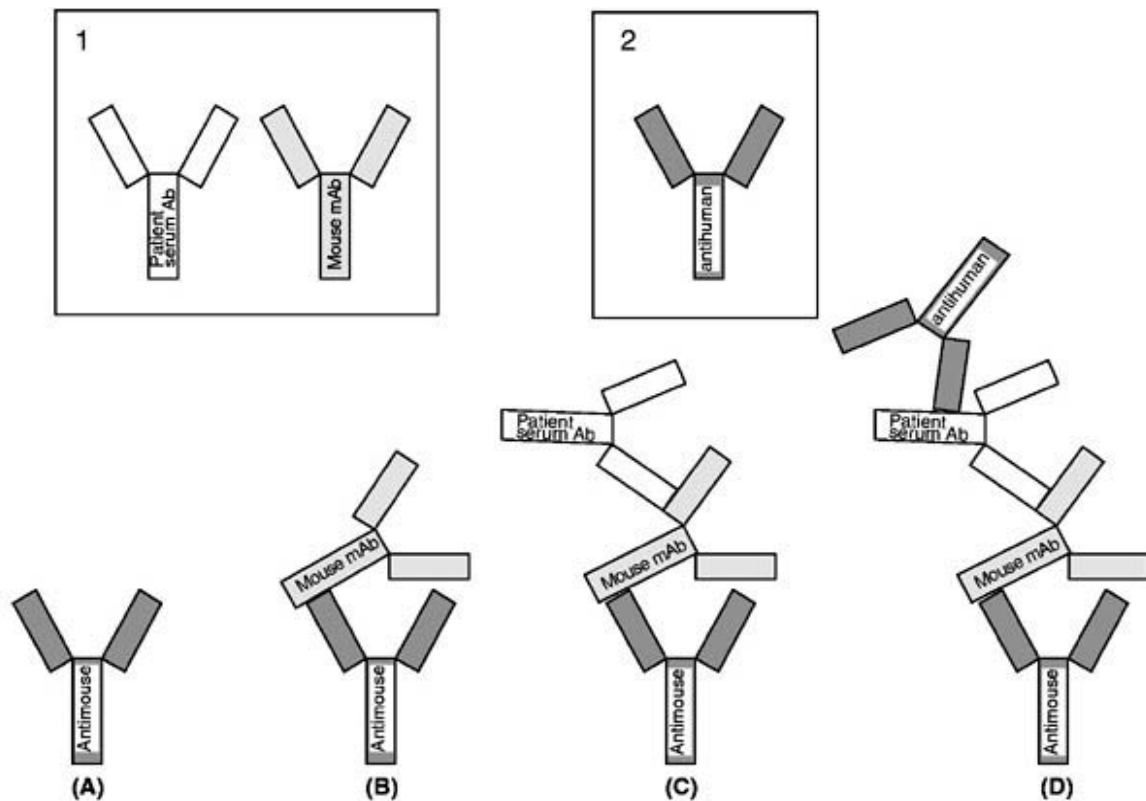


Figure 1 Measurement of a human antimouse antibody response to therapeutic Mabs. An antimouse antibody is first bound to a surface (A). The therapeutic mouse Mab is then added, together with the human serum to be examined (1; B, C). Lastly, an antihuman Ig antibody is added (2; D). The binding of this last antibody is dependent on the presence of antimouse antibodies in the patient's serum.

cytokine release and do not result in so many side-effects. More rarely, symptoms from the central nervous system may occur. There is a risk of lymphoproliferative conditions with both ALS and OKT3, at least after extended use. It has been suggested that side-effects of ALS may be minimal with a remaining therapeutic effect if the administration of ALS is adjusted according to the daily level of T lymphocytes in the blood. Of course, the immunosuppressive effects of both polyclonal and monoclonal lymphocyte-specific antibodies are associated with a risk of opportunistic infections.

Mechanism of action and methods of monitoring ALS before and during clinical use

There is a great need for reliable methods of evaluating the effectiveness of various ALS preparations before they are used on patients. However, as long as the mechanism of action is not clearly established, it is hard to identify the method of choice. The immunosuppressive effect of ALS may be mediated by depletion of lymphocytes from blood and lymph-

oid tissue. This is accomplished by the opsonizing effect of antibodies, stimulating phagocytosis of the lymphocytes. Other possibilities are blocking or modulation of receptors on lymphocytes. While OKT3 causes depletion of blood T lymphocytes, these cells, although antigenically modulated, may persist in peripheral lymphoid organs. It has also been reported that ALS may induce expansion of unusual subpopulations of lymphocytes. Also, donor-specific antigen-dependent suppressor cells may maintain the allograft tolerance induced by ALS. The effect of ALS on severe aplastic anemia has been ascribed to the release of hematopoietic growth factors (such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3), stimulation of hematopoietic cells via CD45RO, blocking of Fc receptors for IgG on T cells, or interference with activated T lymphocytes which inhibit hematopoiesis.

Among the *in vitro* methods used for tests of ALS activity are the study of specificity for different epitopes, the monitoring of cytotoxic or mitogenic effects, inhibition of the binding of sheep erythrocytes to T cells via CD2 (rosette inhibition), opsonizing effect on lymphocytes, quantitation of antibodies

bound to lymphocytes or of macrophage-cytophilic antibody, or methods based on complement binding. The potential of an ATG preparation can be estimated by titration of the dose which gives a 50% inhibition of binding of a panel of mAbs to lymphocyte markers. Owing to relatively poor correlation between *in vitro* tests and the immunosuppressive effect of ALS, *in vivo* testing of antihuman ALS preparations has also been performed. For instance, effects on graft-versus-host reactions in rats, and the rejection of skin allografts in monkeys, were shown to correlate well with immunosuppressive effects on patients. It is possible to examine the effectiveness of treatment by measuring target cell concentrations in the blood, or the density of target antigens on such cells. Also, the immunological response to the administered ALS or mAbs may be quantitated by various methods such as ELISA. Such measurements are important in the planning of a repeated treatment with ALS.

Experimental use of ALS

In addition to the wide clinical use of ALS, it can be used for both the identification of lymphocyte subsets and for isolation of such populations. This can be achieved either by negative selection (complement-mediated lysis) or positive selection (e.g. by means of adherence to antibody-coated surfaces). However, mAbs are mainly used for these purposes today. ATS-treated mice can be used in an assay for tumorigenicity and metastatic capacity of cancer cells.

Natural and disease-associated lymphocyte-specific antibodies

In the blood of normal individuals one finds lymphocyte-specific antibodies which, under certain conditions, may be cytotoxic to all, or to subpopulations of, lymphocytes. These antibodies are part of the natural antibodies found in nonimmunized individuals. The significance of these antibodies is not clear, but their presence may induce acute rejection of a transplanted tissue. Autologous antibodies against lymphocytes seem to be induced by virus infections. They also occur in increased frequency in autoimmune disease (systemic lupus erythematosus, rheumatoid arthritis, ankylosing spondylitis), cancer, and in patients exposed to alloantigen, such as multiple transplanted uremic patients, multiparous dialysis patients, hemophiliacs and male homosexuals. A large percentage of patients with Behçet's syndrome have lymphocytotoxic antibodies, as do some of their healthy children. The presence of lymphocyte-

specific antibodies in human immunodeficiency virus (HIV) infection is associated with increased risk for developing acquired immune deficiency syndrome (AIDS). Such antibodies may have been produced as a result of an immune response against lymphocyte-bound HIV determinants (such as gp41) and may directly cause the lymphopenia in AIDS patients. Autoantibodies to CD4, found in some HIV patients, do not however appear to have a prognostic significance. Autoantibodies to the GM3 ganglioside on AIDS patients' lymphocytes have been detected which do not bind to normal GM3.

Sera with natural lymphocytotoxic antibodies to heterologous cells have not been used therapeutically but may have an experimental value. For instance, natural thymocytotoxic antibodies from rabbits have been used in experiments on guinea pigs to deplete thymus cells of immature populations.

See also: Antiserum; Bone marrow and hematopoiesis; CD antigens; Graft rejection; Graft-versus-host reaction; Immunosuppression; Lymphocytes; Monoclonal antibodies (mAbs); Natural antibodies; Plasma; Serum sickness; Transplantation.

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ANTI-NUCLEAR ANTIBODIES

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Antibodies against antigens of cell nuclei probably occur in the blood of all human beings. Since such antibodies generally react with all kinds of nuclei, whatever their origin, they are also directed against antigens in the nuclei of the host, and are therefore considered to be 'autoantibodies'. If the anti-nuclear antibodies (ANAs) occur in titers elevated significantly above the normal serum level, the employed test is considered 'positive'. Such high titered autoantibodies are especially associated with the autoimmune disease systemic lupus erythematosus (SLE), although they also occur in high frequencies in patients with rheumatoid arthritis (RA), Sjögrens syndrome, scleroderma, polymyositis, mixed connective tissue disease (MCTD), myasthenia gravis and chronic active hepatitis.

ANA were first demonstrated in 1957 by Holborow and by Friou, using the indirect immunofluorescence technique (IFT) as developed by Coons. This method is still the method of choice as a screening technique, before more specific methods are used. The distinct ANAs and the assays used to demonstrate their presence are listed in Table 1.

Nuclear antigens

Nucleus

Many distinct structures in the cell nucleus serve as antigen for different populations of ANAs. Most ANAs are directed against nucleic acids or against proteins associated with nucleic acids. Only nucleoli and centromeres of chromosomes can readily be distinguished by the IFT as separate antigenic entities, all other antigens for autoantibodies have to be determined with more specific methods. The centromere antigen complex contains at least three antigenic proteins: CENP-A (18 kDa), CENP-B (80 kDa) and CENP-C (140 kDa).

Nucleosome

The nucleosome is the basic structure of chromatin and has an important function in the compaction of DNA in the nucleus of the cell. It consists of dimers of the four core histones, H2A, H2B, H3 and H4, together forming a histone-octamer around which 146 base pairs of DNA are wrapped twice (Figure 1). Two nucleosome subunits are connected via a

Table 1 Assays used for the detection of ANAs

ANA	Assay							
	LE cell test	IFT	CIE	IBT	RNA precipitation	Farr assay	PEG assay	ELISA
Anti-DNP	✓							
Antinucleosome								✓
Anti-DNA		✓				✓	✓	✓
Antihistone				✓				✓
Anticentromere		✓		✓				
Anti-Sm			✓	✓	✓			✓
Anti-U1RNP			✓	✓	✓			✓
Anti-Ro/SS-A			✓	✓	✓			✓
Anti-La/SS-B			✓	✓	✓			✓
Anti-Scl70			✓	✓				✓
Anti-Jo1			✓	✓	✓			✓
Anti-PL12				✓				
Anti-PL7				✓				
Anti-rRNP			✓	✓				✓
Anti-RA-33				✓				
Anti-Ku				✓				
Anti-PCNA			(✓)	✓				

IFT, Immunofluorescence technique; CIE, Counterimmunoelectrophoresis; IBT, immunoblotting technique; PEG, polyethyleneglycol.

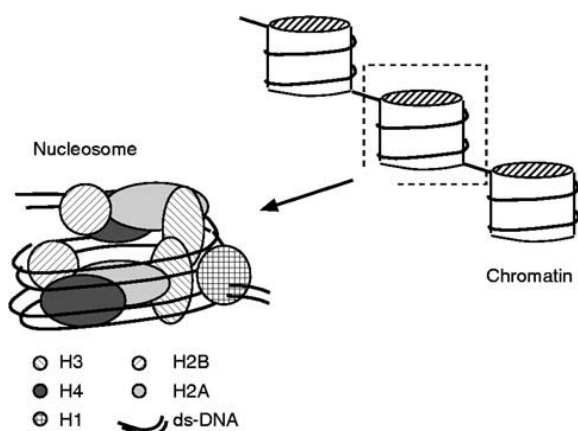


Figure 1 Schematic drawing of chromatin and its subunit, the nucleosome.

stretch of linker DNA to which histone H1 is bound. The four core histones are single-chain polypeptides with molecular weights ranging from 11 to 15 kDa. Clustered at the N-terminal regions of the core histones are basic residues, which, therefore, are located at the outside of the nucleosome. These positive charges on the cylindrical surface of the histone octamer form a left-handed spiral, situated along the DNA and are partly involved in the binding of DNA.

Small nuclear RNP (snRNP)

Eukaryotic cells contain a number of distinct small RNA molecules, which can be broadly divided into two classes: the capped small nuclear RNAs (snRNAs or U-RNAs) and the uncapped small cytoplasmic RNAs (scRNAs). Mammalian cells contain at least 14 different U-RNAs, organized into ribonucleoprotein particles (U-snRNPs; **Figure 2**). The

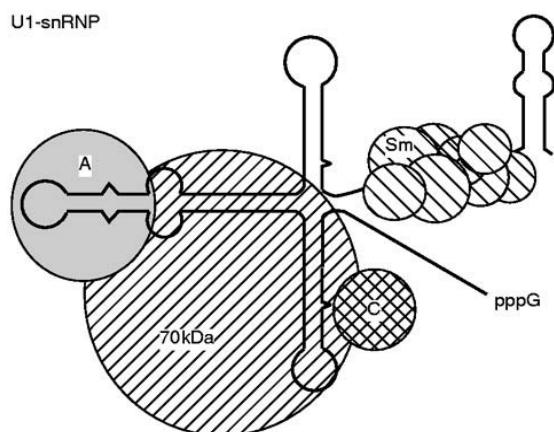


Figure 2 Schematic drawing of a U-snRNP particle. To the U1-RNA molecule the proteins 70 kDa, A, C and those of the Sm complex are bound; the Sm complex encompasses the proteins BB', D1, D2, D3, E, F and G.

U1–U6 snRNPs, with the exception of U3 snRNP, have an important role in messenger RNA processing.

More than 25 different polypeptides have been identified as constituents of the major snRNPs U1, U2, U5 and U4/U6. Of these proteins, nine are present in each of the individual snRNPs, and are designated as Sm or core proteins (**Table 2**). These include the BB' doublet, the D1/D2/D3 triplet and the E, F and G polypeptides. In addition to the core proteins, most of the snRNPs contain a number of unique polypeptides, designated 70 kDa protein, A and C (found in U1 snRNP only), A' and B' (found only in U2 snRNP).

RA-33

So-called heterogeneous nuclear ribonucleoproteins (hnRNPs) are complexes of RNA-polymerase II transcripts and a number of proteins. In these complexes, the processing of hnRNA into mature RNA takes place. Amongst the proteins in the hnRNP-complexes are the hnRNP-A1 and -A2. The latter has been shown to be identical to the RA-specific antigen RA-33.

Ku

The Ku antigen is localized in a particle composed of two noncovalently linked proteins of 70 and 80 kDa that bind DNA. The antigen is identical to nuclear factor IV (NF IV). Both proteins have been cloned and epitope mapping studies indicate that the major epitopes recognized by anti-Ku antibodies are conformational.

PCNA

Proliferating cell nuclear antigen (PCNA), also named cyclin, is a protein of 29 kDa that is primarily present in dividing cells. It has been identified as an auxiliary protein of DNA polymerase δ , a particle involved in DNA replication.

Nuclear enzymes

Several nuclear enzymes serve as antigen for auto-antibodies. Topoisomerase I is the most important of these. It is an enzyme of 100 kDa which is involved in the relaxation of supercoiled DNA, by nicking and releasing one strand of the DNA duplex.

Cytoplasmic antigens

ANAs are, by definition, directed to antigens in the cell nucleus. Since many patients with generalized autoimmune diseases not only have antibodies to nuclear antigens but also to cytoplasmic antigens,

Table 2 Presence of proteins in U-snRNP particles

	70 kDa	A	A'	B	B'	B''	C	D1-3	E	F	G
U1-RNP	✓	✓		✓	✓		✓	✓	✓	✓	✓
U2-RNP			✓			✓		✓	✓	✓	✓
U5-RNP				✓	✓			✓	✓	✓	✓
U4/U6-RNP				✓	✓			✓	✓	✓	✓

these are generally also loosely included under the term 'ANA'.

Small cytoplasmic RNP (scRNP)

One of these cytoplasmic antigens is called La/SS-B. It functions as a termination factor for RNA polymerase III. Therefore, La/SS-B scRNPs are composed of an RNA polymerase III transcript to which the La/SS-B protein is bound (Figure 3). The RNA transcripts include 7S RNA, 5S RNA, tRNA, U6-RNA, the Y-RNAs as well as a number of viral RNAs.

Ro/SS-A scRNPs are composed of several proteins complexed with a subset of the La/SS-B associated RNAs, the so-called Y-RNAs (Figure 3). In human cells four different Y-RNAs have been identified, together with two different Ro/SS-A proteins, designated Ro52 and Ro60. The Ro60 protein is unusual in containing both an RNP consensus motif for binding RNA and a zinc finger motif for binding to DNA. The Ro52 protein contains multiple zinc fingers and a leucine zipper sequence, but no RNP consensus motif. Most probably, this protein is complexed to Y-RNPs via binding to the Ro60 protein. The 50 kDa La/SS-B protein contains, as expected, an RNP consensus motif.

About 30% of both Ro/SS-A and La/SS-B is localized in the nucleus of the cell, which explains the nuclear immunofluorescence of anti-Ro/SS-A and anti-La/SS-B. Contrarily, Y-RNAs are predominantly

cytoplasmic. Presumably, Ro/SS-A and La/SS-B bind to newly synthesized Y-RNA in the nucleus, after which the mature complex, termed scRNP is transported to the cytoplasm.

No function has as yet been associated with the Ro/SS-A proteins.

rRNP

Ribosomal P-proteins are phosphorylated proteins that are present, often in multiple copies, on the ribosome. P1 (19 kDa) and P2 (17 kDa) interact with the eukaryotic elongation factors EF1 and EF2 and play a role in aminoacyl-tRNA binding and polypeptide synthesis. These proteins share a 22 amino acid sequence at the C-terminal that contains an epitope recognized by so-called anti-rRNP or anti-P antibodies.

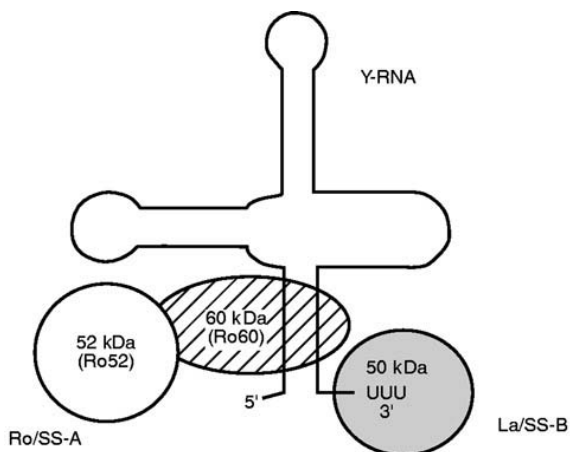
Cytoplasmic enzymes

Aminoacyl-tRNA synthetases are a group of cytoplasmic enzymes that catalyze the binding of tRNA to their respective amino acids. A number of them have been identified as antigens for a special class of ANA.

Detection, identification and clinical significance of ANAs

ANAs with IFT

The IFT is generally used as a screening assay for the presence of ANAs. The method is highly sensitive and detects almost all ANA specificities. Most laboratories now use cultured cells as substrate instead of tissue sections. Sera from patients with generalized autoimmune diseases mostly have several distinct ANAs at the same time. Since the titers of these antibodies differ, titration of the serum can reveal the strongest ANAs giving a certain pattern of nuclear fluorescence. Homogeneous patterns are frequently observed in patients with SLE or rheumatoid arthritis (RA), speckled or granular patterns in sera from patients with Sjögren's syndrome or scleroderma. Nucleolar fluorescence is often seen in patients with progressive systemic sclerosis (PSS). These associations, however, are of limited value to the clinician.

**Figure 3** Schematic drawing of an scRNP particle.

Only the discrete speckled pattern of nuclear fluorescence is informative, since this points to the presence of antibodies to centromeres. This should be confirmed by performance of the IFT on mitotic cells or chromosome spreads. Also the presence of other types of nuclear fluorescence is mostly the start of studies to specify the kind of the ANA.

Nearly all patients with SLE have ANAs, mostly in high titers. In RA, Sjögren's syndrome, PSS, MCTD, myasthenia gravis and chronic active hepatitis the frequency of the ANAs and the titers of these antibodies are considerably lower (Table 3).

A negative result in the IFT does not necessarily mean that ANAs are not present. It is known that low titered reactivities to Ro/SS-A, rRNP and Jo-1 are relatively difficult to detect in this assay.

Most laboratories have tested sufficient sera from healthy individuals to be aware of the statistical limits between normal and pathological ANA titers. Nevertheless, it should be noted that not only generalized autoimmune diseases show a preponderance for women and adults, but that also healthy elderly women have a far greater tendency to show these antibodies than young boys. A positive ANA in a boy, therefore, draws more attention than the same ANA in a woman over 60 years of age.

LE cell test

In 1948 Hargraves described for the first time the

occurrence of LE cells. The demonstration of these cells was a great help to define the complicated clinical picture of SLE (Table 3). Later on, the 'LE cell factors' were identified as autoantibodies and the 'LE cells' as granulocytes which had phagocytosed complexes of these autoantibodies and nuclei of other cells. Nucleosomes are able to inhibit the formation of 'LE cells', in contrast to free dsDNA or histones. Thus, it appears that the LE cell phenomenon is related to the presence of antinucleosome autoantibodies. After 45 years the test is still in use but with the introduction of more specific methods, it has become somewhat redundant.

Anti-DNA

Antibodies to DNA can be found in the circulation of the majority of patients with SLE. They are quite specific for this disease, which makes their detection an important diagnostic aid to the clinician (Table 3). Fluctuations in the level of anti-dsDNA in an individual patient generally parallel the clinical status of that patient. Under defined conditions, increases in the level of anti-DNA predict an upcoming flare of the disease. Indeed, it has recently been shown that instalment of (prednisone) treatment on the basis of increasing levels of anti-DNA may prevent the exacerbation from becoming overt. Furthermore, the presence of anti-dsDNA may precede the diagnosis of SLE by more than a year.

Table 3 Frequencies of ANA in various autoimmune diseases (percentages in round figures)

ANA	Autoimmune disease						
	SLE	RA	SS	MCTD	PSS	CREST	PM-DM
ANA	90	35	30	100	60	70	15
LE cell test	45	5	nk	20	nk	nk	nk
Anti-dsDNA	60	10	0	15	0	0	5
Anti-ssDNA	75	10	0	10	0	0	30
Anti-nucleosome	nk	nk	nk	nk	nk	nk	nk
Anti-histone	60	15	nk	nk	nk	nk	nk
Anti-centromere	2	0	0	5	10	95	nk
Anti-Sm	20	0	0	5	1	1	0
Anti-U1RNP	20	3	0	100	5	2	0
Anti-Ro/SS-A	40	5	70	50	30	nk	nk
Anti-La/SS-B	15	1	60	0	5	0	nk
Anti-Scl70	0	0	0	0	35	5	0
Anti-Jo1	0	0	0	0	0	0	30
Anti-P	10	2	2	0	0	0	0
Anti-RA-33	20	40	4	40	6	0	10
Anti-Ku	6	0	0	30	3	nk	4
Anti-PCNA	3	0	nk	nk	nk	nk	nk

nk, not known. SLE, Systemic lupus erythematosus; RA, rheumatoid arthritis; SS, Sjögren's syndrome; MCTD, mixed connective tissue disease; PSS, progressive systemic sclerosis; CREST, CREST syndrome (variant of scleroderma); PM-DM, Polymyositis-dermatomyositis.

A multitude of assays have been developed to detect the presence of antibodies to DNA. Currently, the most commonly used assays are the IFT on *Crithidia luciliae*, radioimmunoassays (RIAs) such as Farr assay and PEG assay, and enzyme-linked immunosorbent assays (ELISAs). These methods can either be obtained in kit form or be employed as in-house assays. The hemoflagellates *Crithidia luciliae* which are used in the IFT contain a giant mitochondrion, the kinetoplast, which consists of only double-stranded DNA. To facilitate discrimination between nuclei and kinetoplasts, the slides are counterstained by propidium iodide, which intercalates in the dsDNA helices. Fluorescence of kinetoplasts is taken as an indication of the presence of anti-DNA. After 20 years, this method still is one of the most frequently employed techniques for the detection of anti-DNA. The method is specific and quite sensitive. In RIAs the choice of antigen again is of great importance. The DNA employed has to be bigger than 10⁵ but smaller than 10⁷ kDa. Furthermore, the DNA must be double stranded and, to allow quantitation of antibody reactivity, monodisperse in size. This indicates that circular double-stranded bacteriophage DNA (such as from PM2) or plasmids (such as pUC9) are to be preferred.

In ELISA systems, DNA has to be coated to plastic. ssDNA can easily be coated directly, but dsDNA is mostly coated via intermediates such as poly-L-lysine, protamine or methylated BSA. Such precoats introduce problems related to the binding of immune complexes and/or immunoglobulins not directed against DNA to the plates (via the intermediate molecule). An alternative is to make use of biotinylated DNA and coat this via streptavidin to the plates.

Different assay systems are not always comparable, for the following reasons:

1. The source of antigen differs: DNA may be from

eukaryotic or prokaryotic origin, be double stranded or single stranded, be polydisperse in size or homogenous, etc.

2. Presentation of the antigen to the antibody differs: in RIAs it is generally in solution, in ELISAs it is coated to plastic; in the *Crithidia* test DNA is mostly presented intact in cells.
3. Reaction conditions are different: e.g. due to the employed ammonium sulfate precipitation step used in the Farr assay, anti-dsDNA of low avidity is missed with this method; in second antibody techniques such as IFT and ELISA the choice of conjugated antibody is of importance; often, only IgG anti-DNA is measured with these techniques.

General comparison of four different assays using sera of patients with defined SLE leads to high levels of correlations between the various assays (Table 4). However, upon routine screening of sera from patients suspected as having generalized autoimmune diseases, large discrepancies between the different assays are also seen: out of 16 possible reactivity combinations, 11 actually occurred. Sensitivity differences can only partly explain the discrepancies observed between the assays. An important cause is to be found in the avidity of the antibodies (Figure 4). Furthermore, histones or nucleosomes complexed to antinucleosome antibodies may also cause a positive reaction in the Farr assay. It is advisable to quantitate anti-DNA levels and express these in International Units, to allow comparison between different studies. A World Health Organization (WHO) standard serum to serve this purpose is available from the Central Laboratory of the Blood Transfusion Service (CLB) in Amsterdam. The CLB is the custodian of WHO standards for rheumatology.

Of the methods described, the ELISA is the most

Table 4 Comparison of the disease specificity of four anti-dsDNA assays

Disease	Number	Percentage of sera positive in			
		Farr assay	Crithidia	PEG assay	ELISA
Active SLE	96	98	96	96	100
Rheumatoid arthritis	62	1	0	5	3
Sjögren's syndrome	14	0	0	7	20
Scleroderma	12	0	0	0	30
Autoimmune hepatitis	61	0	0	20	15
Myasthenia gravis	25	0	0	32	20
Autoimmune thyroiditis	66	0	0	7	13
Autoimmune gastritis	32	0	0	0	18
Autoimmune hemolytic anemia	40	0	0	10	8
Normal donors	192	0	0	0	0

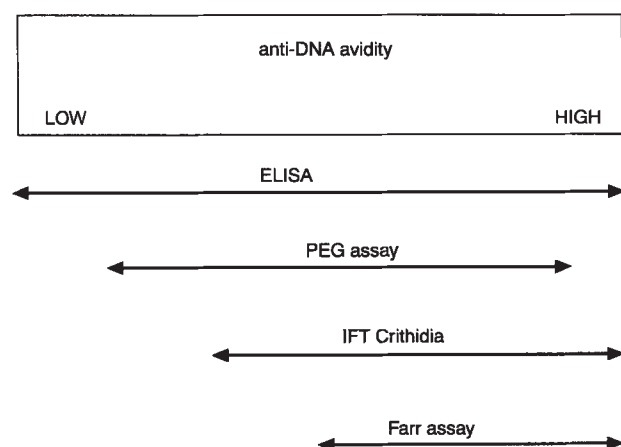


Figure 4 Relation between anti-DNA avidity and assay behavior.

sensitive, whereas the Farr assay is the most specific for SLE. Using the latter method, only antibodies of a relative high avidity for DNA are detected. Mild forms of SLE, where patients only have anti-dsDNA of a low avidity in their circulation, may easily be missed by this technique. On the other hand, if screening for the presence of anti-DNA takes place using an assay that is not selective for anti-DNA of high avidity, then a positive assay result does not always indicate that the patient has SLE: anti-DNA of lower avidity occurs in diseases other than SLE. An evaluation of the diagnostic value of low avidity anti-dsDNA has shown that half of the patients in whom anti-dsDNA is PEG positive but Farr negative have SLE. When high-avidity anti-dsDNA is present as well, nearly 90% of the patients were found to have SLE. Therefore, screening using a 'broad-spectrum' method should be followed by an assay selective for high-avidity anti-dsDNA.

Because the Farr and PEG assays may readily be compared, as they only differ with respect to the way DNA/anti-dsDNA complexes are precipitated, a relative avidity index can be acquired by calculating the ratio between results (in IU ml⁻¹) of both assays. An optimal discrimination between low- and high-avidity DNA was obtained using a cut-off Farr:PEG ratio of 5. Using this approach anti-dsDNA of SLE patients with nephritis was found to have significantly higher avidity than that from patients with CNS involvement.

Antibodies to DNA have always been claimed to play an important role in the pathogenesis of SLE. Traditionally, SLE is considered to be an immune complex disease. In this concept, anti-DNA binds DNA and the resulting immune complexes are deposited in the tissues. This binding of DNA by antibodies may occur in the circulation, but it may

also happen *in situ*. At the site of deposition, subsequent complement activation then leads to inflammation and the characteristic disease features of SLE. In the past few years this concept of the pathophysiology of SLE has been challenged, and a modified hypothesis concerning the pathogenesis of SLE has been proposed. This new concept is based on studies that have shown that anti-DNA may interact with tissue structures such as heparan sulfate (HS), the major glycosaminoglycan side-chain of the glomerular basement membrane (GBM). Although it was originally thought that the binding of anti-DNA to HS was based on cross-reactivity, more recently it has been shown that this binding is in fact mediated by nucleosomes. Increased levels of nucleosomes have been demonstrated in the plasma of SLE patients, perhaps due to increased or defective apoptosis leading to their release into the circulation. Renal perfusion studies in the rat have shown that anti-DNA can only bind to the GBM after prior perfusion with histones followed by DNA, or with nucleosomes. Thus, complexes composed of antibodies and nucleosomal material interact via their histone part with heparan sulfate in the GBM. In contrast to anti-nucleosome and anti-DNA antibodies, antihistone monoclonal antibodies do not localize in the GBM after complexation with nucleosomes. Therefore, the specificity of the antibody bound to the nucleosome appears to be a critical determinant for the nephritogenic potential of the complex.

Anti-nucleosome

Anti-nucleosome antibodies are defined as antibodies which are directed exclusively (or predominantly) against nucleosomes or subnucleosomal structures, consisting of DNA plus core histones. These antibodies were in fact the first autoantibodies described in association with SLE, being the LE factors that were responsible for the so-called 'LE cell phenomenon'.

For the specific detection of antibodies to nucleosomes, ELISAs have been developed in which purified nucleosomes are used as antigen. Several purification protocols have been described, all using micrococcal nuclease digestion which causes internucleosomal cleavage. All eukaryotic cells can be used as starting material, but most frequently calf thymus or rat liver is used. After preparation, the purity should be checked, both for the histones by PAGE analysis and for DNA by extraction and analysis on agarose gels. For screening purposes, H2A-H2B/DNA can be used as a substitute for nucleosomes. To this end, commercially obtained H2A and H2B can be mixed in equal amounts; the

dimer is then coated to ELISA plates, subsequently followed by addition of dsDNA.

A problem in the measurement of antibodies to nucleosomes is, of course, that both anti-dsDNA and antihistone will also react with nucleosomes. So far, this can only be solved by prior absorption of anti-DNA and anti-histone from the serum or plasma sample. In this way, the presence of nucleosome-specific antibodies has unequivocally been demonstrated in SLE patients. From analysis of the epitope specificity of a number of lupus-derived nucleosome-specific monoclonal autoantibodies, it is clear that these antibodies recognize a multitude of conformational epitopes formed by the interaction between DNA and core histones.

Anti-histone

Anti-histone antibodies occur in a number of autoimmune diseases, including SLE, drug-induced lupus, juvenile rheumatoid arthritis and RA (Table 3). The frequency of autoantibodies to individual histones vary according to the type of autoimmune disease. In general, antibodies to H1 are the most frequent in SLE, followed by anti-H2B, anti-H2A, anti-H3 and anti-H4, respectively. Antibodies to histones are generally measured by means of specific ELISAs, using purified histones as antigens. Care has to be taken regarding the purity of commercial histone preparations. Antihistone reactivity is also readily detectable in immunoblotting assays, using nuclear extracts as antigen.

Anti-centromere

These antibodies can be demonstrated with the IFT on cultured cells. A more sophisticated assay makes use of chromosome spreads on slides, to allow the specific localization of the centromeres.

Antibodies to the centromere antigens CENP-A and CENP-B are the most common and can be found in about 95% of the patients with the CREST syndrome (Table 3). They occur less frequently in patients with diffuse scleroderma, and are, therefore, important diagnostic markers for scleroderma with limited skin involvement. They also appear to have prognostic significance. Anti-centromere reactivity is readily measured by IFT and immunoblotting; CENP-B has been cloned and can be used in ELISA.

Anti-snRNP (Sm, U1-RNP, U2-RNP)

Sm-specific antibodies are directed to the D proteins (either all three or to a subset of them) and to the BB' doublet of the U-snRNPs (Figure 2). Since these proteins are common to all major snRNPs except U3, Sm-specific antibodies precipitate U1, U2, U4,

U5 and U6 snRNPs. Whereas these antibodies to Sm are exclusively found in patients with SLE, and are, therefore, disease specific, antibodies to U1-RNP are considered to be a marker antibody for MCTD (Table 3).

Anti-U1-RNP sera precipitate U1-snRNPs; they predominantly recognize U1-70 kDa and U1-A, and with lower frequency also U1-C. In about 50% of the cases U1-RNP sera also react with BB'. Antibodies to U2-RNP react predominantly with the U2-snRNP-specific B'' protein, and in some cases also with the U2-A' protein.

Apart from detection by counterimmunoelectrophoresis (CIE) and immunoblotting, specific ELISAs have also been developed for the measurement of anti-Sm and anti-U1-RNP. Most of the relevant proteins are available in recombinant form, for use in ELISA systems. For some unexplained reason, the recombinant D protein(s) is (are) not immunoreactive in ELISA and cannot be used.

Presently available data on the incidence of anti-Sm and anti-U1-RNP in autoimmune diseases has been compiled using 'older' assays, such as CIE. Data obtained using immunoblotting and ELISA are not readily available.

Anti-RA-33

Antibodies to RA-33 were originally described to be quite specific to RA. More recently they have also been found in other autoimmune diseases (Table 3). They can be detected by immunoblotting, where they generally give rise to a trimer of around 33 kDa.

Anti-Ku

Ku-specific antibodies were originally described in Japanese patients with scleroderma-polymyositis overlap syndromes. Later, it was observed that these antibodies also occur in patients with SLE, scleroderma and MCTD (Table 3). They can be detected by immunofluorescence, CIE and immunoblotting.

Anti-PCNA

Antibodies to PCNA are found in the sera of about 3% of SLE patients, and can be detected by IFT in dividing cells (Table 3). The antibody is also readily detectable by CIE and immunoblotting, where it shows a band at 35 kDa. Epitope mapping studies have shown that the antibodies primarily bind conformational epitopes in this antigen.

Anti-topoisomerase I (Sci70)

Antibodies to topoisomerase I, mostly termed anti-Sci70, are found frequently in sera of patients with diffuse cutaneous systemic sclerosis and are quite

specific to this disease (Table 3). Therefore, anti-Scl70 is an important marker antibody for systemic sclerosis. Anti-Scl70 can be detected by CIE, immunoblotting or a specific ELISA. The presence of this antibody in patients with Raynaud's phenomenon that eventually develop scleroderma suggests that anti-Scl70 may have prognostic significance.

Anti-scrNP (Ro/SS-A, La/SS-B)

Antibodies to La/SS-B are predominantly found in patients with Sjögren's syndrome, but also in SLE and in RA (Table 3). Differences in reported percentages are to be explained on the basis of patient selection and use of different methods of the detection of anti-La/SS-B. Analysis of immunoglobulin heavy and light chain usage has revealed that these antibodies are probably the result of an oligoclonal B cell expansion.

Anti-La/SS-B are almost always found in combination with anti-Ro/SS-A. The reverse is not always the case: antibodies to Ro/SS-A often occur alone in SLE and systemic sclerosis (Table 3).

Classically, antibodies to Ro/SS-A and La/SS-B are detected by immunodiffusion and CIE. More recently, immunoblotting, ELISA and RNA precipitation have been used for the detection of anti-Ro/SS-A and anti-La/SS-B. Autoantibody levels against Ro/SS-A and La/SS-B in patients with SLE do not correlate with disease symptoms or activity. This is in contrast to anti-dsDNA activity which correlates very well with disease activity in these patients. This also implies that the anti-Ro/SS-A and anti-La/SS-B responses are regulated independently from anti-DNA. Anti-Ro/SS-A and anti-La/SS-B responses significantly correlate. Thus, most of the time anti-Ro/SS-A and anti-La/SS-B fluctuate together. One of the few clear-cut clinical correlations between an antinuclear antibody and a clinical syndrome is found in the congenital heart block (CHB). CHB was originally reported as a rare but potentially fatal complication of offspring from mothers with connective tissue disease, especially SLE. Maternal anti-Ro/SS-A are the largest single risk factor for the development of intrauterine or neonatal complete heart block in a child. The period of pregnancy when cases of fetal bradycardia are first noted (from the 20th week of gestation) coincides with the start of transplacental immunoglobulin G (IgG) transport. The autoantibodies are thought to mediate disease by eliciting an inflammatory reaction in the conduction tissue of the fetal heart. Parameters that would allow discrimination between 'pathogenic' (i.e. CHB-inducing) and nonpathogenic anti-Ro/SS-A have not yet unequivocally been identified.

Anti-rRNP

These antibodies react with the ribosomal phosphoproteins P1 and P2. Mostly, they also recognize the neutral phosphoprotein P0 (38 kDa) that is found in the large subunit of the ribosome.

Anti-P can be found in 10% of patients with SLE (Table 3). Initially, its occurrence was related to the presence of lupus psychosis, but later studies have not been able to confirm this relation. Anti-P can be readily detected by CIE and immunoblotting. In the latter assay, anti-P reactivity generally gives rise to three stained bands. Anti-P reactivity can also be detected using an ELISA in which a synthetic peptide of the C-terminal 22 amino acids serves as antigen. Longitudinal studies of anti-P in SLE have shown that quantitative determination has no practical value in the follow-up of SLE patients.

Anti-Jo1 and other anti-tRNA synthetases

Antibodies to tRNA synthetases often occur in patients with polymyositis. The best known of these ANAs is anti-Jo1, which is found in 30% of the patients with adult polymyositis (Table 3). The antibody is directed to the histidyl-tRNA synthetase (54 kDa). Anti-Jo1 can be detected by CIE, immunoblotting and specific ELISA. Because the synthetase is associated with a tRNA molecule, RNA precipitation results in a tRNA band upon gel electrophoresis.

Antibodies related to anti-Jo1 are anti-PL12, which is directed to alanyl-tRNA synthetase (110 kDa), and anti-PL7, directed to threonine-tRNA synthetase (80 kDa). Antibodies reactive with isoleucine-tRNA synthetase and glycine-tRNA synthetase have also been reported.

Concluding remarks

It is the current view that autoantigens themselves drive the autoimmune response against them. In that view, nucleosomes are to be considered the relevant autoantigen for the genesis of antibodies to nucleosomes, histones and DNA. Indeed, it would not be expected that DNA occurs freely in the circulation or the organs of the immune system, since in the nucleus of the cell it is so tightly bound to the core histones. Evidence implicating the role of nucleosomes as the driving antigen has been obtained by several different groups. Datta and colleagues, studying the role of T cells in the induction of autoantibodies, found that in lupus mice 50% of the pathogenic T helper cells respond to nucleosomes. These nucleosome-specific T helper cells not only induced the production of nucleosome-specific autoantibodies by syngeneic B cells, but also the formation of

anti-DNA and antihistone. Comparable results were obtained in studies with human T cell clones. These observations assign a central and dominant role to the nucleosome in the induction of the ANA response. The validity of this concept is underlined by observations made by Rubin and colleagues, that the formation of antibodies to nucleosomes precedes the development of anti-DNA and antihistone in MRL/lpr mice ('antigen spreading'). Sequencing of ANAs has taught us that ANAs, just like 'ordinary' antibodies show somatic mutations with increasing antibody avidity. This too, is taken as evidence for a driving role of the (auto)antigen in the immune response towards autoantigens.

See also: Anti-inflammatory (nonsteroidal) drugs; Autoantibodies, tests for; Glucocorticoids; Molecular mimicry; Prostaglandins; Rheumatological disorders; Sjögrens syndrome; Skin, autoimmune diseases; Systemic lupus erythematosus (SLE), human.

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ANTI-OXIDANT MICRONUTRIENTS AND THE IMMUNE SYSTEM

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In the last decade there has been growing evidence that oxidative stress impairs immune responses and that anti-oxidants can reduce many aspects of oxidant-mediated immune suppression. Oxidative stress is directly related to the concentration of highly reactive molecules, most of which contain reactive species of oxygen, which reduce the anti-oxidant capacity of the body's cells.

Free radicals, highly reactive molecules with one or more unpaired electrons, often contain reactive oxygen species. Free radicals are generated during cellular metabolism, can be ingested or inhaled as environmental pollutants, or can be generated during the metabolism of certain drugs or xenobiotics.

Essential micronutrients with anti-oxidant activities

Antioxidants interfere with the production of free radicals and/or inactivate them once they are formed. There are four enzymes which have anti-oxidant capacity and contain essential minerals. These include two types of superoxide dismutases: a manganese-containing enzyme and an enzyme containing both copper and zinc. The third enzyme, catalase, an iron-containing enzyme, catalyzes the decomposition of hydrogen peroxide. Selenium is an essential component of the fourth enzyme, glutathione peroxidase, important in the decomposition of hydrogen peroxide and lipid peroxides, termination products of free radical attack on lipids.

Three other essential micronutrients can directly interfere with the propagation of, as well as scavenge, free radicals. Vitamin E (α -tocopherol), the major lipid-soluble anti-oxidant present in all cellular membranes, protects against lipid peroxidation and prevents the loss of membrane fluidity. Vitamin E has been characterized as the most critical anti-oxidant in blood. Vitamin C (ascorbic acid) is water soluble and, along with vitamin E, can quench free radicals as well as singlet oxygen. Ascorbate can also regenerate the reduced, anti-oxidant form of vitamin E. Recent work has shown that β -carotene, a pigment found in many photosynthetic plants, is an efficient quencher of singlet oxygen and can function as an anti-oxidant. β -Carotene is the major dietary carotenoid precursor of vitamin A. Vitamin A cannot quench singlet oxygen and has less anti-oxidant activity than the other nutrients discussed; however, its importance for the immune system is well recognized.

Assessment of oxidative stress and anti-oxidant status

Noninvasive measures

The most commonly used noninvasive measure of oxidative stress is the determination of the concentration of the volatile lipid oxidation products, pentane and ethane, in the breath. Dietary recall is the most commonly used noninvasive measure of intake of anti-oxidants.

Invasive measures

Serum concentrations of lipid peroxides and other oxidative products of proteins, DNA and carbohydrates are used to assess levels of oxidative stress. Serum concentrations of the anti-oxidant micronutrients are used as indicators of *in vivo* anti-oxidant status.

Free radicals and immune cell function

There are numerous links between free radical reactions and immune cell functions. White blood cell membranes are composed of lipids containing saturated and unsaturated fatty acids. Unsaturated bonds in fatty acids are highly susceptible to free radical attack, one consequence of which is to adversely affect the integrity of the cell membranes. For instance, oxygen-containing radicals and the products of their reactions have been shown to decrease the fluidity of white blood cell membranes and synovial fluids, consequently reducing their function.

Effects of immune-generated oxidants

The most abundant circulating white blood cell, the neutrophil, utilizes reactive oxygen species to kill invading organisms. When stimulated, neutrophils have the capacity to take up molecular oxygen and generate oxygen-containing free radicals and other reactive molecules. This is often called the oxidative burst. Free radicals and singlet oxygen, along with other reactive molecules, can kill pathogens. Neutrophils can also generate highly toxic halogenated molecules (e.g. hypochlorous acid) when the myeloperoxidase halide enzyme system is activated during the oxidative burst. The halogenated species can also lyse the phagocytosed pathogen. Recently, another reactive oxygen species, peroxynitrite, has been identified as an important component in immune cell killing of pathogens.

Under normal circumstances, the reactive oxygen species generated by neutrophils and other immune cells are used for control of infection; however, in circumstances of chronic activation the reactive oxygen products can result in destruction of normal tissue, as seen in rheumatoid arthritis.

Effects of anti-oxidant deficiency

One of the most obvious factors that could reduce anti-oxidant status is an induced dietary deficiency of, for instance, vitamin C. The effects of marginal vitamin C deficiency on immune and other parameters have been determined under highly controlled conditions in healthy males. Serum, white blood cell and sperm vitamin C levels were significantly reduced when the diet contained 5–20 mg of vitamin C for 2 months. Delayed-type hypersensitivity (DTH) responses were significantly depressed during the period of low vitamin C intake.

In addition to DTH responses, an index of oxidative damage to sperm DNA was also measured; these levels were significantly increased when vitamin C intakes were low. This carefully controlled study clearly demonstrated the importance of the balance between anti-oxidant status, oxidant levels and immune response. The study is especially noteworthy since all other dietary anti-oxidants, such as vitamin E and β -carotene, were provided throughout the study at recommended daily allowance (RDA) levels.

Clinical examples of free radical, anti-oxidant and immune function interactions

Examples of immune dysfunction associated with increased oxidative stress exemplify the interactions between the immune system and the oxidant/anti-oxidant status of the individual.

Rheumatoid arthritis

Rheumatoid arthritis (RA), an autoimmune disease of unknown cause, is characterized by a chronic inappropriate immune response in articular joints, resulting in inflammation and destruction of joint tissues. At some point, the initial insult and inflammation becomes a chronic process. Infiltration of the fluid-filled joint space (synovium) with inflammatory neutrophils occurs and structural changes appear in the synovium, forming an inflamed layer called a pannus. Oxidative products reduce the viscosity of the synovial fluid, thus hindering joint movement further.

Animal models have documented the increased production of pentane in arthritic animals; vitamin E supplementation lowered the pentane exhalation as well as joint swelling. Breath pentane concentrations correlates with the severity of symptoms in RA patients and clinical improvement is associated with a significant decrease in breath pentane. Patients with RA have significantly lower values in the total radical-trapping anti-oxidant parameter ('Trap') assay, based on serum levels of anti-oxidant nutrients, than do control subjects.

With regard to intervention studies, vitamin E has been examined to the greatest extent in patients with RA and other arthritic conditions. Three studies in RA patients suggest that vitamin E at high levels, but not at dietary intake levels, reduced pain. Vitamin E supplementation produced significant pain relief in four other studies. There was also a consistent finding of vitamin E-related antiinflammatory activity, although the studies involved a limited number of subjects.

Aging

The cumulative effects of free radical damage throughout the lifespan are seen in the pigmented age spots of the elderly, which are a consequence of lipid oxidation. The overall increased oxidative stress associated with aging also adversely affects many aspects of immune responses. The consequences of suboptimal immune responses are particularly detrimental in the elderly who have an increased risk of infections as well as of autoimmune diseases.

The cell-mediated immune responses involving T lymphocyte functions (cytotoxicity, interleukin 2 (IL-2) production, proliferation) are the most sensitive to the age-related decline in immune responses. As a consequence, DTH responses to skin test antigens are diminished in the elderly, and can often result in complete loss of response to antigen challenge (anergy) in the most immunosuppressed. Clinical studies have shown that DTH can be used as a pre-

dictor of morbidity and mortality in the elderly; i.e. elderly with anergy had twice the risk of death from all causes as elderly who responded to the antigens. Moreover, in hospitalized elderly who had undergone surgery, anergy was associated with a greater than tenfold increased risk of mortality and a fivefold increased risk of sepsis.

Recently, in a placebo-controlled study, intake of a one-a-day type multivitamin/mineral supplement for 12 months significantly enhanced DTH in healthy elderly. Because the multivitamin included β -carotene, vitamin E, vitamin C and all other essential vitamins as well as several minerals, it is not possible to determine whether the anti-oxidants or the other components in the multivitamin supplement were responsible for the improved DTH responses. In another recent placebo-controlled intervention trial, elderly were given a multivitamin, daily for 1 year, which contained approximately eight times the standard level of intake of β -carotene as well as higher levels of vitamins C and E. The supplemented group had significantly less infections than the placebo group. Responses to vaccines were also improved in the supplemented group.

With respect to individual anti-oxidants, an epidemiological study in an elderly population found that the higher the plasma vitamin E levels, the lower the number of infections. Two placebo-controlled, double-blind studies showed that vitamin E supplementation alone significantly enhanced DTH responses and/or T cell proliferative responses in the elderly. Lymphocyte vitamin E levels increased over threefold with supplementation and were correlated with enhanced IL-2 production and decreased prostaglandin E_2 as well as decreased serum lipid peroxides.

Cigarette smoking

Approximately 30% of US adult women and 25% of adult men smoke. Cigarette smoke contains millions of free radicals per inhalation. Other harmful products in cigarette smoke can stimulate the formation of highly reactive molecules that further increase the free radical burden; smokers have significantly higher breath pentane levels than nonsmokers. The micronutrient most affected by cigarette smoking appears to be the anti-oxidant, vitamin C. Smokers require about four times as much vitamin C per day to reach the same blood levels as nonsmokers. In addition to vitamin C, serum levels of vitamin E, folic acid and β -carotene, as well as lung vitamin E concentrations, are significantly lower in smokers compared to nonsmokers.

Cigarette smokers have depressed immune responses compared to nonsmokers, which may in

part be due to the overproduction of immunosuppressive free radicals by neutrophils and macrophages in their lungs. The lung of the healthy non-smoker contains very few neutrophils. In smokers, there is a constant activation of neutrophils and a consequent overproduction of reactive oxygen species.

Supplementation with high levels of vitamin E resulted in a significant decrease in smoker breath pentane levels and significantly reduced the overproduction of oxidant radicals by their circulating phagocytic cells.

Summary

Immune function is dependent upon a balance between the free radical and anti-oxidant status of the individual. In healthy adults, exposure to high levels of oxidants is associated with reduction in clinically relevant immune responses; exposure to low levels of dietary anti-oxidants also reduces immune responses such as DTH.

Anti-oxidant status becomes particularly critical in conditions which result in increased oxidative stress and immune dysfunction, as illustrated in RA, aging and cigarette smoking. In all three examples, evidence of free radical damage to lipids and other cellular components has been demonstrated. At the

same time, anti-oxidant status is reduced in arthritic patients and smokers compared with controls. Data suggest that elderly individuals may require higher levels of anti-oxidants to maintain DTH responses.

Several clinical studies have reported that supplemental anti-oxidants reduced indices of oxidative stress and improved various parameters of immune function.

See also: Fatty acids (dietary) and the immune system; Phagocytosis; Vitamin A and the immune system; Vitamin C and the immune system.

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ANTIBODIES: ANTIGENICITY OF

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The term anti-antibodies (AAs) refers, in a broad sense, to all antibodies combining with other antibodies, the latter acting as antigens. This also includes antibodies against immunoglobulins of unknown antibody specificity. As early as 1900, Ehrlich and Morgenroth theorized about the formation of 'true AAs' directed against antibody-combining sites and postulated that such antibodies may exert a homeostatic mechanism preventing the formation of autoantibodies that could be harmful for the host. Subsequently, since the beginning of the twentieth century several investigators performed immunization of animals with antibodies of foreign species origin, with the objective of obtaining true AAs. All these studies, however, resulted in production of AAs directed against immunoglobulin structures other than the antibody-combining sites.

AAs to native antibody molecule other than the combining site

Moreschi (1909) used AAs to achieve agglutination of erythrocytes sensitized by nonagglutinating antibodies. He showed that rabbit erythrocytes sensitized by such antibodies of goat origin would undergo agglutination when exposed to rabbit antiserum against goat serum proteins. This was the first description of what has been called later antiglobulin reactions which were introduced into routine laboratory practice by Coombs, Mourant and Race in 1945. AAs active in these tests, frequently referred to as antiglobulin antibodies, are usually produced by immunization with immunoglobulins of animals of foreign species (e.g. rabbits or goats are immunized with human immunoglobulins). These AAs

recognize clearly the species origin of the antibody with which they are reacting.

Allotypes are inherited antigens of immunoglobulins originally described by Grubb (1956). They are usually recognized by means of AAs produced by an animal of the same species. Production of such AAs occurs spontaneously in some humans, although the reasons for this are unclear, or may be achieved by intentional immunization of animals, e.g. rabbits, mice or sheep with allogeneic immunoglobulins.

AAs to altered antibody molecules

Rheumatoid factor (RF) was described by Waaler in 1940 and by Rose and his associates in 1948. It was found in sera of a majority of patients with rheumatic arthritis; it combined with rabbit antibodies sensitizing sheep erythrocytes and caused agglutination of these erythrocytes. In the second part of the 1950s, a general consensus was reached among workers in the field that RF detected in sera of patients with rheumatoid arthritis is an antibody of the immunoglobulin M (IgM) class combining with the Fc fragment of IgG originating from a variety of species; the most frequently studied IgGs were of rabbit and human origin. It was shown that RF reacting with rabbit IgG is different from RF reacting with human IgG and that there also exists a cross-reacting RF, presumably a multispecific antibody combining with IgG of both of these species. Later, in addition to IgM, RF was shown in the IgG and IgA classes. The heterogeneity of RF justified the use of the plural – RFs.

Some investigators have referred to all RFs as autoantibodies. This does not seem justified at all with reference to the RFs combining with IgG of foreign species. Furthermore, RFs combining with human IgG frequently react only with aggregated IgG or, otherwise, with IgG of the allotypic specificity other than that of the patient. Again, neither of these RFs should be called an autoantibody. The term autoantibody should be reserved for those RFs that react with the patient's own native IgG; some evidence for the existence of such RFs has been presented.

An important observation concerning specificity of RFs was made by Hannestad and his associates in the 1970s. They demonstrated RFs which in addition to IgG reacted with other unrelated antigens such as nuclear antigens or cardiolipin. Significantly, the same molecule of RF could react with IgG and these unrelated antigens. It has been proposed that such a cross-reacting RF molecule recognizes shared epitopes on these antigens; alternatively, it has been suggested that the cross-reacting RF is a multispecific

antibody with separate or overlapping combining sites for different epitopes.

Another type of serum factor was described by Milgrom, Dubiski and Wozniczko in a few samples (less than 1%) of human sera in 1956. Similar to RFs, it is an IgM antibody combining with IgG antibodies, however this factor reacts only with human antibodies and only if they appear in the form of immune complexes; it does not react with free antibodies. Accordingly, this factor provided evidence for the long disputed thesis about molecular transformation of an antibody molecule in the course of its reaction with the antigen. Furthermore, the characteristics of this factor encouraged the hypothesis that this factor as well as RFs are formed in response to a stimulus exerted by the individual's own IgG antibodies altered during *in vivo* reactions with their antigens. According to this hypothesis, RFs as well as other AAs in human sera would represent stereotypes of reactions of subjects exposed to prolonged immunizing stimuli. This was consistent with the demonstration of RFs in several pathologic conditions other than rheumatoid arthritis, primarily in subacute bacterial endocarditis. This hypothesis was further supported by studies on experimental animals, which showed that immunization with partially denatured autologous IgG as well as prolonged exposure to any strong antigen resulted in formation of RF-like antibodies. Alternatively, several investigators postulated that RF may be formed as a result of polyclonal stimulation of B cells by mitogens released or created under various pathologic conditions.

The pathological role of RFs in rheumatoid arthritis has been repeatedly suggested but never substantiated convincingly. In recent years, more and more studies have been conducted on IgG RFs. It has been shown that these RFs have a propensity for self-aggregation in that each RF molecule may act as an antigen through its Fc fragment and as an antibody through its Fab fragment. Evidence has been obtained that such aggregates may form immune deposits responsible for or contributing to glomerulonephritides.

AS to the antibody-combining sites

As mentioned, early attempts to elicit production of true AS met with failure. Immunization with antibodies originating from a species foreign to the immunized animal resulted in formation of AS of antiglobulin nature. In 1957, however, immunization of rabbits with rabbit antibodies to *Proteus* and *Escherichia* was shown by Milgrom and Dubiski to result in the formation of AAs that distinguished

the anti-*Proteus* from the anti-*Escherichia* antibody and accordingly, appeared to have properties of true AAs. Subsequently, in 1963 Kunkel, Mannik and Williams as well as Oudin and Michel, used various protocols of immunization of animals and succeeded in eliciting formation of what appeared to be true AAs. They called them idiotypic antibodies. Further studies, however, showed that the idiotypic specificity cannot be equated with the structure of the antibody-combining site, even if it is closely related to this structure.

See also: Allotypes, immunoglobulin; Antibodies, specificity; Antiglobulin (Coombs') test; Idiotypic; Natural antibodies; Rheumatoid arthritis, animal models; Rheumatoid arthritis, human.

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ANTIBODIES, BISPECIFIC

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Bispecific (bifunctional) antibodies represent a category of artificially created immunoglobulins exhibiting a double, well-defined antigen specificity. The major application of bispecific antibodies is to focus the activity of an effector cell on a target cell by virtue of their ability to bind with one combining site to effector cell and with another to target cells.

Antibodies against the T cell receptor (TCR) of helper and cytotoxic effector cells are able to mimic the antigen and to activate the T cells to proliferate, to release lymphokines or to become cytotoxic. Bispecific antibodies which bind with one arm to the effector cell and with another to the target cell can activate the effector cell and ensure intimate contact between effector and target cells.

The preparation of bispecific antibodies was helped by the development of monoclonal antibody technology which can provide unlimited amounts of homogeneous antibody. There are three basic methods used to prepare bispecific molecules with 'à la carte' desired specificity (Figure 1).

Heteroconjugates

Heteroconjugates are bispecific antibodies obtained by chemical linkage of two immunoglobulin (Ig) molecules with different binding specificity. The chemical linkage of two monoclonal antibodies is carried out using cross-linkers such as *N*-succinimyl-3-(2-pyridyldithio)-propionate (SPDP). High-pressure liquid chromatography (HPLC) is used for the purification of heteroconjugates made up of only dimers.

In vitro experiments demonstrated that such heteroconjugates are able to focus the activity of effector T cells on target cells bearing different antigen specificity. The presence of monocytes which can bind the heteroconjugates via Fc receptor can supply additional signals for the activation of T cells. It remains to be tested whether these antibodies are effective *in vivo*, in particular whether they could achieve the activation of local T cell contact with the target cells.

Heteroconjugates, although relatively easy to produce, are likely to be cleared quickly and may function as immune complexes, eventually causing immunopathological phenomena *in vivo*. Their stab-

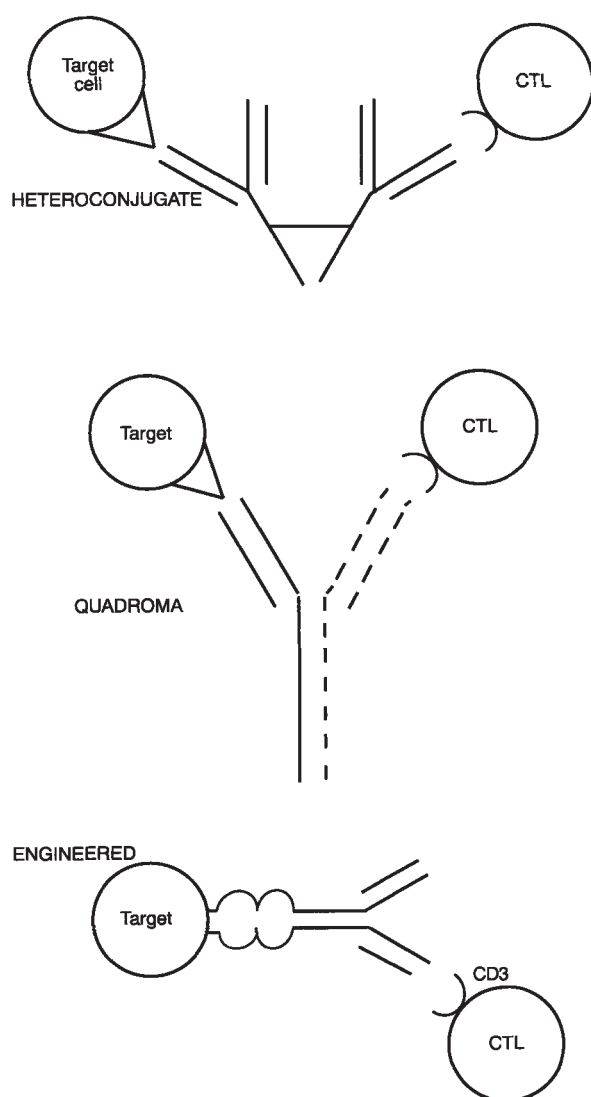


Figure 1 Structural characteristics and binding properties of various types of bispecific antibodies.

ility *in vivo* is questionable and since they are dimeric molecules they can cause antigenic modulation subsequent to cross-linking of TCR molecules associated with the surface of effector cells or of antigen molecules expressed on the surface of target cells.

Quadroma bispecific antibodies

Quadromas are bispecific antibodies synthesized by hybrid hybridomas obtained by fusion of two lymphocytes producing antibodies with different specificity. Because in hybrid hybridomas the expressed V_H and V_K genes of the parental cells are all produced there are ten molecular species of hybrid antibodies which theoretically can be formed as expected from the random association of the product of each gene. However, immunochemical studies of Ig mol-

ecules produced by quadromas showed that only six species were secreted and indicated a marginal preference for heavy-light chain homologous combination. The theoretical yield of a bispecific antibody with desired specificity produced by quadromas is 8–17% of the total Ig synthesized.

The production of quadromas is based on the principle of fusing two parental cells, each carrying a different drug resistant marker and selecting the hybrids of both type of cells in the presence of both drugs.

There have been only a few successful attempts to produce quadromas by fusing cells sensitive or resistant to various drugs such as hypoxanthine-aminopterin-thymidine (HAT)-sensitive, ouabain-resistant, neomycin (G418)-resistant, or resistant to iodoacetamide damage. Utilization of these drugs requires tedious experiments to select drug-sensitive or -resistant mutants in the population of hybridomas which will be used to generate quadromas. A new and easier method takes advantage of defective retroviruses carrying genes conferring resistance to neomycin or methotrexate.

Each partner of the fusion is infected with a retrovirus which confers a stable drug resistance at a very high frequency. The yield of quadromas using retrovirus-infected cells is generally higher than those using cells sensitive or resistant to various drugs. Utilization of hybridomas producing antibodies with different isotypes or idiotypes facilitates the purification of bispecific antibodies using anti-isotype or anti-idiotypic immunoadsorbents.

Bispecific antibodies specific for the TCR of cytotoxic T lymphocytes (CTLs) and for viral antigens budding from the surface of target cells or against tumor antigens have been shown to be very efficient in small concentrations to focus CTL activity on target cells and to cause their lysis. The Fab fragment binding to the TCR is sufficient to activate the CTL, and the other Fab fragment brings the target cell in intimate contact with the CTL thereby causing lysis of the target.

It has been clearly shown that the redirected lysis mediated by bispecific antibodies circumvents the genetic restriction requirement of T cells. The efficacy of bispecific antibodies can be increased by preactivation of CTLs with specific antigens or superantigens. Bispecific monoclonal antibodies have numerous advantages when compared with chemically constructed heteroconjugates. These advantages include greater uniformity, stability and purity, and the absence of aggregates which may be cleared from the circulation as immune complexes. They also have a longer half-life *in vivo* because of their normal structure. In addition, because of the monomeric structure of the combining site it is unlikely that it

will cause antigen modulation, a phenomenon which requires cross-linking of receptors or antigens associated with the membrane of effector or target cells.

Bispecific molecules produced by genetic engineering

The development of molecular biology techniques provided a new approach which permits the insertion, within or adjacent to the genes encoding an immunoglobulin molecule, of oligonucleotides encoding another immunoglobulin, a desired immunogenic epitope or an epitope responsible for interaction with a viral antigen. For example, it is possible to create a bispecific molecule in which the coding sequence for the V region of a CD3-specific antibody is combined with the sequence for the CD4 molecule which is able to bind HIVgp120. Such a bispecific molecule could activate a CTL and bring it into intimate contact with an HIV-infected cell.

Bispecific antibodies and heteroconjugates have a potential application in antitumor therapy and in the treatment of infectious diseases caused by bacteria or viruses in which the microbial antigen is expressed on the surface of infected cells. In the case of antitumor therapy, a bispecific antibody could be constructed using a monoclonal antibody specific for the TCR-CD3 complex (i.e. anti-CD3, anti-idiotypic or anti-allotype) and a monoclonal antibody specific for a tumor-associated antigen. Bispecific antibodies have also been used to deliver isotopes or toxins to tumors which are difficult to remove by surgery or to tag remaining tumor cells after surgery. The efficiency of bispecific antibodies as antitumor immunotherapeutic agents are currently being evaluated in clinical trials in humans.

In the case of the treatment of infectious diseases the heteroconjugates or bispecific antibodies can be constructed using monoclonal antibodies against the TCR-CD3 complex of CTL and monoclonal antibody specific for a viral antigen. Evidence has been presented in a number of viral diseases that viral-specific CTLs reduce viral replication and dissemination. Therefore, the focusing of CTLs on virally infected cells by bispecific antibodies can prevent viral replication and spreading. It has already been shown that heteroconjugates or bispecific antibodies can redirect CTL activity against cells infected with

viruses such as human immunodeficiency virus (HIV), herpes or influenza.

Thus, it appears that bispecific antibodies have the potential to become a new category of immunotherapeutic reagents.

See also: Antigen-binding site; Cytotoxic T lymphocytes; Hybridomas, B cell; Monoclonal antibodies (mAbs).

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ANTIBODIES, DETECTION OF

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The widespread use of diagnostic tests which depend on the use of antibodies, together with the increasing use of antibodies in research laboratories for the detection and quantitation of all manner of biochemicals, has resulted in the development of a vast array of different techniques which can be used to detect antibodies. The nature, abundance and purity of the antigen recognized by the antibody and the number of assays to be performed will be the main factors which determine the selection of one technique rather than another. The most commonly used techniques and their relative advantages and disadvantages are summarized below.

Immunoprecipitation techniques

Antibodies were first detected by their ability to precipitate antigen when mixed at equivalence. This classical precipitin reaction of Heidelberger and Kendall is seldom used in its original form but precipitation tests adapted in many different ways are still widely used for the detection of antibody. In the double diffusion or Ouchterlony technique, antigen and antibody are placed in adjacent wells punched out of an agarose gel. Precipitation occurs when the two meet at appropriate concentrations. The simplicity of this technique makes it ideal for the initial screening of antisera raised in animals. Under appropriate conditions the test can also be used for comparing the specificity of antisera or determining the relatedness of two antigens. The test is commonly used for determining the class or subclass of monoclonal antibodies (in this case the monoclonal antibody is behaving as the antigen).

Many other techniques involving precipitation in gels are used primarily in the detection and quantitation of antigen. Counterimmunoelectrophoresis, in which the antigen and antibody are concentrated together by the effect of an electric current, is one modification of the Ouchterlony technique used for the detection of antibodies. Precipitation techniques are, in general, rather insensitive, detecting antibodies or antigens in the $\mu\text{g ml}^{-1}$ range. They are more useful for the detection of antibodies in polyclonal antisera rather than monoclonal antibodies which precipitate poorly.

The sensitivity of the basic precipitin reaction can be increased considerably by the use of complex optical detection systems to determine the amount

of precipitation. Highly sensitive turbidometers and laser-based nephelometers are widely available. Though the equipment is expensive, these techniques are commonplace in diagnostic laboratories where high throughput can justify the initial investment.

Precipitin reactions can be accelerated by the inclusion of suitable concentrations of reagents such as ammonium sulfate or polyethylene glycol which will encourage precipitation of antibody or antibody-antigen aggregates but not free antigen. If the antigen is radiolabeled, this can be used as a sensitive and simple test for the detection of antibody – the Farr test. Radioimmunoassays have been used extensively for many years for the quantitation of antigens such as hormones and drugs which are present in very low concentrations.

Agglutination assays

If an antigen is particulate, e.g. a bacterial surface antigen or eukaryotic cell surface antigen, agglutination of the cells by the addition of antibody can provide a simple detection system. Agglutination is the standard test for blood group serology and it has other specialized applications. The technique can be extended to antibodies against soluble antigens by coupling of the antigens to particulate carriers. Initially the carrier of choice was the erythrocyte and detection systems depended on coupling of antigen on to the red cell using tannic acid. Hemagglutination assays remain the basis of several diagnostic test systems, quantitation being possible in terms of the antibody dilution of titer at which agglutination stops.

A number of synthetic carriers have been developed, including latex particles and a new generation of polymerized gelatin particles. Agglutination assays are simple to perform, requiring little or no expensive equipment. Modern versions are very sensitive and highly suitable for use outside the laboratory and in less-developed countries. They are widely used for detection of antibodies in the diagnosis of infectious disease. Tests for the human immunodeficiency virus (HIV) based on agglutination techniques compare favorably with other techniques. One drawback is that the tests can be more sensitive to the detection of polymeric immunoglobulin M (IgM) antibodies. This can be advantageous in detecting recent infections but might

be a problem in autoimmune serology. Sensitivity can be down to $1 \mu\text{g ml}^{-1}$.

Complement-dependent assays

The lytic activity of complement can be used in sensitive tests for the detection of antibodies recognizing cell surface antigens. Although now of limited application, it is still used widely in certain specialized situations, for example, the detection of antibodies recognizing histocompatibility antigens. Complement-mediated cytotoxicity is detected either by dye exclusion or by the release of ^{51}Cr from labeled cells. Complement fixation tests, once widely used, depend on the fact that antibody-antigen interaction will consume complement and the remaining complement can then be measured in a hemolytic assay. Though sensitive, these assays have declining usage.

Detection of antibodies using labeled anti-immunoglobulin reagents

The most widely used tests are those which use a second antibody, labeled in some way, to detect the binding of primary antibody to antigen. The availability of excellent, highly specific reagents from many commercial sources has made these techniques simple to apply in any laboratory. 'Second antibodies' are available which recognize immunoglobulins from humans and from most laboratory and veterinary animals. The second antibody can be labeled with radioactivity (usually ^{125}I), a fluorescent or luminescent dye, or coupled covalently to enzyme molecules. Each type of assay has its own advantages and disadvantages.

In quantitative assays, the antigen is usually coated on to plastic tubes or, more frequently, 96-well microplates to provide a solid phase on which to capture antibody. Sensitive detectors measure enzyme activity colorimetrically or detect chemiluminescence or fluorescence in this format. Flexible plates can be cut up for counting radioactivity. The enzyme-linked immunosorbent assay (ELISA) is the most widely used technique. Bound antibody is usually detected using alkaline phosphatase, horseradish peroxidase or β -galactosidase-conjugated second antibody. All of these enzymes cleave suitable substrates to yield highly colored products allowing detection of antibody in the ng ml^{-1} range.

Blotting techniques

A variation on the ELISA involves immobilization of antigen on sheets of nitrocellulose or a similar carrier. Again, filtration devices in the 96-well format

give easy sample management. These 'dot blots' provide rapid, sensitive and simple tests with wide application in research laboratories. Enzyme-linked antibody systems are again most commonly used, utilizing the same conjugates as ELISA but substrates which give highly colored insoluble products. This allows easy measurement by eye, with the option that reflectance readers can be used for quantitation in a manner analogous to conventional ELISA readers. Related blotting techniques are used increasingly in rapid diagnostic tests. Most 'dipstick' techniques use this approach. Purified or recombinant antigens can be coated easily on to plastic or cellulose matrixes as the support for these rapid tests. Several antigens can be applied as discrete spots or bands to allow differential detection of specific antibodies in the same sera; ideal for many applications in infective or autoimmune serology.

In the research laboratory, the immunoblot or western blot is one of the most widely used techniques for the determination of antibody specificity. Separation of a complex mixture of antigens, for example a cellular extract, on polyacrylamide gels is followed by transfer of the separated proteins to a rigid medium such as nitrocellulose or nylon. Antibody is then added and after washing, the blot is developed with labeled second antibody and substrate. This allows characterization of the specificity of an antiserum or monoclonal antibody at the molecular level. The major drawback is that many antigenic determinants are destroyed by the SDS and denaturants used in the electrophoretic procedure. Blotting techniques can also be used for detecting antibodies against glycolipid antigens.

Immunohistochemistry

When the antigen is cell-associated, immunohistochemistry allows the detection and characterization of antibodies. For tissue sections, immunofluorescence is favored in many laboratories because of its simplicity. It is particularly useful for the detection of antibodies, including autoantibodies, recognizing antigens in intracellular organelles or where double labeling with two different antibodies (detected using two dyes of different color) is advantageous. Fluorescein and rhodamine are the standard conjugates, but a wide range of different colored dyes are available. Laser-based confocal microscopes have greatly increased the sensitivity and specificity of these techniques.

Immunofluorescent techniques for the detection of antibodies are widely used in conjunction with the flow cytometer or fluorescence-activated cell sorter. CD numbers describe leukocyte cell surface antigens

recognized by monoclonal antibodies, defined initially by their cytofluorometric staining patterns on a large panel of different cell types. A wide range of different laser dyes are now available which are designed for use with the lasers commonly used in flow cytometers and confocal microscopes.

For tissue immunohistochemistry, enzyme-labeled antibodies are the choice in most laboratories. Peroxidase- and alkaline phosphatase-based techniques are used especially where the antigen detected will withstand the harsh fixation techniques often necessary to maintain tissue integrity. At the electron microscope level, immunogold techniques are used. Small, specifically sized gold particles are attached to antibody to allow localization of antigens. Colloidal gold conjugates can also be used successfully at the light microscope level, particularly in association with silver enhancement techniques. Immunogold can also be applied to blotting techniques giving sensitivity similar to that of enzyme-based detection systems.

Increasing sensitivity

Enzyme-based immunoassays, blotting techniques and immunohistochemistry have become the norm for the detection of antibodies and for characterization of their specificity. These systems have been developed in several ways to increase their sensitivity and ease of application. Biotin, a small molecule (244 Da) which binds with very high affinity to the proteins avidin or streptavidin, can itself be bound covalently to immunoglobulins without the loss of activity. A single biotinylated reagent can then be used in any test system with suitably labeled avidins. Since avidin can bind up to four biotin molecules, this gives the possibility of multilayered systems which offer increased sensitivity.

Alternative approaches to the detection of antibody utilize protein A, a bacterial surface protein which binds specifically to certain classes of IgG. Protein A in its natural or recombinant form or protein G, an IgG-binding protein from streptococci, are widely available. Protein G has broader specificity for subclasses of IgG and binds better to sheep and goat IgG than protein A. Second antibody systems do, however, remain the method of choice, particularly with the availability of good monoclonal antibodies specific for individual immunoglobulin classes or subclasses.

Although conventional detection systems are usually for sufficient sensitivity for the detection of antibodies, the detection of trace antigens such as growth factors or their receptors can require the necessity for an amplified detection system which will quantitate

the binding of a primary antibody. Increased sensitivity is possible by the use of sandwich techniques involving several layers of antibody, enzyme-antibody complexes, biotin-avidin complexes or by newly developed specific amplification systems. One example of an enhancement system uses alkaline phosphatase conjugated to the detecting antibody to generate NAD from NADP. The NAD is then used in a pair of cycling redox reactions to reduce a colorless precursor to a colored formazan dye. The reaction product can be detected at 492 nm by conventional readers. The amplification step results in an increase in sensitivity of over 100-fold and is the basis of several rapid dipstick-type tests.

Chemiluminescence-based techniques have replaced colorimetric ELISAs and immunoblotting techniques in many laboratories because of their much greater sensitivity. A number of substrates which yield chemiluminescent products upon cleavage by peroxidase (luminol) or alkaline phosphatase (dioxetane phosphate esters) are available, usually in the form of kits containing all necessary reagents. For ELISA-based assays 96-well microplate luminometers are used. In chemiluminescence-based blotting techniques the signal can be detected using photographic film or specially designed plate detectors.

Time-resolved fluorescence techniques such as the DELFIA (Pharmacia) system, have been developed to replace the radioimmunoassay for the measurement of hormones etc. in large diagnostic laboratories. These assays are equally applicable to detection of antibodies, for example, in the diagnosis of infectious diseases. DELFIA utilizes Europium³⁺ bound to proteins through a covalently-linked EDTA derivative. Excess of the chelators naphthoylethyltrifluoroacetone and trioctylphosphine are then used to form a fluorescent complex in solution at low pH.

Future advances

Techniques developed for the detection of antibodies during the last few decades have sufficient sensitivity, diversity of application and robustness that they serve the vast majority of requirements. The widespread availability of highly specific, conjugated second antibodies and novel detection systems means that major gains in sensitivity are not necessary for the detection of most antibodies. It is advances in speed and ease of application which will determine the tests of the future. To this end, it is important to appreciate the potential of the instantaneous electrochemical tests based on biosensors and microelectronics which are already being developed and could

make the detection of antibodies as simple and quick as the measurement of pH.

See also: Agglutination; Autoantibodies, tests for; Complement fixation test; Enzyme-linked immunosorbent assay (ELISA); Flow cytometry; Fluorochrome labeling; Immunoassays; Western blotting.

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ANTIBODIES, SECRETION

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As established by the classical experiments of Palade and coworkers, proteins destined to be secreted (as well as cell surface proteins) are synthesized in the endoplasmic reticulum (ER), and then transported through the Golgi complex to the extracellular space. Distinct post-translational modifications (assembly, glycosylation, etc.) take place sequentially along this pathway, allowing the intracellular location of a protein to be determined. The stepwise nature of the secretory pathway is confirmed by the existence of drugs which block intracellular transport at given stages. Similarly, several yeast temperature-sensitive mutants have been characterized in which secretion is blocked at different levels. Since mutations or drugs block the export of most secretory proteins, there appears to be only one main secretory pathway, utilized also by proteins destined to be expressed on the plasma membrane. An alternative pathway has been identified, which allows the selective secretion of certain cytosolic proteins lacking a conventional 'signal sequence' (see below).

Antibody secretion will be discussed in the context of the general problems of protein secretion. Among the many exciting questions in this field, the ones related to selectivity are maybe the most intriguing. How do cells decide what to secrete, when, and for how long?

Antibodies as a model system for protein secretion

Like all proteins destined to be synthesized in the ER,

immunoglobulins are characterized by an N-terminal stretch of hydrophobic amino acids, the signal (leader) sequence, first inferred by Milstein and coworkers in 1972 in studies on immunoglobulin (Ig) light chain secretion. The signal sequence interacts with a multimeric ribonucleoprotein, the signal recognition particle (SRP). This interaction arrests translation until the complex formed by ribosome, nascent protein and SRP binds to the SRP receptor or docking protein on the ER membrane. Translocation of the nascent chain across the ER membrane occurs cotranslationally and the signal sequence is then cleaved by specific proteases located on the luminal face of the ER membrane. The first steps of N-linked glycosylation also occur cotranslationally (Figure 1).

The signal sequence is a paradigmatic example of 'positive' sorting: only the proteins that express it enter the secretory pathway. However, not all proteins synthesized within the ER will reach the extracellular space: some of them accumulate in the ER, some in the Golgi. Specific C-terminal sequences have been shown to cause *retention* within the ER of the proteins possessing them. This has led to the proposal of the bulk flow model, according to which all proteins in the secretory compartment will be secreted 'by default' unless they are marked with specific 'retention signals' which block them within a given organelle.

Protein secretion may be constitutive or regulated. Antibodies are secreted by plasma cells through the constitutive pathway. In the regulated pathway, pro-

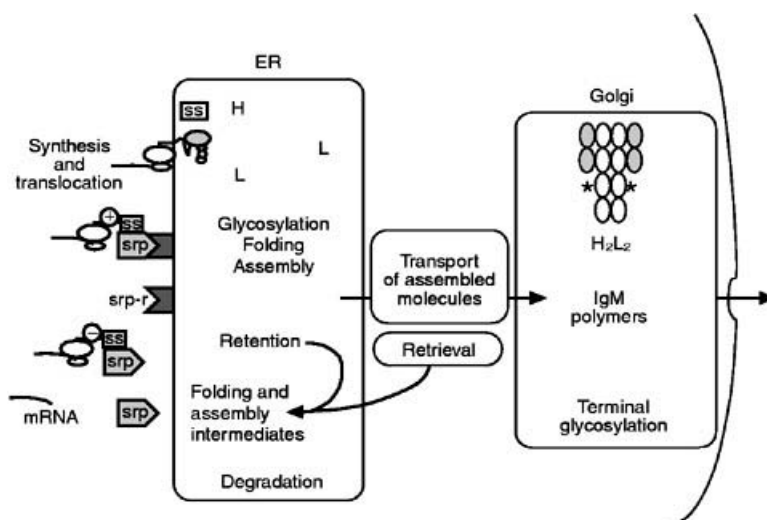


Figure 1 Assembly and secretion of immunoglobulins. The N-terminal signal sequence (ss) is recognized by the signal recognition particle (srp) on cytosolic nascent proteins. This interaction causes an arrest in translation (–). The complex binds to the srp receptor (or docking protein, srp-r), targeting the ribosome to the ER membrane, and allowing elongation of the nascent polypeptide (+). Translocation of the ER membrane and the first steps of N-linked glycosylation (addition of the $\text{GlcNac}_2\text{Man}_9\text{Glu}_3$ precursor) occur cotranslationally. Folding and assembly begin when the nascent H and L chains are still on the polysome, and are rapidly completed under the assistance of ER chaperones and folding enzymes (BiP, calnexin, PDI, ERp72, etc.). Only fully assembled molecules are transported to the Golgi, where they undergo terminal processing of their sugar moieties (*) before being released into the extracellular space. Folding and assembly intermediates are retained in the ER, or retrieved in this organelle from the early compartments of the cis Golgi network. A proteolytic system, independent from that of lysosomes, maintains homeostasis in the ER. All transport steps occur within membrane-sealed vesicles that are thought to bud from one compartment and fuse with the next along the exocytic pathway.

teins are concentrated within intracellular stores (secretory granules) and bursts of secretion can be triggered at rates much higher than the synthetic one. In constitutive secretion there is normally little intracellular accumulation, and secretion depends largely on the rate of synthesis. However, IgM antibodies are an exception to this scheme as their secretion is developmentally regulated (see below). Constitutive secretion is common to virtually all cells, although at different levels. Like most cells specialized in secretion, plasma cells have abundant ER and a developed Golgi apparatus. By contrast, B cells have a very scarce ER.

Secreted proteins are remarkably uniform, and *only* fully assembled molecules are released extracellularly. This selectivity implies the existence of quality control steps in protein synthesis, folding and assembly, which appear to be coupled to intracellular transport. In addition, to maintain cellular homeostasis, proteins which fail these editing steps, and are hence not transported, must be degraded. Antibodies are an excellent model for understanding how cells manage to obtain high secretion efficiency and stringent quality control of the released products. This will be of interest in the general context of protein secretion, as well as for some more specific problems, namely how the onset, duration and intensity of the antibody responses are controlled.

Antibody secretion during B cell differentiation

Before it was discovered that membrane and secreted Ig contained distinct forms of heavy chains, differing at their C-termini, the problem of the differential expression of Ig during B cell differentiation was a dilemma for both immunologists and cell biologists. It was known that small, resting B lymphocytes expressed IgM on the cell surface (10^4 – 10^5 molecules per cell), but did not secrete IgM molecules. The same cells can be induced by mitogens or antigen to differentiate into plaque-forming cells (PFC), which lack surface Ig and secrete large amounts of antibodies (over 10^3 copies s^{-1} per cell). Since it was thought that constitutive secretion could be regulated only by changing the rate of synthesis, it was not easy to explain these results. In 1978–1981 it became clear that μ chains, as well as all other heavy chain isotypes, may be produced in a membrane (μm) or a secretory (μs) form by alternate RNA processing. The $\mu\text{m} : \mu\text{s}$ ratio varies during B cell differentiation, μm predominating in the early (pre-B, B cells), and μs in the late stages (activated B cells and plasma cells). However, in many B lymphomas, abundant μs mRNA is present, and the rate of synthesis and assembly to light (L) chain is quite similar for μm and μs , indicating that the block in IgM secretion is

due to post-translational mechanism(s). The molecular mechanisms preventing IgM secretion in B cells are related to the general problem as to how cells make sure that only fully assembled molecules are secreted or expressed on the cell surface, and will be discussed below.

Only fully assembled immunoglobulins are secreted

Antibodies are multimeric glycoproteins, made of two L and two heavy (H) chains. To maintain the clonal organization of the immune system, allelic exclusion ensures that only one H and one L chain specificity are produced by a B lymphocyte. However, the specificity of a V_H region might differ if H chains were not assembled with L chains. The mechanisms ensuring that only the fully assembled forms of antibody molecules are secreted by plasma cells (or expressed on the surface of B lymphocytes) have been understood recently, and once again information gathered on antibodies has proven of general relevance. It has been shown that, in the absence of L chains, H chains interact with BiP (heavy chain binding protein, grp 78), an abundant resident protein of the ER. BiP belongs to the protein family of chaperonins, which, together with oxidoreductases (such as PDI or ERp72) and other enzymes, assist the folding and the assembly of newly synthesized proteins in the ER. Since BiP is itself retained in the ER lumen via its C-terminal sequence Lys-Asp-Glu-Leu (KDEL), unassembled H chains are not able to leave the ER. The interaction with BiP involves the first constant domain of the H chain (C_{H1}), which normally interacts with the constant domain of L chains. Thus, assembly with L chains masks the BiP binding site, allowing assembled molecules to exit the ER and reach the Golgi apparatus, where terminal glycosylation takes place (Figure 1). Deletion of the C_{H1} domain by recombinant DNA techniques allows secretion of free γ heavy chains, a finding that explains why, in the rare cases of heavy chain disease, secreted free heavy chains always lack the C_{H1} .

Developmental control of IgM secretion

In the case of IgM and IgA, the basic H_2L_2 structure is further assembled into polymers which contain J chain, a small cysteine-rich glycoprotein synthesized by plasma cells, but not by resting B lymphocytes. Only polymeric IgM is secreted, despite the fact that the major intracellular form is μ_2L_2 . Thus, IgM (and IgA) undergo a further level of control, which blocks secretion of nonpolymeric molecules. This editing step depends on disulfide interchange reactions,

which involve the cysteine residue found at the penultimate position of both μ_s and α_s chains. This residue is responsible for the intersubunit disulfide bonds which stabilize the polymeric structure. In assembly intermediates the unbound SH group may interact with ER resident proteins, thereby blocking their transport to the Golgi. Thus, IgM is subject to a dual editing process which allows plasma cells to secrete only polymers. First, μ -L association is controlled by interactions between BiP and sequences in the $C_{\mu 1}$; then, disulfide interchange reactions control polymerization.

In B lymphocytes, polymerization is very inefficient, a finding that explains why IgM is not secreted. The requirement for polymerization might also explain why, also in myelomas, secretion of IgM is rather slow and inefficient compared with IgG. If the C-terminal cysteine of the μ_s chain is replaced by serine, the rate and efficiency of IgM secretion is increased, and intracellular degradation reduced. Furthermore, these mutant monomeric IgMs are secreted also by B cells, indicating that the presence of a developed ER is not *per se* necessary for secretion of immunoglobulin molecules.

As polymeric IgM contains J chains, it has been proposed that the failure of B cells to secrete IgM correlates with the absence of J chain synthesis. However, since B cell lines have been isolated which synthesize J chain and yet do not secrete IgM, and nonlymphoid transfectants secrete polymeric IgM lacking J chains, the latter appear to be neither essential nor sufficient for IgM secretion. Knock-out mice that lack J chains have high levels of IgA (mainly monomeric) and of hexameric IgM in the serum. Hence, J chain seems to play a key role in 1) determining the size of polymeric IgM (pentamers predominate in the presence of J chain), and 2) facilitating the transcytosis of polymeric Igs across epithelia. The problem of why B cells do not secrete IgM may thus be reduced to the single question as to why in B cells polymerization is so inefficient. Differentiation into IgM secreting plasma cells is accompanied by major changes in the ER and by a much higher rate of synthesis of μ and L chains. These events might include modification(s) of the redox potential along the secretory pathway which would favor polymerization.

Glycosylation and secretion

Despite the abundant literature on the issue, the precise function of the carbohydrate moieties of glycoproteins remains to be established. Selective inhibitors (e.g. tunicamycin) or site-directed mutagenesis of the relevant asparagines have been employed to

prevent *N*-linked glycosylation of many glycoproteins, including IgS. In those systems where the presence of tunicamycin blocks secretion, folding and assembly are slower, and intracellular degradation is increased. Glycosylation might increase solubility and stability of antibodies. Since many deglycosylated proteins are exported, glycosylation does not appear essential for secretion. However, the recent findings of several compartmentalized molecules with lectin activity within the secretory pathway have awakened the dormant hypothesis of a role of glycans in facilitating exocytosis.

Intracellular degradation of newly synthesized proteins

To maintain the steady state, proteins which cannot be secreted must be degraded. On the other hand, unassembled or misfolded proteins are retained in the ER via interactions with BiP or disulfide interchange reactions, and may thus not reach the lysosome. This apparent paradox was solved when it became clear that a proteolytic system, independent from the lysosomal one, exists in a pre-Golgi compartment. Heavy chains are degraded more rapidly when not assembled to L chains. Assembly might decrease the susceptibility to proteases, hide signals which target to degradation, or both.

The biochemistry of ER degradation is still largely unknown. Evidence has been recently obtained that ubiquitin and proteasomes are involved in the degradation of CFTR, a transmembrane protein retained in the ER in cystic fibrosis patients. As to the specificity of the process, in many cases, including T cell receptors and Igs, the signals that lead to degradation have been shown to coincide with those that mediate the assembly as well as the retention of unassembled subunits. However, there must be a lag while nascent proteins, that are bound to express many degradation targeting signals, are protected from proteolysis. A distinct subcompartment, specialized in proteolytic activity, may exist in the ER. Alternatively, nascent proteins maybe shielded by chaperones.

Pathologic conditions related to Ig secretion

Defects in immunoglobulin secretion might be at the basis of certain pathologic conditions such as common variable immunodeficiency (acquired hypogammaglobulinemia). In this disease, the number of membrane Ig-expressing B cells is normal but very little antibody is present in the serum: defects in γ chain glycosylation have been reported, but their pathogenic relevance remains to be established.

Immunodeficiency with hyper IgM is characterized by high levels of serum IgM and virtual absence of IgG and IgA. In these patients, mutations in the CD40 ligand perturb T cell-B cell interactions, resulting in impaired isotype switching and affinity maturation. Although it is worth recalling that considerable amounts of monomeric IgM are present in the serum of these patients, the significance of this observation is at present unclear.

Many observations on myelomas and heavy chain disease related to defects in or unbalanced Ig secretion have already been mentioned. Secretion of free L chains (Bence Jones proteins), does not appear to be a characteristic of neoplastic plasma cells, but probably reflects an important feature of the Ig assembly line, where the limiting factor is the heavy chain. While cells producing only L are frequent, myeloma mutants producing only H are exceedingly rare, an observation which led to the proposal of heavy chain toxicity. Assembly of complete Ig molecules requires an equal number of L and H chains. Although it might be thought more economical for the cell to synthesize equimolar amounts of the two chains, L chains are produced in excess to optimize HL assembly. The excess L chains can be secreted mainly as L₂ homodimers.

Mott cells are plasma cells defective in Ig secretion found in multiple myeloma and certain chronic inflammatory and autoimmune conditions. Mott cells are characterized by cytoplasmic inclusions normally containing immunoglobulin molecules, called Russell bodies, and representing dilated cisternae of the ER. Mott hybridomas have been obtained by fusing splenocytes from NZB mice, a strain highly susceptible to autoimmune diseases. Since these hybridomas express several membrane antigens, a general defect in the secretory pathway may be excluded. That the Mott cell phenotype is due to mutations in the Ig chains is indicated by the observation that Russell bodies are formed upon transfection of certain mutated heavy chains into myeloma cells. Not all nonsecretable chains induced the Mott phenotype. Thus, Russell bodies probably reflect the presence of nontransportable molecules resistant to pre-Golgi degradation.

Ig secretion by nonlymphoid cells

Immunoglobulins have been expressed in several heterologous systems, such as nonlymphoid transfectants, *Xenopus* oocytes, or transgenic plants. In most cases, immunoglobulin molecules were properly assembled and secreted. Mutants were handled similarly by lymphoid and nonlymphoid cells, and Russell bodies could be detected in glioma transfectants.

Thus, unlike the rearrangement of Ig genes, the complex and developmentally regulated processes of Ig assembly and secretion do not appear to require tissue-specific factors.

Secretory immunoglobulins

Antibodies in external fluids (milk, bile, saliva, lacrimal, nasal, intestinal, cervical fluids) are often called secretory Ig, as opposed to serum Ig, a definition that may generate confusion now that the same term is used to discriminate the soluble form from the membrane-bound form of antibody molecules. Polymeric immunoglobulins (IgM and IgA) in external secretions contain, in addition to J chain, a polypeptide of approximately 25 kDa, called the secretory component. The latter is not produced by lymphoid cells, but represents a portion of the poly-Ig receptor which is proteolytically cleaved during the process of transcytosis across epithelial cells.

See also: Allelic exclusion; Antibodies, synthesis; Bence Jones proteins; Glycosylation of immune system molecules; Heavy chain diseases; IgA; IgD; IgE; IgG; IgM; Immunoglobulin, cell surface; Joining J chain; Lymphoma; Plaque-forming cell (PFC) assays; Secretory component (the polymeric Ig receptor).

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ANTIBODIES, SPECIFICITY

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Quantitatively, the specificities of antibodies are determined by their relative binding affinities, the intrinsic property of each antibody combining site, expressed as equilibrium dissociation (K_d) or association constants (K_a) for their interactions with different antigens. The K_d values may vary significantly among different antibody–antigen interactions, ranging from 10^{-3} M to 10^{-11} M or higher, but never reach the value of the binding of biotin to avidin (K_d 10^{-15} M).

Different classes of antibodies may differ in their ‘avidities’, i.e. the number of combining sites on each antibody. As illustrated in Figure 1, an antibody molecule has a four-chain structure built of two identical heavy (H) and two identical light (L) chains. Pairing of each H and L chain forms the basic unit of antibody combining sites. In monomeric antibodies such as immunoglobulin G (IgG), IgD, IgE and the majority of the serum IgA, two pairs of H and L chains, linked by disulfide bonds in the hinge region

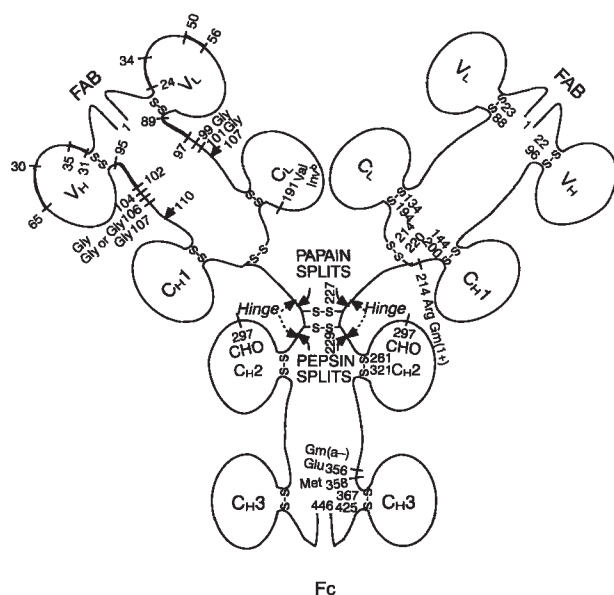


Figure 1 Schematic view of four-chain structure of human IgG1 molecule. Numbers on right side denote actual residues of protein Eu (Edelman *et al*, 1969). Numbers of Fab fragment on the left side are aligned for maximum homology; light and heavy chains are numbered according to Wu and Kabat (1970) and Kabat and Wu (1971). Heavy chains of Eu have residue 52A and 82A,B,C and lack residues termed 100A,B,C,D,E,F,G,H,I,J,K and 35A,B. Thus, residue 100 (end of variable region) is 114 in actual sequence. Hypervariable or complementarity-determining regions (CDRs) are shown by heavier lines. V_L and V_H denote light- and heavy-chain variable regions; C_{H1} , C_{H2} and C_{H3} are domains of constant region of heavy chain; C_L is constant region of light chain. The hinge region in which two heavy chains are linked by disulfide bonds is indicated approximately. Attachment of carbohydrate is at residue 297. Arrows at residues 107 and 110 denote transition from variable to constant region. Sites of action of papain and pepsin and locations of a number of genetic factors are given. (Reproduced from Kabat EA *et al* (1987).)

of H chains, form two identical antibody combining sites; secretory IgA may be dimers or trimers with four or six binding sites; IgM isotype is pentameric, bearing ten identical sites. For a given Ig isotype, the amino acid sequences in the C-terminal domains are basically identical, termed the constant region (C); those of the N-terminal domains are highly variable, called the variable region (V). Avidities of antibodies are associated with H chain C region isotypes; affinities or binding specificities are determined by V region structures.

Many kinds of substances, when introduced into the body, may induce the formation of antibodies. These include infectious agents such as bacteria and viruses, allo- or xenoantigens of blood cells or other cell types and many substances in the environment or derived from foods, e.g. pollens, shellfish and others to which some people may be allergic. Natural antigens may frequently be complex mixtures of

many antigenic substances. Even highly purified substances such as proteins, polysaccharides, glycolipids or nucleic acids may contain multiple antigenic determinants (epitopes). B lymphocytes comprise an enormous population of distinct antibody-producing cells. Each cell and its descendants synthesize one antibody in response to a single epitope but different cells may recognize different aspects of the same epitope and vary in their binding affinities. Thus, the antibody response of an individual to a bacterium or virus, etc. may involve multiple B cell clones recognizing different antigenic determinants and is said to be polyclonal.

Early studies on specificity carried out on polyclonal populations of antibody yielded much information but the most definitive studies are those using monoclonal antibodies from humans or mice with multiple myeloma or macroglobulinemia and with monoclonal antibodies produced by the hybridoma technique of Köhler and Milstein. Hybridoma antibodies may be produced to any epitope; in practice this may be more difficult, particularly for producing human monoclonals. Cell-cell hybridization for making hybridomas is inefficient and often limited to certain stages of B cell differentiation. Alternative strategies for producing monoclonal antibodies, their Fv, Fab fragments and the engineered single-chain antibodies (scFv), have recently been developed by combining PCR amplification and gene expression. Using advanced flow cytometry, a single B cell, with an Ig receptor of defined specificity as well as other surface markers, may be isolated and its original pair of V_H and V_L chains identified, allowing further exploration of the repertoires of monoclonal antibodies.

Binding specificities of antibodies may be investigated using rationally designed antigens in which one or more chemically defined small substances may serve as antigenic determinants. Thus, diazotized arsanilic acid, dinitrofluorobenzene, etc. may be coupled to a macromolecule to produce an artificial antigen. The antibodies induced will generally react best with, or be most specific for, the group introduced. Thus antibodies to the arsanilic acid-containing macromolecule generally bind arsanilic acid azotyrosine, and those to the dinitrophenyl macromolecule often bind only dinitrophenyllysine. Binding specificities may be further illustrated by competitive inhibition of the reaction by corresponding low molecular weight substances. Competition with structural derivatives differing from the active molecule by defined fine modifications at given positions may allow identification of the critical structures involved in specific recognition. Such low molecular

weight substances are termed haptens; the macromolecules are called carriers.

A potential difficulty in these studies is that the introduced groups are frequently insufficient in size to fill the antibody combining site so that the new epitopes may include a portion of the carrier molecule to which it is linked. Studies with small haptenic determinants, such as phosphorylcholine (PC), 2-phenyloxazolone (phOx), 4-hydroxy-3-nitrophenyl (NP) or dinitrophenyl (DNP) and *p*-azophenylarsonate (Ars) coupled to proteins, may thus not be clearly definable as to what constitutes the antigenic determinant. For example, one may not be able to determine whether sequence differences in the V regions of antibodies induced by these conjugates reflect specificity for different antigenic determinants or are a consequence of specificity differences to a single epitope.

Better understanding of antibody diversity and specificity requires a single site-filling antigenic determinant of well-defined structure. Native dextran N279 is a near-linear macromolecule of glucose linked by 90% $\alpha(1, 6)$ and 5% $\alpha(1, 3)$ glucopyranosidic bonds and has 5% $\alpha(1, 6)$ or other linkages for the glucoses at terminal nonreducing ends. The relative structural simplicity of the carbohydrate antigen, $\alpha(1, 6)$ dextran, provides a model system for such studies. The availability of oligosaccharides of $\alpha(1, 6)$ -linked glucose, ranging from isomaltose (IM2) to [Glc($\alpha(1, 6)$)]₉ (IM9), allow us to 'measure' the sizes of antibody combining sites by competitive inhibition of the dextran-antidextran interaction. The combining site is considered to be complementary in size to the smallest oligosaccharide giving maximum competition. Such an oligosaccharide is defined as an epitope or antigenic determinant. It was estimated by such immunochemical mapping that the antibody combining sites to dextran may vary in size from a lower limit of 1–2 glucoses to an upper limit of 6–7.

To accommodate epitopes with distinct structural properties, different 'shapes' or 'types' of antibody combining sites may be required. For the polysaccharide antigen $\alpha(1, 6)$ dextran, either the nonreducing end sugar or the internal chain structure may function as antigenic determinants. They may be specifically recognized by either cavity- or groove-type combining sites. This was first established by immunochemical mapping of the combining sites of two monoclonal myeloma proteins, W3129 and QUPC52, both specific for $\alpha(1, 6)$ dextran. W3129 had a site saturated by isomaltopentaose, whereas QUPC52 accommodated isomaltohexaose. With W3129, the terminal nonreducing glucose contributed 50–60% of the total binding energy but with QUPC52 less than 5%. The two antibodies also dif-

fered in their ability to precipitate a synthetic linear dextran with about 200 glucoses. QUPC52 but not W3129 forms precipitins in saline. These observations indicate that the combining sites of QUPC52 might be 'grooves' into which internal chain epitopes of $\alpha(1-6)$ -linked glucose could fit; in contrast, the nonreducing ends of dextran may be held by the cavity-type site of W3129. Monoclonal antibodies (mAbs) for 3-fucosyllactosamine, i.e. Le^x, may also accommodate either the nonreducing ends (PM81), or may bind to the side of the sugar chain (PMN6). Crystal structures for these mAbs are currently not available but a few antibodies for other carbohydrate antigens have been crystallized. A $\beta(1,6)$ galactan-specific myeloma protein J539 displays a shallow surface pocket accommodating the polysaccharide antigen; a monoclonal antibody, Se 155-4, binds a trisaccharide of *Salmonella* with the terminal abequose totally buried in a deep pocket of the combining site. Antigen-binding sites, which may be classified as either groove- or cavity-type, are also seen in antibodies specific for other antigens. For example, BV04-01 Fab binds ssDNA with a large, irregular groove and Fab McPC603 has a cavity to accept the hapten phosphorylcholine.

Antibodies may interact with protein antigens with a relatively flat surface. Proteins differ from carbohydrate antigens in their behavior in aqueous solution. Proteins tend to fold to bring their hydrophobic side-chains together, forming an oily core with the polar side-chains exposed. Such folding may allow discontinuous amino acids clustered on the solvent-accessible surface to form discontinuous epitopes. Interior residues are usually not accessible to antibody interactions. A protein epitope may thus be formed either by several contiguous amino acid residues or by discontinuous residues. Of three lysozyme-antilysozyme complexes whose X-ray structures have been determined at high resolution, two antibody combining sites, D1.3 and HyHel-5, each interact with amino acids from two discontinuous stretches of the lysozyme polypeptide chain; the third, HyHel-10, is complementary to the exposed surface of a helix including some surrounding structures. With influenza neuraminidase, four segments of its polypeptide chain were found to interact with the antibody combining site in a resolved crystal structure of their complex. It is thus said that antibodies recognize 'conformation' rather than 'sequence' of a protein epitope.

A discontinuous conformational epitope may be mimicked by molecules having different primary structures. Indeed, antibodies which mimic the structures of other molecules exist. They may be specifically induced by idiotypic-anti-idiotypic interactions.

Antibodies to various antigens may have distinct epitopes in their V regions termed idiotopes, which may be antigenic upon injection. Such induced antibodies are termed anti-idiotypic antibodies. Some anti-idiotypic antibodies may mimic the external antigen that elicited the first antibody reacting with the anti-idiotypic. A complex of antilysozyme D1.3 and its anti-idiotypic E5.2, an internal image type, has been crystallized and its three-dimensional structure clarified. By comparison with the crystal structure of D1.3-lysozyme complex, it was shown that D1.3 contacts lysozyme and the internal image antibody E5.2 through essentially the same combining site residues. In addition, E5.2 interacts with D1.3, making contacts similar to those between lysozyme and D1.3. Functional mimicry of a small biologically active compound, taxol, by an anti-idiotypic antibody 82H has been recently reported. Like taxol, this anti-idiotypic antibody can promote and stabilize microtubule assembly. We must still learn how the three-dimensional structure of 82H can mimic a ligand molecule that bears no structural similarity to immunoglobulins.

There is also evidence that variable regions of antibodies with radically different primary structures may accommodate identical epitopes. A mouse mAb specific for blood group substance A and a human antibody to the same antigenic determinant were found to have very different amino acid sequences in their V_H and V_L domains. Their fine specificities to substance A are indistinguishable by current immunochemical analysis. More examples are seen in anti- $\alpha(1, 6)$ dextrans. mAbs to $\alpha(1, 6)$ dextran may be either groove-type or cavity-type, and sites defined as the same could be very similar immunochemically. Their Ig-V region sequences were determined, showing at least 18 distinct combinations of V region genes and segments of different primary structures, 15 combinations for the groove-type combining sites and three for the cavity-type sites. A definitive answer to the paradox of 'diverse sequence-similar site' will come from X-ray crystallographic analysis of these antibodies and their ligand complexes as well as from a dynamic analysis of their solution conformers applying nuclear magnetic resonance (NMR) and other advanced methods.

To understand better the relationships of structural diversity of antibody combining sites and their complementarity and specificities, the critical structures defining the prototypes or shapes of antibody combining sites and their fine specificities should be identified. Analysis of V region sequences led to recognition of hypervariable or complementarity-determining regions (CDRs) in V domains of antibodies. In the early 1970s, Wu and Kabat examined

about 70 human V_K and V_L and mouse V_K sequences and introduced an equation, termed a variability plot for analysis:

$$\text{Variability} = \frac{\text{Frequency of the most common amino acid at a given position}}{\text{Number of different amino acids at that position}}$$

When variability was plotted against amino acid residue number for the 107 residues in the V region, three peaks of high variability were seen at positions 24–34, 50–56 and 89–97, bracketed by invariant or almost invariant residues with length variations at residues 27A–27F and 95A–95F (Figure 2). As V_H

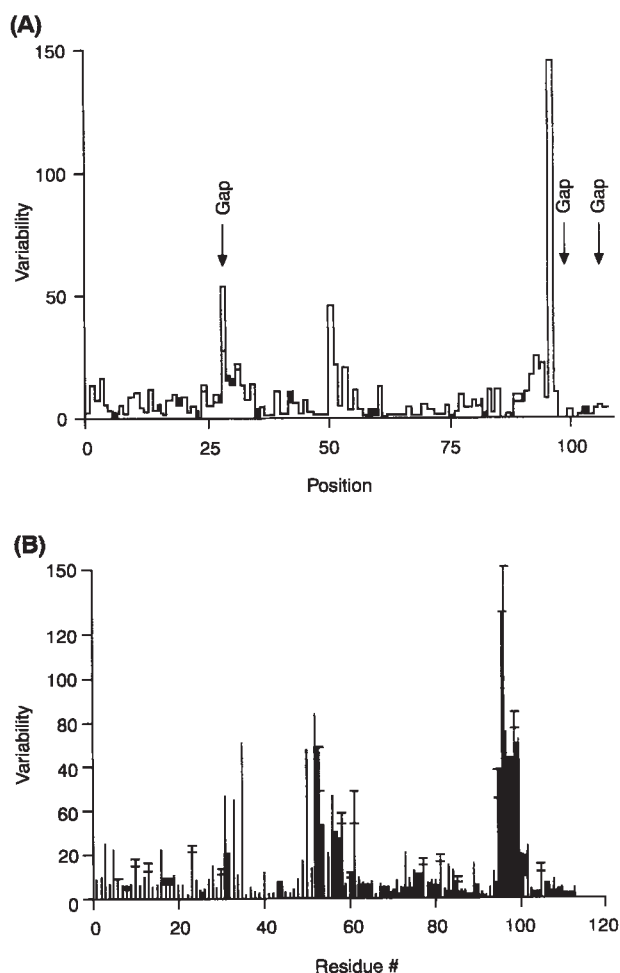


Figure 2 (A) Variability at different positions for the variable region of light chains. GAP indicates position at which differences in length have been found. (Reproduced from Wu TT and Kabat EA (1970) by copyright permission of The Rockefeller University press.) (B) Variability at different positions for the variable region of heavy chains. The plot was made by the PROPHET computer system. (Reproduced from Kabat EA, Wu TT and Bilofsky H (1979) Government Printing Office Publication NIH 80-2008. Washington DC.)

sequences of various species become available, three hypervariable regions were also found at positions 31–35, 50–65 and 95–102; with length variations listed as 35A and 35B, 52A to 52C and 100A to 100K (Figure 2B). Considerable three-dimensional information has now become available by X-ray crystallography. All high-resolution X-ray structures verified the prediction and showed that the hypervariable regions, as defined, formed the antibody combining site. These are termed CDRs and the rest of the V_H and V_L domains form a framework (FR). Comparison of the three-dimensional structures of variable domains of different antibodies reveals that the nonhypervariable or framework regions of these domains are essentially superimposable. Structural variations related to antibody specificity are mainly confined to the hypervariable segments. Amino acid residues of antibody in actual contact with antigenic determinants are found predominantly in the CDR regions. The antibody combining site can thus be viewed as being formed by a relatively small number of segments of variable structure grafted on to a scaffolding of essentially invariant architecture. In three dimensions, these variable segments or CDRs are seen as a loop structure situated at the N-terminal tip of an antibody. Their combination forms a continuous solvent accessible surface, the CDR surface, approximately $2800 \text{ \AA}^2$ in area.

In the crystal structure of antibody–ligand complexes, a portion of the CDR surface may be buried by the ligand; likewise, those of the ligand covered by the antibody. Such a buried area is termed a contacting surface, representing the actual sizes and shapes of antibody combining sites. Different types of interaction, such as reacting to protein, to polysaccharide or to small haptens, may differ significantly in the area of their contacting surface as well as the nature of their three-dimensional complementarity. In Fab-Se 155-4 complexed with a branched trisaccharide α -D-Galp(1–2)[α -D-Abep(1–3)]- α -D-Manp of *Salmonella*. The terminal abequose fills the hydrophobic pocket of the antibody, with contacting surfaces of $255 \text{ \AA}^2$ for the buried sugar epitope and $304 \text{ \AA}^2$ for the antibody, corresponding to the lower limit of site sizes estimated by immunochemical mapping of antidextran. For a carbohydrate–lectin complex, the interface of ligand Le^b and its receptor OMe.GS-IV shows complementarity of polyamphiphilic properties of their contacting surface, with buried areas of $237 \text{ \AA}^2$ and $265 \text{ \AA}^2$, respectively. The changes of contacting area of these carbohydrate receptors is similar to that for binding of haptens by antibodies but much smaller than the interfaces of proteins and their antibody combining sites. McPC603 contacts phosphorylcholine by $151 \text{ \AA}^2$

and $138 \text{ \AA}^2$; Fab AN02 and DNP-spin-label by $363 \text{ \AA}^2$ and $230 \text{ \AA}^2$; and Fab 4-4-20 with fluorescein by $338 \text{ \AA}^2$; and $247 \text{ \AA}^2$. Protein antigens may interact with antibody combining sites with a relatively larger surface. In Fab–lysozyme complexes, lysozyme was bound by HyHEL-5 with buried surface areas of $768 \text{ \AA}^2$ and $723 \text{ \AA}^2$, the HyHEL-10 complex of $699 \text{ \AA}^2$ and $753 \text{ \AA}^2$, and the D1.3 complex by $512 \text{ \AA}^2$ and $524 \text{ \AA}^2$. Their binding constants (K_d) are $5 \times 10^{-11} \text{ M}$, $2 \times 10^{-10} \text{ M}$ and 10^{-10} M , respectively. Binding of carbohydrates by antibodies or by lectins is specific but binding constants are often several logs lower than antibody–protein interactions, generally 10^{-3} M to 10^{-6} M . Possible correlations between their limited contacting areas and the low binding affinities may be evaluated when more crystal structures become available.

The topographic properties of the contacting surface are mainly determined by the loop configuration of individual CDRs and their relative disposition. These, in turn, are determined by the number and nature of amino acid residues in CDRs. There are residue positions in CDR which are more conserved than others and were postulated to be structural or conformational elements. On the basis of comparative studies of known crystal structure of antibodies and sequences, Chothia and colleagues found that the loop conformation of CDRs is determined by a few conserved residues in a CDR segment and suggested that there is a small repertoire of main chain conformations for at least five of the six CDRs of antibody, termed canonical structures. The length variation of CDR segments is also important for their loop conformations. Among six CDRs, only the V_L -CDR2 is conserved in length, with seven amino acid residues (Figure 2) in all productive V_L chains so far identified. A unique canonical structure fits 95% of 69 human V_L and of 183 murine V_L analyzed. The V_H -CDR3s vary extensively not only in residue composition but also in the number of their amino acid residues. No canonical structures can be assigned to this region. For the rest of CDRs, distinct canonical structures can always be identified among segments of a given CDR when they differ in length by as little as a single amino acid residue.

To form a given prototype of antibody combining sites, a certain combination of CDR loops may be required. For example, a long V_H -CDR3 and a long V_L -CDR1 are common to the cavity-type combining sites of different specificities, including all the cavity-type anti- $\alpha(1, 6)$ dextran so far sequenced, anti-phosphorylcholine mAb McPC603 and anti-3-fucosyllactosamine PM81. Modeling of these sites showed that this CDR loop combination is required to form the cavity wall of the combining sites. With

the groove-type sites, a short V_L -CDR1 lacking amino acid residues 27 and 28 is predominantly seen in anti-dextran; a few exceptions have either a long V_L -CDR3 or a long V_H -CDR, but not both. Lara-Ochoa and coworkers analyzed the combinations of the canonical CDR structures of murine Ig sequences provided by the on-line server of the KABAT database. Of 519 murine antibodies studied, only 33 combinations of at least 300 total expected combinations are actually found. About 85% of the antibodies fall into only six different combinations of canonical structures. These distinct groups correlate to the types of antigen with which they interact. It was suggested that relatively small numbers of canonical structural combinations of CDR segments may primarily determine the 'prototypes' or 'gross' specificities of the antibody combining sites. Further diversification of these structures are required to generate a wide variety of antigen-binding specificities.

The molecular mechanisms underlying antibody diversity have been extensively studied. The intact V_H or V_L chains are assembled by genetically separated elements, V_H genes, D and J_H segments or V_L genes and J_L segments, by a site-specific recombination event the V(D)J rearrangements. CDR1 and CDR2 are encoded by the V_H or V_L genes; the CDR3 regions are generated by recombination events, V_L -CDR3 joined by V_L and J_L , and the V_H -CDR3 by V_H , D and J_H . N or P element insertions may occur at either V_H -D or D- J_H junctions. D-D fusion may also occur during the V_H -D- J_H recombination but at lower frequency. Sequences and lengths of V_H -CDR3 are therefore the most variable. A rearranged antibody molecule may be further diversified by another somatic molecular process termed 'hypermutation', which may be induced during an immune response to antigenic stimulation, typically those elicited by T-dependent protein antigens. In the nonimmunized repertoire or the 'natural antibody' population, antibodies with low affinity and cross-reactivity are frequently seen. They are usually encoded by germline V region genes without or with few somatic mutations. Antibodies with relatively high affinity may be obtained during secondary immune responses or later but generally not at the stage of the primary response. Point mutations are frequently observed in their V regions. The process by which an antibody with low affinity is further diversified by point mutations to high affinity is termed 'affinity maturation'. This process is believed to occur in the germinal centers of lymphoid tissue and requires help by T lymphocytes and other accessory cells. The molecular mechanisms underlying hypermutation and how it contributes to the affinities and specificities of antibody combining sites are less understood.

Since V(D)J recombination occurs at the pre-B cell stage and secondary rearrangements appear very infrequently, a 'matured' high-affinity antibody may preserve the lengths of its six CDR loops and the canonical structural combination of its parent antibody, i.e. the rearranged unmutated germline antibody, unless mutations occurred at the conserved CDR residue positions which are considered as those critical for the canonical structure of CDRs. Comparison of such matured high-affinity sites and their low-affinity germline antibody may allow us to investigate how somatic mutation contributes to the affinity maturation. Patten and colleagues studied a catalytic antibody 48G7 and its germline partner. They reconstructed and expressed the Fab segments of 48G7 as well as the germline antibody. The active catalytic antibody 48G7 differs from the germline partner by nine replacement mutations, six in CDRs and three in FRs. None of the six CDR residues, two in V_L -CDR1, one in V_L -CDR2 and three in V_H -CDR2, examined using Chothia's definition, can be classified as 'key residues' for the canonical structure of the CDRs. Thus, the two antibodies have the same combination of canonical CDR structure. These mutations, however, resulted in increased affinity for the transition state analog by a factor of 10^4 and also enhanced catalytic activity with the rate K_{cat}/K_m , from $1.7 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$ to $1.4 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$. The three-dimensional crystal structure of the 48G7 Fab-hapten complex was resolved to 2.0 Å, allowing the identification of amino acid residues which contact the bound hapten. Surprisingly, none of the nine residues generated by somatic mutation contacted the ligand directly. They are all noncontacting residues. It was suggested that these mutants played roles in fine tuning of the combining site, by optimizing the geometries for hapten binding. These studies emphasized that the relative contribution of the CDR contacting and noncontacting residues and their cooperative interaction to antibody specificity need further investigation.

Since the discovery of antibody more than 100 years ago, much has been learned about this molecule. It has become possible to ask whether an antibody combining site of a given specificity may be designed and synthesized. To realize this long-term goal, additional efforts must be made to understand further the relationship of structural diversity and complementarity, to identify the critical structures which define three-dimensional complementarity in overall shapes or gross specificities, and to define those which may further optimize the fine geometries of antibody combining sites to achieve directed specificities. To facilitate these studies, a combination of advanced approaches, including X-ray crystallogra-

phy, NMR, computer modeling, molecular engineering, immunochemical analysis and other methods for determination of fine specificities should be applied in rational designed experiments.

See also: Affinity maturation; Antigens; Carrier; Diversity, generation of; Domains, immunoglobulin-type; Epitopes; Hapten; Idiotypic; Immunoglobulin class switching; Immunoglobulin structure; Antibody-antigen complexes, three-dimensional structures.

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ANTIBODIES, SYNTHESIS

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The human immune system contains 10^{11} – 10^{12} B lymphocytes, which synthesize the 10^{20} antibody (immunoglobulin, Ig) molecules found inside and on the surface of these cells and, most of all, in the serum. Other mammalian species contain B cells and Ig molecules in numbers relative to their body weight, e.g. mice have 10^9 B cells and 10^{17} Ig mol-

ecules. Both B cells and Ig molecules turn over; both are synthesized and degraded throughout life.

Antibodies are glycoproteins composed of glycosylated heavy (H) chains and (mostly unglycosylated) light (L) chains. The amino-terminal domains of both H and L chains are variable (V) from antibody to antibody; they bind antigen. The other, more car-

boxy-terminal, constant (C) domains are involved in functions of antibodies which are unrelated to their individual antigen-binding specificities, but are carried out by antibodies of a given class or subclass, e.g. complement binding and activation, binding to Fc receptors on surfaces of cells, or transport through cells. Genes encoding H and L chains are encoded by segments which have to be rearranged before the genes can be transcribed and translated. The rearrangements of gene segments occur when B cells develop from B lineage-committed progenitor cells. Therefore, rearranged Ig genes only exist in cells of the B lineage, but in no other cells of the body.

Development of B lineage cells and rearrangements of Ig gene loci

In humans (and mice) three Ig gene loci can be rearranged. The H chains on human chromosome 14 (chromosome 12 in mouse) with the μ -, δ -, γ_{1-4} , α_{1-2} and ϵ C-region genes, κ L chains on human chromosome 2 (chromosome 6 in mouse) and λ L chains on human chromosome 22 (chromosome 16 in mouse). During B cell development (in humans at around 10 weeks, in mice between day 10 and 18 of gestation) B cells are generated in waves. In the mouse a first wave in embryonic blood and placenta is succeeded by a second wave in fetal liver. Throughout life B cells continue to be generated in bone marrow at a rate of 5×10^7 cells per day in the mouse (approximately 10^3 -fold more in humans), of which 0.5–5% become mature, surface Ig (sIg)-positive cells of the periphery. Most newly generated B cells therefore die at the site where they have been generated.

From pluripotent stem cells which are the ancestors of the different lineages of blood cells, progenitors develop which are committed to the B lineage.

A collection of intracellular and surface-bound marker molecules has allowed the separation of subpopulations of B lineage cells from fetal liver and bone marrow, and single-cell PCR analyses of the status of the Ig gene loci have been useful to order these cell populations on their way from a pluripotent stem cell to a mature, antigen-reactive B lymphocyte. Many early cells of B lymphocyte development find themselves, and function, in close contact with an environment of stromal cells which provides specific contacts and cytokines that regulate the proliferation and differentiation of B lineage cells. Interleukin 3 (IL-3) and IL-7 have been identified as such cytokines in the mouse, and c-kit and flk-2 are membrane-bound tyrosine kinases on B lineage precursors

which interact with their corresponding membrane-bound ligands on stromal cells.

The earliest signs of a cell to be committed to the B lineage of development is the transcription of the nonrearranged, germline μ H chain gene locus, the transcription of the V_{preB} and λ_5 genes encoding the surrogate light (SL) chain and the activation of the machinery involved in rearrangements of segments of the Ig gene loci, i.e. the activation of terminal deoxynucleotidyl transferase (TdT) and the recombination activating genes RAG1 and RAG2. This occurs in cells which express B220 (CD45R), but do not yet express CD19, the two markers most commonly used in the characterization of B lineage cells. The first Ig gene rearrangements, D_H segments to J_H segments, are already detectable in these cells. TdT is responsible for N-sequence insertions at the joints of the rearrangements. TdT is active in bone marrow but not in fetal liver. Therefore antibodies derived from bone marrow-generated B cells are N-region diverse (in their H chains); those derived from fetal liver are not.

This ordered rearrangement process continues in the next distinguishable B lineage cell population, which is B220 (CD45)⁺, CD19⁺, also expresses c-kit and SL chain on the surface, and now has practically all H chain alleles D_HJ_H rearranged.

The next rearrangement in-line is V_H to D_HJ_H on one of the two H chain alleles. Whenever this rearrangement occurs in-frame, a μ H chain can be made. The μ H chain combines with SL chain and thus forms a pre-B receptor. This development has several consequences which lead to a new cell population. It induces cells into cycle, hence the pre-B receptor-expressing, so-called pre-B II cells are large. Since nonproductively rearranged cells cannot form a pre-B receptor they do not expand by proliferation. Hence, productively rearranged, μ H chain expressing, pre-B receptor-positive cells outgrow this compartment, i.e. are positively selected. They no longer express c-kit and have completely downregulated the rearrangement machinery, i.e. TdT, RAG1 and RAG2. As a new surface marker, they now express CD25 (TAC), the α chain of the IL-2 receptor. Downregulation of the rearrangement machinery is likely to be part of the mechanisms by which the second H chain allele is inhibited from V_H to D_HJ_H rearrangement. Hence it secures allelic exclusion, i.e. that one B cell makes only one H chain sequence.

Large pre-B II cells proliferate for an estimated 3–5 cell cycles, lose pre-B receptor expression, begin to re-express RAG1 and RAG2, and begin to transcribe the nonrearranged L chain gene loci κ and λ . While doing so they fall into a resting state, become small, and hence are called small pre-B II cells. The

rearrangement-active genes RAG1 and RAG2 but not TdT are active; all cells now contain V_L to J_L -rearranged L chain gene loci, but no N-region insertions can be made. Half of them are productively rearranged, express the L chains in their cytoplasm, but not (yet) on their surface. The other half contain nonrearranged, or out-of-frame rearranged L chain loci. Since the pre-B II cells have an active rearrangement machinery, they can engage in secondary V_L to J_L rearrangements which in some cells might change nonproductive to productive L chain gene loci, and which in other cells might change a productive L chain locus encoding an autoantigen-reactive Ig receptor (termed receptor editing). Secondary L chain gene rearrangements might allow a B cell to express two L chains, whenever the rearrangement process allows both L chain alleles to be used. It has been seen that no more than 1–3% of all peripheral mature B cells express two L chains. Since less than 1% of all B cells have been seen to express two H chains, it remains true that for most B cells the rule ‘one B cell makes one antibody’ is valid. This is the basis for clonal selection in the immune system, in which a given antigen selects from all the lymphocytes those which have a fitting receptor on their surface.

The rearrangement machinery continues to be active in the next cell population in-line, the immature, surface IgM-expressing B cells. This is probably useful in editing autoreactive B cells.

The expression of an autoreactive Ig receptor on immature B cells leads to the arrest of differentiation of this cell. Since the cell remains short lived, it will die at this site of development in the primary lymphoid organ unless it can change its receptor specificity through editing. In this way of arrest of differentiation, the emerging B cell repertoire is purged of all B cells which are autoreactive to antigens present and presented in the bone marrow. A classical case for such an autoantigen is double-stranded DNA, present in large quantities due to extensive cell death at this site of development.

Of the sIgM-expressing immature B cells which do *not* recognize an autoantigen at this point of development, a small proportion (2–3%) are chosen each day to become mature, resting antigen-sensitive, sIgM/sIgD double-expressing B cells which are allowed to migrate to the periphery and become eventually longer lived. Whether sIg is involved in this ‘positive selection’, i.e. whether self antigen or foreign antigens are mandatory to make all immature B cells mature, remains to be seen. However, B lineage cells which are defective in T-independent as well as T-dependent B cell activation through mutations in their *btk* and *CD40* genes are unable

to establish longer-lived peripheral, mature B cell compartments, indicating that such stimulatory pathways are involved in the establishment of the peripheral, mature B cell compartments.

It should be mentioned that other species with an immune system might use different strategies to generate antibody diversity. In chicken, as one example, there is only one functional rearrangement of one V_H to a series of different D_H segments and to one J_H segment, and one V_L to one J_L segment generates a very limited original Ig repertoire which is then altered by gene conversion with many pseudo- V_H and pseudo- V_L gene segments, respectively.

As another example, fish carry partially and fully V(D)J-rearranged genes in many copies; hence they rely much less on rearrangements and more on combinations of different H and L chains for antibody diversity.

Selection of the B cell repertoire by self antigens

Once sIg-positive cells have been generated they are subjected to selection processes by self antigens in their own environment, probably presented by cells in the B cell-generating organs. Self reactive B cells appear to be either deleted or anergized. Whether *positive* selection for self antigens can also occur – maybe in a special B lineage, the $Ly-I^+/CD5^+$ B cells – needs to be elucidated.

Those B cells which have been untouched by these selection processes then enter the mature B cell compartment of virgin cells involved in primary B cell responses. Such cells still turn over rather rapidly, with half-lives of less than 1 week. They can be stimulated by foreign antigens, either with the help of T cells (helper T cell dependent) or without such help (T cell independent). Stimulation results in the activation from a resting, G_0 state and entry into the proliferative cell cycle. One cell cycle lasts 20 hours at 37°C and is controlled by several interactions (see below) of antigen, helper T cells, accessory cells and cytokines. Proliferating B cells also mature to Ig-secreting cells. A single activated B cell can balance between proliferation and maturation to Ig secretion. With every successive cell cycle the contribution to Ig secretion increases, so that finally mature plasma cells develop which no longer divide.

Helper T cell-dependent stimulation of B cells

T cell-dependent antigens are often monomeric protein molecules. Stimulation of B cells by such T cell-dependent antigens begins with the binding of anti-

gen to sIg on fitting B cells. The complex of sIg and antigen is taken up by the cell, processed by proteolytic degradation, and the processed antigen in the form of peptides combines with class II major histocompatibility complex (MHC) molecules, so that the peptides of the antigen appear associated with the MHC class II on the surface of B cells. Upregulation of MHC class II expression might be necessary to mediate effective recognition and interaction of the complex with the T cell receptor of antigen-specific helper T cells, and IL-4 produced by helper T cells (or other cells) has been recognized as one major cytokine involved in the upregulation of MHC class II expression. This interaction might result in cross-linking of MHC class II molecules followed by signaling to B cells to proceed in the cell cycle. The same helper T cells have also seen the processed antigen on accessory cells such as macrophages, again an association with MHC class II molecules. In response to the T cell-induced signaling, these accessory cells may have produced cytokines which now interact with B cells at the entry into S phase. T cell-dependent B cell stimulation is, therefore, dependent on accessory cells. Activated helper T cells contribute to B cell stimulation in various ways. Activation of resting helper T cells by the recognition of the MHC class II-antigen-peptide complex on antigen-presenting accessory cells (dendritic cells, macrophages), leads to the upregulation of expression of the ligand for CD40 and to the upregulation of cytokine expression such as IL-2 (by helper T cells of type 0 and type 1), and of IL-4 and IL-5 (by helper T cells of type 2). In addition, different cell-cell contacts mediated by, for example, CTLA-4 and CD28 on helper T cells with B7-1 and B7-2 on B cells appear to guide the responses of B cells to IgM production (T_H0 and T_H1), or IgG and IgE production (T_H2), and the memory responses of B cells. In summary, T cell-dependent activation controls the B cell cycles at four points: 1) by antigen via sIg; 2) by T cell receptor interaction via processed antigen and MHC class II molecules, both in the G_1 phase; 3) by cytokines from accessory cells and by CD40 ligand from helper T cells at the entry of S phase; and 4) by IL-2 or IL-5 from helper T cells via IL-2 and IL-5 receptors before mitosis, and by additional cell-to-cell contact.

Synthesis of antibody with different H chain classes: the problem of H chain gene switching

T cell-independent antigens stimulate IgM- and IgG-synthesizing and secreting B cell clones, without any strong switching to other classes of IgH chains. T cell-dependent antigens, however, stimulate IgM-

secreting B cell clones and induce the switching to other classes of IgH chains in these clones. Different helper T cells in different lymphoid organs may induce the switching to different classes of H chains. T cells secreting IL-4 induce switching to IgG1 and IgE.

Somatic hypermutation of Ig genes

T cell-dependent stimulation generates memory for the antigen in the immune system. This may be so because T and B cells specific for the antigen are now present in higher numbers, because B cells have a longer life in the system and because B cells with better fitting sIg have been made by hypermutation of Ig V regions. With a hypermutation rate estimated to be as high as 10^{-3} per base pair per division, one nucleotide of a V gene is mutated with every B cell division. Antigen continues to select any better fitting Ig on a mutated B cell in the T cell-dependent fashion of clonal stimulation described above. Much of this appears to occur in germinal centers. T cell-dependent stimulation to germinal center formation, Ig class switching and hypermutations is abolished when CD40 is made nonfunctional in B cells by targeted disruption of the gene.

Mutations of B cells to specificities recognizing self antigens may occur, but these autoimmune B cells are not dangerous as they are not selected for further growth. This is because any processed self antigen on MHC class II is not recognized by the helper T cells which are specific for foreign antigens, and because helper T cells for self antigens are absent from the immune system due to deletion in the thymus during their generation.

T cell-independent B cell stimulation

Two types of T cell-independent antigens (types I and II) stimulate B cells without involving MHC class II molecules, i.e. in a non-MHC-restricted fashion, to proliferation and to maturation into antigen-secreting cells. Memory to these antigens is normally not induced; the responses remain mainly IgM response and little, if any, hypermutation of IgV genes is observed. The stimulated B cells remain short lived. Typical type I antigens are those connected to lipopolysaccharide (LPS), while type II antigens are often polysaccharides.

T cell-independent antigens usually consist of repeating determinants with the capacity to cross-link sIg, probably without ever being taken up and processed by the B cells. Three restriction points control the cell cycle of a B cell activated by a T cell-independent antigen: 1) antigen binds to sIg early in

the G₁ phase; 2) at entry into S phase CR2 receptors are ligated; and 3) at entry into mitosis IL-2 or IL-5 bind to their corresponding receptors. T cell-independent antigens of type I do not need IL-2 or IL-5 to stimulate B cells at the third restriction point. Other cytokines, such as IL-1, IL-4, IL-6 and interferon γ (IFN γ) have positive and negative modulating functions for proliferation and/or maturation of B cells.

Synthesis, intracellular transport, surface deposition and secretion of Ig chains

When the Ig gene segments have been rearranged, the Ig gene loci can be transcribed, and the primary transcripts processed into mature mRNA. Transcription is controlled by B lineage-specific promoters and enhancers, so that 50–100 mRNA molecules for μ H chains, and later for L chains, are found in a pre-B cell. This suffices to maintain the synthesis of approximately 5×10^4 μ H chains of membrane bound type (μ_m), and later the same amount of L chains, in 24 hours. As long as pre-B cells divide, most of the newly synthesized molecules are incorporated into newly generated surface membranes used in cell replication. Some of the 7–8S μ H₂L₂ sIgM-positive B cells shed these molecules rapidly, i.e. with a half-life of 2–4 hours, from the pre-B cells. At early stages of B cell development, before the expression of L chains, these 7–8S sIg molecules contain V_{preB} λ_5 as a surrogate L chain. Later, when B cells fall into the G₀ resting phase, the same amount of Ig μ H and L chain synthesis provides the 7–8S μ H₂L₂ molecules shed from the cells with a slow turnover (half-life of 24 hours). The transmembrane portion of μ H_m determines its deposition in the surface membrane.

Within 2 hours of stimulation, resting B cells change the rate of IgM synthesis by a factor of 5, and within the next 2 days by a factor of 20–100. This is achieved by stabilization of mRNA and an increase in the rate of transcription. The increase in IgM synthesis is devoted entirely to the synthesis of the secretory form of μ H chains (μ_s). Splicing of precursor RNA into mature mRNA is altered from μ_m to μ_s type. Within 4 hours of stimulation, 19S (μ H₂L₂)₅ pentameric IgM is secreted from stimulated B cells. Synthesis of J chain is stimulated, and J chains become associated with 19S IgM. Beyond 2

days of stimulation, the rate of IgM synthesis increases by another fivefold. In these later phases of B cell maturation, plasma blasts enlarge their endoplasmic reticulum (ER) and accumulate intracellular Ig (from 5×10^4 – 5×10^6 molecules per cell).

IgH and L chains are synthesized and transported through the cell by the conventional pathway for secreted proteins. H and L chains are synthesized on separate polyribosomes. It takes 30–40 s to synthesize an L chain, and around 90 s for an H chain. Translation is initiated on free ribosomes. As soon as the amino terminal leader sequences of H and L have been synthesized, they allow the binding of the ribosomes to the rough endoplasmic reticulum (RER) and then, the translocation of the nascent chains through the ER membrane. On the cisternal side, the leader sequence is enzymatically cleaved, and the H chain is glycosylated by the transfer of core oligosaccharides in the form of dolichol-phosphate intermediates to Asn-X-Ser/Thr acceptor sites. The H and L chains are successfully S–S bonded, the carbohydrate portions first trimmed, then terminal sugars added, as the Ig travels from the RER to the smooth ER (SER) and the Golgi apparatus. In mature plasma cells Ig is held intracellularly, particularly in the SER. At the late stage of plasma cell maturation, a single Ig synthesizing and secreting cell contains between 1 and 4×10^6 mRNA molecules for H and L chains, from which it synthesizes and secretes 10^3 – 10^4 Ig molecules per second.

See also: Antigen, T dependent and independent; B lymphocyte differentiation; Diversity, generation of; Glycosylation of immune system molecules; Immunoglobulin genes; Immunoglobulin structure; Plaque-forming cell (PFC) assays; Somatic mutation.

Further reading

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ANTIBODY-ANTIGEN COMPLEXES, THREE-DIMENSIONAL STRUCTURES

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The advent of monoclonal antibody technology has made possible structural studies of antibody-antigen complexes by providing large quantities of unique molecular entities. Since the mid-1980s, structural studies of antibody-antigen complexes have encompassed a wide variety of antigens ranging from small molecules through peptides and oligosaccharides to proteins, including several anti-idiotypic-idiotypic complexes (Table 1). All antibody-antigen complexes have shown that the antibody has achieved specificity and affinity through interactions of an antigen-binding site, made up principally, but not exclusively, of residues from the complementarity-determining regions (CDRs), with the antigen. However, the shape of the antigen-binding site and specific interactions have varied considerably depending on the size, shape and surface polarity of the antigen.

Extent and nature of the interface between antibody and antigen

The amount of surface buried in the interaction between antigen and antibody varies with the size of the antigen, from $\sim 1.7 \text{ nm}^2$ to $\sim 9 \text{ nm}^2$ on each surface (Figure 1). The surface area of interaction may also be limited by the size of the antigen-binding site for the largest protein antigen studied (neuraminidase). In the case of protein antigens only a small percentage ($\sim 10\%$ to $\sim 15\%$) of the total antigen surface area is buried in the interaction. Conversely, for most small molecules, the interaction buries 50–90% of the total antigen surface. The surfaces of interaction vary from deep pockets (Figure 1A) to broad shallow pockets (Figure 1B) to grooves (Figure 1C) for small molecules including carbohydrate and peptides. In contrast, antigen-binding sites are relatively flat for protein antigens (Figure 1D). However, in all cases the surfaces are rather complementary with limited space for water molecules in some antibody-protein complexes.

The number of residues involved in the interaction between antibody and antigen also varies with size of the antigen. For smaller antigens, as few as eight residues may be involved on the part of the antibody. For protein antigens, typically 14–21 residues are in contact on both molecules. The antibody-binding

sites on protein antigens (epitopes) consist of 2–5 sequentially discontinuous segments that are spatially contiguous (Figure 1D). Similarly, antigen-binding sites on antibodies (paratopes) consist of 4–6 CDRs that are spatially contiguous (Figure 1A–D).

In the typical complex the heavy chain contributes more surface area and residues to the interaction with antigen, ranging from just over 50% to almost 80%. One exception to this rule is the antibody AN02, where the light chain contributes a majority of the interactions. The CDRs that typically make a preponderance of the interactions are the third CDR of the light chain and the second and third CDRs of the heavy chain. Most complexes with small molecules and some complexes with proteins do not involve the second CDR of the light chain. In contrast, the NC41-neuraminidase complex does not involve the first CDR of the light chain and a few protein complexes have rather minimal involvement of either the third CDR of the light chain or the third CDR of the heavy chain.

Chemical nature of the interactions between antibody and antigen

The interactions of antibody with antigen are generally van der Waals interactions involving surface residues, which despite being solvent exposed are relatively hydrophobic in nature. However, most antibody-antigen interactions contain at least one hydrogen bond or salt link and typically contain many. For example, protein antigens form on the order of 15–20 hydrogen bonds and/or salt links with antibody. However, it is impossible to extrapolate from the number of hydrogen bonds and salt links to the strength of the interaction between antibody and antigen. For example, the 26-10-digoxin complex has no hydrogen bonds and no salt links, but its association constant ($\sim 1 \times 10^{10} \text{ M}^{-1}$) is as strong as any whose three-dimensional structure is known. In the antibody-protein complexes, some water molecules have been found to be part of the interface between antibody and antigen.

In the antigen-binding site, tyrosines and tryptophans occur more frequently than in the rest of the antibody. These two residues are both capable of forming hydrogen bonds to solvent or antigen, but have

Table 1 Antibody-antigen complexes and their Protein Data Bank (PDB) codes

Antibody ^a	Antigen ^b	PDB code	
		Complexed	Uncomplexed
<i>Antiprotein</i>			
<i>Antilysozymes</i>			
D1.3 Fv	Hen egg lysozyme	1VFB, 1FDL	1VFA
D1.3 Fv	Turkey egg lysozyme	NA	
HyHEL-5	Hen egg lysozyme	3HFL	
HyHEL-5	Bobwhite egg lysozyme	1BQL, 2IFF	
HyHEL-10	Hen egg lysozyme	3HFM	
D11.15 Fv	Pheasant lysozyme	1JHL	
D44.1	Hen egg lysozyme	1MLC	1MLB
F9.13.7	Guinea fowl egg lysozyme	1FBI	
cAb-Lys3	Hen egg lysozyme	1MEL	
<i>Antineuraminidases</i>			
NC41	N9 neuraminidase	1NCA, 1NCB, 1NCC, 1NCD	
NC10	N9 neuraminidase	1NMA, 1NMB	
<i>Antiidiotopes</i>			
E225	D1.3	NA	
E5.2 Fv	D1.3 Fv	1DVF	
409.5.3	730.1.4	1IAI	1AIF(409.5.3)
T91AJ5	Yst9.1	NA	1MAM(Yst9.1)
<i>Others</i>			
JEL 42	HPr	1JEL	
HC19	Hemagglutinin	1VIR(C α)	1GIG
N10	Staph nuclease	1NSN	
28	HIV rt-DNA	NA	
7E2 Fv	Cytochrome <i>c</i> oxidase	NA	
<i>Antipeptide</i>			
B13I2	Myohemerythrin, 69-87	1IGF	2IGF
17/9	Hemagglutinin, 75-110	1HIM, 1HIN, 1IFH	1HIL
26/9	Hemagglutinin, 75-110	1FRG	
131	Angiotensin II	NA	
TE33	Cholera toxin, 50-64	1TET	
50.1	HIV gp120 V3	1GGI	1GGB, 1GGC
59.1	HIV gp120 V3	1ACY	
8F5	Rhinovirus VP2, 156-170	NA	1BBB
C3	Poliovirus, 86-103	1FPT	
SD6	FMDV, G-H loop	NA	NA
<i>Anticarbohydrate</i>			
Se155-4	<i>Salmonella</i> O antigen	1MFA, 1MFB, 1MFC, 1MFD, 1MFE	
BR96	Le ^y	1CLY, 1CLZ	1UCB
<i>Anti-DNA</i>			
BV04-01	dT ₃	1CBV	1NBV
<i>Anti-small molecule</i>			
McPC603	Phosphocholine	2MCP	1MCP
4-4-20	Fluorescein	1FLR	
NQ10/12.5	2-Phenyloxazolone	NA	
AN02	TEMPO-DNP	1BAF	
R454511	Cyclosporine	1IKF	
DB3	Progesterone etc.	1DBB, 1DBJ, 1DBK, 2DBL, 1DBM	1DBA
26-10	Digoxin	1IGJ	1IGI
40-50	Ouabain	1IBG	
JEL 103	Nucleotides	1MRD, 1MRE, 1MRF	1MRC
CHA255	EOTUBE (In ³⁺ , Fe ³⁺)	1IND, 1INE	
NC6.8	NC174	2CGR	1CGS
N1G9	(4-Hydroxy-3-nitrophenyl) acetate	1NGP	1NGQ
1F7	Transition state analog	1FIG	
17E8	Transition state analog	1EAP	
CNJ206	Transition state analog	1KNO	2GFB
48G7	Transition state analog	1GAF	

NA = not available, although paper describing the structure has been reported. Blank in the uncomplexed column means structure has not been reported.

^aAntibodies are Fabs unless noted as Fv.

^bAntigen names: HPr, histidine-containing protein of the phosphoenolpyruvate:sugar phosphotransferase system; FMDV, foot-and-mouth disease virus; TEMPO-DNP, 2,2,6,6-tetramethyl-1-piperidinyloxy dinitrophenol; EOTUBE, 4-[N'-(2-hydroxyethyl)thioureido]-L-benzyl-EDTA; NC174, N-(p-cyanophenyl)-N'-(diphenylmethyl)-N''-(carboxymethyl)guanidine.

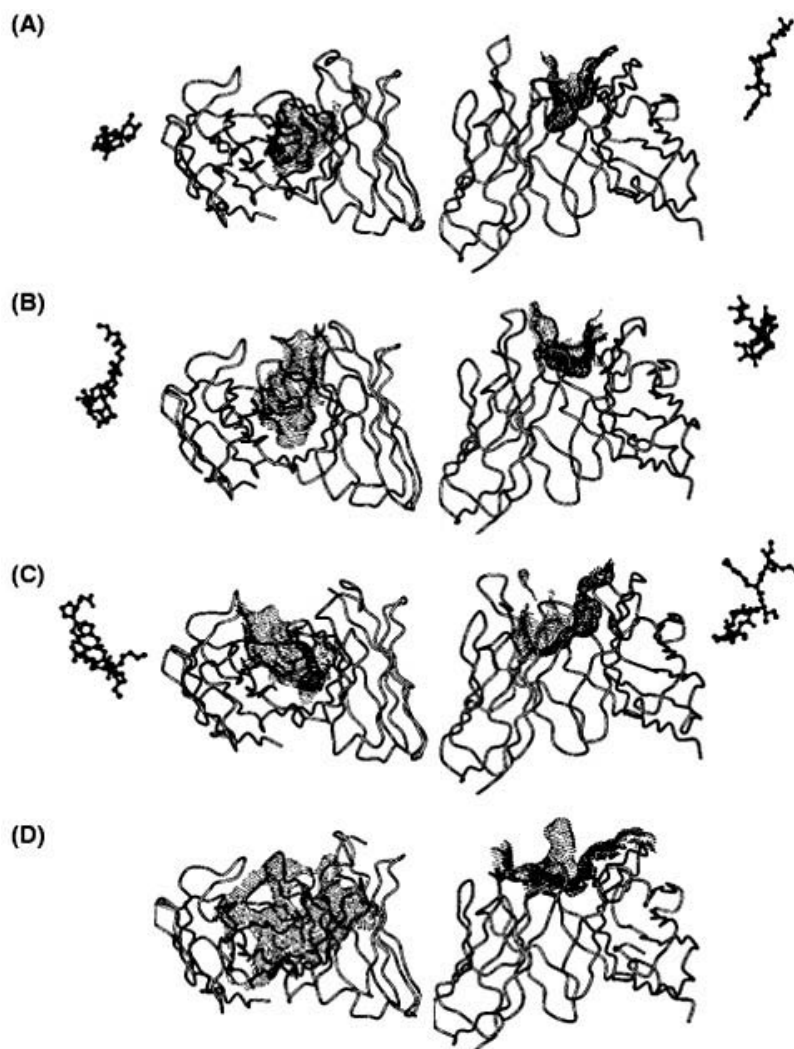


Figure 1 Binding sites and buried surfaces (represented as dots) of selected antibody-antigen complexes demonstrating different sizes and shapes of binding interfaces. For each complex, on the left the antibodies are shown with antigen-binding site face-on and on the right rotated 90° about the horizontal relative to the figure on the left. In both views the light chain is on the left. In the right image, the viewer is looking from CDR-L3 towards CDR-H3, which is in the back. Antibody backbone is represented as a ribbon. To the far left and far right of the antibody structures the small molecule antigens for the first three complexes are represented as ball-and-stick in the orientation that they are bound to the antibody. (A) 26-10-digoxin; (B) BR96-Lewis Y; (C) 50.1-HIV gp120 V3; (D) HyHEL-5-lysozyme. Note the deep pocket of 26-10, the broad pocket of BR96, the groove of 50.1 and the relatively flat and extensive surface of HyHEL-5. (Figure produced with MOLSCRIPT by Per Kraulis.)

large hydrophobic surfaces. Moreover, these large hydrophobic surfaces can be immobilized with limited entropy loss due to restricted rotation of bonds in the complex. Thus the preference for tyrosines and tryptophans is most likely due to the unique nature of the antigen-binding site, which spends part of its time accessible to solvent and part of its time buried in a complex.

Conformational change upon complexation of antibody and antigen

A range of motions have been found for antibody and for antigen upon complexation. From most global to most local these include: rotation of V_H relative to V_L , conformational rearrangement of the polypeptide backbone, rigid-body motion of polypeptide backbone segments, and side-chain motion. Examples of antibody motion have been principally found with antibodies to small molecules including peptides and carbohydrate, but this may be due to

the limited number of structures of uncomplexed antibody available for protein-specific antibodies (Table 1). An example of rotation of V_H relative to V_L and of conformational rearrangement of the third CDR of the heavy chain may be seen in Figure 2A. The effects of these motions on opening the antigen-binding site may be seen by a comparison of Figure 2B with Figure 2C.

Anti-idiotypic complexes

A number of anti-idiotypic complexes have been reported (Table 1). However, the lack of availability of the structure of the original antibody in complex with antigen has limited the ability to describe mimicry of the antigen by the idiotypic antibody. In the case of the D1.3 antibody, whose structures both uncomplexed and in complex with antigen (lysozyme) have been determined, two anti-idiotypic complex structures have also been determined. One (E225) blocks the antigen-binding site, but does not

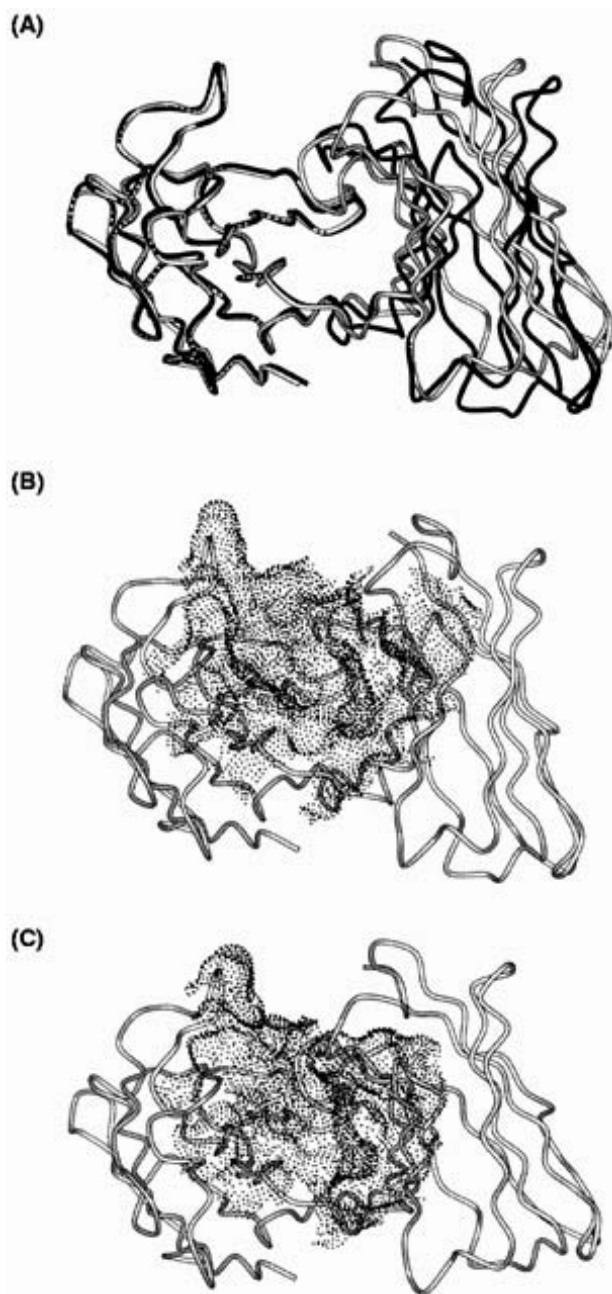


Figure 2 Comparison of complexed and uncomplexed forms of 50.1 showing the change of the binding pocket between the uncomplexed and complexed forms. (A) Superposition of V_L domains of 50.1, showing changes due to differences between relative position of V_H and conformational differences of the third CDR loop of the heavy chain (complexed antibody in black and uncomplexed antibody in white). (B) Binding site (as represented by a dot surface) of complexed form of 50.1. (C) Binding site (as represented by a dot surface) of uncomplexed form of 50.1. The third CDR of the heavy chain is towards the top center of the figures. (Figure produced with MOLSCRIPT by Per Kraulis.)

mimic the surface of the antigen. In contrast, the other (E5.2) blocks the antigen-binding site and appears to mimic the interactions of lysozyme with D1.3 to a high degree.

Future directions

The future of structural work in the antibody-antigen complex field will probably be driven by four forces: 1) the use of antibody-antigen complexes as model systems for examining protein-protein interactions, which includes the ability of the immune system to find multiple solutions to binding to the same surface (epitope); 2) the study of camelid antibodies, which have single, heavy-chain binding domains rather than the more common $V_L:V_H$ heterodimers (an example of a camel antibody-lysozyme complex has recently been determined (Table 1)); 3) the use of antibodies to crystallize complexes of otherwise refractory proteins, such as human immunodeficiency virus (HIV) reverse transcriptase and cytochrome *c* oxidase (Table 1); and 4) a desire to understand the structural basis of catalytic antibodies of which four examples already exist (Table 1).

See also: Antibodies, specificity; Antibody-antigen intermolecular forces; Antigen-binding site; Diversity, generation of; Domains, immunoglobulin-type; Idiotypic; Idiotypic, internal image; IgA; IgE; Immunoglobulin structure; Monoclonal antibodies (mAbs).

Further reading

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ANTIBODY-ANTIGEN INTERMOLECULAR FORCES

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The different types of bonds occurring in the specific interaction between antigen (Ag) and antibody (Ab) molecules are all of the weak physical variety. The three different classes of bonding forces in question are (not necessarily in the order of their relative importance): 1) van der Waals or electrodynamic forces; 2) hydrogen bonding or polar forces; 3) electrostatic forces. Covalent bonds are not involved in Ag-Ab interactions. These physical, noncovalent forces also provide the bonds between other biological entities, such as various ligand and cell surface receptors, carbohydrates and lectins, and enzymes and their substrates. (In enzyme-substrate reactions however, covalent bonds can also occur, in addition to the three types of physical forces mentioned above.)

Human Ab molecules (immunoglobulins) have from two (IgG, IgA, IgD, IgE), to ten identical valencies (IgM), while secretory IgA (S-IgA) has four valencies. Each paratope on an immunoglobulin molecule represents one valency. Ag molecule (or particles) can have from a few to a great many valencies (each epitope represents one valency). The multiple valencies of such Ag molecules may be all different, or all identical, or some epitopes on a given Ag may be different and others the same. Ag and Ab molecules of a given specificity can combine into Ag-Ab complexes in virtually every conceivable proportion: the Ag-Ab reaction is essentially nonstoichiometric. Thus, the valency of an Ag molecule can only be determined under conditions of excess Ag. In both cases the resulting Ag-Ab complexes will tend to be soluble; insoluble complexes only occur at intermediate Ag-Ab ratios, i.e. close to the optimal, or equivalence ratio.

Nature of antigen-antibody intermolecular forces

Van der Waals forces

Also known as electrodynamic interactions, these occur universally between all atoms and molecules, when brought close enough together. Three different but related phenomena contribute to van der Waals interactions: 1) randomly orienting dipole-dipole (orientation) interactions, or van der Waals-Keesom forces; 2) randomly orienting dipole-induced dipole (induction) interactions, or van der Waals-Debye forces; 3) randomly fluctuating dipole-induced dipole (dispersion) interactions, or van der Waals-London forces. Keesom and Debye interactions only occur with molecules which have permanent dipole moments. The van der Waals-London interaction occurs universally and is also present in atom-atom interactions. In condensed media (i.e. in the liquid and the solid phase), on a macroscopic scale (i.e. on a supra-atomic level), it was first shown by Lifshitz that all three varieties of van der Waals forces can be grouped together; they should be treated in the same manner, and the forces decay with distance at the same rate. On a macroscopic scale, which is also the realm of Ag-Ab interactions, the three varieties of van der Waals interactions, grouped together, are alluded to as Lifshitz-van der Waals (LW) interactions. Due to the fact that the total LW interaction between Ag and Ab, in water, not only must account for the interaction between Ag and Ab, but also for the LW interactions between the Ags and the water molecules, and between the water molecules among one another, the *net* LW attraction between such biopolymers is rather strongly attenuated when occurring in water. The net free energy of LW interaction of the Ag-Ab system in water is of the order of: $\Delta G^{LW} = -0.5$ to -5 mJ m^{-2} . For the interaction between one epitope and its corresponding paratope, given an average surface area of the interaction between epitope and paratope: $A = 3 \text{ nm}^2$, the LW

contribution to such an Ag-Ab interaction then corresponds to $\Delta G^{LW} = -0.2$ to -2 kcal mol⁻¹. Thus LW interactions typically represent only between 2 and 20% of the total interaction energy of average Ag-Ab reaction (which is of the order of: $\Delta G^{TOT} = -10$ kcal mol⁻¹).

Hydrogen bonding, or polar forces

These are nonelectrostatic polar (Lewis) acid-base (AB) interactions, which are of considerable importance in strongly polar media such as water. In water, these polar interactions are principally of the hydrogen-bonding variety; their net outcome can be attractive (hydrophobic attraction) or repulsive (hydration repulsion), depending on the circumstances. In either case, net AB interactions are long-range, i.e. they act at a distance of up to about 10 nm. Analogous to LW or apolar interactions, taking place between two different moieties immersed in a liquid, AB interactions taking place in water are the net outcome of the polar interactions between Ag and Ab, and those between Ag and water molecules, as well as those between Ab and water molecules, and between the molecules among one another.

Hydrophilic AB interactions (also known as hydration forces) are the major cause of the net long-range macroscopic scale repulsion between cells and/or biopolymers. This is the principal repulsion force which epitopes and paratopes have to overcome before engaging in the specific microscopic scale attraction that culminates in the Ag-Ab bond.

The equally long-range hydrophobic AB interactions usually represent the principal microscopic scale attractive force between epitope and paratope. Contrary to a still fairly widely held belief, hydrophobic interactions in water need not exclusively take place between two hydrophobic sites. Hydrophobic attractions between, for example, a hydrophilic epitope and a hydrophobic paratope are quite common among Ag-Ab interactions *in vitro* and *in vivo*. As all immunodominant epitopes tend to be hydrophilic, the corresponding paratopic sites usually are hydrophobic. Such hydrophobic paratopic sites are necessarily located in cavities or clefts, thus obviating undesirable aspecific interactions between unduly exposed hydrophobic loci. The driving force for the hydrophobic attraction is the cohesive hydrogen-bonding interaction between the surrounding water molecules. Hydrophobic Ag-Ab interactions, at closest approach, range from -3 to -25 kcal mol⁻¹ and as such usually play the dominant role in the total Ag-Ab interaction.

In contrast to hydrophobic interactions, direct hydrogen bonds are extremely short range in nature, i.e. direct hydrogen bonds only act at a distance of

about 0.1–0.3 nm. Direct hydrogen bond energies range from -1.3 to -3.4 kcal mol⁻¹.

Electrostatic interactions

Electrostatic interactions between Ag and Ab are caused by the attraction between one or more ionized sites on the epitope and one or more oppositely charged sites on the paratope. These charged sites typically are the COO⁻ and the NH₃⁺ groups on ionized amino acids of the Ag (in such cases usually a protein or a peptide) and of the Ab. Of the three classes of forces involved, the electrostatic (or Coulombic) interaction forces are the most influenced by the pH and by the ionic strength of the liquid medium. The pH is important, as it largely determines the degree of dissociation of the COO⁻ or the NH₃⁺ ions. The ionic strength is important, as it governs the value of the electrokinetic, or zeta-potential of the charged sites, as well as of the (Debye) thickness of the diffuse ionic double layer surrounding these sites. At the physiologic ionic strength of 0.15, electrostatic interactions have a maximum range of about 8 nm; at lower salt concentrations the range can be much longer. The free energy of attraction between one COO⁻ and an NH₃⁺ group, at a distance of about 0.3 nm, at physiological ionic strength, is about -7 kcal mol⁻¹; at a distance of 0.5 nm, the electrostatic (EL) energy of attraction is about -5.5 kcal mol⁻¹.

Whilst electrostatic Ag-Ab interactions occur principally between oppositely charged sites on the epitope and paratope, rare instances have been reported of interactions between two negatively charged sites, by the intermediary of Ca²⁺ ions. Ag-Ab systems involved in Ca²⁺ bridging are, for example, polyglutamic acid Ags, recombinant proteins and (occasionally) DNA-anti-DNA complexes.

The obligatory long-range attraction between epitope and paratope

Normally all biopolymers and cells dissolved or dispersed in aqueous media under physiological conditions repel one another, which prevents them from approaching each other more closely than about 3–5 nm. This makes it necessary for specifically interacting epitopic and paratopic sites to be able to attract each other from a distance. Three factors can contribute to make this possible: 1) The specifically interacting sites are small (surface areas usually between 1 and 10 nm²) and the epitope tends to lie on a prominent edge or peak with a small radius of curvature, which enables it to pierce the general repulsion field and to bring it within less than 3 nm of the paratope. 2) The two sites have a significant

electric charge of opposite signs. 3) At least one of the two (usually the paratope) is fairly hydrophobic.

The rate of decay of Ag-Ab interactions with distance

The rate of decay as a function of distance, d , varies considerably in the three different classes of physical bonds. LW interaction energies in the range of virtual contact, up to $d = 10$ nm, varies at $1/d^2$, in a configuration of two flat parallel slabs. Both polar (AB) and electrostatic (EL) energies decay exponentially with distance, d . AB energies decay as $\exp(-d/\lambda)$, where λ is the decay length of water; λ (water) has a value of about 1.0 nm. EL energies decay as $\exp(-\kappa d)$, where $1/\kappa$ is the thickness of the diffuse electrical double layer; at the physiological ionic strength of 0.15, $1/\kappa = 0.8$ nm. As such exponentially decaying forces usually are measurable at distances that are up to ten times longer than the decay length, thus epitope-paratope interactions begin to make themselves felt at a distance, d of about 8–10 nm. Beyond that distance, the generally prevailing AB and EL repulsion acts between Ag and Ab, as it does between most biopolymers and cells, dissolved or immersed in water. At very short distances between Ag and Ab (i.e. $d_0 = 0.16$ nm), the pronounced increase in strength in all three modes of physical bonding, explains why the best 'fit' between epitope and paratope correlates so strongly with the highest binding affinity.

Hybrid intermolecular Ag-Ab forces

Combinations of LW, AB and EL forces all contributing to the Ag-Ab bond in a variety of proportions are more the rule than the exception. In aqueous media, systems interacting exclusively through Lifshitz-van der Waals forces do not occur; such systems only are possible in apolar media such as alkanes, in which all immunoglobulins and most antigens would be insoluble. Exclusively interfacial interactions, i.e. those involving only LW and AB forces, do occur, for example with (electro-) neutral carbohydrates such as dextran as Ags, or with haptens such as 3-azo-pyridine, *p*-azo-toluidine, dinitrophenol, etc. Exclusively electrostatic Ag-Ab interactions also occur, e.g. in the case of dsDNA-anti-DNA. Otherwise, most Ag-Ab interactions involve LW + AB + EL forces in the primary binding interaction between epitope and specific paratope. Thus, in general, $\Delta G^{\text{TOT}} = \Delta G^{\text{LW}} + \Delta G^{\text{AB}} + \Delta G^{\text{EL}}$.

Primary and secondary Ag-Ab bonds

Once the repulsion front is pierced and epitope and paratope have made contact, the epitope-paratope

bond strengthens, either through the sheer effect of the small distance between the two, or because of a qualitative change in the nature of the bond; for example, through expulsion of water of hydration, or (relatively rarely) through formation of direct hydrogen bonds. Also, once epitope and paratope have approached each other closely, in many cases secondary bonds can ensue between biopolymer moieties that are in close proximity to epitope and paratope. Secondary Ag-Ab bonds occur in all cases where the energy of association is smaller than the energy required for dissociation, once the bond has been formed. Secondary bonds tend to be of the interfacial (or LW + AB, or hydrophobic) variety and hardly ever of an EL nature. If one defines ΔG^{TOT} as $\Delta G_{\text{primary}} + \Delta G_{\text{secondary}}$, and $\Delta G_{\text{primary}}$ as the free energy one needs to furnish to prevent the Ag-Ab complex from forming ($\Delta G_{\text{primary}} = \Delta G_{\text{prevention of association}}$), while ΔG^{TOT} equals the energy needed to dissociate the complex, then:

$$\Delta G_{\text{secondary}} = \Delta G_{\text{dissociation}} - \Delta G_{\text{prevention of association}} \quad (1)$$

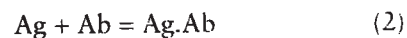
As one can measure both the energy needed to prevent association, and the energy of dissociation (i.e. ΔG^{TOT}), $\Delta G_{\text{primary}}$ as well as $\Delta G_{\text{secondary}}$ can be determined. Consolidation of intraepitope-paratope binding (e.g. through direct H-bonding or direct EL interactions) also involves $\Delta G_{\text{secondary}}$. The bovine serum albumin (BSA) system: BSA-anti-BSA, is an example of a primary EL bond, and a secondary hydrophobic (LW + AB) bond.

Dehydration of the epitope-paratope interstitial space (which gives rise to the increase in entropy typically observed in Ag-Ab interactions) is another important aspect of the secondary part of the Ag-Ab interaction. Due to that dehydration, the distance between epitope and paratope decreases, which strengthens their attraction. Upon the disappearance of interstitial water, the epitope-water-paratope interaction becomes, at least in part, a direct epitope-paratope attraction that mainly involves high-energy direct LW forces.

Thermodynamics of antigen-antibody interactions

Law of mass action

Writing the antigen-antibody reaction as:



according to the law of mass action the equilibrium

association constant is expressed as (simplified for the case of monovalent haptens, or single epitopes, and monovalent, or Fab-type paratopes):

$$K_a = K_a/k_d = (\text{Ag} \cdot \text{Ab})/[(\text{Ag}) \times (\text{Ab})] \quad (3)$$

where (Ag) is the molar concentration of free Ag sites, (Ab) the molar concentration of free Ab and (Ag·Ab) the molar concentration of the Ag·Ab complex. k_a is the association kinetic constant; it tends to be rather uniform in value and fairly high (10^3 – $10^8 \text{ M}^{-1} \text{ s}^{-1}$); k_d is the dissociation kinetic constant; it varies considerably from case to case (10^4 – 10^6 s^{-1}). To determine K_a as well as the antibody valency (n), one can use either a Scatchard or a Langmuir plot. In the Scatchard approach one plots (Ag-bound)/(Ag) $\times$ (Ab) on the ordinate versus (Ag-bound)/(Ab). The slope then yields $-n \times K_a$ and the intercept with the abscissa, n . With the Langmuir approach one plots (Ab)/(Ab-bound) on the ordinate versus 1/(Ab-bound). Here the slope yields $(1/n) \times K_a$ and the intercept with the ordinate, $1/n$. If the line obtained in a Langmuir plot is not a straight line, the (Sips) heterogeneity index α , may be obtained by determining α such that the plot of (Ab)/(Ag-bound) versus $1/(\text{Ag-bound})^\alpha$, forms a straight line. K_a values usually vary between 10^4 and $10^{11} \text{ l mol}^{-1}$. For plurivalent antigens (i.e. for most antigens), eqn (2) and concomitantly, eqn (3) become more complex. In addition, it should be kept in mind that each of the valencies of a typical protein antigen usually represents a different epitope. A polyclonal antiserum to such a protein antigen then comprises a whole collection of antibodies with different specificities, as well as with widely differing affinities. Thus, for the interaction of such a polyclonal collection of antibodies with a protein Ag, a large series of eqns (2) should be used for which, however, most of the parameters are unknown and probably unknowable. One therefore usually reverts to eqn (3) in its simple form, and tests for heterogeneity in the manner indicated above.

Energetics

For purposes of energetics, the equilibrium binding constant, K_a (see eqns (4) and (5), below) has to be expressed as a dimensionless number (K'_a), in terms of inverse mole fractions. For instance in water, which comprises $55.56 \text{ mol H}_2\text{O l}^{-1}$, a concentration of $1 \text{ mol l}^{-1} = 1/55.56 = 0.018$ mole fraction. Thus, starting with the unit in which K_a is habitually expressed (i.e. in l mol^{-1}) it should be noted that 1 l mol^{-1} becomes $55.56 (\text{mol fraction})^{-1}$. Conversely, to find K_a (in l mol^{-1}) from K'_a , one has to multiply K'_a by 0.018.

From the dimensionless equilibrium association constant, K'_a , the free energy of the Ag–Ab bond can be obtained via:

$$\Delta G^{\text{TOT}} = -RT \ln K'_a \quad (4)$$

where ΔG is expressed in kcal mol^{-1} , R is the gas constant $= 1.986 \times 10^{-3} \text{ kcal K}^{-1} \text{ mol}^{-1}$ and T is the absolute temperature in degrees Kelvin. To obtain ΔG in joules, $R = 8.3144 \text{ J K}^{-1} \text{ mol}^{-1}$. To obtain ΔG in SI units per epitope–paratope bond, one divides R by Avogadro's number N ($N = 6.022 \times 10^{23}$), to obtain Boltzmann's constant k , where k replaces R in eqn (4). To obtain both the enthalpy (ΔH) and entropy (ΔS) components of the binding energy, $\Delta G = \Delta H - T \Delta S$, K'_a must be measured at a number of different temperatures, in which case one can use, as an approximation:

$$(d \ln K'_a)/dT = \Delta H/RT^2 \quad (5)$$

where R again may be replaced by k when working on the molecular scale of single bonds. Once ΔH is known, one obtains $T \Delta S$ from $T \Delta S = \Delta H - \Delta G$. ΔH can also be obtained by microcalorimetric measurements of Ag–Ab reactions.

Contrary to a rather widely held belief, hydrophobic interaction energies are not necessarily mainly entropic: they can be enthalpic, or entropic, or a combination of these in any proportion. With an increase in temperature the total interaction energy tends to change from predominantly enthalpic to mainly entropic (see following section).

Temperature effects

As the reaction usually is exothermic, at room temperature or lower, ΔH is mostly negative, but it can vary from $+10$ to $-20 \text{ kcal mol}^{-1}$. The entropy contribution can vary widely, from -40 to $+80$ entropy units per mole. In Ag–Ab interactions a positive entropy is usually taken to be due to the randomization of previously organized (oriented) water molecules of hydration which, upon closer approach between epitope and paratope, are squeezed out and become part of the bulk liquid.

In many Ag–Ab reactions, an increase in temperature causes ΔH to become less negative. However, this effect tends to be compensated by a concomitant increase in $T \Delta S$, thus leaving ΔG practically unchanged over a fairly wide range of temperatures. The increase in $T \Delta S$ with an increase in T is a consequence of the following phenomenon: At increased T , the molecular hydration energy decreases, which favors the escape of interstitial water of hydration

into the bulk liquid and thus causes an increase in the entropy of the system caused by the randomization of formerly more organized water of hydration. This loss of interstitial water of hydration also causes a decrease in the distance between the epitopic and paratopic sites, which increases their mutual energy of attraction and ultimately becomes, at least in part, a higher energy direct LW bond.

Measurement of Ag-Ab interaction energies

There is a large variety of equilibrium methods by which the parameters on the right-hand side of eqn (4) can be determined, allowing the calculation of K_a and from this value, of K'_a and of ΔG . Among these methods are equilibrium dialysis or ultrafiltration, precipitation methods, analytical ultracentrifugation, gel filtration or size exclusion chromatography, affinity chromatography, fluorescence, fluorescence quenching, fluorescence polarization, immunoassay methods, hapten inhibition methods, nuclear magnetic resonance, electron spin resonance, etc. The powerful new bioaffinity sensor methods should be specially mentioned.

Bioaffinity sensors

The bioaffinity sensor methodology has, since the early 1990s, developed into one of the most powerful approaches to determining the kinetic association (k_a) and dissociation (k_d) constants, and from these the equilibrium binding constant, K_a (see eqn (3), above), and its inverse, the equilibrium dissociation constant, $K_d = k_d/k_a$. By optical means, the thickening of a layer of, for example, Ab, which is continuously deposited by means of a thin stream of an Ab solution moving across a thin layer of Ag molecules, can be monitored in real time. The graph of Ab thickness versus time thus obtained yields the kinetic association constant, k_a . Upon subsequent replacement of the Ab solution with a buffer solution, the kinetic dissociation constant, k_d , is obtained. From these the equilibrium binding constant, K_a , is derived (see eqn (3)).

Concentration dependence of K_a

With polyclonal Abs, for example, in the BSA-anti-BSA system (BSA = bovine serum albumin), 100-fold dilution (at Ag/Ab equivalence) causes an almost 40 000 fold increase in K_a , i.e. from 1.7×10^7 to

$6.3 \times 10^{11} \text{ l mol}^{-1}$. Even with monoclonal Abs a significant increase in K_a with dilution must be expected because greater dilution minimizes steric hindrance. Thus, if one aims to characterize an Ag-Ab system by measuring the K_a value, it is advisable to operate at the lowest practical reagent concentration. For instance in a Biosensor setting, with a constant Ag concentration in the immobilized phase, a 100-fold decrease in the mobile phase (monoclonal Ab solution) typically gives rise to a 70- to 100-fold decrease in k_a and K_a . In general, under similar circumstances, only the lowest feasible Ab concentration in the mobile phase will yield accurate k_a and K_a values.

See also: Affinity; Antigens; Immunoglobulin structure; Precipitation reaction; Surface plasmon resonance; Valency of antigens and antibodies.

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ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

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It was first shown by Moller in 1965 that a wide variety of target cells, coated with low amounts of specific immunoglobulin G (IgG) antibodies, can be lysed *in vitro* by lymphocytes not previously sensitized to target cell antigens. Further studies established that nonlymphoid cells such as polymorphonuclear leukocytes, monocytes and macrophages can also mediate this cytotoxic mechanism, known as antibody-dependent cellular (or cell-mediated) cytotoxicity (ADCC). The ability of a given effector cell population to perform this response, however, appears to be strongly dependent on several factors. They include the nature of the target cells, the isotype and density of antibodies coating the target cell surface, the class of receptor for the Fc portion of immunoglobulin molecule (FcR) involved, and the activation state of effector cells. In all cases, ADCC is induced without major histocompatibility complex (MHC) restriction and therefore it is operative in syngeneic, allogeneic and xenogeneic systems.

It should be noted that the specificity of ADCC is conferred by antibodies, which at extremely low concentrations, far below those required for complement-mediated lysis, are able to induce cytotoxicity. In fact, target cells coated with as few as 100–500 immunoglobulin molecules can be destroyed through ADCC.

Although the mechanisms involved in ADCC are still not completely understood, it is well established that ADCC is triggered by the interaction of target cell-bound IgG antibodies with the receptors for the Fc fragment of IgG (FcγR) expressed on the effector cells. These receptors, which act as signal-transducing molecules, are all members of the immunoglobulin-like superfamily of membrane receptors. They are expressed by human hematopoietic cells, including natural killer (NK), B and T lymphocytes, as well as by monocytes, macrophages, polymorphonuclear leukocytes, mast cells and platelets. Most of them express multiple types of FcγR, which fall into three main classes based on structural analysis of the genes and proteins for these receptors: the high affinity FcγRI (CD64), the low affinity FcγRII (CD32) and the low/intermediate affinity FcγRIII (CD16), which involve at least 12 different isoforms.

FcγR mediate a wide array of responses. They range from effector functions, such as ADCC, phago-

cytosis, pinocytosis and release of inflammatory mediators, to immunoregulatory signals, such as modulating antigen presentation, lymphocyte proliferation and antibody production. Triggering of biological responses through FcγR normally requires cross-linking of these receptors, which is usually induced by IgG immune complexes.

ADCC has been defined and characterized in several experimental models performed *in vitro*. Although its relevance *in vivo* has not been clearly established, several lines of evidence suggest that it plays an important role in host immune defense.

Nature of effector cells and antibodies involved in ADCC

NK cells are a subset of lymphocytes, morphologically identified as large granular lymphocytes, which lack typical T and B cell markers and usually express CD16 (FcγRIII) and CD56. They are able to destroy a variety of cell types, in the absence of prior stimulation, through a mechanism known as natural killer cytotoxicity. This cytotoxic response is not restricted by MHC gene products and is induced without antibody participation. NK cells, which comprise about 5% of human peripheral blood lymphocytes, also account for most lymphocyte ADCC activity. Thus, the same lymphocyte subpopulation seems to be capable of mediating distinct cytotoxic mechanisms, i.e. NK cytotoxicity and ADCC, through the activation of different recognition systems.

The ability of NK cells to perform ADCC has been demonstrated employing a wide variety of IgG-coated target cells such as normal, tumor and virus-infected cells, as well as bacteria, fungi and parasites. It is worth noting that in several human infectious diseases the presence of high levels of specific serum antibodies capable of inducing NK-mediated ADCC against the infectious agent has been described. Similarly, high concentrations of serum antibodies, competent to induce ADCC against tumor and allogeneic cells, have also been found in cancer and kidney transplant patients, respectively. These observations suggest that ADCC mediated by NK cells may play an important role in controlling some infection diseases, in antitumor immunity and, probably, in organ transplant rejection.

Although it is well established that NK cells constitute the major lymphocyte subpopulation capable of performing ADCC, the relative contribution of T and B lymphocytes, as effector cells, should be considered. Studies performed with resting T cells have shown that, with few exceptions, they are unable to mediate ADCC. However, since some T cell subsets express Fc γ R only during a narrow window following cellular activation, the possibility that activated T cells mediate ADCC cannot be ruled out. Regarding B lymphocytes, despite the fact that most of them bear Fc γ RII, they are unable to perform ADCC, suggesting that in B cells these receptors do not act as ADCC-triggering molecules.

Phagocytes can mediate the destruction of antibody-coated target cells by two major pathways: either by intracellular killing of target cells following phagocytosis or by ADCC-mediated extracellular lysis of targets. ADCC performed by mononuclear phagocytes has been largely studied employing peripheral blood monocytes, tissue macrophages and monocyte/macrophage-like cell lines as effector cells. Polymorphonuclear-mediated ADCC has been analyzed using peripheral blood neutrophils and neutrophils collected from inflammatory sites. There is general agreement that mononuclear phagocytes and neutrophils are able to mediate ADCC against a wide variety of target cells. In most of the models described, ADCC involves the participation of IgG antibodies, all IgG subclasses being able to mediate cytotoxicity. In contrast, IgM antibodies seem to play no role in the induction of ADCC. IgA antibodies are also unable to induce cytotoxicity, but they are capable of synergizing with IgG in mediating ADCC.

The relevance of ADCC performed by phagocytic cells in defense against disease is still not completely defined. Several lines of evidence strongly suggest that it plays a major role in host acquired immunity against a wide variety of infectious agents. A possible participation of macrophage-mediated ADCC in antitumor immunity has also been proposed. This possibility was recently supported by several studies which demonstrated that the therapeutic efficacy of some monoclonal antibodies, directed against tumor cell-associated antigens, strongly depends on the number of tumor-infiltrating macrophages.

For a long time it was thought that cellular interactions of IgE molecules were restricted to basophils and mast cells, which express a high-affinity receptor for the Fc fragment of IgE (Fc ϵ RI). The discovery of receptors of low-affinity for IgE (Fc ϵ RII; CD23) on mononuclear phagocytes, eosinophils, B cells and platelets suggested that these cellular populations may also be able to respond to IgE. Thus, Capron

and Dessaint showed that macrophage-mediated ADCC induced by specific IgE antibodies against *Schistosoma mansoni* schistosomula led to the death of the parasite. More recent findings have indicated that not only macrophages but also eosinophils and platelets are able to perform IgE-dependent ADCC against helminths. While several lines of evidence strongly suggest that ADCC induced by IgE antibodies plays a major role in resistance to schistosomiasis, its relevance in host immunity against other parasites remains to be established.

Mechanisms involved in ADCC

The mechanisms involved in ADCC are still not well defined. As mentioned above, the cytotoxic response is triggered by the cross-linking of FcR expressed by the effector cells, which is induced by antibodies that coat target cells. It is also well established that cytotoxicity does not involve the participation of a diffusible cytotoxic factor. In fact, when mixtures of antibody-coated and uncoated target cells are incubated together with effector cells, lysis of non-sensitized targets is not observed.

Different metabolic requirements are involved in the induction of ADCC, depending not only on the nature of the effector cells but also on their activation state, the properties of antibodies and the nature of target cells. The role of Ca²⁺ in the induction of ADCC appears to differ for distinct effector cells. It is well known that the stimulation of human neutrophils and monocytes with immune complexes triggers Ca²⁺ mobilization. The increase in [Ca²⁺]_i appears to play an important role in neutrophil-mediated ADCC; however, it represents a secondary phenomenon in monocyte-mediated ADCC. Studies performed to evaluate the role of the cytoskeleton have also demonstrated major differences between neutrophils and monocytes. Thus, integrity of microfilament and microtubule systems is required for neutrophil-mediated ADCC, but it plays no role in cytotoxicity performed by monocytes.

The cytolytic mechanisms responsible for target cell destruction also seem to be quite different in distinct ADCC models. The generation of reactive oxygen intermediates (ROI), such as superoxide anion and hydrogen peroxide, plays a major role in some models of phagocytic cell-mediated ADCC. By contrast, ROI are not involved in cytotoxicity performed by lymphoid cells, which appears to be dependent on nonoxidative mechanisms, including the release of cytoplasmic granules containing perforin and granzymes. Furthermore, employing monocytes from chronic granulomatous disease patients, which generate very limited amounts of ROI, it was found that

oxidative mechanisms play an important role in ADCC mediated by monocytes against red blood cells but not against lymphoblastoid cells. Interestingly, these data indicate that a single type of effector cell is able to perform ADCC towards different target cells via distinct cytolytic mechanisms.

It is possible that many of the variables observed in different models of ADCC may result from the participation of distinct types of FcγR in the induction of cytotoxicity. In this regard, it is noteworthy that FcγR share structurally related ligand-binding domains, but differ in their transmembrane and intracellular domains which mediate intracellular signaling.

Modulation of cytotoxic activity of effector cells

ADCC can be modulated by several cytokines and inflammatory products. Interferon γ (IFNγ) is one of the most important agents capable of increasing ADCC mediated by both polymorphonuclear leukocytes and mononuclear phagocytes. It causes a 5–15-fold increase in the number of FcγRI on monocytes, macrophages and neutrophils, and, moreover, significantly enhances effector cell oxidative metabolism. Both mechanisms seem to be responsible for the increase in ADCC observed after treatment with IFNγ. While interleukin-10 (IL-10) is also able to stimulate FcγRI expression and ADCC in mononuclear phagocytes, both IL-4 and IL-13 downregulate the expression of all three FcγR and strongly inhibit ADCC mediated by either untreated, IL-10- or IFNγ-treated monocytes. Tumor necrosis factor α (TNFα) can also activate ADCC mediated by neutrophils and eosinophils towards tumor cells and schistosomes, respectively. Interestingly, IL-5 has been shown to enhance antibody-dependent lysis of tumor cells mediated by eosinophils but not by neutrophils.

Colony stimulating factors are also powerful stimulators of ADCC mediated by phagocytic cells. Granulocyte-CSF (G-CSF) has been used in clinical studies for the improvement of circulating neutrophil counts. During G-CSF therapy it was observed that the expression of FcγRI is induced on circulating neutrophils. This effect, which results from G-CSF action on myeloid precursor cells, enables neutrophils to mediate high levels of ADCC against tumor cells. Monocyte-mediated ADCC, on the other hand, can be increased by treatment with macrophage-CSF (M-CSF). Measured on a cell-for-cell basis, treated monocytes are 10–100-fold more efficient than untreated monocytes. The ability of granulocyte-macrophage CSF (GM-CSF) to increase ADCC has

also been established. GM-CSF treatment enables eosinophils, neutrophils or monocytes to display high levels of ADCC against antibody-coated tumor cells and significantly increases antibody-dependent killing of parasites by eosinophils.

Other factors are also able to modulate ADCC. The C5a split product of complement activation increases both the expression of FcγRI on phagocytic cells and ADCC. Phagocytic cell-mediated ADCC can also be significantly increased by opioid peptides, such as met-enkephalin. By contrast, glucocorticoids and several compounds able to increase intracellular levels of cAMP, strongly inhibit ADCC.

Even though *in vitro* studies have provided some information about the characteristics of ADCC, much more remains to be learned. As our understanding of the mechanisms involved in the regulation of ADCC increases, this knowledge should be applied to improve the therapeutic efficacy of monoclonal antibodies, not only in cancer immunotherapy but also in the treatment of some infectious diseases, which depends on the recruitment of host effector systems including ADCC.

See also: Cytotoxicity, assays for; Cytotoxicity, mechanisms of; Fc receptors; Granzymes; Immunoglobulin, functions; Macrophage activation; Natural killer (NK) cells; Parasites, immunity to; Perforin; Tumors, immune response to.

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ANTIGEN-BINDING SITE

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Different estimates indicate that the immune system of higher vertebrates can produce antibodies capable of reacting with more than 10^8 different external antigens. Given this physiological diversity of antibody molecules, the molecular structure of their antigen-combining sites has been the object of much speculation and research. Amino acid sequences of immunoglobulin light (L) and heavy (H) chains from human and mouse myeloma proteins indicated the presence of hypervariable regions, located around sequence positions 30, 50 and 100 in each chain. These hypervariable sequences constitute the antigen-combining sites of antibodies and thus are termed complementarity determining regions (CDRs). Because of their sequence hypervariability, the CDRs must possess great conformational diversity, reflecting the potential of antibodies to respond to many different external antigens. Antibody diversity is generated at the genetic level by multiple V_H and V_L genes and segments (D and J), by a somatic recombination process and an antigen-driven somatic mutation mechanism.

The determination of the three-dimensional structure of Fab fragments of myeloma immunoglobulins by X-ray crystallographic techniques indicated that the folding of the polypeptide chain brings the CDRs into close proximity at an end of the antibody molecule. The β -barrel structures of the V domains provide a nearly constant, conformationally stable framework on which different external polypeptide loops, the CDRs, can be placed without adversely affecting the stability of the folding. Their variable lengths, in particular those of the V_L CDR1 and V_H CDR3, have a profound effect on the stereochemistry of the combining site. In addition, part of the interactions between V_H and V_L mediated by the CDRs are important in shaping antigen-combining sites and in determining the preferential pairing of H and L chains.

Molecular structures of Fabs and Fab-hapten complexes

The determination of the first three-dimensional structures of Fab complexes with the haptens phosphorylcholine and vitamin K_1OH , confirmed that the CDRs of both V_H and V_L participate in binding. The contacts of the ligands with the combining site included salt bridges, hydrogen bonds and van der Waals interactions. No conformational changes in the combining site or in other regions of the antibody could be seen by comparing free with liganded Fabs. Thus, an allosteric mechanism by which conformational signals would be transmitted from the combining site to the Fc region was not supported by these structure determinations. Such mechanisms were postulated as a way of activating secondary (effector) functions of antibodies. However, since haptens represent only a fraction of the antigenic determinants recognized by antibodies, the complete antigen-binding site could not be defined by that approach. Moreover, it was thought possible that conformational changes would be observed only if a complex with a macromolecular antigen, contacting a wider area at the combining site, could be studied.

Antibody-antigen complexes

The introduction of cell hybridization techniques leading to the development of immortalized lymphocytic cell lines (hybridomas) secreting antibodies of predefined specificity allowed the production of monoclonal antibodies (mAbs) in large quantities. These made it possible to study specific antigen-antibody reactions in greater detail, eventually providing a deeper understanding of antigenic determinants and combining site structure. Moreover, the elucidation of the antigen-binding site has been greatly facilitated by the production of antibody variable domain fragments (Fv). As the volume of Fv fragments is half that of the Fab, Fv fragments generally crystallize in smaller asymmetric units and thus diffract to higher resolution.

By 1996 more than 50 structures of antibody fragments, free and complexed with antigen, had been determined by X-ray diffraction techniques. By far the

most widely studied antibodies have been of mouse origin. The mAbs were obtained after secondary responses and can be assumed to be highly specific – that is, they recognize only one antigen (and related, cross-reacting antigens sharing the antigenic determinant). Thus, their combining sites are uniquely defined by stereospecific contacts with the antigens. Several BALB/c mouse mAbs have now been studied by X-ray diffraction, including six which form complexes with hen egg-white lysozyme (HEL), two with influenza virus neuraminidase, one with HPr (a histidine-containing phosphocarrier protein), one with NC10 staphylococcal nuclease and four with anti-idiotopic antibodies. These results can be correlated with those of other crystallographic, immunochemical and sequence studies related to antigen and hapten recognition by specific antibodies. In most of the complexes above, the combining site is formed by amino acid residues from each of the CDRs of the L and H chains, although the H chain may in several cases contribute more contacts than the L chain. About 15 residues, mostly from the CDRs but including also one or two from the framework regions, are in contact with a similar number from the antigen. The general topology of the site is not that of a big cavity, but rather flat with interspersed depressions and protuberances that closely complement the surface topology of the antigenic determinant.

The nature of the chemical contacts made by the combining site includes charge bridges between ion pairs, hydrogen bonds and van der Waals interactions. If hydrogen bonds are inaccessible to solvent they should contribute appreciably to the stability of antigen–antibody interactions. In the complex of mAbs HyHEL-5 and D44.1 with HEL, two glutamic acid residues of the combining site interact closely with two arginines of the antigen. The crystal structure of HyHEL-5 complexed with a site-directed mutant of HEL (Arg68→Lys) demonstrated the specificity of electrostatic interactions. While generally considered a conservative mutation, the HEL mutant binds to HyHEL-5 with a 10^3 -fold reduction in affinity. The crystal structure demonstrated that lysine is unable to form both salt bridges to a HyHEL-5 glutamate, therefore the decrease in affinity of the HyHEL-5 reaction with the mutant HEL was ascribed to the loss of one hydrogen bond. Further evidence of the electrostatic complementarity defining antibody–antigen interactions came from the crystal structure of D1.3 complexed with turkey egg-white lysozyme (TEL). In this case, an important residue in the antibody–antigen interface (HEL Gln121) is replaced by a histidine. Gln121 makes two hydrogen bonds to carbonyl oxygen and amide nitrogen main-chain atoms of D1.3 V_L CDR-3 (O ϵ 1-Ser93 N; N ϵ 2-Phe 91 O). The orien-

tation of TEL His121 is such that its N ϵ 2 atom is positionally equivalent to the O ϵ 1 atom of HEL Gln121 and therefore unable to form a hydrogen bond to the amide nitrogen of D1.3 Ser93. D1.3 was observed to compensate for this difference in the electrostatic character of HEL121 by undergoing a flip of the main-chain peptide orientation such that the carbonyl oxygen of Trp92 is exposed at the interface, allowing formation of a hydrogen bond to TEL His121 N ϵ 2 (Figure 1).

Since hydrogen bonds and van der Waals interactions require a somewhat strict complementarity, they can be taken as the basis of antigen–antibody specificity. In the combining sites there is a high relative proportion of aromatic residues Tyr and Trp. Given their bulky size, these contribute a maximum of van der Waals contacts as well as affording the possibility of hydrogen bond formation to their polar atoms. The area of contact between antigen and antibody can be defined as that which becomes inaccessible to solvent after the formation of a complex. The surface buried by protein antigens is of about 600 Å² and higher, compatible with the requirements for the formation of a stable complex. This observation is in agreement with that made in other protein-interacting systems in which subunits make contacts through a closely packed interface occupying a large surface area. The crystal structure of a Trp→Asp mutation of D1.3

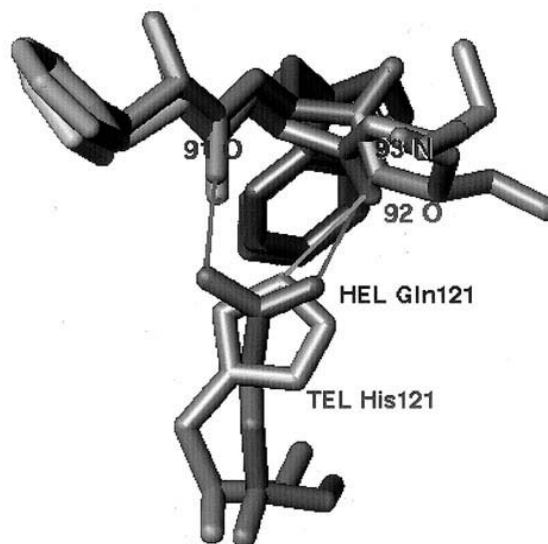


Figure 1 Conformational differences in antibody D1.3 V_L CDR3 induced by differing antigen side-chains. HEL residue Gln121 makes two main-chain hydrogen bonds; to the carbonyl oxygen of D1.3 V_L Phe91 and to the amide nitrogen of D1.3 V_L Ser93. Replacement of the glutamine by histidine in TEL induces a conformational change in the backbone of V_L CDR3 which allows the formation of a hydrogen bond between the histidine and the carbonyl oxygen of V_L Trp92. (See also color Plate 8A.)

exemplifies the importance of interacting surface complementarity and van der Waals interactions. The V_L Trp92→Asp mutation results in a 10^3 reduction in the affinity of D1.3 for HEL. Trp92 makes extensive van der Waals contacts with the antigen. With the substitution of Asp about 150 Å² of surface area are lost to the antibody-antigen complex, resulting in the reduction of affinity (Figure 2).

While a few buried water molecules have been reported in other antibody-antigen complexes, the D1.3-HEL complex contains a large number of bound water molecules which bridge the antibody and antigen. This water contributes to the stability of the antibody-antigen complex in two important ways: 1) the network of water molecules contributes to a favorable free energy of complex formation by mediating hydrogen bonds between antibody and antigen; and 2) the water molecules fill cavities in the interface which are destabilizing to complex formation (Figure 3).

No major conformational change seems to take place at the antibody site upon the binding of antigen. Small rearrangements of side-chains and CDR loops have been observed, particularly upon the binding of small antigens, most notably peptides, as well as relative displacements of V_H and V_L . Thus, antibody-antigen complexes can be viewed as a form of 'lock-and-key' association and small displacements can provide potential diversity in the conformation of the

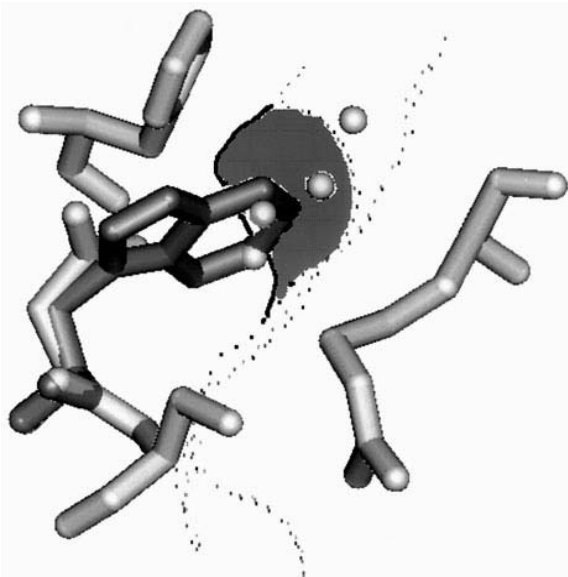


Figure 2 The effect of a Trp→Asp mutation on the interaction surface area of the D1.3-HEL complex. A thin slice through the buried surface of the wild-type D1.3-HEL complex (dots) and the mutant D1.3-HEL surface (solid line) demonstrates the loss of interaction surface area in the mutant complex. A 150 Å² loss in surface area accounts for the reduction in affinity of the mutant D1.3-HEL reaction. (See also color Plate 8B.)

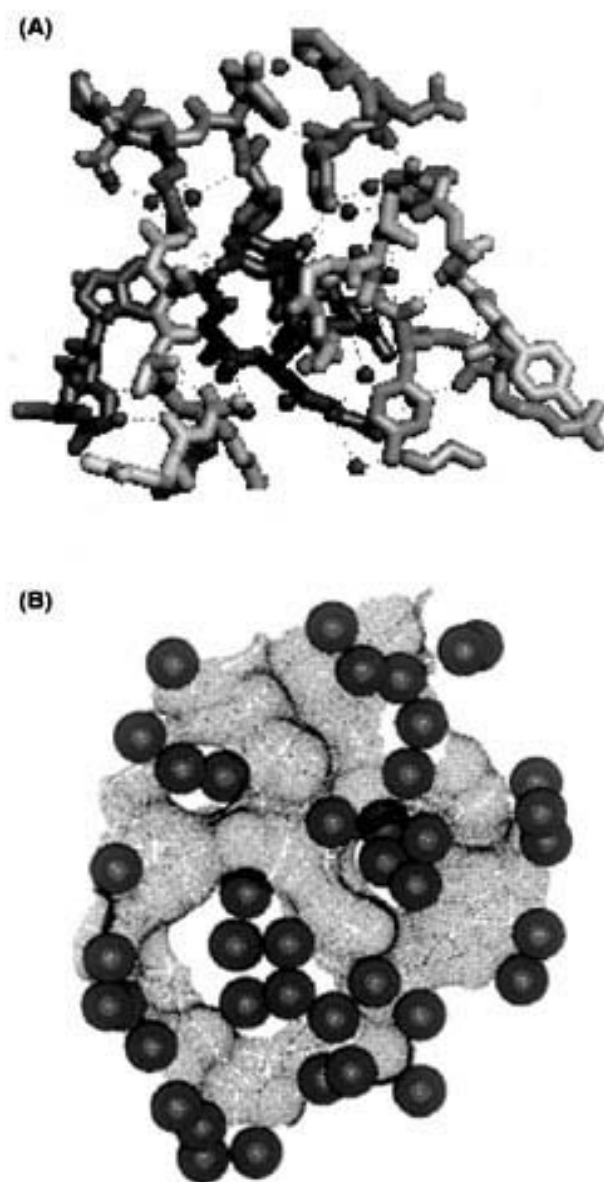


Figure 3 (A) Hydrogen bonding network of the D1.3-HEL interface mediated by bound solvent molecules; 25 water molecules form hydrogen bonds linking the antibody and antigen, directly or through other water molecules. (B) Water molecules in contact with the D1.3-HEL buried surface. Including the 25 bridging water molecules, nearly 50 solvent sites are in contact with the buried surface defined by the D1.3-HEL interface. Many of these water molecules fill internal cavities, further stabilizing the complex. (See also color Plate 8C and D.)

combining site and, perhaps, a mechanism by which the site could adapt to an antigenic determinant without large loss of entropy.

Molecular modeling of combining sites

Modeling of the CDRs of antibodies of unknown three-dimensional structure was attempted in several

laboratories, using different energy minimization and computer graphics approaches. The proposed models were subsequently compared with the determined crystallographic structures. The apparent occurrence of 'canonical structures' for most of the CDRs allowed successful approximation of the folding of the main polypeptide chain for the unknown antibodies. Establishing the orientation of side-chains is generally beyond the reach of these procedures, although in several cases they were correctly predicted. The biggest problems for modeling are posed by 1) the less predictable structure of the CDR3 of V_H ; 2) the relative positioning of V_H and V_L ; 3) the difficulty in placing solvent molecules which could be tightly bound to the combining site or its surroundings and thus contribute to its conformational stability; and 4) predicting possible conformational change that may take place upon antigen binding. Modeling attempts have been partially successful in determining the conformation of some of the CDRs compared with those observed in the crystal structures of the complexes with antigen. Thus they suggest agreement with the experimental observation that there is no major conformational change in going from the uncomplexed to the complexed antibody-combining site.

Genetic engineering of antibody-combining sites

Site-directed mutagenesis experiments performed on an antilysozyme antibody showed that a double mutant V_L Glu28→Ser, V_H Lys56→Gln increased 8- to 9-fold its affinity for the antigen. One interesting conclusion of this work was that removal of electrostatically charged side-chains and their replacement by noncharged residues with hydrogen bond potential allows formation of additional hydrogen bonds with the antigen and leads to an increase in affinity. Certainly, a prime consideration in the production of genetically engineered antibodies is an improvement

in affinity for antigen. However, important questions are yet to be answered in this regard: 1) what are the structural mechanisms which lead to the increase in affinity, and 2) why don't the natural diversification mechanisms produce these same hypervariable sequences?

See also: Affinity; Antibodies, specificity; Antibody-antigen intermolecular forces; Immunoglobulin structure; Antibody-antigen complexes, three-dimensional structures.

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ANTIGEN-PRESENTING CELLS

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Antigen-presenting cells (APCs) refers to the leukocytes responsible for antigen presentation. These include three sets of cells – the mononuclear phagocytes, the Langerhans-dendritic cells and the B cells.

Other cells can be made to have APC function under special conditions.

Antigen presentation is an obligatory step in the recognition of protein antigens by the T cell lineage.

The APCs bear the molecules encoded in the major histocompatibility complex (MHC), the class I and II molecules. These MHC molecules bind peptides resulting from intracellular processing of the protein. The MHC-peptide complex constitutes the antigenic determinant, the epitope, that engages the T cell receptor. Thus APCs are essential cells for the initiation and maintenance of all T cell responses. (See Antigen Presentation via MHC Class I molecules and Antigen Presentation via MHC Class II molecules.)

APCs need to have several properties in order for them to be involved in presentation to CD4 and CD8 T cells: 1) the capacity to take up the antigen; 2) the capacity to internalize the antigen and process it; 3) the synthesis and expression of MHC class I and II molecules; 4) the expression of adhesion molecules, to promote and insure the APC-T cell interaction; and 5) the expression of costimulator molecules (i.e. those molecules that promote the growth and differentiation of T cells during antigen presentation) and the release of cytokines. The APC-T cell interaction affects both cells; it is symbiotic. The APC changes its biology, becoming activated both in the case of macrophages and B cells; and the T cells respond by entering cell cycle and/or expressing cytokine genes. The activation of T cells is a 'two signal' event, dependent, first, on the engagement of its antigen receptor by peptide-MHC complex (signal 1) and, second, on interactions with the costimulatory mol-

ecules (signal 2). The major cell interaction molecules are shown in Table 1.

Not all these properties are expressed to the same degree by all the APCs. The differentiation state of APCs, the environment of a given tissue and the nature of the antigen molecule influence APC function. The T cells may also vary in their requirements, depending on their natural history (i.e. unstimulated versus memory cells). Finally, the antigen molecules themselves dictate the extent to which one or another property becomes important, in three ways. First, the physical and chemical form of the antigen determines the extent of uptake by APCs. Whether the antigen is a small molecule or part of a complex structure like that of a bacterium or a virus makes a difference. In the former case the antigen may enter the cell by fluid phase or receptor-mediated endocytosis, while the latter case involves a phagocytic process with internalization of a large extracellular ligand. Along these lines the capacity to internalize a microbe is limited for some APC-like B cells, in contrast to macrophages. Second, the complexity of the antigen will determine the number of peptides derived from its component proteins that become available for binding to MHC proteins. There is no direct estimate of the number of peptides that can be occupied by an MHC molecule when dealing with a bacterium, a complex protein or a simple polypeptide. But the limited studies indicate that the more protein taken up by the APCs, and the more complex the protein,

Table 1 Molecules involved in APC-T cell interaction

APC	T cells	Interaction
Class I and II MHC molecules	T cell receptor for antigen (TCR)	Engagement of the TCR provides first signal for activation
Class I and II MHC molecules	CD8/CD4 molecules	Function to promote the interaction of TCR with peptide-MHC class I (CD8) or peptide-MHC class II (CD4)
ICAM-1 (CD54) ICAM-2 (CD102) ICAM-3 (CD50)	LFA-1	Major sets of adhesive molecules
LFA-3 (CD58) (in humans), CD48 (in mice)	CD2	Promotes cell interaction and costimulatory function
B7-1 and B7-2	CD28 CTLA-4	CD28 involved in early T cell activation while CTLA-4 inhibits activation
CD40	CD40L	Primarily involved in B7 cell activation → promotes B cell activation
Cytokines IL-1 α and β IL-6 IL-12 TNF IL-10	Cytokine receptors	Interaction Secretion of early cytokines promotes vascular and tissue responses. Secreted by macrophages and DCs. Release of IL-12 promotes T _H 1 differentiation. IL-10 inhibits the system
IFN γ receptor	IFN γ	IFN γ released early by T _H 1 cells promotes macrophage activation

the larger is the number of peptides bound to MHC molecules. Last, the capacity of an antigen to stimulate the APC is a major factor that may lead to the enhanced response to some microorganisms. Indeed, many bacteria trigger the induction of cytokines, costimulatory and adhesion molecules that markedly influence the T cell response. In contrast, bland protein antigens are considerably less effective.

The three main APCs

Brief comments on three main APCs now follow. Macrophages are ubiquitous cells found in most tissues. They are highly active in internalization of a wide range of proteins as well as microorganisms. The extent of expression of class II MHC molecules varies among macrophages of various tissues and also among species. For example, most human monocytes and macrophages express class II MHC molecules, and are active in presenting antigens; in contrast class II MHC molecules are expressed on only a percentage of macrophages in the mouse. Here the percentage of class II-bearing cells varies from most of spleen macrophages to just a few of peritoneal macrophages. Accordingly, antigen presentation varies among them. Regardless, macrophages from all species express high levels of class II MHC molecules after their exposure to interferon γ (IFN γ).

Macrophages are highly effective in presentation of proteins and bacteria, most likely as a result of their highly developed endocytic capacity. They take up proteins by fluid-phase pinocytosis and/or receptor-mediated uptake, i.e. by their binding to specific surface receptors. Macrophages have complement receptors and Fc receptors that enable them to take up opsonized antigens as well as surface receptors for denatured proteins (scavenger receptors) or glycosylated proteins (such as the mannose receptor).

Macrophages are also highly secretory cells. Some of the secretory products are important during antigen presentation and early inductive events. These include interleukin 1 α (IL-1 α) and IL- β , tumor necrosis factor α (TNF α), IL-12, IL-6, IL-8 and a number of other chemokines. This group of early cytokines has effects on T cells as well as on connective tissue and vascular cells and they are believed to be involved in the early inflammatory changes in immunity. Macrophages also express adhesive and costimulatory molecules such as ICAM-1 (CD54), B7-1 (CD80) and B7-2 (CD86), respectively. Their level of expression is increased, particularly after the uptake of microbial antigens.

The Langerhans-dendritic cells constitute a single lineage, abundant in the skin and epithelia and thymus-dependent areas of lymphoid tissues. Langerhans cells are part of the family of dendritic cells (DCs). In the epidermis they constitute about 2–8% of the cells and are found between the keratinocytes, where they were first identified by morphological studies. Langerhans-dendritic cells arrive by way of afferent lymphatics to the thymic-dependent areas of lymphoid organs. In the skin they represent the local APC that takes up antigen molecules that contact the epidermis and carry the antigen by way of the lymphatic circulation to the draining lymph nodes. For example, application of protein-reactive compounds to the skin, such as dinitrofluorobenzene, ultimately results in their binding to the class II MHC of the Langerhans-dendritic cells, and with it the subsequent activation of T cells in the lymph nodes. Ultraviolet irradiation of the epidermis results in their disappearance; if such sensitizing compounds are applied to such areas, no T cell activation takes place.

DCs originate from bone marrow precursors, circulate, and seed the different lymphoid tissue and epithelia. DCs and macrophages have similar progenitors, with monocytes being a common cellular stage prior to the differentiation of the two lineages. In culture, the immature DC can differentiate to a mature state by the action of two cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4.

Another important immunological reaction where Langerhans-dendritic cells participate is in the allograft reaction. Their high content of MHC proteins make the Langerhans-dendritic cells the most powerful allogeneic cell in immunizing the host. Langerhans-dendritic cells are found in variable amounts in the interstitial and connective tissue of most transplanted organs (including liver, kidney, heart and, of course, skin).

DCs constitutively express high levels of class II MHC proteins, which makes them very active as APCs and as stimulator cells in the mixed leukocyte reaction. Langerhans-dendritic cells are active in presenting proteins but their degree of internalization and processing of protein antigens varies as a function of their state of maturation. As with macrophages, they mature in their antigen-presenting capacity. For example, their antigen-presenting capacity is limited when freshly harvested from the epidermis or blood but improves upon brief culture, which is one of the criteria for defining immature and mature DCs. DCs can express costimulatory molecules and release early cytokines such as IL-12.

B cells have an important antigen-presenting func-

tion that forms the basis for their interaction with T cells. B cells bind the specific antigen by way of their membrane immunoglobulin, internalize it and process it to peptides that become bound to their class II MHC molecules. The encounter with T cells reactive to the peptides results in a reciprocal activation of both cells. B cells function best as APCs if they are first activated, as they express only small amounts of costimulatory and adhesion molecules in their resting state. *In vitro* B cells become active in antigen presentation upon culture with anti-immunoglobulin antibodies and cytokines such as IFN γ . Such treatment does not affect the level of class II MHC on B cells but increases the level of adhesion molecules, including ICAM and of B7-1 and B7-2. Expression of class II MHC molecules is constitutive in B cells. This basal level is increased by IL-4 but not by IFN γ .

APC-T cell interaction in tissues

The dynamics of the encounter in tissues between a cloned T cell and the APC bearing the peptide-MHC complex is not entirely known and may depend on a number of conditions. For example, a bacterium that enters a nonlymphoid tissue, the lung alveoli, will be taken up by alveolar macrophages and an inflammatory reaction ensues as the macrophages release early cytokines. This inflammatory reaction is conducive to the encounter between APCs and T cells. A different situation may develop when a contact-sensitizing molecule reaches the skin. Here the Langerhans cells located in the epidermis will take up the sensitizer and will now move via the afferent lymphatic to the draining lymph nodes. Thus, in this reaction part of T cell induction is in part mediated by the transport of the antigen directly to the lymphoid organs.

The anatomy of APC-T cell interaction in lymph nodes and spleen has been difficult to study. A network of APCs are found in the deep cortex of the lymph nodes where presumably the productive trapping of antigen and encounter with the T cell takes place. DCs constitute many of the 'interdigitating cells' found in histological examinations. The relative importance of, and the interplay among, the three APCs described above in a physiological immune response is not entirely understood. A likely scenario is one where antigen is first trapped by the macrophages and DCs of the thymus-dependent areas of lymphoid organs and there recruit and activate antigen-specific CD4 T cells. The subsequent stage envisions B cells binding the antigen molecule via their surface immunoglobulin and interacting with the activated CD4 T cells. In follicles and germinal centers B cells interact with the follicular dendritic

cells that bear antigen-antibody complexes on their surface. This interaction is part of the reactions that lead to B cell activation and differentiation.

The case of CD8 T cells

APCs are involved in the early activation of CD8 T cells by presenting peptides via class I MHC molecules. Once activated, the CD8 T cells will be able to interact and react with class I MHC-peptide complex presented in various target cells. Thus, in the antiviral response the first encounter that recruits and activates specific antiviral clones will be that of infected APCs (either macrophages or DCs) with the CD8 T cells. Once clonal expansion and activation takes place, the CD8 T cells can kill the virally infected target cells. APCs also have the ability to capture proteins from other cells (i.e. tumor antigens, alloantigens, for example) and process it into the class I MHC presentation pathway, a process that needs to be better defined in mechanistic terms. (See Antigen Presentation via MHC Class I Molecules.)

Most epithelial, vascular and mesenchymal cells do not express class II MHC molecules or costimulatory molecules; however, there are exceptions: thymic epithelium normally expresses class II MHC molecules, and some mucosal cells of the gastrointestinal tract and proximal tubular cells of the kidney also express some levels of class II MHC molecules. During inflammation, as a result of local IFN γ production, many cells are made to express class II MHC molecules. Whether this aberrant expression is involved in tissue autoimmunity needs to be settled. Tissue cells express limited amounts of costimulatory molecules which makes them poor APCs for initiating T cell activation.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; B lymphocyte, antigen processing and presentation; B lymphocytes; Dendritic cells; Immunogen; Langerhans cells; Macrophage activation; Microenvironment; Monocytes; Mononuclear phagocyte system; Second signals for lymphocyte activation.

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ANTIGEN-SPECIFIC CELLS: ENRICHMENT AND ISOLATION

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Burnet, in his clonal selection theory of antibody formation, proposed that the precursors of antibody-forming cells carried receptors of unique specificity for a particular antigen. After 'selection' by antigen, the antigen-specific cell would proliferate and eventually give rise to a clone of antibody-secreting cells. The antibody produced would be specific for the original antigen. Given the large number of different antigens in the environment, for any single antigen the frequency of antigen-binding cells would have to be very low, and an individual antigen-binding cell should bind only one antigen. The validity of the clonal selection theory was first demonstrated for B lymphocytes, but was subsequently shown to be true for T lymphocytes, which are also antigen specific.

Antigen-binding cells were first demonstrated and enumerated with large particulate antigens such as bacteria and red blood cells. Binding of soluble antigen by lymphocytes was first visualized by Naor and Sulitzeanu, who used ^{125}I -labeled albumin and autoradiography. The first antigen-binding lymphocytes studied were B cells, which recognize antigen with a unique antigen receptor comprising surface immunoglobulin. The fact that B cells bear this antigen receptor which recognizes antigenic epitopes in their native form has made isolation of antigen-specific B cells relatively successful. In contrast, T cells recognize antigen after processing to peptide form and in the context of major histocompatibility complex (MHC) molecules, and do not bind native antigen. This has made their isolation considerably more difficult. Problems with the isolation of antigen-specific cells are compounded by the relative scarcity of these

cells. Even at the height of the B cell response to haptens such as (4-hydroxy-3-nitrophenyl)acetyl (NP) in the mouse, the antigen-specific B cells constitute no more than 1% spleen cells, declining to <0.01% of cells in the memory cell population. The other problem is that antigen-specific lymphocytes may differ only subtly from their colleagues, and detection of this subtle difference is critical to their isolation.

Early attempts to deplete antigen-specific cells were made to provide evidence in support of the clonal selection theory. These techniques are no longer in common use and will be considered only briefly. Detailed study of the immune response to both experimental immunogens and to pathogens is greatly enhanced by the isolation of antigen-specific lymphocytes. Techniques for such enrichment and isolation are more advanced for B cells, which will be considered first. Recent techniques employed for the isolation of antigen-specific T cells will then be examined.

Enrichment and isolation of B lymphocytes

Depletion

The first attempts to deplete antigen-binding cells were made by Wigzell and colleagues using antigen-coated beads in affinity columns, which depleted antigen-binding cells but also a significant number of nonspecific cells. An alternative approach was to deplete antigen-specific cells by allowing them to bind radioactive antigen of high enough specific activity to kill them; a technique sometimes referred to as 'antigen suicide'. Ada and Byrt demonstrated

that incubation in the cold of lymphoid cells with ^{125}I -labeled T-independent antigens specifically abolished the response of these cells to the unlabeled antigen on adoptive transfer into irradiated syngeneic recipients, establishing that antigen-binding B cells were the precursors of antibody-forming cells. These experiments provided strong support for the clonal selection theory but did not yield purified populations of antigen-specific cells for further study. Such methods are no longer widely used.

Enrichment: historical methods

A number of approaches have been taken to enrich lymphocyte populations for antigen-specific cells. Some are now of historical interest only. Such techniques include the use of affinity matrices and columns. Two major problems with these modalities were nonspecific adsorption of cells to affinity matrices and difficulty in recovering viable, functional cells after interaction with antigen.

Another early method for isolating antigen-specific cells was the use of hapten–gelatin-coated Petri dishes ('panning'). Cell suspensions were added to the dishes, binding of hapten-specific B cells occurred, unbound cells were washed off and bound cells recovered by melting the gelatin layer at 37°C. A major disadvantage of this method was its relatively low cell yield, but combined with limiting dilution assays, it was a useful method for studying antigen-specific B cell activation.

Rosetting has also been used to enrich for antigen-specific B cells. For example, haptenated horse red blood cells (HRBCs) were allowed to bind to hapten-specific B cells, forming rosettes. The rosettes could then be separated from nonrosetted cells by buoyant density and/or sedimentation velocity centrifugation, and the HRBCs removed from the antigen-binding cells by protease digestion, followed by overnight culture of the cells to allow for receptor regeneration.

Enrichment: 'Pre-enrichment' by depletion of nonantigen-specific cells

Partial purification or enrichment of antigen-specific cells subsequently allows more rapid and accurate isolation of these cells by more time-consuming or expensive procedures, such as fluorescence-activated cell sorting (FACS: see below). The removal of unwanted cells can be achieved by physical means, such as by taking advantage of the propensity of T cells to bind to nylon wool columns or of macrophages to adhere to plastic. Depletion of unwanted cells using complement fixation is a commonly used and effective technique. To enrich for immunoglobulin G-positive (IgG⁺) B cells, for example, spleen cells are cultured in a cocktail of antibodies against

T cells (e.g. anti-CD3), macrophages and other B cells (e.g. anti-IgM). Provided the chosen antibodies fix complement efficiently (IgM antibodies are best) and have been properly titrated to reduce nonspecific killing, considerable enrichment and high yields can be achieved. Similar cocktails of antibodies conjugated directly or indirectly (via biotin-avidin) to magnetic beads can be used to remove unwanted cells, as cells binding the paramagnetic beads will stick to steel wool in a column or to the side of a container when a magnetic field is applied, while the desired cells will pass through the column (Figure 1). A potential hazard of this technique is the risk of saturating the column: if the surface area of the steel wool is inadequate to bind the number of cells to be depleted they too will pass through the column causing substantial contamination. One advantage of these 'negative selection' methods of enrichment is that they do not require antibody binding to, and thus potential activation of, the antigen-specific cells.

Enrichment: positive 'pre-enrichment'

Paramagnetic beads can also be used to enrich for antigen-specific B cells in a 'positive' fashion. If, for example, the antigen-specific B cells have a characteristic immunoglobulin isotype (such as the γ heavy and λ light chains in the response to NP) antibodies to these can be used to attach magnetic beads to the cells of interest. These cells will then attach to the column as described above, and can be washed by passage of medium through the column, followed by their removal by flushing after withdrawing the column from the magnetic field (Figure 1). Saturation of the column will not result in impurity of the resulting population, but only a reduced yield. In the example above, enrichment of spleen cells using anti-IgG1 and paramagnetic beads can result in a 40-fold enrichment for anti-NP-specific B cells, with a proportionate reduction in the flow cytometry time needed to complete the purification.

One problem with positive enrichment using these columns is nonspecific adhesion of dead cells to the column. If this is a problem, dead cells may have to be removed in a separate initial step, for example using density gradient separation. Direct conjugation of antigen to paramagnetic beads is currently being attempted which should improve isolation of antigen-specific cells by this method.

The flow cytometer can itself be used to pre-enrich cells prior to a further round of flow cytometry. Very rapid sorting by FACS results in a predictable number of errors and therefore impurity. Substantial enrichment is nonetheless achieved during this very rapid sorting, and the time needed for the subsequent

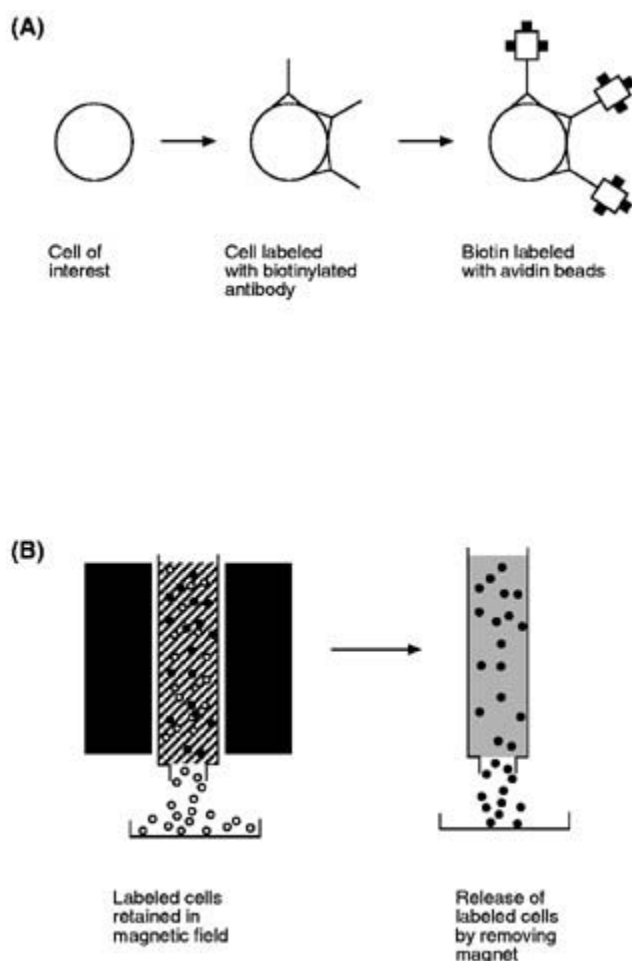


Figure 1 Schematic representation of the use of paramagnetic beads for 'positive' enrichment. (A) The desired cells are labeled with a biotinylated antibody, washed, then attached to avidin beads. (B) Labeled cells are retained when a magnetic field is applied, and unwanted cells are washed through. The desired cells are then recovered after removal of the column or tube from the magnetic field.

slower and more accurate sort is reduced resulting in an overall saving of time.

Isolation: fluorescence-activated cell sorting

FACS provides the most reliable and accurate means of purifying antigen-specific B cells. B cells can be identified by a number of markers, but the ability to detect binding of a specific antigen is crucial to identifying antigen-specific cells. Initial success using this method was achieved by Hayakawa and colleagues, who immunized mice with the fluorescent protein phycoerythrin (PE), later isolating PE-binding B cells on the FACS. This technique was later advanced by Lalor, McHeyzer-Williams and Nossal, who identified NP-specific B cells by detecting binding of NP conjugated to allophycocyanin, allowing further dissection of a well-characterized stereotypic immune response.

A major drawback of this technique has been the time (and therefore expense) taken to sort substantial number of cells if these are very rare. This can now be largely overcome by using one or a combination of the pre-enrichment techniques described above.

Another advance has been the advent of techniques allowing analysis of single cells, which removes the need to sort large numbers of cells. For example, a polymerase chain reaction (PCR)-based strategy can be used to amplify single genes from cDNA which can subsequently be sequenced. This has allowed analysis of patterns of somatic hypermutation in very rare subpopulations of antigen-specific B cells, such as the bone marrow antibody-forming cells described below.

Another major problem is the potential difficulty in ensuring the purity of very rare populations of cells, as relatively few cells which are incorrectly sorted can reduce the purity of such populations dramatically. This has been overcome by making maximal use of the number of detection parameters available on modern FACS machines. Consider the example of the isolation of NP-specific bone marrow antibody-forming cells shown in Figure 2. First, all cells which do not have the forward and side scatter characteristics (which measure size and 'complexity' respectively) of lymphocytes are excluded. One channel is then used to identify and exclude unwanted

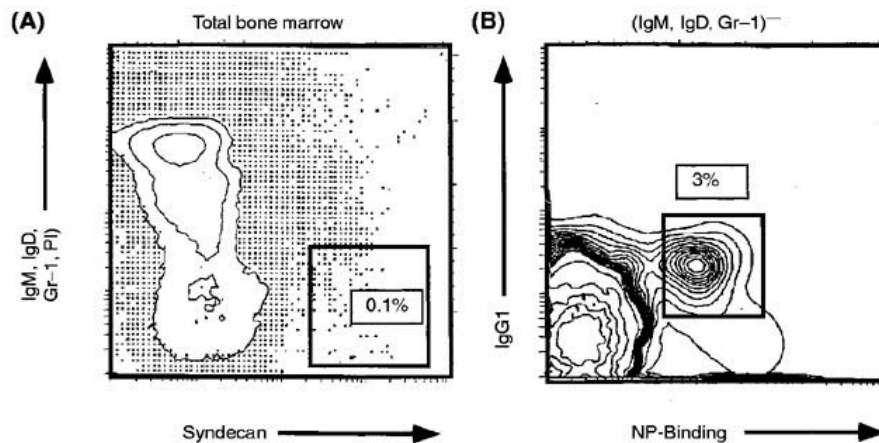


Figure 2 Identification of NP-specific antibody-forming cells in the bone marrow after 14 days of the primary immune response. (A) Cells staining for IgM, IgD and Gr-1 and propidium iodide were excluded. Antibody-forming cells were identified by their expression of syndecan. (B) This population was further partitioned into cells expressing IgG1 and able to bind the immunizing hapten NP, coupled to a fluorochrome. In the example shown, the NP-specific antibody-forming cell population represents 0.003% of nucleated bone marrow cells. Boxes indicate the criteria used for sorting cells for V_H gene sequencing.

cells. Antibodies to IgM, IgD and the myeloid-macrophage lineage marker Gr-1 are conjugated to PE, and dead cells are labeled with propidium iodide (which fluoresces in the PE channel), and all of the cells so labeled can be excluded from further analysis (a 'dump' channel). The addition of a dump channel to a single positive selection channel can decrease nonspecific contamination to less than 1 in 10^6 cells. Two further antibodies identify markers characteristic of the cells required – syndecan (a marker of antibody-forming cells, in the fluorescein channel) and surface IgG1 (in the Texas Red channel). Finally a sixth parameter is used to ensure antigen specificity, that is, the ability of the $\text{IgM}^- \text{IgD}^- \text{Gr-1}^- \text{syndecan}^+ \text{IgG1}^+$ cells to bind the original immunizing antigen NP bound to allophycocyanin. The probability of a nonspecifically staining cell having the same characteristics as the required cells in all six parameters is so low that populations of antigen-specific cells as rare as 1 in 200 000 cells can now be reliably and consistently sorted to >95% purity, even in the absence of pre-enrichment (see Figure 2).

Isolation: histology

The groups of Kelsoe, Rajewsky and others have recently isolated antigen-specific B (and germinal center T) cells by micromanipulation of single nuclei from histological sections. While this can only isolate small numbers of nuclei for PCR analysis, it does permit correlation of the molecular information obtained by PCR with the exact location of the cell in a stained histological section. This has allowed

elegant analyses to be performed of the development of the postantigenic B cell response which would not have been possible using conventional techniques.

Isolation: transgenic mice

Another approach to isolation of antigen-specific cells is the creation of mice bearing transgene-encoded antigen receptors of known specificity. Well-known examples are the mice transgenic for a T cell receptor specific for the male HY antigen, and those transgenic for immunoglobulin binding hen egg lysozyme. Thus the vast majority of the lymphocytes in such mice are of known specificity. This avoids the need to isolate rare cells, but requires careful interpretation of results since they have been obtained from mice with a markedly skewed lymphocyte repertoire.

Enrichment and isolation of T lymphocytes

T cell receptors (TCRs) recognize processed antigen (peptides) in the context of MHC. It is therefore currently not possible to isolate antigen-specific T cells by detecting antigen binding using the FACS or paramagnetic bead technology. Clearly, however, all the pre-enrichment techniques described above for B cells also apply to T cells. A traditional approach to isolating antigen-specific T cells has been to stimulate T cell populations with antigen and subsequently to clone cells activated by that antigen. The option is then available to make hybridomas from single cloned antigen-specific T cells.

A number of novel approaches have been taken to the isolation of antigen-specific T cells in the absence of an *in vitro* activation and cloning step. Monoclonal antibodies have been raised which recognize a single clonotype of TCR and which, in principle, could be used to identify antigen-specific cells in responses utilizing this clone. Some T cell responses use a limited and well-defined TCR, and detection of these cells by FACS can serve as the basis for identifying antigen-specific cells. A recent example is the identification by McHeyzer-Williams and Davis of T cells responding to pigeon cytochrome C in draining lymph nodes after immunization by staining for V α 11V β 3 TCR.

See also: Cell separation techniques; Clonal selection; Flow cytometry; Fluorochrome labeling; Rosetting techniques; T cell receptor, recognition by.

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ANTIGEN CLEARANCE

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The clearance of exogenous antigens constitutes one of the primary functions of the immune system. Antigens are mainly eliminated by the cells of the fixed mononuclear phagocytic system. The mechanism of removal depends on the biological and physico-chemical properties of the antigen concerned, its mode of presentation to the immune system, and its ability to elicit a specific humoral or cellular immune response.

Antigen clearance by immune complex formation

Immune adherence, complement receptor type 1 and immune complex transport

Immune complexes that form within or enter the circulation may have to travel some distance around the body before reaching one of the organs of the

fixed mononuclear phagocytic system. The majority of immune complexes travel through the circulation bound to receptors on the surface of circulating cells rather than free in plasma. On arrival in the fixed mononuclear phagocytic system, they are transferred from the carrier cell to fixed macrophages.

The binding of immune complexes to receptors on carrier cells was first described for complement-coated microorganisms many years ago. Pneumococci injected intravenously into immune rabbits were shown to cluster around platelets in blood taken by cardiac puncture, and it was also shown in early studies that the serum of rats which had recovered from infection with *Trypanosoma brucei* would cause blood platelets to adhere to *T. brucei* *in vitro*. It was subsequently found in humans and other primates that erythrocytes, rather than platelets were the main cell in blood to which opsonized trypanosomes would bind. The role of complement

in mediating these binding reactions was initially suggested by Kritschewsky and Tscherikower, working in Moscow, who observed that sera inactivated at 55°C would not mediate binding. Subsequently, it was shown that erythrocyte adherence reactions could be abolished by heat treatment of sera and by dilute ammonia (inactivating C4). The observation that treatment of serum with cobra venom factor (CVF) prevented adherence reactions showed an essential role for C3. Nelson and colleagues in the 1950s rediscovered this 'immune adherence' phenomenon in studies of the reactions between human erythrocytes and specifically-opsonized treponemes and pneumococci.

Our present understanding of the immune adherence phenomenon followed the isolation by Fearon of the molecule responsible for the adherence reactions of human erythrocytes, the C3b/iC3b receptor (complement receptor type 1, CR1, CD35). This receptor bound large immune complexes and was shown to play a role in the transport of soluble immune complexes *in vivo* in primates. CR1 is a receptor for C3b and iC3b with diverse activities including: 1) the uptake by phagocytic cells of C3b- and iC3b-coated immune complexes and particles; 2) acting as a cofactor for factor I-mediated cleavage of C3b to iC3b and then C3dg; and 3) acting as a transport molecule for immune complexes, both soluble and particulate. Relevant to antigen clearance is the role of CR1 as a transport molecule.

There is considerable interspecies variation of the sites of expression of CR1. In humans and other primates the majority of CR1 in the circulation is located in a clustered form on erythrocytes. The actual number of CR1 molecules per erythrocyte is extremely low, varying between 50 and approximately 1000 receptors per cell in humans. This compares with 5000 to 50 000 receptors per neutrophil depending on the state of cellular activation. However, in spite of this unfavorable comparison, red cells play an important role in the binding and transport of C3b- and iC3b-coated immune complexes and particles through the circulation, partly as a result of the vast numerical majority of red cells over other cell types (approximately 1000 erythrocytes for every neutrophil) and partly as a consequence of the clustered nature of CR1 on erythrocytes which promotes very high avidity interactions with ligand, in comparison to neutrophils and lymphocytes on which the receptor is expressed as cell surface monomers. In other species a hybrid molecule sharing the activities expressed by human CR1 and CR2 is expressed on leukocytes and platelets, and no complement receptor is expressed on erythrocytes.

***In vivo* studies of soluble immune complex processing in animals**

The major site of clearance of immune complexes is the fixed mononuclear phagocytic system. In most species, including lagomorphs, primates and rodents, the liver and spleen are the primary sites in the circulation in which tissue macrophages are located. However, pulmonary intravascular macrophages are also found in pigs, cows, sheep, goats and cats, and these have been shown to be important both in the clearance of particles and soluble immune complexes.

In lagomorphs and rodents soluble immune complexes, injected intravenously, were predominantly removed in the liver and spleen, as in humans, but the complexes were not transported in the circulation bound to erythrocytes, which in these species do not bear CR1. Platelets in these species bear C3b receptors and rapid *in vivo* binding of immune complexes to platelets has been observed following intravenous injection.

Evidence for the importance of antigen as well as antibody in determining the clearance kinetics of immune complexes came from studies of the clearance in mice of immune complexes containing as antigen either orosomucoid or ceruloplasmin or their desialylated derivatives. The asialo-orosomucoid-containing complexes were cleared 20-fold more rapidly than those containing the sialylated molecule, and blocking studies showed that the rapid clearance phase was mediated by a hepatocyte carbohydrate receptor.

Antigen clearance by immune complex formation in primates was first shown in a series of experiments in baboons. Large radiolabeled immune complexes comprising BSA/anti-BSA were initially employed. Following intra-aortic infusion, these complexes bound rapidly to erythrocytes, and were subsequently cleared during transit through the hepatic, but not renal circulation. The effect of complement-depletion was evaluated by CVF treatment. In decomplemented animals, the immune complexes did not bind to red cells, and were cleared more rapidly, depositing in other organs, including the kidney. It was subsequently shown that immunoglobulin A (IgA)-containing complexes, which fixed complement poorly, also failed to bind to baboon erythrocyte CR1, were cleared rapidly, and localized in other organs.

The liver and spleen are not the main sites of immune complex clearance in all species. In pigs, ruminants and cats, the anatomy of the fixed mononuclear phagocytic system is different and intravascular pulmonary macrophages play an important

role in the clearance of immune complexes and also in the clearance of bacteria. Immune complex processing in pigs was associated with a fall in peripheral blood mononuclear cell numbers not seen in studies of immune complex clearance in humans.

***In vivo* studies of soluble immune complex processing in humans**

The *in vivo* processing of exogenously administered soluble immune complexes in humans has been studied in three main model systems. These are heat-aggregated IgG, tetanus toxoid (TT)/anti-TT, and hepatitis B surface antigen (HBsAg)/anti-HBsAg immune complexes. ^{125}I -labeled heat-aggregated IgG injected intravenously into normal subjects was shown by γ scintigraphy to be cleared in the liver and spleen. The complement- and CR1-dependent natures of soluble immune complex clearance mechanisms were first studied *in vivo* using ^{125}I -labeled tetanus toxoid/anti-tetanus toxoid complexes. Either native complexes or complexes preopsonized *in vitro* with autologous serum were injected into normal volunteers, and into patients with immune complex disease or hypocomplementemia. Immune complexes bound to erythrocyte CR1 receptors in a complement-dependent manner, and CR1 number correlated with the level of uptake. Two phases of clearance were seen. In subjects with low CR1 numbers and hypocomplementemia there was a very rapid initial disappearance of immune complexes. The second phase of clearance was approximately mono-exponential, and the observed elimination rate correlated inversely with CR1 numbers and the binding of immune complexes to red cells. Similar *in vivo* studies of soluble immune complex clearance have been performed using ^{123}I -labeled HBsAg/anti-HBsAg immune complexes. The fate of these immune complexes was followed by blood sampling and γ scintigraphy to define the site and kinetics of processing in normal subjects, patients with systemic lupus erythematosus (SLE) and a single patient with homozygous C2 deficiency. In normal subjects, complexes were cleared in the liver and spleen, with a median clearance half-time of 5 min. At 10 min around 40% of the injected complexes were bound in the liver in normal subjects. The majority of the injected complexes bound to red cell CR1 at 2 min. In all subjects there was a very close correlation between *in vivo* binding to red cells and CR1 number. In hypocomplementemic SLE patients there was more rapid initial immune complex uptake into the liver, but subsequent re-release of complexes back into the circulation. Defective splenic immune complex uptake was also observed in these patients, and repletion experiments in a C2-deficient subject

showed that the splenic uptake of soluble labeled HBsAg/anti-HBsAg immune complexes was entirely complement dependent. The early work of Pepys and Klaus and colleagues demonstrated that complement is critical for facilitating the localization of certain antigens to follicular dendritic cells, and for the development of a memory immune response. Defective antigen processing and splenic localization of immune complexes in hypocomplementemia may be a factor in the development of autoimmunity in SLE.

A significant criticism of all of the studies of immune complex processing described above is that they all involve large immune complex prepared *in vitro* in the absence of complement. Physiologic antigen clearance *in vivo* will frequently involve the formation of complexes between an exogenous antigen and pre-existing antibody. A number of attempts have been made to model this situation. The successive infusion of human anti-dsDNA antibodies and dsDNA into monkeys and rabbits led to rapid formation of immune complexes which bound to red cell CR1. The formation and fate of immune complex formed *in vivo* in human subjects receiving radio-immunotherapy has also been studied. Successive administration of a radiolabeled mouse antitumor antibody and a human anti-mouse antibody resulted in the formation of immune complexes comprising the two antibody species. Rapid clearance of complexes was observed with binding of approximately 10% of the complexed material to CR1. Systemic complement activation, and a 30% fall in erythrocyte CR1 numbers were observed. External γ counter monitoring indicated that clearance took place primarily in the liver.

Clearance of an autoantigen – erythrocytes

The class of antibody bound to the antigen influences the site of clearance of particulate immune complexes. This is illustrated by studies of autoimmune hemolytic anemia. Cold agglutinin disease is mediated by IgM anti-I antibodies, which stimulate efficient classical pathway activation and fixation of C4 and C3 to erythrocytes. The role of anti-I and complement in mediating cell lysis was first studied in a rabbit model. IgM cold agglutinin was injected into C3-depleted and C6-deficient rabbits. The latter developed thrombocytopenia, neutropenia and a fall in hemoglobin, with only minimal hemoglobinemia, but a sharp fall in plasma C3. Circulating red cells could be readily agglutinated with antibodies to C3, and *in vivo* immune adherence of platelets to red cells occurred. In the C3-depleted animals injection of the anti-I IgM produced no significant hematological changes. The fate of erythrocytes in cold agglutinin disease has been modeled in primates using

radiolabeled cells coated with an IgM cold agglutinin. The liver was the main site of red cell uptake. Transient retention of cells in this organ occurred, thought to be mediated by reversible binding to complement receptors. In patients with cold agglutinin disease, circulating red cells are characterized by a change to a microspherocytic form, accompanied by the presence of many thousands of C3dg molecules bound per cell. Three possible conclusions may be drawn from these observations. The first is that phagocytic uptake of erythrocytes which have bound IgM and fixed C3 and C4 is very inefficient. The second is that, during the transient retention of red cells in the liver, it is probable that the C3 is catabolized to C3dg and erythrocyte shape changes occur. The third is that there is no clearance receptor in the circulation for C3dg-coated particles.

By contrast, in warm hemolytic anemia mediated by IgG, uptake of erythrocytes occurs predominantly in the spleen by Fc-dependent pathways. ⁵¹Cr-labeled incompatible red cells, transfused into recipients with noncomplement fixing antibodies, were removed from the circulation monoexponentially with a half-time of 18–20 min. Splenic uptake was also monoexponential, with a similar half-time. No uptake was detectable elsewhere, notably in the liver. As the spleen exclusively removed cells, and this organ receives only a small fraction of the cardiac output, it was reasoned that the splenic extraction efficiency was very high. It was subsequently demonstrated that the site of sensitized red cell destruction was dependent on the degree of antibody coating, with more heavily coated cells being destroyed predominantly by the liver.

Radiolabeled erythrocytes, coated with IgG, have been extensively studied as a probe of the capacity of the mononuclear phagocytic system to remove particulate antigens. In humans and other species, these cells are cleared largely in the spleen. The mechanisms whereby this probe exhibits primarily splenic clearance, while cells coated with IgM and C3, and soluble immune complexes, discussed above, localize predominantly in the liver, are poorly understood. Tissue macrophages in the liver and spleen bear both Fc and complement receptors (CR1, CR3 and CR4), while follicular dendritic cells within the spleen and lymph nodes also bear CR2. The Fc receptors primarily involved in interaction with complexes or aggregated immunoglobulin are the relatively low-affinity receptors FcγRII and FcγRIII. The binding and retention of an immune complex within the liver and spleen will partly reflect the specific receptor(s) with which it interacts.

Nonclonal receptors in antigen clearance

Growing evidence suggests that initial interaction of pathogens with the innate immune system can initiate and direct the development of the adaptive immune response. As discussed, pathogens opsonized by serum proteins are phagocytosed and cleared from the body by cells of the immune system. In addition, germline-encoded receptors with the capacity directly to recognize nonopsonized microorganisms have been identified on cells of the innate immune system. Janeway proposed that such nonclonal receptors, termed 'pattern-recognition molecules', bind common constituents of pathogenic microorganisms, providing a first line of defense in the preimmune host. The wide spectrum of molecules recognized would not be expressed on the surface of normal cells, thus allowing discrimination of non-self from self. Scavenger receptors, CD36, CD14, and mannose receptors have been proposed as candidate pattern-recognition receptors.

Scavenger receptors

Initially identified for the ability to bind low-density lipoproteins (LDLs), macrophage scavenger receptors (SRs) were found to exhibit broad polyanionic ligand binding specificity, a characteristic of pattern-recognition molecules. Three independent subclasses of receptor, SR-A, B and C have been described, with expression limited to monocytes, macrophages and hemocytes. Type I and II class A SRs bind to and remove endotoxin (lipid A of LPS) from plasma. Whole gram-positive bacteria and lipoteichoic acid (LTA), a ubiquitous gram-positive cell wall constituent, were shown to be additional ligands for type I SR-As. A third recently described SR-A, 'MARCO', expressed on splenic and lymph node macrophages was also reported to interact with *E. coli* and *Staphylococcus aureus*. A primary function of SR-As on macrophages, monocytes and hemocytes appears to be the clearance of toxic shock-inducing microbial cell surface constituents and pathogenic microorganisms from the body.

The rapid phagocytosis of damaged and apoptotic cells prevents the release of noxious intracellular molecules into the body and prevents exposure of the adaptive immune system to unseen components of self. Ailing cells express surface molecular structures not characteristically found on normal cells. Growing evidence supports the proposal that SRs function in the clearance of apoptotic and damaged cells. COS cells acquired the capacity to phagocytose apoptotic cells when transfected with the class B SR CD36. CD36 has also been implicated in mediating the

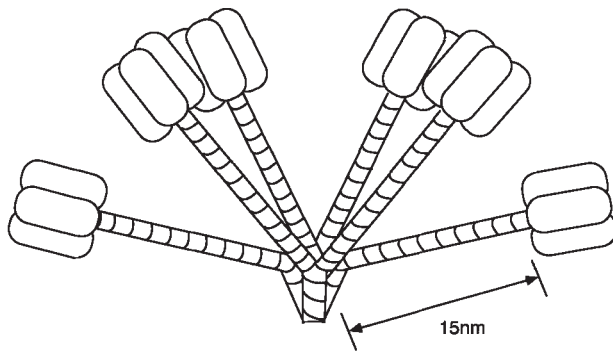


Figure 1 The structure of mannose-binding lectin.

clearance of *Plasmodium falciparum*-infected red blood cells, apoptotic neutrophils and leukocytes, and as a rhinovirus receptor. Recent reports indicated that class A SRs facilitated the uptake of

oxidatively damaged erythrocytes. The uptake of approximately 50% of apoptotic thymocytes *in vitro* was recently reported to be SR-A dependent, a finding yet to be demonstrated *in vivo*. Therefore, SRs appear to be candidate receptors for molecules expressed on damaged or apoptotic cells.

CD14

CD14 is a 55 kDa glycoprotein found as a glycosyl phosphatidylinositol (GPI)-anchored species on monocytes, macrophages and as an abundant protein in plasma. It interacts with soluble lipopolysaccharide (LPS) released from gram-negative bacteria in combination with a plasma protein, LPS-binding protein (LBP). Blockade by mAb or elimination of CD14 expression by gene knockout technology lowered the sensitivity of animals to LPS by 100–10 000 fold. CD14 exhibits many characteristics of a pattern-recognition receptor. The binding of many

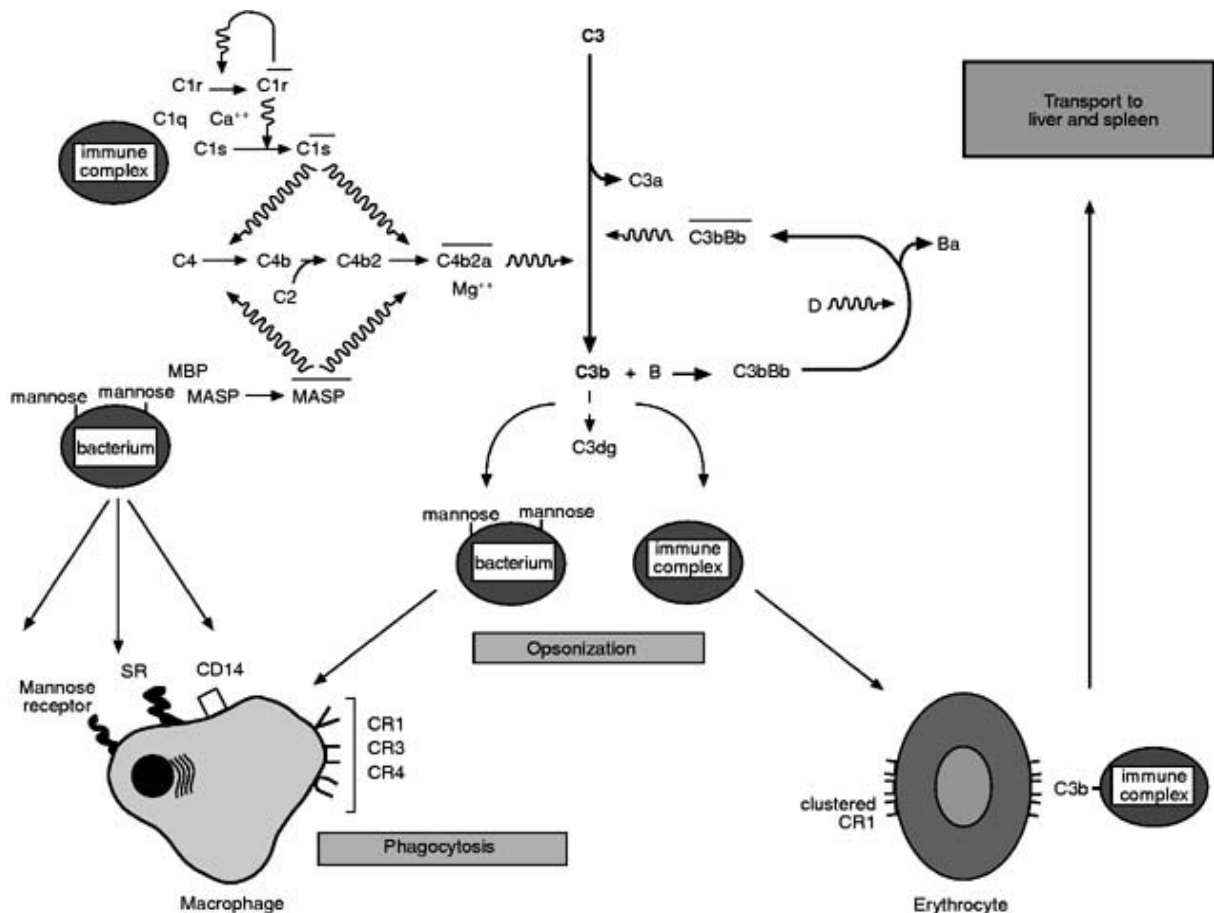


Figure 2 Adaptive and innate immune mechanisms of antigen clearance.

different components of both gram-negative and gram-positive bacteria to macrophages and monocytes has been shown to be CD14 dependent. Indeed, a nonmonocytic cell line acquired the capacity to bind intact gram-negative bacteria following transfection with the CD14 gene. Furthermore, CD14-dependent phagocytosis of whole gram-negative bacteria was recently demonstrated *in vitro*.

CD14/bacterial interaction induces activation of macrophages. The cytokines produced act to recruit lymphoid cells and possibly direct the resulting adaptive immune response. A recent report has suggested that interleukin 12 (IL-12) production following macrophage contact with LTA from gram-positive bacteria can be CD14 dependent. IL-12 is a potent stimulator of T_H1 CD4 T cells. Therefore CD14-dependent signals might exert an early influence on the nature of any resultant adaptive immune response. Furthermore, the release of the potent inflammatory cytokines tumor necrosis factor α (TNF α), IL-1 β and IL-6 following bacterial activation of macrophages appears in part to be CD14 dependent. These mediators recruit phagocytic cells, principally neutrophils, to the site of infection which may enhance the clearance of antigen and influence disease progression. However, if uncontrolled, these same cytokines can contribute to the development of sepsis syndrome in humans. CD14 plays a major role in this disease as CD14-deficient mice are highly resistant to toxic shock. Numerous anti-LPS antibody reagents have been used clinically in an attempt to modify the development of septic shock in infected patients, with to date disappointing results, presumably because the cytokine-mediated physiological response responsible for the syndrome is already established in most cases by the time the therapeutic agent is administered.

Mannose receptors

The mannose receptor is a well-characterized macrophage membrane lectin expressed on tissue macrophages throughout the body but not on circulating monocytes. Evidence strongly suggests that mannose receptors play a role in the clearance of pathogens. The mannose receptor is known to bind to mannose- and fucose-containing microorganisms by carbohydrate recognition domains. Numerous reports have detailed mannose receptor recognition of yeast, bacteria and certain protozoa. Furthermore, non-phagocytic COS cells gained the capacity to bind and ingest microorganisms following transfection with mannose receptor genes, a clear indication that the mannose receptor is a professional receptor for phagocytosis. The engagement and phagocytosis of microorganisms by the macrophage mannose recep-

tor actively stimulates the release of secretory products including IL-1, TNF α and reactive oxygen intermediates, further enhancing the clearance of antigen.

It is of interest that dendritic cells express the mannose receptor and a membrane lectin (DEC-205) with structural similarities and binding specificities to that of the macrophage mannose receptor. These receptors have a potential role in the enhancement of the capture, processing and presentation of antigen to the adaptive immune system by dendritic cells.

Mannose-binding protein/lectin (MBP/L)

MBP/L is a soluble serum protein and belongs to the collectin family of proteins, which is becoming increasingly recognized as an important component of the nonclonal immune system. MBP/L is a C-type lectin (Figure 1) which mediates calcium-dependent binding of certain carbohydrates. The protein recognizes mannose and N-acetylglucosamine residues on gram-positive and negative bacteria as well as yeast and certain parasites, protozoa, mycobacteria and viruses. Acting in concert with an MBP-associated serine protease (MASP), MBP/L can activate the classical complement pathway by cleaving the C4 and C2 molecules generating C4b2a complexes with C3 convertase activity. Indeed, MBP interaction with rough *E. coli* induces complement activation and bactericidal lysis. MBP can also act as an opsonin, either by inducing C3b deposition and subsequent clearance by phagocytic cells or independently by binding to C1q receptors.

The different mechanisms of antigen clearance we have described, involving both the innate and adaptive immune systems are summarized in Figure 2.

See also: Bacteria, immunity to; Bacterial cell walls; Carbohydrate antigens; Chemotaxis; Complement receptors; Endotoxin (lipopolysaccharide (LPS)); Fc receptors; Immune adherence; Immune complexes; Innate immunity; Mononuclear phagocyte system; Opsonization.

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ANTIGEN, ENTRY INTO THE BODY

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The epithelia of the skin and the mucous membranes of the respiratory, gastrointestinal and urogenital tract represent an extensive surface area. They are constantly exposed to a great variety of microorganisms and other antigenic substances. The skin constitutes a strong mechanical (keratin layer) and physiological (gland products, in particular sebum) barrier towards antigen. In the mucous membranes, which are only covered by a thin specialized epithelium, other mechanisms such as ciliary function and nonspecific and antimicrobial factors (e.g. mucin and lysozyme) are involved in preventing or combating infections. In addition, specific immune reactions play a part. The hallmark of specific mucosal immunity is the production and action of secretory immunoglobulin A (sIgA), which displays its functions at the mucosal surface ('outside' the body), and of effector T cells. Thus, the epithelia of the skin and the mucous membranes are primarily engaged in antigen exclusion; only under pathological conditions (e.g. wounding, infections) is there a 'free' exchange of antigens between the external and internal environment. Both types of epithelia possess antigen handling (uptake, processing and presentation) systems.

Antigen uptake by the skin

Several types of nonepithelial cells can be found in the skin, such as lymphocytes, macrophages and, in the epidermis, Langerhans cells. Langerhans cells are involved in the processing and presentation of antigens that enter the skin. They form an extensive network in the basal layers of the epidermis and are efficient antigen-processing cells, capable of taking

up exogenous antigens by virtue of several specialized surface receptors. The display of antigenic peptides by major histocompatibility complex (MHC) molecules on the surface of the cell permits presentation to passing T cells. This process can take place in the epidermis, but Langerhans cells can also, under the influence of factors produced in the skin as a result of antigenic stimulation, leave their sentinel position in the basal layers of the epidermis. As shown in kinetic studies using fluoresceinated dyes, they can be found in lymphatic vessels and migrate into the draining lymph nodes. During this migration they show characteristic, extended membrane processes, for which they are termed veiled cells. Once they have arrived in the lymph node they localize in the paracortical areas where, as dendritic, interdigitating cells, they play a pivotal role in the primary activation of T lymphocytes. Thus Langerhans cells, veiled cells and interdigitating cells are different stages of the dendritic cell lineage (Figure 1). While Langerhans cells are extremely efficient at processing antigen, once they arrive in the lymph node they differentiate into potent presenting cells at the expense of their processing capacity. This is reflected in the expression of MHC molecules and accessory molecules, which increase during the differentiation into interdigitating cells.

It is only under pathological conditions that cells in the skin other than Langerhans cells are involved in antigen handling. In such cases keratinocytes can express MHC class II molecules and are able to present antigen. Studies on squamous (mucosal) epithelia have shown that antigen-processing and -presenting dendritic cells also occur in the non-keratinizing epithelia of the oral and nasal mucosa,

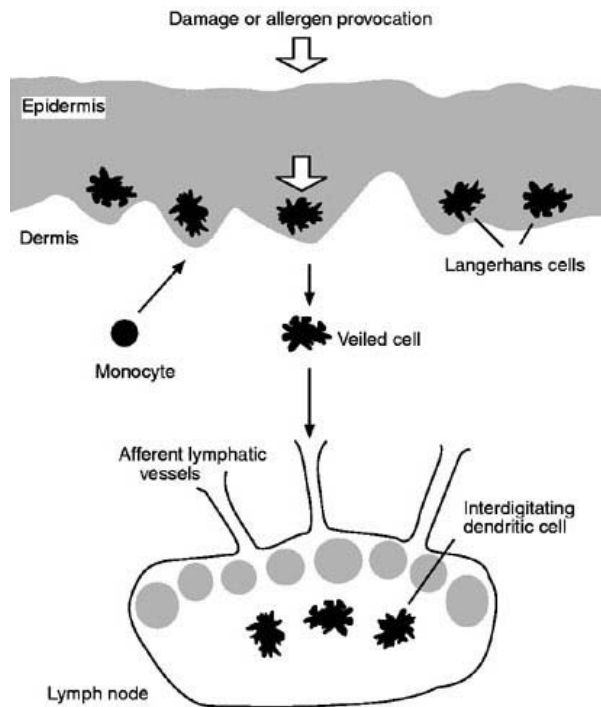


Figure 1 Migration of Langerhans cells from the epidermis into the draining lymph node. Antigens are processed in the skin by Langerhans cells, and stimuli produced by keratinocytes induce the cells to migrate to the lymph nodes. Here they differentiate into antigen-presenting dendritic cells. Langerhans cells are replaced by monocyte-derived precursors.

the trachea and bronchi, the esophagus and the tonsils, and can form comparable networks at these sites. In simple epithelial membranes, such as the epithelium lining the lung, the stomach and the gut, there is no proof for the existence of extensive networks of dendritic cells, although solitary dendritic cells have been demonstrated. In these organs other antigen-handling systems are operative (see below).

Antigen uptake by mucosal surfaces

The structure of mucosal surfaces (epithelium with tight junctions and a mucous layer) itself is an important determinant of antigen exclusion. Macromolecules are shown to transgress this barrier by endocytosis. Granulocytes, macrophages and plasma cells in the lamina propria interact with these absorbed antigens, thus forming a secondary barrier of defense against penetration of antigens into the circulation. Certain diseases or altered states (e.g. malnutrition, vitamin A deficiency, decreased gastric acidity, allergy) may allow for increased amounts of macromolecular transport. It is thought, for example, that tight junctional complexes present between the mucosal epithelial cells are broken down during malnutrition and vitamin A deficiency. In

addition to the epithelial barrier, the mucosa contains a high proportion of lymphoid cells, the so-called intraepithelial lymphocytes (IELs). These cells are mainly localized in the basal area of the epithelium and bear either cytotoxic T cell or natural killer cell markers.

In the gastrointestinal and respiratory tracts a specialized antigen transport system exists which allows for the efficient transport of macromolecules from the lumen to the organized gastrointestinal and respiratory tract lymphoid tissue (gut-associated, nose-associated and bronchus-associated lymphoid tissue – GALT, NALT and BALT, respectively). The epithelium covering these lymphoid tissues contains specialized antigen-transporting membranous (M) cells.

Antigen uptake by the gut

The gut is constantly challenged with large doses of soluble and particulate antigens. Although it is highly antigen-impermeable, the gut possesses specialized antigen transport mechanisms in the epithelium covering the Peyer's patches (PPs), the appendix and scattered lymphoid follicles. M cells are commonly interspersed between enterocytes; their numbers vary between species. M cells are physically attached to enterocytes via conventional junctional complexes. Although the intermediate filament composition and glycosylation state of M cells is slightly different from that of the surrounding enterocytes, the junctional complexes and the presence of epithelial-specific vimentin and cytokeratin expression in M cells provide evidence for an epithelial origin of M cells, in contrast to all other types of antigen-handling cells which are bone marrow derived. M cells differ from the rest of the epithelial cells in histochemical and ultrastructural respects. At the microscope level these cells can be recognized by their close association with IELs. M cells possess large basolateral pocked domains which harbour specialized IELs. In ultrathin sections, an M cell is seen as a rim of apical cytoplasm that bridges the space between the adjacent enterocytes (Figure 2). This M cell forms a kind of umbrella above a space in which IELs, and sometimes macrophages and dendritic cells, are present. Antigen is absorbed at the apical side of the M cell; absorption may be aided by the lack of a 'fuzzy coat' and the absence of mucous (goblet) cells in the PP epithelium. Following absorption, antigen is endocytosed and transported into vesicles to the lateral and basal cell membrane; by fusion the antigen is released into the basolateral space, where it comes into contact with lymphocytes and macrophages or dendritic cells. Antigen is transcytosed by M cells

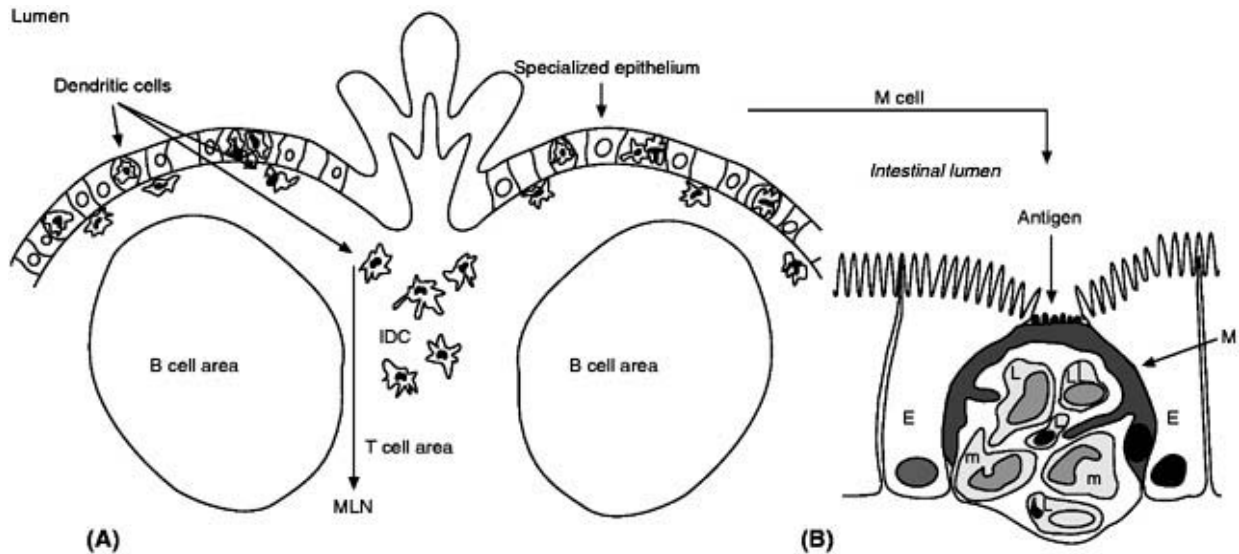


Figure 2 (A) A Peyer's patch; (B) detail of an M cell within the Peyer's patch epithelium. After uptake and transport by the M cell, antigen is processed by macrophages or by dendritic cells which transform into interdigitating cells (IDCs) and is then presented to lymphocytes in the T cell areas of Peyer's patches and mesenteric lymph nodes (MLN). E, enterocyte; L, lymphocyte; m, macrophage/dendritic cell.

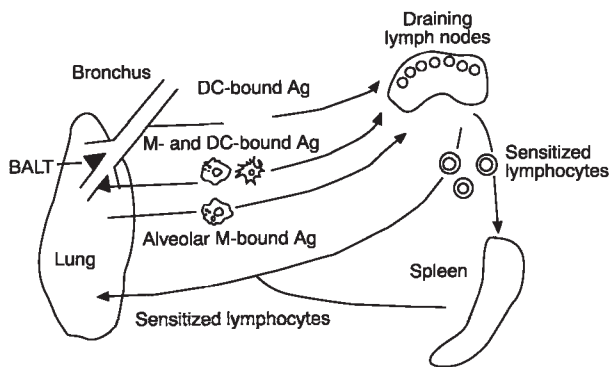


Figure 3 Interactions between the immune compartments of the lung (alveoli with alveolar macrophages, BALT macrophages (M) and dendritic cells (DC) in the lamina propria, lung-draining lymph nodes). Ag, antigen.

with little or no degradation. It is picked up by macrophages or dendritic cells and presented to local lymphocytes or transported to the interfollicular T cell areas of PPs or mesenteric lymph nodes where antigen is presented by interdigitating cells (IDCs). These antigen-presenting IDCs are derived from the dendritic cells that had picked up antigen within the epithelium.

There are indications that antigen uptake by the M cells in the PP epithelium induces a secretory IgA immune response. In addition, a systemic suppression is exerted by the generation of suppressor T cells inside PPs. It is suggested that part of the intestinal antigens – in particular soluble, low molecular weight antigens – are absorbed by the

enterocytes of the villous epithelium. Enterocytes, in particular those of the small intestine, constitutively express MHC class II molecules and it has been shown that these cells are able to present antigen. There are indications that antigen presentation by enterocytes leads to the generation of T suppressor cells which are involved in systemic suppression and tolerance induction. These findings have significant implications in local gut immune responses and may explain the poorly understood phenomena of oral tolerance induction and systemic suppression.

Antigen uptake by the upper and lower respiratory tract

The nasal and oral cavities are covered by a (pseudo)stratified epithelium, that acts, due to surface protection by mucus and sIgA, as an adequate barrier. This epithelium possesses antigen-presenting Langerhans-like (dendritic) cells. Painting of this area with fluoresceinated dyes indicates that dendritic cells can bind antigen and transport it to the draining lymph nodes (in particular the cervical mandibular lymph nodes) for an adequate immune response. The epithelium of the mucosal surface of the oral and upper respiratory tract, except for the epithelium above lymphoid structures (Waldeyer's lymphoid ring), is involved in antigen exclusion rather than in antigen uptake. The epithelium above the ring of Waldeyer – in the nose the lymphoid tissue has been described under the name NALT – contains, just as BALT and GALT do, antigen-transporting M cells

by which the local mucosal immune system is allowed to react adequately.

Antigen-presenting dendritic cells which constitutively express MHC class I and II molecules have also been described in the pseudostratified epithelium of the trachea and the bronchi. The majority of them are localized in the mucosa, either above or below the basement membrane, and in the lamina propria. Although there are no clear data about the migration route of these cells, there are indications that after antigen uptake dendritic cells migrate to the draining lymph nodes for antigen presentation. The lower respiratory tract, in particular the lung, is thought to be involved in antigen exclusion rather than in antigen uptake. Clearance of antigens from this part of the respiratory tract mainly depends on mucociliary activity and alveolar macrophages. The alveolar macrophage population, however, appears to be heterogeneous, both morphologically and functionally. Some macrophages act as real scavenger cells (they phagocytose and digest antigen, and leave the lungs via the mucociliary trap), some are apparently inhibitory, while others display a stimulating effect on the local immune response by presenting antigen to lymphoid cells. Most probably this latter effect is brought about by migration of alveolar macrophages from the alveoli into the lung tissue. It has been shown that antigen-laden alveolar macrophages cross the lining of the alveoli and migrate to and present antigen in the draining lymph node. In this way alveolar macrophages act as dendritic cells forming a specialized antigen handling system in the

lungs. Figure 3 illustrates the interactions between the immune 'compartments' of the lower respiratory tract. Antigens can reach the draining lymph nodes via diverse routes and there are indications that the site where antigen is taken up, processed and presented determines the outcome of the immune response.

See also: Antigen-presenting cells; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; HLA class II; Mucosa-associated lymphoid tissue (MALT); Oral immunity; Skin, contribution to immunity.

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ANTIGEN PRESENTATION VIA MHC CLASS I MOLECULES

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As described in Antigen Presentation via MHC Class II molecules, the T cell receptor for antigen, either of the CD4 or CD8 subset, must interact with peptides bound to the major histocompatibility complex (MHC) molecules. Both the class I and II MHC molecules inform the T lymphocytes of the intracellular content of peptides. CD8 T cells interact primarily with peptides bound to class I MHC molecules. As was initially shown, CD8 T cells directed to viruses can be triggered by viral peptides. Two major differences between presentation by the class I and the class II MHC molecules are, first, that the class I molecules serve to select primarily, but not exclusively,

peptides derived from cytoplasmic proteins – the class II molecules select peptides from proteins found in the vesicular system; and, second, that the primary site of coupling of class I molecules with peptides is the endoplasmic reticulum (ER) – the major site of peptide coupling by class II molecules is in vesicles. In this form, the lymphocyte recognizes products from viruses and some bacteria by way of the MHC class I system, and products of intracellular bacteria or parasites (and of endocytosed/phagocytosed antigens) by the class II MHC system. Details on the biology of antigen-presenting cell (APC)–CD8 T cell interaction have been given under Antigen-Presenting Cells.

Cellular events

The interactions of CD8 T cells first involves a recognition of the peptides bound to class I MHC molecules of APC and the activation of the lymphocyte. This interaction is similar in principle to that involving CD4 T cells with APCs bearing class II MHC molecules. The interaction of the naive CD8 T cells involves the presence of adhesion molecules and the activation of the T cells by costimulatory molecules. Once activated, CD8 T cells can kill different types of target cells bearing the peptide–class I MHC complex. It is thought that very few peptide–MHC complexes are needed for recognition and cytolysis by CD8 T cells (i.e. less than a hundred per cell).

The cellular events involved in the presentation of peptides by class I MHC molecules involve four major steps. First is the catabolism of the protein antigen, which takes place in the cytoplasm. Second is the transport of peptides from cytosol to the ER, and third is the assembly of the peptide–MHC complex at that site. The last step is the transport to the cell surface.

That the cytosol is the site of generation of peptides first came from the studies on viral infections where viral peptides were found to be the main target of CD8 T cells. This recognition brought about protective antiviral immunity. Subsequently, proteins were introduced directly into the cytosol of target cells, and peptides derived from them were found to be associated with class I molecules. In contrast to presentation of peptides by class II molecules, presentation by class I molecules was not sensitive to lysosomotropic alkalinizing agents. The latter, however, was inhibited by peptide derivatives that inhibited the proteasome. Tri- or dipeptide aldehydes inhibited proteasome catabolic activity *in vitro* as well as *in vivo* generation of class I bound peptides. The proteasome is a large multicatalytic protein complex composed of multiple subunits that together form a 26S particle (~1500 kDa). The proteasome subunits are uniquely assembled into a large cylindrical structure, that is responsible for the ATP-dependent proteolysis of ubiquitin-conjugated proteins. Proteasome catabolism controls the turnover of a number of important cytosolic proteins, including transcription factors and cell cyclins. Ubiquitin is a small polypeptide that is attached by way of several enzymatic steps to lysine in cytosolic proteins. Ubiquitin marks the protein for proteasomal catabolism. Whether all peptides bound to class I MHC molecules are derived from ubiquitination of proteins is not clear at present. Peptides akin in structure to those generated by APCs have been generated *in vitro*.

Many of the class I bound-peptides are derived from proteasome catabolism. Two of the enzymes

that form the proteasome are, in fact, expressed, particularly after exposure of cells to interferon γ . These two subunits, LMP2 and LMP7, influence the nature of the catabolic process favoring hydrolysis of peptides after hydrophobic or basic residues. Cells that do not express either of these two enzymes show different profiles of presentation of peptides. LMP2 and LMP7 together with the TAP transporters described below are encoded in the MHC gene locus.

Once generated, peptides cross into the lumen of the ER using the TAP molecules (Transporter Associated with antigen Processing). TAP1 and TAP2 are homologous subunits molecules belonging to the family of transporters containing ATP-binding cassettes found in both eukaryotes and prokaryotes. These two membrane-spanning molecules act in concert by binding to cytosolic peptides of a given length, usually 8–12 amino acids, and transporting them into the ER in an ATP-dependent process. The binding of peptides to TAP transporters has been studied using free microsomes or permeabilized cells. Peptides bind to TAP in an ATP-independent process, with μM affinity constants. There is some degree of specificity of peptides–TAP interaction which varies among species and different class I alleles. Interestingly, there is some concordance in peptide preference for TAP transport and that for binding to a class I MHC molecule. The importance of TAP transporters was first brought into attention by cell lines defective in class I MHC expression caused by mutations in TAP molecules. This indicated that TAP molecules were essential for the transport of peptides which is necessary for the assembly of MHC class I–peptide complex.

The site of interaction between class I MHC molecules and peptide occurs in the ER. The formation of a stable trimolecular complex (i.e. heavy chain of class I MHC molecules, β_2 -microglobulin and peptide) takes place following transport of peptides by the TAP molecules. Several steps have been identified in the ER leading to the formation of the peptide–class I complex. Chaperone molecules that normally are found in the ER, such as BiP and calnexin, help in the formation of a correctly folded heavy chain. BiP is a member of the heat shock family of proteins. Calnexin resides like BiP in the ER and participates in the correct folding of many glycoproteins. The heavy chain and the β_2 -microglobulin then associate physically with the TAP molecules. Involved in the final assembly are recently identified helper molecules, such as calreticulin and tapasin, which probably serve to bridge the interaction of the assembled class I molecule with TAP. Calreticulin is a resident ER chaperone molecule of 45 kDa. The precise role of tapasin is yet to be determined. The MHC class I molecules associated

with TAP then establish contact with the recently transported peptides.

Once the MHC-peptide complex is assembled, it is transported to the Golgi and then rapidly to the plasma membrane. Class I MHC molecules devoid of peptide are unstable molecules at physiological temperatures. The fraction of peptide-free MHC molecules that reach the plasma membrane varies depending on the form and species of it.

Class I MHC molecules clearly present peptides derived from the cytosolic catabolism of proteins such as viral proteins or autologous cytosolic proteins. But class I molecules can also be loaded with peptides from proteins that are internalized in vesicles. These include aggregated proteins, proteins from inactivated viruses, bacteria or particulate bound proteins. The pathways that these proteins follow in the cells have been much discussed recently. It may involve transposition of the proteins from vesicles into cytosol, leading to a sequence of events similar to those described above. But an alternative pathway may result in peptides generated in endosomes or lysosomes that in some form become accessible to class I molecules. Regardless of the exact pathway of processing, the point is that proteins taken in by endocytosis/phagocytosis can also lead to peptide loading of class I molecules.

It is noteworthy that viruses have been shown to produce proteins that inhibit presentation by the class I MHC system. This may be one of the ways in which viruses try to evade the immune system. For example, adenovirus E3/19K glycoprotein binds to the class I MHC molecule directly and inhibits its transport to the cell surface. Herpes virus type I ICP47 protein binds to TAP and inhibits transport. Cytomegalovirus also produces proteins that inhibit class I presentation.

Peptide binding

Class I MHC molecules bind to peptides usually of 8 or 9 residues in length. The binding specificity is broad: many different peptides bind to a given class I MHC molecule, although there are clear preferences depending on the peptide sequence, and these vary among class I molecules of a given genotype. Binding of peptides to class I MHC molecules has been studied using purified molecules in solution but most frequently using cells deficient in TAP transporters. These cells have poor basal expression of class I MHC molecules but will increase this expression when cultured with peptides.

In common with the class II molecule, class I molecules do not discriminate between foreign and self peptides. Peptides have been extracted from class I

molecules of different target cells. These peptides were isolated, fractionated, usually by reverse phase high-pressure liquid chromatography, and then sequenced. Class I molecules were found to contain hundreds of peptides, many of them representing autologous cytosolic proteins.

The biochemical basis of peptide binding has been extensively analyzed following the pioneering studies by Bjorkman and colleagues from Wiley and Strominger's laboratories. There are features in common between the structure of the binding site of both class I and II molecules. The binding site is located at the top of the molecule. It contains a cleft or 'groove', the peptide-combining site, which is made by a platform of β -pleated sheet surrounded by two helices. The groove is about 30 Å in length and 12 Å in its center. Amino acid residues responsible for the extensive allelic polymorphism contact the peptides and are responsible for their binding specificity. For class I MHC molecules, both the α_1 and α_2 domains contribute to the combining site. Each domain contributes to half the number of β sheets and one of the helices. The α_3 domain of the heavy chain and β_2 -microglobulin serve to hold and give support to the top two domains.

Peptides are bound to class I molecules as a result of two sets of interaction. One is that of conserved amino acid residues of the class I heavy chain, with amino acids at each end of the peptide. Amino acid residues at the ends of the peptide form extensive hydrogen bonds with conserved residues at subsites located at each end (termed pockets A and F). The peptides bound at each end will run through the groove as an extended conformation. Depending on the particular peptide and class I molecule, the peptide may or may not bulge or kink towards the middle of it. The extensive hydrogen bonding of the main chain atoms of the peptide with the conserved residues of the MHC molecule stabilizes the complex. A second set of interactions takes place with side-chains of the peptide. These side-chain-specific interactions are responsible for the sequence binding motifs that have been identified. Particularly prominent for most class I bound peptides are interactions at the second and last residue.

Some of the residues in the peptide are solvent exposed and will establish contact with the T cell receptor. The X-ray structure of the T cell receptor bound to a class I MHC molecule shows the receptor contacting the α helices of the MHC molecules as well as the peptide. The peptide occupies about the center of the peptide-binding structure available to the T cell. The receptor is oriented slightly diagonal to the main axis of the peptide, with the CDR3 region of their α and β chains contacting the middle

of the peptides and the CDR1 and CDR2 contacting the peptide ends and the MHC. Thus the structural analysis confirms the dual specificity of the T cell receptor for peptide and MHC molecules, as was predicted by the findings of MHC restriction.

See also: Adhesion molecules; Antigen-presenting cells; Antigen presentation via MHC class II molecules; MHC peptide-binding specificity; CD8; Cytotoxic T lymphocytes; H2 class I; HLA class I; Interferon γ ; MHC, evolution of; MHC, functions of; MHC restriction; T cell receptor, recognition by.

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ANTIGEN PRESENTATION VIA MHC CLASS II MOLECULES

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During antigen presentation a protein is taken up and processed by antigen presenting cells (APCs), and is then recognized by T lymphocytes. The basic patterns of protein recognition apply to both CD4 and CD8 T lymphocytes. Both cells do not recognize protein antigens in their native state but only after the antigens are taken up by an APC. The role of the APC is to process the protein into denatured fragments or peptides that complex with their class I or II major histocompatibility complex (MHC) molecules. The peptide–MHC bimolecular complex represents the antigenic determinant recognized by the $\alpha\beta$ T cell receptor of CD4 or CD8 T cells. However, there are notable differences between recognition by CD4 or CD8 T cells in that CD4 T cells recognize the peptides associated with class II MHC molecules, while CD8 T lymphocytes recognize the peptides associated with class I molecules. The CD4 or CD8 molecules interact with either the class II or class I molecule, respectively, favoring the interaction of the T cell receptor with the MHC–peptide complex.

Historical background

The early studies on responses to protein antigens established a relationship between the degree of uptake of the antigen by the ‘reticuloendothelial system’ and its immunogenicity. This was most evident

in the responses to foreign albumins or gamma globulins where the antigenic preparations differed in their content of polymeric molecules. Albumin or gamma globulins with a heavy content of polymers (or aggregates) were strongly immunogenic while, in contrast, preparations made mostly of monomers were poorly immunogenic or even tolerogenic. The degree of uptake by spleen macrophages of polymers was much higher than of monomers. That these results could be ascribed to the uptake by macrophages was ascertained in experiments in which macrophages that had taken up the protein were isolated and transferred to syngeneic hosts. The recipients made strong responses to the macrophage-associated antigen. Therefore, the macrophage, a cell responsible for protein catabolism, was linked to the immunogenicity of the antigen.

An understanding of this phenomenon required the introduction of cell culture techniques. In culture, APCs were strictly required for antibody responses, or for responses by T lymphocytes to protein antigen or to plant lectins. The depletion of APCs, usually by their property of adherence to culture dishes, resulted in an absolute lack of T cell proliferation or of cytokine secretion. It was in 1973, through the studies of Shevach and Rosenthal, in the inbred guinea pig strains 2 and 13, that it became apparent that T lymphocytes not only required the presence

of macrophages but of macrophages that were syngeneic. The class II MHC molecules were essential elements in this interaction. Further experiments, also first in the guinea pig, led to the concept of determinant selection: that the T cell recognizes antigenic determinants selected by APCs of a given MHC genotype. Thus, antigen presentation was related to the immune response gene phenomena reported in inbred strains of mice and guinea pigs. The 'immune response' gene experiments and those of MHC restriction and determinant selection set the base for the cellular and molecular understanding of this very early inductive phase of T cell recognition and of antigen presentation.

Antigen processing was first inferred from observations on the specificity of antigenic recognition by B and T cells. B cells usually recognize protein antigens in their native state. Much of the antibodies found in blood after immunization with a globular protein antigen bind to the protein in its three-dimensional conformation and not to the denatured protein. This indicates that the native protein antigen was selected by B cells with specific immunoglobulin (Ig) receptors for it, prior to its unfolding by the host's catabolic processes. In contrast, T cells recognize the denatured protein or fragments of it. This was first ascertained by examining a variety of T cell reactions (delayed hypersensitivity, secretion of cytokines, proliferation). In such examples there was no difference if the protein was presented in its native state, unfolded or as a peptide fragment. These phenomena are now explained by the more recent findings indicating that protein antigens are processed by APCs, that some but not all processed peptides bind to a given allelic form of class II MHC molecules, and that the MHC-peptide complex represents the antigenic determinant that engages the T cell receptor.

Cellular events

Antigen presentation takes place very rapidly upon entry of antigen into lymphoid tissues. Presumably macrophages and Langerhans-dendritic cells take up the antigen and are responsible for the early recruitment and activation of CD4 T cells. B cells that have surface immunoglobulin molecules with specificity for the antigen also participate by binding the antigen, processing it and presenting it to the CD4 T cells.

Antigen presentation comprises other events besides processing of the antigen and formation of the peptide-MHC complex. First, the antigen-specific T cells must establish physical contact with the APCs. Second, T cells require to interact with two sets of molecules provided by APCs: costimulatory

molecules, in order to enter cell cycle and to activate cytokine-specific genes; and cytokines that modulate their expression of different genes. Clearly T cell activation is a multistage process involving a 'signal 1', the engagement of the T cell receptor, and various 'signal 2'. The molecules that primarily promote physical contact are termed cell adhesion molecules (or CAMs), while those that primarily promote growth and differentiation are termed costimulators. Details of the APC-T cell interaction are given in the Antigen-Presenting Cell article which contains a table showing the molecules involved.

The interaction between the APCs and the CD4 T cells is physically manifested by close cell-to-cell contact. This intimate APC-CD4 T cell contact is favored by cell adhesion molecules the lymphocyte functional antigen-1 (LFA-1) on the T cells with its corresponding ligands, ICAM-1, ICAM-2 and ICAM-3 on the APCs; and the CD2 molecule with its counter-receptor. The functional effects of the interaction are reciprocal – macrophages will be stimulated to release cytokines, B cells will proliferate and differentiate, while the CD4 T cells will also proliferate and release cytokines.

Costimulator molecules are key in determining that the engagement of the T cell antigen receptor to the MHC-peptide complex is a productive interaction. These include the B7-1 and B7-2 molecules on APCs (and their coreceptors CD28 and CTLA-4 on T cells), and the CD40 molecule on B cells in particular, and the CD40 ligand coreceptors on T cells. These costimulator molecules may act by inducing early release by T cells of growth factors such as interleukin-2 (IL-2) and/or by regulating the expression of receptors for growth factors, and by inhibiting apoptosis. *In vitro* experiments indicate that exposure of T cells to fixed APCs (which cannot provide costimulatory molecules) results in an absent response. Such T cells, in fact, may develop an unresponsive or anergic state. Costimulatory molecules of APCs are expressed in low amounts. The representation of their expression is one component that initiates productive presentation. Finally to note is that several cytokines from APCs modulate the T cell response during antigen presentation. Included are IL-1 α and β , tumor necrosis factor- α (TNF α), IL-6 and IL-12. This latter cytokine is key in the differentiation of T cells to the T_H1 subset.

Antigen processing

Processing refers biochemically to the unfolding of the proteins and/or the generation of peptides as a result of their partial proteolysis. Globular proteins in their native state do not bind to MHC class II

proteins: proteins are required to have conformational flexibility in order to be able to bind and mold into the combining site of an MHC molecule. This flexibility is brought about either by denaturing the protein or partially fragmenting it to small peptides. The denatured proteins or the peptides, in contrast to the native unfolded molecule, interact and complex to the class II MHC molecules.

Processing by APCs involves several successive stages: 1) the internalization of the antigen into acid intracellular vesicles; 2) its partial proteolysis; 3) its coupling to intracellular MHC II molecules; and 4) its transport to the plasma membrane.

Protein antigens internalized by APCs are taken to deep intracellular vesicles where unfolding of the protein and/or its partial catabolism to peptides then takes place. The denatured protein and/or peptides are thus available to bind to class II MHC molecules. The most recent evidence suggests that this encounter takes place preferentially, but not exclusively, with class II MHC molecules that have been recently synthesized, and that this encounter takes place in vesicles with the properties of early lysosomes.

Class II MHC molecules assemble in the endoplasmic reticulum (ER) and traverse the Golgi vesicles associated with a nonpolymorphic polypeptide, the invariant chain. Invariant chain and the $\alpha\beta$ dimers of class II molecules form a complex that is transported to vesicles outside the Golgi vesicles. The estimate is that a trimerized invariant chain binds to three class II molecules (i.e. three $\alpha\beta$ dimers), thus forming a nonameric complex. The invariant chain has signal sequences in its cytosolic portion that serves to guide the complex out of the Golgi into late vesicles. The invariant chain also has the important property of covering the combining site of the class II molecule during its travel through the ER. Thus, during their sojourn through the ER and Golgi the class II molecules have less opportunity to interact with peptides derived from catabolism of self peptides. The invariant chain has different isoforms that may be involved in different ways in the fate of the complex.

Once the invariant chain- $\alpha\beta$ complex leaves the Golgi, the invariant chain is catabolized in a proteolysis rich, acidic compartment; however, a segment of the invariant chain comprising residues 81–104 remains associated with the combining site. This peptide, termed CLIP, must be removed to make the $\alpha\beta$ dimer competent to bind foreign (or autologous) peptides. A secondary auxiliary molecule (HLA-DM in humans or H2-M in the mouse) then associates with the $\alpha\beta$ dimer containing the CLIP peptide. This association leads to the removal of CLIP. How this protein associates with the $\alpha\beta$ dimer, and the basic

interaction that favors a fast off-rate of the bound peptide still requires analysis. As the CLIP peptide is removed, high-affinity binding peptides found in the vesicular 'loading' compartment then bind. Thus, there is an exchange of peptides, with CLIP leaving, and strong binding peptides occupying, the class II MHC molecule. Class II molecules with bound peptides are then transported to the plasma membrane, the site of interaction with the T cell and its receptor.

Many of the class II MHC bound peptides have a very slow off-rate and, therefore, remain bound during their entire span in the APC. In fact the half-life of the class II MHC molecules reflects their content of different affinity peptides. Some of the class II molecules bearing weak binding peptides can exchange peptides in endosomal vesicles or at the plasma membrane.

Thus, a scenario that is favored is that an internalized protein (i.e. a soluble protein or a microbe containing a large array of proteins) is taken to a deep lysosomal compartment, and it is there that peptides are generated from partial proteolysis. Nascent class II molecules meet with these peptides and an effective peptide exchange reaction takes place, as described above. Peptides that are bound, i.e. 'selected', are protected from further proteolysis. Class II molecules are, therefore, peptide-binding molecules that protect from extensive catabolism. Figure 1 illustrates the antigen-processing events depicted using the protein lysozyme.

The nature of the proteolytic event that results in the degradation of the invariant chain and of the protein antigen is still under much consideration. Most likely, cathepsins are involved in the degradation. A strong case has been made for the involvement of cathepsin S in invariant chain degradation. Concerning the protein antigen, one way of viewing the processing event is to postulate the unfolding of the protein, after which the denatured unfolded polypeptide binds to class II molecules through the segment displaying sequences that favor high binding to MHC molecules. Once bound, the portion of the unfolded peptide that spills out of the peptide-combining site is catabolized, perhaps by amino- and carboxypeptidases, up to the limit of the combining site.

There has been much discussion about the nature of the MHC-peptide loading compartment (called MIIC). An organelle containing lysosomal proteins and class II MHC molecules, and which receives internalized protein late, has been identified by electron microscopy, and also defined by subcellular fractionation. Thus, for many protein antigens this late vesicular and lysosomal-like compartment may be the site of loading to nascent class II molecules. However, it is also clear that peptides can bind class

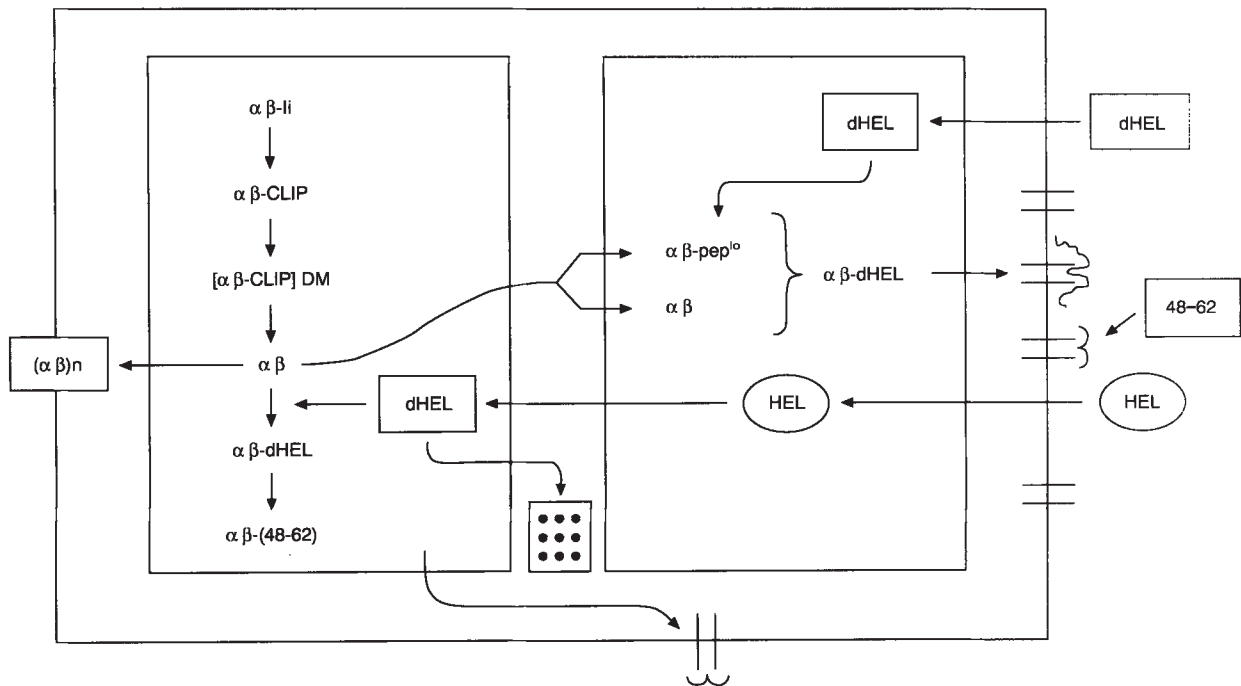


Figure 1 Processing of the protein hen egg white lysozyme (HEL). There are two functional compartments in APCs, depicted as the two squares: a deep compartment (left) and a recycling or endosomal compartment (right). HEL is a globular protein that requires denaturation by reduction of its four disulfide bonds (dHEL). This takes place after HEL reaches the deep compartment. The dHEL binds to the $\alpha\beta$ dimers of class II MHC, after their exit for ER–Golgi. $\alpha\beta$ dimers exit bound to the invariant chain ($\alpha\beta$ -Ii). The invariant chain is degraded leaving CLIP peptides bound to it. The HLA-DM molecules remove CLIP making the $\alpha\beta$ dimers competent to bind dHEL. The dHEL bound to class II molecules is trimmed by amino- and carboxypeptidases to a peptide of 15–16 residues. The peptide is transported to plasma membrane. The dHEL not bound is catabolized to amino acids (box with dots). If APCs are given HEL already denatured, it can bind to $\alpha\beta$ dimers in endosomes where peptide exchange takes place. Peptides can also bind directly to some peptide-free $\alpha\beta$ dimers at the cell surface.

II molecules in early endosomes or, if available, at the plasma membrane. The nature of the protein, and the ease by which the protein is subjected to denaturation and partial proteolysis, clearly are factors in determining the site of loading. Autologous proteins, which are rapidly secreted from APCs, can also load into class II molecules during their short travel through APCs. This is an indication that there is some degree of proteolysis taking place during secretion and that the loading process is very effective. Lastly, peptides derived from cytosolic molecules have been found bound to class II molecules, an indication that cytoplasmic-derived peptides are also accessible to the class II MHC system. However, whenever a comparison has been made, peptides derived from cytosolic proteins are less effective than those found in vesicular proteins in reaching the class II MHC system.

Binding properties of class II MHC molecules

The binding properties of class II MHC molecules can be studied using purified molecules, usually in

detergent solutions, or directly in the APC. Purified class II molecules bind peptides in a homogeneous saturable process with binding affinity constants in the 10^{-6} – 10^{-8} M range. The rate of association of peptide with the MHC molecule is slow, but once the peptide is bound it can form a very stable and long-lived complex. The complex of MHC with peptide can be assembled and isolated in free solution, and can directly trigger CD4 T cells these experiments have been done with T cell hybridomas that are triggered by the engagement of their receptor without the need of costimulatory molecules. Binding of peptides to class II MHC molecules can also be directly determined on APCs by using peptides tagged with a radioactive or fluorescent label, or with photoreactivatable probes. In live APCs the proportion of class II MHC molecules that need to be occupied by a given peptide to trigger T cells is small, usually about 0.1% of total class II MHC (about 10^{-2} in the APCs studied so far).

The binding of peptides takes place in the antigen-binding site of the MHC molecules, which was defined through structural studies of purified complexes pioneered in the laboratory of Don Wiley and

Jack Strominger. The main architectural features are found for both the combining site of class I and II MHC molecules. For class II molecules the binding site is at the top of the molecule and consists of a groove or pocket made up of a platform of β -pleated sheets, derived from the α_1 and β_1 domains, surrounded by α helices, also made from portions of α_1 and β_1 domains. Most of the amino acid residues responsible for allelic polymorphism, found in the α_1 and β_1 domains, are located in the helices and in the platform. These residues are believed to be involved in interaction (charge or ionic, or hydrophobic) with residues in the processed protein antigen.

Peptides are found in an extended conformation with a slight twist typical of polyproline II-like conformations. Two types of interaction determine the affinity and specificity of peptide binding: those between the peptide backbone and conserved residues of the MHC molecules; and those between side-chains of critical amino acids with sites of the MHC molecule. The first set of interactions include hydrogen bonds between the conserved residues in the MHC molecules and the peptide backbone. This extensive array of hydrogen bonds contributes to the affinity of the peptide and is dependent on the length of the peptides. In contrast, side-chains of amino acids interacting with critical areas in the combining site give specificity to the interaction. The sites or areas have preference for different amino acids side-chains. The amino acid residues of the MHC pockets characterize a given MHC genotype.

Binding kinetics and competition experiments indicate the single peptide-binding site can be occupied by peptides of 8–30 amino acid residues. The specificity of peptide binding is broad, assuring that many different peptide structures can be recognized by the T cell system. Several peptide motifs that favor binding to class II MHC molecules have been identified. Not all of these motifs so far identified predict all T cell determinants. A peptide contains amino acid residues that contact the MHC binding site and other residues that contact the T cell receptor. The contact residues for MHC and T cell receptors are intermingled in the peptide. Usually 1–3 residues are solvent exposed and serve primarily to contact the T cell receptor. To note is that although there is broad binding specificity, not all peptides bind to a given allelic form of an MHC class II protein. The weak binding, or lack of it, results, as expected, in very poor activation of the specific T cells. It is the weak or poor binding of peptides to a given allelic variant of class II MHC molecules that explains many of the immune response gene effects described from early studies on inbred mice.

An important observation first made with lysozyme peptides is that the class II MHC binding site does not discriminate between self and nonself peptides. Self peptides have been shown to bind or not to bind, depending on their structural features. Hence, self and nonself peptides behave in the same way insofar as their binding properties to a given class II protein. Moreover, it has now been shown that APCs isolated from different tissues, including the thymus, contain self peptides associated with them. Indeed, the number of different self peptides isolated from class II molecules is in the hundreds. Some peptides derive from uptake of extracellular molecules but the majority derive from membrane proteins of the vesicular system.

The binding of self peptides to MHC molecules results in three potentially important effects: 1) on the thymus stromal cells it may be instrumental in causing the death or negative selection of maturing T cells; 2) on APCs of peripheral lymphoid tissues, self peptides may compete for the binding of foreign peptides to MHC class II molecules; and 3) such MHC-peptide complexes on APCs can potentially trigger autoreactive T cells, to cause autoimmunity. Of course, reactivity to foreign or self antigens comprises multiple steps and factors of which the generation of the MHC-peptide complex is only one.

See also: Antigen-presenting cells; Antigen presentation via MHC class I molecules; Antigens; Antigens, T dependent and independent; B lymphocyte, antigen processing and presentation; CD28; CD40 and its ligand; B7 (CD80 & CD86); Dendritic cells; H2 class I; H2 class II; HLA class I; HLA class II; Invariant chain (Ii); MHC, functions of; MHC restriction; Phagocytosis; Second signals for lymphocyte activation.

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ANTIGENIC VARIATION

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Antigenic variation is a well-established mechanism among infectious organisms for escaping the immune response of the host. It is a Darwinian process in which alteration of the antigenicity of surface molecules enables infectious organisms to escape immune elimination. These alterations are the result of random mutations, and organisms that acquire surface changes that allow them to escape from host recognition will have a selective advantage and predominate. Antigenic variation is the principal means of persistence of protozoans such as African trypanosomes, *Plasmodium* spp., *Giardia* and several species of bacteria, including *Neisseria gonorrhoeae* and *Borrelia* spp., in an infected individual. This phenomenon also allows for the persistence of animal viruses. The classic example of antigenic variation of viruses is observed among the influenza A viruses. Influenza viruses do not establish persistent infections in individuals, but alterations in the surface glycoproteins as the virus spreads through a population allow for reinfection of individuals and therefore the persistence of influenza viruses in a population and worldwide.

Antigenic variation of viruses within an individual host occurs with the lentiviruses. Lentiviruses include the human immunodeficiency virus (HIV) and an increasing number of animal viruses which cause persistent infections with chronic disease. Antigenic variation has also been observed for the enteroviruses (poliovirus, Coxsackie virus, echoviruses) and for the common cold viruses (rhinovirus types 22 and 51). Even monotypic viruses, such as measles, exhibit genetic diversity in surface proteins. More detailed descriptions of the molecular mechanisms of antigenic variation of trypanosomes (an example of protozoans), influenza A viruses and lentiviruses are provided here.

Differential gene expression in trypanosomes results in antigenic variation

Trypanosomes are eukaryotic flagellates that cause sleeping sickness in humans and nagana in cattle. These protozoan parasites are transmitted by insects to the bloodstream of the host. The trypanosome has a number of developmental stages. The bloodstream-stage trypanosomes are covered with a dense coat composed of a single type of glycoprotein, the vari-

ant surface glycoprotein (VSG). Antigenic variation in trypanosomes occurs through the sequential expression of different VSG genes. During the course of infection, trypanosomes with serologically unrelated coats are periodically generated, thus allowing evasion of the humoral immune response produced against previously circulating coat VSG.

Each trypanosome has a large repertoire of different VSG genes (100–1000, depending on the species). The switch from one coat to another occurs at a low frequency (10^{-6} or 10^{-7} per cell division) and appears to be independent of immune selection. Only a single VSG gene is usually expressed at one time. The active VSG gene is invariably in a telomeric location in a VSG expression site. Although there are multiple expression sites, located on a few specific chromosomes, only one site is active at any given time. Nonexpressed or silent VSG genes are clustered at internal positions on the chromosomes and are also found at many of the chromosome telomeres. Activation of specific VSG genes occurs by two genetic mechanisms. The first is gene replacement, by duplicative transposition of a new VSG gene into a transcriptionally active expression site. This is thought to occur via homologous recombination. Selection of VSG genes is dictated by the degree of homology between two loci, which may explain the loosely defined order of appearance of trypanosome variants during an infection. The second mechanism for activation of VSG genes is the activation of an alternative expression site. The promoters of expression sites are negatively controlled, and transcriptional activation is thought to be mutually exclusive. Transcription of VSG genes is insensitive to α -amanitin, but the polymerase involved is not known. There is evidence that the presence of an unusual DNA base, pdJ, may be involved in the inactivation of expression sites.

Despite the enormous VSG gene pool available, the reproduction of variant antigenic types is limited in the trypanosome: this prevents the exhaustion of the surface glycoprotein repertoire at an early stage of disease. Antigenic variation also occurs in trypanosome surface receptors involved in food uptake. These variants are limited in number, making these proteins a better target for vaccine development. The bacterium *Borrelia hermsii*, which causes relapsing fever, uses DNA rearrangement

mechanisms for producing antigenic variants similar to those seen with trypanosomes.

Antigenic 'shift' and 'drift' in the influenza viruses

Among the influenza A viruses, both of the surface glycoproteins (the hemagglutinin and neuraminidase) undergo two types of variation: antigenic drift and antigenic shift. Antigenic drift involves minor changes in the antigenicity of the surface protein and is thought to be responsible for the persistence of the virus in a susceptible population. Antigenic shift involves major genetic changes that are responsible for the development of the 'new' viruses in the host species. Antibodies to the hemagglutinin neutralize the infectivity of the virus, and variation in this protein is mainly responsible for recurring outbreaks of influenza and for the failure to produce an effective vaccine. Extremely high concentrations of antibodies to the neuraminidase can also neutralize the infectivity of influenza viruses. This neutralization may occur by a saturation of antibody-binding sites on the neuraminidase resulting in steric inhibition of the hemagglutinin.

Antigenic drift of either surface molecule occurs by the accumulation of point mutations in the respective gene; however, single point mutations appear to have little effect on the antigenic properties of the proteins. Nucleotide sequence analyses of epidemiologically significant strains suggest that single amino acid changes in two or more epitopes of the hemagglutinin are necessary for a significant antigenic change. The most likely mechanism for mutations in multiple epitopes is single mutations occurring sequentially during the spread of the virus from person to person. Strains with single mutations in one epitope must escape the immunity of an individual, possibly because the humoral immune response in humans is heterogeneous and the limited antibody repertoire against influenza virus in children probably permits such selection.

Antigenic shift involves replacement of at least the hemagglutinin gene via genetic exchange with an avian or animal virus. The new shift variant can cause a pandemic, as no neutralizing antibodies are present in the human population. There is evidence for genetic reassortment between human and animal influenza viruses *in vivo*. A shift virus which emerged in 1968 contained a human neuraminidase gene, but the hemagglutinin was 98% homologous at the nucleotide level with an avian influenza A virus. It is believed that new pandemic strains are produced by co-infection of pigs with avian and human influenza virus strains.

Antigenic variation in lentiviruses

Antigenic variation in an individual host occurs with animal lentiviruses. The first report of antigenic drift was in equine infectious anemia virus (EIAV). Horses infected with EIAV develop sequential episodes of acute hemolytic crises during persistent infections. Sera taken at various times from an infected animal were able to neutralize isolates from previous clinical episodes, but failed to neutralize subsequent viral isolates. Visna virus in sheep also causes persistent infection, although episodic disease is not observed. Like EIAV, antigenic variants of visna virus emerge over time in persistently infected sheep. This antigenic drift can be duplicated in cell culture using plaque-purified virus and monospecific antibody for the selection of variants.

The molecular mechanism of antigenic variation in the lentiviruses is the accumulation of single nucleotide changes in the surface glycoprotein genes. In visna virus the accumulation of point mutations appears to be progressive. Antigenic variant viruses sequentially isolated from a single animal contained the same point mutations when compared to the input virus. Antibodies produced by the animal to a particular variant recognized all changes found in the other preceding variants, supporting the cumulative and progressive nature of antigenic drift in visna virus.

In both visna virus and EIAV, point mutations are seen throughout the surface glycoprotein gene, but hypervariable regions have been identified. Antigenic variation in visna virus can be detected 1 year after infection and continues throughout the infection. In contrast, antigenic drift in EIAV occurs more rapidly, in the first weeks after infection, and continues until the host develops a broad antibody response against the virus. Distinct EIAV variants can be associated with each clinical episode. However, unlike visna virus, no accumulation of point mutations has been observed; variation is more random.

Extensive genetic heterogeneity of HIV isolates from infected individuals has also been observed, and may provide evidence for antigenic drift *in vivo*. Genetically diverse viruses, or 'quasispecies', are produced in infected individuals due to the infidelity of the viral reverse transcriptase. Recent studies have shown that the evolutionary dynamics displayed by these heterogeneous virus populations *in vivo* is consistent with adaptive evolution. Also, antigenic variants of HIV have been isolated in cell culture using a molecularly cloned virus and sera from an immune individual. Host neutralizing antibodies appear to be at least one of the selective pressures responsible for the emergence of antigenic variants. However, other

selective forces, such as cell-mediated immune responses, may also play a role in the production of variants. The role of these antigenically-distinct viruses in the pathogenesis of HIV is unclear.

Antigenic variation and immunity

Antigenic variation of protozoans, bacteria and animal viruses provides a mechanism for persistence of the organism in its host, but makes immunological control difficult. The strategy used for the development of influenza A virus vaccines has been to identify the circulating strains early in the influenza season and to rapidly produce a vaccine against the predominant strains. In lentiviruses this strategy cannot be used because mutation and selection occur in a single infected host. Thus a wide repertoire of neutralizing epitopes must be included in a vaccine to prevent the evolution of lentiviruses that can evade the host immune response.

See also: Influenza virus (*orthomyxovirus*), infection and immunity; Parasites, immunity to; Rhabdovirus, infection and immunity; Trypanosomiasis, African; Vaccines; Viruses, immunity to.

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ANTIGENS

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Antigen is any substance (molecule) that provokes the production of specific antibody or immunocyte (immune cell), or that interacts specifically with these products of the immune response, when penetrating the body of a vertebrate. The first part of this definition, namely, the capacity to provoke an immune response is also called immunogenicity and the substance provoking such a response is called immunogen. The second part, the specific interaction, is called antigenic specificity. A chemical or physical

change in a molecule that will result in an increased immune response will increase its immunogenicity, even though it may or may not change its antigenic specificity. It is the antigenic determinant (also called epitope) which is responsible for the specificity.

The ability to mount an immune response depends ultimately upon the interplay between the chemistry of the antigen and the physiologic state of the host. Thus, immunogenicity is operationally dependent on the experimental conditions of the system, including

parameters such as the antigen, the mode of immunization, the organism being immunized and its genetic background, as well as the sensitivity of methods used to detect a response.

It is worth stressing that we are using the word antigen sometimes for a molecule, sometimes for a virus or a bacterium, and sometimes for an organ or a tissue, but the antibodies have a *combining site* (also defined as *paratope*), with all its distinctive features, of a more or less similar size and cavity. These combining sites are not complementary to a complete bacterium or a complete heart, but they are always complementary to a unique antigenic determinant which is of a limited molecular size.

Among the immunocytes we recognize B cells (the precursors of antibody-producing cells) and T cells. The combining sites on B cells are essentially the same as those of the antibodies which their progeny will produce. On the other hand, the combining sites of the T cell receptors are distinct from those of antibodies. What is characteristic of the T cell receptors is that they recognize generally the antigen after it has been processed within a cell and presented to the T cell receptor in conjunction with either class I or class II antigens. These are antigens defined by immune response genes which are part of the major histocompatibility complex (MHC). Most antigens are thymus dependent, which means that they need to be recognized in the above manner. A minority of antigens, called thymus independent, do not need the recognition by T cells, and may lead to efficient antibody formation after interacting exclusively with B cells.

The notion of antigen includes also the capacity to induce specific immunological tolerance, or anergy, defined as a state of T lymphocyte unresponsiveness characterized by the absence of proliferation (human T cell clonal anergy).

The recognition of antigens by the T cell compartment of the immune system is a multistep process culminating in the formation of a ternary complex between the T cell receptor for antigen (TCR) and the binary complex of class I or II MHC encoded molecules, with short peptides derived from the antigen. Thus, the specificity of the TCR is for a *neoantigen* determinant composed of both, the genetically restricting MHC molecule and the bound peptide. The recognition process may therefore be considered as consisting of two distinct phases, the first in which peptides are produced and get bound to the MHC encoded proteins, and the second where the TCR binds this binary complex. Since MHC encoded molecules may bind peptides derived from both self and nonself proteins, the fundamental implication of the above is the need for a selection process where cells

with TCR that bind self-peptides are eliminated during the development process in the thymus, yielding a repertoire of mature T cells that recognize MHC complexes with nonself peptides.

The antigenic determinants leading to the formation of antibodies by B cells may be parts of proteins, nucleic acids, polysaccharides, lipids or glycolipids, or other biological macromolecules. Very often they have unique steric conformations and are part of a native structure. We have thus to distinguish between sequential determinants (epitopes) and conformation-dependent (or conformational) determinants (epitopes). The operational definition holds that if antibodies against, e.g. a protein, react well with a tetra-, penta- or hexapeptide derived from that protein, then the antibody is against a sequential determinant. The antibody, on the other hand, is against a conformation-dependent determinant if it is made against a juxtaposition of atoms in space which results from a unique conformation of the macromolecule, and any peptide derived from such a protein will not be able, after denaturation, to react with the antibody.

It is of interest that for most globular proteins and native nucleic acids, almost all the antigenic determinants are conformation dependent, whereas for most polysaccharides, fibrillar proteins such as silk fibroin, and single-stranded nucleic acids, the determinants are sequential. The use of homopolymers of amino acids or sugars, and of peptidyl proteins as antigens has established that the sequential determinant is composed of 4–6 amino acid or sugar residues which contribute unequally to binding with the antibody combining site.

Not all the antigenic determinants express themselves all the time: some are more immunopotent than others. Some do not express themselves at all under a certain set of conditions, and we call them immunosilent, even though under other conditions they may provoke an efficient immune response. Thus a determinant may be immunosilent within a complete macromolecule, but may be quite immunopotent when a segment of that macromolecule on which it is present is used for immunization. Situations are also known where a determinant is immunosilent but becomes immunopotent in animals made tolerant to other parts of the immunogenic macromolecule of which the determinant discussed forms a part. We may thus define immunopotency as the capacity of a region of an antigen molecule to serve as an antigenic determinant and induce the formation of a specific immune response.

The term hapten, in its strictest sense, designates any substance, large or small, which does not elicit an immune response by itself but can be shown to

react with antibody provoked by immunization with a complete immunogen of which the hapten formed a part. In practice, most haptens investigated are small chemical substances, in most cases of a size definitely smaller than that of a complete antigenic determinant. When attached to a protein, haptens such as dinitrophenyl or penicilloyl might be defined as immunodominant parts of an antigenic determinant (an epitope). In studies of determinants of polysaccharide or polypeptide nature, it is of interest to establish which is their immunodominant portion.

Generally an organism distinguishes between self (material which is its own) and nonself (any foreign material). The immune system of an organism reacts against any foreign compound (antigen), and is tolerant (unable to react) towards its own body components, which may be good immunogens in other organisms. This self-tolerance is acquired during fetal or neonatal development, and the immune system can be made tolerant even to foreign material or tissue introduced during this period. Such material, which can induce immunological tolerance or unresponsiveness, is called a tolerogen.

Another phenomenon which should be mentioned here is antigenic competition. This may occur between different antigens (intermolecular), or between different specificity determinants on the same antigen (intramolecular), in which case we define it as competition between antigenic determinants. The existence of this phenomenon may account for some determinants being immunosilent under certain circumstances. Antigenic competition may be defined as the inhibition of the immune response to one antigen or determinant by the administration of another antigen or determinant.

Molecules as antigens

The two types of natural macromolecules most investigated as antigens are proteins and polysaccharides. These also include glycoproteins, nucleoproteins, lipoproteins, etc., as well as peptidoglycans, glycolipids and other conjugates. Nucleic acids are also antigenic. Lipids are poor immunogens, but antibodies against them can be obtained, and liposomes play a role here, as they do in enhancing the immunogenicity of various other antigens. Synthetic antigens, especially synthetic polypeptides, have played an important role in the elucidation of the molecular basis of antigenicity and many other immunological phenomena, and they will be discussed here in more detail below. Other synthetic polymers have also been shown to be immunogenic, e.g. polyvinylpyrrolidone.

All proteins are probably immunogenic, although

individual proteins differ markedly in their immunogenicities. Denatured proteins are often less immunogenic than the corresponding native proteins. Self-aggregation of a protein is usually associated with a negligible change in its antigenic specificity, but with a considerable increase in immunogenicity. Like other antigens, proteins possess a continuous spectrum of antigenic determinants that correspond to discrete portions of the surface structure and that are preferentially located in those regions most exposed to the external environment. The relationship between structure and antigenicity is, however, more complex for globular proteins than for other antigens, in that it depends to a very large extent upon the overall conformation of the molecule. The exploration of antigenic regions on the surface of proteins has become easier with the advent of monoclonal antibodies and rapid methods of peptide synthesis. The antigenic sites may be described as surface domains made up from amino acid side chains which may be distant in sequence but close in space (conformation-dependent determinants). Such domains are probably overlapping and cover most of the protein surface. On the other hand, fibrous proteins possess sequential determinants, of three to six amino acid residues.

Special attention has been paid by immunologists to those proteins which possess distinct and easy-to-measure biological properties. These include enzymes and enzyme inhibitors, protein hormones, toxins, antibodies (i.e. immunoglobulins as antigens), as well as proteins composing viral coats. Immunologic studies have also been successful in following the evolution of proteins.

The other class of substances that was readily shown to be antigenic comprises the polysaccharides. Although complex, they nevertheless provide antigens of relatively simple structure by which many of the detailed structural aspects of antigenic determinants and antibody combining sites have been worked out. Microbial polysaccharides are located on the cell surface and are, therefore, of importance in recognition and immune responses of a higher organism to microbial infection. The simplest polysaccharide antigens are dextran, a polymer composed entirely of glucose, and levan, composed entirely of fructose. Another important group contains the capsular polysaccharides of pneumococci. Complex lipopolysaccharide antigens, endotoxins, are found in a large variety of microorganisms, notably in gram-negative Enterobacteriaceae, such as *Salmonella* and *Shigella*. The polysaccharide determinants are predominantly sequential, consisting usually of 4–6 sugars, but some antibodies may have combining sites which are smaller.

Blood group antigens are the other important category of polysaccharide antigens. They are gene-dependent structures expressing the individuality of cell surfaces, body fluids and secretions. Chemical characterization of blood group structures has been fully developed. The chemical structures of ABH and Lewis antigens have been elucidated using water-soluble blood group substances isolated from secretions (ovarian cyst mucin, gastric mucin). These structures, when on erythrocytes and other cells, are part of more complete glycolipid or glycoprotein antigens.

Antigenic functions of nucleic acids were only recognized much later than those of proteins or polysaccharides. No immunogen has yet been prepared that would be capable of inducing in experimental animals antibodies to double-stranded DNA. Such antibodies are, nevertheless, available in humans suffering from systemic lupus erythematosus, as well as in mice and dogs with similar diseases. Their sera usually also contain antibodies to single-stranded, denatured DNA, as well as to RNA, double-stranded RNA, histone and nucleoprotein. It is possible to induce antibodies to single-stranded RNA or DNA, either by immunizing these, complexed with a macromolecule of opposite electrical charge, or by preparing conjugates of nucleosides, nucleotides or oligonucleotides, with proteins or synthetic polypeptide antigens, and using them as immunogens. Complex formation of methylated bovine serum albumin with synthetic polynucleotides, including some that were double-stranded and triple-stranded, yielded a mosaic of antigens, some of which recognized the higher order structure of these macromolecules.

Progress in the field of lipid immunology is more recent, and largely due to advances in lipid and membrane chemistry. This led to a heightened awareness of lipids as important cellular antigens. Among the lipids, most important immunologically are phosphatides, such as sphingomyelin and cephalin, as well as glycosphingolipids such as galactocerebroside. Antibodies against lipoidal extracts of various tissues appear during the course of syphilitic infection, and this led to a standard serological test for syphilis. Another lipid antigen is the Forssman antigen, mainly responsible for hemolysis of sheep erythrocytes in the presence of antiserum and complement. The greatest barriers to advancement in the field of lipid immunology have always been the chemical and physical properties of the lipids themselves, particularly water insolubility. The problem of reactivity of soluble antibodies with lipid antigens was partially overcome by the inclusion of 'auxiliary lipids' such as lecithin and cholesterol in the antigen

suspension. Mixtures of lipids in the form of liposomes, consisting of concentric spherules of lipid bilayer membranes, can mimic precisely many of the immunological aspects of intact cell membranes. The availability of liposomes has permitted the study of the immunogenicity of membrane-associated lipids.

Immunoglobulins

Immunoglobulins (Igs) are mentioned here exclusively in terms of their antigenicity. Usually immunological data preceded structural information, and they were crucial, e.g. in the discovery of allotype and idiotypy. The availability of monoclonal antibodies permits today a detailed antigenic analysis of immunoglobulins of every class and type. The epitopes within one immunoglobulin molecule vary much in their relative immunopotencies. Thus some determinants on the Fc fragment of an IgG are immunodominant compared with determinants on Fab, but when Fab is injected, in the absence of Fc, either by itself or as the dimer (Fab)₂, antibodies are produced efficiently against epitopes on the Fab fragment of IgG.

Antigenic analysis helped not only to distinguish between the various classes (IgG, IgM, IgA, IgE, IgD) and subclasses of immunoglobulins, but also to define their phylogeny. Allotypes are immunologically detectable genetic differences in particular constant regions, whereas idiotypes are the unique antigenic determinants found on the variable regions of antigen binding receptors, i.e. they represent paratopes of different specificities (idiotypes) in terms of their antigen variation. The regulation of the immune system through idiotypic-anti-idiotypic interactions can be achieved without the presence of the antigen, and, thus, is particularly well suited to the maintenance of steady states in lymphoid populations after the antigenic stimulus has been removed. Indeed, idiotypic-anti-idiotypic relationships have the potential to link diverse members of the immune system into a network, so that the activation of one set of clones within the system may have far-reaching and quite unanticipated effects elsewhere within the 'immunological network'.

Synthetic antigens

Landsteiner first showed that small molecules (e.g. 2,4-dinitrophenol) when injected into animals do not elicit antigen formation. However, dinitrophenyl proteins, in which dinitrophenyl is attached to a macromolecular carrier, elicit the formation of antibodies reacting specifically with the dinitrophenyl group. Such small molecules were termed haptens.

Thus a new antigenic specificity may be grafted on an antigen. On the other hand, a limited enrichment of gelatin with tyrosine increased its immunogenicity without significantly changing its specificity. Thus by chemical modification it is possible to change both immunogenicity and antigenic specificity. Increase in immunogenicity by means of appropriate adjuvants has developed significantly due to synthetic adjuvants which may also be attached covalently to the antigen.

Synthetic polypeptides (polymers of amino acids), linear and branched, have been used extensively in immunological research, as their structures were both simple and well known. This permitted the construction of literally hundreds of antigens for the purpose of elucidating the molecular basis of antigenicity, and later the molecular basis of manifold immunological phenomena.

Knowing the chemistry of the copolymers made it possible to arrive at conclusions concerning the role of various structural features in their antigenic function. It is, nevertheless, important to stress that the immunogenicity of a chemical substance depends not only on its structure but also on the biological background of the animal immunized, including its genetic features. It was thus learned that the immunogenically important area of the molecule must be readily accessible to the B cell and cannot be hidden in the interior of the molecule. By chemical modification, antigenic materials may be converted into nonantigens (e.g. by attachment of poly(ethylene glycol) or by poly(DL-alanylation)), whereas nonantigenic materials may become immunogenic.

The overall shape of the molecule does not seem to be a crucial factor in immunogenicity, whereas the size seems to be important: very few molecules of less than 2000 Da are immunogenic, and the immunogenicity increases steadily with the molecular size. The presence of electrical charges on a macromolecule is not a minimum requirement for it to be immunogenic.

Appropriately constructed polymers of D-amino acids may be immunogenic in a similar way to polymers of the natural L-amino acids, but to detect this antigenicity, it is necessary to immunize animals with very low doses, as in the dose range required to prove the immunogenicity of L-amino acid polymers, the D-polymers induce immunological tolerance, also called paralysis. This is probably due to their being very slowly, if at all, metabolized. Through studies of structurally related immunogens, it was possible to establish that those antigens, such as pneumococcal polysaccharides, *Escherichia coli* lipopolysaccharides or D-amino acid polymers, which possess repeating antigenic determinants and are slowly

metabolized, are T independent, i.e. they do not need the cooperation of the helper T cells with B cells, whose progeny produce antibodies, for an efficient immune response. In contrast, most antigens – including L-amino acid copolymers – are T dependent. The purely cellular immune response is limited to the T cell population, of which several subpopulations are now known, and it probably involves the cooperation of helper T cells with effector T cells.

Antigens leading to an immune response of essentially any specificity desired can be prepared synthetically, and this includes the production of antibodies against peptides, oligosaccharides, oligonucleotides, tRNA, lipids, as well as haptens such as penicillin, prostaglandin, dinitrophenol, pyridoxal or ferrocene. Similarly, antibodies have been obtained against many biologically active peptides such as angiotensin, bradykinin or vasopressin. Moreover, in recent years totally synthetic immunogens, including peptide segments of viral coat proteins, e.g. MS2 bacteriophage, hepatitis virus or bacterial toxins (diphtheria, cholera) have been produced, and they have led to the production of antibodies capable of neutralizing the virus, or inducing in experimental animals protection against diphtheria and cholera.

The spatial folding of proteins plays a decisive role in determining their antigenic specificity. With synthetic models, it has been possible to build with the same peptides, immunogens which possessed either exclusively sequential or conformation-dependent antigenic determinants. In globular proteins, most of the antigenic determinants are conformational, as is evident by loss of reaction with their antibodies after denaturation. Antibodies are, therefore, good probes for the conformational state of proteins, and there are many reported cases in which antibodies have been used for detection of different conformations in proteins. Working from these considerations, we have shown that a peptide analogous to a stretch of the hen eggwhite lysozyme sequence can be synthesized, closed into a 'loop' by a disulfide bridge and attached to a synthetic branched polymer. The resulting macromolecule leads to production of antibodies which cross-react efficiently with a unique conformation-dependent region of a native protein. In conceptual terms this opened the road to the synthetic vaccines of the future as it pinpointed the possibility of synthetically preparing relevant determinants that could lead to protection against diseases.

To give just one of the many examples where synthetic antigens were of crucial importance in detecting, or elucidating, a defined immunological phenomenon, the genetic control of immune response should be mentioned. It is clear today that

the capacity to respond well or poorly to a certain antigenic stimulus is under strict genetic control. This observation has been made largely thanks to synthetic antigens (simple chemically) and inbred strains of animals (simple genetically). Using defined branched synthetic polypeptides, differing only in a limited manner within their antigenic determinants, it was possible to prove conclusively that the genetic control of the immune response is determinant specific. McDevitt showed later that the immune response to these synthetic antigens was linked to the MHC locus of the species. The newly discovered immune response (Ir) genes and their products (Ia) have been extremely important in enabling us to understand the phenomena of immunity and resistance to diseases. The Ir gene products (Ia antigens) are present on the cell surface and are important in controlling interactions between cells of the immune system.

Complex antigens

Antigens of importance in practical immunology are mostly not dispersed molecules but cellular or multicellular structures. Of course, the specificity is ultimately definable in all cases in molecular terms. Viruses, sometimes even crystallizable, are among the simplest of such structures, with most of the antigenic specificity residing in their coats, sometimes of purely protein nature, but often including lipids or polysaccharides. In some cases, inner core proteins are also efficient antigens, capable of providing a protective immune response. More has been learned about immunology from studies on bacteria than from any other group of natural antigens, and their antigenic determinants have been in many cases elucidated in great detail, most of it of a polysaccharide nature, but also involving proteins and teichoic acids. Lipids often participate in these specificities. More limited progress has been made in the investigation of mycoplasmas and chlamydiae. On the other hand, recent years have seen a tremendous progress in the immunology of parasite diseases, due to both helminths and protozoa, including the great antigenic variations characteristic of some agents. Fungi are another group of antigens that express an enormous number of antigenically different entities, about whose molecular nature relatively little is known.

Vaccines used in medical practice include four main types of antigen preparations: toxoids (the soluble exotoxins of bacteria such as diphtheria and tetanus bacilli, which have been modified and rendered less toxic by the addition of formalin or gentle heating); antigens isolated from infectious

agents (such as the capsular polysaccharide of the pneumococci); killed vaccines (cultured organisms killed by heat, ultraviolet irradiation or chemicals such as phenol, alcohol or formalin); and attenuated living vaccines (made from strains of organisms that have lost their virulence by growth in culture).

Allergens are antigens which cause allergic reactions either of the immediate or delayed type. They may be of widely different origins such as dust, fungi, hair, pollen, bacterial proteins, food or drugs. The immediate-type allergy is induced mainly through a mechanism triggered by IgE class antibodies, whereas the delayed-type allergy is T cell mediated.

All plant and animal cells possess antigens that can express themselves in a foreign host. Many animal and human antigens may trigger autoimmune phenomena. Some antigens may be organ specific, whereas others are present essentially on all cells (e.g. histocompatibility antigens). In the past two decades the central role of the MHC in immune processes has been recognized. The MHC, a cluster of diverse genes which mediate and regulate a variety of immune mechanisms, appears to exist in all higher vertebrate species (HLA in humans; H2 in mice). Genetic, structural and functional studies of the multiple MHC products have defined three broad classes of genes and molecules. The class I products are glycoproteins expressed on the membranes of all nucleated cells (histocompatibility, or transplantation, antigens). These are the main targets of the graft rejection reaction, and they mediate the recognition and destruction of virus-infected or neoplastic cells. Class II products, defined by immune response (Ir) genes, are expressed principally on the membranes of antigen-presenting cells (e.g. macrophages, dendritic cells, B lymphocytes) and mediate the regulation, through so-called helper and suppressor effects, of a variety of humoral and cellular immune responses. When incompatible, these Ir gene products also play a potentiating role in transplant rejections. Class III genes determine the structures of several discrete proteins including certain components of the complement system which cause destruction and elimination of bacteria and other foreign cells.

No listing of natural antigens would be complete without mentioning all the cellular markers, receptors and tissue antigens. There is a growing number of immunologically and chemically defined antigens characteristic of various types of T cells, and also B cells and macrophages. There are also all the cytokines, molecules provoked in cells and secreted into body fluids, such as interleukins, interferons, thymic and tumor factors, etc.

There are many indications that the cancer patient's immune response may affect tumor growth.

In the study of tumor immunity, the identification of new tumor-specific antigens not present in normal tissues is a primary task. One has to distinguish them from organ-specific antigens and histocompatibility antigens. Tumor antigens are often tumor selective rather than tumor specific, i.e. their concentration may be much higher on cancer cells than on normal cells. Tumor antigens are classified according to the origin of the tumor: experimentally induced (by chemical, physical or viral carcinogens) or spontaneous. The category of oncodevelopmental antigens is of special interest. These, exemplified by α -fetoprotein and by the carcinoembryonic antigen of the colon, are present in normal individuals before birth but disappear thereafter (or stay at an extremely low level), and reappear in the body fluids in adult life only concurrently with specific cancers. Their quantification may, therefore, be of diagnostic value. Antitumor antibodies may be used for immunotargeting of toxins (immunotoxins) and chemotherapeutic drugs.

See also: Allergens; Allotypes, immunoglobulin; Antibodies, specificity; Antigen-binding site; Antigens, cell surface; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Antigens, T dependent and independent; Autoimmunity; Carbohydrate antigens; Carrier; Epitopes; Hapten; Idiotype; Immune response; Immune response (Ir) genes; Immunogen; MHC restriction; Specificity; Tolerance, peripheral; Vaccines; Valency of antigens and antibodies.

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ANTIGENS, CELL SURFACE

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The basic structure of the plasma membrane is a lipid bilayer with intercalated proteins and carbohydrate side-chains extending from the external membrane surface. Chemical studies with SDS-polyacrylamide gel electrophoresis, lectin chromatography and glycolipid analysis have shown that plasma mem-

branes isolated from various tissues differ widely in their protein and carbohydrate composition. However, by far the most detailed picture of plasma membrane diversity, especially with regard to the diversity displayed on the external membrane surface, has come from immunologic studies.

Immunogenetic classification of cell surface diversity

Four genetically distinct categories of cell surface diversity have been identified with serologic and cell-mediated immune reactions (Table 1). First, the discovery of the human ABO blood group antigens and mouse H-2 major histocompatibility antigens established precedents for allogeneic diversity. Allogeneic diversity is specified by genetic polymorphism, i.e. the presence and mendelian inheritance of different alleles of a common gene in members of the same species. The allelic variation may reside in 1) the structural gene for a cell surface protein, as illustrated by major histocompatibility complex (MHC) class I and class II molecules; 2) the activity of an enzyme involved in carbohydrate synthesis, as illustrated by blood group antigens; or 3) a regulatory gene or DNA sequence controlling the expression of a structural gene, as suggested for the TL antigens of TL⁺ and TL⁻ mouse strains.

Second, xenogeneic diversity reflects genotypic differences between members of different species and includes instances in which homologous cell surface molecules in disparate species show structural diversity (e.g. mouse versus human Thy 1) and instances in which an antigen in one species has no apparent structural homolog in another species (e.g. apparent lack of genes homologous to mouse Tla in several other species). A special case of xenogeneic diversity detected on the plasma membrane of mammalian cells is antigens encoded by viral genes integrated into the host cell genome. Despite the clear genetic distinction, the observed patterns of viral antigen expression may be deceptively complex, as illustrated by the murine leukemia virus (MuLV)-encoded gp70 cell surface antigens of mouse leukemias. MuLV genetic information is ubiquitous in the mouse, but whether these endogenous viral genes are expressed or not is determined by a variety of viral and host

gene functions. For example, the expression of specific gp70 determinants differs between mouse strains with low and high leukemia incidence and between differentiated normal tissues in the same animal, thus mimicking the distribution of alloantigens or restricted differentiation antigens encoded by host genes. Apart from direct insertion of viral proteins into the plasma membrane, peptides generated from the intracellular degradation of viral proteins may bind to MHC molecules and be presented on the cell surface, where they can be recognized by T cells.

Third, clonogenetic diversity is determined by somatic genetic changes in gene structure, such as mutations and recombinational events. Genetic recombination is a normal step in immunoglobulin (Ig) and T cell receptor (TCR) gene expression, whereas mutations due to hits by external mutagens or errors in replication may affect any surface antigen gene in somatic cells. Such changes will escape notice unless an affected cell undergoes clonal expansion. For example, malignant transformation of individual B cell or T cell clones has allowed serologic detection of clonotypic determinants on surface Igs and TCRs. The individually distinct tumor rejection antigens of chemically induced sarcomas of mice, the unique antigens of human melanoma detected by autologous typing, and the tum⁻ antigens of mutagenized mouse tumors may represent clonogenetic diversity of this sort on nonlymphoid cells. In the case of unique antigens of human melanoma, the serologic specificity has been traced to a variant of a normal melanocyte surface antigen, melanotransferrin. In the case of tum⁻ antigens, the mutations affect genes encoding for intracellular rather than cell surface components, and the clonotypic determinants appear to reside on endogenous peptides that are presented on the cell surface by MHC molecules and recognized by T cells.

The fourth category of cell surface diversity can

Table 1 Immunogenetic classification of cell surface antigen diversity

<i>Designation</i>	<i>Genetic basis</i>	<i>Prototype antigens</i>	<i>Reagents (examples)</i>
Allogeneic	Allelic differences	MHC class I, II antigens Blood groups	Alloantisera mAbs T cell clones
Xenogeneic	Species divergence for homologous genes Endogenous viral genes	Most surface proteins Virus-coded tumor antigens	Heteroantibodies Heteroantibodies
Clonogenetic	Gene recombination Somatic mutations	TCR; Igs Unique tumor antigens	mAbs Autologous antibodies T cell clones
Epigenetic	Regulated expression	CD antigens	mAbs Heteroantibodies

be referred to as epigenetic ('differentiation-related') diversity and reflects differences in gene expression, rather than disparity of gene structure. (In this context, the term differentiation can be used for all processes that lead to diversity in the patterns of proteins synthesized in cells harboring the same genome, such as embryonic and fetal development, normal self-renewal and terminal differentiation in adult tissues, inflammation, tissue repair and malignant transformation.) Prior to the impact of serologic analysis, there was a general notion coming from transplantation studies with tolerant and chimeric animals that cells from different lineages shared a common surface antigenic phenotype. The first examples of cell surface antigens that distinguish different cell types in the same animal were discovered some 25 years ago in the search for tumor-specific antigens of mouse leukemias. At that time, H-2 was the only known cell surface antigen on these tumor cells. However, specific immunization and testing schemes devised to obviate anti-H-2 responses, led to the detection of several new antigenic systems, including TL, Thy-1, Lyt-1 and Lyt-2,3. Like H-2, these antigens were found both on leukemic cells, which in the mouse are commonly of T cell origin and arise in the thymus, and on normal thymocytes. Unlike H-2, the newly defined antigens showed a restricted distribution among other lymphoid cells and in non-lymphoid tissues and came to be known as differentiation antigens. Enormous interest was generated by the finding that specific cell surface phenotypes not only permitted a distinction between T and B cells, but also between T cell subsets with cytotoxic (Lyt-2,3⁺) and helper cell (Lyt-2,3⁻) activities. This gave rise to the now widely accepted idea that the surface of cells reflects their differentiated state and that cells undergoing distinct pathways of differentiation or serving disparate functions have unique surface phenotypes.

The terms used here to classify cell surface diversity apply to the epitopes defined by specific antibodies or T cells rather than to entire molecules. Any individual cell surface molecule, be it a differentiation antigen or not, may combine structural features that are xenogeneic, allogeneic and clonogenic in nature.

Test systems and approaches to cell surface antigen characterization (Table 2)

Initial interest in cell surface antigens came from studies of blood group antigens, histocompatibility antigens and tumor rejection antigens and their role in blood transfusion, organ transplantation and

tumor immunity. The discovery of differentiation antigens established cell surface serology as a new approach to studying general aspects of cellular differentiation, first in cells of hematopoietic origin and subsequently in other cell lineages. With recent developments in serologic, biochemical and genetic techniques and test systems, the study of cell surface differentiation has been vastly expanded and it is now possible to carry out a comprehensive survey of tissue-specific cell surface antigen display. First, hybridoma technology permits sampling of common and rare immune responses and permits rapid analysis of large numbers of monoclonal antibodies (mAbs) without the elaborate specificity controls required for complex hetero- and xenoantisera. Thus, over the past years, mAbs have been used to define hundreds of new cell surface antigens, primarily in humans and mice. Second, indirect immunofluorescence staining combined with cytofluorometry and cell sorting and other techniques allow detailed analysis of hematopoietic cells and other tissues that can be prepared as free-cell suspensions. Cytofluorometry does not lend itself to the analysis of intact tissues. To test substrate-adherent cultured cells, procedures such as radiobinding assays or immunocytochemistry are available. For solid tissues, highly sensitive immunohistochemical procedures have been developed, including indirect immunofluorescence, avidin-biotin complex immunoperoxidase, and alkaline phosphatase (ALP)-anti-ALP procedures. These largely replace absorption assays and other methods that do not permit antigen localization to specific cell types or subcellular structures within tissues. Third, a large number of human and rodent tumor cell lines have been established, representing a wide range of distinct cellular lineages, and much progress has been made in culturing differentiated normal cells. They are used as standard typing panels for antigen specificity and as a source of homogeneous cell populations for biochemical and genetic studies. Fourth, the development of sensitive immunochemical techniques in conjunction with mAbs has led to the definition of a host of plasma membrane proteins, glycolipids and carbohydrates and it has permitted their chemical characterization. For a host of proteins, this analysis has yielded information on their primary structure and facilitated the cloning of antigen-coding cDNAs. In addition, it has allowed distinct cell surface proteins to be placed into families with shared structural features and possibly, common evolutionary ancestry, as exemplified by the immunoglobulin superfamily. Some cell surface antigen genes show alternative mRNA splicing to generate transmembrane, glycosylphosphatidylinositol

Table 2 General approach to cell surface antigen classification

<i>Antigen characteristics</i>	<i>Examples and methodologies</i>
Cell-type specificity	
Cultured cells	Binding assays with short-term cultures of normal cells and tumor cell lines (cotyping)
Tissues	Immunohistochemistry; immunofluorescence staining and flow cytometry (cell suspensions)
Biochemistry	
Proteins	Immunochemistry (immunoprecipitation, Western blotting, purification, amino acid sequencing) Biosynthesis and subunit composition Membrane anchoring
Glycolipids	Carbohydrate and lipid analysis (immuno-thin layer chromatography)
Carbohydrates	Sugar analysis, carrier analysis (lipids, proteins)
Epitope structure	Competition studies with multiple mAbs (radiobinding assay, surface plasmon resonance) Species comparison Molecular structure (amino acid sequence, carbohydrate determinants, conformation-dependence, combinatorial epitope for multimeric antigens) Active site mapping for biochemical functions
Membrane dynamics	
Internalization	Coated pits, caveolae, macropinosomes Surface recycling and processing/degradation of internalized antigen
Extracellular release	Secretion of splice variants lacking membrane anchors Proteolytic release of membrane-bound forms
Genetics	
Molecular cloning	cDNA cloning, sequence analysis for homologies (gene families) and functional domains Alternative mRNA splicing
Gene locus	Chromosome mapping Exon-intron structure, regulatory elements, species comparison Disease-related mutations, deletions and polymorphisms
Biochemical function	Signaling receptors or ligands (cell-bound versus soluble forms) Adhesion molecules Membrane-bound enzymes
Biological function	Growth, differentiation of cultured cells Transgenic animals, knockout animals Altered structure or regulation during development, physiologic activation, or in diseased tissues (inflammation, cancer, immunologic diseases)

(GPI)-linked, and secreted forms of the antigen, or antigens that differ in the length of their cytoplasmic or extracellular domains. Examples of antigens that show this sort of isoform diversity include the neural cell adhesion molecule (NCAM) and the leukocyte common antigen (CD45). Additional diversity within antigenic systems may result from cell type-specific patterns of glycosylation or from differences in subunit assembly in the plasma membrane, as illustrated by the combinatorial diversity of T cell antigen receptor complexes or integrin heterodimers. Analysis of this level of structural diversity is greatly facilitated by the availability of mAbs specific for individual subunits or isoforms carrying shared and unique epitopes. Finally, chromosome mapping and genetic linkage studies that employ 1) serologic analysis of somatic cell hybrids, 2) cytogenetic analysis of inbred and congenic animals, 3) cytogenetic analysis with DNA probes (in situ hybridization to somatic and meiotic chromosomes), and 4) physical mapping with yeast artificial chromosomes and other large

DNA fragments have permitted the construction of genomic maps for cell surface antigen genes.

The plasma membrane is a dynamic structure, with extensive lateral diffusion of surface antigens in the lipid bilayer. Nevertheless, many membrane antigens are not evenly distributed over the entire cell surface (Figure 1). Thus, the paradigm of a 'fluid mosaic' of cell surface molecules freely diffusing within the plane of the plasma membrane has been modified. Complex assemblies of multiple cell membrane components that appear to be organized into specialized cell surface domains have been identified, some of which are anchored to intracellular structures via cytoskeletal proteins. Spatial restriction of cell surface molecules has been documented in detail for polarized epithelial cells and vascular endothelial cells, in which many antigens show preferential localization either to the apical (luminal) surface or to the basolateral surface membranes. Other antigens are targeted to sites of cell-cell or cell-extracellular matrix contacts, to membrane protrusions of invad-

ing cells (invadopodia), or to the leading or trailing edges of migrating cells.

Cell surface antigens are not strictly confined to the outer surface of cells. Instead, they are taken up into intact cells by one of several modes of vesicular internalization (Figure 2). The best-studied uptake mechanism is via clathrin-coated pits and vesicles, a pathway utilized, for example, by the receptors for epidermal growth factor and transferrin. Additional pathways of internalization use caveolae, as shown for the membrane-bound folate-binding protein (FBP) and other GPI-linked surface molecules, or macropinosomes, as shown for the A33 colon cancer antigen. Viruses exploit a number of internalizing cell surface antigens as receptors to gain access to the cell interior, with human immunodeficiency virus (HIV) binding to CD4 being one example. Finally, the clear distinction between surface-bound antigens and secreted antigens has been abandoned because genes encoding *bona fide* integral membrane antigens may also encode soluble antigens found in the extracellular space, either as a result of alternative splicing of membrane-anchoring, transmembrane domains or due to cleavage of the extracellular domains of the membrane-bound forms by specific proteases. These principles apply to a number of cell surface receptors for cytokines that are also found as soluble cytokine-binding proteins and for cytokines that are derived from membrane-bound precursors.

With the new techniques and test systems, the following questions can now be addressed.

1. What is the size of the surface antigen repertoire expressed on normal cells?
2. Which surface molecules are constitutively expressed on all cell types and which are differentially expressed?
3. Of the differentiation antigens, how many follow a single lineage or phase pattern and how many show complex patterns?
4. How is the biochemical function of a cell surface antigen correlated with its cell type specificity?
5. Are the repertoires of cell surface antigens conserved in different species, and are structural homologs expressed with the same tissue specificity and serve the same functions?
6. What is the hierarchy of intrinsic and extrinsic factors that control cell surface differentiation and the coordinate expression of multiple antigenic systems?
7. What is the significance of quantitative variations in the expression of individual antigens on different cell types?
8. Do the various antigens that make up the cell surface have preferential locations as part of a

prescribed organization of surface elements, and what information is encoded by such supramolecular associations?

9. Which alterations in surface phenotype are associated with malignant transformation and other perturbations in normal cell growth and function?

Emerging view of cell surface differentiation

The wealth of information coming from serologic studies of cell surface antigen expression in different species and in diverse cell and tissue types has led to the demise of earlier concepts about how the surface of cells is constructed. Chief among these is the notion that developmental cell lineage patterns provide the general organizing principles for differentiation-related cell surface diversity. The expectation was that distinct cell lineages in developing embryonic and fetal tissues and in self-renewing adult tissues would be marked by unique surface antigens (e.g. lymphoid or neuroectodermal lineage markers) and that discrete phases in these lineages would be marked by differential expression of additional antigens (phase markers). These molecules would serve cell functions unique to the respective lineages and phases and provide convenient histogenetic markers for the study of embryonic development, tumor classification and other cell type analyses. One early exception to this concept was the mouse Thy-1 antigen, which was found not only on thymocytes and peripheral T cells but also on fibroblasts, in brain tissue, and on a few additional cell types. Thus, Thy-1 shows the restricted expression of a differentiation antigen, yet its tissue distribution is complex, overlapping several distinct embryological lineages and showing differences in developmental regulation within these lineages. Subsequent studies have shown that lineage-restricted antigens are the exception and antigens like Thy-1, whose distribution does not conform to embryonic derivation or lineage are the ones commonly found. The reluctance of differentiation antigens to fit into established cell lineage patterns means that comprehensive maps of antigen distribution are required before conclusions can be reached with regard to cell type, tissue type or disease-related specificity.

Apart from normal differentiation antigens, tumor cell surface antigens have been the subject of much research, and the question of their normal tissue distribution has been of key importance. One suggestion has been that tumors re-express molecular and biological traits of fetal cells that are absent from normal adult cells. This theory has not held up for

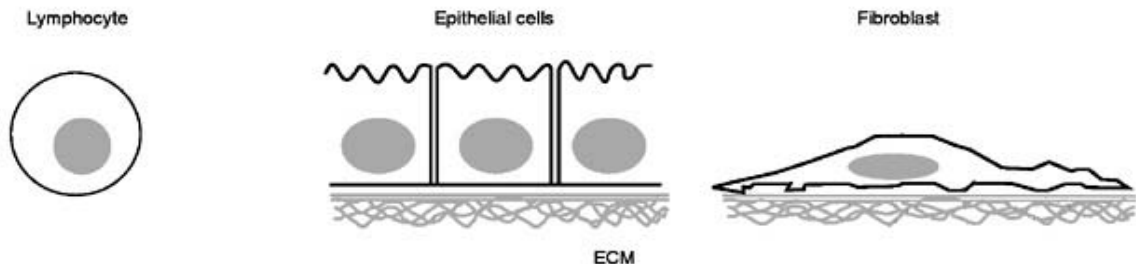
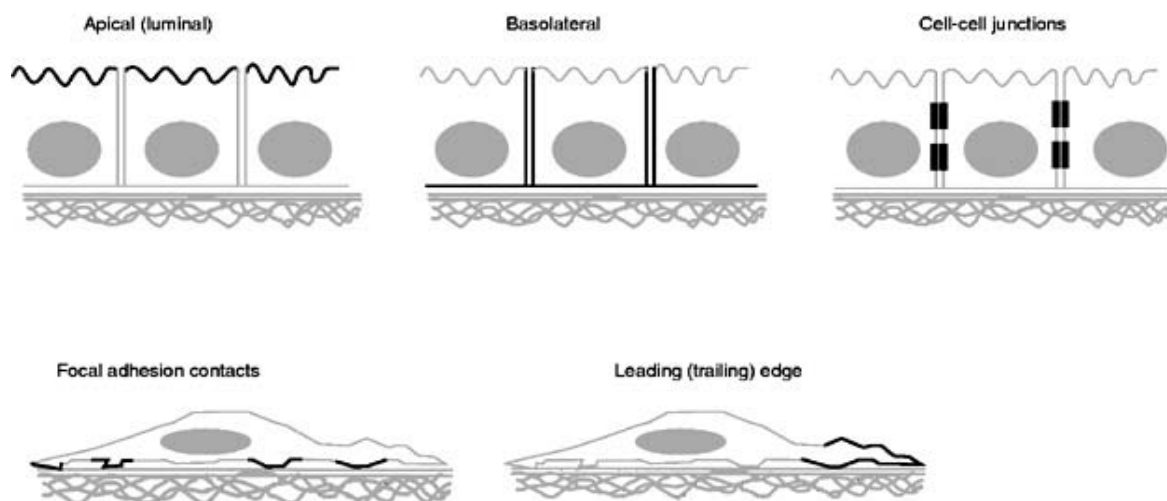
(A) General cell surface distribution**(B)** Restricted cell surface distribution**(C)** Cell surface shift of cryptic antigens

Figure 1 Differential localization of cell surface antigens. Examples of antigen expression on lymphoid, epithelial and fibroblastic cells are illustrated as follows. (A) Uniform antigen distribution on the surface membrane, as seen for MHC class I molecules. (B) Spatially restricted cell surface distribution on polarized cells growing on extracellular matrix (ECM). In epithelial cells, apical, basolateral and cell junctional patterns are distinguished. For fibroblastic cells, antigen localization to focal adhesion contacts and the leading (or trailing) edge of migrating cells are indicated. (C) Cryptic antigens may be present in intracellular compartments of some cells but become exposed on the surface of other cells. The example shown here represents a mAb-defined carbohydrate structure, lacto-*N*-tetraose (type I blood group precursor), carried by glycolipids and glycoconjugates, that is detectable in the Golgi apparatus of normal adult cells, but becomes further glycosylated and 'cryptic' before reaching the cell surface. By contrast, in fetal endoderm and teratocarcinomas, the glycosylation of lacto-*N*-tetraose moieties is deficient and large numbers of the precursor structure reach the cell surface.

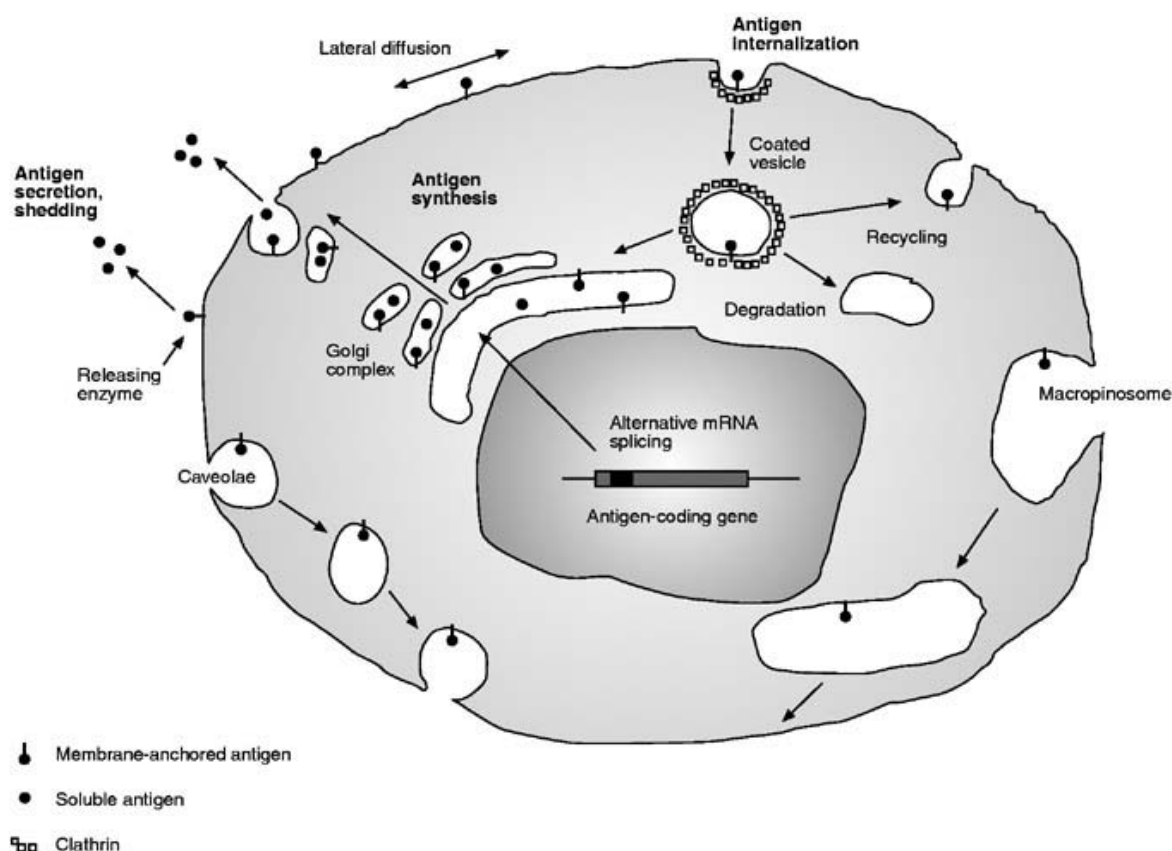


Figure 2 Dynamic view of cell surface antigen expression on the plasma membrane. Using immunocytochemical staining methods with permeabilized cells, some antigens are detected as biosynthetic precursors in the cytoplasm and Golgi complex, prior to their appearance on the cell surface. Similarly, antigens that are internalized through clathrin-coated vesicles, macropinosomes, or caveolae can be followed by these methods until they are either degraded (e.g. lysosomal pathway) or recycled in intact form to the cell surface. Alternative splicing of antigen-coding mRNAs with removal of membrane-anchoring transmembrane domains or cleavage of membrane-bound antigens by specific proteases may result in secreted or shed variants of the antigen.

most cancer antigens when the analysis of normal adult tissues has been sufficiently detailed. Antigens that fit an 'oncofetal' pattern in one system are invariably found in other systems with a different mode of expression and control. Similar departures from the presumed modes of expression have been found for other categories of 'transformation-related' or 'proliferation-related' antigens of cancer cells. (Much of the current interest in tumor-specific antigens focuses on clonogenetic determinants on cell surface differentiation antigens detected by the host immune system, as described above.) Even for those antigens for which a biochemical function has been identified, such as various cell surface receptors, membrane-bound enzymes and adhesion molecules, the unpredictable cross-lineage patterns are observed.

Understanding the genetic and biochemical control of the complexity in cell surface antigen display will require decoding the signals that initiate, regulate and maintain the differentiated state. Understanding

the physiologic role of a cell surface antigen in the organism, even if it has been assigned an *in vitro* biochemical function and its mode of genetic control is known, may require highly specialized test systems. A search for genetic diseases caused by abnormalities in a single cell surface antigen locus may facilitate the study of physiologic function for some molecules, as illustrated by the analysis of patients with leukocyte adhesion deficiency due to defects in the CD18 gene. However, it is tempting to speculate that many common biological functions of the cell surface are not assigned to individual proteins. They are the responsibility of integrated circuits comprising multiple components, both in the plasma membrane and inside and outside of the cell, in which individual proteins serve as structural and functional modules. The same module may be used over and over in different cell types and different circuits to serve different functions, and similar functions may be mediated, in parallel or alternatively, by separate molecular mechanisms. Such a concept is consistent

with the experience that targeted inactivation of genes in knockout mice is commonly associated with unexpectedly subtle phenotypes.

What lessons can be drawn at this stage of the analysis?

1. From the large pool of genes coding for cell surface antigens, distinct but widely overlapping sets are activated during differentiation in disparate lineages.
2. The distinctive surface phenotype of differentiated cells is achieved not by the display of unique lineage-restricted antigens but rather through unique combinations of antigens that are drawn from a common pool.
3. Although the surface phenotype of differentiated cells is quite stable, there is considerable flexibility in reprogramming individual antigens. Surface antigens are not irreversibly silenced or expressed during differentiation but can be induced or suppressed by specific extrinsic/intrinsic signals.
4. Individual surface antigens can be viewed as modular elements that together generate complex and unique surface patterns. This concept of modularity in the construction of cell surfaces could explain unique functions achievable with common elements, as the same module in different cell types would come under different

regulatory control and could impart different information.

See also: ABO blood group system; Alloantigens; B-lymphocyte differentiation; Carbohydrate antigens; CD antigens; Flow cytometry; Immunocytochemistry and enzyme markers; Monoclonal antibodies (mAbs); T lymphocyte differentiation; Thy-1; Tumor antigens.

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ANTIGENS, T DEPENDENT AND INDEPENDENT

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Defense mechanisms against microbial invaders form a spectrum ranging from relatively primitive innate immunity to highly sophisticated acquired immune responses. The innate immunity uses only invariant, germ line-encoded receptors for the recognition of foreign substances. The T cell-dependent B cell response, on the other hand, produces somatically mutated, high-affinity antibodies with an enormous range of specificities and a variety of constant regions for different effector functions. The B cell response to T-independent antigens occupies an intermediate position in this scheme; it generates essentially low-affinity antibodies of the immunoglobulin M (IgM) class with germ line specificities. The elaboration of high-affinity antibodies by somatic mutation carries

the risk of creating dangerous self-reactive antibodies; the B lymphocytes must be controlled by helper T lymphocytes via 'cognate' T–B interaction (linked recognition of T and B epitopes of the same antigen). Most natural proteins are T-dependent antigens, and B cells can not respond to such antigens without costimulatory signals from cognate T help. T-independent antigens bypass the requirement for cognate T help. They do this by reacting with both B cell antigen receptors and receptors of innate immunity (on B cells or accessory cells) which provide other costimulatory signals. Typical T-independent antigens are microbial sugars, lipid structures and certain nucleic acids which can not be presented to T cells on major histocompatibility

complex (MHC) molecules for linked recognition and must be handled otherwise. Type 1 T-independent antigens (such as endotoxin, lipopolysaccharide) are directly mitogenic for B cells of certain species, while type 2 antigens (e.g. polysaccharides) activate B cells in conjunction with accessory cells, cytokines and other factors, such as complement. However, the T-independent antibody responses, compared to those benefiting from cognate T help, are limited with regard to affinity, isotype pattern and immunologic memory.

Role of costimulatory signals

The immune system should discriminate between self and nonself. It copes with this problem at different levels. Immature lymphocytes which react with self antigens undergo apoptotic cell death, but not all self antigens are present in the lymphopoietic micro-environments. Therefore, the cellular activation processes during immune responses depend on the cotriggering of lymphocytes by different signals, via the antigen receptor and other receptors; triggering at the antigen receptor only, leads to apoptosis or tolerance. Usually, microorganisms or their products generate costimulatory signals for T cells at the level of the antigen-presenting cells. In the case of B cells responding to T-dependent antigens, the critical costimulatory signals are then provided by helper T cells. In particular, the functions of B cells, 1) to generate high-affinity antibodies by somatic mutation (with the risk of producing novel self-reactive antibodies), 2) to switch from IgM to other immunoglobulin isotypes, and 3) to extensively proliferate and generate a memory cell population in addition to antibody secreting cells – depend on cognate T help and take place in the germinal centers of secondary lymphoid organs. However, cognate T–B interaction only occurs if the antigen, after recognition via a B cell epitope, and then internalization and processing by the B cell, produces a T cell epitope (a short peptide) which can then be presented as a complex with an MHC class II molecule on the surface of the B cell. The most important costimulatory signal during cognate T–B interaction is mediated by CD40 ligand (CD40L) on the T cell binding to CD40 on the B cell. CD40 knockout mice generate no germinal center responses and only respond to T-independent antigens. Humans with nonfunctional CD40L mutations suffer from the X-linked hyper IgM syndrome: the germinal center response is lacking and the immune system is overstimulated by T-independent bacterial antigens.

T-independent antigens can provide other costimulatory signals for B cells, directly or via

accessory cells (Figure 1). On the other hand, obligatory T-independent antigens do not induce a germinal center response and, consequently, do not induce either somatic hypermutation or memory. Only certain isotype switches occur (see below).

Type 1 T-independent antigens

Type 1 antigens are directly mitogenic for B cells from certain species. LPS (lipopolysaccharide, endotoxin) of gram-negative bacteria is the prototypic type 1 antigen for murine B cells. CD14, a 55 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein, is the major cell surface receptor for the activating effects of LPS on macrophages, B cells and various other cells. Serum lipid transfer protein or LPS-binding protein (LBP) accelerates the transfer of LPS to CD14 in a catalytic fashion. LPS then probably exerts its mitogenic activity by incorporating itself into the cell membrane. Because the lipid A/core portion of LPS bears structural resemblance to the second messenger ceramide, LPS could interact with ceramide-activated protein kinases. However, while LPS is a potent activator of human macrophages, it is not directly mitogenic for human B cells; it acts like a type 2 antigen for human B cells. For murine B cells, one can distinguish between the polyclonal B cell activator function of LPS, which occurs at a high LPS concentration, and the proper type 1 antigen function which occurs at a 1000-fold lower concentration. At the low dose, B cells with the appropriate antigen receptors have the advantage of focusing the mitogen on to themselves; the response, e.g. to DNP-haptenated LPS, then occurs only in hapten-specific B cells.

CD14 functions as a 'pattern recognition receptor' for a range of microbial constituents from gram-negative as well as gram-positive bacteria which, like LPS, activate macrophages. The B cell mitogenic properties of these substances are not yet well characterized. On the other hand, LPS reacts with a variety of receptors, including the CD11b/CD18 integrin, the animal lectin galectin-3 (expressed by activated macrophages) and others. *In vivo*, cytokines, inflammatory mediators and cell adhesion/cell interaction molecules from various accessory cells (macrophages, dendritic cells, natural killer (NK) cells, mast cells, activated bystander T cells, etc.) can participate in a T-independent antigen type 1 response. *In vitro*, high concentrations of appropriate cytokines induce all types of immunoglobulin class switches in LPS-activated B cells.

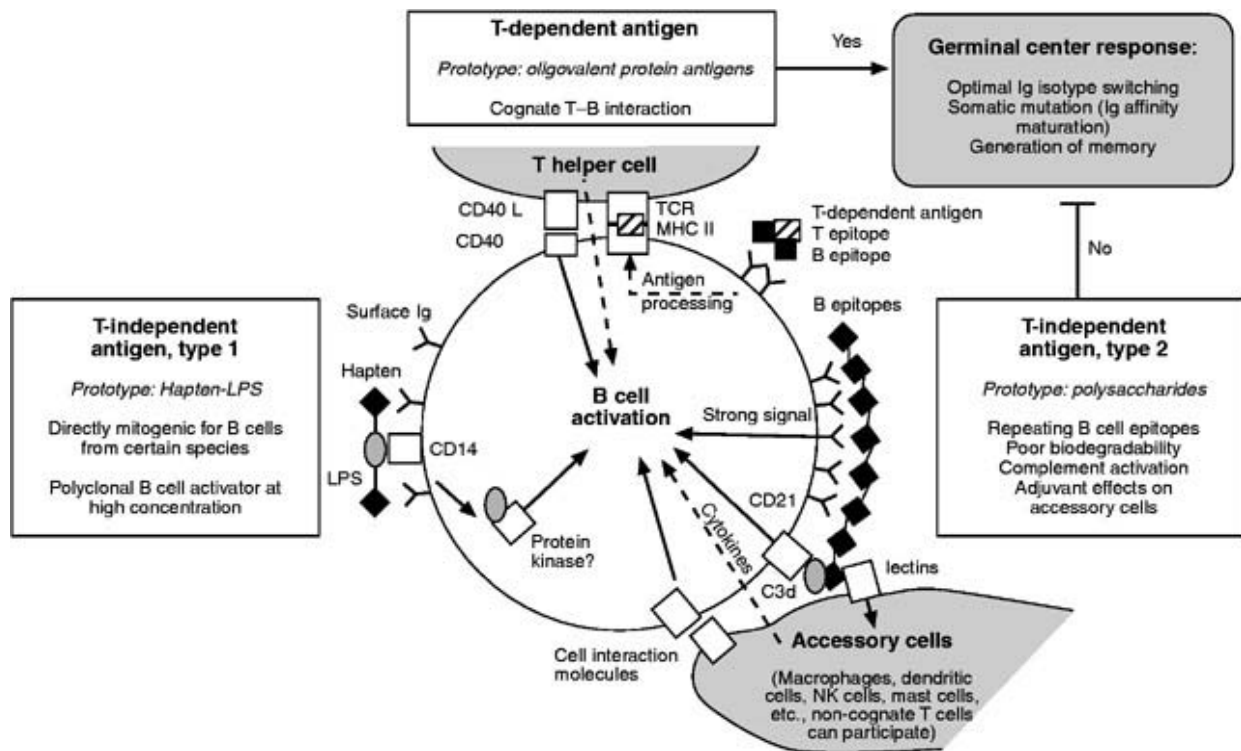


Figure 1 T-dependent and -independent B cell activation: properties of type 1 and type 2 T-independent antigens compared to T-dependent antigens.

Type 2 T-independent antigens

Although the presence of repeating B cell epitopes, leading to extensive cross-linking of B cell antigen receptors, is a common feature of type 2 antigens, they are not by themselves mitogenic for B cells. They bypass cognate T help only by synergy with the soluble factors and/or cell interaction molecules from accessory cells like those mentioned above; their effects on the B cell antigen receptor and on nonantigen receptors are equally important. Polysaccharides from encapsulated bacteria are the prototypic type 2 antigens in nature.

Effects of type 2 antigens on the B cell antigen receptor

Type 2 antigens are high molecular weight substances with many repeating B epitopes; they can be sugars (dextran, inulin), proteins (polymerized flagellin, viral capsids) or synthetic polymers (polyacrylamide). The number of identical epitopes is an important variable for B cell activation. Monovalent antigens simply occupy the antigen receptors. Bivalent/low-multivalent antigens can be internalized and their T cell epitopes presented to helper T cells. Cognate T help is required for these antigens, to which most natural proteins belong. Too extensively multivalent antigens can inactivate B cells. However,

somewhere in between, multivalent antigens optimally sensitize B cells to various T-independent costimulatory signals. Cross-linking of surface Ig leads to intracellular clustering of protein kinases, phosphatases and other receptor-associated proteins. The outcome depends on the extent of cross-linking and additional variables, such as the rigidity of the antigen backbone, allosteric effects on individual antigen receptors, and persistence of the interaction; type 2 antigens usually have a poor biodegradability.

Historically, type 2 antigens were defined as antigens which produce no antibody response in those mice which later turned out to have the xid defect (X chromosome-linked immunodeficiency). Interestingly, whereas humans with X-linked agammaglobulinemia, caused by mutations of the tyrosine kinase Btk, have a profound B cell maturation defect, the xid mice, with another Btk mutation, only show a defect of B cell activation by type 2 antigens. The signaling defect after Ig cross-linking is irrelevant for the response to T-dependent antigens in these mice.

Effects of type 2 antigens on nonantigen receptors

Polysaccharides are activators of the alternative pathway of the complement system. Via bound complement fragment C3d, they can react with the complement receptor 2, CD21 (the Epstein-Barr

virus receptor on human B cells). CD21 is associated in the B cell membrane with the CD19 signal transduction complex, and thus can generate an activating signal. In addition, B cells, macrophages and other cells express carbohydrate receptors of various kinds/specificities: C-type lectins (selectins, collectins) and others (e.g. galectins). These animal lectins play a role in cell adhesion, phagocytosis and cell activation as well as in interactions of cells with glycosylated serum proteins. In addition, they can recognize carbohydrates on microbial antigens, and thus probably participate in T-independent polysaccharide-specific antibody responses (e.g. the B cell signaling molecules CD23 and CD72 have C-type lectin domains. More generally, the 'adjuvant effect' of type 2 antigens is their capacity to activate accessory cells and induce cytokine secretion by such cells (phagocytes, inflammatory cells, etc.). In principle, activated bystander T cells can also participate in type 2 antigen-dependent B cell activation via cytokines. Moreover, activated B cells themselves are now known to produce a variety of cytokines, including typical 'T cell cytokines' like interleukin 2 (IL-2).

Immunodeficiencies for type 2 antigens

Neonatal mammals generate antibody responses to type 2 antigens either not at all or weakly; human infants under 2 years old respond very poorly to polysaccharides. Thus, some maturation of B cells and/or accessory cells (microenvironments) is necessary. Poor antipolysaccharide responses also occur in common variable immunodeficiencies and in the Wiskott-Aldrich syndrome. In the latter, a mutation in the WASP gene prevents correct expression of a whole series of cell surface molecules. In normal adults, antipolysaccharide responses are greatly compromised after splenectomy. Splenic B cells are important, particularly marginal zone B cells and CD5⁺ B cells. Some investigators consider that CD5⁺ (also called B1) B cells belong to a separate B cell lineage which preferentially expresses certain germ line-encoded (low-affinity, polyreactive) antibody specificities. Alternatively, since CD5 is an (early, pregerminal center) activation marker for B cells, one could conceive that conventional B cells expressing certain (i.e. polyreactive) antigen receptor specificities would be frequently activated *in vivo*. In any case, antipolysaccharide responses, in accordance with their generation outside germinal centers, typically produce only germline antibody specificities, are oligoclonal and primarily restricted to IgM. But isotype switching to IgG2 in humans and IgG3 in mice does occur; interferon γ (IFN γ), IL-3 and granulocyte-macrophage colony-stimulating factor

(GM-CSF) synergistically induce the IgG3 switch in murine B cells exposed to polysaccharides. Conjugate vaccines of polysaccharides linked to proteins induce cognate T-B interaction. Their utilization to enhance defense against encapsulated bacteria in infants and splenectomized or otherwise immunocompromised adults is of medical importance.

Immunogenicity of microbial DNA

dG polynucleotides can form four stranded conformations which are recognized by the scavenger receptor of macrophages. Moreover, certain palindromic DNA motifs, with sequences rarely present in mammalian DNA, can activate macrophages as well as be directly mitogenic for B cells. The mitogenic effect occurs via interaction with presently unknown intracellular receptors following internalization. The presence of an unmethylated CpG core in the active motifs seems to be relevant to self-nonself discrimination; the CpG content is higher in bacteria than in mammalian DNA and cytosine is not methylated in bacteria. The possibility (still theoretical) exists that bacterial surface molecules could be converted into T-independent antigens by complex formation with a mitogenic DNA motif.

B cell superantigens

Other microbial substances act as superantigens by binding to the B cell antigen receptors outside the antigen binding site. *Staphylococcus aureus*-derived protein A is the prototype for this interaction at the B cell level. Polymerized protein A induces strong B cell proliferation and Ig secretion in conjunction with cytokines such as IL-2. Superantigens are polyclonal activators but, rather than stimulating protective immunity, they could deviate the B cell response away from important antigens in the interest of the microorganism.

See also: B lymphocyte activation; B lymphocyte repertoire; B lymphocyte, antigen processing and presentation; CD5; CD40 and its ligand; Cytokines; Endotoxin (lipopolysaccharide (LPS)); Germinal center; Helper T lymphocytes; Immunoglobulin class switching; Innate immunity; Lectins; Memory, immunological; Polyclonal activators; Selectins (CD62-E/L/P); Somatic mutation; Superantigens; Vaccines.

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ANTISERUM

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An antiserum is simply a serum obtained from an immunized animal or vaccinated human. Immunization is the process of introducing an antigen, often repeatedly, and via a variety of routes (intraperitoneal, subcutaneous, intramuscular, intravenous or oral) into an experimental animal. An immunogen is a molecule which is recognized by the immune system and is capable of inducing an immune reaction. The immune response elicited usually consists of a humoral component and a cellular component. The term antibody was coined to designate the serum proteins that are responsible for humoral immunity. Antibodies are synthesized by plasma cells (terminally differentiated B cells) and can be detected in the serum. Thus a serum that contains a greater than usual concentration of antibodies to a particular antigen, usually as a result of immunization (although natural infection can also generate convalescent antisera), is an antiserum against that antigen. Serology is the term classically used to refer to the study of reactions between an antigen and its antiserum.

The antibody response

Initial immunization of an animal results in a slowly rising, limited response consisting mainly of immunoglobulin M (IgM) class antibodies in the serum. Further injections of antigen boost the antibody level, resulting in a long-lasting enhanced response. This secondary response is composed primarily of IgG class antibodies. Antibodies that comprise the secondary response possess a higher affinity for antigen and hence most sera are collected after the onset of a secondary response.

Passive transfer of immunity

Specific immunity can also be conferred upon an animal or human by the passive transfer of antiserum from a specifically immunized donor. The recipient of such an adoptively transferred antiserum becomes immune to the particular antigen, without ever having been directly exposed to the antigen or having responded to the antigen itself. The immunity thus acquired is of short duration and no memory cells are generated. This approach is useful for conferring immunity rapidly, without the time delay needed to generate an active immune response. The main disadvantage of passive immunization was first observed by a physician named Clemens von Pirquet who reported on a phenomenon we now call serum sickness. He observed that patients repeatedly treated by passive immunization with horse serum, containing antibodies to diphtheria toxin, developed fever, skin rashes and joint pains.

Specificity, titer and affinity

Several parameters have to be evaluated to establish the quality and usefulness of an antiserum. These are specificity, titer and affinity.

The most outstanding feature of a good antiserum is specificity. Most antigens express a large number of epitopes and, against each of these, different antibodies may be elicited. A highly specific antiserum is one that reacts only with epitopes on the antigen used to raise it and not to other antigens that resemble it. In practice an antiserum reacts with antigens that in one way or another resemble the immunizing antigen. Extensive absorption against these

cross-reacting antigens will yield a monospecific antiserum.

A high titer antiserum is one which has a large number of antibodies that are specific for the antigen being tested. The titer of an antiserum is generally expressed as the reciprocal of the highest dilution that reacts with antigen to a predetermined end-point in a given assay system.

The affinity of an antibody reflects the strength of binding between a single antibody-combining site (paratope) and an antigenic determinant (epitope). The term avidity is thus used to describe the overall strength of binding between the antigen and antibody. Avidity is influenced by the intrinsic affinity of the antibody for an epitope on its antigen and the valency of both the antibody and the antigen. As a rule, when an antibody and antigen can form multivalent complexes, the overall strength of the interaction (avidity) is greatly increased.

Making antigens immunogenic

There is no perfect recipe for the generation of a good antiserum. Many foreign molecules (viruses, bacteria and cells) elicit strong antibody responses but some foreign substances fail to induce a good antiserum. In some of these instances the immune system can be manipulated to generate a response, and this can be achieved either by modifying the antigen or varying the host animal. Many of these modifications have been established empirically over the years; however, our increased understanding of the molecular events controlling an immune response should allow more rational approaches for the generation of high titer, specific antisera. Before attempting to produce an antiserum, consideration should be given to the following points: the nature of the antigen and its purity, the site/route of immunization, the dose of the antigen, the choice of adjuvants and the genetic background of the animal to be immunized.

In general, particulate antigens and higher molecular weight proteins tend to be more immunogenic than soluble antigens and proteins of low molecular weight. Converting small soluble molecules into large aggregates is an effective way of enhancing their immunogenicity. The physical coupling of a small molecule which is nonimmunogenic on its own (hapten) to a larger immunogenic carrier molecule also achieves the same result. The most common routes of immunization are subcutaneous, intramuscular, intradermal, intraperitoneal and intravenous. Other routes of immunization include oral ingestion of antigen, antigen inhalation, topical application to the skin and the direct injection into an organ such

as the spleen. The dose of the antigen to be administered can profoundly influence the outcome of the procedure. Thus either very low or high doses of antigen may induce tolerance rather than the successful production of an antiserum. One should also bear in mind that with multiple injections, trace contaminants within an antigenic preparation may eventually elicit responses which will be detected in later bleeds collected from the animal and may result in an antiserum with multiple specificities. The degree of specificity of an antiserum depends largely on the degree of purity of the antigen used. The decision about antigen purity is often based on the intended use of the antiserum. If a highly specific antiserum that will only recognize the appropriate antigen is needed, then before commencing immunization the antigen must be purified to homogeneity.

Choice of species

Antisera can be raised in a wide variety of vertebrate animals: mice, rats, guinea pigs, hamsters, rabbits, chickens, sheep, pigs, goats, donkeys and horses. The choice of animal for immunization tends to be determined by how much serum is needed, how much antigen is available and the extent of foreignness of the antigen to the immunizing animal. Sheep, goats, pigs, donkeys and horses yield larger quantities of antiserum but are expensive to maintain and are generally used only for the production of commercially available antisera. Rabbits are most commonly used in the laboratory setting when individual investigators need to generate larger volumes of a particular antiserum. Exsanguination is the recommended method for collection of large amounts of antiserum from an animal to be sacrificed but this should only be performed by individuals well versed in the correct technique and as approved by institutional and national guidelines. The production of limited quantities of antisera in the mouse can be overcome by the production of ascites fluid where high antibody titers, equivalent to those achieved in serum, can be obtained. When only tiny amounts of antigen are available, the use of mice is also a clear advantage. A further advantage is that most investigators use inbred mouse strains and the genetics of the immune response for most of the strains have been well documented. If a weak response to an antigen is obtained in a particular mouse strain, changing the strain may help overcome the problem. If the amount of antigen is not limiting, several animals should be used as, even in inbred animal strains, a single antigen preparation will produce differing responses. The most important rule in the choice of animals is that immunization should be done in

animals that are furthest in evolutionary distance from the source of antigen.

Adjuvants

The judicious use of adjuvants (L. *adjuvare*, to help) allows for the generation of early, strong and sustained immune responses. In fact the use of an adjuvant can often determine the difference between success and failure. The adjuvants that are available are highly heterogeneous agents and exert their effects in diverse ways, such as causing depot formation at the site of injection (e.g. oil-based adjuvants), causing immunostimulation (e.g. lipopolysaccharide) and serving as delivery vehicles by facilitating the targeting of antigens (e.g. liposomes, biodegradable polymers). The most commonly used adjuvant is Freund's complete adjuvant, in which the immunogen is prepared as a water-in-oil emulsion. The main disadvantage of Freund's complete adjuvant is that it evokes aggressive granuloma formation in the animal and is thus completely unacceptable for use in human vaccine programs. A common alternative, and one licensed for human use, is alum. Here antigen is adsorbed on to an aluminum salt, aluminum hydroxide being most commonly used. Other adjuvants include Freund's incomplete adjuvant, N-acetylmuramyl-L-alanyl-D-isoglutamine (common name MDP) and its derivatives, lipid A, lipopolysaccharide, saponin, Quil A, ISCOMS, liposomes and micropheres made of biodegradable polymers. It should be stressed that the use of adjuvants is not only important for the humoral arm of the immune response (i.e. in generation of a superior antiserum) but also for activation of cellular immune responses. The single most important issue determining the acceptability of an adjuvant for human use is safety and the severity of side-effects, especially when implemented for routine childhood immunizations.

Uses of antisera

A final word about the use of antisera. They are used in medicine both for diagnostic purposes and for patient treatment, and in the laboratory they are used as analytical and preparatory tools. Thus the injection of antisera against toxins such as diphtheria and tetanus, against the venoms of snakes, and against rabies still remains a mainstay of treatment. Equally, the use of specific antiserum allows for the detection and quantitation of minute quantities of telltale antigens such as meningococcus in the cerebrospinal fluid or hormones and drug levels in urine or sera. Specific antisera are used for tissue typing and cells can be phenotyped using antisera raised against specific cell surface molecules. Antisera coupled to radionuclides are used in the detection and treatment of some tumors.

See also: Adjuvants; Affinity; Antigens; Antilymphocyte serum; Carrier; Epitopes; Hapten; Immunoassays; Immunogen; Liposomes; Plasma; Serum sickness; Specificity; Tissue typing; Tolerance, peripheral; Toxins; Vaccination, methods of administration; Venoms.

Further reading

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APOPTOSIS

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In a multicellular organism cell death occurs in many contexts – during development, tissue homeostasis, terminal differentiation and disease. In most of these cases cells are eliminated due to the triggering of a process that results in the dismantling of the cell from within. This demolition process results in a very

distinctive and easily recognizable cellular phenotype that, in 1972, led John Kerr, Andrew Wyllie and Alister Currie to propose that this mode of cell death was different from that which occurred in response to severe cellular insults (necrosis), and for which they coined the term apoptosis. Apoptosis – a word

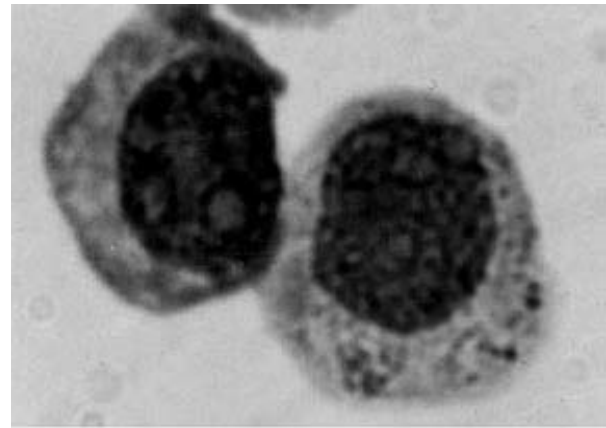
derived from the Greek where it is used to describe the process of leaves falling from trees or petals from flowers – perfectly encapsulates a process where cells quietly shrink and fall away from the tissues they were once an integral part of, with little damage to the main organism. Although the features of apoptosis were originally described in mammalian cells, the morphologic characteristics of dying cells are strikingly similar in birds, amphibians and insects. This suggests that at least some of the genes controlling this process have been well conserved through evolution, and indeed, evidence for this is now emerging.

Apoptosis can be triggered by diverse stimuli, both physiologic and pathologic, all of which seem to engage the same cellular machinery that is responsible for the destruction of the cell from within. Thus, although the proximal signaling events that can lead to apoptosis may vary from one stimulus to another, it is likely that different signals converge on a common set of effector molecules at some point. Recent developments in this field heavily implicate members of the interleukin 1 β (IL-1 β)-converting enzyme (ICE) family (caspases) as the end-stage effectors of apoptosis.

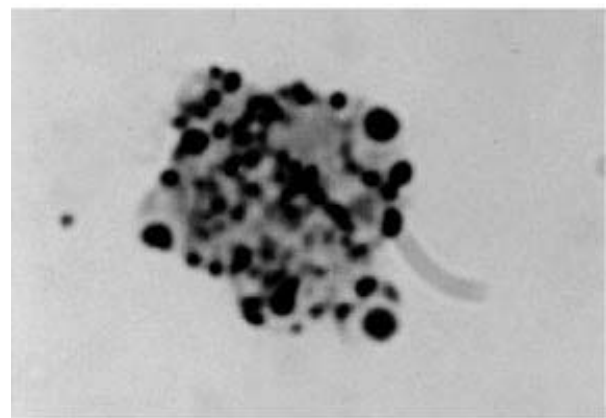
Apoptosis: form and function

Morphology

During apoptosis, a cell typically condenses its nucleus and cytoplasm, its DNA is degraded into many small (200 bp–250 kbp) pieces, marked blebbing of the plasma membrane occurs, and, in the final stages, the cell collapses into multiple intact fragments that are eaten by phagocytes *before* the cellular contents have had a chance to escape (Figure 1). Probably the most striking morphologic feature of apoptosis is the marked condensation and disintegration of the nucleus into numerous, intensely staining, spheres scattered throughout the cytoplasm



(A)



(B)

Figure 2 Morphological features of (A) a live versus (B) an apoptotic cell. Note particularly the disintegration of the nucleus into numerous discrete fragments.

(Figure 2). This contrasts starkly with the process of necrosis, where the nucleus typically swells to occupy most of the cytoplasm, and the chromatin decreases rather than increases in staining intensity. Plasma

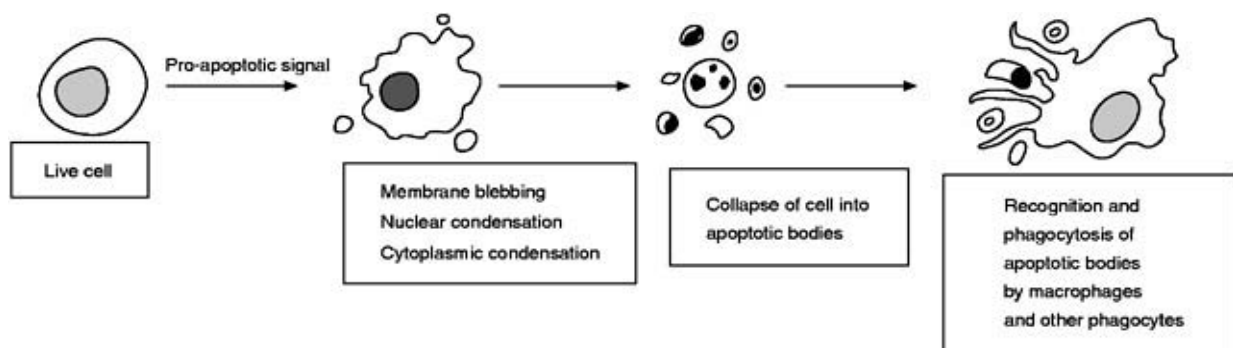


Figure 1 Schematic representation of apoptosis.

membrane blebbing and disintegration of the cell into numerous apoptotic bodies are other highly characteristic features of apoptosis (Figure 3). The collapse of the cell into apoptotic bodies, that retain membrane integrity for several hours, likely facilitates phagocytosis and removal of the dying cell by surrounding phagocytes (Figure 1). In essence, apoptosis represents a clean and efficient way of removing unwanted or diseased cells while minimizing an inflammatory response.

Recognition and removal of apoptotic cells by phagocytes

Changes to the composition of the plasma membrane have long been suspected to mediate early recognition of apoptotic cells by phagocytes – thereby triggering their safe removal. In many ways this stage of apoptosis is the most critical, since the essential functional difference between an apoptotic and a necrotic cell is that the former is somehow recognized as being in the throes of death several hours before any cytoplasmic contents leak out. Necrotic cells, on the other hand, undergo early lysis, thereby

causing damage to surrounding cells and eliciting an inflammatory response. When apoptotic cells are formed in culture, where there are no phagocytic cells around to eat them, they eventually undergo secondary necrosis, which would probably be just as damaging as primary necrosis were it to occur *in vivo*. Thus, the membrane changes that signal to phagocytes that a cell is undergoing apoptosis are of paramount importance in this process.

Membrane changes that occur on apoptotic cells have proved elusive. To date it appears that there are two main alterations that occur on the surface of apoptotic cells that may be mutually exclusive in their usage. One mechanism used by apoptotic cells to signal their plight to surrounding cells is to export phosphatidylserine – a lipid normally confined to the inner plasma membrane leaflet on healthy cells – to the external surface of the plasma membrane. This appears to trigger phagocytosis of these cells, presumably by recognition of surface phosphatidylserine via a putative phosphatidylserine receptor on the macrophage. The other mechanism that has been comparatively well characterized involves the appearance of, an as yet unidentified, thrombospondin-binding ligand on the surface of the apoptotic cell. This ligand is bound by thrombospondin-bearing macrophages (thrombospondin being bound to the macrophage via a vitronectin receptor-CD36 interaction) and facilitates uptake of the apoptotic cell by the latter. Interestingly, the CD36-associated mechanism also appears to operate in *Drosophila*, as recent studies have discovered a CD36-related molecule (Croquemort) involved in macrophage removal of apoptotic cells in this organism. Other apoptosis-associated membrane changes have also been described, such as alterations to the carbohydrate composition of the plasma membrane, but these changes remain poorly characterized.

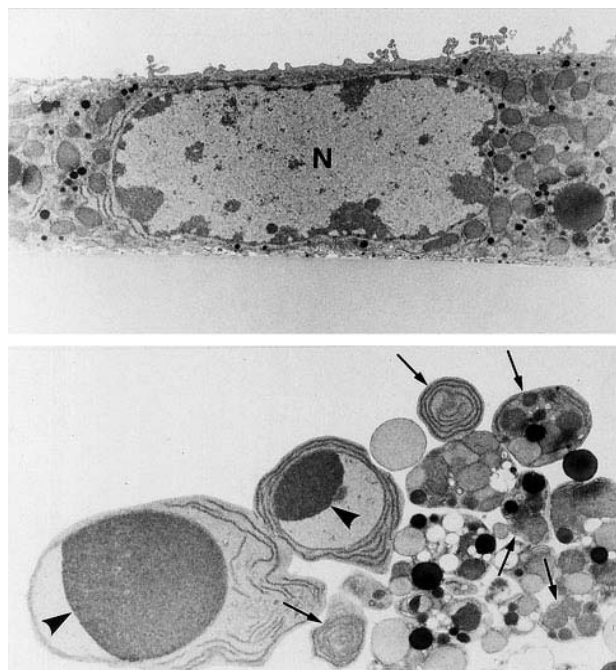


Figure 3 Transmission electron micrographs of a healthy (top) versus an apoptotic (bottom) mouse hepatocyte. Apoptosis was induced by ligation of CD95 (Fas/APO-1) on these cells with cross-linking antibody. Note the dramatic collapse of the apoptotic cell into many discrete apoptotic bodies (arrows) and the marked condensation of the chromatin (arrowheads). N, indicates the position of the nucleus in the live cell. (Reproduced with permission from Ni *et al.* (1994) *Experimental Cell Research* 215: 332–337. Figure kindly provided by Professor Shigekazu Nagata.)

Towards a molecular mechanism

Due to the widespread importance of apoptosis in many biological processes there has been intense interest in understanding how this process works at a molecular level. By 1993, although much of the cell biology of apoptosis had been established and several key apoptosis regulatory molecules, such as Bcl-2 and p53, had been discovered, very little was known concerning the nature of the cell death machinery itself. In 1993 a major breakthrough occurred when it was reported that CED-3, one of the genes that was known to be required for all the developmental-related programmed cell deaths in the nematode worm *Caenorhabditis elegans*, was homologous to ICE (caspase-1). ICE is a cysteine protease that has

been implicated in the processing of IL-1 β to its mature form and is unusual as it was the first protease described that cleaves after aspartate (Asp) residues. Recent studies suggest that ICE itself probably does not play a pivotal role in apoptosis: rather several of its mammalian homologs (including CPP32 (caspase-3), Mch2 (caspase-6), Mch3 (caspase-7) and mch5 (FLICE/Caspase-8)) are involved in the cell death signal. These caspases, which cleave after Asp residues, appear to be constitutively expressed in most cells as inactive precursors that require proteolytic processing to achieve their active forms (Figure 4).

Evidence that proteases are central to apoptosis

The evidence that proteases may be key components of the cell death machinery is derived from several lines of investigation. Early indications that proteases may participate in apoptosis was suggested by observations that several proteins underwent proteolytic cleavage during this process. Additional evidence was provided by the discovery that certain broad-spectrum protease inhibitors could block or delay apoptosis. Strong support for a role for caspases in apoptosis has subsequently been provided by studies that have examined the effects on apoptosis of CrmA, a cowpox virus-derived serpin (serine protease inhibitor) that was known for its ability to act as a pseudosubstrate for, and thereby neutralize, ICE. Several groups have independently reported that CrmA potently blocks several forms of apoptosis, including that induced by ligation of the CD95 molecule (Fas/APO-1) as well as due to CTL attack. Another pseudosubstrate for caspases was recently discovered in the form of the baculovirus

p35 protein, which was already known for its ability to act as a potent repressor of apoptosis.

Many recent studies have also found that specific peptide inhibitors of caspases (YVAD, DEVD, DEAD, VAD) inhibit many forms of apoptosis. All of this evidence for protease involvement in apoptosis is supported by the ongoing discovery of new proteins that are cleaved during this process. Together these data heavily implicate proteases as central components of the cell death machinery and suggest that the caspases play a critical role in apoptosis (Figure 5).

How protease activation may lead to the apoptotic phenotype

So how do proteases produce the apoptotic phenotype in a cell? The short answer is that we do not know as yet. Some informed guesses can be made by looking at the nature of the substrates that are cleaved during apoptosis. To date, most of the substrates that have been documented to undergo proteolytic cleavage during apoptosis are localized in the nucleus. It is likely that many of these cleavage events are not contributory to the apoptotic phenotype but are merely bystander effects due to these proteins possessing the appropriate cleavage sites. This idea is strongly supported by the observation that enucleated cells can also undergo apoptosis. However, it is relatively easy to conceive how cleavage of some of these proteins may directly lead to changes in the cell that frequently occur during apoptosis. For example, cleavage of the nuclear lamins, proteins that are largely responsible for the maintenance of the integrity of the nuclear envelope, could be directly responsible for the collapse (condensation) of the nucleus that is seen during apoptosis. Proteolysis of fodrin, a cytoskeletal protein that plays a major role in the cortical cytoskeleton, could lead to the plasma membrane blebbing that is typical of apoptosis (Figure 3). Proteases could also serve to degrade an inhibitor of the endonuclease that cleaves DNA at internucleosomal sites during apoptosis. Indeed, a protease with nuclease-activating properties has been described. Further studies are required in order to fully investigate the impact of each of the cleavage events upon cell integrity and to elucidate how they impact upon the cell death process.

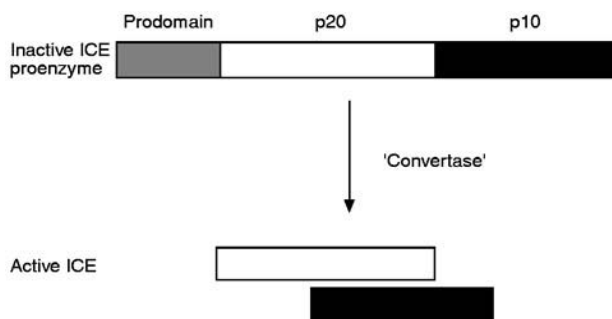


Figure 4 Caspase (ICE/CED-3) family proteases require proteolytic processing to achieve their mature forms. Most caspases are probably constitutively expressed as inactive precursor proteins that require further proteolytic processing at Asp residues in order to achieve their active heterodimer forms (that may further associate to form dimers of heterodimers). Maturation of some caspases is seen during the early stages of apoptosis (CPP32 and Mch3, for example).

Apoptosis in immunity

In the immune system, apoptosis is used as a means to dispose of unwanted cells time and again. In many cases the CD95 molecule (Fas/APO-1), a member of the TNF/NGF receptor family, is used to transduce the death signal. Some of the situations where

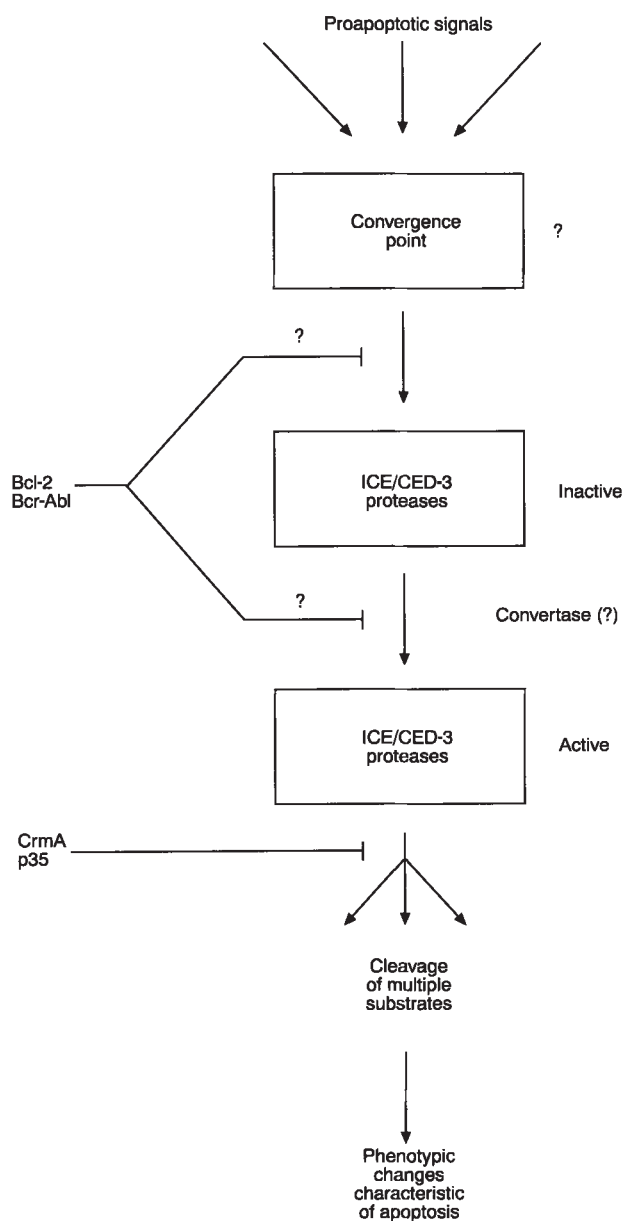


Figure 5 Molecular events in apoptosis. Members of the caspase (ICE/CED-3) protease family are depicted as being at a point just below the convergence point for diverse proapoptotic stimuli. Activation of ICE/CED-3 family proteases may lead directly to the apoptotic phenotype through cleavage of specific substrates, although this is speculative. From current evidence, some apoptosis-repressor proteins (Bcl-2) appear to act upstream of the point of activation of at least some of the members of the ICE/CED-3 family, while others (CrmA, p35) act directly as inhibitors of these proteases.

apoptosis plays a key role in immunity are described below.

Thymic selection

It has been estimated that approximately 95–98% of thymocytes that enter the thymus are eliminated

there. Thymocytes die at several stages of the maturation process; either because they fail to rearrange their TCR genes successfully, because they exhibit strong reactivity with self (negative selection), or because they fail to recognize self-MHC at all (death by neglect). Studies in the late 1980s and the early 1990s established that thymocytes die in all of these cases due to the induction of apoptosis in these cells. Most of the cell deaths that occur in the thymus do so in the cortex and at the corticomedullary boundary at the double-positive stage ($CD4^+CD8^+$) of thymocyte development. Upon entry into the medulla, single-positive thymocytes ($CD4^+CD8^-$ or $CD4^-CD8^+$) become much more resistant to the induction of apoptosis. The precise nature of the signal that provokes death of cortical thymocytes has not been established. The possible involvement of CD95–CD95 ligand interactions is ruled out due to the fact that thymocyte maturation is largely unaffected in mice that carry loss-of-function mutations in CD95 (*lpr/lpr*) or the CD95 ligand (*gld/gld*). The thymocyte death signal is similarly unaffected by overexpression of Bcl-2, a 26 kDa protein that blocks many other forms of cell death.

Despite the enormous amount of cell death that goes on in the thymus, it is remarkable how few apoptotic cells are actually detectable in tissue sections of this organ. This puzzle can be explained if it is recalled that apoptotic cells are readily recognized by cells with phagocytic capability – by virtue of the membrane changes on these cells – and are eaten before they exhibit the morphologic features of apoptosis. Recent studies have implicated resident $F4/80^-$ macrophages as the effectors of this efficient clearance process for thymocytes undergoing negative selection, whereas $F4/80^+$ macrophages appear to remove thymocytes that have died by neglect.

Peripheral deletion

The small minority of thymocytes that are positively selected exit the thymus and migrate to the peripheral lymphoid organs. Here they are faced with the possibility of reacting with previously unencountered self-antigens. Should this happen, the autoreactive mature T cell, all going to plan, should then undergo apoptosis, probably as a result of receiving an inappropriate activation signal (such as TCR engagement in the absence of CD28 costimulation). Should this peripheral deletion process fail for any reason, then autoimmunity can result. Unlike in the thymus, CD95–CD95L interactions do appear to be important for peripheral deletion, as both *lpr* and *gld* mice are impaired in peripheral deletion and develop a systemic lupus erythematosus (SLE)-like autoimmune disease as a result.

Peripheral T and B lymphocytes are also eliminated after an immune response, otherwise the numbers of these cells would steadily increase after each successive encounter with antigen. While such an increase does happen to some extent (i.e. precursor frequencies to recall antigens are higher than to previously unencountered antigens), clearly the majority of clonally expanded lymphocytes do not survive long after an immune response has ended. Studies in recent years have established that these excess lymphocytes die by apoptosis. Once again, CD95–CD95L interactions have been implicated in this process. While mature peripheral T cells constitutively express CD95 on their surface, they are largely resistant to CD95L-mediated killing until they are activated, whereupon they now acquire sensitivity to CD95 ligation. Thus, activated T cells die upon encounter with CD95L-bearing cells. Since the expression of CD95L is strictly restricted to certain tissues, it is not clear what cells do the actual killing. It may be the activated T cells themselves, since CD95L is upregulated on lymphocytes upon activation, or a subset of dendritic cells or other antigen-presenting cells.

CTL and NK killing

Cytotoxic lymphocytes (CTLs and natural killer (NK) cells) are capable of killing target cells via two distinct pathways: by delivering cytotoxic granules on to the plasma membrane of the target cell, or by cross-linking the CD95 molecule on the target cell surface via CD95 ligand. In both cases, the end-result is the activation of the endogenous death machinery in the target cell, resulting in death from within – a kind of assisted suicide.

Granule-dependent killing is thought to be the major mechanism of cytotoxic lymphocyte killing. Cytotoxic cell granules contain perforin, a pore-forming protein similar to the C9 component of complement, as well as a series of enzymes – several of which are serine proteases – called granzymes. Perforin is thought to act as a conduit or channel to allow the entry of granzymes into the target cell cytoplasm. Of the seven or so granzymes that have been characterized to date, only one of these, granzyme B, has been shown to be both necessary and sufficient, in combination with perforin, to induce apoptosis of target cells. Granzyme B is an unusual protease in that it cleaves after Asp residues, a property that it shares with the caspase family of cysteine proteases discussed earlier. Recent studies have shed light on the mechanism whereby granzyme B activates the endogenous death machinery in the target cell. A number of groups have found that granzyme B is capable of directly cleaving and activating several

members of the caspase family, but not ICE itself. Thus, granule-dependent killing operates by directly activating the endogenous cell death machinery in the target cell by processing members of the caspase family to their active forms.

The other mechanism of cytotoxic lymphocyte killing involves a CD95 ligand-bearing cell interacting with, thereby cross-linking, CD95 on the target cell surface. As mentioned above, CD95 is a member of the TNF/NGF receptor family that transduces, upon trimerization, a death signal into cells bearing this receptor. CD95 was originally identified in 1989 by two separate groups and is commonly called two different names as a result: Fas or APO-1. The molecular mediators of the CD95-associated death signal have recently begun to emerge. Briefly, the cytoplasmic tail of CD95 contains a stretch of amino acids that are critical for the CD95-mediated death signal (the death domain), but this region does not have any obvious signaling function (such as kinase or phosphatase activity). Other death domain-containing proteins have since been discovered, such as FADD (Fas-associated death domain), that can associate with the CD95 death domain, thereby propagating the apoptotic signal. Thus, the death domain appears to be a protein–protein interaction domain. FADD then recruits caspase-8 (MACH-1/FLICE) which becomes processed to its active form, thus propagating the death signal.

Maintenance of immune privilege

It has been known for over a hundred years that certain sites in the body enjoy immune privilege. That is to say, immune responses do not generally occur at these sites. Classically, an immune-privileged site is defined as an area where introduction of allogeneic or xenogeneic cells does not result in immediate rejection as it would in other areas of the body. This definition has subsequently been broadened to include infectious organisms and tumors. Examples of immune-privileged sites include the eye, the testis and the central nervous system – all of which are tissues where the benefits of an immune response are outweighed by the dangerous consequences of a vigorous immune reaction in such delicate and vital tissues. While it was initially thought that immune privilege was maintained by physical barriers that contained antigen and prevented infiltration of the site by myeloid cells and lymphocytes, this is now known to be untrue because these cells do enter privileged sites but are somehow inactivated. Recent studies on the eye and the testes appear to have solved this puzzle. In these organs, it transpires that CD95 ligand is abundantly expressed such that infiltrating lymphocytes and neutrophils – both of which

express surface CD95 – are induced to undergo apoptosis upon entry into these sites. As elegant proof of this, mice carrying a non-functional CD95-ligand (gld) do not enjoy immune privilege and, in the case of the eye, display marked infiltration upon introduction of a virus (herpes simplex virus type 1, HSV-1) into the anterior chamber of this organ – resulting in keratitis. In stark contrast, introduction of HSV-1 into the eye of wild-type mice results in death of infiltrating lymphocytes and neutrophils and containment of the infiltrating cells to the anterior chamber. Similarly elegant transplantation experiments of testes from *lpr* and *gld* mice yielded a similar conclusion that CD95 ligand was responsible for maintaining immune privilege in this organ also. Other studies have demonstrated that CD95L is also abundantly expressed in other immune-privileged sites such as the brain, ovary, uterus, adrenal gland and prostate, suggesting that a similar protective mechanism from immune responses operates in these tissues also.

B cell development and affinity maturation

Like T lymphocytes, the majority of newly formed B lymphocytes fail to migrate to the peripheral lymphoid organs due to the induction of apoptosis in these cells. B cells are produced throughout life in the bone marrow but only a small minority of these cells, as is the case with thymocytes, will be allowed to enter the peripheral blood. Most developing B-lineage cells die by apoptosis, either because of aberrant immunoglobulin (Ig) gene rearrangements or due to encounter with high concentrations of high-affinity antigens (akin to negative selection). It has been estimated that approximately 75% of developing B cells die in the bone marrow at the transition between large pro-B to small pre-B cell stage. The small fraction of virgin B cells that exit the bone marrow travel to the spleen and then on to the lymph nodes. In the peripheral lymphoid tissues B cells fall into two compartments, short-lived (2–3 days) and long-lived (several weeks). Short-lived B cells probably die (by apoptosis) through failure to encounter antigen and are rapidly replaced by newly formed B cells from the bone marrow. B cells that receive a survival signal (antigenic stimulation) likely switch to the long-lived state, although there has been some debate concerning whether short-lived and long-lived B cells are derived from separate bone marrow precursors or a common progenitor. In either case, long-term survival of B cells appears to require repeated re-stimulation with antigen. Long-term B cell survival appears to be regulated, at least in part, by expression of the Bcl-2 protein. Bcl-2 transgenic mice

display elevated peripheral B cell numbers and extended B cell memory.

B cells that manage to persist also run the gauntlet of apoptosis during affinity maturation in the germinal centers. During the germinal center reaction, activated B cells acquire point mutations in the V regions of their immunoglobulin genes that may or may not increase their affinity for antigen and can even result in autoreactivity. The majority of B cells that undergo this hypermutation process are eliminated via apoptosis, with mutants that have a high affinity for antigen being rescued from a similar fate due to encounter with their specific antigen in the context of CD40 ligand (gp39) costimulation. CD95 also appears to play a role in this elimination process because activated B cells acquire sensitivity to CD95 unless they receive concurrent engagement of the CD40 molecule. This may also explain why *lpr* mice (that are defective in CD95) also display a B cell defect and develop an SLE-like autoimmune disease.

Resolution of inflammation

One of the earliest events in an immune response involves the dramatic accumulation of infiltrating neutrophils and other cells of the myeloid lineage at the site of infection. Neutrophils form a very important first line of defense and these cells appear to be well equipped for this task, with a barrage of toxic enzymes and reactive oxygen intermediates within their secondary granules. But what happens to all of these cells when they have completed their task and are no longer required? It is well known that neutrophils have a very short half-life of approximately 12–18 hours. But where do they die and how are they disposed of? By now it will come as no surprise to learn that old unwanted neutrophils die by apoptosis and that damage to cells in the vicinity of dying neutrophils is minimized as they are programmed to die around the time that the wave of macrophage infiltration reaches its peak. Thus, inflammatory macrophages are charged with the task of removing the dying neutrophils and of safely disposing of their potentially destructive contents. The trigger for macrophage recognition of senescent neutrophils, as discussed earlier, appears to be either phosphatidylserine externalization at the surface of the plasma membrane or the appearance of a thrombospondin-binding moiety on the surface of these cells – depending on whether the phagocytic cells are inflammatory or resting macrophages, respectively. Interestingly, macrophages respond very differently when they phagocytose apoptotic cells, as opposed to yeast particles, for example, as the former do not elicit the release of inflammatory mediators by the

macrophage. Whether a distinct phagolysosomal compartment is used for digestion of apoptotic cells has not been explored as yet.

Apoptosis in disease

We now know that proper control of cell death is just as important for the maintenance of dividing cell populations at equilibrium as control of cell division is. Therefore, just as loss of normal controls on cell division can result in disease, so can defects in the regulation of cell death. Obviously, cell death defects can take two forms – too much or too little. Situations where excessive or inappropriate apoptosis may contribute to disease include neurodegenerative diseases such as Alzheimer disease, Parkinson disease and spinal muscular atrophy. In the latter case, this appears to be due to loss-of-function mutations in an endogenous inhibitor of apoptosis (neuronal apoptosis inhibitory protein). It has also been proposed that the loss of CD4⁺ T cells that is observed during human immunodeficiency virus (HIV)-1 infection is due to excessive apoptosis (although this is a debatable point). The development of some forms of autoimmunity may either be the result of a block in apoptosis – that allows autoreactive lymphocytes to survive – or defective apoptosis – that allows the release of proteins that could potentially break self-tolerance. On the other side of the coin, over-expression or dysregulation of genes that can block apoptosis (such as bcl-2 or bcr-abl) or mutations in genes that can facilitate apoptosis (p53) may also contribute to the development of malignancy by synergizing with proliferation-associated oncogenes (such as c-myc). Thus, intensive investigation is currently underway to understand how apoptosis works at a molecular level and to explore how this process can be modulated in situations where this may be desirable.

See also: B lymphocyte activation; Cytotoxic T lymphocytes; Thymic epithelium: potential role in regulatory T cell tolerance; Fas (CD95) and fas ligand; Germinal center; Lifespan of immune cells and molecules; Membrane-associated cytoskeleton: role in regulating immune cell function; Memory, immuno-

logical; Natural killer (NK) cells; Perforin; Privileged sites; T lymphocyte activation; T lymphocytes, autoreactive.

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ARACHIDONIC ACID AND THE LEUKOTRIENES

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Arachidonic acid (AA) is an important constituent of mammalian cell membranes. Following membrane perturbations or receptor-mediated signaling which leads to activation of phospholipase A₂ (PLA₂), AA is released from membrane phospholipids. It may undergo oxidative metabolism through cyclo-oxygenase, which results in the formation of the various prostaglandins, through the cytochrome P450 system, or through one of several lipoxygenases. Borgeat and Samuelsson first identified a novel class of molecules derived from this latter metabolic pathway of AA, which they named leukotrienes, because of their leukocytic origin and their characteristic triene structure. Leukotrienes (LTs) are derived from the combined actions of 5-lipoxygenase (5-LOX) and 5-LOX-activating protein (FLAP), with initial formation of 5-hydroperoxyeicosatetraenoic (5-HPETE) acid, followed by LTA₄. LTB₄, which is an enzymatic, hydrolytic product of LTA₄, differs from the other leukotrienes, LTC₄, LTD₄ and LTE₄, in that it has no peptidic component. It is a more stable molecule than the peptidoleukotrienes, but can be rapidly degraded through ω -oxidation by polymorphonuclear leukocytes (PMNs) to the relatively inactive metabolites, 20-OH-LTB₄ and 20-COOH-LTB₄.

While sharing some of the myotropic properties of the other leukotrienes, LTB₄ has been found to exert very strong leukocytotropic activities. It is one of the most powerful chemokinetic and chemotactic agents and it can induce neutrophil aggregation, degranulation, hexose uptake and enhanced binding to endothelial cells. It can also induce cation fluxes, augment cytoplasmic calcium concentrations from intracellular pools and activate phosphatidylinositol hydrolysis. LTB₄ can also synergize with prostaglandins E₁, (PGE₁) and PGE₂ in causing macromolecule leakage in the skin, through increased vascular permeability, and, when injected into guinea pig skin, it induces leukocytoclastic vasculitis. Two sets of plasma membrane receptors for LTB₄ have been described on human PMNs. The high-affinity receptor set mediates aggregation, chemokinesis and increased adherence to surfaces, while the low-affinity receptor set mediates degranulation and increased oxidative metabolism. In contrast to human cells, rat leukocytes lack the lower affinity receptor set. Signal transduction via high-affinity receptors appears to involve

a guanine nucleotide regulatory protein. In contrast to PMNs, little is known at present about potential LTB₄ receptors on lymphocytes, although a fraction of CD4⁺ and CD8⁺ T cells bind LTB₄.

Cellular sources of LTB₄

LTB₄ is rapidly synthesized by phagocytic cells, principally PMNs and alveolar macrophages, upon challenge with a variety of stimuli such as microbial pathogens, toxins, aggregated immunoglobulin, tumor necrosis factor α (TNF α), particulate material or ionophores, especially when exogenous AA is available. Its synthesis can also be induced by platelet-derived 12-HPETE or platelet-activating factor (PAF). Induction by natural stimuli of LTB₄ production by PMNs *in vitro* is relatively modest, unless the cells are primed by granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF α or bacterial lipopolysaccharide (LPS) (Table 1). Under these conditions, enhanced LTB₄ production appears to be the result of both an immediate activation of PLA₂, with subsequent generation of AA, and a later, transcriptional induction of 5-LOX and FLAP gene expression. In contrast, PMNs exposed to transforming growth factor β (TGF β) lose the capacity to produce LTB₄ through a selective downregulation of 5-LOX (but not FLAP) mRNA expression. Such an interaction between priming events and direct stimuli of LT generation may also be operative *in vivo* in the context of infection and inflammation.

LTB₄ is predominantly a product of cells of myeloid origin. PMNs are the main source of the mediator: when stimulated by LPS, PMNs can produce five times more LTB₄ than monocytes. In con-

Table 1 Production of LTB₄ by PMNs as a function of stimulus and priming

Stimulus	Priming	LTB ₄ production
Ca ²⁺ ionophore	–	++
PAF or fMLP	–	±
PAF or fMLP	GM-CSF, TNF α or LPS	++
PAF or fMLP	TGF β	–

fMLP, N-formylmethionine leucine phenylalanine.

trast, the latter produce a wider variety of AA metabolites, including the peptido-LT and prostanoids. B lymphocytes can also produce LTB₄ and 5-HETE. Unlike PMNs or monocytes, they require particular stimulation conditions, including an oxidative environment, in addition to a source of AA. T lymphocytes, on the other hand, do not produce LTs, as they lack 5-LOX. Similarly, endothelial cells and epithelial cells of different origins cannot directly synthesize LTB₄ from AA, because they lack both 5-LOX and FLAP (Table 2). They can and do, however, produce LTB₄ when supplied with LTA₄, through what is known as transcellular metabolism, since they have LTA₄ hydrolase. That the genes involved in LT synthesis can be derepressed is suggested, however, by the observed production of LTC₄ by a human hybridoma formed by fusing CD8⁺ T cells with CD4⁺ CEM-6 lymphoma cells.

LTB₄ has been detected in significant concentrations in inflammatory synovial exudates, psoriatic skin lesions, peritonitis and inflammatory bowel disease. In an animal model of colitis, local production of LTB₄ and LTC₄ correlates with inflammatory cell infiltrates. Globally, LTB₄ thus appears to be a potent proinflammatory agent. It may also affect many immunoregulatory functions via interactions with a variety of cell populations, as presented in the next sections.

Cellular actions of LTB₄

T lymphocytes

When LTB₄ is added to human peripheral blood mononuclear leukocyte cultures stimulated with mitogenic lectins, an inhibition of the proliferative response can be observed. Similar findings are also observed for LTD₄ and LTE₄, and for 15-HETE in mice. A significant suppressor cell activity can indeed be generated at picomolar concentrations of LTB₄.

Table 2 Expression of 5-LOX and FLAP in various cell populations

Cell population	FLAP	5-LOX
PMNs	+	+
Monocytes/macrophages	+	+
Myeloid cell lines		
THP-1	—	+
U937	+	—
HL-60 (differentiated)	+	+
B cells/B cell lines	+	+
T cells	±	—
T cell lines (Jurkat, MOLT)	+	—
Endothelial cells	—	—

LTD₄ induces no such suppressor cell activity. The magnitude of the suppressor cell activity generated by LTB₄ is comparable to that seen with histamine, but the concentrations at which it occurs are orders of magnitude lower than those needed with the latter. LTB₄-induced suppressor T cells can also inhibit immunoglobulin production by B cells and leukocyte migration inhibitory factor production by T cells.

When LTB₄ induces the appearance of suppressor cells, the effective suppressor cells bear finally the CD8⁺ phenotype. Proliferation of CD4⁺ cells is inhibited by LTB₄, whereas the proliferation of CD8⁺ cells is enhanced. Further analysis of the cellular requirements for LTB₄-induced suppressor cell activity reveals that, whereas only T cells need to interact with LTB₄, the expression of their suppressor activity requires the presence of monocytes in the responding cell population. Only purified CD8⁺ T cells can bypass this requirement. For preincubated CD4⁺ T cells or unfractionated T cells, little suppression is seen in the absence of monocytes, whereas a helper effect is evident in the presence of monocytes plus the cyclo-oxygenase inhibitor indomethacin. This phenomenon could be explained by a dual stimulation of monocytes to produce both PGs and interleukin-1 (IL-1) by LTB₄-preincubated T cells. Indeed, LTB₄-pulsed T cells release a soluble factor which stimulates monocytes to produce PGE₂ and/or IL-1, and most of the activity of such T cell supernatants is attributable to interferon γ (IFN γ). LTB₄ can indeed enhance, at nanomolar concentrations, IFN γ production by CD4⁺ T cells and inhibit IFN γ production by CD8⁺ T cells. In the mouse, LTB₄ (as well as other leukotrienes and AA itself) can replace a requirement for helper cells or IL-2 in IFN γ production. LTB₄ can also induce proliferation of IL-2-dependent T cells in the presence of suboptimal IL-2 and restore such proliferation after inhibition with lipoxygenase inhibitors or hydrocortisone. LTB₄ can also induce T cells to produce IL-2 and IL-5 (Figure 1).

B lymphocytes

Purified human B cells show amplified proliferative responses to IL-2, IL-4 and B cell stimulatory factor, when exposed to picomolar concentrations of LTB₄. Immunoglobulin G (IgG) production is also enhanced by LTB₄, but not by LTC₄. Moreover, in the presence of IL-4, LTB₄ stimulates IgE production, suggesting a central role for this mediator in allergy. In contrast, LTC₄ can affect B cells directly and suppress Ig production. LTB₄ also induces enhanced CD23 and major histocompatibility complex (MHC) class II antigen expression on B cells (Figure 1).

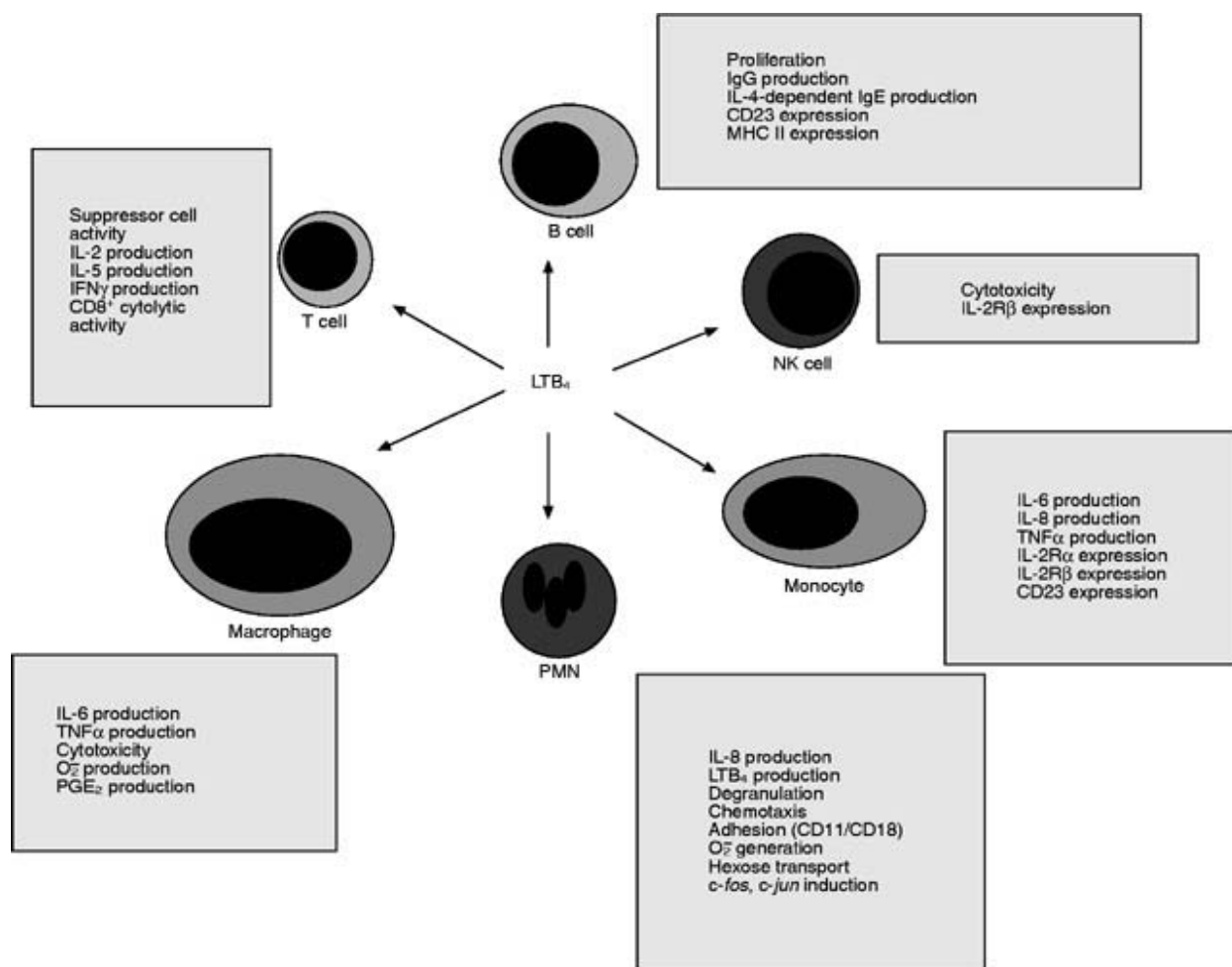


Figure 1 Stimulatory proteins of LTB_4 on cells of the immune system.

NK cells

LTB_4 can modulate the activity of natural killer (NK) and natural cytotoxic (NC) lymphocytes. At picomolar concentrations, LTB_4 and, to a lesser extent, LTA_4 , markedly augment NK and NC activities. Lipoxygenase inhibitors can reversibly block NK and NC activity, and this inhibition can be effectively reversed by LTB_4 , indicating that exogenous lipoxygenase products can compensate for endogenous lipoxygenase inhibition. However, whereas human NK cells pretreated with interferons or poly I:C are partially resistant to suppression by lipoxygenase inhibitors, lymphokine (IL-2)-activated killer (LAK) cells can be reversibly inhibited by these drugs. LTB_4 acts on effector NK or NC lymphocytes rather than on their target cells, increasing both the binding of effector lymphocytes to their target cells and the rate of target cell killing. No IL-2 or IFN γ is detectable in these cultures.

LTB_4 can also enhance IL-2-induced cytolytic activity on both NK (CD56 $^+$) cells and cytotoxic T

(CD8 $^+$) cells. Twenty-four hours after exposure to 10^{-10} M LTB_4 , the cells respond to 100-fold lower concentrations of IL-2 with enhanced cytotoxicity. This is accompanied by an LTB_4 -dependent increase in expression of IL-2R β on CD56 $^+$ and CD8 $^+$ cells, suggesting a receptor-mediated sensitization of NK and cytotoxic T cells (Figure 1).

Monocytes/macrophages

Cytotoxicity The potential regulatory role of endogenous and exogenous LTs on functions of monocytes/macrophages has also come under study, in view of the findings that LTs are synthesized and released by these cells after receiving immunological and nonimmunological stimuli. For instance, as assessed by use of lipoxygenase inhibitors, activation of macrophage phagocytic capacity by endotoxins appears to be dependent on lipoxygenase metabolites of AA. LTs can also induce the release of PGs and lysosomal enzymes by macrophages. When appropriately stimulated, monocytes/macrophages can pro-

duce a variety of cytokines, as well as inhibit tumor cell growth or destroy susceptible target cells by a number of different mechanisms. For instance, when mouse-resident peritoneal macrophages are cocultured with MOPC-315 myeloma cells, the cytostatic activity of the former is enhanced by either LTD₄ or indomethacin, whereas both LTD₄ and indomethacin together are additive in causing a more than 80% inhibition of tumor cell proliferation.

Human peripheral blood monocytes can also lyse several types of tumor target cells. When they are incubated with LTB₄, a very significant augmentation of cytotoxicity is observed. This is associated with enhanced binding of monocytes to target cells and augmentation in the rate of killing of target cells by the culture supernatants. Inhibitors of lipoxygenase cause a marked inhibition of monocyte-mediated cytotoxicity. Exogenous LTB₄ can restore the activity, suggesting that both endogenous and exogenous LTs have a positive effect on cytotoxic functions of mononuclear phagocytes.

Cytokine production LTB₄ is a powerful stimulator of IL-6 production by monocytes. At nanomolar concentrations, LTB₄ induces a fivefold increase in IL-6 gene transcription and a threefold extension of IL-6 mRNA half-life. This effect is dependent on activation of a tyrosine kinase and involves induction of the transcription factors NF- κ B and NF-IL6. Although LTB₄ also stimulates the accumulation of IL-1 β mRNA, it preferentially augments IL-6 production as compared to IL-1. LTB₄ can also affect cytokine production indirectly, via T lymphocyte activation: human T cells pulsed with LTB₄ modulate IL-1 production by human monocytes by secreting IFN γ .

TNF α is a potent mediator of cytotoxic activity exerted by monocytes and macrophages, as well as NC lymphocytes. LTB₄ markedly enhances cytotoxicity mediated by NC cells and monocyte-mediated cytotoxicity is also augmented by LTB₄. Addition of LTB₄ to monocyte cultures enhances TNF α activity of their supernatants. Addition of indomethacin to the cultures augments TNF α production, whereas lipoxygenase inhibitors diminish TNF α production. LTB₄ may thus affect cytotoxic activity of human monocytes by augmenting their production of the cytolytic cytokine TNF α .

Alveolar macrophages can play a crucial role in the pathogenesis of pulmonary disease via their ability to produce potent inflammatory and fibrogenic mediators. When alveolar macrophages are cultured with silica particles or asbestos fibers they produce TNF α and LTB₄ in a concentration-dependent fashion. Increased amounts of LTB₄ precede the

rise in TNF α activity. The lipoxygenase inhibitors NDGA and AA861 reduce in a concentration-dependent fashion asbestos- or silica-stimulated TNF α release. On the other hand 'reconstitutive' experiments in which exogenous LTB₄ is added to alveolar macrophages treated with lipoxygenase inhibitors show partial restoration of TNF α production induced by chrysotile or silica. Alveolar macrophages are also stimulated by PAF to produce IL-1 β , TNF α and IL-6. Lipoxygenase inhibitors, including the FLAP antagonist MK-886, prevent this effect of PAF, whereas exogenous LTB₄ partially reconstitutes the responses.

LTB₄ can induce the expression of IL-2R α in human monocytes by activating IL-2R α gene transcription. It can also stimulate the expression of IL-2R β through post-transcriptional regulation. This augmented expression of both α and β chains of IL-2R is associated with enhanced sensitivity of monocytes to IL-2 in terms of TNF α production, and may be relevant to some of the proinflammatory actions of LTB₄.

In addition to modulating cytokine production and cytokine receptor expression, LTB₄, as well as LTC₄ and LTD₄, can stimulate human myelopoiesis and play a role as essential intermediates in GM-CSF-stimulated myeloid colony formation.

Polymorphonuclear leukocytes

AA induces PMNs to aggregate, generate superoxide anion and release the contents of lysosomes, by mechanisms which do not appear to involve any of its metabolites. LTB₄ also activates PMNs in a number of ways: it induces chemotaxis, aggregation, degranulation, cation fluxes and superoxide anion production. LTB₄ induces migration and adhesion of PMNs to endothelium, secondary to expression of CD11/CD18 adhesion molecules. Moreover, LTB₄ constitutes a powerful stimulus for its own production, as well as for the production of PMNs of certain cytokines, in particular IL-8.

AA and LTs as second messengers

AA can exert a variety of direct effects on leukocytes that do not depend on the metabolism via cyclooxygenase or lipoxygenase pathways. AA can directly activate protein kinase C, an effect it shares with diacylglycerol. AA can also directly activate a pertussis toxin-sensitive GTP-binding protein in the PMN plasma membrane and may thereby act as a second messenger in signal transduction.

LTB₄ and possibly other 5-LOX metabolites may also play central roles as internal 'second messengers'. LTB₄ can induce *c-fos* and *c-jun* proto-

oncogene expression in monocytes and PMNs. This is accompanied by enhanced binding of LTB₄-induced nuclear proteins to the AP-1 consensus site, suggesting that LTB₄ may play a role in the transactivation of genes containing the AP-1 binding site in their promoter region. Through intracellular formation of H₂O₂, LTB₄ can also activate the transcription factors NF-κB and NF-IL6.

LTB₄ may also play a role as an endogenous Ca²⁺ ionophore, and the sensitivity to LT synthesis inhibition of a number of cellular responses to a variety of stimuli strongly suggests that LTs may act as second messengers for the regulation of certain immune functions, including cytokine production. The recent development of a 5-LOX knockout mouse model should prove particularly useful in defining the selective involvement of LTs in immune and inflammatory conditions.

Conclusion

An increasing body of evidence suggests that LTB₄ may be an important modulator of many immune cell functions, either as an internal messenger within

some cells, or as an external signal, linking inflammatory cells to lymphocytes. LTB₄ and, to some extent, other LTs, are known protagonists of numerous inflammatory processes. They can also mediate varied and powerful effects on many cells involved in immunological reactions.

See also: Anti-inflammatory (nonsteroidal) drugs; B lymphocytes; Cytokines; Glucocorticoids; Lymphokine-activated killer (LAK) cells; Macrophage activation; Monocytes; Natural killer (NK) cells; Prostaglandins; T lymphocytes; Tumor necrosis factor α.

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ARENAVIRUS, INFECTION AND IMMUNITY

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Discussions of cell-mediated immunity and immunopathogenesis frequently cite the prototype arenavirus, lymphocytic choriomeningitis virus (LCMV). The 1996 Nobel Prize in Physiology or Medicine went to Peter Doherty and Rolf Zinkernagel who used LCMV-infected mice to show that cytotoxic T lymphocytes (CTLs) only kill cells that are syngeneic at the major histocompatibility complex (MHC) (murine H2) locus. Two properties of LCMV facilitated this landmark finding: first, LCMV is non-cytopathic in cell culture, enabling 15 h chromium-release assays in which cell lysis is entirely due to CTLs, and second, LCMV elicits a vigorous CTL response, peaking 7 days after infection and causing very high specific lysis in chromium-release assays. Several other viruses elicit cell-mediated immunity, but usually their virus-specific CTL activity cannot be detected without secondary stimulation. This entry summarizes research primarily on the less viru-

lent arenaviruses – LCMV, Pichinde and Tacaribe – and, to a lesser extent, on the virulent ‘emerging viruses’ such as Lassa, Machupo, and Junin.

Characteristics of the arenaviruses and their antigens

The arenaviruses are enveloped viruses with two single-stranded RNA genomic segments. They are classified as ‘negative-strand viruses’ because their genomic RNA cannot function as a messenger RNA, i.e. transcription by the viral RNA-dependent RNA polymerase (RdRp) must precede translation. The arenaviruses encode four gene products; a nucleocapsid protein (NP) that encapsidates the genomic RNA, an envelope glycoprotein (GP) that undergoes a maturation cleavage to become GP1 and GP2, a small zinc-binding protein (Z) and a large RdRp (L). The small (S) RNA segment is 3.4 kb and encodes

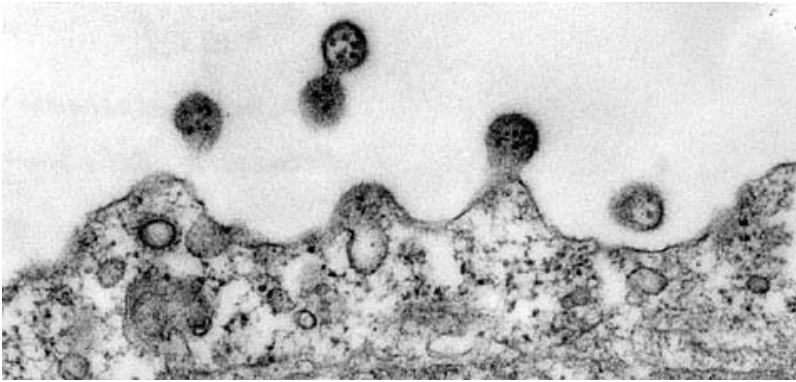


Figure 1 The arenaviruses are noncytopathic and bud from the host cell. They have a granular appearance from which the name (*L. arena*, sandy) is derived. (Reproduced by permission from M Buchmeier, La Jolla, CA.)

GP and NP in an ambisense arrangement (using sense and antisense (complementary) mRNAs). The large (L) RNA segment is 7.2 kb and encodes the Z and L proteins, also in an ambisense arrangement. The morphological characteristics of arenaviruses are depicted in Figure 1.

Immune responses of the host

Arenaviruses are generally carried by rodents with little ill-effect to the carrier; however, transmission from rodents to primates can cause severe encephalitis, hepatitis or hemorrhagic syndrome. Thus, rodent and humans can have drastically different immunologic and pathologic responses to infection. We will describe the classical studies of cell-mediated immunopathology in the LCMV/murine model with the caveat that it does not apply to arenavirus disease in primates.

LCMV was first isolated in the early 1930s from a man who died of encephalitis in St Louis, Missouri. It was never entirely clear whether the virus came from the deceased person or from the laboratory mice used for passage, because similar stocks of LCMV were isolated from mice in other laboratories around the same time. Electron microscopy and serological studies confirmed the familial relatedness of the various laboratory isolates and some new viruses from outbreaks in Africa (Lassa virus) and South America (Machupo and Junin).

Ever since the 1960s, LCMV was used to study virus-induced cell-mediated immunity. There are two types of murine infection: an acute infection that correlates with vigorous cell-mediated immunity (as measured by CTL assay) or a persistent infection that correlates with little cell-mediated immunity (Figure 2). The immune response to the acute infection either kills the mouse by engendering meningitis, or subsides and leaves the mouse LCMV-immune. Adoptive transfer of splenocytes from an LCMV-immune mouse can clear infection in a persistently infected mouse. Clearance is mediated by CD8⁺ and not

CD4⁺ splenocytes, as shown by antibody depletion of lymphocyte subsets. In contrast to their beneficial effects in clearing virus infection, the CD8⁺ cells mediate pathology (choriomeningitis) when injected intracerebrally. Thus the LCMV/murine model offered a paradigm for the positive and negative effects of CTL-mediated immunity. Even though LCMV has an acid-sensitive envelope, it has recently been shown to infect the gastric mucosa at very low doses; thus expanding its usefulness to studies of mucosal immunity.

LCMV-specific cell-mediated immunity was defined by studies with transgenic mice, synthetic peptides, and recombinant vaccinia expression vectors. BALB/c (H-2^d) mice make a CTL response primarily to epitopes on the viral envelope, GP, whereas C3H (H-2^b) mice respond primarily to epitopes on the NP. Synthetic peptides were used to define the most important residues for these CTL epitopes. Transgenic mice lacking CD8⁺ expression (either by CD8 gene knockout or by knockout of β_2 -microglobulin) have greatly reduced CTL responses, resulting in virus persistence. Intracerebrally infected mice lacking CD8⁺ lymphocytes die later than normal mice, thus the CD8-mediated immunopathology is reduced but not entirely eliminated. Knockout mice lacking perforin are also unable to clear infections, indicating that virus clearance is dependent on the perforin-mediated destruction of virus-infected cells. Mice lacking CD4⁺ cells or B cells are able to clear virus-infected cells initially, but virus reappears and the mice then succumb to acute infection.

LCMV infection induces inflammatory intermediates such as interferon γ (IFN γ) and tumor necrosis factor α (TNF α), both of which influence the host's immune response to other pathogens. IFN γ stimulates the proliferation of natural killer (NK) cells that serve to eliminate certain pathogen-infected cells and some tumors. TNF α produced in the liver as a result of LCMV infection can clear hepatitis B virus infection, even in perforin-negative mice, probably by

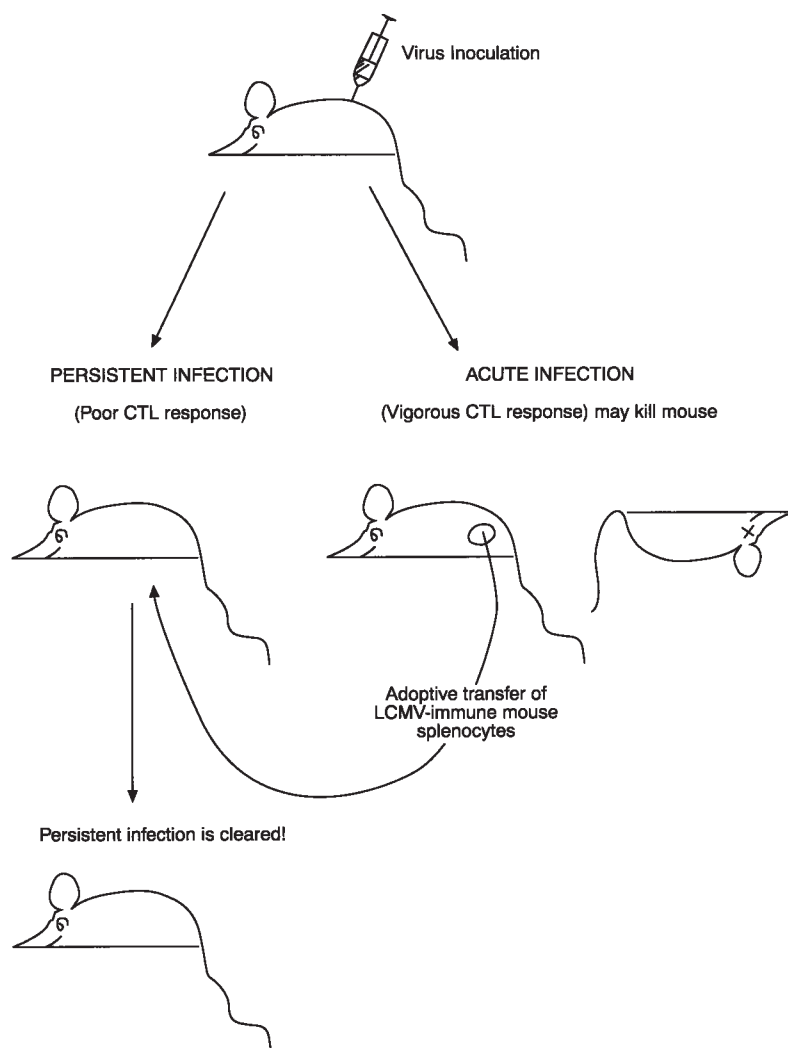


Figure 2 In the murine/LCMV model, acute and persistent infections are correlated with vigorous and poor virus-specific CTL responses, respectively. Acute infection generally requires an adult immunocompetent mouse, whereas persistent infection generally occurs in a neonatal or immunosuppressed mouse, or in an adult mouse given an immunosuppressive variant of LCMV. Vigorous CTL response can mediate lethal immunopathology, but adoptive transfer of CTL can also mediate virus clearance.

inducing an RNase that destroys the hepatitis viral nucleic acid.

Junin, Machupo and Lassa viruses can cause hemorrhagic fever in primates, the severity of which is directly proportional to virus titer. Arenavirus disease in primates is not alleviated by immunosuppressive cyclophosphamide. The Junin and Machupo diseases are primarily treated with convalescent serum, whereas Lassa disease is primarily treated with ribavirin, since antisera are only effective early in the disease.

Virus evasion of host immune responses

The ability of arenaviruses to persist in their long-term carriers is facilitated by antigenic variation that allows evasion of host immune responses. Neutralization-resistant variants of Junin and Tacaribe viruses escape the humoral immune response, and certain

epitope variants of LCMV escape the CTL response. In addition to viral variants that can escape host immune surveillance, variants have been described that suppress the immune response and thereby persist. Docile is a variant of the Aggressive isolate of LCMV (UBC), and Clone 13 is a variant of the LCMV (Armstrong strain). Both Docile and Clone 13 replicate very well in the spleen and poorly in the brain in contrast to their parental viruses. Both Docile and Clone 13 elicit poor CTL responses when tested 7 days after infection, and consequently, both persist in the mouse longer than their parental viruses. Docile and Clone 13 isolates actually do elicit CTL by day 3 after infection, but these viruses replicate so vigorously in the spleen that they cause high-dose immune suppression, i.e. high immune stimulation leading to massive apoptosis of immunocytes, leading to little CTL response by day 7. Thus, virus isolates that were thought to be immunosup-

pressive are in fact evading host immune responses by overstimulating and destroying the immune response.

Vaccines

Mice can be protected from lethal LCMV challenge either by peptide vaccination or by vaccinia vectors that express viral epitopes. Guinea pigs can be protected from lethal Lassa virus challenge by inoculation with vaccinia vectors that express either Lassa glycoprotein or Lassa nucleoprotein; however primates are only protected by the vector expressing glycoprotein. Primates are protected from the Junin virus disease, Argentine hemorrhagic fever, by injection with attenuated strains of Junin or with the related strain Tacaribe. Tacaribe or its subunits would make an excellent vaccine for Argentine hemorrhagic fever because it is unlikely to revert to pathogenicity, and causes a brief infection.

Vaccine research using arenaviruses established important negative as well as positive results. Vaccination of guinea pigs with both the Lassa NP and GP-expressing vector does not give double protection against virus challenge, but instead gives less protection than a single vaccination. Vaccination of mice with a GP-expressing vector can sometimes exacerbate virus-mediated pathogenesis. Despite the setbacks, there is considerable optimism that effective subunit vaccines may eliminate some problems that arise when attenuated viral strains are used for vaccination.

See also: Antigen presentation via MHC class I molecules; Cell-mediated immunity; Fever; Cell-mediated lysis; Immunopathology; Immunosuppression; MHC

restriction; Mucosal immunity; T lymphocyte activation; Transgenic animals; Viruses, immunity to.

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ARGININE AND IMMUNITY

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Arginine, a dibasic nitrogen-rich amino acid, is classified as semidispendable. In health it is synthesized in adequate amounts for the maintenance of nitrogen balance. Dietary requirement is essential only for the optimal growth of the young of some species. In certain circumstances, such as immaturity and severe stress (sepsis, trauma, nitrogen overload), arginine becomes indispensable, being required for maintenance of nitrogen balance and physiologic functions in humans. More importantly, arginine has

several unique and remarkable pharmacologic properties, including a marked immunomodulatory function.

Immune function

Supplemental administration of arginine (approximately 500 mg kg⁻¹ per day) to rodents either enterally or parenterally enhances thymic size, lymphocyte count and lymphocyte mitogenesis in

response to mitogens and alloantigens. This is accompanied by enhanced interleukin 2 (IL-2) synthesis. Following injury, arginine reduces or abrogates post-traumatic thymic involution and the impairment of T lymphocyte function. Other immune effects of arginine include enhanced delayed-type hypersensitivity reactions, amplified *in vitro* and *in vivo* allogeneic responses and increased survival following lethal septic challenges, experimental burn injuries and trauma. Arginine also promotes host antitumor responses in a variety of tumor models (solid transplanted, chemical- or viral-induced) by decreasing the rate of tumor growth and metastatic spread, and by increasing the latency period to tumor appearance following induction. Survival is also increased. Similarly, in humans, supplemental arginine stimulates T lymphocyte mitogenesis, increases T helper to T suppressor cell ratio and improves wound healing.

Arginine is required *in vitro* for optimal lymphocyte mitogenic response, cytokine production, and for induction of cytotoxic T lymphocyte (CTL) and natural killer (NK) cell function. Maximum mitogenic stimulation and IL-2 production occur at an arginine concentration of 0.04 mM, further increases do not result in enhanced responses. Maximal NK and CTL generation and activation *in vitro* is observed at an arginine concentration between 0.4 and 0.9 mM. Plasma arginine concentrations in human adults range from 0.04 to 0.1 mM, suggesting that the effects observed with *in vivo* arginine supplementation are not related to elevation of plasma levels, since they are already optimal for lymphocyte function.

Within the rodent immune system macrophages, neutrophils and lymphocytes utilize the arginine-nitric oxide (NO) pathway. In macrophages this pathway is essential for the expression of cytotoxicity against tumor cells and pathogenic fungi. Inhibition of NO generation using a specific inhibitor (L-mono-N-methylarginine) results in abrogation of cytotoxic response. Cytotoxic macrophages generating NO destroy target cells by inhibiting aconitase activity (a citric acid cycle enzyme), DNA synthesis, and mitochondrial respiration. A similar pathway has been identified (*in vitro*) in cloned T cells. In these cells NO has an autocrine function in the regulation of cell mitogenesis. The role of the arginine-nitric oxide pathway in humans is less well defined.

The presence of arginine is essential for the function of a variety of immunoregulatory proteins such as thymosin, thymopentin and tuftsin. Production of activated C3a and C5a (both powerful vasoconstrictors and fundamental in the inflammatory process) occurs when the C3 and C5 α chains, respectively,

are split at the N-terminal end by convertase enzymes, thus exposing a C-terminal arginine molecule. Subsequent arginine removal by carboxypeptidase B inactivates C3a. Removal of the arginine from C5a produces C5a-desarginine, which maintains (at a much reduced level) the cell-activating properties of C5a (neutrophil chemotaxis, activation of neutrophil leukotrienes and mast cell degranulation), but there is loss of vasoconstrictive properties.

Mechanism of action

The mechanism for the diverse actions of arginine on the immune system are not fully understood. Nonetheless several hypothesis exist. Arginine via its secretagogue effects promotes growth hormone and prolactin release from the pituitary, and insulin and glucagon release from the pancreas. Convincing *in vivo* and *in vitro* evidence suggests an immunopermissive role for prolactin. Hypophysectomized rats fail to mount an adequate immune response to antigenic challenge, a failure reversed by giving them prolactin or growth hormone. Prolactin-like receptors have been identified on the surface of T cells, as has the production of a prolactin-like protein by stimulated T cells. It is likely that arginine (*in vivo*) exerts some effect on the immune system via increased pituitary release of prolactin and/or growth hormone. However, hypophysectomized rats supplemented with growth hormone, thyroxine and testosterone do not demonstrate increased immune responses secondary to arginine supplementation. There are no similar data available for the human immune system. The *in vitro* arginine requirement for optimal lymphocyte responses to lectins also suggests a local cellular response to arginine, possibly via the nitric oxide pathway.

It has been postulated that arginine is required as a precursor for polyamines which are necessary for lymphocyte proliferation. This does not explain why many of the *in vivo* immune effects of arginine are replicated by ornithine but not by citrulline, spermine or putrescine (other precursors of polyamine synthesis). Finally, when looking at macrophage function, intracellular NO formation from arginine explains much of their activities. Within the liver (in rodents), activated Kupffer cell secretory function (TNF α , IL-6) but not phagocytosis is enhanced up to twice normal following dietary supplementation with arginine. Again, the exact mechanism of action is not fully understood. Elsewhere within the immune system the arginine NO pathway is responsible for inhibition of neutrophil aggregation and may on the basis of *in vivo* studies be a requirement for T cell mitogenesis. However, the exact mech-

anism by which arginine influences lymphocyte function *in vivo* remains unknown.

Despite insufficient knowledge regarding its mechanism of action, it is clear that arginine has a potent effect on multiple cells and molecules involved in immune responses. In the future it is likely that arginine may be used as a pharmacologic agent in immunocompromised states.

See also: Thymic hormones and peptides.

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ARTHUS REACTION

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The Arthus reaction was first described by Maurice Arthus as an acute inflammatory response induced in rabbit skin by a local injection of horse serum in rabbits sensitized by previous injections of the same substance. Although the reaction described originally could have had components of anaphylactic and delayed hypersensitivity responses, the term Arthus reaction best describes the acute response initiated by local deposition of immune complexes and is an example of type III hypersensitivity. Strictly speaking, the Arthus reaction is restricted to the skin and Arthus-like or Arthus-type reactions occur in other organs.

Antibody–antigen complexes are believed to be principal in the inflammatory response associated with a number of diverse diseases which are listed in Table 1. Some of these are well known while others are more obscure and are complications of other dis-

eases (e.g. erythema nodosum leprosum is a serious complication of lepromatous leprosy) or can be adverse reactions to drugs. In the Arthus reaction as originally described, antigen was injected intradermally into sensitized animals and, as antibody diffused from cutaneous microvessels, immune complexes were deposited in and around the vessel wall. Experimentally it is convenient to induce the reaction passively and in reverse, such that antibody is injected intradermally and antigen intravenously – the so-called reversed passive Arthus reaction.

The Arthus reaction can be dissected into three fairly distinct stages: a rapid increase in vascular permeability and extravasation of plasma protein, which is maximal at 1–2 hours, infiltration of leukocytes (initially neutrophils) and hemorrhage which peak in intensity after 4–8 hours. Longer reactions are sometimes associated with necrosis. Experimentally, hemorrhage is the most notable feature of the reaction in transparent tissues such as skin.

Table 1 Diseases and conditions which have features of the Arthus reaction

Systemic lupus erythematosus
Rheumatoid arthritis
Immune glomerulonephritis
Vasculitis
Kawasaki disease
Erythema nodosum leprosum
Inflammatory demyelinating neuropathies
Hypersensitivity pneumonitis
Reaction to immunization with diphtheria toxoid
Drug hypersensitivity

Role of complement

Immune complex-mediated tissue injury is thought to be mediated in the initial stages by activation of primarily the classical pathway of the complement system. In the Arthus reaction the formation of immune complexes in and around the wall of microvessels (Figure 1) results in the activation of complement and the generation of C3a and C5a as well as assembly of the membrane attack complex C5–C9. Depletion of complement (e.g. with cobra

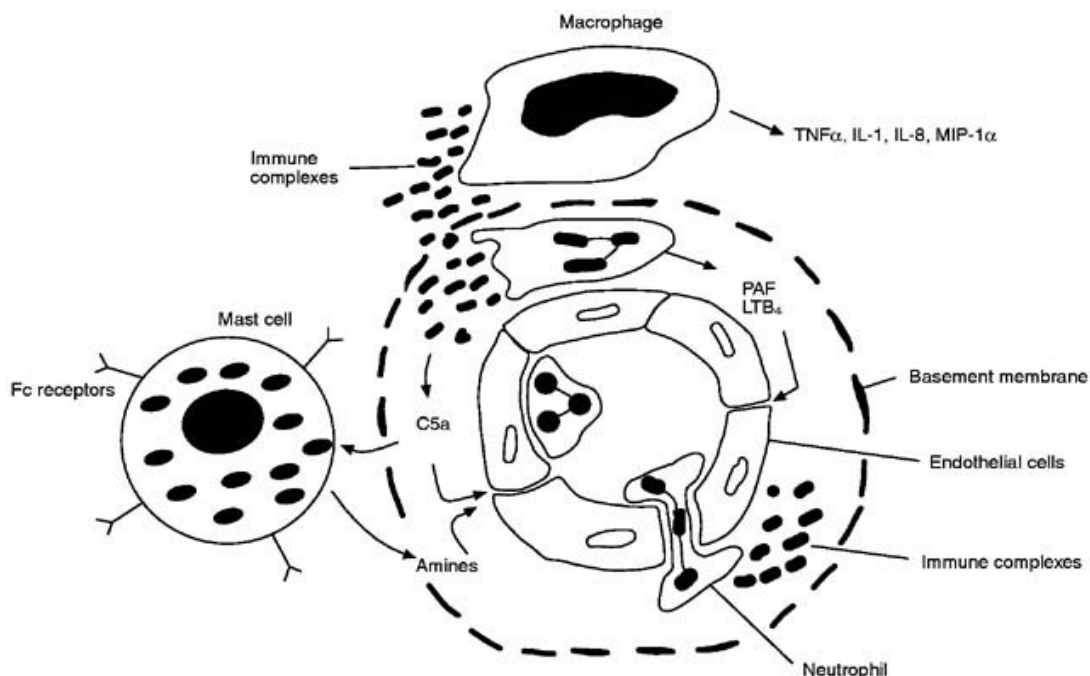


Figure 1 Some of the mechanisms involved in the pathological changes associated with an Arthus reaction. Although considered to be driven primarily by complement components (in particular C5a), recent studies suggest that the mast cell Fc receptor plays a crucial role (see text for details). Evidence that amines, PAF and LTB₄ contribute to the edema formation and neutrophil accumulation has been obtained from pharmacological studies. A similar approach (using neutralizing antibodies) has demonstrated a role for the cytokines and chemokines listed.

venom factor) reduces the magnitude of the Arthus reaction and has led to the idea that C5a plays a key role in orchestrating the early stages of the reaction. Thus, generation of C5a could act on mast cells to release bioactive amines which increase vascular permeability and C5a itself increases permeability by a mechanism that depends on circulating neutrophils. This would supply more plasma proteins, including complement components, leading to further C5a generation. In support of this idea, both antibodies to C5a and soluble CR1, which inhibits the generation of C5a, provide partial protection against immune complex-induced lung and skin inflammation in animal models. However, this concept has been challenged recently by studies in mice genetically deficient in complement components C3, C4 or C5. In these animals the Arthus reaction is indistinguishable from that in wild-type controls, suggesting that complement is not required.

Role of blood elements and adhesion molecules

One of the characteristic features of the reaction is the accumulation of neutrophils. Experimentally this peaks 4–8 hours after initiating the reaction and an early observation was that edema formation and

hemorrhage in the Arthus reaction could be suppressed by depletion of circulating neutrophils. Similar findings have been made using monoclonal antibodies directed against neutrophil β_2 -integrins and endothelial ICAM-1, which prevent neutrophil recruitment to sites of Arthus reactions, presumably by preventing firm adhesion and migration. Neutrophils contribute to inflammatory changes in the Arthus reaction in several ways: neutrophil-dependent edema formation induced by C5a; neutrophil-derived proteases which could cleave C5 in tissue fluid leading to the generation of C5a; neutrophil proteases such as elastase which could lead to tissue destruction and hemorrhage; neutrophil production of lipid mediators (such as platelet-activating factor (PAF) and leukotriene B₄ (LTB₄)) and chemokines (e.g. interleukin-8, IL-8).

Arthus reactions are also suppressed by reagents that interfere with selectin function, for example an anti-E-selectin monoclonal antibody, which reduce neutrophil accumulation and the attendant pathological changes. This has been attributed to the capacity of selectins to mediate neutrophil rolling, the first stage in the process of leukocyte recruitment. An unexpected finding was the ability of a monoclonal antibody against the leukocyte β_1 -integrin VLA-4 to decrease neutrophil accumulation in

immune complex-induced lung inflammation. As neutrophils do not express VLA-4, the protective effect of the antibody might be due to suppression of macrophage function.

Platelet depletion also reduces neutrophil accumulation in the Arthus reaction. The reason for this is not entirely clear but platelets are suggested to facilitate efficient recruitment of neutrophils into tissues, perhaps via lipid mediator interactions and P-selectin-mediated adhesion.

Mediators involved in the Arthus reaction

A list of inflammatory mediators demonstrated (using a pharmacological approach) to have a role in the pathology of the Arthus reaction is given in Table 2. Biogenic amines (histamine and 5-hydroxytryptamine) released from mast cells, usually in response to the anaphylatoxins C3a and C5a, can increase vascular permeability, although this action is greatest in rodents. Antagonists of these amines have no effect on neutrophil recruitment and hemorrhage. A role for PAF in the Arthus reaction was discovered almost a decade ago and it appears to be an important mediator of plasma leakage. The site of deposition of immune complexes may be important in this regard; in the Arthus reaction neutrophil phagocytosis of immune complexes in the vessel wall releases PAF adjacent to endothelial cells leading to plasma leakage. In contrast, phagocytosis of immune complexes away from the vessel wall does not reveal a role for PAF. The same consideration may apply for LTB₄ because specific receptor antagonists reduce edema formation in an Arthus reaction. Vasodilator prostaglandins (e.g. PGE₂, prostacyclin) produced in the Arthus reaction may modulate edema formation and neutrophil accumulation by augmenting the blood supply; this increases hydrostatic pressure in postcapillary venules and increases the delivery of neutrophils to the tissue.

Of the cytokines, IL-1 is involved in the edema formation, neutrophil accumulation and hemorrhage

in the Arthus reaction and immune complex-induced lung injury. In contrast, tumor necrosis factor α (TNF α) appears to have a more important role in the lung as a number of TNF α inhibitors have no effect on Arthus reactions in skin. The source of these cytokines is speculated to be macrophages which are stimulated by immune complexes. Macrophages are also likely to be one of the sources of chemokines, including IL-8 and macrophage-inflammatory protein 1 α (MIP-1 α), which appear to play a role in the Arthus reaction. However, other cells, including endothelial cells, fibroblasts and even neutrophils themselves, could contribute to production of these mediators.

Other neutrophil-derived factors that contribute to the pathological changes in the Arthus reaction include proteases, particularly elastase which may cleave C5 but also destroys tissue and leads to hemorrhage. Indeed, an elastase inhibitor prevents immune complex-induced hemorrhage in the lung. Nitric oxide (NO) can enhance blood flow and exacerbate the Arthus reaction in a similar manner to PGE₂. Moreover, on interaction with superoxide anion, NO forms the highly reactive peroxynitrite anion (ONOO⁻), which in turn can be protonated to form the tissue damaging hydroxyl radical.

Recent advances in mechanisms underlying the Arthus reaction

As discussed above, recent data suggest that in the mouse the presence of an intact complement system is neither necessary nor sufficient to trigger or propagate an Arthus reaction. This is a surprising finding given that antibodies to C5a are effective at attenuating the reaction in rat and rabbits, and treatment with an anti-C5 mAb for 6 months prevents the spontaneous development of an autoimmune syndrome with features of systemic lupus erythematosus (SLE) in NZB/W mice. In addition, a soluble form of the complement regulatory protein CD46 (membrane cofactor protein), which inhibits complement activation *in vitro*, reduces inflammatory cell influx and edema formation in the Arthus reaction. In the same model, soluble CD35 (CR1) and CD55 (decay-accelerating factor) were also effective as anti-inflammatory agents.

An alternative mechanism has been proposed based on the greatly diminished capacity of mice deficient in the γ subunit of Fc receptors to develop Arthus reactions. Mast cell reconstitution of the mast cell-deficient mouse strain W/W^v reveals that Fc γ RIII on this cell is responsible for triggering the inflammatory response in the Arthus reaction. Other Fc γ RIII-expressing resident tissue cells (macrophages

Table 2 Mediators demonstrated to be involved in the Arthus reaction

Class	Examples
Lipid-derived	PAF, LTB ₄ , prostaglandins
Amines	Histamine, serotonin
Complement-derived	C5a
Cytokines	TNF α , IL-1
Chemokines	IL-8, MCP-1, MIP-1 α
Others	Nitric oxide

and Langerhans cells) and neutrophils could be involved in later stages of the reaction. Similarly, Fc γ RII is involved in aspects of the Arthus reaction. A soluble form inhibits hemorrhage and perivascular neutrophil recruitment; this may be related to suppression of neutrophil activation *in vivo*.

Although much progress has been made in understanding the physiological and cellular mechanisms underlying the Arthus reaction, clearly much has still to be elucidated concerning the relevant inflammatory and hemorrhagic response process to be targeted clinically.

See also: Acute inflammatory reaction; Adhesion molecules; Anaphylatoxins; Autoimmune diseases; Mast cells; Chemokines; Cobra venom factor; Complement, classical pathway; Cytokines; Fc receptors; Histamine; Hydrostatic pressure, effect on immune system; Immune complexes; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Interleukin 8 and its receptor; Neutrophils; Nitric oxide; Phagocytosis; Platelet-activating factor (PAF); Prostaglandins; Selectins (CD62-E/L/P); Serotonin; Systemic lupus erythematosus, experimental models; Tumor necrosis factor α ; Chemotaxis of neutrophils.

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ASCARIASIS

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The organism and its antigens

Ascaris lumbricoides is among the most widespread and common of all pathogens of humans, infecting over 1.3 billion people. It is a nematode, or 'round-worm', and can arguably present the human host with a greater burden of foreign biological material than does any other parasite. Sexually-producing adult worms develop in the intestine to a maximum size of about 400 mm long by 6 mm thick, and females release eggs at the rate of 200 000 per day. These take about 2–3 weeks to embryonate and produce the infective larvae. Following ingestion, the larvae emerge from the egg, invade the mucosa, migrate to the lungs via the liver, break out into the alveoli, move up the trachea and are swallowed. Upon re-entry to the intestine, they develop to mature adult worms, mate, and eggs usually begin to be released in the feces by about 60–70 days of infection.

It has often been written that the vast majority of ascariasis cases are symptomless, but it is becoming clear that chronic malabsorption and malnutrition can result, and even single worms can prove lethal by penetrating the bile or pancreatic ducts. Rupture and/or blockage of the intestine by the sheer mass of worms is estimated to kill about 20 000 children a year, but this is almost certainly a gross underestimate. Treatment by anthelmintics is effective but parasite infections can return to pretreatment levels within 1 year because of contamination of the environment with the highly persistent eggs. There is, therefore, little evidence for the acquisition of a sterile immunity to the parasite in humans, although certain individuals appear to remain consistently uninfected. A characteristic of most helminthiases, including ascariasis, is that the parasite population is overdispersed within a host population. That is, most of the parasites are present in a small proportion of potential hosts, and the majority of people are parasite-free or have only small burdens. Among infected individuals, children tend to suffer infections of greater intensity than do those in older age groups. Recent genetic studies on the parasites have shown that the closely related parasite of pigs, *A. suum*, probably does not reach maturity in humans, but exposure to it might nevertheless influence the immune response to *A. lumbricoides*.

The first clinical signs of infection are associated

with the pulmonary phase and include pneumonitis, cough, dyspnea, substernal pain and sometimes a blood-stained sputum. This is known as the Löfller syndrome and has some of the characteristics of an immediate-type hypersensitivity response. There can be a dense pulmonary infiltrate at this time, and a rising eosinophilia. Pulmonary hypersensitivity can cause significant mortality among children in arid regions where high-level seasonal transmission occurs. The intestinal phase is associated with digestive disorders, nausea and colic. Villous atrophy has been demonstrated in infected children and pigs, and is probably immune-associated.

Until recently, work on the antigens of *Ascaris* has been confined to somatic materials of the parasite. These have been shown to be rich in the phosphorylcholine hapten, and antibody responses to it are a major part of the response to infection in mice. The determinant is found internally and is not thought to be exposed on the parasite surface. *Ascaris* is renowned among laboratory workers for its content of allergens, and proteinaceous allergens ranging between 14.4 and 360 kDa have been described. A nonspecific mast cell-degranulating factor of about 9 kDa has also been described from *Ascaris*. The extent to which these substances contribute to pathology is not clear, but there is one report of lethal intestinal anaphylaxis following chemotherapy for ascariasis, and it is likely that parasite allergens contribute to pulmonary hypersensitivity.

It is now clear, however, that antigens released by the tissue-invasive larvae of the parasite (usually termed 'excretory/secretory' (ES) products) are potentially immunogenic and that the set of (glyco) proteins of which they are comprised changes radically during the migration from intestine to lungs. The larvae also release several proteinases, the biochemical activity of which are inhibitable by antibody elicited by the infection. ES products contain components ranging from 14 to 410 kDa, the 14 kDa component also being found in abundance internal to the worm, and is a potent allergen named ABA-1. This protein is now known to be a fatty acid and retinoid-binding protein which appears to have no homolog in vertebrates, and has highly unusual binding characteristics. It is thought to be a four-bundle α -helix protein with its binding site in its core (Figure 1). ABA-1 is a member of the nematode

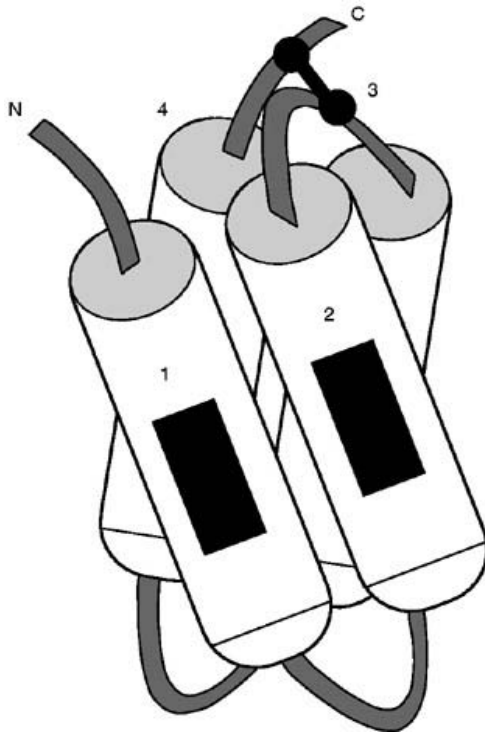


Figure 1 Model of the ABA-1 allergen of *Ascaris*, showing the proposed four α -helix bundle structure. The protein is known to form dimers and the position of putative external hydrophobic patches on helices 1 and 2, which may act as the subunit interface, are shown as black rectangles. (Courtesy of Dr A Brass, University of Manchester, UK.)

polyprotein antigens/allergens which appear to be produced by all nematodes as a polyprotein that is cleaved by proteinases during post-translational processing into multiple copies of the active allergen protein.

Immune response of the host

Infected humans produce strong immunoglobulin G (IgG) and IgE antibodies against the parasite, but most people respond only to a subset of parasite components. For instance, only about one in five infected people appear to respond to the ABA-1 antigen/allergen. This heterogeneity has been modeled in laboratory rodents in which the immune repertoire to *Ascaris* antigens is restricted by the class II region of the major histocompatibility complex. A surprising feature of the restriction is that it applies to whole molecules as large as 220 kDa. A similar genetic restriction probably explains the limited repertoire seen in humans, but heterogeneity in recognition of individual parasite components might mean that people will vary in the specificity of their hypersensitivity reactions to *Ascaris*. Moreover, there is growing evidence that the IgE response is protective

in ascariasis, as is thought to be the case in some other helminth infections. Experiments in mice have shown that the immune response in *Ascaris* infection is dominated by T_H2 cells, which presumably explains the high IgE, mastocytosis and eosinophilia of the infections. In experimental infections in mice with other species of intestinal nematode, the T_H2 response is crucial to the immune elimination of the parasites.

Evasive strategies by the organism

Anything written on evasion strategies in nematodiasis remains speculative. The parasite alters its surface and secreted antigens during its tissue migrations, and it has been suggested that this functions to keep the parasite ahead of the immune response. This would not, however, be compatible with the continuous recruitment of parasites known to occur in infected humans. The larvae secrete proteinases which could cleave bound antibody and/or complement. Parasitic nematodes commonly secrete antioxidants, such as superoxide dismutase, but this has not been examined for *Ascaris*. If, like the related nematode *Toxocara canis*, *Ascaris* has a surface that rapidly sheds antigens, then continuous loss of bound antibody, complement and/or cells would provide an immune evasion mechanism. It also might simply be that it is not possible for the immune system to deal with a rapidly migrating multicellular parasite. The intestinal stage of *Ascaris* is thought to live for at least 1 year, and there appear to be successive waves of recruitment. It is not known how these worms evade the immune response, although it is known from experimental models that some species of gastrointestinal nematodes are able to suppress local and sometimes systemic immune responses. Another possibility is that T cell tolerance to these long-term occupants of the gut is generated.

Vaccines

There is no vaccine, and no immediate prospect of one. ES antigens are known to be partially protective in rodents and pigs, and are therefore attracting attention. It has become conventional that parasite surface antigens be investigated, but their activity as protective antigens has not been examined for *Ascaris*. Only one vaccine against any nematode parasite is currently available, the irradiation-attenuated vaccine against the lungworm of cattle and sheep, *Dictyocaulus viviparus* and *Dictyocaulus filariae*. Ultraviolet-irradiated larvae of *Ascaris* confer partial resistance in pigs, but such a preparation would probably not be acceptable for human or vet-

erinary use because of the associated pathology, particularly in the liver. The design of a vaccine against *Ascaris* (as with all pathogens) comprising recombinant proteins would have to take into account the genetic restriction of the immune repertoire. Some new generation vaccines are thought to circumvent this restriction, but this has not yet been found to be the case for nematode antigens. Likewise, the importance of IgE antibody to protection will need to be considered, although immunization regimens designed to generate an IgE response may be necessary; they would need to be designed to avoid unacceptable hypersensitivity responses in vaccinated subjects who may already have been sensitized by infection.

See also: Allergens; Gastrointestinal tract infections; Hookworm disease; Parasites, immunity to; Toxocariasis; Trichuriasis

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ASTHMA

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Asthma is a disease of the lungs affecting both children and adults and characterized by: 1) airway obstruction that is partially or completely reversible, 2) airway inflammation, and 3) airway hyperresponsiveness. A persistent rise in disease prevalence, hospitalizations due to disease exacerbation, and disease mortality over the last two decades underscores the importance of an increased understanding of disease pathogenesis and the development of improved therapeutic agents. This entry summarizes our current understanding of this disease entity.

Epidemiology

There is a close concordance between asthma, allergic rhinitis and atopic dermatitis; the presence of one of these entities increases the relative risk of the other two by 3- to 30-fold over the lifetime of the subject. All three of these diseases are associated with high levels of nonspecific and antigen-specific serum immunoglobulin E (IgE).

The worldwide prevalence of asthma in childhood ranges from approximately 1.4 to 11.4%, but varies widely by racial and geographic factors and ascertainment methods. The overall prevalence of asthma is approximately equal in industrialized and nonindustrialized nations; however, prevalence tends to be less in rural than in urbanized communities. Moreover, children emigrating from rural to urbanized areas tend to assume the relative risk associated with their new environment, implicating exposure to extrinsic agents in disease pathogenesis. In developed countries, asthma is the second leading cause of hospitalization in children. Finally, greater than 70% of bronchial hyperresponsiveness (BHR) in adults and greater than 85% of BHR in children is associated with IgE sensitivity to various, predominantly inhaled, allergens.

A strong genetic influence on the development of asthma has also been described in studies of mono- and dizygotic twins. Vertical transmission studies show a 25–35% relative risk of asthma conveyed to each child by each affected parent. Finally, a

synthesis of data from various linkage studies in affected kindred have shown associations of the asthma phenotype to microsatellite markers on chromosomes 4, 6, 7, 11, 13 and 16, with more specific mapping to 11q13 (FceR1 β), 5q31 (IL-4, -5 and -13 gene cluster), 6p21 (HLA-DR), 14q11.2 (T cell receptor α/δ chains) and 12q15. Thus, our understanding of disease pathogenesis must take into consideration the multifactorial nature of this clinical entity.

Pathogenesis

The key initiating event in the pathogenesis of asthma is currently felt to be inflammation of the conducting airways. T lymphocyte activation is suggested by increased surface expression of CD25, CD45, HLA-DR and VLA-1 on lymphocytes from bronchoalveolar lavage (BAL) and bronchial biopsy specimens of asthmatic subjects. Moreover, expression of interleukin-4 (IL-4) and IL-5 but not interferon γ (IFN γ), in a pattern consistent with a T_H2 response, has been shown in asthmatic subjects; this expression is greater in symptomatic subjects, increased after allergen challenge, positively correlated with asthma symptoms, and negatively correlated to FEV₁ (forced expiratory volume in 1 second). Early activation of pulmonary mast cells is suggested by elevations of both histamine and tryptase in BAL samples immediately following allergen challenge. IgE-dependent activation of mast cells results in the generation of sulfidopeptide leukotrienes that contribute to mucus hypersecretion, increased microvascular permeability and smooth muscle contraction. The participation of tryptase in neuropeptide metabolism may further contribute to neuronally mediated increases in airway smooth muscle tone. The late inflammatory response is associated with a marked

influx of eosinophils and basophils into the asthmatic airway. Eosinophil degranulation is suggested by increases in major basic protein, eosinophil peroxidase, and eosinophil cationic protein in asthmatic airways. These highly toxic substances may promote BHR through direct damage to airway epithelium, resulting in exposure of mast cells and nerve endings. Activation of basophils recruited to these sites of inflammation result in both the generation of mediators of bronchoconstriction and mucus hypersecretion and the elaboration of proinflammatory cytokines such as IL-4 and IL-13. Selective and coordinated recruitment of these various effector cell types may be achieved through the upregulation of specific members of the integrin and selectin superfamilies of cellular adhesion molecules and the elaboration of specific cellular chemoattractants (Figure 1).

The pathology of asthma reflects these basic principles of allergic inflammation. Those airways most affected in asthmatics are the large and medium conducting airways. Classic changes seen on biopsy of severe asthmatic subjects, as well as postmortem examination of subjects suffering fatal asthma, include vascular congestion, mucosal edema with exudation of fluid and proteins from the microvasculature, and infiltration of inflammatory cells into interstitial tissue. Mucus hypersecretion with disruption of pulmonary epithelial cells results in luminal obstruction. Chronic inflammation may also result in airway remodeling with thickening and fibrosis in the region of the basement membrane. Curschman spirals (expectorated casts of inflamed airways), Creola bodies (compact clusters of sloughed epithelial cells), and Charcot-Leyden crystals (condensed eosinophil granular proteins) are considered classic findings in severe asthma. Squamous metaplasia may occur as a response to epithelial disruption. The functional

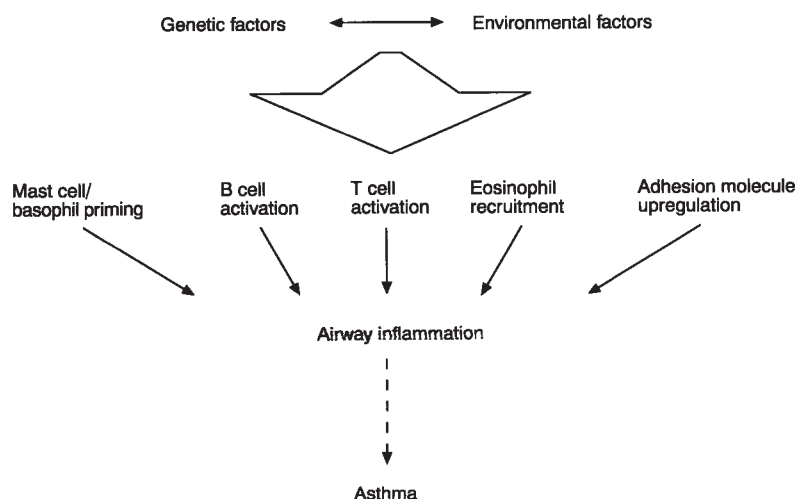


Figure 1 The pathogenesis of asthma.

consequences of these pathological changes include early airway closure with distal air trapping and increased residual volume, reduced FEV₁ values, and abnormal gas distribution and exchange. Increased airway resistance and tidal volumes closer to inspiratory plateau contribute to an increased work of breathing that is only partially compensated by the use of accessory muscles for respiration. Finally, defects in smooth muscle relaxation may contribute to the pulmonary mechanical dysfunction characteristic of asthma.

Clinical presentation

Asthma may present acutely or as a chronic pulmonary disease. Symptoms of acute asthma include shortness of breath, chest tightness, wheezing and cough, often productive of clear or slightly colored sputum. When present, chest pain is usually musculoskeletal in origin. Audible wheezing may not be present in mild asthma, but may be elicited by forced expiratory maneuvers. Increased diurnal variations in pulmonary function are often associated with nocturnal exacerbations. Triggers for worsening asthma include cold air exposure, exercise, viral respiratory infections, sinusitis, gastroesophageal reflux, exposure to seasonal or perennial inhalant allergens, and exposure to inhaled irritants such as cigarette smoke. Seasonal variations in asthma severity often correlate with seasonal allergen exposure. Finally, a number of medications, including β adrenergic blockers and nonsteroidal anti-inflammatory agents, as well as sulfite preservatives, may exacerbate asthma in susceptible subjects. A correlation of total suspended particles and levels of various air pollutants to exacerbation rates for asthma has been reported.

Physical signs of acute asthma include tachypnea, tachycardia, pulsus paradoxicus, hyperinflation of the chest, audible wheezing, and the use of accessory muscles of respiration. Chest roentgenogram may show flattening of the diaphragms bilaterally and increased anteroposterior dimension. Pulmonary function studies reveal an obstructive pattern which may be partially or completely reversed by the administration of β -adrenergic agonists. Diurnal variations in peak expiratory flow rate greater than 20% are highly suggestive of asthma. Bronchoprovocation with a variety of agents, including methacholine and histamine, may identify subjects with BHR. However, it should be noted that the perception of asthma by an affected subject may not correlate to the degree of pulmonary dysfunction due to an impaired sensitivity to hypoxia and hypercarbia.

The differential diagnosis of asthma includes mechanical obstruction of the airway (extrathoracic

and intrathoracic), laryngeal dysfunction, chronic obstructive pulmonary disease, cardiac dysfunction, pulmonary embolism, pulmonary infiltrates and eosinophilia (PIE syndrome), cough due to drugs (angiotensin-converting enzyme ACE inhibitors), and carcinoid tumors. The majority of these entities may be readily differentiated from asthma on the basis of clinical presentation, physical examination, roentgenographic studies and pulmonary function testing.

Treatment

Nonpharmacologic

Optimal management for asthma of any stage should include a number of nonpharmacologic measures. Paramount among these is education of the patient and family concerning basic issues of disease pathogenesis, mechanisms of action of medications, and the proper technique for the use of metered-dose inhalers. Avoidance of allergic and irritant triggers of asthma, particularly indoor allergens (dust mite, animal danders, cockroach and mold) and indoor irritants (cigarette smoke, colognes) should be encouraged. Finally, every patient should be instructed in the proper use of step care for asthma exacerbations due to exposures and viral illnesses, with the objective of reducing the necessity for acute and subacute care (Figure 2).

Pharmacologic

Nonacute asthma may be classified as mild, moderate or severe on the basis of clinical parameters. Mild asthma is defined as exacerbations of cough and wheezing less than twice a week with few clinical signs or symptoms between exacerbations, good exercise tolerance, nocturnal symptoms less than

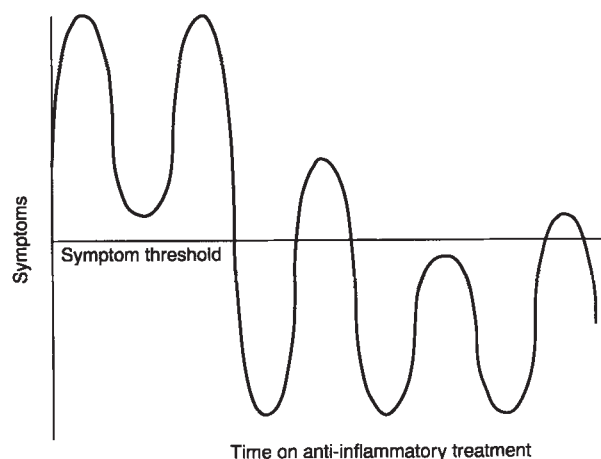


Figure 2 The beneficial effects of anti-inflammatory therapy on frequency, duration and severity of exacerbations in asthma.

twice a month, and a resting peak expiratory flow rate (PEFR) of >80% of predicted with less than 20% diurnal variability. Moderate asthma is defined as exacerbations more than twice a week, resulting in urgent care treatment less than three times a year, with periods of minimal symptoms between exacerbations; exercise tolerance in these patients is slightly diminished, nocturnal symptoms may be present 2–3 times a week, and resting PEFR may be 60–80% of predicted with diurnal variability of 20–30%. Severe asthma is characterized by daily symptoms with frequent exacerbations and frequent utilization of urgent care facilities or a history of respiratory failure. Patients with severe asthma have poor exercise tolerance, frequent nocturnal symptoms, resting PEFR <60% of predicted with diurnal variability >30%. Risk factors for fatal asthma include hospital admission for severe asthma within the preceding year, a history of respiratory failure with hypercarbia due to asthma, and a history of barotrauma. Pharmacologic therapy should be targeted to clinical stage, as summarized in Table 1. A synthesis of available studies suggests that immunotherapy for inhalant allergens reduces BHR, frequency and/or severity of exacerbations, and medication usage in all three clinical stages of chronic asthma. Leukotriene antagonists may be useful for all stages of asthma, and particularly effective for aspirin sensitive asthmatics. Agents considered investigational at this time for the treatment of severe asthma include methotrexate, gold, macrolides and cyclosporine.

The treatment of acute asthma should focus on intensification of pharmacologic therapies and maintenance of respiratory function with the use of ventilatory support and neuromuscular paralysis as necessary. Therapeutic BAL may be useful in selected patients. Mucolytic agents and iodides are contraindicated.

Special considerations

Occupational asthma

Approximately 2% of all asthma is related to occupational exposures. A large and growing list of agents may precipitate this syndrome, including vegetable materials, chemicals, animal materials, metals and pharmaceuticals. While the high molecular weight compounds are complete antigens that elicit IgE synthesis, many of the low molecular weight compounds are haptens, creating a complete antigen only when bound to carrier proteins. There is often a latency period between the onset of exposure and the development of clinical disease. Symptoms tend to be worse on work days and better on weekends and holidays. Bronchoprovocation testing with the specific agent may confirm the diagnosis. Subjects with long exposure histories may suffer chronic pulmonary dysfunction despite removal from the environment.

Asthma during pregnancy

Poorly controlled asthma poses significant risks to both mother and fetus. The primary goals of asthma therapy during pregnancy are stabilization of disease with prevention of exacerbations and rapid, early intervention for acute exacerbations should they occur. Intensification of nonpharmacologic therapies is indicated: risk/benefit analysis suggests a positive profile for most asthma medications, particularly the inhaled medications.

See also: Acute inflammatory reaction; Allergens; Atopic allergy; Basophils; Drugs, allergy to; Eczema; Eosinophils; Food allergy; Glucocorticoids; Helper T lymphocytes; Histamine; Hypersensitivity reactions; Mast cells; Rhinitis, allergic.

Table 1 Pharmacologic therapy of chronic asthma

<i>Asthma grade</i>	<i>β Agonists</i>	<i>Cholinergic blockers</i>	<i>Cromoglycates</i>	<i>Steroids</i>	<i>Methylxanthines</i>	<i>Immunotherapy</i>
Mild	As needed pre-exercise pre-exposure	As needed; may be useful	May be useful pre-exercise pre-exposure	Rarely needed	May be useful for nocturnal symptoms	Indicated
Moderate	As needed or regular use; consider long-acting agents		Regular use	Regular use of inhaled; systemic as needed	May be useful adjunct therapy	Indicated
Severe	As needed and regular use; consider long-acting agents		Regular use	High dose inhaled; systemic as needed	Regular use	Indicated

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ATAXIA TELANGIECTASIA

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Ataxia telangiectasia (AT) is a rare (1:40 000 live births) pleiotropic disorder whose various phenotypic manifestations include a progressive cerebellar ataxia, ocular apraxia, and oculocutaneous telangiectasia (Figure 1). Almost all patients have an elevated serum α -fetoprotein (AFP). Translocations involving chromosomes 7 and 14 are characteristic. About half of the patients have a moderate to severe immunodeficiency, involving both B and T cell compartments. One-third of patients develop cancer, usually lymphoid. Because these patients are hypersensitive to ionizing radiation and are cancer susceptible, they are in grave danger of being overtreated with conventional doses of radiation therapy. The disease is inherited as an autosomal recessive trait. Despite previous work describing four complementation groups, only a single AT gene exists, ATM (AT, mutated). Most patients are compound heterozygotes, i.e. they inherit a different mutation from each parent. The function of the ATM gene is presently unknown; however, much evidence suggests that one of its primary roles is to sense double-stranded DNA breaks and alert the cell, via signal transduction pathways upstream of p53, not to enter the S (synthesis) phase of the cell cycle until the DNA damage has been repaired. No doubt the ATM gene product will have other roles as well. AT cells have G₁, S, and G₂/M checkpoint defects. All DNA repair

mechanisms that have been tested in AT patients have been normal.

Neuropathology

The most apparent characteristic of the AT syndrome is the ataxia. Shortly after learning to walk, AT children begin to stagger; by 10 years of age, they are confined to a wheelchair for the remainder of their lives. The most obvious lesion in the central nervous system at postmortem examination is a paucity of Purkinje cells in the cerebellum. These cells are present in normal numbers at birth but degenerate progressively. Purkinje cell migration and arborization are also abnormal, lesions that would have had to have been expressed during the third trimester of pregnancy, long before symptoms appear. It is not known whether the Purkinje cell degeneration is central or results from anterograde or retrograde degeneration.

Radiosensitivity

Radiation therapists have observed that when AT patients with cancer are treated with conventional doses of ionizing radiation, they develop sequelae characteristic of much higher dosages. The radiosensitivity can be demonstrated *in vitro* as well, usually

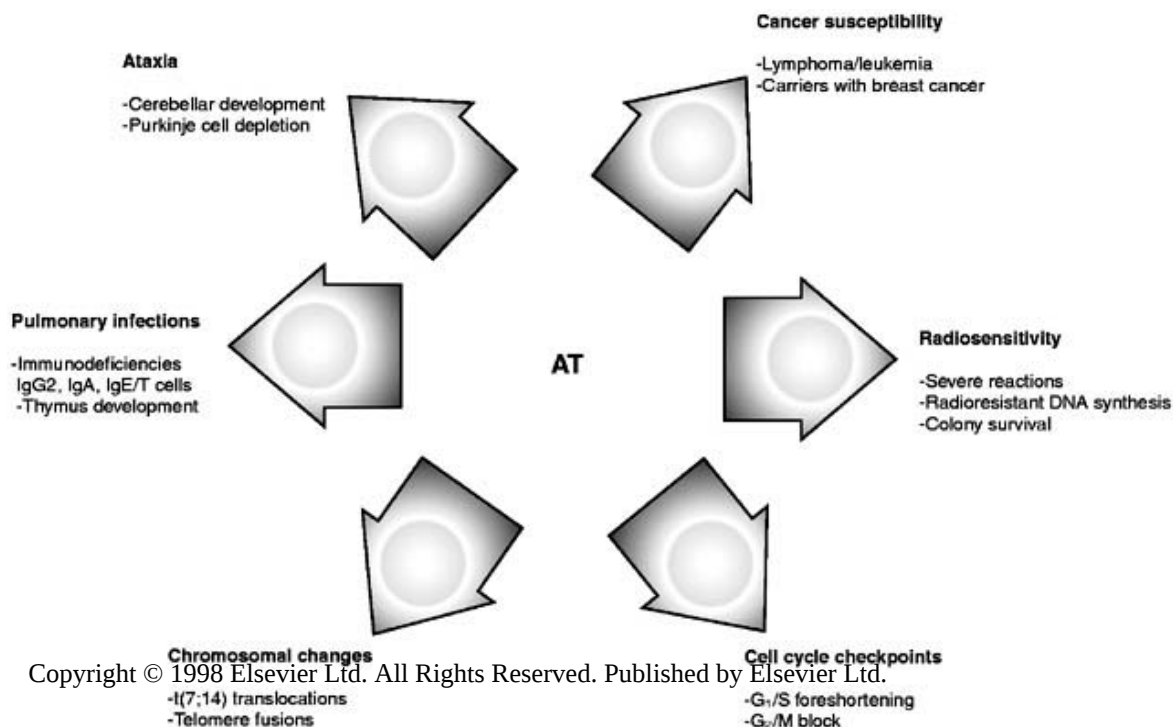


Figure 1 The AT syndrome.

by 'radioresistant DNA synthesis' or by colony survival efficiency following radiation. In the laboratory, carriers are intermediate in radiosensitivity between homozygotes and normals. AT cells are sensitive not only to ionizing radiation but to a variety of radiomimetic agents as well. Testing for radiation hypersensitivity can be used to confirm the diagnosis; however, few laboratories test clinical specimens. Our laboratory uses an Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line (LCL) to measure *in vitro* radiosensitivity (Figure 2).

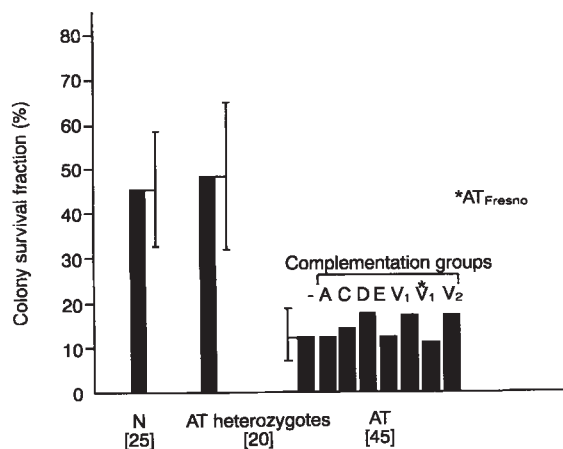


Figure 2 Colony survival assay (CSA) measures radiosensitivity of LCLs to 1 Gy on patients with AT, AT heterozygotes, and normals. Also included are results on patients with NBS (V₁), BBS (V₂) and AT_F (V₃).

Heterozygotes cannot be distinguished by this method under the conditions used.

Cancer susceptibility

During the shortened lifetimes of AT homozygotes, 38% develop a malignancy, usually lymphoid. This represents a 61-fold and 184-fold increase in Euro-American and Afro-American patients, respectively. About half of the malignancies observed are lymphomas, usually of the B cell type. Leukemias account for another quarter of the malignancies. These are usually T cell leukemias. Young AT patients develop a less aggressive T cell leukemia than older patients, who develop a prolymphocytic leukemia (T-PLL); the latter was formerly described as T-CLL because the cells histologically resemble those of chronic lymphocytic leukemia. However, the T-PLL cells are T cells. Myeloid leukemias have not been described in AT patients. AT heterozygotes are also cancer prone, although not to the extent that homozygotes are. Breast cancer was observed to be five-fold higher among heterozygotes in US families. Based on this observation and an estimated carrier frequency of 1%, it has been postulated that between 8 and 18% of all breast cancer patients may be AT heterozygotes. Despite this prediction, recent genetic screening of breast cancer patients for ATM mutations has failed to reveal increased numbers of AT heterozygotes. Further studies are in progress to

answer this question. Meanwhile, ATM knockout mice all die with malignant thymic lymphomas, closely mimicking the cancer susceptibility of AT patients.

Chromosomal instability

Almost all AT patients have translocations involving the T cell receptor gene complexes at 14q11, 7q35 or 7p14, making up about 10% of metaphases counted. AT heterozygotes have similar translocations in about 1% of their T cells. Normals also have such translocations in about 0.1% of their T cells. Chromosomal aberrations in fibroblasts and amniocytes of AT patients are random and do not show the characteristic translocations. The T-PLL leukemia of AT patients is frequently associated with translocations or inversions involving the same sites described above. These translocations juxtapose part of a T cell receptor gene to a site just proximal to either the TCL-1 (T cell leukemia 1) gene at 14q32 or the c6.1B/MTCP-1 (mature T cell proliferation 1) gene at Xq28. Other factors must also be necessary for leukemia to develop since some AT patients have these clonal expansions in 100% of their T cells for many years and yet do not have leukemia. The frequencies of both mitotic and meiotic recombination are increased in AT cells, as is intrachromosomal recombination. Gametogenesis may be abnormal. ATM knockout mice have gonadal streaks, female animals do not ovulate, and male animals are sterile. Despite this, most AT patients reach normal sexual maturity, with normal menstrual cycles in females, and the ability to produce sperm in at least some males.

Immunodeficiency

At postmortem examination, virtually every AT homozygote has had a small embryonic-like thymus. No single uniform immunological abnormality has been found in AT patients. Indeed, even affected siblings often have different degrees of immunodeficiency. IgA and IgG2 deficiencies have been described in 60% and 80% of patients, respectively. Occasional AT patients have hyper-IgM, with hyperviscosity of blood and splenomegaly. T cell levels are normal to slightly reduced in AT patients. A suggestion that a $\gamma\delta$ T cell subset is increased in AT patients has not been generally confirmed. CD4⁺/CD45RA⁺ (naive) T cells are decreased in some patients. Responses to mitogens, antigens and allogeneic cells are usually slightly reduced, although a few patients have profound deficiencies of T cell responses. T cells show abnormally fast capping of FITC-labelled con-

canavalin A. Natural killer (NK) cell activity and NK cell levels have been described as normal, decreased, or increased in various studies. Some of these discrepancies may reflect transient immunological changes during infections. Unlike all other immunodeficiency symptoms, opportunistic infections are not seen in AT patients.

The ATM gene and mutations

The AT gene was isolated in 1995, culminating a 14-year positional cloning effort by an international consortium of investigators. The ATM gene and gene products are very large: 3056 amino acids, 350 kDa protein, and a 13 kb transcript that may be alternatively spliced in some tissues. The ATM gene is expressed in all tissues tested. The gene has 66 exons, each of about 100–300 base pairs in length; these are spread over about 150 kb of genomic DNA at chromosome 11q23.1. Eighty-five per cent of mutations result in a shortened (truncated) protein. Since the gene has a phosphatidylinositol 3-kinase domain at the 3' end, truncated proteins probably will be lacking this important functional domain. Many of these truncations result from deletions of 1, 2 and 3 exons because of mutations at splice sites. About 200 mutations have been characterized thus far (Figure 3); only two potential hotspots can be appreciated at exons 54 and 16. The rest of the mutations are found across the entire gene, making it difficult to identify each patient's mutations without a great deal of screening of the gene. The most useful screening techniques thus far have been protein truncation testing (PTT), single-strand conformational analysis (SSCA), heteroduplex analysis (HA), conformation-sensitive gel electrophoresis (CSGE), RNAase cleavage assay (RCA), and restriction endonuclease fingerprinting (REF). Each of these must then be followed by DNA sequencing to identify the actual site of the mutation. A few common mutations have been identified and can be tested easily among Amish, Central-Southern Italians, Moroccan Jews, Midland English, Norwegians, Costa Ricans and Polish.

Related syndromes

Several related syndromes overlap with AT. Nijmegen breakage syndrome (NBS) shares t(7;14) translocations, radiosensitivity, immunodeficiency and cancer susceptibility with AT but these patients do not have ataxia, telangiectasia, nor elevated AFP; NBS patients are microcephalic and mentally retarded. AT patients are usually not mentally retarded. Berlin breakage syndrome is very similar

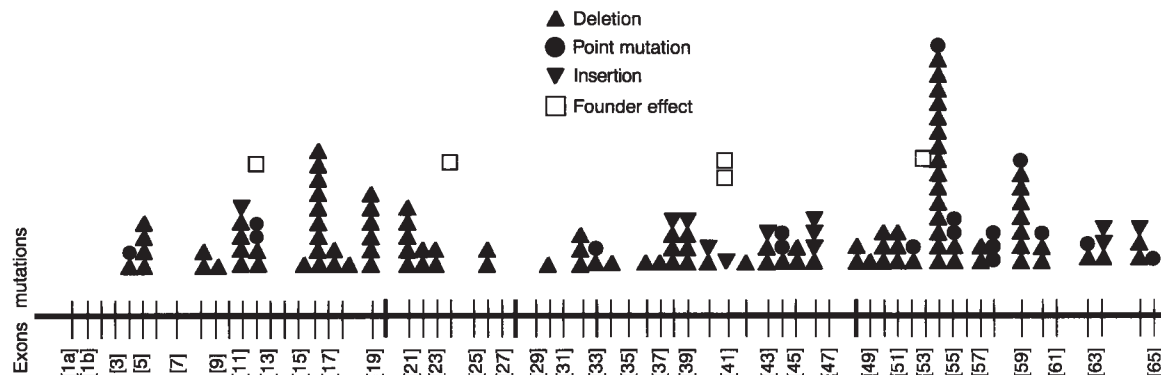


Figure 3 Spectrum of ~120 ATM mutations based on studies of cDNA from cells of patients from many countries. Mutations seen in related (i.e. shared haplotypes) families are indicated only once (boxes) so as not to bias the distribution.

to NBS but sometimes includes syndactyly or anal stenosis. AT_{Fresno} combines the AT and NBS syndromes. AT_{Fresno} patients have ATM mutations. NBS and BBS families do not link to 11q23.1 and, therefore, are probably caused by mutations at another (AT-related) gene.

Therapy

There is no effective therapy for the progressive ataxia. Spasticity can be controlled in some patients with bromocriptine; however, the safety of this medication has not been established for children under 15 years. Valium has been used to ameliorate chronic jerks and tremors but its effects are short-lived. Vitamin E (α -tocopherol) is sometimes prescribed to minimize nonspecific free-radical damage to tissue, although its efficacy is unproven. Immunotherapy is only indicated for patients with frequent recurring infections. Intravenous gamma globulin is effective in reducing the frequency and severity of infections in such patients. Conventional dosages of radiation therapy and radiomimetic agents are contraindicated and life-threatening.

Physical activities such as swimming and calisthenics maintain muscle tone, prevent fractures and slow the side-effects of long-term confinement to a wheelchair – most notably, contractions of hands and feet. Speech therapy helps articulation and voice volume. Good pulmonary hygiene and regular breathing exercises minimize lung impairment, although there is a bit of evidence that the lungs of AT patients may be anatomically compromised by the disease itself. Parental counselling is of paramount importance in alleviating confusion and anxiety. Bringing members of AT families together periodically as a *de facto* support group is also effective therapy. Prenatal diagnosis is now feasible and is quite accurate, with a turnaround time of 1–2 weeks.

See also: Chromosome translocations of immune genes; Immunodeficiency, primary.

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ATOPIC ALLERGY

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Definition

Allergy was originally defined in 1906 by Clemens von Pirquet as 'altered reactivity' to denote the different reaction which occurs on second exposure to an antigen, due to the formation of antibodies, when compared to the first exposure. Used in this way the term covered all immune reactions; however, its use has since become restricted to certain hypersensitivity reactions. The term atopy (from the Greek 'out of place') was introduced by Coca and Cooke in 1923. They had observed that certain disorders, such as asthma, eczema and urticaria, run in families and that affected subjects show positive wheal and flare skin reactions to common inhalant antigens, but lack precipitating antibodies.

Prausnitz and Kustner in 1921 demonstrated the transfer of atopic reactivity to normal skin by means of serum. The serum factor, called 'reagin' was identified as a new class of immunoglobulin, IgE, in the 1960s.

Immunoglobulin

IgE has the usual immunoglobulin structure of two heavy and two light chains and is heavily glycosylated, containing about 12% carbohydrate. There are five domains in the heavy chain, compared to four in IgG1. A part of the Fc region of IgE (C_H3 and

C_H4) is the binding site for high affinity Fcε receptors on mast cells and basophils. This activity is destroyed by heating at 56°C for 30 min; however, antigen binding activity, which resides in the Fab part of the molecule is not heat labile. Serum levels of IgE are far lower than for other immunoglobulins, being measured in nanograms as opposed to milligrams per milliliter. The half-life of IgE in serum is short (2.5 days); once cell bound, this is increased to several weeks.

Individuals produce IgE in response to certain stimuli, such as parasitic worms, but atopic individuals produce it in response to harmless airborne and/or ingested antigens, which are termed allergens. These include grass pollen, animal dander, house dust mite feces, moulds, food antigens and also industrial substances such as toluene di-isocyanate, plicatic acid and platinum salts. Guidelines for allergen nomenclature recommend the use of the first three letters of the genus, plus the initial of the species, followed by a roman numeral which usually reflects the order in which the allergen was isolated. Major allergens are however designated I. Thus the major house dust mite allergen is known as Der p1, the major cat allergen as Fel d1, etc.

Basis of atopy

The incidence of atopy is closely related to the serum IgE concentration, with less than 5% of the

individuals with levels less than 60 iu/ml (1 iu = 2.4 µg) being affected, but over 95% of those with values over 450 iu/ml. There is also an obvious genetic influence, with over 50% of individuals having two allergic parents being atopic, compared with 15% in the normal population. There are several genetic influences determining IgE production: an autosomal dominant gene codes for low IgE levels, HLA-linked genes control IgE and IgG production against specific allergenic proteins (most noticeable for minor determinants when total IgE is low), and HLA-B8 and DRW3 are associated with general immune hyper-responsiveness, possibly via decreased T suppressor activity. Genetic linkage of atopic IgE responses to chromosome 11q13, now observed by several groups, is strongest in maternally derived alleles and may contribute to the maternal inheritance of atopy seen in many studies. The gene for the β chain of the high affinity IgE receptor is a candidate for the chromosome 11q13 effect. A polymorphic variant Leu181/183 was present in 4.5% of the population of Busselton, Western Australia, and when inherited maternally carried a significant risk of atopy and bronchial hyper-responsiveness, but accounted for only a part of the atopy and asthma in that population. Other candidate genes are the TCR α region on chromosome 14 and the interleukin-4 (IL-4) cluster on chromosome 5, both of which show linkage to IgE responses.

Environmental influences are also important: smoking and the use of β -blocking drugs in pregnancy increase the incidence of atopy in offspring. Early contact with highly allergenic proteins (as in cow's milk, eggs, fish, nuts, Der pI, Fel dI) before mucosal protective mechanisms, especially IgA, are fully established appears to contribute to allergy in genetically predisposed infants. Environmental risk factors for seasonal rhinitis include higher social class, being the first-born child and not having to

share a bathroom. These suggest that early infections may have a protective antiallergic effect, possibly by interferon γ production.

The incidence of allergic diseases (hay fever, asthma and eczema) in the population is increasing, possibly caused by increased exposure to indoor allergens, or by the adjuvant effect of atmospheric pollutants such as diesel fumes, or possibly by dietary changes. Disruption of mucosal surfaces, allowing increased antigen entry and IgE production can occur following exposure to pollutants such as sulfur dioxide or during viral infections. Viruses can precipitate allergic symptoms, especially following respiratory syncytial virus infections in childhood.

Type I hypersensitivity

The central mechanism of atopic allergy is type I hypersensitivity (Figure 1). Mucosal contact with allergen results in uptake by an antigen-presenting cell (APC) and presentation in the form of an allergenic peptide held in the groove of the major histocompatibility complex (MHC) class II molecule to a helper T lymphocyte. This then provides help for a previously committed B cell, which is capable of recognizing the antigen, to make IgE antibody.

Factors determining IgE rather than IgG production are not fully understood; however, the nature of the antigen-presenting cells may be relevant. In atopic eczema and allergic rhinitis Langerhans cells which are HLA-DR positive and bear high-affinity IgE receptors have been described. Class switching to IgE is promoted by cytokines IL-4 and IL-13 and is inhibited by interferon γ . In atopics increased levels of T_H2 type T cells producing IL-4 have been described, e.g. in vernal conjunctivitis.

A second signal required for IgE expression is delivered by a soluble fragment of Fc ϵ RII (CD23). This is also upregulated by IL-4. Class switching

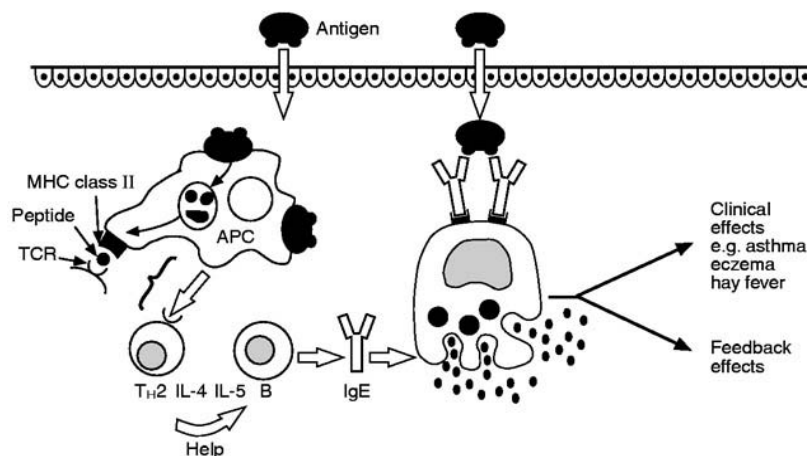


Figure 1 Type I hypersensitivity.

with the production of mRNA for IgE has been described within 30 min of allergen contact. Initial IgE production probably takes place in the local lymphoid tissues. Once produced, the IgE is rapidly and avidly bound by its Fc piece to mast cells and basophils, which are then sensitized. Subsequent contact with the same allergen results in cross-linking of IgE molecules by their Fab portion, calcium ion influx, cell degranulation and mediator release.

Certain mediators exist preformed in granules within the cell, others are formed after activation by phospholipase A₂ breakdown of the cell membrane to release arachidonic acid. This is then metabolized by the cyclo-oxygenase or lipoxygenase pathways, depending on the cell type, to produce prostaglandins and thromboxanes, or leukotrienes respectively. Recent research suggests that there is a third phase of cytokine production taking place in the mast cell during the hours after degranulation. This involves *de novo* protein synthesis and results in the generation of several cytokines, including IL-3, IL-4 and IL-5. Storage of IL-4 has also been demonstrated within mast cells and its release can occur on degranulation.

Degranulation of mast cells and basophils can also occur following cross-linking of adjacent membrane-bound IgE molecules by anti-IgE, by anti-idiotype, or by lectins. Fab portions of anti-idiotype could occupy IgE antigen-binding sites without causing cross-linking and degranulation, and can thus theoretically protect against allergen challenge.

Pseudoallergy

Certain substances can affect mast cell membranes directly, allowing Ca²⁺ ion influx and release of gran-

ule contents. These include the anaphylatoxins, C3a and C5a, secretagogues such as calcium ionophore A23187 and mellitin (a component of insect venom) and drugs such as codeine, morphine and synthetic adrenocorticotropin. Physical stimuli such as pressure, heat, cold and sunlight can cause mast cell degranulation in certain individuals.

Late-phase response

The above mechanism accounts for the immediate allergic reaction; however, in many cases inflammation with ingress of many cell types, including eosinophils, further mast cells, basophils, neutrophils and lymphocytes, occurs within hours of allergen contact. The mechanisms for this depend partly on chemotactic factors released by mast cells, but probably more on cytokine production by T lymphocytes (IL-3, IL-5, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF)), macrophages (GM-CSF) and endothelial cells (GM-CSF), together with mast cells (IL-2, IL-3, IL-4, IL-5) (Figure 2). This cytokine network provides the basis for clonal amplification of T cells, B cells and inflammatory cells such as mast cells, basophils and eosinophils. Cells passing in nearby blood vessels are recruited to the site of inflammation, becoming attached to the vascular endothelium, via expressed integrin molecules on the cell surface which interact with receptors such as ICAM-1 and iC3b on the vascular endothelium. Expression of integrins and their receptors is upregulated by inflammatory cytokines. The cells then diapedese through the vascular endothelium and migrate down a chemotactic gradient to the site of allergic inflammation.

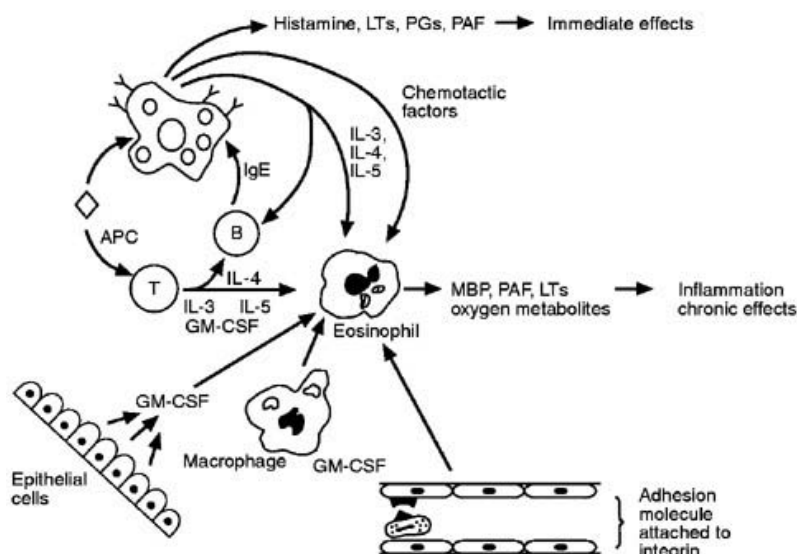


Figure 2 Some of the pathways leading to the late phase inflammatory reaction which is seen in chronic allergic disease.

Physiological effects

The physiological effects of the mast cell-derived mediators fall into three main groups: spasmogens, activators and chemoattractants. In humans the major vasoactive amine is histamine, which causes vasodilatation, increased capillary permeability and bronchoconstriction. Prostaglandin (PG) D₂, leukotrienes (LTs) C₄ and D₄ and platelet activating factor (PAF) are all newly formed bronchoconstrictors, some of which could be partly responsible for the late phase of the allergic response (see below). PAF also activates platelets, leading to microthrombi and further mediator release. Other activators include tryptase, which is a C3 convertase, and kininogenases, which generate kinins from plasma and tissue kininogens, affecting small blood vessels and leading to inflammation. The chemoattractants include neutrophil chemotactic factor (NCF), eosinophil chemotactic factor of anaphylaxis (ECF-A), which are granule derived, and leukotriene B₄, which is newly formed. These induce a cellular infiltrate which includes neutrophils, eosinophils, basophils and monocytes.

The actual mediators released depend on the cell type involved; for example, basophils do not produce prostaglandin D₂. It is apparent that there are at least two different subpopulations of mast cells in humans, with differences in their morphology and pharmacology.

Clinical effects

The clinical effects of type I hypersensitivity depend upon the site of the reaction: involvement of the nose and/or lungs results in rhinitis and/or asthma; skin involvement causes urticaria. Atopic eczema involves IgE-mediated allergy, although the histology resembles a type IV hypersensitivity reaction. It is possible that an initial type I mechanism causes increased vascular permeability, allowing the influx of lymphocytes. Type I reactions to food can cause immediate reactions involving itching and swelling of the lips, mouth and tongue or may lead to nausea, vomiting and abdominal pain, usually within an hour of ingestion. Where the allergen is injected systemically, as in insect stings and intravenous infusions, and occasionally in a very sensitive subject following ingestion, there may be overwhelming basophil degranulation causing anaphylaxis. This involves massive vasodilatation with a sudden drop in blood pressure and collapse. Other symptoms include bronchospasm, an itchy whealing urticarial rash, gut cramps and diarrhea. Anaphylaxis can prove fatal; the mainstay of treatment is epinephrine (adrenaline), either intramuscular or subcutaneous.

The inflammation of the late phase of the allergic reaction is probably of great importance in chronic allergic disease such as asthma, where the infiltrating eosinophils are thought to damage the bronchial mucosa by means of some of their granule contents: such as major basic protein (MBP) and eosinophilic cationic protein (ECP). Exposure of nerve endings could then contribute, via axonal reflexes, to bronchial hyper-reactivity. In the nose and sinuses the thickened mucosa can act as a predisposing factor to chronic or recurrent infection.

Clinical tests

The onset of the allergic reaction from the time of allergen contact with sensitized mast cells is rapid, taking place within minutes. Skin-prick testing, which is the classical test for allergy, can thus be performed as a rapid diagnostic procedure. Antigen is introduced into the skin, which is examined for the presence of a wheal some 15–20 min later; histamine is used as a positive control, saline as the negative. A positive skin test (wheal 3 mm in diameter larger than saline control) usually correlates with the laboratory test for allergen-specific IgE in blood, the radioallergosorbent test (RAST) and with provocation tests. However, the skin-prick test results must always be interpreted in the light of the patient's history, as IgE reactivity to allergen is not always associated with clinical symptoms. Conversely, a negative skin-prick test can exist where there is only local sensitization in the shock organ; such patients will only give positive results on provocation tests.

Other cells with IgE receptors

Mast cells and basophils possess high affinity ($K_d = 10^{-10}$ M) IgE receptors (FcεRI) which have a six-chain structure (two α, two β, two γ). Recently, other cells, such as Langerhans cells, activated eosinophils, monocytes and macrophages, have also been shown to possess such receptors. Many cells of the immune system bear lower affinity (10^{-6} M) IgE receptors (FcεRII, CD23) of a different structure. This is not a member of the immunoglobulin superfamily but instead shares homology with animal C type lectins such as mannose-binding protein. It is present on B and T lymphocytes, monocytes, alveolar macrophages, eosinophils, platelets and Langerhans cells. Soluble CD23 acts as the second signal for IgE production by B cells. FcεRII-bearing cells increase with allergen exposure. These may have cytotoxic properties when armed with IgE. Similarly, eosinophils and platelets are able to kill *Schistosomula* larvae when sensitized by IgE. This function of parasite killing is

probably the rationale for the existence of IgE and its immune reactions, with atopic disease an unintended byproduct.

Treatment

Treatment of atopic allergic disease falls into three categories: 1) allergen avoidance; 2) pharmacotherapy; and 3) immunotherapy. The major anti-inflammatory drugs used are glucocorticoids, which have a wide spectrum of activity, including reduction of cytokine production and inflammatory cell numbers and activation, a decrease in antigen presentation via a decrease in Langerhans cells in nose and skin, and reduction of mast cell mediator production from arachidonic acid; and sodium cromoglycate, which has multiple effects, including inhibition of mast cell degranulation, sensory nerve function and cytokine secretion. Immunotherapy involves repeated allergen injection; the mechanism of its action may be to induce a T_H1 rather than a T_H2 response locally. Other possibilities such as the use of peptides to produce T cell unresponsiveness, anticytokines and cytokine inhibitors are under investigation.

See also: Allergens; Arachidonic acid and the leukotrienes; Asthma; Basophils; Eczema; Fc receptors; Food allergy; Hypersensitivity reactions; IgE; Mast cells; Prostaglandins.

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ATTENUATED ORGANISMS AS VACCINES

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Attenuation is a process of genetic changes leading to a loss of virulence by pathogenic microorganisms while retaining the capacity to induce an immune response in susceptible hosts, resulting in lasting protection from a disease. Attenuation is a part of the natural evolution of pathogenic microorganisms, and is used by man to create 'live' vaccines against infectious diseases (Table 1), in contrast to 'killed' vaccines consisting of inactivated pathogens or their immunogenic subunits. Both types of vaccines have advantages and shortcomings; these will be compared after a brief historical overview and discussion of the general principles behind the process of attenuation. The evolution of pathogens involves several concurrent trends, including immunological drift to escape host defense mechanisms, expansion into new populations (including cross-species adaptation), acquiring new pathogenic factors and properties, and, finally, reduction and loss of pathogenicity (attenuation). While the adaptive value of the former processes is self-evident, as they expand the habitat

of the species, the driving force behind the latter is less apparent. Such 'altruistic' behavior can, perhaps, be explained by the advantages of replicating and spreading without killing a host (which in many cases is a dead end for the microorganism).

Brief history of live vaccines

The oldest example of the use of naturally attenuated organisms for protection against diseases is provided by smallpox. 'Variolation', i.e. deliberate inoculation of susceptible people with virus-containing lymph from pustules of patients with mild forms of the disease, goes back to the early cultures of Africa and Asia. As a result of variolation, patients usually developed the disease with limited lesions, but lasting resistance to smallpox. A few of them, however, had severe disease and some died. By the seventeenth century it was being practised in Europe with the modification of using pustular liquid from cows having a closely related disease, cowpox. In 1796 the British

Table 1 Attenuated organisms as live vaccines (currently licensed)

Bacterial vaccines	Viral vaccines
Anthrax ^a	Adenovirus types 4 and 7
Chicken cholera ^a	Hepatitis A ^b
Cholera ^b	Influenza A and B ^b
Salmonella ^b	Measles
Shigella ^b	Mumps
Tuberculosis (BCG)	Parainfluenza type 3 ^b
Typhoid ^b	Poliomyelitis types 1, 2 and 3
	Rabies ^{a,b}
	Rotavirus ^b
	Rubella
	Smallpox
	Tick-borne encephalitis ^b
	Varicella
	Yellow fever

^a No longer used.^b Still under investigation.

doctor, Edward Jenner, became aware of this procedure, practised by farmers in southern England, and took the next step by propagating the cowpox through patient-to-patient transfer; he called this procedure 'vaccination' (*L. vacuum*, cow). Thus, he created the first biological and called it a 'vaccine'. This term was later broadened to include all biologicals (live, killed or synthetic) used to induce protective immunity against various pathogenic microorganisms, and to treat noninfectious diseases by stimulating the immune system.

The introduction of smallpox vaccine was responsible for a dramatic reduction of the incidence of smallpox in the nineteenth century and led to its complete eradication in the 1970s. Jenner's work was also important for demonstrating the feasibility of preventing infectious diseases and for identifying, for the first time, a method for achieving artificial attenuation. The 'jennerian' approach, involving the growth of pathogenic microorganisms in a non-human host and the use of closely related animal pathogens, was the basis for the creation of many other live vaccines. Recent examples are experimental vaccines made with bovine and rhesus rotaviruses which protect against human rotaviruses, and bovine paramyxovirus type 3 vaccine which protects man against human parainfluenza.

The next step in the attenuation of microorganisms was taken by Louis Pasteur, who, in 1880, developed a live vaccine against chicken cholera by exposing a bacterial strain of *Pasteurella multocida* to air. The strain could be cultivated *in vitro* and was the first wholly artificial vaccine prepared outside a living organism. In 1881, by extending this approach based on adverse treatment of the microorganism, he proposed the use of a weakened strain of anthrax

bacillus as a vaccine and demonstrated its remarkable effectiveness. In 1885, together with Emile Roux and others, he proposed to immunize against rabies, using a series of successive inoculations of virus preparations with gradually increasing virulence, a procedure similar to variolation but carried out with a vaccine artificially prepared from the virus weakened by air-drying the spinal cords of infected rabbits.

Another method of achieving artificial attenuation of pathogenic organisms by adaptation to growth in culture is exemplified by the BCG (bacille Calmette-Guérin) vaccine. This attenuated strain of *Mycobacterium bovis* was developed in the early twentieth century by the French doctors Albert Calmette and Camille Guérin. They found that the mycobacteria could be grown in culture on potatoes, cooked and supplemented with glycerol and beef bile. After 231 consecutive passages over the course of 13 years, the strain lost its virulence for animals but retained its antigenic properties and was able to stimulate protective immunity against infection with the virulent strain.

In 1935, a combination of growth in nonnatural hosts and in culture was used by Max Theiler to prepare two strains of a live vaccine against yellow fever. The French neurotropic strain (FNS) was attenuated by serial passaging in the brains of mice, and the 17D strain by intermittent passaging in rhesus monkeys and mosquitoes, followed by passaging in whole mouse embryonic tissue cultures, and then in chick embryos. Since the FNS retained a greater degree of virulence, only the 17D strain is currently used in manufacturing the vaccine.

In addition to the above-mentioned principles of attenuation, it was noted that passaging natural viral isolates in cell cultures almost invariably leads to temperature-sensitive (ts) mutations, suggesting that these may be linked to attenuation. This was one of the principles used by Albert Sabin for the creation of oral poliovirus vaccine (OPV) (Figure 1). In many respects OPV is a paradigm for other live viral vaccines and deserves special attention. Sabin noticed that when polioviruses were grown at a low multiplicity of infection, the wild-type strains retained their neurovirulence even after prolonged serial passaging. However, if the virus was passaged at high multiplicity, the degree of neurovirulence dropped. Sabin was also aware of the fact that natural populations of poliovirus consist of a mixture of viral particles with differing 'genetic potentialities'. The term 'quasispecies' is now used to describe this genetic diversity of viral populations. Sabin hypothesized that, while virulent virus particles in a natural mixture replicate to a higher yield and have a replicative advantage in the long run, attenuated particles may

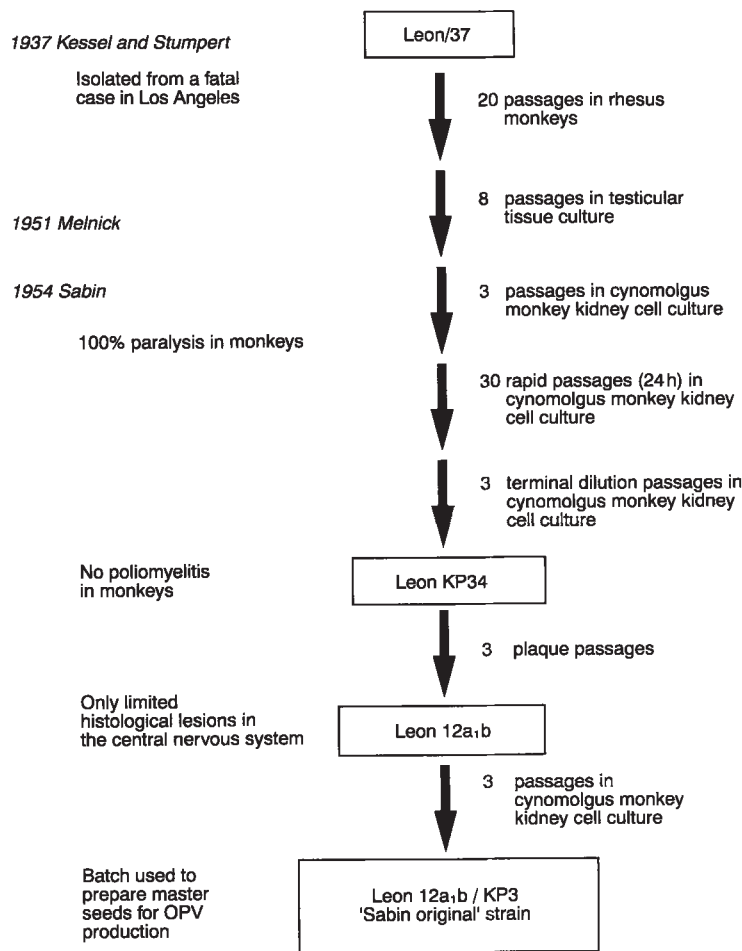


Figure 1 Attenuation of type 3 poliovirus by Albert Sabin.

replicate faster and be selected if replication of the virus is limited in time. Thus he carried out a series of 'rapid' (24 hours) passages in primary monkey kidney cell cultures, nonneuronal in origin, to suppress viral neutropism. The passages were done at 34°C to select for ts mutants. He was able to create strains of all three serotypes of poliovirus with markedly reduced neurovirulence. The strains were then cloned and plaques with the lowest levels of neurovirulence for monkeys were selected for making the vaccine. The attenuated strains had a number of characteristic genetic markers, including a reduced capacity to replicate at high temperatures (rct40) and at a decreased concentration of bicarbonate (d-marker), and, most importantly, very low neurovirulence even when inoculated directly into the central nervous system of monkeys. These strains were highly immunogenic and have become a major tool in the worldwide eradication of the disease, which is anticipated to occur by the year 2000.

Genetic stability

While the attenuated poliovirus strains comprise the basis of a remarkably effective vaccine, they are prone to partial reversion to neurovirulence. Attenuated virus passaged in cell cultures, and the virus excreted by healthy vaccine recipients, were found to have gradually lost their attenuation markers. This required rigorous neurovirulence safety testing during vaccine production. This potential for reversion back to virulence is a general problem inherent in all live vaccines. A similar process occurs in some strains used in the live mumps vaccine, and to a lesser degree in the yellow fever vaccine. A reduction in, and eventual elimination of, this potential for reversion can be accomplished by creating genetically stable vaccine strains using our knowledge of molecular mechanisms of virulence and attenuation.

Molecular basis of attenuation

A reduction in pathogenicity can occur either through the loss of external pathogenic factors (toxins), as is the case with some bacterial vaccines, or by impairing the invasiveness of a pathogen and limiting its access to target organs. In many cases attenuating mutations occur in capsid or envelope proteins of a virus, presumably reducing its affinity for the target cells. Detailed studies of attenuated polioviruses identified a number of mutations that distinguished vaccine viruses from their wild-type progenitor strains. The mutations linked to attenuation were located in genes for both capsid and non-capsid proteins, as well as in the untranslated regulatory regions (UTRs) of the genome. The mutations in a stem-and-loop domain within the internal ribosome entry site (IRES) of the 5'-UTRs were responsible for the attenuation of all three types of poliovirus. These mutations impaired the initiation of viral protein synthesis by decreasing the affinity of viral RNA to cellular initiation factors. This impairment was cell-type specific, as the difference in replicative capacity between attenuated and wild-type strains was much more pronounced in cultures of cells of neuronal origin than in those of nonneuronal cells.

Viruses serially passaged *in vitro* or isolated from recipients of OPV were found to have reverted at some of the attenuated sites (including the one in the IRES) back to the wild-type state. Other mutations were found which boosted the replicative capacity of the attenuated strains *in vitro* but did not contribute to increased neurovirulence in monkeys. While attenuation is generally associated with lowering the replicative capacity, 'weakening' of the virus does not necessarily lead to a loss of pathogenicity. Attenuation is linked to an inability of the virus to replicate in specific target cells (motor neurons in the case of poliovirus), while retaining the ability to replicate in noncritical organs.

Rational vaccine design

Ideally, an attenuated organism would possess the ability to replicate vigorously and induce an immune response, while being completely devoid of virulent properties. Strains with hyperattenuation (reduced vitality) and substantial or unstable residual virulence are unacceptable for use as vaccines. A knowledge of the genetic basis of virulence allows us to target virulence determinants and to create strains lacking the genes required for pathogenicity. Another challenge in vaccine development arises when dealing with viruses that have a very high rate of antigenic drift, e.g. influenza. In this case, standardized tech-

niques for virus attenuation would be desirable in order to prepare vaccines with consistent effectiveness against the new influenza strains that emerge each year. It was found that passaging influenza virus at low temperatures results in attenuation and that the determinants of attenuation are located on the PA, M, PB2, and PB1 genes of the cold-adapted influenza A virus. Genes of influenza viruses are located on separate RNA segments, and so reassortment of the segments bearing the determinants of attenuation with the ones carrying the genes for hemagglutinin and neuraminidase, which are responsible for immunogenicity, resulted in vaccine strains with the desired antigenic structure and a predictably high degree of attenuation.

New genetically engineered bacterial vaccines provide other examples of a rational approach to vaccine design. Hybrids of *Shigella* and *Escherichia coli*, and *Salmonella* and *shigella*, devoid of toxin genes, but expressing important antigens, have been suggested for use in live vaccines. Selective mutagenesis of the cholera toxin gene results in a strain which possesses all of the antigens important for protective immunity, but with much lower pathogenicity.

Recently it has been found that artificial rearrangement of gene order in vesicular stomatitis virus led to strong attenuation of the virus, even though no changes of amino acid sequences was involved, suggesting a new rational way for creating stable vaccine strains of negative-strand RNA viruses.

Attenuated organisms versus killed vaccines

Arguments about the relative advantages of live and killed vaccines are endless and started with the invention of vaccines themselves. Live vaccines are generally less stable than killed vaccines and require freezing during transportation and storage. The possibility of contamination with live adventitious agents, including the unknown ones, is a consideration that is hard to discount. But the greatest shortcoming of live vaccines is their potential for reversion to virulence. Because of this, in the nineteenth century, variolation was considered a felony in England. Modern vaccines, especially the ones created with a knowledge of the molecular basis of virulence, are much safer than the attenuated strains of the past. In addition, sophisticated methods of quality control are now used to ensure the safety of live vaccines.

The advantages of using attenuated organisms as vaccines are numerous. Since they replicate in the body of a vaccinee, the dose of a live vaccine is usually small, which means its cost and potential for antigenic overload and allergization are low. Many

live vaccines can be administered simply by oral or intranasal routes, thus avoiding the hazards of injection. In most cases, vaccination with a live vaccine results in antigenic presentation that closely mimics the natural infection. As a result, immunity is more comprehensive than that induced by killed vaccines, involving both systemic and local factors. For example, immunization with inactivated poliovirus vaccine (IPV) results in high seroconversion rates and high titers of antibodies in the blood of vaccine recipients, but does not create adequate intestinal immunity. Consequently, while the individual immunized with IPV is completely protected from poliomyelitis, the wild-type poliovirus can still replicate in the intestinal tract of the vaccinee and be excreted. Thus IPV cannot break the chain of transmission of wild-type polioviruses, in contrast to OPV, which induces intestinal immunity and results in comprehensive resistance to the poliovirus. OPV stops the circulation of wild-type polioviruses in the human population, and spreads to contacts, thus expanding the reach of immunization. Nonspecific immunity factors such as interferons are produced by live vaccines, which therefore can protect against a wide range of other diseases. For example, OPV has been successfully used to stop outbreaks of diseases caused by other serologically unrelated enteroviruses and has been shown to interfere nonspecifically with infection by other viruses.

Conclusions

As stated earlier, attenuation is a natural part of the coevolution of a host and its microorganisms. As always in symbiotic relationships, both parties benefit. The host provides a substrate for a microorganism's subsistence, which in exchange refrains from causing extensive damage to its host. As an additional payoff, this endosymbiotic microorganisms may help its host to fight off pathogenic microorganisms. This occurs with normal enteric bacterial microflora, and may also be true for naturally attenuated viruses which can temporarily enter into symbiotic relationship with their hosts. A good example of this is the large variety of nonpathogenic enteroviruses (Sabin strains of poliovirus should be regarded as members of this group) that asymptotically infect the intestinal tract and, by stimulating a host's immune system, interfere with a wide range of pathogenic viruses and help the host fight other diseases. Thus, by acting within the framework of existing micro/macroorganism interactions, artificially attenuated viruses and bacteria are a manmade addition to the natural arsenal with which the human body protects itself against diseases.

See also: Antigen; Bacteria, immunity to; BCG; Cell-mediated immunity; Humoral immunity; Interferon α ; Interferon β ; Interferon γ ; Mucosal immunity; Vaccination, methods of administration; Vaccines; Vaccines, adverse reactions to; Viruses, immunity to.

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AUTOANTIBODIES, TESTS FOR

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Autoantibodies are antibodies that have the capacity to bind to self antigens. Autoantibodies are evaluated for a number of reasons and may be used as an aid in diagnosis of disease, either by inclusion or exclusion. In some cases the autoantibodies may actually cause disease. However, they are more frequently used as markers in a variety of ways. They may help distinguish between different forms of a condition, which may result in using different therapeutic strategies. Changes in antibody quantity may be used to monitor successful therapy or disease exacerbation. They may be used as indicators of individuals at risk for future diseases in disorders that are familial. Autoantibody tests are never used by themselves for diagnosis of disease. The total picture of the patient must always be considered as autoantibodies can also be found in normal individuals. Autoantibodies in normal individuals range in frequency according to age and sex; generally, the lowest frequency is in young males, the highest in elderly females.

Methods for identifying autoantibodies

Techniques for demonstrating autoantibodies are frequently ordained by the location and properties of the antigen and the required level of sensitivity. Early assays, some of which are still used today, included agglutination and precipitation techniques. Agglutination employs stable suspensions of beads (e.g. latex) or erythrocytes coated with the appropriate soluble antigen. Autoantibodies cross-link the coated particles forming the complex lattice structure of agglutination. Thyroid autoantibodies are frequently detected by indirect hemagglutination (i.e. by using erythrocytes coated with thyroid antigens), while latex agglutination remains the most widely used test for the measurement of rheumatoid factor (i.e. by using latex beads coated with gammaglobulin). Although precipitation in fluid or gelified media (e.g. immunodiffusion) is less sensitive than agglutination, it is still appropriate for the demonstration of antibodies to complex mixtures of soluble antigens, such as certain nuclear antigens that have not yet been purified.

Indirect immunofluorescence (IIF) is the most commonly used test for the detection of tissue-specific autoantibodies because of its wide range of applications. Briefly, serum is placed on a tissue section

(frozen section, 4 μ m thick). If autoantibodies are present, they will bind to the appropriate antigen. Excess serum is washed away. A second reagent, antihuman immunoglobulin conjugated to a fluorescent dye (usually fluorescein isothiocyanate, FITC), is then incubated with the tissue section. Unbound reagent is again washed away. Autoantibodies are detected using a microscope (transmission or incident light illumination) with an appropriate light source (usually mercury vapor or halogen quartz) and filter system that will enable the fluorescent markers to be seen. IIF can detect multiple antibodies on a single tissue or on a multiple tissue block, and thus has considerable value as a screening test. It is particularly valuable in testing for autoantibodies for which the antigen has not yet been characterized or for unexpected antibodies. These latter antibodies may be missed by an assay employing only purified antigens. With IIF, the reaction site can be seen, and thus the location of the cellular antigen can be determined. In a few cases, however, the major autoantigen associated with a particular autoimmune disorder has been isolated. Subsequently, more sensitive quantitative tests, such as hemagglutination, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), have been developed using the isolated antigen fraction and have replaced IIF. Autoantibody isotype can be evaluated by using heavy chain-specific anti-immunoglobulin, although this is also true for any of the indirect detection tests.

Indirect immunoperoxidase (IIP), although similar to IIF in performance, utilizes an enzyme (e.g. horseradish peroxidase) to hydrolyze a substrate to form a colored precipitate at the site of antibody binding, rather than a fluorescent marker. Another enzyme, alkaline phosphatase, can also be used in a similar fashion. Although IIP has certain advantages over IIF, as the reaction can be seen under a standard light microscope and the slide can be stored indefinitely, it is not totally without problems. Endogenous peroxidases may cause false-positive results. The same time and effort must go into establishing this technique as for IIF. IIP is reported to be more sensitive than IIF, but each individual assay model must be evaluated independently.

RIA is a highly sensitive technique for the detection of autoantibodies; however, it requires defined antigens and the use of radioisotopes, and the trend

is to replace radiolabeled compounds with enzyme-labeled substances and many of the former RIAs have therefore been converted to ELISAs.

The western immunoblot has recently been introduced for clinical diagnosis. It does not require a pure antigen but is relatively slow and expensive. Briefly, the antigen solution is separated by electrophoresis in polyacrylamide gel on the basis of relative molecular weight. The proteins in the gel are electrotransferred to a nitrocellulose membrane and cut into strips. Individual strips are reacted with patient serum, washed and incubated with an anti-immunoglobulin conjugated to an enzyme. Finally, the strips are treated with an enzyme substrate and the site of antibody localization is identified by a color precipitate. Western blot will indicate the approximate molecular weight of the antigen that is recognized by the autoantibody, but will not identify the protein itself.

Autoantibodies

There is a spectrum of autoantibody reactivities ranging from organ-specific to non-organ-specific autoantibodies. Organ-specific autoantibodies react to antigens of only one tissue, such as to thyroid tissue. Non-organ-specific autoantibodies react with antigens common to all tissues, such as to different nuclear antigens, and are often associated with more generalized disorders, such as systemic lupus erythematosus. There is also a small group of intermediate autoantibodies with specificity to particular tissues but with a widespread distribution, such as smooth muscle. The description of the autoantibodies and their tests, presented below, will feature this range of reactivities, summarized in Table 1.

Thyroid-specific antigens

Thyroglobulin

Thyroglobulin autoantibodies can be demonstrated by several procedures, such as precipitation in agar, IIF, indirect hemagglutination of cells coated with thyroglobulin, RIA and ELISA. At present, hemagglutination is the most commonly employed technique for the detection of antibodies against thyroglobulin. The ELISA is increasingly used for the measurement of antibodies to thyroglobulin and will additionally detect antibodies that are nonagglutinating. Thyroglobulin antibodies are found in individuals with chronic thyroiditis, myxedema, Graves disease, goiter, thyroid tumors and other polyendocrine disorders. The highest titers are found in patients with autoimmune thyroid disorders. Low titers of thyroglobulin antibodies may be found in normal

individuals. The prevalence of such antibodies in subjects without overt thyroid disease is higher in women. The incidence also increases with age, so that 18% of women over 40 years old may have antibodies to thyroglobulin. Total thyroglobulin antibody is not a specific marker of autoimmune thyroiditis as so many other conditions also show the presence of this antibody. Recent evidence suggests, however, that there may be disease-related epitope specificity of the autoantibody. The disease-related antibodies were detected by competitive inhibition ELISA experiments using murine monoclonal antibodies.

Microsomal antigens (TPO) of thyroid epithelial cells

The microsomal antigen has been identified chiefly as the thyroid peroxidase enzyme (TPO) of the thyroid epithelial cell. However, purified TPO is not readily available for use in diagnostic tests. The most commonly used tests for the microsomal autoantibodies are either IIF, using primate thyroid tissue, or hemagglutination. ELISA, RIA or a chemiluminescence-based assay may also be employed. The antibodies to the microsomal antigens belong predominantly to the immunoglobulin G (IgG) class and, when detected by IIF, stain the cytoplasm of thyroid cells. The IIF test for antibodies to thyroid microsomal antigens is positive in approximately 70–90% of patients with chronic thyroiditis. It is also positive in 64% of patients with primary hypothyroidism, 50% of patients with thyrotoxicosis, 10% of patients with simple goiters, and 17% of patients with thyroid tumor. It can also be found in normals at a low frequency and titer.

Thyrotropin receptor

Autoantibodies binding to the thyrotropin (TSH) receptor on the surface of thyroid epithelial cells are believed to be the direct cause of the hyperthyroid state in Graves' disease. The thyroid-stimulating autoantibodies (TSAbs) bind near to or with the TSH receptor and mimic TSH activity, resulting in uncontrolled stimulation as there is no negative feedback control. Tests for TSBAs are primarily of two types: bioassays or binding assays. Most bioassays have limited utility because they are cumbersome and require scarce materials, such as viable slices of fresh human thyroid tissue. Bioassays, however, evaluate functional aspects of the antibodies as their endpoint. Binding assays are based on competition between ¹²⁵I-labeled TSH and patient autoantibodies for binding to thyroid preparations. Some confusion has been created by the heterogeneity of the autoantibodies and the diversity of the assays for their

Table 1 Detection of autoantibodies

<i>Autoantibody to:</i>	<i>Primary method of detection^a</i>	<i>Primary associated disorders</i>
Thyroid		
Thyroglobulin	Hemagglutination ELISA	Autoimmune thyroiditis, primary myxedema
Microsomal (thyroid peroxidase)	IIF (mk-thyroid) Hemagglutination	Autoimmune thyroiditis, primary myxedema
TSH receptor	Bioassay Binding assay	Graves' disease
Parietal cell	IIF (ro-stomach)	Atrophic gastritis, pernicious anemia
Adrenal	IIF (mk-adrenal)	Idiopathic Addison's disease
Ovary	IIF (mk-ovary)	Infertility
Testes, sperm	IIF (mk-testes) Agglutination Immobilization	Infertility
Pancreas islet cell	IIF (hu group O or mk-pancreas)	Insulin-dependent diabetes mellitus
Parathyroid	IIF (mk-parathyroid)	Idiopathic hypoparathyroidism
Pituitary	IIF (hu- or mk-pituitary)	Growth disorders
Skin		
Basement membrane zone	IIF (hu-skin, mk-esophagus)	Bullous pemphigoid
Intercellular substance	IIF (hu-skin, mk-esophagus)	Pemphigus vulgaris
Muscle		
Smooth	IIF (ro-stomach)	Chronic active hepatitis
Cardiac striated	IIF (ro-heart)	Autoimmune cardiomyopathy
Skeletal striated	IIF (ro-skeletal)	Myasthenia gravis
Endomysial	IIF (mk-esophagus)	Celiac disease
Acetylcholine receptor	RIA, ELISA	Myasthenia gravis
Neutrophil cytoplasm (C-ANCA, proteinase 3, P-ANCA, myeloperoxidase)	IIF (hu-PMNs), ELISA	Wegener's granulomatosis, microscopic vasculitides
Nuclear antigens (ANAs)	IIF (ro-liver, Hep-2 cells), ELISA Hemagglutination Immunodiffusion	Systemic lupus erythematosus, connective tissue disorders
Mitochondria	IIF (ro-kidney)	Primary biliary cirrhosis
Phospholipids	ELISA	Antiphospholipid syndrome
Rheumatoid factor	Latex agglutination Nephelometry	Rheumatoid arthritis

PMN, polymorphonuclear leukocyte.

^aSpecies and tissue substrate used: mk, monkey; ro, rodent; hu, human.

demonstration. Different specificities of autoantibodies to the thyroid epithelial cell surface on or near the TSH receptor may be represented.

The detection of these antibodies in Graves' patients ranges from 55 to 95%, depending on the assay. The TSAb assay is of greatest utility in conditions where conventional test results are equivocal or where clinical signs and symptoms are not readily apparent (e.g. ophthalmopathy in the absence of other features). Moreover, the TSAb assay is usually negative in patients with thyrotoxicosis in association with thyroid nodules, with cancer, or in patients with subacute thyroiditis. Therefore, a positive test confirms a diagnosis of Graves' disease with a high degree of confidence. The assay can be used

to monitor patient therapy, as successful treatment of Graves' disease with propylthiouracil may result in lowering levels of TSAb antibodies. The assay has also proven useful in the diagnosis of neonatal thyrotoxicosis.

Gastric parietal cells

Autoantibodies to antigens of gastric parietal cells (parietal cell antibodies) occur with high frequency in patients with pernicious anemia and atrophic gastritis. Autoantibodies to gastric parietal cells are frequently found simultaneously with thyroid antibodies, although these cells are not usually considered as part of the endocrine system. These

autoantibodies are readily detected by IIF with sections of rodent stomach (mucosa) as tissue substrate. The pattern of staining resembles that seen with mitochondrial antibodies; therefore, a control test on kidney should also be performed. Parietal cell antibodies are found in 90% or more of patients with pernicious anemia. Recently, the autoantigen has been identified as the α and β subunits of gastric H^+,K^+ -ATPase. An ELISA using this enzyme and a western blotting technique using gastric extracts have been developed. These antibodies are also present in a number of other conditions, such as chronic thyroiditis (33%), Sjögren's sicca syndrome (15%), atrophic gastritis (60%), gastric ulcer (22%), etc. The antibodies are also found in the normal population, with an incidence that varies according to age and sex; i.e. from 2% in subjects under 20 years old to 16% in subjects over 60 years old. They are more frequent in women than in men.

Other endocrine organs

Autoimmunity also develops against other endocrine organs such as the adrenal gland, pancreas islet cells, parathyroid, pituitary and ovary. The autoantibodies are much less prevalent than with thyroid disease but, if present, help in diagnosis. The reactivities, assays and primary associated disorders are summarized in Table 1.

These autoantibodies share certain characteristics. Antibodies bind to antigens associated with the cytoplasm of cells, usually to unidentified antigens. The antibodies belong predominantly to the IgG class and, in general, have rather low titers (not higher than 100) and are rarely found in normal individuals.

The presence of antibodies to particular endocrine organs suggests that the disease is idiopathic and not of malignant, infectious or other nature. However, it is not uncommon for patients with autoantibodies to one endocrine organ to have multiple endocrine reactivities. Furthermore, not all patients with a particular disorder have the autoantibodies, so that a negative test obviously does not exclude a particular diagnosis. On the other hand, a positive test is not necessarily diagnostic, in that the patient might have other autoantibodies to endocrine organs without clinical disease. An interesting feature of autoantibodies to islet cells of the pancreas is that the percentage of positive sera decreases with time after onset of disease.

Skin antigens

Patients with autoimmune skin disease, such as pemphigus vulgaris or bullous pemphigoid (BP) develop autoantibodies that bind to epidermal antigens. These antibodies are identified by IIF, using tissue sections of skin (e.g. foreskin) or other squamous epithelium (e.g. monkey esophagus) as tissue substrate. Patients with pemphigus vulgaris show serum autoantibodies to the intracellular area of the epidermis in 80–90% of cases. These antibodies are IgG, most of them bind complement, and they are pathogenic, as demonstrated by serum transfer experiments. Low levels of pemphigus-like autoantibodies are sometimes found in other conditions, such as burns and systemic lupus erythematosus, as well as in normal individuals. These antibodies appear to correlate with disease activity and may be used to monitor therapy.

Over 70% of BP patients exhibit autoantibodies that bind to the basement membrane zone between the dermis and the epidermis, appearing as a linear band of staining. False positives are rare. Evidence from *in vitro* and *in vivo* studies shows that these autoantibodies are pathogenic.

Muscle antigens

Smooth muscle

Sera from patients with chronic active hepatitis contain antibodies to smooth muscle antigens that are detectable by IIF and bind smooth muscle of all organs. The major antigen of the smooth muscle is actin. The antibodies belong mainly to the IgG class, but they can also be found in the IgM class. The test is performed on unfixed cryostat sections of rodent stomach as substrate. Smooth muscle antibodies are found in 40–70% of patients with active chronic hepatitis, with lower titers found in 50% of patients with primary biliary cirrhosis, and 28% of patients with cryptogenic cirrhosis. These antibodies are also found at low titer in patients with acute viral hepatitis, infectious mononucleosis, asthma, yellow fever and malignant tumors (carcinomas of the ovary, malignant melanoma). They have been found in less than 2% of the normal population.

Cardiac muscle

The presence of antibodies to cardiac striated muscle in patients with cardiomyopathy may help to distinguish autoimmune myocarditis from other forms of myocarditis (e.g. viral). This finding, in turn, may influence therapy. These antibodies are most readily detected by IIF with sections of rat heart as tissue substrate and bind to fibrillary antigens as well as

to sarcolemma or subsarcolemma antigens. The most severe cases of myocarditis or dilated cardiomyopathy have been associated with high titers of IgG antibodies specific for cardiac and not skeletal striated muscle. Many normal individuals may have low titers of antistriated muscle antibodies, but they are generally of the IgM class and react with both skeletal and cardiac striated muscle.

Endomysial

Autoantibodies to endomysial membranes, the outside lining of smooth muscle bundles, are found in patients with dermatitis herpetiformis and with celiac disease, and are primarily IgA. The major test is IIF using the lower part of monkey esophagus as tissue substrate. It appears to be a more specific and sensitive marker of celiac disease than testing for reticulin autoantibodies. In a few patients with celiac disease that have an IgA immunodeficiency, the antibody to endomysial antigens were found to be IgG. Isotype-specific anti-immunoglobulins conjugated to FITC are used as specific reagents to determine the relevant autoantibodies.

Acetylcholine receptor

Autoantibodies to acetylcholine receptor (AChR) are present in 80–95% of sera from patients with myasthenia gravis. Variations in the reported incidence of these antibodies are due to the different methods (complement fixation, passive hemagglutination, and various RIAs and ELISAs) used for their detection. In addition, there are heterogeneous specificities of antibodies to AChR; i.e. some that react to determinants other than the neurotoxin-binding site, others that react with the neurotoxin-binding site or the extrajunctional receptors, and others that are species specific. Since the cross-reactivity of human antibodies to AChR with receptors from other species is limited, human skeletal muscle is preferred as the source of AChR.

The procedures usually employed to detect circulating antibodies to AChR are RIAs. A direct binding assay is based on the inhibition of toxin binding to the receptor by the antibodies. A double antibody assay is based on the use of a labeled neurotoxin–receptor complex and thus quantitates receptor antibodies by measuring their binding to sites other than the acetylcholine- or neurotoxin-binding site.

Neutrophil cytoplasmic antigens

Antineutrophil cytoplasmic autoantibodies (ANCA) have recently become an important diagnostic tool for vasculitis associated with Wegener's granuloma-

tosis and certain other microscopic vasculitis and glomerulonephritis disorders. The antibodies are detected primarily by IIF on isolated human neutrophils as substrate. Differences in fixation of the cells can separate the antibodies into two distinct groups with different specificities: C-ANCA and P-ANCA. Ethanol fixation causes a redistribution of the antigens of the P-ANCA specificity causing a 'perinuclear' (P) picture of the fluorescent staining, while C-ANCA remains cytoplasmic (C). Formaldehyde fixation prevents the redistribution of the P-ANCA antigens so that the binding of both antibodies appears cytoplasmic. The major antigen of the C-ANCA specificity has been reported as proteinase 3 of the α granules, while the major antigen of P-ANCA has been identified as myeloperoxidase. ELISA tests using specific antigens are currently being developed. The appearance and change in titer of these antibodies appear to be good indicators of remission/exacerbation and severity of disease.

Mitochondrial antigens

Autoantibodies to mitochondrial antigens, detectable by IIF on rat kidney sections, are primarily associated with patients with primary biliary cirrhosis. The major antigen is reported to be a component of the pyruvate dehydrogenase enzyme complex. The antibodies belong to the IgG, IgA and IgM classes. The mitochondrial antibodies stain the cytoplasm of cells from different organs, such as thyroid or kidney, that varies with the mitochondrial content of the cells. Distal tubules of kidney and gastric parietal cells show a bright, uniform, cytoplasmic fluorescence, whereas renal proximal tubules show a characteristic coarse, granular, duller staining.

Mitochondrial antibodies are detected in 87–98% of patients with primary biliary cirrhosis. They are also observed in 25–28% of patients with active chronic hepatitis and in 25–30% of patients with cryptogenic cirrhosis. They are only seldom observed in patients with extrahepatic biliary tract obstruction and occur very rarely in normal subjects.

Nuclear antigens

A wide range of antinuclear antibodies (ANAs) occur against antigens associated with cell nuclei, including autoantibodies to DNA, histones, small nuclear RNP (snRNP), Ro/SS-A, La/SS-B, and tRNA synthetase. Many fine specificities have been defined amongst the various types of ANA. Diseases in which ANAs are particularly prevalent include mixed connective tissue disease, systemic lupus erythematosus, the CREST syndrome and progressive systemic sclerosis.

They are also commonly seen, although at a lower frequency, in rheumatoid arthritis and Sjögrens syndrome. IIF is used as an initial screening test for the occurrence of ANAs. Antibodies to many of the specific antigens can be measured in ELISA, although RIA (including the Farr assay and PEG assay), immunoblot, immunoelectrophoresis, RNA precipitation, and the LE-cell test are all commonly employed.

Phospholipid antigens

Antiphospholipid autoantibodies are a heterogeneous group of antibodies with specificity to a phospholipid-protein complex. The protein associated with cardiolipin has been identified as a β_2 -glycoprotein (β_2 -GPI). Some investigators suggest that this is the relevant autoantigen in patients with antiphospholipid syndrome, although the relative importance of these two molecules has not yet been fully clarified. The most sensitive test used is an ELISA using cardiolipin or other phospholipids to coat ELISA plates. Standardization of the various tests for the different phospholipids has been a problem. However, even so, the clinical utility has been established for antiphospholipid syndrome.

Rheumatoid factor

Rheumatoid factor represents a group of antibodies characterized by their ability to react with antigenic determinants on the Fc portion of immunoglobulin. Serum from patients with rheumatoid arthritis agglutinate sheep red blood cells (SRBCs) sensitized by rabbit anti-SRBC and this is the basis of the Waaler-Rose test. However, latex beads coated with Cohn fraction II as the source of IgG antigen, devised by Singer and Plotz, is the most widely used test today.

Other techniques for demonstrating rheumatoid factor include nephelometry and ELISA. Laser nephelometry and rate nephelometry utilize the changes in light-scattering properties of aggregated IgG when exposed to sera containing rheumatoid factor. These tests are reported to be equally sensitive and specific and more reproducible than established agglutination techniques, and they lend themselves well to automation.

See also: Agglutination; Antibodies, detection of; Antiglobulin (Coombs') test; Antinuclear antibodies;

Autoantigens; Autoimmune diseases; Autoimmunity; Enzyme-linked immunosorbent assay (ELISA); Immunoassays; Immunodiffusion, single radial; Nephelometry; Precipitation reaction; Western blotting.

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AUTOANTIGENS

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Antigens, and autoantigens, are detected by their capacity to participate in immunological reactions. When antigens provoke an immune response they behave as immunogens. Immunogenicity depends on various properties of the antigen, including its mode of presentation, local concentration and the immune capacity of the responding individual. For autoantigens there is an additional constraint on the response, this being the degree of natural tolerance to the autoantigenic particle.

Just as there is reciprocity between antigen and antibody, so too is there reciprocity between autoantigen and autoantibody. In addition, autoantigen is a self constituent, and thus the autoantibody reactive with it *in vitro* is assumed to be reactive *in vivo* as well. The individual's immune system scrutinizes a 'universe' of potential antigens: those that are foreign, extrinsic or nonself, and those that are constitutive, intrinsic or self, i.e. autoantigens. However, differences between 'foreign' and 'constitutive' antigens may be slight. Thus, some bodily constituents are absolutely specific or unique to an individual but others may be shared, at least within the species, or across species, or may be so conserved as to be represented throughout most living organisms.

When speaking of autoantigens, the context is usually that of reactivity with autoantibody, i.e. a product of the B cell lineage; however, most autoantigens would also need to be reactive with a T lymphocyte for induction of an immune response and for expression of pathogenic effects. T cell reactivity with antigen and especially with autoantigen, compared with B cell reactivity, is harder to detect and quantify; however, current assays based on proliferative responses or release of cytokines are becoming increasingly reliable.

The interaction *in vivo* between autoantibody and antigen is a potential cause of disease – autoimmune disease. Whether all self constituents behave as autoantigens is uncertain; for some, autoantibody is never demonstrable, so that tolerance to them is absolute. For many self constituents there may be autoantibodies demonstrable but seldom or ever is there any associated disease; indeed there is an increasing awareness of the wide range of 'natural' autoantibodies in health to various autologous constituents. In other instances autoantibody is only transiently present, e.g. after injury. The development of disease

after an autoimmune response may thus depend on additional factors, of which many are under genetic control. These include abundance and accessibility of the autoantigen, the capacity for presentation by antigen-presenting cells, the appropriate stimulation of cognate T cells, the efficiency of regulation of the immune response, the affinity and subclass of the autoantibody produced by B cells, and others.

Autoantigens have accrued a rather loose terminology among clinical immunologists who, hitherto, have referred to large cellular organelles as 'antigens', for example nuclear, mitochondrial, microsomal, etc. This parlance was convenient for clinical diagnostic purposes, since antinuclear, antimitochondrial and other tissue antibodies had disease associations, but it reflected ignorance of the constituents of cellular organelles to which autoimmune responses are directed. This is changing with molecular immunology leading to the cloning of cDNAs which code for autoantigenic polypeptides. An antigen, of whatever provenance, is actually a mosaic of determinant groups or epitopes, described by N. K. Jerne as follows: 'An antigen particle carries several *epitopes* (= surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns, specific areas). An antibody molecule normally carries, apart from its epitopes, two paratopes (= combining sites, reactive segments, complementary cavities, specific receptors). Epitopes are potentially immunogenic when present on an appropriate carrier: they may induce the formation of paratopes.'

The several or more epitopes of autoantigens are called autoepitopes. These may be reactive with paratopes of the autoantibody, and with the corresponding cell surface immunoglobulin molecule which serves as the antigen receptor for B cells: these are B cell epitopes. Also there are epitopes which engage T cells, T cell autoepitopes; the (moderately) predictive algorithms proposed for identifying T cell epitopes of foreign antigens are applicable also to autoantigens.

Epitopes, including autoepitopes, for B cells may be represented by linear sequences of peptides but more often are created by the conformational structure of the protein, and may require a substantial sequence of the antigenic molecule for their formation. Examples among the autoantigens include the

Ro(SS-A) and La(SS-B) ribonucleoproteins, the E2 subunit of pyruvate dehydrogenase complex (PDC-2) and glutamic acid decarboxylase.

Epitopes for T cells are represented by short linear sequences, nine or more amino acids, that are products of the processing of antigenic molecules, yielding peptides that bind into the polymorphic antigen-binding cleft created by the molecular configuration of major histocompatibility complex (MHC) molecules; particular residues, 'anchor' residues, are critical for this binding. A conformation of amino acids, known as a disease susceptibility motif, in the antigen-binding cleft of the MHC molecule, that can effectively bind a T cell epitope of an autoantigen may explain some of the well-known HLA associations with disease. Molecular sequences that bind to MHC molecules (in reality T cell epitopes) were formerly called agretopes but, with newer knowledge, this term has lapsed. Epitopes of antigens including autoantigens that react with and stimulate T cells can be recognized by assays that depend on cellular proliferation or release of lymphokines; however, progress has been slow.

The question often arises as to whether autoantigens actually function as immunogens. The focused nature of autoimmune responses strongly suggests that these are 'antigen driven' and, if this were so, the drive would be directed to the helper CD4 T cell. It remains uncertain whether the antigen drive, for B or T cells, is derived from autoantigen or a cross-reactive extrinsic structure with a closely similar molecular shape, as postulated by the mimicry hypothesis; alternatively, anti-idiotypic antibody against, say, antibody developed to an infectious agent could act as an 'internal image' of an autoantigen and so provide an immunogenic stimulus.

Autoantigens are so numerous and diverse that classification is hardly feasible, but this could be based on their site of origin (Table 1) as follows: cell membranes; cell cytoplasm; cell nuclei; specialised tissue constituents; secreted molecules; neural products.

From the standpoint of pathology, the autoantigens listed in Table 1 have varying degrees of relevance. The reactivity of various 'natural' autoantibodies present in normal sera is irrelevant to actual pathogenesis. Then there are autoantibodies that can be termed 'marker' antibodies, as they point to a disorder of immune tolerance as a cause of the disease with which they are associated, but do not *per se* appear to be pathogenic; and there are other autoantibodies which are truly pathogenic, such as those reactive with cell membrane structures including receptors for neurotransmitters and hormones.

Autoantigens associated with intracellular organelles, nuclear or cytoplasmic, raise interesting issues

in that many of these have a close if not specific relationship with a particular disease, and are therefore useful diagnostically. Examples include autoantibodies to centromere or Scl-70 antigens which occur in scleroderma, to ribonucleoprotein antigens in lupus-related diseases, and to mitochondrial antigens in primary biliary cirrhosis (PBC). However, for pathogenicity to be ascribed to such antigens, one or even both of two requirements should be fulfilled. One would be that the autoantigen becomes translocated to the cell surface, and here the PDC-E2 autoantigen of primary biliary cirrhosis is an interesting example as it is demonstrable at the surface of biliary ductular cells of patients with PBC; this observation foreshadows interests for autoimmunity in mechanisms that may influence intracellular transport of molecules between nucleus, cytoplasmic organelles and cell membranes. The other requirement would be a capacity for autoantibody to penetrate the membrane of intact cells to access intracellular organelles; there are data supporting this but the matter is controversial.

The category 'neural products' (Table 1) contains reactants for interesting autoantibodies in paraneoplastic autoimmune neurodegenerations, central or peripheral, that are rare accompaniments of ovarian, lung and breast cancers. The cancer itself presumably generates the neural antigen and facilitates its immunogenic presentation.

A better understanding of the structure of autoantigens has come from advances in analytical biochemistry, including gel chromatography and transfer western immunoblotting, and also molecular biology, including antibody probing of gene expression libraries and phage display libraries. These techniques have identified the reactive polypeptide antigens in various of the cellular organelles and have yielded cloned cDNAs which allow for gene sequencing and mapping of autoepitopes; screening of phage display libraries may occasionally reveal linear mimotopes of conformational autoantigens. There have been interesting outcomes, as follows:

1. There is an increasing availability of recombinant proteins for diagnostic assays, ELISAs and radioimmunoprecipitation which, by reason of greater sensitivity and versatility, are supplementing detection of autoantibodies by immunofluorescence.

2. Epitope mapping is revealing that, for most autoantigens, there are multiple and apparently structurally different epitopes recognized by antibody. This impinges on the concept of 'molecular mimicry', which holds that resemblances between a sequence in a host protein and a microorganism can result in production of autoantibody. The presence

Table 1 Examples of autoantigens and disease associations

<i>Autoantigen</i>	<i>Related diseases</i>
<i>Cell membranes</i>	
Blood cells	Hemolytic diseases: hemolytic anemia, thrombocytopenic purpura, neutropenia
Receptor structures for acetylcholine thyrotropin (TSH) insulin	Myasthenia gravis Graves disease (thyrotoxicosis), goiter (?) Diabetes, insulin-resistant type
Nerve cells: myelin of oligodendroglia, Schwann cells	Experimental autoimmune encephalomyelitis, neuritis
Liver cells: asialoglycoprotein receptor	Autoimmune hepatitis, type 1 (?)
<i>Cytoplasm, organelles</i>	
Tissue specific	
gastric H ⁺ , K ⁺ -ATPase, α and β subunits	Autoimmune gastritis, pernicious anemia
thyroid peroxidase (microsomal antigen)	Hashimotos thyroiditis
adrenal 21-hydroxylase	Addisons disease
pancreatic islet glutamic acid decarboxylase; ICA512/IA-2/37-40k; insulin	Type I diabetes mellitus
salivary duct cell	Sjögrens syndrome with rheumatoid arthritis
neutrophil cytoplasmic proteinase 3 and myeloperoxidase (ANCA)	Wegener's disease, vasculitic diseases and others
Non-tissue specific	Autoimmune hepatitis, type 2
cytochrome P450 2D6 (liver-kidney microsomes)	Primary biliary cirrhosis
2 oxo-acid dehydrogenase enzymes (mitochondria, M2)	
phosphoproteins P0, P1, P2 (ribosomes)	Systemic lupus erythematosus (SLE)
cytoskeletal actin (smooth muscle antibody)	Autoimmune hepatitis, type 1
stress (heat-shock) proteins	Rheumatic diseases (?)
transfer RNA synthetases (Jo1)	Polymyositis and variants
<i>Nuclei</i>	
DNA	SLE
Histones	SLE, particularly drug related; various rheumatic diseases
U (uridine-rich) RNA-protein complexes	
U1-RNP	Mixed connective tissue disease
U1, U2, U4-6 RNPs (Sm antigen, various epitopes have been mapped)	SLE
Ro-La	
Ro(SS-A)	Primary Sjögrens syndrome; cutaneous LE; fetal heartblock
La (SS-B)	Primary Sjögrens syndrome; SLE
Centromere	Scleroderma, CREST variant
Topoisomerase I (Scl-70)	Scleroderma, systemic sclerosis
Nucleolus	Systemic sclerosis; undifferentiated rheumatic diseases
fibrillarin	
nucleolar RNP	
Proliferating cell nuclear antigen (cyclin)	SLE (rarely)
<i>Specialised tissue products</i>	
Squamous epithelium	
desmosomes (desmoglein 1,3)	Pemphigus vulgaris, pemphigus foliaceus
hemidesmosomes (BPAG 1 & 2)	Bullous pemphigoid
Striated muscle, sarcomere, cytoskeletal proteins	Myasthenia gravis, with thymoma
Cardiac muscle, sarcolemma	Myocarditis, rheumatic carditis
Chondrocytes, collagen type II	Rheumatoid arthritis
Glomerular basement membrane, collagen type IV	Glomerulonephritis (Goodpasture type)
Retina, S antigen	Uveitis
Sperm antigens	Male infertility; orchitis (?)
Colon mucosal cell	Ulcerative colitis (?)
β_2 glycoprotein 1 (cardiolipin)	Antiphospholipid syndrome, thromboembolism
<i>Secreted products</i>	
Gastric intrinsic factor	Atrophic gastritis, pernicious anemia
Immunoglobulin G, Fc region	Rheumatoid arthritis
Thyroid colloid	Atrophic hypothyroidism; Hashimotos thyroiditis
Insulin	Type I diabetes mellitus

Table 1 Continued

Autoantigen	Related diseases
<i>Neural products</i>	
Glutamic acid decarboxylase	Stiff man syndrome
Calcium channels, synaptotagmin	Lambert-Eaton myasthenic syndrome
Purkinje cell cytoplasm (Yo)	Paraneoplastic (PN) cerebellar degeneration
Cerebellar neuronal nuclei	
ANNA 1 (Hu)	PN sensorimotor neuropathy
ANNA 2 (Ri)	PN midbrain encephalitis/ataxia/myelopathy

(?) Indicates incomplete consensus.

of several epitopes on autoantigens, and/or the existence of conformational epitopes, could limit the appeal of the molecular mimicry hypothesis because it is unlikely that the several epitopes of the autoantigen would be conformationally similar, or that the extrinsic antigen would have multiple mimicry epitopes; however, mimicry could exist for a single shared or cross-reactive epitope recognized by the CD4 helper T cell that could then recruit successive B cell epitopes into the autoimmune response, a process known as epitope spreading.

3. The epitope recognized by autoantibody is frequently associated with an active site of an enzyme or other functional molecule, and the antibody can be shown to inhibit the function *in vitro* of the cognate autoantigenic enzyme. For example, the major epitope of PDC-E2, the M2 autoantigen of PBC, is the functional lipoyl domain, and the reaction of PBC serum with this domain specifically abrogates the catalytic function of the PDC enzyme *in vitro*.

4. There is a possibility that autoantigens may have 'disease-specific' epitopes that differ in site or structure from epitopes that are reactive with non-disease-specific natural autoantibodies.

5. Various intracellular antigenic molecules have been functionally characterized at the molecular genetic level by the use of autoantibodies as research reagents. Examples, of which there are many, include the U-ribonucleoproteins which splice out introns from mRNA, the La ribonucleoprotein which participates in the termination of transcription of RNA polymerase III, and the Ku antigen which participates in recombination and repair of DNA.

One major need at present is to verify T cell responses to autoantigens and map the relevant epitopes, noting that autoepitope-responsive T cells have a low representation among peripheral blood cells and, even in affected tissues, will be 'diluted' by nonspecifically reactive cells in inflammatory exudates. Another need is to understand why particular molecules or related groups of molecules are singled out among numerous others as autoantigenic reac-

tants, i.e. why there is failure of tolerance to these particular tissue constituents. This will require better understanding of how 'nascent' self reactive T or B lymphocytes are deleted in the thymus or bone marrow respectively during ontogeny, and how self reactive lymphocytes that escape this process are tolerized in the periphery in postnatal life.

See also: Adrenal autoimmunity; Anemia, autoimmune hemolytic in human; Anemia, pernicious; Antiglobulin (Coombs') test; Antinuclear antibodies; Autoantibodies, tests for; Autoimmune disease, induced experimental models; Autoimmune disease, pathogenesis; Autoimmune disease, spontaneous experimental models; Autoimmune diseases; Autoimmunity; Cardiac disease, autoimmune; Epitopes; Experimental autoimmune encephalomyelitis (EAE); Eye, autoimmune disease; Goodpasture's syndrome; Idiopathic thrombocytopenic purpura; Insulin-dependent diabetes mellitus, animal models; Insulin-dependent diabetes mellitus, human; Molecular mimicry; Neuromuscular junction autoimmunity; Neurological autoimmune diseases; Polyendocrine autoimmunity; Rheumatoid arthritis, animal models; Rheumatoid arthritis, human; Sjögrens syndrome; Skin, autoimmune diseases; Systemic lupus erythematosus, experimental models; Systemic lupus erythematosus (SLE), human; Thyroid autoimmunity, experimental models; Thyroid autoimmunity, human; Vitiligo; Primary biliary cirrhosis; Chronic active and autoimmune hepatitis.

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AUTOIMMUNE DISEASE, INDUCED ANIMAL MODELS

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The first indication that the immune system could be experimentally provoked into dangerous self reactivity stems from the early 1930s when Rivers and his colleagues induced encephalomyelitis in monkeys by the injection of brain material. Some 20 years later, Voisin and coworkers made the, quite literally, seminal observation that testicular lesions could be similarly induced by testicular extracts including spermatozoa. Although these studies provided the prototype animal models of autoimmune diseases, the foundations of experimental autoimmunity were not truly laid until Rose and Witebsky demonstrated in a series of classical studies that both antibodies to thyroid constituents and thyroiditis could be induced by immunizing rabbits with thyroid extracts in Freund's complete adjuvant. Since then, many experimental models have been developed in a broad range of animal species and, consequently, the 1975 prediction of Wick – that autoimmune disease may be induced in any organ provided the investigator tries hard enough – seems ever more likely. In addition, new experimental approaches not involving specific immunization have been introduced and these have widened the scope and range of disease models now available for study. These later models have opened alternative avenues for investigation and, in consequence, new conceptual insights into the autoimmune process have been made.

As a group, induced models may be defined as those in which an autoimmune process is superimposed on otherwise normal, unaffected animals by a variety of treatments or manipulations. They contrast with the spontaneous models where disease occurs without intervention and is largely, although not entirely, determined by genetic composition. In consequence, whilst spontaneous models are limited to a particular genome, induced models are well suited to the investigation of genetic influences by their imposition on inbred strains of differing genetic constitution. They also have the particular advantages that normal syngeneic animals are available for parallel control studies, as recipients in adoptive transfer experiments, and also for the provision of cells for reconstitution studies in depleted animals.

The extensive range of induced animal models that is currently available has resulted from an expanding variety of induction strategies and can be con-

veniently grouped on this basis. Since the methods employed often reflect fundamental differences in both the triggering and pathogenic mechanisms, each type provides its own insight into autoimmune pathogenesis and has particular advantages for research in selected areas. Despite these widely differing inductive procedures, however, it is likely that the terminal pathogenic pathways coincide when a common organ is involved. These aspects will be discussed in the following sections which briefly review the various models currently available.

Methods of experimentally inducing autoimmunity

1. Induction of autoimmunity by immunization ('immunization models')

This approach, the first to be introduced, has been of primary importance in establishing the validity of the autoimmune concept. Furthermore, since it has been possible by this means to raise autoimmune responses to an extensive range of self components (Table 1), it is evident that potentially autoreactive cells are not only a normal component of the immune repertoire of every individual but also that they are quite capable of being activated under appropriate conditions. As a corollary, it also follows that, under normal circumstances, these cells are effectively subjugated by one or more regulatory mechanisms. In consequence, it is generally necessary to inject native autoantigens in combination with powerful nonspecific immunostimulants such as Freund's water-in-oil adjuvant in order to override these inhibitory influences. As an alternative procedure, the immunogen can be altered self components or material derived from other species which may provide a more effective autoimmune stimulation by way of 'modified self' or cross-reactive antigenicity. Weigle, for example, used rabbit thyroglobulin to induce thyroiditis in mice and the injection of rat erythrocytes into mice also induces antibodies to mouse erythrocytes and transient anemia. Presumably, under these circumstances, the inclusion of new antigenic determinants can provide a bypass mechanism permitting new T cell help for the generation of autoimmune responses.

Immunization models have been extensively used

Table 1 Induction of experimental autoimmunity by immunization with autoantigens

<i>Target organ/tissue</i>	<i>Animal species</i>	<i>Analogous human disease</i>
Thyroid follicles	Rabbit, mouse, guinea pig, rat, monkey, chicken, dog	Hashimoto's thyroiditis
Adrenal cortex/medulla	Guinea pig, rabbit, rat (Lewis, BN), mouse (CF1, BSVS), monkey, dog	Autoimmune adrenal failure (Addison's disease)
Pituitary/adenohypophysis	Rat, rabbit	Lymphocytic adenohypophysitis
Parathyroid	Rat, dog, chicken, mouse	Autoimmune parathyroiditis
Pancreas (islet cell)	Rat, mouse, rabbit	Type I diabetes
Central nervous system neurons	Rat (Lewis), guinea pig (strain B), mouse, sheep, monkey	Multiple sclerosis
Eye (uvea/retina)	Guinea pig (Hartley and NIH)	Autoimmune uveitis
Kidney (tubules/glomeruli)	Rat (BN) Rat	Autoimmune tubulointerstitial nephritis Autoimmune glomerulonephritis
Joint (articular surface)	Mouse (DBA), rat, rabbit	Rheumatoid arthritis
Liver (hepatocytes)	Mouse	Chronic active hepatitis
Testes (spermatozoa)	Mouse (C57 black)	Autoimmune orchitis
Heart (myocardium)	Guinea pig, mouse	Autoimmune myocarditis
Thrombocytes	Rat (BN)	Idiopathic thrombocytopenia
Erythrocytes	Mouse	Autoimmune hemolytic anemia
Neuromuscular junction	Rat, guinea pig	Myasthenia gravis

to study genetic influences on autoimmune susceptibility. As might be expected, strong major histocompatibility complex (MHC) influences have been observed in some models as, for example, in mouse thyroiditis and experimental autoimmune encephalomyelitis (EAE) in rats. However, this does not seem to apply to the mouse antierythrocyte/anemia model mentioned above, although non-H2 genetic associations are evident.

One major disadvantage of this procedure is that the pathological effects induced are often transient in nature, presumably due to the imposition of autoimmunity on the normal animal with intact immunoregulatory feedbacks. As a consequence, overt clinical disease is not observed in many immunization models, even though short-term tissue infiltration is readily apparent histologically. However, EAE is a notable exception in that severe nervous signs can be readily induced by immunization of susceptible animals with myelin basic protein or its peptide derivatives. Clearly, the nervous system is more sensitive to the effects of immunological attack and these are more apparent clinically than, for example, those involving the endocrine tissues.

Although the relapsing form of EAE can be induced in guinea pigs by this method, in most experimental systems autoimmunity is rarely reactivated even by further immunization. There is evidence in a number of models that this effect may be due to the generation of antigen specific 'suppressor' cells, as demonstrated by adoptive cell transfer stud-

ies. Evidence for such activity has been found in mouse thyroiditis, mouse antierythrocyte autoimmunity and EAE in rats.

Although the injection of autoantigens in adjuvant can clearly have little relevance to the natural triggering events leading to human autoimmune disease development, immunization models continue to provide a wealth of information on many other aspects of autoimmunity. Currently, with the rapid accumulation of information at the molecular level concerning the subcellular components involved in autoimmunity, this procedure is likely to be utilized as a requisite step in the evaluation of purified recombinant polypeptides derived from such structures as potential B or T autoepitopes.

2. Induction of autoimmunity by impairment of immunological function ('immune depletion models')

It is now accepted that partial impairment of normal immunological function can lead to the onset of autoimmune reactivity, particularly of the organ-specific type. The earliest evidence in this context was the observation that thymectomy of certain mouse strains leads to the appearance of antinuclear antibodies. However, this relationship was firmly established in 1973 by two groups who independently reported the induction of organ-specific autoimmunity by procedures causing partial immunodepletion. Penhale and coworkers found that autoimmune thyroiditis could be induced by thymec-

tomy of 3-week-old Wistar rats followed by repeated low-dose irradiation. In a similar vein, Nishizuka and colleagues made the observation that thyroiditis spontaneously developed in mice following thymectomy alone, provided that this was carried out in the neonatal period. The autoimmune basis of these conditions was evident from the presence of infiltrating lymphocytes in the target tissue, circulating tissue-specific autoantibodies and the ability to transfer the disease to naive recipients with lymphocytes from affected animals. From 1975 onwards a number of other manipulations and treatments involving the immune system were shown to induce organ-specific autoimmune disease in rodents. A feature of all these procedures is either a deficiency of circulating T lymphocytes or their functional impairment as, for example, by a reduction in the range of the T cell receptor (TCR) repertoire. The spectrum of autoimmune diseases induced by this means is now impressive and includes diabetes, thyroiditis, gastritis, orchitis, oophoritis, prostatitis and coagulating adenitis. These conditions are rarely, if ever, seen in normal unmanipulated rodents of the same strains. The various depletion methods employed to induce these diseases are shown in Table 2.

A central question in understanding the process of autoimmunity is how the aberrant immune response is initiated. The development of immunodepletion models has been conceptually important in demonstrating the critical requirement for regulatory elements in the control of potential autoimmune reactivity in the normal individual. They also strongly suggest that neither aberrant antigen modification nor presentation are likely to be necessary antecedents of the autoimmune process. In consequence, they provide a powerful tool for studying the basis for the loss of self tolerance to peripheral self antigens and the development of destructive autoimmune lesions. In this regard they have proved particularly suitable for studying the immunological regulation of autoimmunity by reconstitution studies

using syngeneic lymphocytes derived from normal animals.

Immunodepletion models do not require immunization with putative autoantigens in adjuvant, as in the previous model above, but presumably involve the appropriate targeting of natural autoantigens rather than surrogate model antigens. In addition, they are also similar to their clinical counterparts in humans in that, once initiated, the disease is sustained and chronic. Furthermore, an association between various immunodeficient states and clinical autoimmunity in humans has been recognized. Because of these similarities in many pathological and immunological features they are providing invaluable insights into the pathogenesis of their human disease counterparts.

3. Induction of autoimmunity by infectious agents ('infection models')

The natural history of autoimmune disease in some individual patients strongly suggests the involvement of environmental or infectious agents in the inductive process. For example, postmeasles encephalomyelitis may result from an autoimmune response to myelin basic protein, and infection with Coxsackie virus group B has been associated with myocarditis in which there are autoantibodies to heart antigens. Despite this circumstantial evidence in isolated instances, direct evidence for the involvement of infectious agents in human autoimmune disease is unavailable. In contrast, experimental studies in animals provide some compelling evidence of the involvement of infectious agents, particularly viruses, in the triggering of certain forms of autoimmunity. Several viruses have been shown to induce diabetes-like conditions in inbred strains of mice with evidence of associated autoimmunity. Polyendocrine syndromes and diseases of nonendocrine organs have also been observed in certain infections. Certain bacteria or their products may also cause experimental autoimmune effects. For example, the injection of mycobacterial proteoglycan may induce arthritis, several bacterial products may similarly cause myasthenia (see below) and the injection of live *Listeria* directly into the testes can provoke an autoimmune orchitis.

The phenomenology of infection models require further investigation before a detailed understanding of their mechanisms can be gained and an autoimmune basis for their pathogenesis is unequivocally established. However, a number of immunological processes may underlie their pathogenicity, including:

1. The effect infectious agents have on host cells.

Table 2 Manipulations of the immune system leading to the development of organ-specific autoimmunity

<i>Experimental manipulation</i>
Combined sublethal irradiation/thymectomy of adult rodents
Neonatal thymectomy of rodents
Neonatal administration of cyclosporine in mice
Combined cyclophosphamide/thymectomy of adult rodents
High dose (42.5 Gy), fractionated total lymphoid irradiation of adult mice
Single-chain TCR transgenic mice
T cell transfer to syngeneic, T cell-deficient mice

Apart from the expression of virus gene products in persistently infected cells which may target them for host immune responses, virus may also have subtle effects on cell genes and the resulting enhanced or altered expression of host cell proteins could induce an autoimmune response. A further possibility is interaction of microorganisms with immunocompetent cells, leading to their polyclonal activation or reduced susceptibility to regulation. They may also cause a loss of function of regulatory cells with autoimmune consequences.

2. Molecular mimicry. Partial sequence homology between microbial antigens and self determinants may lead to the generation of host-specific responses by T helper cell bypass.
3. Idiotype-anti-idiotypic interactions (see below).
4. Tissue injury induced by infection may lead to the breaking of tolerance to self antigens and subsequent perpetuation of injury by autoimmune mechanisms. This mechanism could account for the induction of autoimmune disease by an acute, nonpersisting infection.
5. Enhanced expression of MHC antigen by microbial influences directly on the target cell or indirectly via lymphocytes.

4. Induction of autoimmunity by chemical agents ('chemically induced models')

A number of chemical substances appear to give rise to autoimmune effects in patients after prolonged use as therapeutic agents; for example, methyl dopa may cause a Coombs'-positive hemolytic anemia. Similarly, in the experimental context, several chemical agents have been shown to induce autoimmune-like syndromes, such as streptozotocin-induced diabetes in mice and mercuric chloride-induced glomerulonephritis in brown Norway rats. There are several possible mechanisms whereby chemicals may be involved in generating autoimmune manifestations, including: 1) modification of tissue components, particularly cell membrane structures; 2) breaking of self tolerance following liberation of cellular constituents by direct tissue damage; and 3) modification of immune function, particularly affecting regulatory cells. In general, withdrawal of the chemical agent results in recovery and diseases induced by this means have few clear analogies with naturally occurring human disorders. Low dose streptozotocin-induced diabetes mellitus in mice is a possible exception to the above statement, although the actual pathogenesis, whether direct toxicity or true autoimmunity, is still in doubt.

5. Induction of autoimmunity by anti-idiotypic (Id) responses ('anti-idiotypic models')

Theoretically, several mechanisms of induction of autoimmunity through Id interactions appear to be possible, although few examples of such are currently documented and none established as regularly utilized models for inducing autoimmunity. First, an anti-Id antibody may resemble an autoantigen sufficiently to be cross-reactive. This is exemplified in the insulin system, where it has been found that an antibody to the Id of anti-insulin antibody may interact with and actually trigger insulin receptors. Another, more dramatic example of the same mechanism appears to be the myasthenia gravis-like syndrome observed when rabbits are injected with a synthetic agonist (Bis Q) of the acetylcholine receptor. A second way in which the Id network can stimulate autoimmunity is through the development of 'parallel sets'. These may arise when immunologically distinct antibodies share a 'public' idiotypic. As an example, extensive idiotypic has been discovered between the antibodies to the acetylcholine receptor and the DEX antigen (1,3-dextran) present on the surface of many bacteria. Thus, the injection of such bacteria may induce autoantibodies to the receptor and hence myasthenia gravis. It may be no coincidence that many examples of autoimmune anti-Id effects involve receptor molecules.

6. Induction of autoimmunity by genetic manipulation ('transgenic models')

Transgenic technologies have provided new opportunities for developing animal models of autoimmune diseases. To date their development has been largely directed at obtaining new insights into the fundamental questions of autoimmunity, such as the nature of self tolerance, rather than the provision of relevant models of human disease. Expression of well-defined molecules can be induced in specific cell types by this technology and three broad approaches have been utilized to study aspects of autoimmunity:

1. The genes selected may code for a normally foreign antigen which will be synthesized at a particular development stage and behave essentially like a self antigen. The gene may be linked to a tissue-specific promoter which ensures its expression in a particular tissue while simultaneously avoiding thymic expression. Thus it is possible to generate cells expressing a genuine self molecule without concurrent thymic deletion of the complementary lymphocytes.
2. The genes selected may be rearranged T cell receptor or immunoglobulin genes coding for recognition molecules with specificity for a self

component. This procedure has the potential to generate antiself lymphocytes at high frequency, allowing the fate of such cells to be followed during and after ontogeny. Alternatively, the TCR repertoire can be greatly restricted by introducing the gene coding for an irrelevant nonself specificity. Thus the T cell compartment can be functionally depleted, leading to autoimmunity (see method 2 above).

3. The transgene may code for MHC molecules in order to address the question of the significance of their upregulation on target tissues during autoimmunity. This has led to the surprising finding that diabetes of nonimmune origin can be induced by overexpression of these molecules on the islet β cells. This is presumably due to derangement of cell metabolism as a consequence of high class II expression.

These strategies can also be combined to produce multiple transgenic animals in which, for example, an antigenic molecule can be directed to a specific cell type and the complementary TCR introduced into lymphocytes simultaneously.

The β cell of the pancreatic islet currently appears to be the most popular location for transgenic modification. Apart from the importance of type I diabetes as a human autoimmune syndrome, this cell is particularly convenient in that the transgene can be linked to the specific promoter for insulin production. Studies with transgenically modified β cells have been revealing in a number of respects. For example, experiments with lymphocytic choriomeningitis virus (LCMV) glycoprotein antigen expression on these cells point to immunological ignorance rather than tolerance as the mechanism responsible for the absence of reactivity to islet cell antigens, as diabetes does not occur spontaneously in this model and can only be induced by subsequent infection with LCMV. Furthermore, a number of additional lines of evidence indicate strongly that the virus is not breaking immunological tolerance in this experiment. New generations of these transgenic models are likely to revolutionize our understanding of this central phenomenon of autoimmunity.

Conclusions

Study of human autoimmune disease presents the considerable problem of deciding whether the changes observed are the cause or consequence of the disease process. The use of animal models avoids this difficulty and their manipulative potential has yielded many valuable insights into the autoimmune process. The disease range of induced models now available covers a wide spectrum of human autoimmune diseases and, although differing in detail from the human conditions, they have been instrumental in clarifying basic pathogenic mechanisms and, perhaps more importantly, in demonstrating that multiple and converging pathways to autoimmune disease development exist. They have also demonstrated that, in addition to a specific triggering event, disturbances of normal regulatory function may be prerequisite for chronic, sustained autoimmunity.

Finally, despite the multifactorial nature of autoimmune disease clearly revealed by modeling, this approach also offers the prospect that autoimmune disease might be controlled by interfering with the final common pathways without necessarily eliminating all the antecedent immune abnormalities.

See also: Adjuvant arthritis; Anemia, autoimmune hemolytic in animal models; Autoantigens; Autoimmune disease, spontaneous experimental models; Autoimmunity; Contrasuppression; Experimental autoimmune encephalomyelitis (EAE); Idiotype network; Immunosuppression; Insulin-dependent diabetes mellitus, animal models; Multiple sclerosis; Neuromuscular junction autoimmunity; Rheumatoid arthritis, animal models; T cell vaccination; Thyroid autoimmunity, experimental models; Transgenic animals.

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AUTOIMMUNE DISEASE, PATHOGENESIS

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Now as ever, autoimmune diseases constitute one of the main unsolved problems in human clinical medicine. This is because our knowledge of their etiology and pathogenesis is still not sufficient to provide new concepts towards specific therapy. They are considered to be pathological consequences of immune reactions directed towards autologous antigens.

Over 30 years ago, Witebsky and Milgrom formulated their well-known criteria for autoimmune diseases, the most important of which are that: 1) antibodies or lymphocytes specifically reacting with a defined autoantigen can be detected; 2) immunization of animals with an autoantigen results in an autoimmune disease corresponding to its original tissue distribution, and 3) experimental autoimmune disease is inducible in healthy recipients by passive transfer of autoreactive antibodies or lymphocytes. These postulates, essentially still valid today, pointed to the importance of animal models, and our present knowledge about etiopathogenesis is indeed mainly derived from the study of animals, in which autoimmune diseases are either experimentally induced by immunization with autoantigen, or occur spontaneously at high incidence.

Autoimmune diseases are a heterogeneous group of diseases. According to manifestation we can distinguish systemic diseases affecting various different tissues from organ-specific diseases, where the autoimmune process is restricted to single organ systems. There is good evidence that these two types differ considerably in etiology and pathogenesis.

Breakdown of autotolerance

The ability to tolerate autologous antigens, a central feature of the adaptive immune system, is guaranteed in redundant ways at the level of both the B and T lymphocyte system. It is thought to be the result of negative selection within the central lymphoid organs, i.e. bone marrow and thymus, where sensitive stages of developing B (sIgM⁺ sIgD⁻) or T (CD4⁺ CD8⁺) cell clones are eliminated by apoptosis due to high-affinity binding to autologous antigens. Autoreactive cell clones that escape these central selection processes for whatever reason are effectively controlled in their reactivity in the periphery, either passively by virtue of insufficient costimulatory signals or by active suppressive immunoregulation. These peripheral mechanisms are not specific for autoantigens, but are identical to those that

induce tolerance against any given foreign antigen. With regard to autoimmunity the interest has focused primarily on T lymphocytes, because of the central regulatory role of T helper cells in the immune response against protein antigens, and in view of the well-known association of autoimmune diseases with major histocompatibility complex (MHC) alleles differing in effectivity of antigen presentation to T cells.

Defective differentiation

According to the aforementioned concepts a breakdown in autotolerance can occur due to failures in thymic selection, and any factors that influence the survival of thymic lymphocytes could principally be expected to result in shifts in the peripheral T cell repertoire. As MHC molecules play an important role in the presentation of thymic autoantigens, changes in thymic selection may be one mechanism by which genes of the MHC influence autoimmune diseases. Nevertheless, it has to be stated that experimental data directly proving the occurrence of autoimmune reactions as a consequence of a disturbed intrathymic differentiation are lacking as yet.

Based on the finding that autoreactive lymphocytes of patients and experimental animals with organ-specific autoimmune diseases exhibited a limited number of V genes, it was debated whether autoimmunity may be the result of an abnormal immunoglobulin (Ig) or T cell receptor (TCR) repertoire. However, restriction fragment length polymorphism (RFLP) analysis performed in humans with autoimmune diseases did not indicate associations to certain Ig or TCR genes, and no consistent differences were detected in germline Ig genes of autoimmune mouse strains.

Peripheral loss of autotolerance

As to peripheral mechanisms, the loss of anergy against autoantigens may be the result of abnormal activation of T cells by antigen-presenting cells (APCs), such as dendritic cells, macrophages and B cells, that increase the expression of costimulatory surface molecules as a result of irritation, e.g. in the course of infections or local inflammatory reactions. In accordance with this notion is the established fact that the induction of experimental autoimmune diseases in animals requires immunization with autoantigens together with strong adjuvants that lead to

activation of APCs. Also, nonprofessional APCs, such as epithelial cells, may lead to local activation of autoreactive T cells by virtue of inappropriate expression of immunostimulatory molecules, such as MHC class II determinants. Another strong indication for a crucial role of T cell activation comes from results proving that enhanced levels of interleukin-2 (IL-2) are able to abrogate a state of anergy against tissue autoantigens. Further peripheral mechanisms, not related to T cell activation, may be a resistance of lymphocytes to apoptosis as described in the MLR-*lpr/lpr* mouse with systemic lupus erythematosus (SLE) symptoms and a defect in the expression of the fas apoptosis gene. However, no such association has been found in human SLE patients.

Peripheral tolerization of B lymphocytes results from antigenic stimulation without proper T cell help. Thus, a stimulus from autoreactive T helper cell clones may end the state of B cell autoanergy. Alternatively, certain foreign antigens may exhibit shared epitopes with autoantigens, and T helper cells specific for these shared epitopes may drive autotolerant B cells into activation.

Immunological cross-reactions between foreign microbial antigens and autoantigens are well known to be able to elicit humoral or cellular autoimmune responses, the classical example being rheumatic fever due to a 'normal' immune response against streptococcal antigens that is cross-reactive with myocardial protein. More recently, mycobacterial heat shock protein (hsp65) has gained interest as an antigen of possible relevance to pathogenic autoimmune responses in several diseases, including atherosclerosis.

Polyclonal, antigen-independent lymphocyte activation has been implicated in the activation of autoreactive lymphocytes and elicitation of autoimmune responses. Injection of mice with bacterial lipopolysaccharide (LPS), which is a polyclonal B cell activator in mice, has been shown to lead to the production of autoantibodies of various specificities. Even though these antibodies, due to the lack of T cell help, are of low affinity and nonpathogenic, the polyclonal B cell activation by 'LPS-like' bacterial substances may be at least one of the reasons for the close association of autoimmune and infectious diseases. Similarly the binding of bacterial superantigens to certain V_{β} chains of the T cell receptor has been discussed as a possible mechanism of autoimmunity at the T cell level. Finally, autoimmune phenomena are observed in the course of graft-versus-host reactions, supposedly due to polyclonal B cell activation induced by alloactivated donor T helper cells.

A disturbed balance in helper and suppressor influences exerted by T regulator cells has long been implicated in the pathogenesis of autoimmune diseases. While the classical T helper/suppressor cell model was abandoned several years ago, it is now thought that the balance between T helper1 (T_H1) and T helper2 (T_H2) cells is relevant to the maintenance of autotolerance, whereby T_H2 cells under normal conditions are thought to keep autoreactive effector T_H1 cells in line. Accordingly, quantitative or functional abnormalities in this balance may allow autoimmune reactions to occur. Investigations of joint lesions in rheumatoid arthritis (RA) patients seem to support this concept, as cloning studies identified the vast majority of infiltrating T cells as belonging to the T_H1 subtype, whereas T_H2 clones were only rarely found. In contrast, synovial specimens from more benign, nondestructive types of arthritis were found to contain high levels of IL-4 mRNA. From this it was concluded that the local presence of functional T_H2 cells may be critical for the self-limitation of the autoimmune process in the affected joints.

Besides their central roles in the effector phase of autoimmune processes (see below) cytokines are certainly critically involved in systemic and local immunoregulation and thereby modulate the clinical outcome of an autoimmune disease. Furthermore, the above mentioned data on IL-2 suggest that they may also take part in the breakdown of self-tolerance. Recently, enhanced systemic levels of inflammatory cytokines (IL-1 β , IL-6, tumor necrosis factor α (TNF α)) and soluble cytokine receptors (sIL-2R, sTNFR) were reported to significantly correlate with the clinical activity of RA in children, whereby the different clinical subtypes displayed different patterns.

Effector mechanisms

As to effector mechanisms, autoimmune diseases are no different from any other pathogenic immune response against foreign antigens involving reactions of types II (cytotoxic antibodies), III (immune complexes), IV (cellular-mediated immunity) and V (stimulating antibodies) according to the Gell/Coombs nomenclature. Furthermore, antigen non-specific mechanisms secondarily activated by autoantigen-activated lymphocytes play an important pathogenetic role.

The role of autoantibodies

The detection of circulating and/or tissue-bound autoantibodies is undoubtedly one of the most consistent findings in autoimmune diseases in human

and animal models. Although low levels of autoantibodies can be detected in healthy individuals, and, on the other hand, autoimmune diseases can exist without detectable autoantibodies, there is in general a good correlation between peripheral levels of autoantibody and activity of an autoimmune disease, whereby the spectrum of specificities may be characteristic, sometimes even diagnostic, for a particular autoimmune disease. There are several ways by which autoantibodies can be involved in the pathogenesis of autoimmune diseases:

- Tissue-specific autoantibodies may activate complement and lead to cytolysis of the target cell (type II), as is observed for example in spontaneous or drug-induced autoimmune lysis of red or white blood cells. In organ-specific diseases cytotoxic autoantibodies are thought to contribute to tissue destruction, e.g. in autoimmune diseases of endocrine glands, in Goodpasture's syndrome and in myasthenia gravis.
- The binding of autoantibodies to tissues leads to triggering of the natural immune system by attracting and activating Fc receptor and complement receptor bearing cells to phagocytosis, cytotoxicity and antibody-dependent cellular cytotoxicity. Furthermore, the amplification system of inflammatory mediators involving the clotting fibrinolysis and kallikrein-kinin systems can be induced by autoantibodies and complement via activation of factor XII.
- Autoantibodies specific for cell surface structures, e.g. hormone receptors, can stimulate or block the growth and functions of target cells. Stimulatory autoantibodies were first recognized in autoimmune disease of the thyroid gland being directed against the thyroid-stimulating hormone (TSH) receptor, blocking autoantibodies are found for example in myasthenia gravis, and bind specifically to the motor end-plate.
- Autoantibodies specific for soluble cell products can give rise to high levels of circulating immune complexes (type III reactions) causing inflammatory tissue damage at the sites of deposition as seen in both systemic and organ-restricted autoimmune diseases. The localization of immune complexes is dependent on hemorheological factors, Fc binding, and on nonspecific binding characteristics of certain tissues. Hence, an enhanced binding of DNA to basement membranes appears to be responsible for the characteristic deposition of DNA-anti-DNA complexes.
- Autoantibodies with specificity for the idiotype of autoantibodies have been detected in several auto-

immune diseases. Treatment with such autoantibodies has been shown to suppress spontaneous autoimmune disease in animal models, which points to the potential of these autoantibodies to control the course of an autoimmune disease. On the other hand, as anti-idiotypic antibodies can mimic the primary antigen (i.e. autoantigen) autoantibodies could enhance autoimmune disease by eliciting and/or perpetuating an autoimmune response.

The role of T cells

For a long time autoantibodies were thought to play the major role in autoimmune effector mechanisms. Results in both experimental and spontaneous animal models, however, revealed that autoreactive T lymphocytes are both necessary and often sufficient to cause autoimmune disease. This was shown for example in the obese strain of chickens afflicted with spontaneous Hashimoto-like thyroiditis, where eradication of the T cell system by neonatal thymectomy plus treatment with T cell-specific antibodies resulted in prevention of disease. On the other hand, it was shown that the disease could be effectively transferred to healthy recipients of the same genetic background by injection of autospontaneous thymocytes, or purified T cells derived from infiltrated thyroid glands of B cell-depleted (bursectomized) obese strain chickens. The dominance of the autoreactive T effector cell also emerged from the work on experimentally induced autoimmune diseases in rats and mice, where autoantigen-specific T cell lines could be developed that were instrumental in the study of the pathogenetic role of T cell immunity. Transfer of low numbers of viable autoreactive T cells induces vigorous autoimmune disease in several models. If such cells are growth arrested by irradiation prior to transfer, the recipient becomes protected against induction of the disease by immunization with the autoantigen. Taken together, these data clearly point to the fact that both the induction and effector phases of an autoimmune disease can primarily involve autoantigen-specific T lymphocytes. The appearance of autoantibody may often be secondary and have only a modulatory role in the disease.

As to the effector mechanisms by which autoreactive T cells mediate tissue damage, the evidence obtained suggests that it is mainly the CD4⁺ T_H1 phenotype (T_{D1H}) that upon activation by autoantigen in the context of MHC class II molecules releases cytokines, such as IL-2, IFN γ , TNF α/β and others, and which by themselves or by activation of other specific (autoreactive CD8⁺ cells) and nonspecific (NK cells, macrophages) inflammatory cells destroy the target tissue. T_H2 cells, as mentioned above,

supposedly suppress T_H1 effector cells, but, on the other hand, may exert helper functions to autoreactive B cells via IL-4, IL-5, IL-6, and may keep autoreactive T cells alive by IL-10, which – besides its immunosuppressive effects – prevents apoptosis.

Nonspecific mechanisms

Pathogenetic mechanisms of natural immunity were long ago recognized as important in autoimmune disease. This is confirmed by a number of more recent investigations showing the crucial roles of inflammatory monokines IL-1 β , IL-6, and particularly of TNF α , as well as several chemokines in autoimmune tissue destruction. Furthermore, IL-12 produced by several professional antigen-presenting cells has been shown to be critical in the development of autoimmune disease, as it promotes the differentiation of T_H1 cells. The essential role of the monocyte/macrophage series is further underscored by more recent findings in certain animal models, where autoimmune diseases were effectively controlled by antioxidants or by inhibition of nitric oxide synthesis. Until recently, it was thought that nonspecific effector cells were secondarily activated by autoantibodies, autoimmune complexes with or without activation of complement, and cytokines derived from activated T helper cells. The newer concepts of how autotolerance can get lost in the periphery (see above), however, make it likely that a primary abnormal activation of monocytes/macrophages may lead to loss of autotolerance and thereby initiate an autoimmune disease. This view is supported by recent data in the obese strain of chickens with spontaneous autoimmune thyroid disease, and by the induction of lupus-associated autoantibodies in BALB/c mice by i.p. injection of pristane.

It should be stressed that the tissue destruction process in autoimmune diseases with few exceptions has to be regarded as the result of a combination of several humoral and cellular pathogenetic mechanisms.

Modulating factors in autoimmune disease

In discussing factors or conditions that can modify the severity and course of autoimmune disease a clear distinction between etiology and pathogenesis becomes often difficult. This is due to the fact that autoimmune diseases have a multifactorial etiology requiring both genetic features and environmental conditions to occur. The question as to whether a given genetic or epigenetic factor is permissive to an

autoimmune disease or only modifying the pathogenesis is open in most cases.

Genetic predisposition

The first evidence for an involvement of certain genes in the development of autoimmune diseases came from clinical experiences in humans revealing familial incidence of certain autoimmune diseases. This was confirmed by the observations that certain strains of experimental animals spontaneously exhibit enhanced frequencies of certain autoimmune disorders, and that selective breeding for the autoimmune phenotype resulted in further enhanced incidence and severity. The best-established animal models for spontaneous autoimmune diseases are strains of mice with manifestations of systemic lupus erythematosus, and the obese strain chickens afflicted with an organ-specific disease, i.e. autoimmune thyroiditis. Also the susceptibility to the induction of experimentally induced autoimmune disease has been shown to be genetically determined.

Various loci have been found to be involved in the development of autoimmune diseases, some of them are associated with the major histocompatibility complex (MHC). The best example for the association of an autoimmune disease with a certain MHC allele is HLA-B27, which confers an 85–90-fold relative risk of ankylosing spondylitis. Further examples include rheumatoid arthritis associated with HLA-DR4 and insulin-dependent diabetes mellitus (IDDM), with HLA-DR4 and DR3. In the latter it was shown that a certain allele, i.e. HLA-DR2, confers protection with a relative risk of 0.2. Mechanisms that are discussed to explain MHC associations of autoimmune diseases include 1) insufficient thymic presentation of autoantigens, 2) abnormal presentation of autoantigens to peripheral T helper cells, 3) association with alleles of non-MHC loci, such as the genes for TNF α/β , that map to the MHC and are linked with certain MHC alleles, and 4) antigenic mimicry between microbial antigens and autologous MHC molecules.

Another set of genes – not associated with the MHC – is involved in different mechanisms of immunoregulation, and, finally, the susceptibility of the respective target organ in organ-specific autoimmune diseases appears also to be under genetic control. The latter two types of genes have been defined by classical genetic analysis of experimental and spontaneous autoimmune diseases in animal models.

Endocrine and neuroendocrine influences

Immune responses in general are not only regulated by mechanisms intrinsic to the immune system, but also by endocrine and neuroendocrine signals. It has

long been recognized that many autoimmune diseases preferentially occur in females, whereas males appear protected probably due to immunosuppressive properties of androgens. Similar observations were made when obese strain chickens were first developed. However, with continuous selective breeding this sex difference disappeared, which suggests that sex hormones only have a modifying role in this disease.

Hormones involved in stress responses, such as glucocorticoids, catecholamines, endogenous opiates and most of the anterior pituitary hormones, have strong effects on specific and natural immune functions. It is well known that acute or chronic stress can exacerbate certain autoimmune diseases in humans. Chemical sympathectomy (by means of 6-OH-dopamine) has been shown to influence the course of experimental autoimmune disease in animals. Obese strain chickens were found to have enhanced plasma levels of corticoid-binding globulin leading to a diminished availability of free glucocorticoids. Early *in vivo* supplementation with free corticosterone prevented the onset of thyroiditis. Further to this, obese strain chickens were shown to have a central defect in the responsiveness of the hypothalamo-pituitary-adrenal axis to signals of the activated immune system, resulting in the lack of the peripheral glucocorticoid response after immunization as it is observed in healthy controls, and it was concluded that the defect in this negative feedback may predispose to autoimmunity. Findings in two experimentally induced models, i.e. experimentally induced encephalomyelitis and experimentally induced arthritis in rats, strongly supported this concept, and there is now good evidence that human patients suffering from rheumatoid arthritis show endocrine abnormalities indicating altered pituitary functions, which may contribute to etiology and/or pathogenesis of the disease.

Age

Aging is known to be associated with increased autoimmune phenomena. However, these increased 'forbidden' responses as manifested in mildly elevated levels of autoantibodies are not necessarily pathogenic. Some authors have assigned this age-associated autoimmunity even a physiological role as a possible scavenger mechanism for cell and tissue constituents that accumulate due to enhanced tissue damage at higher age. Most of the acute severe autoimmune diseases usually start at young to middle ages, only certain chronic processes, e.g. chronic polyarthritis, tend to severe at higher ages.

Environmental influences

Little is known about environmental influences on autoimmune disease. In general, any exogenous agent or condition that affects the immunogenic properties of autoantigens and/or the regulation of the immune response can be expected to modify autoimmune diseases. Modification of autoantigens and/or immune reactivity can occur by several bacterial and viral infections, and the outbreak of several autoimmune diseases indeed appears to be associated with certain infectious diseases, such as IDDM or multiple sclerosis following certain viral infections. Another way by which bacterial or viral infections may induce autoimmune disease is the abovementioned antigenic cross-reaction with autoantigens.

Autoimmune diseases as side-effects of drugs comprise type II reactions against drug-modified autologous white or red blood cells, but also lupus-like phenomena in patients taking β -adrenergic blockers or drugs like hydralazine and procainamide. While the mechanism(s) of action of β -blockers in this respect is not clear, *in vitro* investigations have shown that hydralazine and procainamide induces self-reactivity in cloned, heteroantigen-specific T cell lines by DNA methylation. This suggests that DNA methylation as a mechanism of gene expression plays a role in the specificity of immune responses, and that drugs or substances that interfere with the regulation of gene expression in general may have the potential to induce forbidden immune responses leading to autoimmune disease.

See also: Aging and the immune system; Autoantigens; Autoimmune disease, induced experimental models; Autoimmune diseases; Autoimmunity; Glucocorticoids; Goodpasture's syndrome; Hypersensitivity reactions; Immune complexes; Insulin-dependent diabetes mellitus, human; MHC disease associations; Neuromuscular junction autoimmunity; Neuroendocrine regulation of immunity; Sex hormones and immunity; Systemic lupus erythematosus, experimental models; Systemic lupus erythematosus (SLE), human; Thyroid autoimmunity, experimental models; Thyroid autoimmunity, human; Tissue typing.

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AUTOIMMUNE DISEASE, SPONTANEOUS ANIMAL MODELS

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Autoimmune diseases can be experimentally induced by immunization of normal animals with the respective antigens combined with appropriate adjuvants. By this means organ-specific experimental autoimmune diseases can be produced in animals of many different strains and in nearly every organ. There is no doubt that such experimentally induced autoimmune diseases, e.g. experimental allergic encephalomyelitis (EAE) or experimental autoimmune thyroiditis (EAT), have contributed considerably to our understanding of the pathogenesis of autoimmune diseases in general and these models in particular. It is, however, also clear that most of these artificially produced autoimmune diseases often do not parallel their human counterparts with respect to all essential characteristics.

Fortunately, there are several animal strains available that – based on many years of selective breeding – develop certain organ-specific or systemic autoimmune diseases spontaneously, i.e. without any experimental manipulation. These models mimic the human situation much more closely and thus serve as apt objects of study for the pathogenetic mechanisms underlying the development of these diseases as well as the establishment of new diagnostic and therapeutic strategies. In this entry, spontaneous animal models for four types of diseases, two systemic and two organ specific, will be presented. This selection

is based on the large body of literature on these models, but it should be emphasized that several other promising strains exist which have not yet been investigated in such depth but may also turn out to be very useful in the future, such as the Buffalo (BUF) rat that develops an autoimmune thyroiditis in high frequency, the autoimmune glomerulonephritis, arthritis and orchitis emerging in Aleutian minks, the delayed amenalosis (DAM) chicken with autoimmune depigmentation, and several others.

In principle, spontaneous autoimmune models arose due to a combination of serendipity (spontaneous mutations causing autoimmune phenotypes) and inbreeding plus selection procedures. As a consequence, inbred animal strains afflicted by spontaneous autoimmune diseases usually manifest disease phenotypes that are compatible with breeding programs, i.e. that do not cause severe pathology before animals reach sexual maturity, although they sometimes may need special dietary, hormonal, etc. supplementation. Alternatively, autoimmune phenotypes are studied in severely diseased F1 crosses of relatively normal parental inbred strains. This particular feature of spontaneous autoimmune disease models may explain why certain laboratory-produced mutations (e.g. disruption of the CTLA4 gene) that give rise to an acute, fulminant autoimmune disorder have not been observed spontaneously.

Spontaneous animal models for organ-specific autoimmune diseases

Obese strain of chickens as an animal model for Hashimoto's thyroiditis

During the first few weeks after hatching, obese strain (OS) chickens develop a hereditary spontaneous autoimmune thyroiditis (SAT) that resembles human Hashimoto's disease in all clinical, histopathological, endocrinological and immunological aspects. The thyroid glands of OS chickens become heavily infiltrated by mononuclear cells, finally resulting in complete destruction of their architecture entailing clinical symptoms of hypothyroidism (Figure 1), such as small body size – albeit with relatively high body weight due to the deposition of subcutaneous and abdominal fat – lipemic serum, long silky feathers, cold sensitivity, low laying capacity, diminished fertility and poor hatchability. In common with Hashimoto's thyroiditis, the infiltrated thyroid glands show high numbers of well-developed germinal centers as a characteristic hallmark that is unique to this model and absent in EAT. The serum of OS chickens contains autoantibodies to thyroglobulin (Tg-AAb), to thyroid microsomal antigens (MSAs) and to tri-iodothyronine (T_3) and thyroxine (T_4). The hypothyroid symptoms can be prevented or reversed by appropriate supplementation of the diet with thyroid hormones.

The infiltrated thyroid gland itself has been identified

as a major Tg-AAb producing organ and complement-binding Tg-AAbs are also vertically transferred from the mother hen via the egg yolk into the newly hatched chick. The main initial effector mechanism for the development of SAT is, however, the infiltration by interleukin-2 receptor (IL-2R)⁺ CD4⁺ T cells, and Tg-AAbs only play an accelerating role.

OS chickens also develop autoantibodies against a variety of nonthyroid organ-specific or systemic autoantigens, but without concomitant histopathological or functional lesions. Adoptive transfer experiments have shown that thyroid-infiltrating lymphocytes are most effective in transferring the disease into histocompatible recipients of the Cornell C strain (CS), the nearly normal mother strain from which the OS was originally developed by R.K. Cole by selective breeding. Functional studies revealed a general hyperreactivity of the OS immune system against autoantigens, but also against exogenous antigens, such as sheep red blood cells and Rous sarcoma virus-induced tumors, as well as the T cell mitogens concanavalin A (con A) and phytohemagglutinin (PHA). This hyperreactivity is paralleled by a significantly increased production of IL-2 by OS peripheral blood lymphocytes and spleen cells compared with normal controls.

OS macrophages also show signs of hyperreactivity, such as an increased oxidative burst activity. Coculture experiments combining thymocytes and autologous peripheral blood lymphocytes revealed a

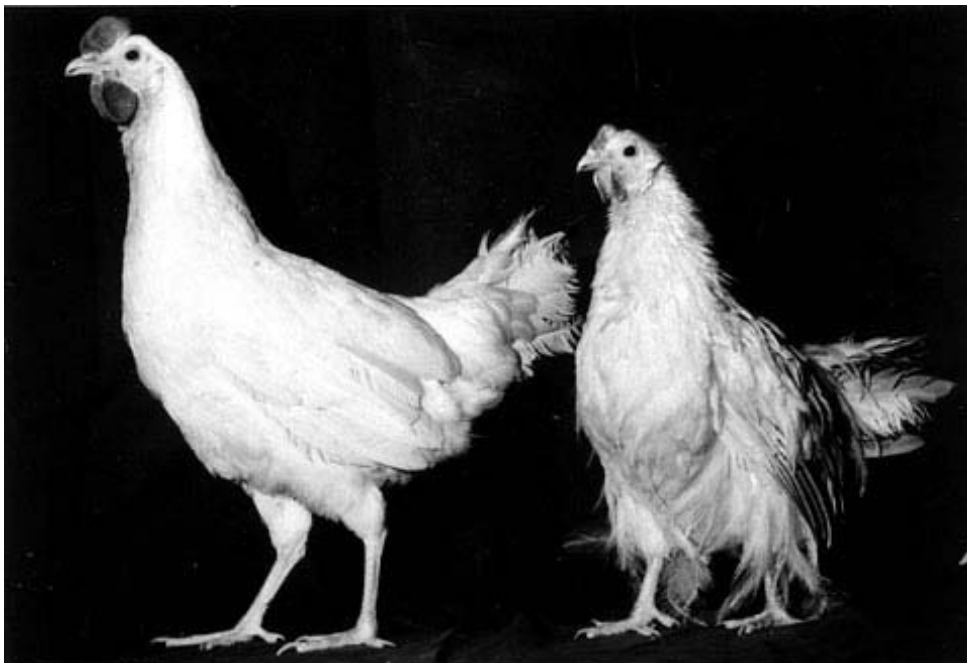


Figure 1 Normal White Leghorn chicken (left), and 6-month-old female OS chicken (right). Note the smaller size and the long silky feathers of the OS bird.

defect of the suppressive activity in the OS thymus that contributes to the general immunological hyper-reactivity in this strain.

Furthermore, OS chicken thymocytes are relatively resistant against glucocorticoid-induced thymocyte apoptosis, even if high pharmacological doses are applied. This phenomenon may be involved in the inefficient thymic negative selection process in this strain, leading to insufficient deletion of auto-reactive clones.

In addition, factors extrinsic to the immune system that also affect immunoregulation are altered in OS chickens, such as a significantly increased serum concentration of corticosteroid-binding globulin (CBG) and, therefore, decreased free, metabolically active glucocorticoids. Furthermore, OS chickens show a malfunctioning immunoendocrine feedback loop: injections of antigen or IL-1 do not lead to the surge of blood corticosterone levels observed in normal birds. This altered glucocorticoid response is due neither to a defective production of glucocorticoid increasing factors (mainly IL-1) by the immune system of the OS, nor to a disturbed release of adrenocorticotrophic hormone (ACTH) by the pituitary, nor to insufficient responsiveness of the adrenal glands, but can rather be attributed to an altered responsiveness of the hypothalamus to glucocorticoid-increasing factors or of the pituitary to corticotropin-releasing hormone (CRH).

Transfer experiments and cross-breeding studies between OS and inbred normal chickens led to the assumption that *both* autoreactivity of the immune system and a genetically determined primary susceptibility of the target organ are prerequisites for the development of severe SAT.

Various functional abnormalities of OS thyroid glands, such as an increased ^{131}I uptake before the onset of infiltration, an autonomous thyroid function that cannot be suppressed by high doses of T_4 , and a decreased growth rate of OS thyroid epithelial cells *in vitro* with insufficient release of growth-promoting autocrine factors, can be demonstrated, but have not been found to be essential for the development of SAT. There is also no indication for an abnormal composition of OS thyroglobulin.

The question of whether there is a genetically determined primary aberrant expression of major histocompatibility complex (MHC) class II antigens on thyroid epithelial cells of OS chickens has not yet been unequivocally clarified. OS thyroid epithelial cells do, however, clearly show a lower threshold for interferon γ -induced MHC class II expression compared with those of normal chickens, a phenomenon that is restricted to the thyroid gland and cannot be

found with cell suspensions of other organs, such as the adrenal gland and the kidney.

Investigations on the putative role of viruses during the development of SAT led to interesting findings in two directions. First, C-type particles have been demonstrated electronmicroscopically in the thyroid gland of OS but not of normal chickens, and this indication for a possible presence of a thyrotropic virus has recently been corroborated by the demonstration of 2', 5'-adenylate synthetase in the thyroid, an enzyme that is induced by interferon γ and activated by double-stranded RNA. Second, parallel studies have led to the discovery of a new endogenous virus (*ev22*) locus that seems to be specific for the OS and the presence of which (in Southern blots of OS DNA digests) correlates significantly with the above-mentioned decreased corticosterone response to IL-1 or antigen. It is not yet clear if this latter observation has any pathophysiological significance.

OS chickens possess three MHC (*B*-locus in the chicken) haplotypes (*B5*, *B13* and *B15*) but each of the homozygous sublines carrying these haplotypes still segregates for other major and minor histocompatibility antigens. The haplotypes were found to have only a modulating effect on the severity and incidence of SAT.

Genetic analyses that were aimed at the identification of the number and nature of genes that possibly contribute to the development of SAT have led to the formulation of a hypothesis postulating the contribution of both essential and modulatory genes. From these studies it became clear that *one essential recessive autosomal 'thyroid susceptibility gene'* is a prerequisite for the development of SAT. So far, this gene or the product of it, has not been identified. Possible candidates for additional modulating, non-essential genes are those that regulate CBG levels and T cell hyperresponsiveness, those determining excessive macrophage function, the *ev22* locus which cosegregates with the altered corticosterone response to cytokines, the primary altered iodine thyroid metabolism, and MHC-linked immune response genes.

Finally, it should be mentioned that the OS model has proven to be of great value for the assessment of new immunosuppressive drugs, such as synthetic androgen analogs that possess immunosuppressive and anti-inflammatory properties but are devoid of undesired endocrinological side-effects.

Animal strains with spontaneous insulin-dependent diabetes mellitus (IDDM)

Many animal species may be afflicted with spontaneous IDDM, but so far only two inbred models

of IDDM have been subjected to detailed immunological and genetic analysis: the nonobese diabetic mouse (NOD) mouse and the diabetes-prone biobreeding (DP-BB) rat. In both cases the autoimmune etiology of IDDM has been clearly established based on histological (mononuclear cell infiltration of the islets of Langerhans before the development of clinically manifest diabetes) as well as functional evidence. DP-BB rats and NOD mice exhibit both humoral and cellular autoimmune responses against islet β cells, although the pathogenetically relevant antigens remain to be identified. Disease development is inhibited by immunosuppressive intervention (neonatal thymectomy, administration of cyclosporin A, monoclonal antibodies against CD4, class II molecules, or the IL-2R knockout of the β_2 -microglobulin gene) and autoimmune insulinitis and β cell destruction can be adoptively transferred to healthy histocompatible recipients by bone marrow or spleen cells from NOD or DP-BB donors. The role of T cells is further underlined by the predominance of T cells in infiltrated islets, the absence of diabetes in NOD nu/nu mice, and the efficient transfer of disease into B cell-deprived recipients. Recent studies on the cell type responsible for diabetes transfer indicate that CD4⁺ T_H1 but not T_H2 cells are responsible for β cell destruction, both in NOD mice and in DP-BB mice. Thus, autoimmune diabetes is a T_H1 disease. Whether T_H2-like cells may act as endogenous immunosuppressors is, however, still a matter of debate.

About 40% of male and 70% of female NOD mice develop overt IDDM at 7 months of age, whereas the DP-BB model lacks a female preponderance. Interestingly, a low percentage of DP-BB rats also develop autoimmune thyroiditis. Interindividual differences in the expression of disease may be attributed to unknown environmental factors or to the stochastic process of T cell receptor and antibody gene rearrangement which creates genetic heterogeneity between individuals with identical germline genes. Extensive studies have addressed the genetics of IDDM and the molecular defects implicated in the etiopathogenesis of the disease. In the NOD model, one dominant gene confers predisposition to lymphoid infiltration of pancreatic islets, whereas the development of manifest IDDM requires at least several further genes, one of which is linked to the NOD MHC. NOD mice lack messenger RNA transcribed from the I-E α gene and, consequently, do not express I-E products on the cell surface. Moreover, the NOD class III-A β chain is mutated in positions 56 and 57 of the first external domain. Expression of I-E α transgenes in NOD mice prevents insulinitis, whereas I-A β of a normal haplotype fails to do so, thus

underlining the importance of the I-E defect for IDDM development. In addition to the diabetogenic NOD MHC (termed *Idd1*), at least 10–14 different IDDM susceptibility loci (*Idd2* through *Idd14*) influence the incidence and the severity of insulinitis and diabetes. The best fitting model for these loci is a multiplicative (epistatic) model. Thus far, the pathogenic contribution of these loci remains elusive at the molecular level. It may be significant that one of the insulinitis susceptibility loci has been mapped close to the apoptosis-inhibitory oncogene *bcl-2*. Moreover evidence was obtained for linkage of *Idd3* with IL-2 and *Idd10* with IgG-Fc receptor polymorphisms. In the DP-BB rat, two recessive diabetogenic loci have been postulated. One of them (*Iddm2*) appears to be associated with the class II subregion of the MHC, the other (*Iddm1*) with a gene responsible for a profound T cell lymphopenia which may be caused by defects in prothymocyte differentiation and/or thymic antigen-presenting cells. The pathogenetic relevance of ectopic MHC class II expression on islet β cells is still a matter of debate, but experiments with transgenic animals carrying class II genes under the control of the insulin promoter suggest that the presence of such molecules on β cells *per se* is insufficient to initiate autoimmune insulinitis. In contrast, expression of the costimulatory molecule B7-1 markedly accelerates diabetes in transgenic NOD mice.

Spontaneous animal models with systemic autoimmune diseases

University of California at Davis (UCD) 200 strain as an animal model for progressive systemic sclerosis (SSc) – scleroderma

SSc is a human disease that is classified among the autoimmune connective tissue diseases that also comprise systemic lupus erythematosus (SLE), dermatomyositis and polymyositis, rheumatoid arthritis and several others. While there are several well-established, spontaneously occurring animal models available for SLE, models for the other conditions in this group are either nonexistent or of questionable significance. This is also true for SSc for which homologous disease of rats has been proposed as a model, as it is associated with chronic skin lesions with increased thickening and collagenization of the dermis. Significant titers of antinuclear antibodies (ANAs) are, however, lacking and the presence of immune complexes in vascular walls is also not characteristic for SSc. Another model for human scleroderma is the tight-skin (TSK) mouse, a dominant mutant of the inbred B10.D2 (58N/SN) mouse strain. The TSK mouse is characterized by cutaneous

and skeletal hyperplasia but – in contrast to human SSc – these animals do not show gastrointestinal involvement, particularly the characteristic vascular pathology. There is also no evidence in these mice of early mononuclear cell infiltration of the skin.

The UCD 200 chicken model, described below, has many more similarities with SSc than all the models described so far, but it has to be emphasized that there are also some dissimilarities, including the development of glomerulonephritis, particularly in older animals, that may, however, be a sequel of secondary infection.

The first (male) chickens showing signs of a genetically determined fibrotic disease resembling SSc were discovered in 1942 by P. Bernier at the Department of Poultry Husbandry, Oregon State University, Corvallis. These birds exhibited comb and dermal swelling accompanied by fibrosis. The affected birds were then selectively bred for over 40 years and a new strain, now denoted UCD 200, was developed where males and females are affected to about the same extent.

200 strain UCD chickens exhibit severe alterations of the comb, neck and extremities. The comb already becomes visibly swollen and red 1–2 weeks after hatching, and at the age of 4 weeks about 90% of all birds develop comb necrosis with subsequent resorption ('self-dubbing'). Similar events occur in the digits of 30–40% of the birds, again beginning at 2–3 weeks of age, including polyarthritis of peripheral joints. At 2–4 weeks of age the skin of the neck also becomes involved, but the constant mechanical irritation of the neck in the outlets of the cages to gain access to food and water may also contribute to the lesions at this particular location.

A mononuclear cell infiltration is first observed in the affected skin areas and can be demonstrated in about 70% of line 200 birds at 1 week of age. Mononuclear cell infiltration starts perivascularly and is followed by vessel occlusion, similar to one of the hallmarks of human SSc. This acute inflammatory process reaches a peak at 2 weeks of age and subsides at the age of 5 months, when no more skin inflammation can be shown macroscopically or microscopically. At 5 months of age the esophagus becomes involved with a severe cellular infiltration around vessels and in the lamina propria of mucous glands in about 50% of the animals, increasing to 70% or more at the age of 1 year. Similar changes can be observed in the lamina propria of the small intestine, albeit at a lower frequency of about 30%. Furthermore, mononuclear cell accumulations can be found in the lung, starting at 1 week of age (60%) and decreasing to about 30% at 4–6 weeks, in the heart

and kidney (30–40% at 5–6 weeks of age) and the testes, beginning at 4–6 weeks of age.

The early mononuclear cell infiltrates in the skin are composed mainly of T cells expressing the γ/δ receptor. An influx of α/β T cell receptor-positive cells is first observed later, i.e. at the age of 20 days. While γ/δ -positive T cells occur mainly in the upper layers of the dermis, α/β -positive cells prevail in the deeper areas. Most of these latter cells express B-L, the chicken MHC class II analog.

The vascular changes are very characteristic in UCD 200 birds and can be found in most of the organs mentioned above. They are first noted in the comb and the feathered skin at 1 week of age, where the vessels show hyperplasia of the media and intimal thickening. In the internal organs the vascular changes are found later but always precede the macroscopic and clinical symptoms.

The nature of the increased deposition has not yet been studied in great detail by chemical and molecular biological methods but only by immunohistology on frozen sections, using specific antibodies against the different collagen variants. With this approach an increase of type III and type VI collagens can be noted in the skin and the terminal vascular rarefaction becomes clearly evident when antibodies against basement membrane components, such as type IV collagen or laminin, are applied. The accumulation of connective tissue, as manifested by fibrosis of the lung, severe myocardial muscle fiber destruction, subpericardial fibrosis, thickening of the esophageal wall and effects on other internal organs, leads to the premature death of these birds. In addition, they are prone to secondary infections and show a low fertility rate due to the massive involvement of the gonads, particularly the testes.

As in the human disease, no gross alteration at the genomic level of collagen genes has been found by means of restriction fragment length polymorphism (RFLP) analysis, thus suggesting that the UCD 200 model is an appropriate counterpart for studying the altered collagen metabolism in SSc. Investigation of the putative role of endogenous viruses (*ev*) for the development of SSc led to the discovery of *ev23*, which is found only in UCD 200 chickens. So far, the role of *ev23* in the development of avian scleroderma is unclear.

Similar to human patients with SSc, sera from UCD 200 chickens contain ANAs in high frequency. At 1 month of age over 70% of the birds show ANAs with various staining patterns, including speckled centromeric and nucleolar fluorescence that are also characteristic of human SSc. These antibodies belong mainly to the IgY isotype, homologous to mam-

malian IgG, and can also be demonstrated on mammalian substrates, such as HEp-2 cells.

Although the UCD 200 chickens have now been kept for over 40 years, there are only a very few reports in the literature addressing the possible pathogenetic mechanisms leading to the SSc-like disease in this strain. The group of Gershwin and colleagues has shown that thymectomy on the day of hatching significantly reduces – but not completely abolishes – the comb/neck lesions, when assessed at the age of 8 weeks. Furthermore, the severity of the lesions in general was significantly reduced by this manipulation.

In vitro, UCD 200 peripheral blood T lymphocytes show a significantly decreased mitogen-induced proliferation associated with a decreased capacity to produce IL-2 and to express IL-2 receptors compared with normal White Leghorn chickens. In contrast to the deficient *in vitro* IL-2 production, the sera of UCD 200 chickens contain significantly higher levels of IL-2 bioactivity.

UCD 200 chickens also show a disturbed immunendocrine feedback, characterized by a prolonged secretion of corticosterone after application of certain cytokines. Although the peak serum concentrations of the glucocorticoids are equal to that of controls, UCD 200 have to secrete twice as much ACTH to achieve this corticosterone serum level, owing to an apparent hyporesponsiveness of the adrenal gland to this secretagogue. The altered cytokine-induced glucocorticoid secretion is found in early as well as in chronic, sclerotic stages of the disease.

Recent studies by Sgontz and coworkers brought forward an interesting aspect on the pathogenesis of SSc: simultaneous *in situ* analysis of frozen skin sections for apoptosis and immunofluorescence staining for cell markers show that endothelial cells are clearly the first cells to undergo apoptosis in the skin of UCD 200 chickens, a process that seems to be induced by antibody-dependent cytotoxicity. Endothelial cell apoptosis is found before any other macroscopical or microscopical alterations are visible. Analysis of early lesion skin biopsies from human SSc patients shows a similar course of events.

The UCD 200 chickens are homozygous for a variation of the MHC haplotype B17. In addition they have been typed for some of the other blood groups and found to carry A1, C2, D3, E7 and P2. They are homozygous for the immunoglobulin 7S Ig (homologous to mammalian IgG) allotype 1.7. Since UCD 200 chickens are still segregating at the B locus and some other major blood group loci, attempts have been made in recent years to develop a subline that matches one of the related inbred normal lines

(line 058) in order to allow for mutual cell transfer experiments. Such a subline, denoted line 206, carrying the B15 haplotype has now been established.

In summary, the UCD 200 line of chickens seems to provide the best model for human SSc available so far. It mimics most of the clinical, histopathological and immunological hallmarks of SSc, albeit in an accelerated fashion. With respect to more detailed further studies, particularly the development of new therapeutic strategies, this accelerated course of the disease is an experimental advantage.

Murine strains with SLE-like autoimmunity

Several models of spontaneous SLE in mice have been extensively studied, namely New Zealand Black [NZB], (NZB × New Zealand White [NZW])F1, (NZB × SWR)F1, BXSB, and MRL mice, which exhibit two forms of disease, a late-life variety appearing during the second year of life (male [NZB × W]F1, female BXSB, MRL/Mp-*+/+* of both sexes, NZB mice of both sexes) and an acute form beginning a few weeks or months after birth (female [NZB × W]F1, male BXSB, MRL/Mp mice of both sexes homozygous for the lymphoproliferation [*lpr*] gene, female and male [NZB × SWR]F1 mice). Accelerating factors are female sex hormones in (NZB × W)F1, a gene located on the Y chromosome (*Yaa* = Y chromosome-lined autoimmunity accelerator) of BXSB mice, the *lpr* gene in MRL mice, or genes from phenotypically normal SWR or minimally affected NZW mice in crosses with the NZB strain. All these models are characterized by hypergammaglobulinemia, anti-DNA and other ANAs, antiretroviral gp70 antibodies, circulating immune complexes, glomerulonephritis, and a low percentage of coronary arthritis. MRL/Mp-*lpr/lpr* mice also develop necrotizing polyarteritis and a rheumatoid arthritis (RA)-like disease accompanied by rheumatoid factors in serum. The NZB strain develops autoimmune hemolytic anemia.

Experiments addressing the genetics and the cellular or molecular mechanisms of SLE in these murine strains revealed that murine lupus is a polygenetic and multifactorial process, involving a combination of various genetically determined defects, which at least in part are different in each of the five models mentioned above. Studies of bone marrow irradiation chimeras indicate that murine lupus is caused by abnormalities at the hematopoietic stem cell level. The relative roles of T and B cells in the initiation and perpetuation of autoaggression are still a matter of debate. However, it appears clear that B cell hyperreactivity of lupus-prone mice is not due to defects intrinsic to immunoglobulin genes, but rather depends on T helper cells and/or polyclonal acti-

vation signals. Autoantibodies are of poly- or oligoclonal origin, the nephritogenic ones belonging to the IgG isotype and being cationic in charge. On the cellular level, dependent of the strain investigated and the age of donors, conflicting results have been obtained as far as the production of cytokines (e.g. IL-2), the response to them (e.g. IL-2 and IL-5), the cooperation between T and B cells, and the distribution of lymphocyte subsets are concerned. Both the *lpr* and the phenotypically similar, but non-allelic, *gld* (generalized lymphoproliferative disease) genes result in a massive accumulation of abnormal Thy-1⁺ CD5⁺ CD4⁻ CD8⁻ CD3⁺ TCR- $\alpha\beta$ ⁺ T cells, which anomalously express B cell markers (B220, PC1) and appear to be of polyclonal origin, the V β 8.2 and V β 8.3 T cell receptor genes being over-represented. Homozygosity in the recessive *lpr* or the *lpr^{cs}* mutations affects the expression (*lpr*) or signal-transducing capability (*lpr^{cs}*) of the CD95/Fas/Ap-1 surface receptor that initiates apoptosis-inducing pathways upon interactions with its ligand FasL, the molecule affected by the *gld* mutation. Since Fas is involved in the activation-induced cell death of T lymphocytes it is assumed that peripheral deletion is at least partially compromised in *lpr/lpr* and *gld/gld* mice. Moreover, it is known that Fas expressed on anergic B cells can receive apoptosis-inducing signals via interaction with FasL on T cells. Therefore, the *lpr* or *gld* mutation may compromise both T and B cell tolerance.

A dominant NZW locus contributing to severe SLE in (NZB $\times$ W)F1 maps within the H-2 region. This may be either due to the unique sequence of the NZW I-E β chain gene or to reduce production of the MHC class III product tumor necrosis factor (TNF). This latter finding could be pathogenetically relevant since treatment with TNF postpones the development of nephritis. Heterozygosity for the I-A β haplotypes of NZB and SWR has also been shown to correlate with the development of glomerulonephritis in crosses of the NZB strain with SWR mice. In other strains, no strong correlation with a particular H-2 haplotype was observed. Heterozygosity for the T cell receptor strongly correlates with nephritis in (NZB $\times$ SWR)F1 $\times$ NZB backcrosses, thus suggesting that the deletion of about half the T cell receptor V β genes which is found in healthy SWR parents might contribute to the development of SLE. In contrast, the NZW T cell receptor β chain locus, the C β 1, D β 1 and J β 1 regions of which have been deleted, probably does not contribute to the SLE of (NZB $\times$ W) hybrids.

In summary, the etiology of murine lupus appears to be rather complex and awaits further elucidation. Multiple loci accounting for different disease mani-

festations are currently under molecular study using a combination of classical genetic approaches (phenotyping of intercrosses or backcrosses) and microsatellite-based chromosomal maps. Interestingly, some of the loci determining lupus of (NZB $\times$ NZW)F1 mice map close to loci determining glomerulonephritis in MRL/*lpr* mice or diabetes in NOD mice, suggesting the existence of shared genetic mechanisms for some autoimmune disorders.

Conclusions

Animal models of spontaneous autoimmune disease have substantially contributed to our knowledge on the etiopathogenesis, genetics and molecular defects ultimately responsible for autoaggression. A variety of different mechanisms, including defects in the lymphoid lineages, endocrinological alterations, target organ defects, endogenous viruses or mutations in immunologically relevant molecules (e.g. MHC, T cell receptor genes), have been implicated in animal autoimmune disease and, in part, are also being described in human disease. Furthermore, the avant-garde position of animal models on the frontier of immunopathological research has allowed for the development of new therapeutic strategies for the treatment of autoaggression.

See also: Autoantigens; Autoimmune disease, induced experimental models; Autoimmune disease, pathogenesis; Autoimmune diseases; Autoimmunity; Insulin-dependent diabetes mellitus, animal models; MHC disease associations; Systemic lupus erythematosus, experimental models.

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AUTOIMMUNE DISEASES

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Autoimmunity and autoimmune disease

Autoimmune reactions to autologous molecules occur in health and may well be ubiquitous. Autoimmune disease is defined when such reactions become uncontrolled and result in structural or functional pathology. Normally there exists a physiological (natural) immunological tolerance to self constituents; previously, this was thought to be absolute, such that any immunological reactivity with autologous molecules would be abnormal. However, currently, tolerance can be expressed either as an *absolute absence*, or a *controlled level*, of autologous reactivity. Pathogenic autoimmunity might thus represent either an amplification of naturally occurring autologous reactivity, or be in some way qualitatively different.

Natural autoimmunity could serve physiological purposes: 1) early defense against infection with microorganisms that share cross-reactive epitopes with autologous molecules; 2) disposal of products of injury or tissue catabolism; or 3) facilitation of expansion in early life of immunological repertoires

through mutual stimulation of the antigen receptors on B cells due to anti-idiotypic connectivities. Natural autoimmunity includes both humoral (autoantibody) and cellular (T cell) activities. Natural autoantibodies are usually described as being of immunoglobulin M (IgM) isotype, broadly reactive, non-pathogenic and, to some degree, products of a particular lineage (B-1) of B cells characterized by the CD5 marker; in contrast, disease-related autoantibodies are class-switched to the IgG isotype, of focused reactivity, often pathogenic and are derived from somatically mutated Ig variable region (V) genes. However, some naturally occurring autoantibodies also exhibit these properties and, accordingly, would be difficult to distinguish from disease-related IgG autoantibodies in presymptomatic stages of autoimmune disease. Likewise, there are naturally occurring T lymphocytes reactive to various autoantigens, albeit at much lower numbers in blood than those observed in autoimmune disease. This is relevant to the diagnosis of autoimmune disease (see below), for which a major criterion is the coassociation of an 'appropriate' autoantibody; discriminative cut-off levels must optimally distinguish between

natural background reactivity and pathogenetically significant titers. Where T cell reactivity to autoantigen is applicable to the definition of an autoimmune disease, similar considerations will apply.

Designation of autoimmunity as a cause of a disease

Some authors, in discussing a particular disease, refer to it as 'an autoimmune disorder of unknown etiology'! Others even question whether autoimmunity is a legitimate etiology for any disease, perhaps forgetting Harrington's experiment in the 1950s wherein plasma transferred from a donor with 'idiopathic' thrombocytopenic purpura caused, in volunteer recipients, an immediate fall in blood platelet levels due to pathogenic anti-platelet autoantibodies. Witelsky in 1956 presented four 'postulates' to nominate a disease as autoimmune: 1) demonstration of free circulating antibodies that are active at room temperature, or of cell-bound antibodies detected using indirect means; 2) the recognition of the specific antigen against which these are directed; 3) the production of antibodies against the analogous antigen in actively immunized animals; and 4) pathological changes in the tissues of the actively sensitized animal resembling those of the corresponding human disease. However, the lack of models (components 3 and 4) precludes fulfillment of these postulates in many human autoimmune diseases.

Bayesian reasoning provides clinical and/or pathological features that can be factored in to presume an autoimmune basis for a given disease. These include: female gender; coassociation in the patient or family members of other diseases of autoimmune nature; histological evidence of 'organized' lymphoid infiltrates in the affected tissue including germinal centers, or deposition of immune complexes that contain autoantigen; association of the disease with particular alleles (usually class II) or the major histocompatibility complex (MHC), HLA in humans; and brisk responsiveness to corticosteroid drugs. As further evidence for an autoimmune basis for a given disease, there are models in animals, either spontaneous (lupus, hemolytic anemia or diabetes in particular strains of mice) or induced by immunization with the corresponding tissue in adjuvants (thyroiditis or encephalomyelitis in various species). Finally, in diseases designated as autoimmune, no other provocative causes should be demonstrable. Indeed, in chronic destructive diseases that *are* demonstrably due to a persisting microorganism, hallmarks of autoimmunity tend to be conspicuously absent, e.g. gastric mucosal destruction due to infection with *Helicobacter pylori* does not provoke the

parietal cell antibody response characteristic of autoimmune (pernicious anemia-type) gastritis. But there are exceptions, including spirochetal infection (cardiolipin antibody), hepatitis C virus infection (liver microsomal antibody, occasionally) and others.

In the absence of firm nominative criteria, a listing of likely autoimmune diseases is necessarily provisional (Table 1). A somewhat imperfect classification is as follows:

1. Organ and autoantibody specific, i.e. diseases in the 'thyrogastric cluster'.
2. Tissue and autoantibody specific, i.e. diseases in which a 'disease-relevant' target autoantigen exists, but this is widely 'dispersed' throughout the body e.g. hemolytic anemia (erythrocytes), myasthenia gravis (acetylcholine receptor).
3. Diseases that are organ or tissue specific, and in which there is a disease-specific autoantigen(s), but this is distributed in all cells and thus not 'disease relevant', e.g. primary biliary cirrhosis and mitochondrial autoantigens, or Sjögren's syndrome and ribonucleoprotein autoantigens.

Table 1 A partial list of autoimmune diseases

Hashimoto's thyroiditis
Primary myxedema
Graves' disease
Pernicious anemia
Autoimmune atrophic gastritis, pernicious anemia
Addison's disease
Ovarian failure, premature menopause
Insulin-dependent diabetes mellitus
Goodpasture's syndrome
Myasthenia gravis, Lambert-Eaton syndrome
Male infertility (few cases)
Pemphigus, bullous pemphigoid
Psoriasis (possibly)
Crohn's disease (possibly)
Sympathetic ophthalmia
Autoimmune uveitis
Multiple sclerosis
Autoimmune hemolytic anemia
Autoimmune thrombocytopenic purpura
Autoimmune neutropenia
Primary biliary cirrhosis
Chronic active hepatitis, 'cryptogenic' cirrhosis
Ulcerative colitis (possibly)
Sjögren's syndrome
Rheumatoid arthritis
Dermatomyositis
Polymyositis
Scleroderma
Anti-phospholipid syndrome
Mixed connective tissue disease
Vasculitis
Vitiligo
Systemic lupus erythematosus

4. Diseases that are multisystem and 'nonfocused', with autoimmune reactivity against antigens associated with various non-tissue-specific intracellular organelles, e.g. systemic lupus erythematosus (SLE).

Etiology and pathogenesis

Germline genetic influences

An important genetic component in autoimmune disease is female gender, with the female:male ratio ranging up to tenfold, although a notable exception is insulin-dependent diabetes mellitus (IDDM) that occurs at prepubertal ages with an equal gender incidence. The influence of female sex hormones on immune responsiveness, and perhaps an immunopotentiating gene on the X chromosome, explains the female predisposition to autoimmunity. A strong genetic predisposition to autoimmunity is conferred by alleles of the MHC, in humans and in experimental models, particularly at the class II loci, HLA-DR, -DQ, -DP rather than the class I loci, HLA-A or -B. Class II alleles encode heterodimers that present antigenic epitopes to T cell receptors, and such MHC molecules may contain, in their hyper-variable regions, a sequence of amino acids that represents the disease susceptibility motif (DSM); this explains for example the HLA associations, DR1, DR4, with rheumatoid arthritis. The DSM may confer a high binding affinity for an autoepitope that can engage the T cell receptor, or may act to bias the thymic selection of the T cell repertoire.

Studies on genetic linkages in families with multiple members with an autoimmune disease e.g. IDDM, or in animals using backcrossing and chromosomal localization, e.g. lupus mice, indicate the existence of many other gene loci, encoding either 'immunologically relevant' genes or 'non-immunologic' (background) genes, that confer or alleviate risk for autoimmunity. Examples would be genes that govern expression of cytokines, chemokines or adhesion molecules, or genes that influence expression of molecules that promote or inhibit apoptosis, *fas* and *bcl*. Still further genetic effects on autoimmunity could relate to the antigenic composition of the target organ itself, or to autoantigenic molecules as products of alternatively spliced genes, isoforms of enzymes, or to other structural or functional polymorphisms of potential autoantigens.

Somatic genetic influences

Susceptibility to autoimmune disease may depend on somatic genetic effects (mutations) in postnatal life. The best example is antigenic stimulation of B lymphocytes in germinal centers of lymph nodes, with

ensuring hypermutation of antibody V region genes to generate affinity maturation of antibodies. Thus B lymphocytes with reactivity with autoantigens may emerge as a 'byproduct' of affinity maturation of antibodies to bacterial antigens, and may persist if tolerogenic processes fail, with ensuing 'rescue' of such autoimmune B cells from apoptosis. Various other somatic genetic mutations in postnatal life could predispose to autoimmunity, e.g. those that influence patterns of cytokine secretion, or processes of apoptosis.

Environment

The environment can interact with genetic predisposition to induce autoimmune disease. Viewed most simply, any cause of tissue damage can result in release of cellular products which, if exposed to antigen-presenting cells of individuals with 'susceptibility genotypes', in an appropriate microenvironment such as cytokine excess, could stimulate self-reactive T cells that normally circulate at low levels. In the case of infectious damage, there is the added possibility of antigenic (or epitope) mimicry. This idea is based on molecular homologies between a constituent protein antigen of a microorganism and host being sufficiently close that the 'protective' response to the organism becomes cross-reactive with 'self'. An example of a break in tolerance initiated by molecular mimicry is the provocation by streptococcal cell wall antigens of rheumatic endomyocarditis. Currently, molecular biological techniques for identifying sequences of autoantigenic epitopes have been combined with searches of gene and protein databanks to reveal potentially informative homologies; however, such comparisons often fail to acknowledge that B cell epitopes, including autoepitopes, are usually conformational rather than linear. Thus molecular mimicry may be more applicable to short linear T cell epitopes.

Other relevant environmental influences are ultraviolet radiation, chemicals including mercuric chloride that induce autoimmunity in rodent models, and medicinal drugs that induce various autoimmune syndromes including SLE, hepatitis and myasthenia gravis. Good explanations for the induction of autoimmunity by these influences are lacking; sunlight might act to create neoepitopes in skin cells, mercuric chloride may cause structural alterations in MHC molecules on antigen-presenting cells, and medicinal drugs may yield molecules that generate compound epitopes with cellular constituents.

Induction of autoimmune disease

The critical elements for the induction of autoimmune responses are: 1) the autoantigenic epitope;

2) processes that facilitate the presentation of the autoantigenic epitope to the immune system; and 3) recognition of the epitope by T and B lymphocytes that have evaded tolerogenic influences.

To serve as an autoantigen, a self molecule should activate both T and B lymphocytes. Autologous molecules will not be recognized as autoantigens if deletional processes have completely removed any cognate responsive T cells in the thymus, or B cells in the bone marrow. T cells can escape deletional tolerogenesis in the fetal thymus if the requisite self molecule is either not represented there, or is not adequately processed by antigen-presenting cells, or if its affinity for the antigen receptor on intrathymic T cells is too low. Thus some self-reactive T cells do exit from the thymus to the periphery: however, these are not necessarily dangerous as they may have only a low affinity for autoantigens and are not readily activated; or, being of a naive phenotype, will not express the surface adhesion molecules required for their transit into tissue parenchyma.

Autoreactive B cells can arise in two sites, the bone marrow during lymphogenesis, or lymph node germinal centers by affinity maturation during responses to extrinsic antigens (see above). B cells at both sites are subject to various tolerogenic influences, including antigen in excess, which induces cell death by apoptosis. Again, however, there is 'leakage' in health of self-reactive B cells from these sites into the periphery; evidence for this is the presence in the circulation, particularly in aging, of various autoantibodies, and also B cells capable of binding labeled autoantigens. A low degree of B cell autoimmunity may well be 'innocuous', if not beneficial (see above), provided that there is no source of potent contiguous T cell help.

Effector activities in autoimmunity

Effector activities depend on the various functions of B or T lymphocytes or, probably, both sets of cells, in unison. B cells and circulating autoantibody are the clear cause of autoimmune pathology in hemolytic diseases, or SLE in which damage results from deposition of immune complexes, or diseases in which autoantibody binds to cell surface receptors, either to inhibit a neurotransmitter, e.g. acetylcholine receptor in myasthenia gravis, or to stimulate a hormone receptor, e.g. the thyrotropin receptor in Graves' disease. Another role for B cells is to act as antigen-presenting cells, in a focused way, as autoantigen can be specifically captured by the B cell immunoglobulin receptor for presentation to autoimmune T cells, thus sustaining the autoimmune reaction. CD4⁺ helper T cells are the likely effectors of damage in autoimmune disease of parenchymal

tissues, thyroid gland, stomach, brain, synovium, pancreatic islets, etc., presumably by release of injurious cytokines, particularly tumor necrosis factor (TNF), or injurious enzymes (granzymes). CD8⁺ cytotoxic T lymphocytes (CTLs) are less easily studied functionally as effectors of autoimmune damage and data are scarce. As judged by the infrequency of MHC class I associations with autoimmune disease, CD8⁺ CTL-mediated damage might be relatively less important in autoimmunity. However, immunohistochemical studies of tissue lesions often show a striking infiltration of CD8⁺ T cells in contiguity with damaged cells and, in animal models, cell transfer studies have implicated CD8⁺ CTLs as early effectors, e.g. in autoimmune islet cell damage in nonobese diabetic (NOD) mice.

Regulation of autoimmunity

Self tolerance and avoidance of autoimmunity are of such fundamental importance to the organism that several 'layers' of tolerogenesis are in place. The first and fundamental layer, 'central' tolerance, is deletion of lymphocytes in the primary lymphoid organs, thymus or bone marrow, consequent on high-affinity contact between autoantigen as ligand and antigen receptors on nascent T or B cells, i.e. negative selection. Deletional self tolerance has been elucidated by recent studies on transgenic mice in which cDNA for a marker antigen, e.g. hen-egg lysozyme, is introduced in embryonic life, and hence is treated as self; in 'double' transgenic mice there are additionally introduced genes that encode T cell or B cell antigen receptors for the transgenic molecule.

Processes of 'peripheral' tolerance operate to limit the activity of autoreactive cells that have escaped deletion in the thymus or bone marrow. Thus 1) thymic emigrant T cells have a 'naive' phenotype and lack activation molecules needed for extravasation through vascular endothelium into tissue parenchyma; 2) T cells that encounter autoantigens in the blood or on the surface of tissue cells do not receive a critical 'second signal' that is provided only by 'professional' antigen-presenting cells, and respond by anergy rather than activation; 3) particular cytokines may deflect a damaging autoreactive CD4⁺ T helper cell response from the T_H1 inflammatory type to the more benign T_H2 antibody dominated type; 4) autoimmune B cells remain innocuous if they fail to obtain adequate helper signals from T cells that may be adequately tolerized; and, finally, 5) there are mechanisms of 'dominant' tolerogenesis, also called suppression, which can prevent autoimmunity or even override established self-reactive effector processes. Autoimmunity in states of natural and experimentally induced T cell lymphopenia is evidence for

the existence of a regulatory/suppressive subset of T lymphocytes. Dominant tolerogenesis may also be mediated by anti-idiotypic antibodies; such antibodies are directed against the combining site of the antibody molecule itself. Anti-idiotypic antibodies to autoantibody molecules appear to exist in normal sera, and are considered to explain the beneficial therapeutic effect in certain autoimmune diseases of pooled normal human immunoglobulins given intravenously.

Diagnosis

Autoimmune diseases are usually diagnosed according to the functional deficits that ensue in affected organs, rather than by their immunological features *per se*. This applies to autoimmune disease affecting the thyroid gland, adrenal gland, pancreatic islets, stomach, liver, kidney, muscles, neural transmitters, etc. However, testing for the disease-relevant autoantibody will in many instances provide specific and decisive diagnostic information, exemplified by testing for the antiglobulin reaction in hemolytic anemia, and for autoantibodies to acetylcholine receptor in myasthenia gravis, adrenal cortical cells in Addison's disease, dsDNA in SLE, mitochondrial pyruvate dehydrogenase enzyme complexes in primary biliary cirrhosis, or to the combination of nuclei and F actin in type 1 autoimmune hepatitis. An interesting application of modern sensitive and automated tests for autoantibodies, utilizing recombinant autoantigens, is screening for presymptomatic autoimmune disease, e.g. assays for anti-GAD (glutamic acid decarboxylase) and other islet autoantigens to detect presymptomatic IDDM among first-degree relatives, or even among populations.

Treatment

Many of the organ-specific autoimmune diseases are adequately treated by replacement therapy when terminal destruction ensues, e.g. thyroiditis, adrenalitis, gastritis, insulinitis, etc.; however, the state of autoimmunity must be 'controlled' when disease affects vital organs or tissues, such as the liver in autoimmune hepatitis or muscles in autoimmune polymyositis, or when the disease is systemic and many tissues are at risk, as in SLE.

Traditionally, 'control' over the immunologic activity in autoimmune disease has been with corticosteroid drugs, which have particular efficacy in blocking unwanted effects of antigen-antibody reactions. The action of corticosteroids can be supplemented by 'immunosuppressive' drugs of the types used to prevent rejection of transplanted allografts: azathioprine, cyclophosphamide and cyclo-

sporine; however, because prolonged usage of these drugs can have various adverse effects, therapists are seeking more subtle immunological interventions. These include blockage by monoclonal antibodies of critical components of the immune response, and particularly the receptor complex of CD4 T cells, T cell cytokines including TNF α , or B cell/antibody receptors by infusion of normal polyclonal human IgG that is presumed to contain anti-idiotypes to autoantibody molecules. There is theoretical, and some practical, promise in 'at source' therapy which is to re-establish broken tolerance to culprit autoantigens. This depends on the autoantigen being administered as preparations and by routes that are more conducive to tolerance than to immunity – the 'negative vaccine' idea. Delivery of the autoantigen via the intestine, 'oral tolerance', is currently favored. The obvious benefits of this approach for the prevention of autoimmune disease should justify the years of developmental study that seem needed to bring it into routine use.

See also: **Autoantigens; Autoimmune disease, induced experimental models; Autoimmune disease, pathogenesis; Autoimmune diseases; Autoimmunity; Avian immune system; Insulin-dependent diabetes mellitus, animal models; Rheumatological disorders; Systemic lupus erythematosus, experimental models; Systemic lupus erythematosus (SLE), human; Thyroid autoimmunity, human; Tolerance, peripheral.**

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AUTOIMMUNITY

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At the beginning of this century Paul Ehrlich used the expression 'horror autotoxicus' to describe the body's apparent inability to respond to self antigen and he suggested that nonreactivity may be mediated by the lymphoid system. Evidence that these control mechanisms can sometimes fail was provided afterward by the observation of Donath and Landsteiner that patients with paroxysmal cold hemoglobulinemia possess antibodies against their own erythrocytes.

The experiments of Rivers, Sprunt and Berry in the early 1930s showing that injection of monkey brain antigens back into monkeys provoked encephalomyelitis provided the first experimental model of autoimmune disease. A decade later, Owen's observation that dizygotic cattle twins sharing the same blood supply during embryonic life did not destroy each other's nonidentical erythrocytes (even if these were injected in adult life) suggested that self tolerance is acquired during ontogeny. Such chimeric cattle are able to exchange skin grafts without subsequent tissue rejection. In 1953 Billingham, Brent and Medawar demonstrated that neonatal injection of cells from CBA strain mice into A strain mice allowed the mice to later accept CBA skin grafts. Three years after these experiments, a series of classical papers on thyroid autoimmunity were to firmly establish the role of autoimmune phenomena in producing disease. Rose and Witebsky induced both autoantibodies and thyroiditis by immunizing rabbits with thyroid extract in Freund's adjuvant; Roitt, Doniach and Campbell demonstrated autoantibodies to thyroglobulin in Hashimoto's disease; and Adams and Purves discovered stimulatory autoantibodies directed against the thyroid-stimulating hormone (TSH) receptor.

Consequences of autoimmunity

Autoimmunity is defined as an immune response leading to reaction with self antigen, i.e. any molecule that is a normal body constituent of the animal mounting the response. Self reactivity can arise either

through the triggering of receptors directly by autoantigen or by virtue of cross-reaction between foreign and self antigens. Topographic similarity of B cell epitopes or sequence homologies of linear T cell epitopes may lead to such cross-reactions and it is highly unlikely that the immune system is able to recognize all foreign antigens specifically without some recognition of self epitopes also occurring.

Techniques such as polyclonal activation of cells with mitogens, and the establishment of lymphocyte clones and hybridomas, have established that self reactive T and B lymphocytes are frequently found in individuals who lack readily detectable autoantibodies in their serum. Indeed, at least some degree of self reactivity appears to be a normal physiological phenomenon during lymphocyte development and perhaps may be necessary in preparing the immune system for its future encounters with foreign antigen. The B-1a (CD5⁺, Mac-1⁺, CD23⁻, strongly sIgM⁺) subset of B lymphocytes are found mainly in the peritoneal cavity where they are self-renewing and express germ line V genes encoding polyspecific low affinity IgM autoantibodies bearing cross-reactive idiotypes. This B cell subset is elevated in, for example, the autoimmune NZB mouse and in patients with rheumatoid arthritis. Low affinity IgM autoantibodies are probably not pathogenic. Encounter with nonidentical cross-reacting foreign antigen will, in the presence of T cell help, lead to affinity maturation within germinal centers, thereby generating antibodies with high affinity for foreign antigen which simultaneously lose their original self-reactivity. However, as certain self antigens such as idiotypes form key recognition structures for immunological regulation it is crucial that the immune system retains the ability to recognize these particular self components.

The pathogenicity of an autoimmune response is determined not only by the magnitude but also by the nature of the response. For instance, although 10–20% of clinically normal individuals possess low titers of autoantibodies specific for thyroglobulin,

and although these antibodies are present at higher titers in about 70% of Hashimoto's disease patients, it is autoantibodies to another thyroid component, thyroid peroxidase, which show a stronger correlation with histopathological lesions. These autoantibodies occur in 95% of patients with Hashimoto's disease and, unlike most thyroglobulin autoantibodies, are complement fixing and therefore potentially cytotoxic to thyroid epithelial cells. Another example underlining the distinction between autoimmunity and disease is seen in patients treated for longer than 1 year with the drug procainamide (used to control cardiac arrhythmia). Over 90% of these patients develop IgM antibodies reactive with a broad spectrum of self histones but it is only in the 10–20% of patients who develop high levels of IgG antibodies specific for the H2A-H2B histone dimer bound to DNA that a lupus-like autoimmune disease arises. In the case of immunoglobulin, different parts of the same molecule can elicit both physiological (idiotypic) and potentially pathogenic (rheumatoid factor) autoantibodies.

Animal models have confirmed the importance of cell-mediated immune responses in the development of autoimmune disease; the adoptive transfer of autoantigen-specific T cell clones is often sufficient to induce disease in normal recipients. Most autoimmune diseases probably have both T and B cell components and in some situations other mechanisms involving natural killer (NK) cells or antibody-dependent cellular cytotoxicity may play a role in the pathogenic process.

Mechanisms controlling autoimmunity

Because pathogenic autoimmunity is the exception rather than the rule, mechanisms must exist which normally prevent the development of autoimmune disease. In this respect the immune system is able to distinguish self from nonself and, ultimately, becomes 'tolerant' towards most self antigens. However, as alluded to above, many of the tolerance mechanisms controlling autoimmunity may be less concerned with repression of all self reactive lymphocytes than with preventing the development of pathogenic T cells and autoantibodies. Indeed, there is evidence from studies in transgenic mice to suggest that, in situations in which high-affinity antiself B cells are anergized, low-affinity antiself B cells escape this tolerization process.

The danger model of immune responses proposes that, rather than discriminating self from nonself, the immune system primarily distinguishes between harmless and harmful. In this scenario, self would normally be considered harmless and would fail to

trigger a response. However, a response to harmful situations would occur due to upregulation of costimulatory molecules on antigen-presenting dendritic cells following signals induced by microbial antigens, cell stress or necrotic cell death.

Although evidence for several different tolerance mechanisms exists, the relative role of each of these in controlling autoimmunity remains to be firmly established. For a long time it was thought that many self antigens were sequestered in 'privileged sites' and therefore shielded from the immune system. The advent of sensitive assay techniques allowed the detection of, for example, low levels of circulating thyroglobulin (an autoantigen previously thought to be completely sequestered within the thyroid). Nevertheless, it is believed that for a few self molecules, such as lens proteins, sperm and brain antigens, this concept largely holds true.

The 'forbidden clone' theory put forward by Burnet in the 1950s suggested that deletion of self reactive clones led to self tolerance. Although it is now clear that this is not a universal mechanism for avoiding autoimmunity, clonal deletion is known to occur for some self antigens. Mice bearing, for example, particular Mls antigens eliminate any T cells which are expressing T cell receptor β chain variable region genes associated with recognition of these antigens. In situations where self reactive cells are not deleted they either become anergic or are actively suppressed by regulatory mechanisms which have evolved in order to restrain harmful autoimmunity. T cell-mediated suppression maintains control over both B lymphocyte and effector T lymphocyte responses, so that any defect in antigen-specific or idiotype-specific suppression might allow autoimmune responses to occur. Conversely, increases in helper T cell activity may allow normally quiescent self reactive lymphocytes to mount an autoimmune attack. In instances in which potentially autoreactive B lymphocytes normally remain inactive due to tolerance of the relative helper T cells, the provision of new carrier determinants can provide a 'bypass' mechanism allowing T cell help for autoantibody production. It has been shown that tolerance can be broken experimentally using chemically modified or hapten-conjugated protein antigens and it has been suggested, for example, that the Coombs' positivity of some patients treated with methyl dopa may be due to the drug complexing with erythrocyte antigens, thereby providing carrier determinants which will stimulate cognate T cell help. In other cases, T cell epitopes on the autoantigen may not be effectively presented during tolerance induction in the thymus and so potentially autoreactive T cell clones escape deletion. Only if higher levels of these

normally 'cryptic' epitopes later become effectively presented in the periphery, due perhaps to altered processing or presentation of the self antigen, will autoimmune reactivity manifest itself. Furthermore, once initiated, the autoimmune response may spread to other epitopes on the same or different antigens, a process that could be fueled by the local production of inflammatory cytokines which might further lead to upregulation of cell surface molecules involved in antigen presentation and cellular activation.

Idiotypic networks may be important in the control of immune responses to autoantigen; defects in this regulatory network could provide a pathway by which self reactivity can arise. For example, anti-idiotypes to DNA-specific autoantibodies have been described in the sera of patients with inactive systemic lupus erythematosus (SLE) which were absent in those with active disease, suggesting that the presence of these antibodies may influence the course of the disease. In the anti-insulin response seen in patients with insulin-dependent diabetes mellitus (IDDM), idiotypic determinants have been reported to be cyclically expressed even though the total amount of anti-insulin remained constant, again suggesting that idiotypic interactions are involved in regulating this response. In some instances pathogenic autoantibodies may be internal image anti-idiotypes which are directed against antiviral antibody and bind to the same cell surface molecule that is used as a receptor by the virus.

Major histocompatibility complex (MHC) class II gene products are normally expressed only on 'professional' antigen-presenting cells (APCs) but they are sometimes also aberrantly expressed on a variety of cell types during autoimmune disease. It has been shown that such cells themselves are then also able to present antigen. Aberrant expression of MHC class II may be the result of cytokine secretion by cells already involved in an ongoing inflammatory response, and might provide a mechanism for perpetuation of the disease.

Regardless of the cell type expressing MHC class II it is known that autoimmune disease is influenced by MHC (particularly class II) haplotype. As the majority of autoantigens are likely to be T dependent, the ability of self peptides to associate with self-MHC at a concentration necessary for T cell activation will be central to the induction of an immune response.

Individuals bearing HLA-DR4 are nearly six times more likely to develop rheumatoid arthritis (RA) than those lacking this haplotype. For some autoimmune diseases there appears to be a synergistic effect between MHC genes; individuals who bear HLA-DR3 or HLA-DR4 have an increased risk (of

between 2 and 7) of developing IDDM but for someone bearing both these haplotypes the relative risk increases to 14. In IDDM the particular residue at position 57 of the DQ β chain appears, in concert with other residues, to be important in determining disease susceptibility in some caucasian populations.

Molecular mimicry of self antigen by invading organisms can lead to a breakdown of self tolerance as seen in the cross-reactions between streptococcal M protein and cardiac myosin in rheumatic fever and between both brain and heart tissue and *Trypanosoma cruzi* in Chagas disease. Many other examples of autoantibodies cross-reacting with pathogens have been described. Cross-reactivity between self and foreign antigens is also seen for T lymphocytes as demonstrated by the existence of T cell clones specific for determinants on a mycobacterial hsp65 and a human hsp60 which share 48% amino acid sequence homology. Polyclonal lymphocyte activation occurs in response to some infections, for example with Epstein-Barr virus, and has been proposed as a possible mechanism involved in the development of autoimmunity. However, although some of the clones activated may be specific for self antigen these will usually be short lived and produce IgM antibodies of low affinity. Experiments involving neonatal thyroidectomy of autoimmune obese strain chickens demonstrated that the presence of autoantigen is required for the development of thyroglobulin autoantibodies and therefore in this instance these antibodies are not induced by nonspecific activation of B cells.

Somatic mutation of antibody variable region genes is a further mechanism by which autoantibodies may arise. It is known that a single, naturally occurring, point mutation can change an antibody with specificity for phosphorylcholine into an antibody that recognizes double-stranded DNA, protamine and cardiolipin. This antibody has therefore lost the ability to bind to bacterial antigen but acquired binding for self antigen. Despite the fact that autoantibodies are normally restricted in their epitopic specificity (for example to two or three paired determinants on human thyroglobulin) they are usually polyclonal. It is therefore unlikely that somatic mutation on its own provides an explanation for autoimmune disease, although triggering of idio-type-specific T helper cells might allow a single clone to initiate a polyclonal response via network interactions. It is generally accepted that the development of most autoimmune disease is multifactorial in nature. Some of the animal models suggest a role for ADCC or NK cells. Other factors that may influence the initiation and development of the disease are defects in immune regulation (for example T_H1/T_H2

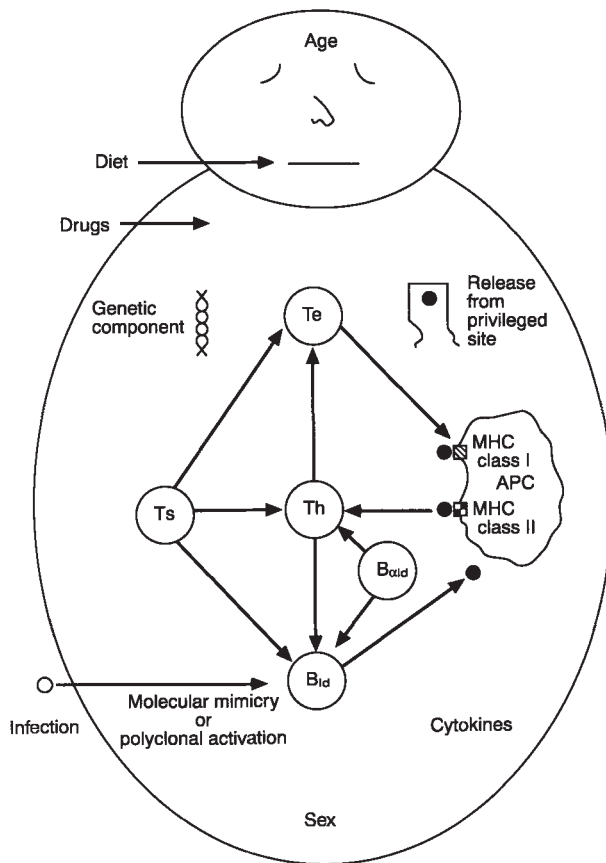


Figure 1 The multifactorial nature of autoimmunity. ●, Autoantigen; APC, antigen-presenting cell ('professional' or aberrant class II expression); Th, helper T cell (antigen- or idiotypic-specific, carrier-specific for T cell bypass); Te, effector T cell (for example cytotoxic T cell); Ts, suppressor T cell; B_{id}, autoantibody-secreting Id⁺ B cell; B_{aid}, anti-idiotypic B cell.

imbalance) and in the target antigen, sex hormones (autoimmunity is more common in females than in males), diet, and the time and nature of exposure to infectious agents. Some of the above concepts are drawn together in Figure 1.

Intervention in autoimmunity

As a clearer picture emerges of the precise mechanisms involved in the breakdown of self tolerance, therapeutic approaches for the autoimmune diseases can begin to address the underlying defect, i.e. the specific autoimmunity. At present, when endocrine tissues are destroyed, as in Hashimoto's disease or IDDM, hormone replacement therapy is utilized. Antithyroid drugs, thyroidectomy and radioactive ablation are used in Graves disease rather than elimination of the TSH receptor autoantibody response. The immunosuppressive regimens used for systemic autoimmune conditions such as SLE and rheumatoid arthritis are crude attempts to dampen the whole

immune system rather than control the specific autoimmune response. More rational approaches to therapy are now beginning to emerge based upon, for example, oral or nasal tolerance induction, the use of cytokine antagonists, and intervention using peptide-based therapeutics to directly block T cell activation.

See also: Adhesion molecules; Autoantigens; Autoimmune diseases; Cytokines; Idiotype network; Oral tolerance; Privileged sites; T cell vaccination; Tolerance, peripheral; Tolerance, central; Immunotherapy of autoimmune diseases; Relative risk.

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AUTORADIOGRAPHY

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Autoradiography is a method of localizing radioactive atoms in a specimen. The method briefly comprises of:

1. *Labeling* of a suitable molecule with a radio-nuclide, serving as a tracer.
2. *Incorporation* of the radioactive tracer into the sample under study.
3. *Processing* of the sample by some suitable means.
4. *Exposure* by placing the prepared sample in close approximation with a special photographic emulsion so that the products of radioactive decay in the sample may interact with the silver halide crystals of the emulsion to form latent images.
5. *Developing* the latent images to convert them into silver grains.
6. *Fixing* the resultant autoradiogram by washing away unreacted silver halide crystals.

The silver grains thus deposited are visualized and their position noted in relation to the underlying sample structure to try and understand the distribution of the label in the sample. Depending upon the nature of the information sought, autoradiography can be carried out at three levels:

1. *Gross level*: in the case of samples such as entire organs, large tissue slices, leaves, gels, etc., where the resolution required is of the order perceived by the naked eye (0.2 mm)
2. *Microscopic level*: cellular and tissue level localization, resolution of around 1–5 μm required.
3. *Ultrastructural level*: subcellular detection and localization with a resolution in the submicron range.

Further discussion will be confined to autoradiography of the microscopic and ultrastructural levels only.

For the successful application of the autoradiographic technique to accurately localize and quantify

a biological molecule of interest it is necessary to have detailed information of the biochemical, physiological and pharmacological processes involved in its uptake, distribution and clearance. Also important is the need to have knowledge of the efficiency and resolution obtainable by the experimental technique. Efficiency is defined as the number of silver grains formed per radioactive disintegration in the sample. It depends upon:

- Dose, nature and energy of the radioactive emission.
- Thickness of the sample and emulsion.
- Developing parameters.

The resolution obtainable can be considered in three ways:

- Distance of separation between the points of radioactive decay and grain formed.
- Ability to separate two points of radioactive decay.
- Ability to separate the individual grains formed.

Resolution is also modulated by the nature and energy of the radioactive emission and developing parameters; it also depends upon the size of silver halide crystals in the photographic emulsion.

Commonly used radioisotopes in biological studies

Not every radioisotope will be suitable for a particular autoradiography procedure. The criteria for determining suitability are:

1. *Nature of emission*. Particle emitters are preferred. Gamma and X-ray emitters have very poor efficiency. Electron emitters are usually the most favorable.
2. *Particle energy*. Generally the higher the energy the lower is the efficiency of detection. This is because high-energy particles have a tendency to

escape from the sample and emulsion. β Particles with energies above 0.5 MeV are usually very difficult to detect. A 'high' energy will also mean that the particle will travel further before forming a latent image, hence resolution will also be poor.

3. **Half-life.** If the half-life of the radioisotope used is too short (less than 30 days), most of the radioactivity will disappear during sample processing. On the other hand, use of isotopes with very long half-lives will mean a very low specific activity (few radioactive decay events per unit time) and hence the exposure time would have to be lengthened unduly to collect the requisite number of latent images in the emulsion.

Some of the commonly used radioisotopes in biology are listed in Table 1. The exact choice of a particular radionuclide is dependent upon its biological relevance. The final decision in determining a suitable isotope should be made keeping in mind the nature of the biological process under study. The radio-labeled biomolecule prepared has to be purified, checked for specific radioactivity and assayed for biological activity before use for autoradiography.

Dose and administration of radiochemicals

The dose of radioactivity to be administered depends upon the detection system and resolution sought. High-resolution autoradiography is usually carried out with thin films/sections of the sample where the mass of the tissue present is very small. It is therefore necessary to use a high radioisotope concentration and extended exposure time. Although methods are available for the estimation of exposure time for a given level of radioactivity in a sample block, it is usually the practice to prepare autoradiographs in batches. Administration of the radiomolecules can be by one of several routes. In the case of animals, oral intake, direct infusion into arteries/veins or ventricular spaces, injection into peritoneum, etc., can all be considered. For labeling cell suspensions, *in vitro* incubation in culture medium is by far the most rapid, efficient and inexpensive method.

Table 1 Radioisotopes used in biology

Isotope	Nature of emission	Energy (MeV)	Half-life
^3H	β	Low: 0.018	Long: 12.26 years
^{14}C	β	Medium: 0.159	Very long: 5760 years
^{35}S	β	Medium: 0.167	Moderate: 87.2 days
^{32}P	β	High: 1.71	Short: 14.3 days
^{125}I	Auger electron from electron capture reaction	Low: 0.008	Moderate: 60 days

Emulsions used

Photographic emulsions used for high-resolution autoradiography are characterized by a small size of silver halide crystal. The larger the crystal diameter, the poorer is the resolution obtainable; however, greater efficiency is obtainable with larger crystals. Apart from small size, for high resolution work in an electron microscope the emulsion of crystals has to be applied in a monolayer over the sample. There are several suppliers of autoradiography emulsions (Kodak, Sakura, Ilford) with various crystal diameters available.

Application of emulsion to radiolabeled samples

Once a sample has been prepared for autoradiography it is brought into intimate contact with the emulsion. There are primarily two methods for forming a monolayer of emulsion on the sample.

Loop method

A drop of liquefied emulsion in the form of a bubble in a wire loop is applied on to the surface of a sample. This method is commonly used in ultrastructural analysis. The sample is in the form of ultrathin sections/film mounted on a grid.

Flat substrate method

Frequently used in light microscopic studies, here samples mounted on glass slides are dipped into a beaker of liquefied emulsion to form a uniform coat of silver halide crystals. A variation of this method involves first casting of the emulsion films on glass slides, from where they are floated on water and allowed to settle on to the sample.

Exposure

Exposure of emulsion-coated samples is carried out in dessicated light tight boxes at 4°C. The emulsions must be shielded from stray light and radiation. Low temperature and humidity is important to prevent

the fading of latent images (resulting in lowered efficiency). The time of exposure can vary from a few weeks to a year and depends upon the nature of the experiment and the radioisotope used. In our laboratories we usually check batches of samples after every few weeks of exposure.

Development

Development involves precipitation of silver atoms at the site of the latent image to form silver grains, which are visualized in a microscope. A generalized method of development involves immersion of the emulsion coated sample in a suitable fine grain developer (for example Kodak D-19 or Microdol X) for a standardized period of time (usually for a few minutes). After rinsing in distilled water the emulsion is fixed in sodium thiosulfate solution for at least 20 minutes and then washed extensively in distilled water. Care has to be taken to ensure that at no point is the surface of the sample contaminated with any precipitate or other material. After washing, the sample can be stained before examination under the light or electron microscope. It should be noted that different developers yield different numbers of grains (different sensitivity); moreover, the shape of the silver grains deposited also varies with developer, a factor which affects the resolution.

Control observations

The successful validation of any autoradiographic experiment requires a proper set of control observations for determination of background and presence of chemography.

Background

Silver grains deposited due to radioactivity of the tracer molecules incorporated into the sample constitute the signal. However, some additional grains can also develop for nonspecific reasons. These constitute the noise or background of the system. Generally the background should not exceed more than 5% of the signal grain density. Background can be estimated by taking the ratio of the grains present over the target tissue to the grains present over the same area of empty section (only embedding material coated with emulsion). The factors contributing to background are:

- Exposure to stray light or radiation.
- Pressure during drying of emulsion film.
- Static discharges in the vicinity (common in low-humidity workplaces).
- Overdevelopment (excessive time and/or temperature).

Chemography

Induction of grain formation by the chemical action of sample components on silver halide crystals is known to occur and is called positive chemography. The reverse process, of eradication of latent images by chemicals in the sample, is known as negative chemography. The former causes an increase in the background, while the latter lowers the efficiency. Interposing a thin inert layer of carbon between the sample and the emulsion usually minimizes the problem. A control for positive chemography is a non-radioactively-labeled, identically-treated sample. For negative chemography a control would be a light-fogged, identically-treated sample.

Interpretation of results

The simplest method is to assume that the grain observed overlies the point of radioactive decay and hence represents the site of the target molecule. For lower resolution studies at the light microscopic level this assumption may work; however, in the case of electron microscopy autoradiography this assump-

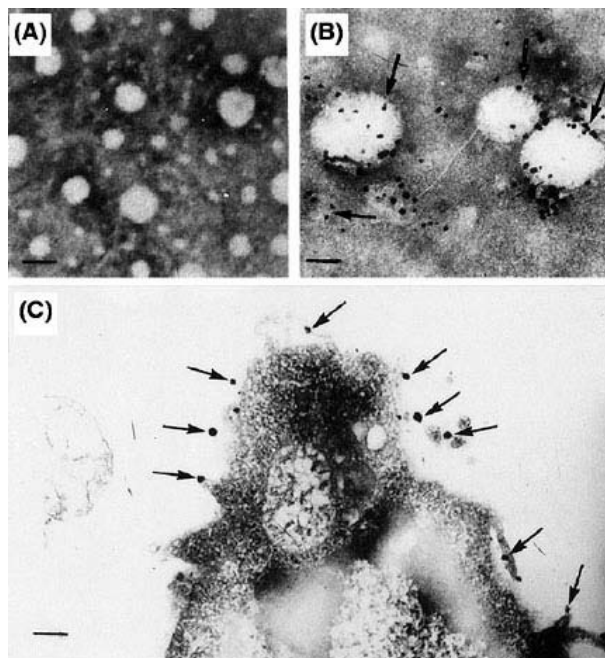


Figure 1 Binding and distribution of B3 (a costimulatory molecule) on the surface of T cells. B3 coupled to liposomes was labeled with ^{125}I and incubated with anti-CD3-activated CD4^+ T cells and electron microscopic autoradiography was performed using Ilford nuclear L4 emulsion. (a) Negative staining of liposomes by phosphotungstic acid (bar = 200 nm); (b) ^{125}I -labeled B3 coupled to liposomes; arrows show the liposomes bearing the iodinated B3 (bar = 200 nm); (c) CD4^+ T cell labeled with B3 (arrows) (bar = 200 nm). (Reproduced from Vinay, Raje, Verma and Mishra (1995).)

tion need not hold true and careful analysis of the images obtained has to be made. There are two methods in common use for localization of the radioactive decay event in a sample with relation to the observed grain density. These are known as the probability circle analysis and the cross-fire method (see Williams (1985) for detailed methodology).

Some areas of application

Autoradiographic techniques have been used extensively to study the binding of a variety of small molecules such as peptides, drugs and hormones to a variety of target sites in cells and tissues. The number

of conserved subunits in chromosomes was demonstrated using autoradiography. Many important observations have been made on macromolecular metabolism, synthesis and secretion using these techniques. The method has also been successfully applied to the study of virus particles and nucleic acids. In our laboratories we have used autoradiography and electron microscopy to demonstrate the binding of a costimulatory protein to its receptor on the surface of CD4⁺ T lymphocytes (Figures 1 and 2).

See also: Immunodiffusion, single radial; Immunoelectrophoresis; Isoelectric focusing; Lymphocyte trafficking; Northern blotting; Radiolabeling; SDS-polyacrylamide gel electrophoresis (SDS-PAGE); Southern blotting; Western blotting.

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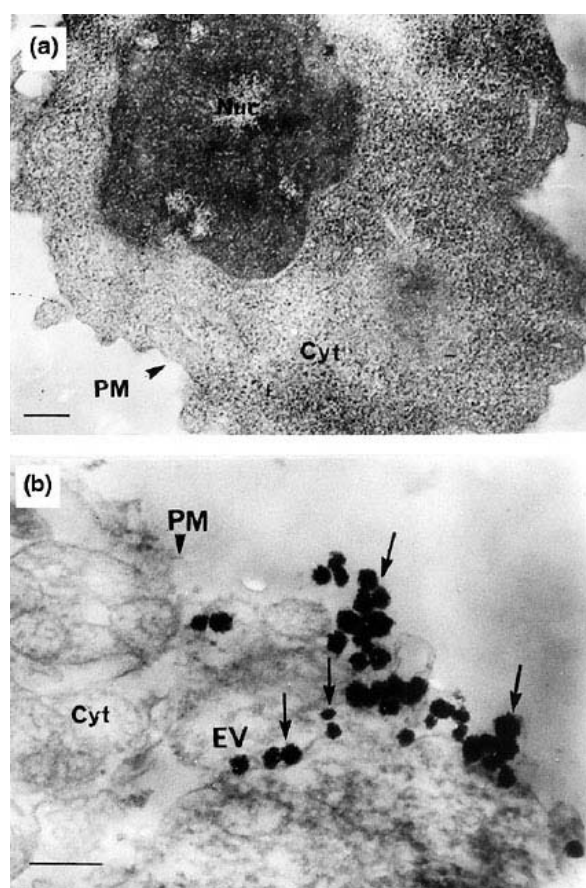


Figure 2 Murine anti-IgM coupled to liposomes and ¹²⁵I-labeled was incubated with T cells and A20 cells. Electron microscopic autoradiography was carried out using ultrathin sections coated with Ilford nuclear L4 emulsion. The period of exposure was 3 weeks and development was carried out using Kodak D-19 developer. PM, plasma membrane; Cyt, cytoplasm; Nuc, Nucleus; EV, endocytotic vesicle. (a) anti-CD3-activated T cells incubated with liposomized and ¹²⁵I-labeled murine anti-IgM (immunoglobulin M) (bar = 200 nm); (b) A20 cells incubated with liposomized and ¹²⁵I-anti-IgM (bar = 200 nm). Note the absence of labeled anti-IgM binding on the T cell membrane. Also note the distribution of labeled anti-IgM in and on the surface of A20 cells. (Reproduced from Vinay, Raje, Verma and Mishra (1995).)

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AVIAN IMMUNE SYSTEM

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The species studied by avian immunologists include turkeys, ducks, pigeons and Japanese quail. However, the contributions of the domestic chicken (*Gallus gallus*) to immunology far outweigh those of other birds, and indeed exceed those of other species except humans and mice. The contributions date back to 1880 (Pasteur's key observation of fowl cholera attenuation, on which much vaccine development is based), and are continuing at present with the para-aortic localization of hematopoietic precursors in very early embryos. Some important immunological results obtained using chickens or chick embryos are the discoveries of tumor viruses (including endogenous viruses) and interferon; the association of the major histocompatibility complex (MHC) with resistance to viral disease; induction of tolerance by embryonic parabiosis; the definition of separate B and T lymphocyte lineages following Glick's elucidation of the role of the bursa; quantitation of graft-versus-host reactions and frequencies of alloreactive T cells; and evidence that the primary lymphoid organs are colonized by bloodborne hematopoietic stem cells, that a gene conversion-type mechanism can play a major role in the development of antibody variability, and that immunoglobulin G (IgG)- and IgA-producing cells are derived from IgM-positive precursors.

Avian and mammalian immune systems have much in common. However, there are important differences, emphasized in this entry, between them, notably in the structure of lymphoid organs, the ways in which antibody variability is generated, and the arrangements of Ig and MHC genes. The main advantages of chickens in immunological experiments are the accessibility of the embryo, the ease of manipulating B cell development in the bursa, and the ready availability of peripheral blood and lymphoid organs from birds with defined genetic background. Young chicks are easy to immunize, bleed and operate, and can be kept in standard animal housing, whereas maintaining adult chick-

ens and establishing a breeding program requires facilities designed for this purpose, and is necessarily a long-term project since the generation time is 6–12 months.

Chicken strains and genetic variability

Over a dozen highly inbred chicken strains, representing at least 10 of the approximately 30 characterized different MHC (B locus) haplotypes are available. Skin grafts between birds of the same inbred strain are permanently accepted, with the exception of female to male grafts, which are slowly rejected, hens being the heterogametic sex. MHC-congenic and recombinant haplotypes are available on some of the highly inbred backgrounds. Four μ chain, 10 γ chain and two light chain Ig allotypic variants are described, and Ig allotype-congenic and recombinant strains exist. Variation also occurs at non-MHC or Ig locus-linked loci of interest to immunologists, such as loci controlling blood group antigens or responsiveness to T cell mitogens.

With very few exceptions, the inbred strains are based on the White Leghorn type of domestic chicken. Attempts to inbreed other types of chicken or species of bird (ducks, Japanese quail) have been made, but have not generally been successful. There are lines of chickens with hypogammaglobulinemia resulting from abnormal bursal development, a line in which a disease similar to systemic scleroderma can be induced, and 'obese' chicken strains which develop a spontaneous autoimmune thyroiditis similar to Hashimoto's disease. Transgenic chickens can be produced by DNA transfer into fertilized eggs; viral vectors which can give high levels of somatic transgenesis are available. A chicken genome mapping project is under way.

Immune responses and tolerance

Chickens are generally good antibody producers, and their evolutionary distance from us enables them to

respond to some proteins (e.g. cytoskeletal elements) whose sequences are highly conserved in mammals. Immune responses develop early in chickens. They can respond to some protein antigens injected on the day of hatching, and in some cases to viruses or allogeneic or xenogeneic cells introduced into the egg. Responses to individual protein antigens develop at different times after hatching. As in mammals, IgM responses precede IgG; however, in some (but not all) antihapten responses, only poor affinity maturation is observed, consistent with the idea that there is not extensive generation of antibody variability in peripheral tissues (see below). Because of the difficulty of preparing monoclonal antibodies from chickens, their idiotypes and antibody repertoire have not been extensively studied.

Avian T cell-dependent responses (mixed lymphocyte reaction (MLR), skin graft rejection, delayed-type hypersensitivity given by cells injected into the wattle, responses to T cell mitogens) can be assayed essentially as for mammalian cells, while graft-versus-host reactions can be quantitated by the embryonic splenomegaly assay or by pock formation on the chorioallantoic membrane. T cell-dependent cytotoxic assays are established, and spleen cells from chickens infected with leukosis or reticuloendotheliosis viruses can kill infected target cells in an MHC-restricted, antigen-specific manner. Cytotoxic CD8⁺ cells play an important role in regression of some virally induced tumors.

Depending on the antigen and mode of injection, tolerance can be induced in chick embryos or newly hatched birds. Several interesting tolerance models have been studied. The antigens used include mammalian serum albumins (large doses given to newly hatched chicks lead to B cell tolerance, which is not suppressor cell dependent); quail embryo wing buds grafted into chick embryos (tolerance is induced only if a quail thymus is also grafted with the wing bud, and may be suppressor cell dependent); parabiosed allogeneic chick embryos (leads to tolerance in early embryos, but to development of leukemias and other abnormalities in later embryos); and allogeneic bursa cells injected into cyclophosphamide-treated 4-day-old chicks, leading to long-lasting and specific B cell chimerism. This model has been useful in studies of B cell development, requirements for T-B cell collaboration and antigen presentation by B cells.

Lymphoid organs

The development of B lymphocytes in a distinct primary lymphoid organ, the bursa of Fabricius, distinguishes the avian from other immune systems. Other differences between the lymphoid organs of

chickens and mammals are the multilobe structure of the bird's thymus, which consists of 6–7 distinct lobes on each side of the neck, making thymectomy technically difficult; the absence of organized lymph nodes in many avian species (they exist in a primitive form in ducks, but are replaced by numerous small lymphoid foci in chickens); the higher percentage of immigrant B cells in the avian than in mammalian thymus; the presence of a distinct gland (Harder's gland), located above the eye, which is full of plasma cells secreting IgM and IgA; and the presence of two appendix-like structures, the cecal tonsils, which branch from the gut and contain many lymphocytes. The thymus develops from an epithelial outgrowth of the pharyngeal pouches, and the bursa from an outgrowth of the cloacal epithelium starting at around day 5 of incubation. Epithelio-mesenchymal interaction is important in formation of the bursa. It has been postulated, but is not firmly established, that soluble factors (bursin, bursapoeitin, thymopoeitin) also play a role in genesis of these organs. Intrabursal B cell development is largely independent of exogenous antigens, although antigens introduced into the bursa can induce a local immune response in the bursal medulla.

Cells

B cell precursors located in embryonic mesenchyme enter the bursa between about days 8 and 15 of incubation. The presence of the Bu-1 antigen on at least some of these precursors (prebursal stem cells) suggests that they are already committed to the B cell lineage. Antibody variability is generated by gene conversion in the bursa (see below), probably until the organ involutes at sexual maturity. There is no evidence for development of B cells from Ig-negative precursors after hatching in any anatomical site. Instead, B cells arise in the bursa from Ig-positive bursal stem cells, and later in the periphery from postbursal stem cells, which can restore the peripheral, but not bursal B cell compartment after transfer to a cyclophosphamide-treated host. In their early formation and capacity for self-maintenance, chicken B cells differ from the majority of mouse and human B lymphocytes, although they do share some properties, e.g. CD5 expression, with the B-1 cells of these species. In spite of these differences, chicken B lymphocytes are functionally very similar to their mammalian counterparts in terms of capacity to differentiate into plasma cells and secrete antibodies at high levels. Chicken B cell subsets differing in surface phenotype and physiological criteria, such as mode of emigration from the bursa and turnover rate, have been described.

Chicken T cells develop from precursors which enter the thymus in several waves, starting at about day 6 of embryonic development, and formation of mature T cells appears to follow a course similar to that observed in mammals. Cells expressing $\alpha\beta$ T cell receptors (TCRs) have helper, cytotoxic or suppressor activities, and their functions, at least for helper and cytotoxic T cells, are MHC-restricted. Cells with a helper phenotype (carrying the CD4 antigen), as well as virally transformed CD8-bearing cells have been cloned. T cell-mediated suppression is most clearly shown in models where transfer of T cells from bursectomized birds into normal birds can induce B cell deficiency in the recipients; however, the mechanism of this suppression is not clear. Natural killer (NK) cell and antibody-dependent cellular cytotoxicity (ADCC) activities are readily detectable in chicken spleen and blood. Chicken NK cells have been identified as cytoplasmic CD3⁺, surface CD3/TCR⁻ and CD25⁺ cells. A subset of these cells expresses CD8 α .

Avian nonlymphoid hematopoietic cells (monocytes, granulocytes, thrombocytes, erythrocytes) are clearly defined morphologically and functionally, and bear characteristic cell surface markers detectable by monoclonal antibodies. All of these are nucleated cells, including the erythrocytes and thrombocytes, so that separation procedures designed for mammalian lymphocytes may need modification for avian cells.

In experiments involving cell or organ transfers, the types of cell marker used to follow cellular origin include strain- or species-specific monoclonal antibodies, the sex chromosome marker, and the quail-chick nuclear marker, useful because of its independence of cell cycle and lineage.

Proteins

The well-defined chicken Ig classes (IgM, IgA and an IgG-like class, also referred to as IgY) are functionally comparable to their mammalian counterparts. A truncated IgG/Y (IgY(Δ Fc)), derived by alternative RNA splicing, is found in ducks and geese. Whether a chicken IgD homolog exists is unclear. The chicken IgG/Y heavy chains have one more domain than mammalian γ chains and show about equal sequence homology to mammalian ϵ and γ chains. The IgG/Y molecules aggregate in high salt concentrations, a property which can be exploited to increase the sensitivity of assays dependent on immunoprecipitation. IgG/Y, but not IgM, is transported into the egg, from which antibody can be purified as readily as from serum. As in mammals, secretory IgA is polymeric and associated with a secretory component.

Chicken β_2 -microglobulin and MHC class Ia (B-F) and class II (B-L) molecules are structurally and functionally homologous to their mammalian counterparts. The β_2 -microglobulin is synthesized in the embryonic thymus around day 6 of incubation. B-F molecules are first detected on lymphoid and myeloid cells in 10- or 11-day embryos, and appear on erythrocytes around the time of hatching, whereas B-L molecules are first seen on bursal cells and macrophages about day 11 of incubation. A third type of polymorphic MHC product, B-G molecules, are disulfide-linked dimers of polypeptides which are not glycosylated and not associated with β_2 -microglobulin, and whose molecular weights vary in different strains between about 30 and 55 kDa. B-G molecules are present on the surface of embryonic and adult erythrocytes and their precursors, and on thrombocytes. On other cells, e.g. lymphocytes and intestinal epithelial cells, B-G or B-G-like antigens have also been detected. The function of B-G antigens is unknown, although they show some sequence homology to myelin oligodendrocyte glycoprotein and butyrophilin.

Chicken T cell surface components which resemble mammalian T cell TCR, as well as the CD3, CD4, CD5, CD6, CD8, CD28 and CD45 antigens and the interleukin 2 (IL-2) receptor α chain (CD25), have been identified. Sequence identities with the corresponding human homologs range from 23% (CD4) through 30–40% (CD3, 5, 8) to 50% (CD28). Three subpopulations of T cells, of which two carry $\alpha\beta$ and one $\gamma\delta$ TCR heterodimers, have been defined with monoclonal antibodies. Many (20–50%) peripheral T cells express $\gamma\delta$ TCRs. Monoclonal antibodies have also been used to characterize B and T cell surface markers without known functions or mammalian homologs, e.g. the Bu-1 (chB6) B cell allo-antigen and the chT1 cortical thymocyte antigen, as well as 'oncodevelopmental' antigens present, for example, on lymphoid tumor cells as well as immature (but not mature) normal erythrocytes.

Chicken IL-1, IL-2, interferon α/β , interferon γ and complement components C1, C3 and factor B of the alternative complement pathway have been characterized and resemble their mammalian counterparts. However, there is no evidence yet for a functional C2- and C4-mediated classical complement pathway in chickens. Fc receptors have been defined by their function on several cell types.

Genes

The Ig and MHC gene complexes of the chicken differ in interesting ways from their mammalian counterparts. In the case of the Ig light (λ) chains,

DNA rearrangement during embryonic development joins a unique functional V_L gene to a single J_L sequence adjacent to the single C_L gene. Light chain variability is generated principally by gene conversion (or a mechanism with similar effect), which leads to replacement of sequences in the rearranged V_L gene by sequences from one or more of a series of 25 V_L pseudogenes located 5' of the rearranged V_L . The whole light chain gene complex has been isolated, and all the pseudogenes as well as the functional V , J and C_L genes sequenced. A similar gene conversion mechanism generates heavy chain V region diversity, although in this case only 30 of an estimated 70 V pseudogenes have been sequenced (in addition to the single functional V_H , D and J_H regions and the unique C_μ and C_γ genes). D regions fused to the pseudogenes also contribute to variability. Recent evidence suggests that gene conversion and somatic point mutations additionally diversify the peripheral B cell repertoire in splenic germinal centers. As in mammals, the light and heavy chain gene complexes are not linked, whereas μ and γ chain genes are closely linked.

Chicken TCR α , β , γ and δ genes have been cloned and sequenced. As in mammals, δ and β genes comprise rearranged VDJC elements, whereas α and γ genes result from VJC rearrangement. There are only two V_α , two V_β , two V_δ and probably three V_γ families in chicken. The TCR α/δ locus organization is similar to that of mammals. Preliminary results indicate that both $V_\alpha 1$ and $V_\alpha 2$ gene segments can be used to encode TCR δ chains. The TCR repertoire is generated, in contrast to the B cell receptor repertoire, by combinatorial and junctional diversity, as in mammals.

The chicken MHC (B complex) is located on microchromosome 16, which carries the nucleolar organizer region (NOR). The B complex is smaller and simpler than mammalian MHCs. The average distance between genes in the complex is only about 10–20 kB, and typical intron sizes are about 100 bp. The class I α and class II β genes (two of each in the B complex of the B12 strain) are closely spaced, interspersed and G+C-rich. The single class II α gene is situated on the same microchromosome, some distance (about 5 cM) away from the MHC. In most MHC haplotypes there is a preferential expression of only one class I α and one class II β gene, although the number of expressed genes may be strain-dependent. Within the MHC, the BF/BL region containing class I α and class II β genes can be separated from a region encoding polymorphic B-G antigens, although the frequency of recombination between the two regions is low (about 0.05%). The B-G region contains multiple B-G genes, of which at least two are

transcribed and presumably give rise by multiple splicing, processing or other mechanisms to the multiple B-G products seen in protein analyses. At least one expressed B-G-like gene is also located within the B-F/B-L region. Other genes, one coding for a protein homologous to a G protein subunit, are also present in this region. In contrast to the mammalian situation, factor B polymorphism is not linked to the MHC in chickens.

Rfp-Y, a second region bearing MHC-type (two class I α and three class II β) genes, is also located on chromosome 16. It is separated from the B complex by a region which contains the NOR and exhibits frequent recombination. These MHC genes seem to be neither highly polymorphic nor highly expressed.

The chicken β_2 -microglobulin gene has been cloned and sequenced. It shows little polymorphism and is located on a large microchromosome (either 10 or 11).

Pathology

Diseases which specifically affect the avian immune system include the avian leukoses, Marek's disease and infectious bursal disease (IBD). The avian leukosis viruses (ALV) are closely related to the acutely transforming, *onc*-gene carrying viruses such as Rous sarcoma virus, which carries the *src* oncogene, and the replication-defective myelomacytosis viruses. The latter carry the *v-myc* oncogene and produce a spectrum of myeloid and lymphoid tumors. The ALVs do not carry their own oncogenes, but early studies of the slowly-transforming leukosis viruses showed that tumors developed in bursal cells when the virus was integrated next to the *c-myc* oncogene, leading to its aberrant activation, as in Burkitt's lymphoma cells. Reticuloendotheliosis viruses are also capable of inducing lymphomas in immunologically compromised birds, but are retroviruses not belonging to the ALV family. Marek's disease is caused by a very contagious DNA virus of the herpesvirus family. The virus infects both B and T cells, but specifically induces T lymphocyte transformation, and also leads to peripheral nerve degeneration, perhaps due to autoimmune attack. Resistance to Marek's disease is strongly correlated with presence of the B21 MHC allele, in both heterozygotes and homozygotes, and with low MHC class I antigen expression. Vaccination against the disease by infection of newly hatched chicks with a turkey herpesvirus is very effective, and provided the first example of a practical antitumor vaccine. The IBD virus is a double-stranded RNA virus which infects

bursal cells, producing extensive necrosis and immunodepression. An attenuated IBD vaccine is available.

Vaccines effective against other diseases of chicks exist: Newcastle disease (caused by an influenza-like virus), fowl pox and cholera, infectious bronchitis, avian encephalomyelitis, egg drop syndrome and coccidiosis, which is caused by a gut parasite. There is considerable interest in defining coccidial antigens which render birds immune after a single coccidial infection, since the current attenuated vaccine needs more care in storage and delivery than would a sub-unit-based vaccine. T cell clones with specificity for coccidial antigens have been cloned and in some cases shown to have helper activity *in vitro*. Defining the mechanisms of resistance to this and other avian diseases is currently an important task.

See also: Autoimmune diseases; Bursa of Fabricius; Immunoglobulin, evolution of; MHC, evolution of; Phylogeny of the immune response; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T cell receptor, evolution of; Thyroid autoimmunity, experimental models.

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B

B7 (CD80 AND CD86)

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Two signals are required for optimal T cell activation. Specificity is provided by the interaction of the T cell receptor (TCR) with major histocompatibility complex (MHC)–peptide but a second, antigen-unrestricted costimulatory signal is also required. The B7 family of proteins, B7-1 (CD80) and B7-2 (CD86 or B70) provide the major costimulatory signal for T cell activation and clonal expansion via their interaction with CD28. Engagement of

the TCR in the absence of B7 costimulation results in T cell clonal anergy.

Structure

B7-1 and B7-2 are type I membrane proteins with an extracellular domain consisting of one immunoglobulin V (IgV)-like and one IgC-like domain, followed by a transmembrane anchor and short cyto-

plasmic tail. Mature B7-1 has 254 amino acids, is heavily glycosylated, and has a molecular weight of 45–70 kDa. Mature B7-2 has 304 amino acids, is heavily glycosylated, and has a molecular weight of 60–100 kDa. The genes for both B7-1 and B7-2 are located on human chromosome 3q21 and mouse chromosome 16B5.

Both B7-1 and B7-2 are counter-receptors for CD28, expressed on resting as well as activated T cells, and for CTLA4 (CD152), expressed on activated T cells. Dimeric CD28 has equivalent low avidity for both B7-1 and B7-2 (2400 nM) but dimeric CTLA-4 has a greater than 400-fold higher avidity for B7-1 than B7-2 (1 and 4 nM). Both B7-1 and B7-2 have very fast on/fast off rates but the binding kinetics differ, with B7-1 binding and dissociating more slowly.

B7-1 and B7-2 bind to the same general region on CD28 and CTLA4 but the amino acids critical for B7-1 or B7-2 binding are different. The CDR3 loop of the IgV-like domain of CD28 and CTLA4 contains a conserved MYPPPY motif (amino acids 99–104) important for both B7-1 and B7-2 binding. Tyrosine 104 is critical for binding to both B7-1 and B7-2. Residues 100, 99, 101 and 102 are important for differential binding of B7-1 versus B7-2.

Though B7-1 and B7-2 have similar structures, their extracellular domains have only 27% amino acid identity and other regions are even less conserved. B7-1 and B7-2 function well across species, suggesting that the ligand binding site is highly conserved. Conserved residues on the GFCC'C" face of the B7 IgV domain and on the ABED face of the B7 IgC domain are important for binding. The eight N-linked glycosylation sites of B7 are on the opposite side from the B7/CD28 interaction surface and do not contribute to binding. Thus, CD28 and CTLA4 use sequences in their CDR3 and CDR1 regions, much like antibodies, to bind to a surface on the side of the B7 IgV and IgC domains.

Expression

B7 expression is tightly regulated and mainly restricted to antigen presenting cells. B7 on the cell surface is a monomer but is locally concentrated in small foci. B7-2 is constitutively expressed on unstimulated dendritic cells and blood monocytes. B7-1 is generally absent from unstimulated cells. B7-1 mRNA transcription is regulated by a cell-type specific enhancer responsive to NF κ B family members. B7 expression is upregulated by 'danger' signals such as bacterial DNA or lipopolysaccharide and by the innate immunity receptor, Toll. On dendritic cells, both B7s are rapidly upregulated by granulocyte-

monocyte colony-stimulating factor (GM-CSF) or CD40L/CD40 signaling. On monocytes, B7 levels are increased by interferon γ (IFN γ). Unstimulated human B and T cells do not express B7. B7-1 and B7-2 are induced in B cells by cross-linking of CD40, surface Ig, or MHC class II, and by interleukin-2 (IL-2), IL-4, IL-5, tumor necrosis factor α (TNF α) and lymphotoxin (TNF β). Memory B cells constitutively express B7-1 and B7-2 and rapidly upregulate them, permitting memory B cells to rapidly activate T cells. B7-1 and B7-2 are induced in T cells following activation and are upregulated by IL-7. Other cells also can express B7, including IFN γ -treated fibroblasts (B7-1), vascular endothelial cells (B7-2), IFN γ -treated astrocytes (B7-2) and gastric epithelial cells. Adjuvants (e.g. Freund's adjuvant, Neisserial porins) strongly induce B7 expression, providing an important mechanism for their stimulation of an immune response. Generally, B7-2 is expressed more rapidly and at higher levels than B7-1. B7-2 is expressed at sites of inflammation and B7-1 expression is found when chronic inflammation progresses to tissue damage.

B7 expression is downregulated by IL-10, transforming growth factor β (TGF β), ultraviolet light, the environmental toxin, dioxin, and cross-linking of the low-affinity IgG receptor, CD32, by immune complexes. Microorganisms (e.g. *Leishmania donovani*, *Mycobacterium tuberculosis*) can inhibit B7 expression. These agents are immunosuppressive, presumably in part because they reduce B7 costimulatory signals.

Function

The B7/CD28 pathway is important for cellular and antibody responses to viral and microbial infections, cancer, graft rejection and the development of autoimmunity. During antigen presentation, B7 bound to CD28 or CTLA4 coassociates on the cell surface with MHC-peptide bound to TCR in the activation cap between T cell and antigen-presenting cell (APC) but engages a distinct signaling pathway. For B7-1, this colocalization depends on the short but highly positively charged cytoplasmic tail which interacts with cytoskeletal components.

Binding of B7 to CD28, in conjunction with a TCR signal, stimulates production of multiple cytokines, including IL-1, 2, 3, 4, 5, 6, 8, 10, 13, TNF α , TNF β , GM-CSF, CSF-1 and IFN γ . B7/CD28 signaling upregulates expression of growth factor receptors including α , β and common γ chain of the IL-2 receptor. B7/CD28 signaling upregulates chemokines (MIP-1 α , MIP-1 β and RANTES) and chemokine receptor CXCR4, but downregulates expression of

chemokine receptors CCR1, CCR2a, CCR2b and CCR5. B7/CD28 interaction stimulates production of the anti-apoptotic proteins bcl-x_L and bcl-2, leading to enhanced T cell survival and upregulates telomerase expression, thereby contributing to T cell clonal expansion. B7/CD28 interaction activates cyclic AMP-responsive element-binding protein which regulates proliferating cell nuclear antigen, DNA polymerase δ and cyclin A. While some of these proteins can be induced by a strong TCR signal alone, B7/CD28 signaling greatly hastens and augments the level of expression. B7/CD28 interaction lowers the threshold for T cell activation by approximately 2 logs of antigen concentration and is thus most important for low antigen doses and weaker antigens. Nevertheless, B7/CD28 interactions are not essential for T cell activation as strong TCR signaling alone can activate T cells. While B7 can prime T cells, it is not essential for T cell priming. B7/CD28 interactions are critical for T cell clonal expansion, high-level lymphokine production, and T cell help for B cells.

The B7/CD28 and CD40/CD40L pathways cross-talk to reinforce immune activation. CD28 signaling induces CD40L expression and CD40 signaling induces B7 expression, and in dendritic cells and macrophages also induces IL-12 secretion. The cross-talk and synergistic function of these pathways is supported by the observations in some transplant models which show that optimal tolerization is achieved by blockade of both the B7/CD28 and CD40/CD40L pathways.

B7/CD28 interaction is also critical for the differentiation of precursor CD8 T cells into cytolytic effectors and induces cytolytic proteins such as granzyme B. Following the development of cytolytic effectors, B7 expression is not needed on the target cell for cytotoxic T lymphocyte killing.

CTLA4-Ig, a fusion protein in which the extracellular domain of CTLA4 is fused to IgG, binds to B7-1 and B7-2, and is a potent blocker of immune responses. While high concentrations of CTLA4-Ig completely inhibit both B7-1 and B7-2 mediated costimulation, lower concentrations inhibit B7-1 but not B7-2-mediated responses.

CTLA4-Ig or a combination of anti-B7-1 and B7-2 monoclonal antibodies (mAbs) have shown great promise in blocking autoimmune disease or tolerizing for transplantation. CTLA4-Ig or anti-B7-1 and B7-2 mAbs have been used to tolerize successfully for pancreatic islet cell xenotransplants, heart transplants and bone marrow transplants. Following cessation of CTLA4-Ig treatment, donor antigen-specific tolerance is seen. The mechanism for this tolerance is hypothesized to be T cell clonal anergy.

A competent immune response to other antigens and infections remains.

B7-2 is the major costimulator for initiating both T_{H1} and T_{H2} immune responses because of its constitutive expression and more rapid upregulation. The need for two B7 proteins is unclear but many studies with murine models of autoimmunity show different effects of blocking B7-1 or B7-2. Whereas complete blockade with CTLA4-Ig or anti B7-1 plus anti-B7-2 mAbs prevents the development of disease in murine models of nonobese diabetes (NOD), systemic lupus erythematosus (SLE) and experimental autoimmune encephalitis (EAE), and alleviates symptoms in established disease, different effects are seen when either B7 is blocked. In NOD and SLE, anti-B7-2 mAb is sufficient to alleviate disease but in NOD, anti-B7-1 mAb alone exacerbates disease. In contrast, anti-B7-1 mAb alleviates EAE, whereas anti-B7-2 mAb exacerbates EAE. B7-1 is a more effective costimulator for weak peptide ligands and this may explain the importance of B7-1 in the progression of autoimmunity to subdominant epitopes.

Alternatively, these differences may be due to timing of expression, as, in NOD and SLE, B7-1 and B7-2 are regulated by normal genetic mechanisms but, in EAE, B7-1 and B7-2 are artificially upregulated by adjuvant. Differences in function also may be due to differences in strength or quality of signal delivered upon binding to CD28/CTLA4. B7-1 and B7-2 can equivalently stimulate IL-2 and IFN γ production, however, the level of IFN γ is only moderate, with IL-12 being critical for the high-level IFN γ production characteristic of T_{H1}. B7-2 costimulation along with antigen concentration and affinity are important for the differentiation of naive T cells into the initial producers of the IL-4 that primes for further IL-4 synthesis. Three studies show that B7-2 more effectively induces IL-4, but two others find that B7-1 and B7-2 induce IL-4 equally. However, after T_{H2} differentiation, a TCR signal alone can be sufficient for IL-4 production. B7/CD28 signaling also regulates proliferation of T_{H2} cells via the induction of IL-1 which makes T_{H2} cells responsive to IL-4.

Studies on antibody production using mice lacking both B7-1 and B7-2 show a loss of germinal center formation and reductions in IgG1 (IL-4 dependent) and IgG2a (IFN γ dependent) of 99% and 95%, respectively. Deletion of B7-1 alone has no effect on antibody production, whereas deletion of B7-2 alone results in a loss of IgG production when mice are immunized in the absence of adjuvant. When B7-2 deficient mice are immunized in the presence of adjuvant, which induces B7-1, IgG levels are normal. These studies show that if it is induced, B7-1 can

replace B7-2 in stimulating help for antibody production.

Productive infection by human immunodeficiency virus (HIV) requires activated memory CD4 T cells. Blockade of B7/CD28 prevents HIV transmission in an *in vitro* T cell/dendritic cell system. CD28 signaling promotes viral cDNA synthesis, transport of viral DNA to the nucleus, and provirus expression, and enhances HIV-Tat protein-mediated IL-2 production. CD28 signaling upregulates expression of CXCR4, the T-tropic fusion coreceptor, but not CCR5, the macrophage-tropic coreceptor. Progression of HIV-infected individuals to the acquired immune deficiency syndrome (AIDS) is associated with loss of M-tropic strains and appearance of T-tropic strains. Before progression, *in vitro* expansion of T cells from HIV-infected individuals with anti-CD3 and anti-CD28 mAbs leads to loss of HIV through downregulation of CCR5, and production of MIP-1 α , MIP-1 β and RANTES, and provides a source of healthy T cells. However, following progression to a T-tropic strain of HIV, CD28 stimulation might increase virus load. Using HIV antigens and either B7-1 or B7-2 in naked DNA vaccines, B7-2 but not B7-1 stimulated both cytotoxic T lymphocyte and delayed-type hypersensitivity responses against HIV antigens in mice.

Tumors generally do not express costimulatory molecules. The B7 gene has been transfected into tumor cells in the hope that the tumor cells will gain costimulatory function and stimulate an antitumor response. Many B7-transfected tumor cell lines are rejected by their host. When an animal that has rejected a B7⁺ tumor is challenged with parental tumor not expressing B7, the tumor is rejected, demonstrating that B7 can stimulate antitumor immunity. Immunity is best induced by live B7⁺ tumor cells and is primarily CD8 T cell mediated. B7-transfected tumor cells can stimulate a successful immune response against established wild-type tumor if the tumor burden is small. Some studies find that B7-1 and B7-2 equivalently stimulate antitumor immunity, whereas others report that B7-1 is more effective.

CTLA4 has a greater than 400-fold higher avidity for B7 than does CD28 but is expressed only after T cell activation and at only 2–5% of the level of CD28 cell surface expression. In contrast to the stimulatory signal delivered by B7/CD28 interaction, the B7/CTLA4 interaction results in a downregulatory signal leading to inhibition of IL-2 production and cell cycle progression and to T cell death. An mAb with dual specificity that recognizes a common epitope shared by CTLA4 and CD28 positively stimulates T cell proliferation, suggesting that

B7/CD28 signaling dominates over B7/CTLA4 signaling.

The differing avidities of B7 for CD28 and CTLA4 and their highly regulated temporal expression has led to a model in which moderate levels of B7 engage CD28 on resting T cells and costimulate T cell activation. Following activation, the T cell expresses CTLA4 and CD28, and with moderate to high levels of B7, both are engaged, with the stimulatory CD28 response dominating. As B7 expression declines to low levels, all available B7 is engaged by higher avidity CTLA4 and CTLA4 signaling leads to downregulation of the T cell response.

See also: Anergy, T cell; Antigen-presenting cells; Autoimmunity; B lymphocyte activation; CD28; CD40 and its ligand; Cytotoxic T lymphocytes; Dendritic cells; Graft rejection; Human immunodeficiency viruses; Humoral immunity; Immunotherapy of tumors; Second signals for lymphocyte activation; T lymphocyte activation; Tolerance, peripheral; Tumors, immune response to.

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BABESIOSIS

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Babesiosis is a malaria-like disease transmitted by parasitized ticks of the genus *Ixodes* during blood feeding. There are over 100 species of *Babesia* which, with *Theileria*, comprise the family Piroplasmorida. Both adult and nymph stages of the *Ixodes* ticks (*I. scapularis*, *I. pacificus*, *I. ricinus*) transmit the disease from reservoir competent-species, principally rodents, to humans (Figure 1). The rodent strain, *Babesia microti* (in the United States), and the cattle strains, *B. divergens* and *B. bovis* (in Europe), are responsible for the majority of human cases. Conditions favorable for human infection include presence of disease reservoir species (rodents), of suitable hosts for blood feeding (deer, cattle) and proximity to human populations. Disease transmission through blood products and transplacental and possible perinatal infection have been described. In the United States, the majority of cases occur in the Northeast, upper Midwest and Pacific Northwest. Recently, a new species of *Babesia*, designated WA-1, has been implicated in human babesiosis in Washington and northern California. Phylogenetic analysis of DNA isolated from this species demonstrates greater similarity with *B. gibsoni*, a canine form, and to theileria species than to species known to infect humans. A fatal case of babesiosis has also been reported from Missouri. The isolate, designated MO-1, was found to have antigenic similarities and ribosomal DNA

sequences similar to *B. divergens*, a strain that causes disease in cattle and humans in Europe.

Infection with the murine strain, *B. microti*, is typically a mild illness in healthy people; however, in the asplenic or immunocompromised patient, such as those with AIDS, lymphoma or transplant patient, the disease may be overwhelming. Infection with bovine strains *B. divergens* and *B. bovis*, as seen in Europe, occurs in asplenic patients and is often fatal. Initial symptoms of babesiosis are nonspecific and include fever, chills, nausea, vomiting, arthralgia, myalgia, headache, fatigue and dark urine. The presence of a rash similar to erythema chronicum migrans probably reflects simultaneous infection with *Borrelia burgdorferi*, which is transmitted by the same vector. Laboratory studies may show decreased haptoglobin, elevated reticulocytes and parasitized red cells on blood films. Leukocytosis is rare; however, thrombocytopenia is common. Patients often have a positive direct Coombs test, and urine studies reveal proteinuria and hemoglobinuria. Liver function tests often show mild elevations of alkaline phosphatase, serum bilirubin, aspartate transaminase, alanine transaminase and lactate dehydrogenase. Successful treatment involves combination drug therapy with clindamycin plus quinine, or atovaquone plus azithromycin. Emergence of resistant strains has been documented following monotherapy. Life-

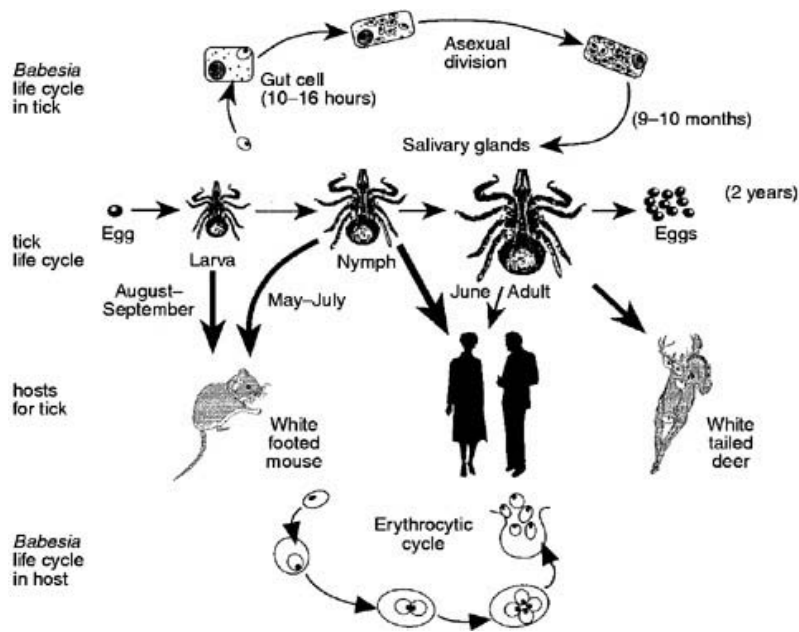


Figure 1 Life cycle of *Babesia*. The parasite requires the presence of *Ixodes* ticks, a reservoir-competent host animal such as rodent species, and large mammals for nymph and adult stages of the tick. Human infection occurs following the bite of infected nymph or adult ticks.

threatening cases may benefit from exchange blood transfusion.

The pathogen

After passage from the salivary glands of the tick into the vertebrate bloodstream, sporozoites penetrate and infect host erythrocytes (Figure 1). Following infection, *B. microti* undergo asynchronous, asexual budding and divide into two or four merozoites (daughter cells) (Figure 2). Intraerythrocytic merozoites often appear in a ring form morphologically similar to *Plasmodium falciparum*, which has resulted in diagnostic confusion (Figure 3). Infection by the rodent species, *B. rhodaini*, has been shown

to involve the alternative complement pathway and the C3b receptor on rat erythrocytes. The parasite activates complement and fixes C3b to its surface, which in turn binds to the rat erythrocyte C3b receptor, leading to intracellular infection. Additionally, erythrocytes with C3 on their surface are infected, thus suggesting that complement facilitates the parasite-erythrocyte interaction. Following intracellular maturation, merozoites exit the erythrocyte, resulting in perforations and damage to the red cell membrane. The actual mechanism of hemolysis is unknown. Circadian hemolysis from synchronous schizogony, typified by the cyclic fevers of *Plasmod-*

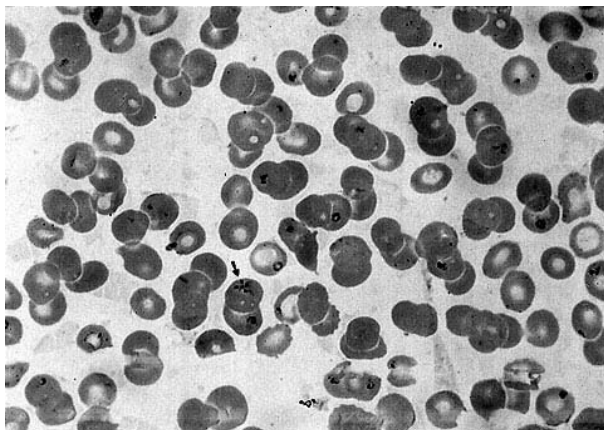


Figure 2 Giemsa-stained thin blood film. Four mature merozoites form the 'tetrad' or 'Maltese cross' prior to schizogony (single arrow). (Courtesy of Philip R. Daoust MD.)

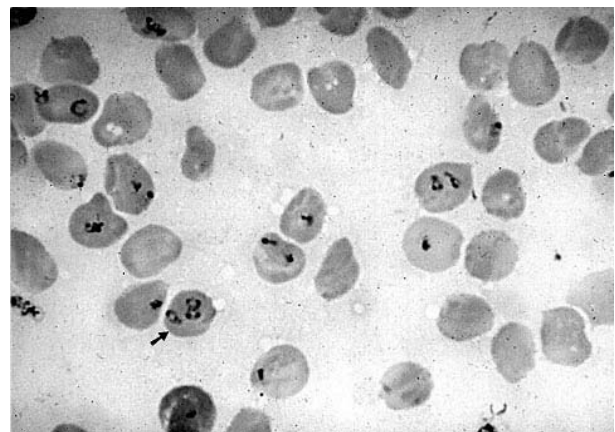


Figure 3 Giemsa-stained thin blood film of a patient with *B. microti*. Parasites often adopt intracellular ring forms that resemble *Plasmodium falciparum*. (Courtesy of Philip R. Daoust MD.)

ium infection, does not occur because schizogony is asynchronous.

Host response to babesia is poorly understood; however, the spleen is known to play a central role in immune defense. Asplenic patients and animals are more susceptible to infection by babesia and have increased parasitemia. Splenectomy and steroid therapy in animals and patients that have recovered from babesiosis may result in recrudescence of disease. As blood percolates through the spleen it is in close contact with splenic endothelium, macrophages and macrophage products. Erythrocytes must squeeze through intraendothelial spaces. Infected, deformed and potentially more rigid infected erythrocytes are more likely to be retained, where ingestion by macrophages or the action of macrophage products may serve to control the infection. Complement-sensitized erythrocytes would likely bind under these circumstances. Furthermore, complement activation by babesia could theoretically lead to the generation of tumor necrosis factor α (TNF α) and interleukin-1 (IL-1), enhancing local defense. Decreased complement levels, presumably secondary to activation, are frequently found in babesiosis. In addition, increased circulating C1q binding activity, presumably due to immune complexes, with a commensurate decrease in C4, C3 and CH50 levels, are seen in patients. The generation of these primarily macrophage-produced mediators (TNF α and IL-1) in turn could explain many of the clinical features (fever, anorexia, arthralgias, myalgias) of babesiosis, especially the fulminant shock syndrome of bovine babesiosis. It is likely that, in the less fulminant situation, TNF α functions to enhance the killing of *Babesia*, as with malaria. In addition to macrophage factors, other cellular immune functions appear to be important in the host response to *Babesia*. Nude mice and mice with greatly reduced T lymphocyte responses due to thymectomy, lethal irradiation or anti-T-lymphocyte serum develop significantly greater parasitemia. The disease itself alters cellular immune function. Patients with acute babesiosis have an increase in T suppressor/cytotoxic lymphocytes and decreased responses to lymphocyte mitogens with a polyclonal hypergammaglobulinemia.

See also: Parasites, Immunity to; Ungulate immune systems.

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BACILLUS, INFECTION AND IMMUNITY

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The best known member of the genus *Bacillus*, *Bacillus anthracis*, the causative agent of anthrax, was a major scourge for humans and animals from antiquity to just a few decades ago. The extensive history associated with anthrax dating back to about 1250 BC has been well reviewed. It features in classical writings, and several pandemics evidently occurred in Europe and Russia from medieval times to the mid-1800s. By the time it was realized at the end of the nineteenth century that one agent was responsible for several manifestations, the disease had become known by a wide variety of names, such as black bane, malignant carbuncle, rag picker's disease, Siberian plague, woolsorter's disease and numerous equivalents in other languages. It was with anthrax that Koch established his famous postulates in 1876, and history's first bacterial vaccines were against anthrax.

Other members of the genus only began to feature in literature in the early part of the twentieth century and, because of the extreme diversity of the species comprising it, organization of the taxonomy and classification within the genus has only developed very slowly and remains a challenge for today's taxonomists. For most practical purposes, the classification and identification system laid down by Gordon and colleagues in 1973 continues to be the most helpful for the principal species encountered in the context of infection and immunity.

Description and distribution of the genus

The family Bacillaceae comprises the rod-shaped bacteria which form endospores. The two principal subdivisions are the anaerobic spore-forming bacilli of the genus *Clostridium* and the aerobic or facultatively anaerobic endospore formers constituting the genus *Bacillus*. Familiarly known as aerobic spore-bearing bacilli (ASB), they are gram positive or gram variable and the majority are motile by peritrichous flagella. The endospores, one per vegetative mother cell, are resistant to adverse physical and chemical conditions. They are mostly saprophytes, having a wide diversity of physiological characteristics, which, together with their ability to form hardy spores, enables them to be widely distributed in nature from Arctic environments to hot springs and desert sands and from fresh water to salt or marine sediments.

Thermophilic and psychrophilic, acidophilic and alkaliphilic, and halophilic representatives exist which are capable of growing at extremes of temperature, pH or salt concentration that would inhibit or destroy life processes in other living organisms. The spores are dispersed in dust, water and on materials of plant or animal origin.

Importance

As a result of their wide-ranging physiological characteristics and the myriad of enzymes, antibiotics and other metabolites they elaborate, *Bacillus* species are important in a host of medical, agricultural, pharmaceutical or other industrial applications and processes. Among the better known antibiotics are bacitracin from *B. licheniformis* or *B. subtilis*, polymyxin from *B. polymyxa* and gramicidin from *B. brevis*. Several are important in antibiotic assays (*B. pumilus*, *B. cereus*, *B. stearothermophilus*) or other health-related assays, e.g. folic acid (*B. coagulans*), aflatoxin (*B. megaterium*) and hexachlorophene (*B. subtilis*). Certain *Bacillus* species are utilized for degradation of pollutants and waste materials. One strain of *B. cereus* is the active ingredient of an antidiarrheal formulation prescribed in certain countries of Europe. The spores of the thermophilic *B. stearothermophilus* are particularly well suited to checking heat sterilization procedures for laboratory, surgical, pharmaceutical and food preservation processes, and *B. subtilis* var *globigii* (NCTC 10073), having a high resistance to heat, radiation and chemical disinfectants, is used for validating alternative sterilization and fumigation procedures. Contrariwise, the resistance of spores to heat, radiation, disinfectants and desiccation results in *Bacillus* species being troublesome contaminants in pharmaceutical products, foods, surgical theatres and dressings, etc. *B. thuringiensis*, *B. popilliae*, *B. sphaericus*, *B. larvae* and *B. lentimorbus* are insect pathogens. The first three are utilized in commercial insecticides for controlling crop-destroying and disease-carrying insects; *B. thuringiensis* has the widest use in this context, providing control of an extensive range of pests on many different crop types.

Bacillus species and diseases

That the etiological agent of anthrax is a *Bacillus* species, namely *B. anthracis*, was mentioned at the outset. The pathogenic potential of ASB other than *B. anthracis* has become increasingly recognized in the past three decades with a proliferation of reports implicating *Bacillus* species, particularly *B. cereus*, in distinct emetic and diarrheal types of food poisoning and in infections of immunocompromised or otherwise debilitated hosts (e.g. alcoholics, diabetics), in mixed or secondary infections or occasionally in primary infections in otherwise healthy humans or animals.

Species in addition to *B. anthracis* that have been implicated on one or more occasions in published reports on infections are *B. cereus* (numerous reports), *B. licheniformis* and *B. subtilis* (several reports), *B. alvei*, *B. brevis*, *B. circulans*, *B. coagulans*, *B. macerans*, *B. pumilus*, *B. sphaericus* and *B. thuringiensis* (a few reports). Among the types of infection involved apart from food poisoning are bacteremia/septicemia, endocarditis, meningitis, ophthalmitis, respiratory and urinary tract infections and severe wound infections.

Virulence factors in anthrax

Bacillus anthracis depends for its virulence on two factors: 1) a polypeptide (poly- γ -D glutamic acid) capsule protecting it from phagocytosis, and 2) a toxin comprised of three protein components termed protective antigen (PA – 83 kDa), lethal factor (LF – 90 kDa) and edema factor (EF – 89 kDa). By the currently accepted model, PA binds to host cell surface receptors and is activated by a host cell protease, furin, which cleaves off a 20 kDa piece, thereby exposing a secondary receptor site for which LF and EF compete to bind on the active PA (PA63). Complexes of PA + LF (lethal toxin) or PA + EF (edema toxin) are then internalized, apparently in heptameric complexes, by receptor-mediated endocytosis, with the LF or EF being released into the cytosol from the endosome following acidification. There is some analogy here to the A–B complex of cholera toxin, with EF and LF acting as alternate ‘A’ (active) moieties competing for the same entry mechanisms and with PA as the ‘B’ (binding) moiety, with PA probably creating ion-conductive channels across the eukaryotic cell membrane through which LF and EF gain access to the interior of the cell. Edema factor is a calmodulin-dependent adenylate cyclase (calmodulin only being present in eukaryotic cells). The only other known bacterial adenylate cyclase toxin component is that of *Bordetella pertussis* but

the two do not appear to be closely related structurally, genetically or antigenically, although some genetic and antigenic homologies have been noted.

LF appears to be a calcium- and zinc-dependent metalloenzyme. It is generally believed that the LF + PA complex is the major cause of tissue damage and death, with systemic shock and death seemingly resulting from the effects of high levels of cytokines (see below).

The precise kinetics of production of PA, LF and EF are not known; they appear to be elaborated concurrently and can be detected in cultures from early log phase onwards. Virulence differences among strains have not been associated with differences in quantity or rate of toxin production by different strains, and the toxin from different strains appears identical in all respects – attributable to it being encoded on a plasmid. The origin of the toxin components in the *B. anthracis* cell has not been elucidated.

The two virulence factors of *B. anthracis*, toxin and capsule, are genetically encoded on two large plasmids, respectively pX01 (175 kb) and pX02 (90 kb). The genes for each of the toxin components *pag*, *lef* and *cya* and for the membrane associated enzymes mediating D-glutamic acid polymerization have all been sequenced.

Pathogenesis and host resistance in anthrax

Lethal toxin (LF + PA) and edema toxin (EF + PA) are now regarded as being responsible for the characteristic symptoms and course of anthrax. LF + PA is cytolytic for mouse peritoneal macrophages and kills certain mouse macrophage cell lines; EF + PA impairs the phagocytic function of neutrophils but EF + PA, LF + PA, or EF + LF + PA stimulate neutrophil chemotaxis.

There has long been fascination over the seemingly inverse relationship between susceptibility to toxin and susceptibility to infection. Rats, for example, are highly susceptible to the toxin (LD_{50} (median lethal dose) of Fischer 344 rat = 0.5 μ g LF + $\approx$ 2.5 μ g PA) but highly resistant to the infection (intramuscular LD_{50} $\sim$ 10^6 spores), while guinea pigs are relatively resistant to the toxin (LD_{50} = 50 μ g LF + $\approx$ 250 μ g PA) but highly susceptible to infection (intramuscular LD_{50} < 10 spores). The underlying host defense mechanisms responsible for this paradox have not been elucidated but it indicates that resistance to infection may involve a separate mechanism than that required for resistance to lethal toxicity. If resistance to infection is due to the number and availability of macrophages then this protective

mechanism would also provide a larger target population of macrophages for the effect of LF toxin.

The *in vivo* depletion of mouse macrophages by the use of repeated injections of silica has elucidated the key role macrophages play in the disease process. BALB/c mice depleted of macrophages are resistant to lethal toxin (LT) but can be made sensitive to it by adoptive transfer of 10^8 RAW264.7 cells (a toxin-sensitive monocyte/macrophage cell line). The *in vivo* transfer of the same number of the LT-resistant mouse macrophage line IC-21 cells does not restore sensitivity. Resistant cells have been shown to possess normal numbers of receptors for PA and are able to activate PA and bind and internalize LF and EF. In addition, introduction of LF into the cytosol of resistant cells does not result in cell death, indicating that these cells may lack the cytosolic target of LF. Genetic studies involving resistance to nonencapsulated bacilli indicate that resistance may be controlled by a single dominant locus or gene complex.

Analysis of cytokine levels produced by RAW264.7 cells *in vitro* has revealed that levels of LT as low as 10^{-9} $\mu\text{g ml}^{-1}$ LF with 0.1 $\mu\text{g ml}^{-1}$ PA induce synthesis of high levels of tumour necrosis factor (TNF) and interleukin-1. Neutralizing antisera to each of these cytokines injected into mice 24 hours before challenge with LT provides partial protection, while complete protection is achieved when the antisera are combined. These results strongly implicate the release of these factors in the cause of death of toxin-treated animals by a septic shock-type mechanism. Whether this and differences in susceptibilities of macrophages to LF + PA/EF + PA are related beyond mouse models to inter- and intraspecies differences in susceptibility to *B. anthracis* or its toxin remains to be determined.

Virulence factors and pathogenesis of other *Bacillus* species

The state of knowledge on virulence factors of *Bacillus* species other than *B. anthracis* is less advanced owing to the relatively spasmodic association between these species and infections and to the absence of adequate models for analyzing the candidate factors and determining their actions in the laboratory.

Bacillus species characteristically produce a host of enzymes and other extracellular metabolites. Among those of *B. cereus*, four groups of what are broadly described as toxins have been distinguished and most of these are believed also to be virulence determinants in infections. These are:

- 1 Three phospholipases C (alias lecithinase, egg-yolk turbidity factor), phosphatidylcholine

hydrolase (23 kDa), phosphatidylinositol hydrolase (phosphatase factor, 29 kDa) and sphingomyelinase (29 kDa). Although under normal *in vivo* circumstances they cannot reach their phospholipid substrates in the eukaryotic cell membrane, they may conceivably act secondarily after exposure of the phospholipids in wounds or other infections. Sphingomyelinase is also hemolytic.

- 2 Hemolysin I (alias cereolysin), a thiol-activated thermolabile protein cytotoxin with a single 518 amino acid polypeptide chain of 55 kDa. The binding-site of the thiol-activated cytotoxins on the eukaryotic cell membrane is cholesterol and binding results in pitting and micropuncturing of the cell membrane, leading to loss of control of ion exchange. The net flow of ions and water into the cell leads to swelling and rupture. However, cereolysin is inactivated by free cholesterol in the blood, which presumably limits its activities in natural infections. It is almost instantly lethal on intravenous injection in mice and causes necrosis if injected into skin.
- 3 Hemolysin II (HBL), consisting of a protein (component B, 36 kDa) which binds to or alters cells, allowing subsequent lysis by a second protein (component L, 45 kDa). This appears also to be the necrotic enterotoxin responsible for diarrheal-type *B. cereus* food poisoning and occasional severe wound, eye and other infections. It is almost instantly lethal if injected intravenously in mice; unlike cereolysin, this activity is not neutralized by cholesterol.
- 4 Emetic principle. This is a highly stable, small molecular weight entity, or group of entities, capable of surviving extremes of heat and pH and not susceptible to proteolytic enzymes. It may be associated with breakdown products from the implicated food, although it has recently been proposed that it is now identifiable as 'cereulide', a novel dodecadeptide.

The protein cytotoxin enterotoxin identified by Japanese workers in 1991 may represent a fifth group. The different toxins appear to be produced to different extents by different strains, which may account for the varying degrees of seriousness of *B. cereus* infections.

Toxins have not been demonstrated in culture fluids or extracts of other *Bacillus* species periodically implicated in infections and, assuming they did play active roles in the infections from which they were isolated, the basis of their pathogenic activity is not clear.

Acquired immunity: vaccines

The polypeptide capsule of *B. anthracis* is only weakly antigenic and there is little evidence to suggest it contributes to naturally acquired immunity or can produce artificially induced protection against anthrax. Pasteur derived his vaccine strains by subculturing at 42–43°C and was inadvertently curing the bacilli of pX01. However, cap⁺/tox⁻ strains are, in fact, not protective and both the partial efficacy and the residual virulence of Pasteur's vaccines are now explained in terms of mixed cultures with residual uncured forms. Cap⁻/tox⁺ strains of *B. anthracis* are protective and one such strain, produced by Sterne in 1937, has remained to this day the basis of a highly successful live-spore animal vaccine, although this vaccine too retains some residual virulence in certain susceptible species (albeit considerably less than wild type tox⁺/cap⁺ *B. anthracis*), such as guinea pigs, a number of inbred strains of mice and certain livestock species, notably goats and llamas. As a result of this, it is considered unsuitable in the West for use in humans, although a live-spore vaccine similar to the animal vaccine is administered by scarification to people in the countries of the former USSR and in China. In the West, human vaccines developed in the 1950s and 1960s are available and consist essentially of aluminum hydroxide-adsorbed cell-free filtrates of cultures of a noncapsulating, nonproteolytic derivative of strain V770 originating from a case of bovine anthrax in 1951 (US vaccine) and alum-precipitated cell-free filtrates of Sterne strain cultures (UK vaccine). Several doses are required, however, for these to take effect and the results of animal tests suggest it may be possible to improve their efficacy; attempts are now being made to do this using the rapidly expanding knowledge of what is and what is not needed for protection.

With recently acquired techniques for purification and genetic manipulation of the toxin components, it has become clear that immune responses to PA, but not to LF or EF, are essential for protection. However, studies have also indicated that, while antibody responses to EF and LF are weak in mice immunized with spores of *B. anthracis* strains capable of only producing either EF or LF, immunization with strains producing either PA or EF or PA and LF resulted in higher antibody responses to EF or LF, respectively. In addition, studies have indicated a synergistic relationship between PA and LF or EF in immunoprotection. These results may indicate a requirement for EF and LF to be internalized, via PA, and intracellularly processed in order to produce substantial antibody responses. Notwithstanding this, the protection induced by PA alone can be

raised to levels equivalent to and as long-lasting as that conferred by the live-spore vaccine if purified PA is administered together with killed whole-cell microbial supplements such as Freund's complete adjuvant, *Bordetella pertussis* or *Corynebacterium ovis*, or complex cell wall extracts from the tubercle bacillus ('Tri-Mix' and 'De Tox', Ribi ImmunoChem Research, Hamilton, Montana, USA). This nonspecific stimulation of protection indicates involvement of some cellular immune mechanisms – more than that simply produced by inciting a coincidental inflammatory response as the performance of PA is not enhanced by an irritant such as saponin.

To date, PA is the only antigen shown to induce substantial protective immunity. Studies on monoclonal antibodies directed against PA, which neutralize lethal toxin activity, either *in vivo* or *in vitro*, have identified at least three nonoverlapping antigenic regions. Two of these regions are involved in the binding of LF to cell-bound PA (the first region mapping between amino acids Ile581 or Asn601 and the second within the PA17 fragment produced by limited chymotrypsin digest of PA63). The third region is involved in the binding of PA to cells and maps between amino acids Asp671 and Ile721 of the carboxy-terminus of PA. The elucidation of the protective motifs on PA may eventually lead to the introduction of a subunit-based vaccine. The relationships between the toxin, the development of the characteristic massive bacteremia of anthrax and the mechanism by which antibodies to PA, a log phase metabolite, prevent this bacterial multiplication, remain to be elucidated. The development of a vaccine dependent on an antigen apparent to the immune system at the outset of the infection, rather than during the log phase of growth, appears to be unachievable.

Passive immunity was tried in Europe in the early part of the twentieth century in the treatment of anthrax but was discontinued many years ago as being of negligible value. In the light of modern plasmapheresis technology, it may be appropriate to reconsider this; it is possible that plasma from vaccinated individuals may offer a life-saving supplement to treatment in individuals diagnosed late in the course of a fulminating anthrax infection. Interestingly, antianthrax (horse) serum is still administered together with penicillin in the treatment of human anthrax in China.

Infections by *Bacillus* species other than *B. anthracis* are too infrequent and varied to merit development of any vaccines; consequently the topic of immunity in relation to such infections is an area that has not been studied.

See also: **Attenuated organisms as vaccines; Bacterial cell walls; Toxins.**

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BACTERIA, IMMUNITY TO

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Bacteria cause disease because of toxicity, invasiveness, immunopathology, or blends of these three mechanisms. Thus much of the interaction between a given bacterial species and the immune response can be predicted by considering the immunological mechanisms available in relation to the mechanism of pathogenicity, and the structure of the bacterium. For a toxigenic bacterium, neutralizing antibody may be all that is needed. Otherwise destruction of the organism itself may be required.

Nonspecific defenses

Nonspecific recognition of common bacterial structures

Much of the defense against bacteria depends on pathways which have nothing to do with the specific antigen receptors of either B cells or T cells, and

probably antedate these in evolution. Many bacteria are simply excluded by barriers such as skin, acidity in gut and vagina, commensals occupying the relevant niche, entrapment by mucus, etc. If not excluded, the organism may be recognized by:

1. Acute phase proteins, C-reactive protein and mannose-binding protein which also fix complement.
2. Receptors for the formyl peptides released by bacteria which are chemoattractants and activators of phagocytes.
3. Receptors for bacterial cell wall components, glycolipids, glucans and other polysaccharides that nonspecifically enhance the immune response, and can be used as immunological adjuvants.
4. Complement, activated in the absence of antibody by a wide range of bacterial components.

5. Receptors that lead to cytokine release. The bacterial products which do this include endotoxin (lipopolysaccharide, LPS) of gram-negative organisms, lipoteichoic acids of gram-positive cocci, and the lipoarabinomannan of mycobacteria. It is probable that all bacteria contain cytokine triggers.

Nonspecific recognition and selection of response mechanism Collectively these nonspecific recognition pathways perform crucial functions. First, the pattern of nonspecific recognition events, cytokines and bacterial products supplies information as to the type of immune response that needs to be activated. This immunoregulatory 'decision' is particularly important because activation of inappropriate effector mechanisms can lead to enhanced susceptibility rather than to protection. These features account for the 'adjuvant' properties of bacterial components, which are often used by immunologists to boost or direct responses to other antigens. For example, the adjuvant properties of *Bordetella pertussis* and *Mycobacterium tuberculosis* impose different balances of T_H1 to T_H2 cytokines on the T cell response to any simultaneously administered antigen. If the immune system activates a T_H2 pattern of response to a mycobacterial pathogen, accelerated disease will result.

Other factors influencing the T_H1/T_H2 balance of the T cell response Dose and the different handling of soluble versus particulate antigen are also important. Low dose soluble antigen selectively primes T_H2 , while low dose particulate antigen selectively primes T_H1 . These relationships reverse as dose is increased. The reason appears to be that presentation of low dose soluble antigen is mostly by B cells, which tend to drive T_H2 , while presentation of low dose particulate antigen (such as bacteria, or *Leishmania*) is mostly by macrophages, which tend to drive T_H1 .

Local metabolism within lymph nodes of glucocorticoids (cortisol), and of dehydroepiandrosterone (which yields metabolites that oppose some effects of cortisol), also profoundly influences T_H1/T_H2 cytokine balance. Cortisol diverts the response to T_H2 , and is partly responsible for increased susceptibility to certain infections in stressed individuals.

Nonspecific recognition and early defense: the protective role of cytokines The nonspecific recognition events cause sufficient activation of the immune system to mobilize a 'holding operation' that can control bacterial growth while specific immunity develops. The cytokines released have

rapid protective effects, but if their release is excessive or prolonged, they can cause severe immunopathology, and even death (see below). Nevertheless tumor necrosis factor α (TNF α) protects mice from *M. avium*, *Legionella pneumophila*, *Streptococcus pneumoniae* and *Klebsiella*. The mechanisms of protection by cytokines, particularly TNF α , include rapid induction (<5 min) of increased adhesion of neutrophils to endothelial cells, increased expression of CR3 and other cell adhesion molecules, priming of neutrophils for enhanced production of oxygen reduction products, and activation of macrophages. Cytokines also affect 'nonprofessional' phagocytes, and exposure of Hep-2 cells (derived from a human carcinoma of the larynx) to TNF α renders them resistant to invasion by *Salmonella typhimurium*. Similarly, interferon γ (IFN γ) derived from natural killer (NK) cells will activate killing mechanisms in macrophages.

Interactions of bacteria with complement Numerous bacterial components can activate the alternative pathway. This results in three categories of protective function:

1. Release of the chemotactic products. C3a and C5a. These cause smooth muscle contraction, mast cell degranulation, and neutrophil chemotaxis and activation.
2. Attachment to the organism of derivatives of C3, which play an important role in the subsequent interaction with phagocytes.
3. Killing of some gram-negative organisms if the lytic complex C5b-C9 gains access to the outer lipid bilayer.

Many organisms have devised strategies to resist these effects of complement. Some capsules are very poor activators of the alternative pathway. Alternatively, long side-chains (O antigens) on LPS may fix C3b at a distance from the vulnerable lipid bilayer. Similarly, smooth gram-negative organisms (*Escherichia coli*, *Salmonella*, *Pseudomonas*) may fix but then rapidly shed the C5b-C9 membrane complex. Capsules rich in sialic acid (like host cell membranes) promote the inactivation of C3b by interaction with factors H and I, and *Neisseria meningitidis*, *E. coli* K1 and group B streptococci resist complement attachment in this way. The M protein of group A streptococci acts as an acceptor for factor H and there is a gene for a C5a protease close to the M protein gene.

Role of antibody

Antibody clearly plays a crucial role during infections with toxigenic organisms. It neutralizes diphtheria toxin by blocking the attachment of the binding portion of the molecule to its target cells. Similarly it may block locally acting toxins, extracellular matrix-degrading enzymes which act as spreading factors, and motility due to flagella. An important function on external surfaces, often performed by secretory immunoglobulin A (IgA), is inhibition of binding of bacteria to epithelial cells. It is also likely that some antibodies to the bacterial surface can block functional requirements of the organism such as receptors for iron-chelating compounds or the intake of nutrients. However, the most important role of antibody in immunity to nontoxigenic bacteria is the more efficient targeting of complement so that even organisms that resist the alternative pathway by the mechanisms described in the previous section are damaged by complement, or become coated with C3 products.

Interactions between bacteria and phagocytic cells

The important consequence of the inflammatory and chemotactic events triggered by bacteria in the tissues is the enhanced exposure of the bacteria to phagocytic cells.

Binding, uptake and the triggering of killing

The first stage in the intracellular killing is the attachment of the organism to the surface of the phagocyte. This important and complex interaction determines whether uptake occurs, and whether killing mechanisms are triggered. In addition to complement and antibody, this interaction can involve C-reactive protein, lectins on the organisms and on host cell membranes, the family of adhesion-promoting receptors CR3, LFA-1, p150,95 and the integrins. Some organisms are able to enter cells via unconventional receptors, such as integrins, and so avoid triggering killing mechanisms.

Antimicrobial mechanisms of phagocytic cells

Activation of antimicrobial functions

Monocytes and polymorphonuclear cells possess a number of oxygen-dependent and oxygen-independent antimicrobial mechanisms. These, while often constitutively expressed, can be activated directly by bacterial products or by cytokines such as IFN γ .

Oxygen-dependent pathways: derivatives of superoxide anion Oxygen is reduced by a membrane-associated cytochrome system which transfers an electron onto molecular oxygen to form superoxide anion ($O_2^{\cdot -}$), usually during phagocytosis. Subsequent interactions give rise to hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), and perhaps singlet oxygen (Δ^1O_2), which is oxygen with one electron in a high energy state. These toxic intermediates are generated in the absence of myeloperoxidase (MPO) and therefore do not require fusion of the MPO-containing lysosomes with the phagosome. If fusion does occur, MPO catalyzes the formation of hypohalous acids from H_2O_2 and halides.

Oxygen-dependent pathways: nitric oxide Nitric oxide (NO) is formed from the guanidino nitrogen of L-arginine, by NO synthase, yielding citrulline as the other product. Formation of NO is enhanced by exposure to IFN γ and a trigger of TNF α release such as endotoxin, or the equivalent cytokine triggers of other organisms. The NO is a relaxant of vascular smooth muscle and has poorly understood roles in intracellular signaling. Within macrophages it probably acts as an antimicrobial agent, though such effects are difficult to distinguish from its signaling role. Whatever the mechanism, agents that block NO synthase enhance growth of *M. tuberculosis* within macrophages both *in vivo* and *in vitro*. It is more difficult to make human macrophages generate high levels of NO, but it is now clear that they can do if stimulated with mixtures of cytokines (interleukin 4 (IL-4) and IFN γ) or further triggered by cross-linking CD23.

Oxygen-independent pathways

Some oxygen-independent killing may be due to the sequential exposure to lysozyme and neutral proteases at pH 7.0–8.0, followed by acid hydrolases after the acidification step, but several more specific pathways have now been defined.

Cationic proteins Human granulocytes contain cationic proteins with microbicidal activity. Six have been sequenced and are cysteine- and arginine-rich peptides of 32–34 amino acids with molecular weights of about 4 kDa. They are representatives of a conserved family of related mammalian ‘antibiotics’ which have been named ‘defensins’. Similar proteins are found in the granules of rabbit alveolar macrophages but not in human macrophages, although these contain other cationic antibacterial substances, some of which are enzymes, which kill gram-positive and -negative bacteria and fungi by mechanisms quite independent of their enzymatic activity. More-

over, it is likely that the uptake of enzymes and cations from other cells plays a significant role in macrophage antibacterial function *in vivo*. The defensins will kill organisms as diverse as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Cryptococcus neoformans* and even the enveloped virus, *Herpes simplex*.

Tryptophan deprivation Exposure to IFN γ causes degradation of tryptophan in monocytes and fibroblasts. This amino acid is essential for some pathogens.

Cell-mediated immunity

This term is usually reserved for immunity that involves direct intervention of T lymphocytes. One of their functions is the release of lymphokines, which has several types of consequence.

1. Lymphokines attract monocytes and polymorphonuclear cells to the site of infection.
2. Lymphokines enhance antimicrobial functions of phagocytic cells. The major lymphokine for these functions is IFN γ .
3. It is likely that the mechanisms described in 1) and 2) constitute the basis for the delayed hypersensitivity reaction to skin-test challenge with soluble antigen, typified by the Mantoux test.
4. When release of lymphokines and accumulation of mononuclear cells is persistent, the characteristic T cell-dependent tuberculoid granulomas are formed. These granulomas can be isolated and the release of cytokines monitored *in vitro*. *In vivo* TNF α also seems to be involved and neutralizing antibodies to TNF α will block granuloma formation in response to BCG. It is likely that granulomas serve to isolate organisms within a focus of activated macrophages.
5. IFN γ also evokes antimicrobial mechanisms in other cells, such as endothelial cells which are incapable of the oxidative burst, as shown with *Rickettsia* and *Chlamydia psittaci*. Many cell types are capable of activation to produce nitric oxide.

Cytotoxic T cells

Cytotoxic T cells are also generated during the cell-mediated response to intracellular pathogens. These can kill infected or antigen-pulsed macrophages. It is becoming apparent that this phenomenon is at least as important as macrophage activation and may serve to release bacteria from macrophages that are failing to kill ingested bacteria and are merely acting as sites for intracellular growth. Following the death

of such cells, the bacteria can be taken up by fresh activated phagocytes.

Until recently the term 'cytotoxic T cell' was almost synonymous with CD8⁺ T cells with α/β receptors, recognizing peptide epitopes presented by major histocompatibility complex (MHC) class I. Now several other T cell types are known to be potentially cytotoxic, including some CD4⁺ cells.

γ/δ T cells In the mouse, mycobacterial infection leads to a disproportionate increase in the γ/δ T cell receptor-bearing population. Moreover γ/δ T cells which respond to mycobacterial antigens can be isolated from the lesions of some types of leprosy, and a strikingly high percentage of the circulating γ/δ T cells from normal donors respond to *M. tuberculosis*. Among the antigens recognized by these cells are heat shock proteins, compounds containing 5'-triphosphorylated thymidine, and phenyl pyrophosphate derivatives. They are cytotoxic and usually IFN γ secreting.

CD4⁻CD8⁻ CD1-restricted α/β T cells These T cells recognize antigens presented by CD1, which is a remotely MHC class I-like membrane glycoprotein. Clones have been identified that respond to mycobacterial glycolipids and lipids, so like γ/δ cells they indicate that recognition of bacteria by T cells does not depend only on recognition of peptide epitopes.

NK cells These lymphocytes kill cells that fail to express self MHC class I. There is unconfirmed evidence that some NK cells can directly damage certain bacteria. NK cells also play a role in macrophage activation through release of IFN γ . This pathway may provide the source of IFN γ in severe combined immunodeficiency (SCID) mice, which are unexpectedly resistant to many infections.

Avoidance of antimicrobial mechanisms of phagocytic cells

Organisms may secrete toxins which repel or kill the phagocytes. Capsules of carbohydrate (*Str. pneumoniae*) or polypeptide (*Bacillus anthracis*) may impede complement deposition and phagocytosis. The M proteins of group A streptococci limit uptake by phagocytes, while permitting specific attachment to the epithelium of the oropharynx. Other organisms are taken up, but fail to trigger the oxidative burst. Secretion of polyanions or ammonia have been reported to block phagolysosome fusion, although the methods used to demonstrate these phenomena have recently been re-evaluated. Superoxide dismutase and catalase can degrade oxygen intermediates,

and the phenolic glycolipid secreted by *M. leprae* may act as a scavenger of oxygen radicals. The lipoarabinomannan released from mycobacteria may inhibit lymphokine-mediated macrophage activation, as murine macrophages heavily infected with *M. leprae* cannot be activated to kill a protozoan. Other organisms avoid antimicrobial mechanisms by escaping from the phagosome. *M. leprae* and *Shigella flexneri* can multiply free in the cytoplasm, while *Brucella abortus* and *Legionella pneumophila* are found in the rough endoplasmic reticulum.

It is increasingly apparent that many bacteria, like viruses, hijack cellular machinery for their own use. *Listeria monocytogenes* uses 'hemolysins' to lyse the phagosome, and then interacts with actin in the cell in order to propel itself towards and into neighbouring cells. Finally, some organisms, such as *M. tuberculosis* tend to kill the macrophage, perhaps by rendering them sensitive to the TNF α which they themselves are induced to release.

Mechanisms of immunopathology

Septicemic shock and the adult respiratory distress syndrome

The cytokine release triggered by bacterial components has a protective and immunoregulatory role as described above, but when excessive, as during septicemia, there can be excessive systemic activation of phagocytes and of endothelial cells. The latter leads to adhesion of phagocytes to the endothelium, initiation of the clotting cascade via expression of tissue thromboplastin, and eventually to diffuse intravascular coagulation that exhausts the clotting system and leads to a clotting defect. Mediators such as platelet-activating factor (PAF) and NO are released, blood pressure falls and hemorrhage may occur. Neutralizing antibodies to tissue thromboplastin or inhibitors of PAF can attenuate this syndrome. A neutralizing antibody to TNF α is protective in a model of septicemic shock in the baboon. The systemic toxicity of TNF α is greatly increased in the presence of IL-1 or LPS and these are both likely to be present during bacterial infections. High serum levels of TNF α correlate with a poor clinical outcome in septicemia, and in the adult respiratory distress syndrome (ARDS), which is a similar condition that targets the lungs.

Shwartzman reaction

Cytokine release can also cause local rather than systemic pathology. Microbial products and certain types of inflammatory response (both T cell dependent and independent) 'prepare' tissue sites so that

they become exquisitely sensitive to cytokines, particularly TNF α , and liable to undergo hemorrhagic necrosis if TNF α release is subsequently induced systemically, or if TNF α is directly injected into the same site. This may explain the rash often seen during meningococcal septicemia. If bacteria released during a previous subclinical septicemic episode have lodged in skin capillaries, these will become 'prepared' sites, and a later septicemic episode severe enough to trigger systemic cytokine release will cause these sites to undergo necrosis via a cascade of events similar to those occurring systemically in endotoxin shock.

Koch phenomenon and necrotizing T cell-dependent granulomas

Robert Koch observed that when tuberculous guinea pigs were skin-tested with tuberculosis bacilli or culture supernatant (tuberculin), there was a necrotic reaction at the skin-test site within 24–48 h, and if a large dose of tuberculin was used there was additional necrosis in distant tuberculous lesions. The same is true in human tuberculosis patients. This phenomenon is described in the section on mycobacteria, and probably represents a pattern of tissue destruction and eventual fibrosis that occurs when there is local release of TNF α and other cytokines into T cell-dependent inflammatory sites mediated by a mixed T_H1 plus T_H2 lymphocyte response. A similar argument holds for tissue damage around granulomas evoked by schistosome ova, so the Koch phenomenon may be a model of an important immunopathological entity.

Heat shock proteins and the possibility of autoimmunity

It was discovered recently that several dominant antigens of infectious agents are 'heat shock' or 'stress' proteins. The sequences of these microbial antigens are similar to the sequences of the mammalian homologs. The fact that the immune response focuses much 'attention' on these conserved proteins may increase the chances of recognizing any pathogen, but also increase the chance of autoimmunity.

See also: **Bacillus**, infection and immunity; **Bacterial cell walls**; **Bacteroides**, infection and immunity; **Bordetella**, infection and immunity; **Borrelia**, infection and immunity; **Brucella**, infection and immunity; **Campylobacter**, infection and immunity; **Complement, alternative pathway**; **Chlamydia**, infection and immunity; **Coccidioides**, infection and immunity; **Complement, classical pathway**; **Complement deficiencies**; **Complement fixation test**; **Complement, genetics**; **Complement, membrane attack pathway**; **Complement**

receptors; Coryneform bacteria, infection and immunity; *Cryptococcus*, infection and immunity; Cytokines; Cytotoxicity, assays for; Cytotoxicity, mechanisms of; Effector lymphocytes; Endotoxin (lipopolysaccharide (LPS)); *Escherichia coli*, infection and immunity; *Francisella*, infection and immunity; *Fusobacterium*, infection and immunity; Granuloma; *Haemophilus*, infection and immunity; *Klebsiella*, infection and immunity; *Legionella*, infection and immunity; *Leptospira*, infection and immunity; *Listeria*, infection and immunity; Microbicidal mechanisms, oxygen-dependent; Microbicidal mechanisms, oxygen-independent; *Mycobacteria*, infection and immunity; *Neisseria*, infection and immunity; *Nocardia*, infection and immunity; Opsonization; *Pasteurella*, infection and immunity; Phagocytosis; *Proteus*, infection and immunity; *Pseudomonas aeruginosa*, infection and immunity; *Rickettsia*, infection and immunity; *Salmonella*, infection and immunity; *Shigella*, infection and immunity; *Staphylococcus*, infection and immunity; *Streptobacillus*, infection and immunity; *Streptococcus*, infection and immunity; Stress and the immune system; T lymphocyte differentiation; *Treponema*, infection and immunity; *Vibrio cholerae*, infection and immunity; Vitamin D and the immune system; *Yersinia*, infection and immunity.

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BACTERIAL CELL WALLS

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The bacterial cell wall is a rigid, complex macromolecular structure which surrounds the bacterial cytosol and determines the characteristic shape of different bacteria. The cell wall and associated membrane(s), often referred to as the cell envelope, in addition to maintenance of cell shape, perform several important physiological functions involved in the regulation of interactions between the organism and its environment. In particular, the bacterial surface is the site of interaction of the organism with host tissues, and the molecular architecture of the cell envelope is of key importance in the pathogenesis of bacterial infection. Some components act as receptors or adhesins and mediate attachment to host cell surfaces. Many cell envelope components are anti-

genic and stimulate host immune responses which may be important in controlling or eradicating infection. In contrast, other components inhibit immune interactions and contribute both to the initiation of infection and persistence of the organism in host tissues. Expression of many envelope components is influenced by environmental conditions. Products expressed under conditions which most closely mimic those found *in vivo* are of increasing interest in understanding the pathogenesis of bacterial infections.

The majority of bacteria can be classified into one of two types, gram-positive or gram-negative, using a simple staining technique which exploits fundamental differences in the structure of the cell envelope.

ope to differentiate between the two groups. The principal components of the cell envelopes of gram-positive and gram-negative bacteria are shown in Figure 1.

Cytoplasmic membrane

This bounds the bacterial cytoplasm. Proteins present in the membrane are involved in selective transport of nutrients and synthesis of peptidoglycan and periplasmic and outer membrane proteins. In gram-negative organisms, the space between the cytoplasmic membrane and the outer membrane – the periplasmic space – contains proteins with catabolic, binding and degradative functions.

Peptidoglycan

This is the major component of the cell wall involved in maintenance of cell rigidity, shape and osmotic stability and may be referred to as murein or mucopeptide. It consists of a polymer of $\beta 1 \rightarrow 4$ -linked *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues which are cross-linked by peptide bridges. The antibiotic penicillin interferes with peptidoglycan synthesis by inhibiting peptide bridge forma-

tion. Peptidoglycan is present in all bacteria except mycoplasmas and some halophilic archaeobacteria. In gram-negative bacteria the peptidoglycan layer may be only one or two molecules thick but in gram-positive bacteria it is much thicker. Removal of the peptidoglycan by treatment of bacteria with lysozyme generates osmotically fragile structures termed protoplasts in gram-negative bacteria and spheroplasts in gram-positive organisms; L-forms are bacterial variants that have lost the ability to synthesize peptidoglycan. Peptidoglycans of many bacteria are antigenic, and a degradation product of mycobacterial peptidoglycan – muramyl dipeptide (MDP) – has adjuvant properties.

Gram-negative outer membrane

In gram-negative bacteria such as *Escherichia coli*, the peptidoglycan layer is surrounded by a second bilayer lipid membrane, the outer membrane. The outer membrane is linked to the peptidoglycan layer by a lipoprotein and contains proteins and a highly immunoreactive molecule called lipopolysaccharide (LPS) or endotoxin.

Some outer membrane proteins (OMPs) function as receptors or ligands, and one group – the porins –

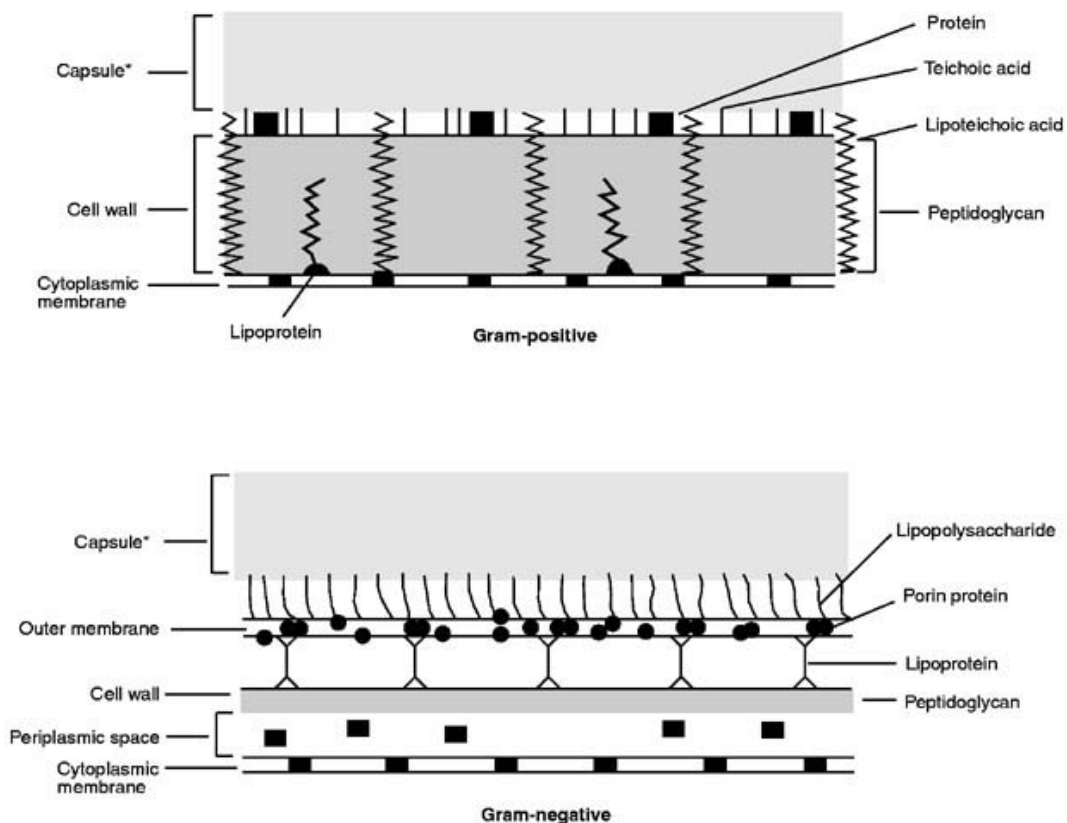


Figure 1 Components of the cell envelope of gram-positive and gram-negative bacteria. *Not always present.

form transmembrane channels involved in the non-specific transport of small hydrophilic solutes across the membrane. Other proteins mediate specific uptake of larger metabolites. Expression of some OMPs is regulated by environmental factors, such as growth medium composition. For example, many gram-negative bacteria express iron-regulated OMPs when growing in iron-deficient media. *In vivo* such proteins are involved in scavenging iron in mammalian tissues, a prerequisite for bacterial growth, and are therefore important virulence determinants of bacterial pathogens. Many OMPs are antigenic, and antigenic variation in these proteins is important in the pathogenesis of diseases such as gonorrhea (caused by *Neisseria gonorrhoeae*) and relapsing fever (caused by *Borrelia* spp.). Some OMPs contain T cell epitopes and stimulate cell-mediated responses which may modulate the immune response during infection. OMPs are also involved in the resistance of some intracellular bacteria to the bactericidal effects of phagocytic cells.

LPS or endotoxin is a complex molecule present in the outer leaflet of the outer membrane. It contains a unique lipid moiety – lipid A – which is responsible for its diverse biological activities including pyrogenicity and activation of macrophages. Many of its effects are nonspecific and are mediated through cytokines such as tumor necrosis factor α (TNF α) and interleukin-1 (IL-1) produced following interaction of LPS with lymphocytes or macrophages. LPS also contains oligosaccharides that confer antigenic specificity on the molecule and constitute the ‘O’ or somatic antigens of gram-negative bacteria such as *Salmonella*. The oligosaccharide side-chains are also important in preventing access of activated complement components to the bacterial outer membrane. Recent studies have shown that LPS of some bacteria, e.g. *Helicobacter pylori*, contain sugar sequences identical to human blood group antigens, which may play a role in modulating the immune response to the bacterium. The LPS of some bacteria, e.g. *Neisseria* spp., naturally lacks the oligosaccharide side-chains and these molecules are referred to as lipo-oligosaccharides (LOS).

Other components of gram-positive cell envelopes

Gram-positive bacteria lack an outer membrane but proteins, lipoproteins and other macromolecules are associated with the cytoplasmic membrane and peptidoglycan. Many wall proteins are anchored directly to the peptidoglycan, and contain a conserved amino acid motif – LPXTG – which is involved in transporting these proteins across the cytoplasmic mem-

brane. Lipoproteins, which may be highly immunogenic, are anchored to the cytoplasmic membrane by the N-terminus which is lipid modified. Expression of both wall and cytoplasmic membrane proteins may be influenced by environmental factors such as iron availability. Also associated with the surface of some gram-positive bacteria are immunoglobulin-binding proteins such as staphylococcal protein A and streptococcal protein G, which bind the Fc region of antibody molecules. Proteins with analogous function are now being detected in some gram-negative bacteria. Release of such proteins may protect the organism by binding to antibody and preventing its deposition on the bacterial surface. Other proteins such as the M protein of *Streptococcus pyogenes* have an antiphagocytic function.

Teichoic and lipoteichoic acids are acidic polymers of glycerol or ribitol and are bound to the peptidoglycan and cytoplasmic membrane respectively. They may be important antigens in staphylococci and streptococci, stimulating both humoral and cell-mediated immune responses.

Bacterial capsules

The outermost layer of many bacteria consists of a capsule. Capsules are usually, but not exclusively, acidic polysaccharides and are only loosely associated with the bacterial surface. Capsules may act as adhesins or have antiphagocytic functions due to their acidity and hydrophilic nature. In addition, capsular material may be poor antigenic (e.g. the hyaluronic acid capsule of *Streptococcus pyogenes*) and may mask more antigenic deeper layers of the cell envelope. The presence of a capsule may also sterically hinder access of complement components to the bacterial outer membrane. Other capsular antigens induce opsonizing antibody which promotes clearance of the bacteria. Although successful vaccines based on conjugated capsular polysaccharides have been developed, e.g. *Haemophilus influenzae* serotype b, some bacteria, e.g. *Streptococcus pneumoniae*, produce more than 30 antigenically distinct capsular types, which makes development of cross-protective capsular vaccines very difficult.

Other components associated with the bacterial cell envelope

Also associated with the surface of many gram-negative bacteria and some gram-positive organisms are filamentous appendages called pili or fimbriae. These are proteinaceous and are involved in mediating specific attachment of some bacteria to cell

surfaces. Antigenic variation in pili may also be important in the pathogenesis of gonorrhea.

In both gram-positive and gram-negative bacteria, flagella – organelles involved in motility – are anchored into the cell envelope. Flagella are antigenic and constitute the 'H' antigens of gram-negative bacteria.

Mycobacteria do not readily stain by Gram's method and have a cell envelope which is unusually rich in lipids. This property confers the property of 'acid fastness' on these organisms since they resist decolorization with acids following staining. The lipid component of mycobacteria probably contributes to their resistance to phagocytic killing and the difficulty encountered in their degradation by these cells. Included among the lipids are mycolic acids or cord factors which may be toxic for mammalian cells.

See also: Bacteria, immunity to; Endotoxin (lipopolysaccharide (LPS)); Muramyl dipeptide; Phagocytosis; Bacterial immunoglobulin-binding proteins.

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BACTERIAL IMMUNOGLOBULIN-BINDING PROTEINS

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Bacterial immunoglobulin-binding proteins represent a family of functional molecules expressed by certain microorganisms that can interact with immunoglobulins (Igs) at sites unrelated to their antigen-combining regions. The study of these molecules was initially driven by their utility as immunochemical reagents but has now expanded to address more basic scientific questions of structure–function and biological activities.

Six types of IgG-binding proteins have been described on gram-positive bacteria. These different types have been classified by their ability to bind with the Fc region of different mammalian species and subclasses of IgG (Figure 1). The most extensively studied IgG-binding protein is protein A (the type I).

Protein A is expressed by the majority of human isolates of *Staphylococcus aureus*. The type II receptors are found on the surface of the majority of human isolates of group A streptococci and display a large amount of variability in their immunoglobulin-binding profiles (Table 1). Protein G, the type III IgG-binding protein, is expressed by the majority of human group C and G streptococcal isolates and displays the widest range of species and subclass reactivities of any of the immunoglobulin-binding proteins identified to date (Figure 1). The type IV, V and type VI immunoglobulin-binding proteins are associated with animal isolates of streptococci and have not been studied extensively.

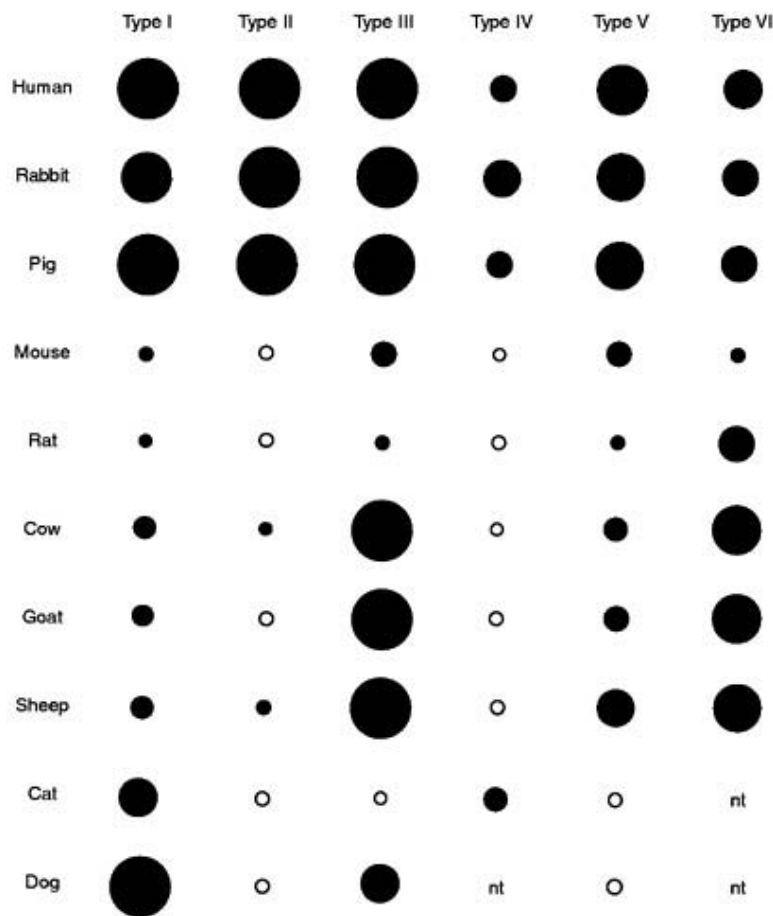


Figure 1 Profile of species reactivities of different bacterial IgG-binding protein types. Larger dots represent greater binding activity; o, no reactivity; nt, not tested. Note these reactivities have been determined using polyclonal IgG preparations. Occasional differences have been noted in the reactivity of samples from individual animals. (Reproduced with permission from Reis *et al* (1988).)

Table 1 IgG-binding profiles of type II IgG-binding proteins expressed by certain group A streptococci

Functional designation	Binding profiles						
	Human IgG1	Human IgG2	Human IgG3	Human IgG4	Rabbit IgG	Pig IgG	Horse IgG
Ilo	✓	✓	✓	✓	✓	✓	✓
II'o*	✓	✓	✓	✓	✓		
Ila	✓	✓		✓	✓	✓	✓
Ilb			✓				
Ilc	✓			✓	✓	✓	

*Also designated protein H.
Adapted from Boyle and Raeder (1993).

IgG Fc-binding proteins have also been described on the surface of other bacteria, including *Haemophilus somnus*, *Clostridium perfringens*, *Brucella abortus*, *Taylorella equigenitalis* and *Gardnerella vaginalis*. In addition, bacterial binding proteins that display nonimmune reactivity with immunoglobulin isotypes other than IgG have been described (Table 2). These include proteins that bind constant regions in IgD or IgA. Other proteins have been recognized that bind regions within constant portions of the

F(ab)'₂ fragments as well as molecules that show selective reactivity with immunoglobulin light chains. To date, a bacterial protein with absolute selectivity for the Fc region of IgM from any species has not been described. A *B. abortus* protein with reactivity for certain bovine IgM allotypes, a *Clostridium difficile* toxin protein that displays preferential reactivity with murine IgM, and a potential IgM-specific lectin-like molecule of *Borrelia burgdorferi* have been reported.

Table 2 Representative bacteria that bind immunoglobulin in a nonimmune fashion

Bacteria	IgG	IgM	IgA	IgD	IgE
<i>Staphylococcus aureus</i>	+				
Streptococcus, group A	+		+		
Streptococcus, group B			+		
Streptococcus groups C and G	+				
<i>Branhamella catarrhalis</i>				+	
<i>Clostridium perfringens</i>	+	+	+		
<i>Taylorella equigenitalis</i>	+	+			
<i>Brucella abortus</i>		(+)*			
<i>Coprococcus comes</i>	+				
<i>Peptococcus magnus</i>	+	+	+	+	+
<i>Haemophilus somnus</i>	+				

*Reactivity limited to a subgroup of bovine IgM antibodies.

Protein A – the type I IgG-binding protein

The initial IgG-binding protein described and characterized was the type I binding protein expressed on the surface of the majority of *Staphylococcus aureus* isolates and more frequently designated as staphylococcal protein A. Protein A and its interaction with the Fc region of reactive IgG antibodies has been extensively studied and detailed structure–function analysis by X-ray crystallography and nuclear magnetic resonance has mapped the key binding amino acids in the selective interaction of protein A with the C_{H2}–C_{H3} domain of IgG.

The interaction of protein A and IgG is of relatively high affinity 10^8 – 10^9 M⁻¹ and a lower affinity interaction has also been noted for protein A binding to F(ab)₂ regions of IgG. In addition, reactivity of protein A with certain subtypes of IgM and IgA have also been described. The gene encoding protein A has been cloned and contains four or five homologous regions capable of binding to IgG.

Protein A is bivalent in its IgG-binding activity and in the presence of excess IgG forms (IgG)₂ (PA) complexes. When protein A is present in excess the predominant complex is (IgG)₁ (PA)₂, indicating the presence of two protein A binding domains on each IgG molecule. These different protein A–IgG complexes display different potentials for binding C1 and activating the classical complement pathway. The (IgG)₁ (PA)₂ complexes do not bind C1 indicating, that protein A and C1q target a similar site on the Fc region of IgG, whereas a dimer of the [(IgG)₂ (PA)₁]₂ complex is extremely efficient in binding and activating C1 and behaves in complement fixation assays with the characteristics of an IgM antibody. The ability to form such IgM-like complexes may explain the ability of protein A to induce wheal and flare reactions when injected into humans intradermally or to induce anaphylactic reactions in guinea pigs.

In addition to effects of protein A on the humoral immune system, the bacterial protein has also been reported to have T cell mitogenic properties and to act as a B cell superantigen. The T cell effects of protein A, however, are based on earlier studies of responses induced by heat-killed and/or formalin-fixed *S. aureus* Cowan strain bacteria or wild-type preparations of protein A. These sources of protein A have the potential to be contaminated with staphylococcal enterotoxins, which could account for many of the observed effects. Studies with recombinant protein A, which demonstrates all of the expected IgG-binding activities, failed to show T cell mitogenic activity, suggesting protein A may not be a T cell mitogen. By contrast, the elegant studies of Silverman and colleagues indicate that protein A can induce proliferation of a subset of human B cells and this is restricted to B cells expressing surface immunoglobulin containing certain V_{H3} Fab regions.

The majority of studies of protein A have focused on its utility as a reagent for detection of antigen–antibody complexes or, once immobilized, as a reagent for affinity purification. The high selectivity of protein A for the Fc region of antibodies in immune complexes has resulted in protein A tracers replacing conventional second antibody reagents in many immunotechnological applications. Protein A has proved to be extremely valuable for procedures involving screening of hybridoma supernatants for a desired monoclonal antibody with a desired specificity as well as for the subsequent purification of that antibody.

Analysis of the infectivity of *S. aureus* isolates and paired mutants in which the protein A gene was non-functional have failed to provide any evidence that protein A is a virulence factor.

Type II IgG-binding proteins

Group A streptococci express an array of functionally distinct IgG-binding proteins (Table 1). The majority of the functional types of type II IgG-binding proteins have been cloned and sequenced. All of these IgG-binding proteins are encoded by M or M-related protein genes that are present in a virulence regulon of group A streptococci, suggesting a potential role for these proteins in pathogenesis. In addition to binding IgG, the majority of type II binding proteins also bind other plasma proteins, e.g. albumin, factor H, fibrinogen, plasminogen and IgA. Analysis of the binding domains of these proteins suggest they consist of different combinations of distinct functional modules that react with different IgG subclasses or other plasma proteins. Expression of type II IgG-binding proteins is associated more frequently with group A streptococcal skin isolates than with throat isolates. Studies in mice have indicated an association between IgG-binding protein expression and skin infectivity, while no such association occurs when the bacteria are injected intraperitoneally.

Type III IgG-binding proteins

The type III IgG-binding proteins are more frequently designated as streptococcal protein G. Protein G is expressed by the majority of human isolates of group C and group G streptococci. A number of variant forms of protein G have been described at both the gene and protein level. All forms of protein G are antigenically related and the majority also bind human serum albumin. The gene encoding protein G has been cloned and the albumin-binding and IgG-binding domains have been shown to be distinct. This has enabled nonalbumin-binding forms of protein G to be genetically engineered.

Protein G demonstrates the widest species and subclass reactivity of any bacterial IgG-binding protein (Figure 1). Protein G, like protein A, can form a number of different-sized complexes with IgG and these demonstrate a similar pattern of complement-fixing potential to that observed with protein A. Protein G has been shown to mediate mitogenic responses when added to human peripheral blood lymphocytes. This activity, however, could not be correlated with either the albumin or IgG-binding domain of the protein.

By virtue of its broad species and subclass reactivity profile, protein G is an extremely useful reagent as a tracer and for affinity purification of antibodies that are not reactive with protein A.

Type IV, V and VI IgG-binding proteins

Type IV, V and VI IgG-binding proteins have not been extensively studied. They are all antigenically distinct proteins that demonstrate different patterns of nonimmune IgG binding (Figure 1). The type IV binding protein is associated with certain β hemolytic isolates of streptococci from cattle; however, this protein demonstrates no detectable reactivity with bovine immunoglobulins. The type V and VI proteins have been isolated from different *S. zooepidemicus* isolates. It is probable that, like type II IgG-binding proteins, there will be extensive heterogeneity in the IgG-binding proteins expressed by different *S. zooepidemicus* isolates. To date, there is no evidence that any type IV, V or VI IgG-binding protein associates with disease in any animal species and none of these proteins have been tested for their utility as immunochemical reagents.

Other bacterial immunoglobulin-binding proteins

In addition to the IgG-binding proteins associated with staphylococci and streptococci, an increasing number of both gram-positive and gram-negative bacteria have been reported to express immunoglobulin-binding proteins with reactivity for IgA, IgD and IgM, as well as a protein that binds to certain κ light chains (Table 2). To date, no disease association has been suggested for any of the putative IgA-, IgM- and IgD-binding proteins. The Fc binding protein associated with *H. somnus* has been implicated as a virulence factor in cattle and an association between expression of protein L, a light chain-binding protein, by *Peptococcus magnus* and vaginal infections has been proposed.

As might be anticipated, based on the utility of protein A and protein G, many of these immunoglobulin-binding proteins may find applications for the purification and detection of specific immunoglobulin classes or subclasses. To date, the light chain-binding protein (protein L), a hybrid of protein L and protein G (LG), and a group B streptococcal IgA-binding protein (protein B) have demonstrated practical utility in certain immunological procedures.

Summary

Immunoglobulin-binding proteins represent a family of diverse molecules capable of interacting with antibody without impairing the ability of the antibody to bind its cognate antigen. By virtue of these properties, these molecules are potentially valuable

immunochemical reagents. There is limited understanding of the role of IgG-binding proteins in bacterial virulence. Studies of *H. somnus* in cattle and *S. pyogenes* and *Peptococcus magnus* in humans have suggested a possible association between expression of IgG-binding proteins and the course of infection.

See also: Affinity chromatography; Bacterial cell walls; IgG; Immunoassays; Immunocytochemistry and enzyme markers.

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BACTEROIDES, INFECTION AND IMMUNITY

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The importance of anaerobic bacteria in clinical human disease, particularly intra-abdominal infection, has been well established over the past two decades. Much of the interest has focused upon the various *Bacteroides* species because of their prevalence in clinical isolates and their requirements for particular antibiotic therapy. Once classified as subspecies of *Bacteroides fragilis*, six distinct species of *Bacteroides* are now recognized based on distinctive patterns of DNA homology: *B. fragilis*, *B. distasonis*, *B. vulgatus*, *B. thetaiotaomicron*, *B. ovatus*, and *Bacteroides* species. Although *B. vulgatus* and *B. thetaiotaomicron* are the numerically dominant species of the fecal flora, *B. fragilis*, a numerically minor member of the normal flora, is the most frequently isolated anaerobic species in many infectious processes, including intra-abdominal sepsis. This sug-

gests that *B. fragilis* must possess virulence factors not present in the other species.

Examination of clinical isolates of *B. fragilis* has demonstrated the presence of a polysaccharide capsule, rarely found on other *Bacteroides* species. The capsular polysaccharide of *B. fragilis* has been well characterized chemically. Prolonged laboratory culture of encapsulated strains of *B. fragilis* may result in loss of the capsule and a marked diminution in virulence.

Much of our knowledge about *Bacteroides* virulence comes from a rat model of intra-abdominal sepsis, in which bacterial isolates are placed in a gelatin capsule along with adjuvants and implanted intraperitoneally. Using complex mixtures of fecal flora, an early peritonitis phase, with bacteremia and high mortality, is followed by an abscess phase. This

latter phase has been shown to be dependent upon the presence of anaerobes in the inoculum. When various isolates were tested in this model, it was shown that encapsulated strains of *B. fragilis* were the only organisms capable of abscess formation as single isolates. Unencapsulated strains of *B. fragilis* and other anaerobes only led to abscess formation when combined with other facultative organisms, such as enterococcus. In fact, heat-killed preparations of encapsulated *B. fragilis* strains led to abscess formation in the majority of cases, as did inocula containing purified capsular polysaccharide, clearly demonstrating the role of the capsule in virulence.

The apparent central role of capsular polysaccharide in *B. fragilis* virulence led to studies using the polysaccharide as a vaccine. Rats immunized with *B. fragilis* capsular polysaccharide with or without complete Freund's adjuvant developed significant antibody titers against the polysaccharide. These animals were protected against abscess formation when challenged with *B. fragilis* or *B. distasonis*. Animals challenged with more complex bacterial inocula still formed abscesses; however, *B. fragilis* was eliminated from the abscesses in those animals. Surprisingly, when serum from immunized animals with high-titer antibody to the capsular polysaccharide was transferred to nonimmunized animals, no protection against abscess formation was conferred. On the other hand, transfer of spleen cells from immunized animals did confer protection and this result could be repeated with spleen cells enriched for T cells with nylon-wool columns. Similarly, immunization of athymic nude rats with capsular polysaccharide, although producing excellent antibody levels, did not prevent abscess formation whereas phenotypically normal littermates were protected. Thus, in the rat model, there was a strong suggestion for the role of T cell immunity in the immune response to *B. fragilis* and abscess prevention.

A murine model of intra-abdominal abscess formation was developed in order to better dissect the cellular immune response to *B. fragilis*. This model has the further advantage that the bacterial inoculum can be introduced without surgical implantation. It was possible to confirm all of the rat observations in the murine system, namely, that immunization with capsular polysaccharide was protective against *B. fragilis* abscesses, that protection could not be transferred passively with serum, but could be conferred by spleen cell transfers. The cell responsible for protection was, in fact, a T cell, of the $\text{Ly-1}^- \text{Ly-2}^+ \text{I-J}^+$ phenotype. The immune T cells are antigen specific, that is, they only protect against *B. fragilis* abscesses, but are not major histocompat-

ibility complex (MHC)-restricted. This immune T cell produces a factor and transfer of cell-free lysate of immune T cells are protective against abscesses as well. The factor is polypeptide in nature and of relatively low molecular weight (<12 kDa).

Because of the immune T cell phenotype and the elaboration of a soluble factor, the possibility that abscess prevention might be mediated by a T cell suppressor circuit was raised. This possibility raised two questions: what was being suppressed, and on what was the T cell factor acting. Both nude mice and rats are able to form abscesses in response to *B. fragilis* challenge, suggesting that T cells are not necessary for abscess formation. It is known that nude animals do have early T cell precursors, however. Treatment of mice with low doses of cyclophosphamide abolished their ability to form abscesses, and reconstitution of the cyclophosphamide-treated animals with $\text{Ly-1}^+ \text{2}^+$ T cells restored this ability. Thus T cells are required at an early stage in the generation of *B. fragilis* abscesses, and the cell required for abscess induction is a relatively early precursor T cell as one might expect from the nude mouse data. It is presumed that this precursor T cell is being acted upon by the T suppressor cells in the prevention of abscess formation.

Although nude mice are capable of developing abscesses in response to challenge with *B. fragilis*, the transfer of the usual small number of immune T cells (2.5×10^6) or immune factor does not induce protection as in normal mice. This suggested that a second T cell might be required to induce abscess protection. This possibility was investigated with reconstitution experiments in nude mice. Transfer of 25×10^6 naive spleen cells into nude recipients reconstituted the ability of these mice to be protected against abscesses by the further transfer of 2.5×10^6 immune cells. The cells capable of reconstituting the nude mice were also $\text{Ly-1}^- \text{2}^+$ by phenotype.

Thus, it appears that abscess production and prevention in response to *B. fragilis* occurs by a T cell-mediated mechanism. Abscess induction requires a precursor-type T cell. Preliminary evidence suggests that the earliest cells entering the peritoneum in response to bacterial challenge may be lymphocytes. The mechanism by which they may act to elicit abscess formation is not yet identified. Prevention of abscesses require at least two T cells, of suppressor phenotypes, which communicate via a small polypeptide factor. These cells are antigen specific, but not MHC-restricted.

One additional question is the potential benefit of abscess formation to both the host and the bacterium. Animals which do not form abscesses do not develop diffuse overwhelming infection and death.

On the contrary, the *B. fragilis* in these animals is cleared without sequelae. This raises the possibility that abscesses are an adaptive mechanism by which the bacteria create a milieu favorable to survival rather than a particularly useful protective mechanism for the host.

See also: **Bacteria, immunity to; T lymphocytes.**

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BARE LYMPHOCYTE SYNDROME

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Bare lymphocyte syndrome (BLS) encompasses a series of rare autosomal recessive disorders in which patients display low or null expression of HLA major histocompatibility proteins. Three types of BLS have been classified, with the severity of these disorders varying both in phenotypic HLA expression and loss of immune function. In BLS type I, low or no expression of HLA class I molecules are displayed on peripheral blood cells. The degree of immunodeficiency is most variable in BLS type I, with some individuals displaying essentially normal immunity. Type II BLS is defined as a reduction or complete loss of HLA class II protein expression on all cells. This disorder typically manifests as a severe combined immunodeficiency. In BLS type III disorders, both HLA class I and class II antigen expression are diminished and these individuals are severely immunodeficient. Studies of BLS patients have proven to be exceptionally informative, yielding insights into the effects of selective disruption of histocompatibility antigen function, as well as offering clues to the transcriptional and post-translational regulation of histocompatibility molecules.

Bare lymphocyte syndrome Type I

Class I histocompatibility antigens are constitutively found on all normal nucleated cells, however surface expression of these proteins may be down-modulated by viruses and in some tumors. In normal peripheral blood lymphocytes, diminished class I antigen expression with normal levels of class II proteins is an established hallmark of BLS type I. The levels of

class I antigen cell surface expression are quite variable among type I patients. Immunodeficiency does not always correlate with the level of class I antigen cell surface expression, and individuals with no or low class I antigen expression and no signs of immune dysfunction have been identified. Remarkably, Epstein–Barr virus transformation of B cells from patients with this latter phenotype resulted in partial restoration of class I expression. In other patients, severe immunodeficiency has been associated with BLS type I, including persistent bacterial and yeast infections, low numbers of peripheral blood T lymphocytes, and death within one year of birth. In the majority of these patients, the specific genetic factors responsible for the loss of class I antigen expression remain unclear.

Molecular studies to identify the genetic lesion in type I BLS have been carried out in patients with very low class I expression (1–3% of normal levels) and recurring sinopulmonary infections as well as bronchiectasis. In all cases, these individuals suffer from chronic bacterial infections and require antibiotic therapy. Investigations of two related type I patients revealed a truncation mutation in TAP2 (transporter associated with antigen processing), a molecule required for class I protein assembly and function. Class I heavy chains are synthesized by these patients; however these molecules are trapped intracellularly and only loosely associated with β_2 -microglobulin. In normal individuals, both surface expression of class I proteins and the stable association of class I heavy chains with β_2 -microglobulin is dependent upon binding of antigenic peptides.

Antigenic peptide delivery to nascent class I proteins typically occurs via a heterodimeric transporter consisting of TAP1 and TAP2 proteins localized in the endoplasmic reticulum. Murine and human mutant cell lines, as well as knockout animals lacking TAP proteins, display a phenotype almost identical to BLS type I patients. A low level of class I antigen surface expression is observed in both these BLS type I patients and the laboratory mutants. Studies with TAP-deficient cell lines indicate a small population of peptides exists within the endoplasmic reticulum which can bind class I complexes and promote stable transport and surface expression.

Clinical investigations of these type I BLS patients are remarkable in that susceptibility to pulmonary bacterial infections predominate. Peripheral blood CD8⁺ T lymphocyte levels are low and there appears to be no or very low natural killer cell function in these individuals. In contrast, high antibody titers against bacteria and viruses, as well as good responses to vaccination are found in these BLS type I patients. The enhanced frequency of respiratory tract bacterial infections in these patients suggests class I antigens on phagocytic cells such as macrophages must play a key role in immunity against these pathogens.

Bare lymphocyte syndrome type II

Class II histocompatibility antigens are expressed on professional antigen-presenting cells such as B lymphocytes, dendritic cells and macrophages. Expression of class II proteins on other cell types can be induced in response to cytokine treatment while a small number of viruses are capable of downregulating cell surface levels of class II antigens. In humans, heterodimers of class II α and β subunits are synthesized from highly polymorphic DR, DP and DQ loci encoded within the major histocompatibility gene complex. BLS type II is classically defined as a global loss of class II antigen expression. However, this definition has been broadened to encompass individuals with a loss of specific class II subunits as well as restricted class II expression on discrete cell types. Patients displaying very low or no class II proteins on their peripheral blood cells all have severe immunodeficiencies and suffer from chronic viral, bacterial, fungal and protozoal infections of the respiratory and gastrointestinal tract. Clinical onset of disease symptoms occurs within the first year of life and most patients do not survive beyond the age of four without therapeutic interventions such as bone marrow transplantation.

BLS type II patients display strong allograft rejec-

tion responses without immunosuppression, presumably because class I antigens are expressed by these patients along with, in some cases, low levels of class II molecules. Thus, the success of bone marrow transplantation is reduced in these individuals compared with other patients suffering from combined immunodeficiencies. Total numbers of peripheral blood T cells are normal in BLS type II patients, with very low numbers of circulating CD4⁺ T lymphocytes and enhanced levels of CD8⁺ T cells. This observation differs from the phenomena observed in mice lacking class II structural genes, where no single positive CD4 T cells mature. Possible explanations may include low expression of class II antigens on thymic cells in BLS type II patients, resulting in limited CD4⁺ T cell selection. Alternatively in humans, class I molecules in the thymus may play a role in the selection of CD4⁺ T cells. Most interestingly, following bone marrow transplantation of these patients, the circulating levels of CD4⁺ T cells remain low, yet immunity is restored. The implications of this finding are twofold: 1) that bone marrow-derived cells are not critical in the efficiency of thymic selection of T cells in these patients; and 2) that restoration of class II antigen expression on bone marrow-derived cells is critical to host defense. The number of peripheral blood B lymphocytes in BLS type II patients are normal, but antibody production is severely deficient in these individuals, presumably due to a lack of helper CD4⁺ T cells. In some individuals, autoreactivity including polyarthritis, eczema and psoriasis is also associated with the absence of class II antigen expression.

Analysis of BLS type II at a molecular level has led to the clustering of patients within multiple complementation groups, each group lacking a distinct gene regulating class II protein expression. In all cases of BLS type II, class II structural genes are intact, yet the transcription of mRNA from these loci is perturbed. Family studies suggest the genetic defects in all patients lie outside the major histocompatibility gene complex on chromosome 6. Fusion of patient cell lines with cells from normal individuals restores the expression of class II alleles derived from the BLS type II individual. This finding supports the conclusion that the defective gene is a *trans*-acting factor that controls the transcription of class II molecules. Fusion of B cell lines from different type II patients has firmly established three genetic complementation groups in this disorder (groups A, B and C (also termed II, I and IV respectively)). A mutagenized B cell line selected for its lack of surface class II antigens has been generated in the laboratory, and this cell comprises a fourth complementation group (group D or III). Additional complementation groups may exist, including patients selectively

expressing only a subset of class II subunits on distinct cell types. The majority of the patients identified fall within complementation groups A and B. In two of the complementation groups (B and C), patients also have substantially reduced class I antigen expression on peripheral blood cells. These individuals have been classified both as BLS type II and type III by different investigators. Immortalization of B cells from group B and C individuals using Epstein-Barr virus typically restores class I antigen surface expression to wild-type levels, but this viral transformation does not influence class II antigen transcription.

Molecular and functional studies have defined in part the genetic defects in BLS type II. *In vivo* analysis of class II promoters from type II groups B, C and D reveals a lack of transcription factors stably binding these regions of DNA, a phenotype referred to as 'bare' promoters. This result suggests that the class II gene promoter regions are inaccessible in these mutants. By contrast in BLS type II group A, normal occupancy of class II gene promoters is observed. To date, only the transcription factors defective in BLS type II groups A and C have been definitively identified. Using complementation cloning, the transcription factor defective in group A patients has been identified as a 1130 amino acid protein, CIITA (MHC class II trans-activator). Studies of several group A patients revealed point mutations, defects in exon splicing and genomic deletions in this protein. CIITA does not bind directly to DNA and may serve instead as a coactivator to facilitate the interaction of other transcription factors. Expression of CIITA in only group A mutants restores class II antigen expression and this transcription factor is critical in interferon γ (IFN γ) induction of class II antigen mRNA. CIITA is the only transcription factor at this time whose expression directly correlates with class II antigen mRNA levels in cells. For example, CIITA synthesis is ablated during the transition of B lymphocytes to plasma cells, coincident with a loss in class II antigen expression. The C-terminus of CIITA encodes a transcription-activating domain while the N-terminus confers specificity for class II promoter regions.

BLS type II group C patients have mutations in a 75 kDa transcription factor, RFX5. This protein serves as a key subunit in a multimeric complex termed RFX which binds the X-box region of class II promoters. Disruption of the RFX complex results in the 'bare' class II promoter phenotype of group C patients. These findings suggest another component of RFX may also be defective in group B or D patients which also display the 'bare' promoters phenotype. Questions remain as to whether RFX

plays any role in regulating class I antigen transcription in groups B and C, where reduced class I levels are observed on peripheral blood cells.

Both CIITA and RFX5 are essential for the synthesis of cell surface class II DR, DP and DQ alleles, however these transcription factors also have been shown to regulate the production of other molecules required for presentation via the class II pathway. The assembly and intracellular transport of class II proteins is mediated by a chaperone protein, the invariant chain. Despite its chromosomal location outside the major histocompatibility gene complex, the invariant chain shares some promoter elements with class II proteins. Expression of class II proteins and invariant chain is tightly linked in all cells and in BLS type II, synthesis of invariant chain mRNA is severely reduced but not completely ablated. The invariant chain must be proteolytically released from class II molecules to allow peptide binding and antigen presentation. Release of invariant chain peptides from the groove of class II proteins is mediated in endosomal/lysosomal compartments by HLA-DM. Functional and molecular analyses indicate that HLA-DM is also deficient in BLS type II patients. The absence of functional DM molecules renders cells unable to present exogenous antigens. While gene therapy and the replacement of class II structural genes has been discussed as a potential curative therapy for BLS type II, the requirement for multiple factors in antigen presentation suggests that restoration of the defective transcription factors is a more promising approach for this disorder.

Bare lymphocyte syndrome type III

Patients with BLS type III display no cell surface class II antigens as well as little or no class I proteins. The phenotype of these patients is most variable and many of these patients are classified as BLS type II due to their residual class I antigen expression. The immunodeficiency of these individuals mirrors that of BLS type II patients including susceptibility to chronic infections and early death without intervention. Peripheral blood levels of CD4⁺ T cells are low and antibody responses dramatically reduced in BLS type III.

While multiple patients with BLS type III have been identified, little progress has been made in elucidating the genetic defect(s) in these individuals. Class I antigen expression in some patients can be induced using IFN γ but this does not restore class II antigen levels. Molecular studies of a transformed fibroblast line from a type III individual revealed interferon treatment induced low levels of mRNA for class I heavy chain, β_2 -microglobulin and the proteasome

subunits LMP2 and LMP7. Interestingly, interferon induced normal levels of mRNA production for both TAP1 and TAP2 in fibroblasts from this type III patient. No induction of class II mRNA and little synthesis of invariant chain were observed in these interferon-treated cells. *In vitro* fusion of these type III fibroblasts with B cell lines from each of the four BLS type II complementation groups restored class II antigen expression. These results suggest that the defect in this type III individual is genetically distinct from BLS type II mutations. Elucidation of the defects in these cells will be important as approaches for gene therapy and transplantation biology are advanced.

See also: Antigen-presenting cells; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; HLA class I; HLA class II; Invariant chain (Ii); Severe combined immunodeficiency.

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BASOPHILS

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Basophils are a member of the granulocytes, the white blood cells in the circulation, and constitute less than 1% of the circulating leukocytes. Basophils share many features with mast cells. They both develop from a common bone marrow-derived hematopoietic precursor cell that is CD34⁺, have granules containing histamine, proteolytic enzymes and other inflammatory mediators, have high-affinity receptors for immunoglobulin E (IgE) (FcεRI) and are stimulated to release inflammatory mediators when they react with secretagogues. Therefore, both types of cell play a role in immediate hypersensitivity

and inflammatory reactions. However, there are a number of differences between basophils and mast cells. In contrast to mast cells, which mature in the tissues where they finally reside, basophils differentiate in the bone marrow and enter the circulation as mature, functionally active cells. Basophils have the capacity for chemotaxis and are recruited into tissues during inflammatory reaction. However, even in tissues they can be identified as basophils and there is no evidence that they transform into mast cells. Basophils are smaller than mast cells, are short lived (<2 weeks) and are probably end-cells. In contrast,

the mast cells are distributed in connective tissues, often adjacent to blood vessels and beneath epithelial surfaces (e.g. the gastrointestinal and respiratory tracts, and the skin). Mast cells normally do not circulate, are long lived and appear to retain the capacity to proliferate. Connective tissue-type mast cells in general contain much more histamine than basophils (a human basophil contains approximately 1 pg histamine). Unlike mast cells, chondroitin monosulfates are the major proteoglycan of basophils. There are also differences in the surface proteins found on basophils compared with those on mast cells. Studies in several mammalian species suggest an inverse quantitative relationship between basophils and mast cells, e.g. species that have abundant mast cells have few basophils. Humans however, have significant numbers of both mast cells and basophils.

The entry on mast cells describes the mediators released from basophils or mast cells, the high-affinity IgE receptor FcεRI, stimuli that activate the cells for secretion and the biochemical mechanisms of the release reaction. The present section describes the unique features of basophils and relies heavily on studies with human cells.

Morphology

Human basophils have a polylobed nucleus with condensed nuclear chromatin and absent nucleoli. They have little endoplasmic reticulum and free ribosomes. The cytoplasm contains a large number of granules that are heterogeneous in size. By electron microscopy some granules are filled uniformly with electron dense material, whereas others lack this dense material. When antigen is added *in vitro*, the basophils lose their oriented motility and extend pseudopodia. The degranulating basophils develop small cytoplasmic 'vesicles' that rapidly increase in size and coalesce. The granular membranes fuse with the plasma membrane, resulting in the development of narrow openings between the cell surface and individual granules. This results in the extrusion of membrane-free granular material through multiple openings in the circumference of the cell. However, there are some rare interconnected chains of granules opened to the exterior at a single point on the cell surface. The granule matrix is released as a whole to the outside, but the granule membrane is left behind. Frequently, membrane-free granular contents are seen attached to the cell exterior. The degranulation of mast cells is of the 'compound' type with granules fusing with each other, whereas in basophils most of

the granules fuse directly with the plasma membrane (Figures 1 A & B).

Basophils infiltrate and occasionally constitute a significant proportion of the total cells at sites of delayed hypersensitivity reactions. Such reactions are termed *cutaneous basophil hypersensitivity*. The T cells in such reactions probably release factors that are chemotactic for basophils and might also induce the basophils to degranulate slowly: the granules never fuse with the cell membrane, but lose their matrix in a 'piecemeal' manner over a period of days. Small packages of granular content appear to bud from granule membranes, traverse the cytoplasm and fuse with the plasma membrane, leaving completely degranulated cells.

Basophil releasability

Releasability of basophils refers to the variation in the extent of histamine release from the cells of different donors. The extent of histamine release from the cells of any one individual can vary from none to values as high as 90–100% of the total cellular content. There is good positive correlation between antigen-induced histamine release *in vitro* with the

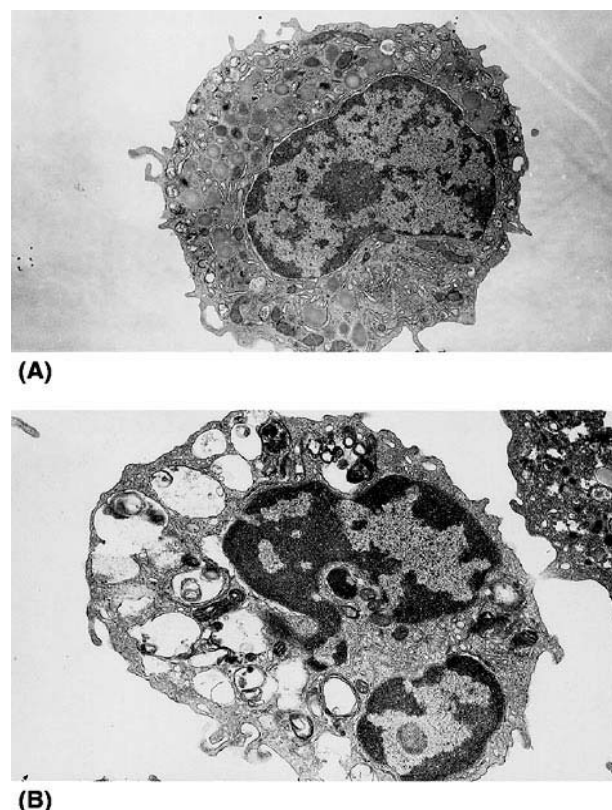


Figure 1 Human basophils; (A) non-activated; (B) after degranulation. Photomicrographs kindly provided by Dr. Connie Oliver.

basophils of allergic patients and the extent of their symptoms. The releasability of the same cells can also vary with different secretagogues. There are also differences in the extent of release from basophils compared with mast cells. There is no correlation between the number of IgE molecules present on the surface of basophils and releasability. Recent evidence suggests that these differences in secretion are regulated by intracellular factors. For example, the cells of donors who are high responders release histamine with chemically cross-linked IgE dimers; whereas the cells of the donors who are low responders release with chemically cross-linked trimers, but very poorly with dimers. The sensitivity of cells to different stimuli can be modified by factors such as adherence of the cells to other cells or extracellular matrix proteins, by cytokines, by steroids or by dietary lipids that are incorporated into the membranes.

Cell desensitization

The cross-linking of the IgE receptors on the basophil or mast cell causes the activation of enzymes that result in secretion of mediators. Some of these biochemical reactions also regulate the extent of the release reaction. This process is called *cell desensitization*. Experimentally, desensitization is initiated by addition of the secretagogue under conditions that do not result in secretion, and after a defined incubation period the permissive conditions are restored. Desensitization is an active process, blocked by some pharmacologic agents, and is secretagogue-specific. The IgE receptor-mediated desensitization requires receptor aggregation, and can be either specific to the one antigen or for all IgE-mediated release reactions. At low cell surface IgE densities there is antigen-specific desensitization, whereas at high surface IgE levels there is desensitization for all IgE-mediated reactions. Desensitization is not due to the loss of cell surface antigen-specific IgE caused by endocytosis or shedding of IgE-antigen complexes. It is probably due to the decay of an unstable intermediate formed

by the cross-linked receptors interacting with a secondary component. The degree of desensitization probably regulates the extent of the release process.

The mechanism for mediator release is similar in the various cell systems studied, e.g. human mast cells, basophils or rat mast cells. All systems respond to the physiologically relevant stimulus of IgE-antigen. However, there are differences in the response to miscellaneous secretagogues that could be related to the presence of surface receptors, and there are differences in the rate of release with various cells. Some pharmacologic agents inhibit release from human basophils but not from mast cells. There are differences in the spectrum of mediators released by mast cells compared with basophils. In contrast to mast cells, human basophils release very little, if any, of the cyclo-oxygenase-derived products of arachidonic acid such as prostaglandin D₂. However, both mast cells and basophils release the leukotriene C₄. Mast cells but not basophils release the enzyme tryptase. There is little evidence to suggest that the secretory process is fundamentally different in the basophils compared with that in the mast cells.

See also: Allergens; Anaphylatoxins; Arachidonic acid and the leukotrienes; Exocytosis; Histamine; Hypersensitivity reactions; IgE; Interleukin 3; Platelet-activating factor (PAF); Serotonin.

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BCG

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Calmette and Guérin first described bacille Calmette–Guérin (BCG) in 1908. A bovine strain of *Mycobacterium tuberculosis* was grown on a piece of potato cooked at 70°C in bile containing 5% glycine. After 231 subcultures on this special medium and 13 years later, the bacilli were found to be nonpathogenic, but otherwise indistinguishable from the virulent strain. In 1921, this bacillus was first advocated as a live vaccine against tuberculosis (TB). Since then, BCG constitutes one of the mainstays of any TB control program. Evidence for successful vaccination is development of a delayed-type hypersensitivity reaction to a purified protein derivative (PPD) of *M. tuberculosis*.

Recommendations, administration and efficacy

The inconsistent policies among countries with regard to the indication for administering BCG vaccine reflect the contradicting and inconclusive data on the prevention of TB and the drawback of a resultant tuberculin seroconversion preventing the interpretation of the tuberculin skin test.

BCG is presently recommended in the USA for tuberculin-negative infants and children who have continuous exposure to isoniazide- and rifampin-resistant active TB, who cannot take isoniazid and are exposed to TB, or who belong to population groups with rates of new *M. tuberculosis* infections exceeding 1% per year. Vaccination is also advised for groups residing in high-prevalence areas such as military and foreign service personnel. Vaccination should be avoided in immunosuppressed individuals, with special regard to patients with established human immunodeficiency virus (HIV) infection because of the increased risk of disseminated infection.

The administration protocol recommended by the World Health Organization's expanded program is a single dose at birth. However, this recommendation is not implemented universally; for example,

in the UK a single dose is administered at the age of 13, whereas in eastern Europe repeated doses are given through childhood.

The efficacy of BCG against TB has been debatable since its initial use in 1921, although, to date, well over 3 billion doses have been administered. A recent comprehensive meta-analysis from 14 prospective trials and, separately, 12 case-control studies, addressing the efficacy of BCG, pointed towards an overall protective effect of 50% against TB infections. The protective effect against pulmonary and disseminated infection was 78%, against tuberculous meningitis 64% and against death 71%.

Antigenic properties and immunologic aspects

The mycobacterium is an acid-fast, slow-growing bacillus. Most of its antigens are firmly attached to the cell wall. These have been classified into three groups: 1) antigens common to all mycobacteria, which can also be found in other bacteria such as *Listeria*, *Corynebacterium* and *Nocardia* spp.; 2) common antigens present on slow-growing bacteria; 3) species-specific antigens. Most studies on the soluble antigens have been based on vigorous experimental methods by which the cell wall was disrupted and solubilized. This crude antigen preparation was found to be a nonspecific stimulant of cell-mediated immunity, as well as a powerful adjuvant to vaccines used in experimental animals.

Repeated injection of Freund's complete adjuvant containing killed mycobacteria results in a response resembling autoimmune disorders (the so-called adjuvant arthritis in rats). BCG itself can induce non-specific resistance to certain infections (or enhancement of others) and tumor growth suppression, attributed to various influences of the vaccine such as enhancement of macrophage cytotoxicity, increased production of natural killer cells, cytokines and cytotoxic T cells. Most of the above-mentioned properties are related to the high lipid content of the bac-

teria, up to 40% of its dry weight. These lipids include a variety of biologically active substances: trehalose 6,6'-dimycolate or 'cord factor', neutral red reactive sulfolipid, 6-O-methylglucose lipopolysaccharide, phosphatides and waxes. A group of substances which have been studied extensively are the mycolic acids. β -Mycolic acid is typical of BCG. It consists of three aliphatic chains of 11–28 carbons, linked by ester or hydroxyl residues to extensive hydrocarbon chains with a carboxyl side-chain at the link and at the end of the chain. A cyclopropyl group is present between the first two short chains. In the cell wall, these molecules are connected to sugars, containing arabinose and galactose, and with wax D. This complex is the molecule that gives the adjuvant activity. The nonspecific immune stimulation has been used in cancer management. Animal studies have demonstrated tumor regression following repeated local BCG infection. Viable BCG cells, injected into metastases of human malignant melanoma, led to marked tumor regression. Probably the most abundant use of BCG in cancer therapy is in bladder cancer. Intravesical administration is used for three purposes: 1) adjunctive therapy as a single treatment after complete tumor resolution to prevent

tumor cell implantation and recurrence; 2) prophylactically, in repeated doses to prevent *de novo* recurrence of tumor following complete excision; 3) therapeutically, in repeated administrations to treat residual unresected tumor. Known adverse effects include regional adenitis, disseminated BCG infection and osteitis due to the BCG organism.

See also: **Adjuvants; Mycobacteria, infection and immunity; Vaccines.**

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BEHAVIORAL REGULATION OF IMMUNITY

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Although immune responses can readily occur and be analyzed *in vitro*, the immune system normally functions in an intact organism where it has the opportunity to interact with other physiological systems. A significant body of recent data points out that indeed such interactions do occur *in vivo*. For example, the central nervous system (CNS) is in dynamic communication with the immune system. Lymphoid tissues are richly innervated, and abrogation of this sympathetic innervation alters expression of natural and acquired immunity. Cells of the CNS produce proinflammatory cytokines (e.g. interleukin-1 (IL-1) and IL-6), and mitogen- or antigen-stimulated lymphocytes produce different hormones (e.g. proopiomelanocortin-derived peptides, growth hormone, prolactin, luteinizing hormone) and neuropeptides (e.g. substance P, vasoactive intestinal peptide). Lymphocytes and macrophages

express cell surface receptors for a diversity of hormones and neuropeptides, suggesting autocrine and paracrine regulation by these molecules. Experimental manipulation of hormone- or neurotransmitter-immunocyte receptor interactions can evoke alterations in immunologically relevant events such as lymphocyte proliferation, activity of natural killer (NK) cells, production of antibody, synthesis and release of cytokines, and expression of cytokine receptors.

There is now incontrovertible evidence that the immunomodulatory interactions between the CNS and the immune system can be 'regulated' by the behavior of the organism. Such evidence has been provided by predominantly phenomenological studies on the influences of classical (Pavlovian) conditioning and on the effects of 'stress' on immunity.

Pavlovian conditioning and immunomodulation

Classical (Pavlovian) conditioning is perhaps the first behavioral approach that has been used experimentally to reveal a relationship between the CNS and the immune system. In the classical Pavlovian paradigm, a stimulus (e.g. food) that unconditionally elicits a particular response (salivation) is repeatedly paired with a neutral stimulus that does not elicit that same response. Eventually, the neutral stimulus becomes a conditional stimulus, in that it will elicit salivation in the absence of food, the unconditional stimulus. Since Pavlov's experiments in the 1920s, a wide range of behavioral, physiologic and pharmacologic processes have been conditioned in a wide diversity of species. The first conditioning studies that involved nonspecific arms of the defense system were conducted in the Soviet Union in 1926, as extensions of Pavlov's work on the conditioning of physiologic responses. In these early studies, Metalnikov and Chorine conditioned an increase in polymorphonuclear leukocytes (PMNs) in a peritoneal exudate by repeatedly pairing the intraperitoneal injection of foreign material, in guinea pigs, with the scratching or heating of the animals' skin. After multiple pairings, and after the number of PMNs returned to baseline levels, re-exposure to the conditional stimulus alone was reported to evoke an increase in the number of PMNs. Several variations on these types of experiments, plus those in which increased levels of specific antibody were attributed to conditioning, appeared in the Russian literature in the next decade and then again in the 1950s.

The first rigorously controlled and immunologically relevant conditioning experiments in the West were published in 1975 by Ader and Cohen. In their initial study, rats were subjected to a single learning trial in which an injection of cyclophosphamide (CY), a potent immunosuppressive drug that served as an unconditional stimulus, was paired with the consumption of a novel, distinctly flavored saccharin (SAC) solution, the conditioning stimulus. When rats conditioned in this fashion were re-exposed to the conditional stimulus at the time of antigenic challenge, conditioned immunopharmacologic effects were revealed by a significantly depressed antibody response relative to the appropriate controls (i.e. nonconditioned animals, or conditioned animals that were never re-exposed to the conditional stimulus). Similar and subsequent conditioning protocols involving mice as well as rats, CY or other immunosuppressive drugs as unconditional stimuli, and different novel tastes as conditional stimuli, have revealed a conditioned: depression of

graft-versus-host reactions; enhancement of delayed hypersensitivity responses; depression of humoral immune responses to thymus-independent as well as thymus-dependent antigens; and depression of mitogen- and alloantigen-induced lymphocyte proliferation, heart and skin allograft rejection, plasmacytoma growth, NK cell activity, numbers of white cells and arthritic inflammation.

Exposure of conditioned animals (conditioned by pairing SAC with CY) to the conditional stimulus appears to create an immunomodulatory environment. This was suggested by experiments in which conditioned rats, exposed to the conditional stimulus a few weeks before they were immunized, still displayed a conditioned immunosuppressive response to the antigen. More direct evidence that conditioning alters the internal milieu (and may primarily affect T cells) comes from the use of classic lymphocyte transfer and cell-mixing protocols by Gerczynski. In these, and in other conditioning studies in which the unconditional stimulus was the injection of either CY or some other immunosuppressive drug, it seems reasonable to propose that the actual unconditional stimuli being perceived by the CNS as deviations from homeostasis are some secondary or tertiary immunologic consequence(s) of the direct effect of CY on the lymphocyte (e.g. changes in lymphocyte number and/or traffic).

That conditioned immunopharmacologic effects are of biological importance is apparent from Ader and Cohen's observations that the development of a systemic lupus erythematosus (SLE)-like autoimmune disease in (NZB $\times$ NZW) F_1 hybrid mice could be markedly delayed by treating animals under a partial schedule of pharmacologic reinforcement. That is, by substituting conditional stimuli that had been paired with CY for a proportion of the trials on which animals would normally have received active drug therapy, conditioned mice showed a significant delay in the onset of lupus using a cumulative dosage of CY that was, by itself, insufficient to alter the course of the autoimmune disease. Moreover, conditioned mice repeatedly re-exposed to the taste stimulus that had been paired with CY following the termination of active drug therapy survived significantly longer than conditioned animals that received no 'medication'.

Although in the not-too-distant future, conditioned immunopharmacologic effects may prove to be significant in clinical situations where one wishes to reduce the amount of actual drug administered to a patient with an autoimmune disease or an organ transplant, this model of conditioned immunosuppression may be of limited help in trying to understand the basic impact and physiology of

conditioning on 'normal' immune responses. More revealed models would be those in which immune responses are altered in animals that are conditioned by pairing a conditional stimulus with a naturally occurring immunologic response modifier (e.g. a cytokine) or with a substance that itself unconditionally elicits an immune response, namely an antigen. Since conditioning is an example of feed forward control in which the organism reacts physiologically based on an anticipated need, a conditioned immune response in the absence of an externally administered cytokine or an immunogenic concentration of antigen would indeed highlight the physiological importance of CNS regulation of immunity during the normal life of an organism. At this time, only a handful of such studies have been performed, and several of these have involved the conditioned release of histamine or protease II from mast cells of animals sensitized with protein antigens. In one study, however, a conditioned change in antigen-specific lymphocytes was reported. In this experiment, Gorczynski, Macrae and Kennedy repeatedly grafted CBA mice with allogeneic skin from C57BL/6J mice. During the 40 day interval between first, second- and third-set grafting, the number of cytotoxic T lymphocyte precursors (CTLp) specific for the alloantigens of the sensitizing donor increased and then returned to baseline levels. Each recipient mouse was anesthetized, shaved, had a graft bed surgically prepared on to which the skin allograft was placed, and was then bandaged. That is, the grafting procedure itself served as a complex of conditional stimuli. On the fourth (test) trial, animals in all groups were exposed to just the conditional stimuli (i.e. they were sham grafted). Only conditioned mice displayed an increase in CTLp in response to the stimulus conditions associated with grafting in the absence of an actual graft. In each of several replications of this experiment, only about half of the conditioned mice showed the conditioned change in CTLp. These 'responder' mice were then divided into subgroups that either experienced two extinction trials (unreinforced exposure to the conditional stimulus) or two additional learning trials (i.e. two separate trials in which there were pairings of the conditional and unconditional stimuli). All 'responder' animals that received additional conditioning trials displayed an increase in CTLp in response to the conditional stimulus; none of the 'responders' given extinction trials showed the conditioned response.

In a recent study by Ader and his colleagues, an enhanced antibody response provided the immunologic readout of a conditioning protocol in which antigen itself served as the conditioned stimulus. In

this study, injection of a very small amount of key-hole limpet hemocyanin (KLH) was paired with a novel taste on each of several learning trials. In between each learning trial, the serum antibody response increased and then decreased. On the test trial, mice were given an amount of KLH (0.5 ng) that only elicited a modest antibody response by itself, but when it was given to conditioned animals in association with the conditional stimulus, KLH-specific antibody titers increased significantly over baseline. To confirm that this was a *bona fide* conditioned response, mice were pre-exposed to the conditional stimulus before the initiation of the first and subsequent learning trials. When such animals were presented with the small antigen boost plus the conditional stimulus, they did not produce antibody, an observation totally consistent with learning theory.

Given that we do not fully understand the mechanisms that underlie these examples of conditioning, one can only speculate about: 1) the nature of the neurochemical consequences of various conditional stimuli in the nervous system; 2) which (and how the) relevant internal physiologic consequences of an antigenic, pharmacologic or physical unconditional stimulus become associated internally with the neurologic sequelae of a conditional stimulus; and 3) how presentation of the conditional stimulus evokes the immunomodulatory responses that many investigators have observed. Regardless of our ignorance of the constellation of mechanisms that may be involved in conditioned alterations of immunity, it is clear that, since learning involves higher centers of the brain, the ability to effect changes in immune responses by conditioning must mean that, *in vivo*, the immune system receives and responds to signals (either directly and/or indirectly) from the CNS.

Stressor-associated regulation of immunity

Stress, as defined by Hans Selye, is the response to deviation from the normal resting condition (i.e. homeostasis). It is generally accepted that stressor administration causes complex neurologic and neurochemical changes as the host attempts to adapt to the demands of the situation. These CNS changes regulate many physiologic processes, including those involved in the generation of an immune response. Immunomodulatory 'stress' protocols have involved different periods of exposure to either physical stimuli (e.g. electric shock, rotation, restraint, temperature, noise) or so-called psychosocial stressors (e.g. differential housing, maternal separation and handling in experimental animals; bereavement, divorce

and school examinations in humans). Immunologic outcomes measured in such studies have been: the development of primary and secondary antibody and cell-mediated immune responses to experimental antigens, microbial pathogens and tumor cells *in vivo*; the numbers of B cells and T cell subsets in various lymphoid compartments; the magnitude of mitogen- and/or antigen-induced lymphocyte proliferation *in vitro*; the production of, and response to, cytokines; NK cell cytotoxicity; and macrophage activation. Considering the variability among these studies with respect to the nature, duration and intensity of the stressor, the age, sex, species and strain of the stressed organism and the parameters of the immune function studied, it is not surprising that different effects of stress on immunity have been reported. Thus, the same stressor can effect enhancement or suppression of different immune responses; different stressors can have different effects on the same parameter of immunity; the same stressor applied for varying periods of time may have different effects on the same parameter of immunity; and changes in a single parameter of immunologic reactivity in response to different stressful stimuli are not necessarily unidirectional. By and large, however, stress in experimental animals and humans has been reported to depress the particular parameters of immunity that were being examined.

Stress has a variety of neuroendocrine effects, and immune responses are affected by the neuroendocrine milieu in which leukocytes function. However, it is far from clear which (and how) stress-associated neuroendocrine changes are responsible for which stress-associated immunomodulatory events. For example, the hypothalamo-pituitary-adrenal (HPA) axis is activated during stressful situations, and there are numerous reports of stress-induced adrenocortically-mediated immunosuppression. However, there are also several reports of stress-induced alterations of immunologic reactivity that occur in adrenalectomized animals. Although hypophysectomy obviates the effects of stress on some measures of immune function, stress-induced suppression of others are potentiated by this procedure. Because of the involvement of the HPA axis in stress-induced analgesia, many investigators have looked for, and found, a role of endogenous opioids in stress-associated immunomodulation in a variety of species including fish, mice, rats and humans. However, not all stress effects on immunity can be blocked by naloxone or naltrexone and/or mimicked by exogenous opioids such as endorphins and morphine. Finally, although levels of catecholamines and other neurotransmitters are known to be altered in response to stress, and many studies have revealed a

role of norepinephrine on immune function, few studies have attempted to correlate stress-induced changes in plasma catecholamines with altered immune function.

For immunologists, the fact that physiologic responses to stressors are immunomodulatory has practical implications that relate to experimental design as well as experimental theory. For example, putative pheromones from stressed (footshocked) mice conveyed to KLH-immunized recipients can affect cytokine production by lymphocytes *in vitro* and the immune responses they regulate *in vivo*. An extension of these observations to a situation in which manipulations of mice in an animal room can be communicated to and effect immune responses of other animals in that same room is not an unwarranted assumption. A second example of practical import for immunologists is that the differential housing of mice (housed one versus four per cage from the time of weaning) reproducibly and significantly influences antigen-stimulated *in vitro* production of T_H1- and T_H2-derived cytokines by splenocytes from mice immunized with either KLH or herpes simplex virus (HSV).

In the pheromone studies mentioned above, the immunologic effects on recipients of 'stress odors' occurred in a manner consistent with the theory that depressed cellular but enhanced humoral immune responses in pheromone-exposed BALB/c mice are reflective of an altered 'balance' between production of T_H1 and T_H2 lymphocyte-derived cytokines. However, the immunologic effects of differential housing on immune responses of either BALB/c or C57/BL6 mice to either KLH or HSV do not fit all predictions inherent in the theory of a differentially 'balanced' cytokine response and strain-associated dominance of humoral or cellular immunity. That is, regardless of strain or antigen, individually housed animals produce more antibody, have greater delayed-type hypersensitivity responses, and make more interleukin-2 (IL-2), IL-4, interferon γ (IFN γ) and IL-10 than do group-housed animals.

The observations that physical and psychosocial stressors can modulate immunity on the one hand, and modify disease progression on the other, has led to popularization of the idea that stress plays an important role in the pathogenesis of immunologically relevant diseases (e.g. cancer, viral and bacterial infections, autoimmunity, allergy). At this time, however, the cause-effect relationships between a stressor-altered immune system and disease progression must be viewed more as a reasonable and attractive hypothesis than as hard fact. Specifically, it has yet to be reproducibly documented in experimental animals (let alone in humans) that stress-

associated alterations of some facet of immunocompetence are, at least partially, responsible for disease pathophysiology.

Immunity can modulate behavior

The previous material has reviewed data indicating that behavioral triggers of the nervous system can regulate immunity. Several lines of evidence also indicate that components of an immune response can also signal the CNS. For example, many investigators have reported electrical changes in the hypothalamus following the administration of antigen. Others have reported that IL-1 stimulates release of corticotropin-releasing hormone from the hypothalamus into the hypophyseal portal circulation, where it can then activate the HPA axis. Moreover, the response to antigenic stimuli appears to be associated with the release of neurotransmitters and hormones produced by lymphocytes themselves. Finally, evidence suggests that these and/or other signals from an activated immune system may be directly or indirectly signaling the CNS to actually modify the behavior of the organism. The most intriguing observations are those suggesting that the immunologic abnormalities of the lupus-prone strains of congenic MRL mice modify (directly or indirectly) the animals' behavior, which in turn can act to 'correct' homeostatic imbalances within the immune system.

Summary

The current data on conditioned and stress-associated alterations of immunologic reactivity produce compelling evidence of a fundamental integration of behavioral, neuroendocrine and immune processes of adaptation. It now appears that, like all other biological systems functioning in the interests of homeostasis, the immune system is integrated with other psychophysiologic processes and therefore is subject to regulation or modulation by the brain. Conversely, immune processes seem to have implications for neural and endocrine function, including behavior. As such, they provide evidence for the *in vivo* bidirectionality of interactions between the immune system and the central nervous system.

See also: Cytokines; Immunosuppression; Neuroendocrine regulation of immunity; Stress and the immune system.

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BENCE JONES PROTEINS

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Bence Jones proteins (BJP), initially known as urinary proteins precipitating at 56–64°C and redissolving on boiling, are now defined as monoclonal free immunoglobulin (Ig) light chains (LC) (polyclonal free LC that may be found in urine are not BJP). Their characterization and quantification is of importance because of their diagnostic and prognostic significance.

Characterization

Bence Jones proteins are usually present in urine as monomers and dimers. Search for BJP often requires a concentration step which is best achieved by vacuum dialysis preferably of daily urine. This results in minimal denaturation and does not bias against certain proteins (including some BJP). Electrophoresis of concentrated urines typically shows a narrow band with a mobility generally different from that of the entire monoclonal Ig which may be present in the serum. Bence Jones proteins are sometimes heterogeneous in charge and are easily and reliably quantified by scanning the electrophoresis. Immunoelectrophoresis shows one or several contiguous narrow precipitating lines with the relevant anti-LC serum and no reactivity with anti-heavy chain sera. Being monoclonal in origin, the BJP are of either κ or λ type. When part of a κ type BJP displays a fast migration, this usually results from an interaction with α_1 -antitrypsin. Immunofixation may be useful in the diagnosis of BJP. Western blotting of high-resolution agarose electrophoresis followed by detection with enzyme-coupled Ig-specific antibodies is more sensitive and more accurate.

Bence Jones proteins are detectable in the serum when they are secreted in very large amounts, when they polymerize and in the case of renal failure. Antisera that react with free LC and not with LC in whole Ig molecules may be helpful.

Diagnostic significance

Normal plasma cells most often produce balanced amounts of heavy and light chains. In contrast, malignant plasma cells often secrete free LC. Hence, the presence of significant amounts of BJP ($\geq 0.3 \text{ g l}^{-1}$ of urine) is indicative of malignancy. Minute amounts of BJP detected after $1000 \times$ concentration of proteins present in urine in physiologic concen-

trations are also mostly observed in malignant diseases. In myeloma, BJP are found as the only monoclonal Ig in about 20% of cases. In IgD myeloma (of the λ type in 9 out of 10 cases), there is usually a large output of BJP, whereas the serum monoclonal IgD is often present in small amounts. Hence, the finding of an apparently isolated λ BJP should lead to a search for IgD myeloma. Bence Jones proteins may be observed in B cell lymphomas and leukemias, in small amounts in virtually every patient with Waldenström macroglobulinemia, and in about 50% of cases of μ heavy chain disease.

Pathogenetic implications

Bence Jones proteins play a direct role in myeloma-associated kidney disease and their measurement is of important prognostic significance. They are also involved in visceral diseases featuring tissue deposition of LC-related material. Combined study of serum and urine by sensitive methods reveals evidence of BJP in almost all cases of AL (amyloid light chain) amyloidosis. In LC deposition disease, tissue deposits of monoclonal LC (and of monoclonal heavy chains in some patients) correlate with the presence of a monoclonal population of bone marrow plasma cells, whatever the clinical context. In some cases, the LC are normal-sized and present in urine but in about 60–70% of patients they display an abnormal (short or enlarged by glycosylation) size, polymerize and are undetectable in serum and urine. In both instances there are numerous mutations in the variable (V) regions of the LC, especially in the hypervariable regions (with probably a direct pathogenic role), together with an abnormal subgroup distribution (V κ IV predominance). V region abnormalities, with a V domain (of the V κ I subgroup) highly resistant to proteolysis and prone to readily form crystals, are also involved in myeloma-associated Fanconi syndrome.

See also: Amyloid; Heavy chain diseases; Immunoelectrophoresis; Immunoglobulin structure; Lymphoma; Western blotting.

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BIOZZI MICE

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Biozzi mice are lines of mice genetically selected for high (H) or low (L) antibody responsiveness. From the initial study in 1968 by Biozzi and his group, five different selections, referred to as selections I, II, III, IV and V, were carried out by Biozzi and colleagues.

The character selected was the maximal or minimal antibody response at a given time after an optimal dose of the various antigens used in the different selections. The antibody response was measured by direct or passive agglutination. In every population the frequency of the log₂ of the agglutinin titer (the highest doubling serum dilution giving a positive agglutination) was normally distributed.

Except for selection II, two non-cross-reactive antigens were alternated in consecutive generations in order to avoid the specific interference of maternal antibodies passively transmitted to the progeny. In selection I the antigen used was sheep red blood cells (SRBC) for the first six generations; subsequently SRBC and pigeon erythrocytes were alternated at each generation. In all generations of selection II, mice were immunized with SRBC but the period between weaning and immunization was prolonged to allow elimination of the maternal antibodies. Selections III and IV were carried out for responses to flagellar and somatic antigens, respectively, of two non-cross-reactive *Salmonellae* (*S. typhimurium* and *S. oranienburg*) given alternately. The antigens used for selection V were bovine serum albumin and rabbit gammaglobulin, alternately.

High and low antibody responder lines were produced by bidirectional selective breeding. Starting from a foundation population (F0) of outbred albino mice displaying great variability in antibody response, the two-way selective breeding was performed for maximal or minimal antibody response and repeated in each consecutive generation. Assortative

mating of the highest responder mice produced the H line, while that of the lowest responder mice produced the L line. In each line several pairs were culled at each generation and were issued from different families to delay the increase in consanguinity. The two lines diverged progressively until the maximal interline separation was reached at a given generation (selection limit).

According to the principles of quantitative genetics as applied in these studies, at the selection limit the two lines are considered homozygous for all independent loci controlling the antibody response, owing to the progressive accumulation of high-effect alleles in the H line and low-effect alleles in the L line.

The total response (TR) to selection is the interline difference at the selection limit, while the response (R) to selection is the difference between the mean values of two successive generations of each line. The selection differential (S) is the difference between the mean of the selected parents and that of the total population out of which they have been culled. The mean realized heritability (h^2) is calculated by the linear regression coefficient of cumulated R versus cumulated S over the generations between F0 and the selection limit, and is equal to R/S .

Once the selection limit was attained, the following interline hybrids were derived from H and L parents: (H × L) = F1; (F1 × F1) = F2; (F1 × H) = BcH; (F1 × L) = BcL. Using mean values ($\bar{x}$) and variances (V) of the character measured in these mouse populations, relevant genetic parameters were estimated. The total additive effect (a) of all the homozygous loci in H and L lines at the selection limit in the absence of dominance is given by

$$a = \frac{1}{2} (\bar{x}_H - \bar{x}_L).$$

The global dominance deviation (d) in F1 hybrids is given by

$$d = xF1 - \frac{1}{2}(xH + xL).$$

The dominance effect is d/a .

The environmental variance (VE) is the mean phenotypic variance of the genetically homogeneous populations: H and L lines at the selection limit and their F1 hybrids. Therefore,

$$VE = \frac{VH + VL + VF1}{3}.$$

The phenotypic variance of the genetically heterogeneous populations, F0, F2, BcH and BcL, is contributed by both genetic and environmental factors. The variance of F2 hybrids is

$$VF2 = VA + VD + VE$$

where VA is the additive variance and VD the dominance variance. The summed variance of the two backcrosses ($VBcH + VBcL$) is

$$VBcs = 2(\frac{1}{2}VA + VD + VE) = VA + 2VD + 2VE.$$

Since

$$VF2 - VA = VD + VE, \text{ then } VBcs = 2VF2 - VA.$$

It follows that $VA = 2VF2 - VBcs$.

Knowing VA , the number (n) of independent loci controlling the character is calculated as

$$n = \frac{a^2}{2VA}$$

Since the h^2 value results from the additive effect of homozygous loci occupied by either high- or low-effect alleles, the heritability of the character can also be measured in the progeny of interline crosses (F2 or backcrosses) by the ratio of the additive variance to the total phenotypic variance, as follows:

$$h^2 = \frac{VA}{VA + VD + VE}.$$

Comparison of the immunogenetic parameters in the five selections (Table 1) revealed a remarkable

similarity in the total response to selection, selection limit and realized heritability. Only in selection V was the total response greater and attained after fewer generations. The number of independent loci controlling antibody responsiveness differed in the five selections, owing to the different nature of the antigens and immunization procedures used. Mapping studies associating polymorphic microsatellite markers with antibody response in selection I have shown that some segregating loci are linked to genes on chromosomes 4 and 8 but also to genes certainly involved in major immune functions, such as genes on chromosome 6 coding for the TCR β chain, Ig κ and CD8, genes on chromosome 12 coding for the Igh allotypes, and genes on chromosome 17 coding for the MHC, TNF α , TNF β , C2 and C4.

The antibody response in H, L lines, and interline hybrids of the five selections (Table 2), indicates that the additive effect (a) was similar, whereas the dominance deviation (d) was different in all selections, yielding a different dominance effect (d/a) in each selection. Thus, the high responsiveness was incompletely dominant, to a variable extent, in selections I, II, III and IV, whereas it was incompletely recessive in selection V.

In all selections, the high or low effects of the alleles at the n loci are not limited to the selection antigen but may also influence the immune response to immunogens non-cross-reactive with the selection antigen. This multispecific effect is not general, as the difference in antibody response between H and L responder mice to various unrelated antigens may be identical, smaller, insignificant or even inverse as compared with the difference in response to the selection antigen. Comparison of the results obtained in the five selections indicates that the multispecific effect is large in selections I and III, intermediate in selections II and IV and restricted in selection V.

Selective breeding was also carried out for mitotic responsiveness of lymph node cells to *in vitro* stimulation by phytohemagglutinin (PHA). The selection limit was reached after ten generations, the realized heritability was 0.24, the low responsiveness was incompletely dominant, and the character was under the control of 10–19 independent loci. These H and L responder mice also displayed a similar difference in responsiveness to concanavalin A, in mixed lymphocyte reaction and graft-versus-host reaction, but produced the same antibody titer when immunized with SRBC.

Whether selective breeding for a polygenic character, such as antibody or mitotic responsiveness, also affects lifespan was investigated in selections I, II, III and PHA. The lifespan was longer in H than in L mice of selections I and II, but no difference was

Table 1 Genetic parameters of the five selections

Selection	TR	Selection limit	h^2	n
I	7.8	F16	0.20	9–11
II	6.7	F13	0.21	5–7
III	6.3	F16	0.20	4–7
IV	6.5	F13	0.21	2–4
V	9.8	F7	0.22	2–4

TR, total response to selection; h^2 , realized heritability; n , number of independent loci.

Table 2 Antibody titers ($\log_2$) in H, L lines and interline hybrids of five selections

Mice	Selection I Mean $\pm$ SD	Selection II Mean $\pm$ SD	Selection III Mean $\pm$ SD	Selection IV Mean $\pm$ SD	Selection V Mean $\pm$ SD
H	12.5 $\pm$ 0.8	11.6 $\pm$ 1.0	12.8 $\pm$ 0.7	11.4 $\pm$ 1.0	14.2 $\pm$ 1.9
L	4.7 $\pm$ 1.1	4.9 $\pm$ 1.2	6.5 $\pm$ 0.8	4.9 $\pm$ 1.2	4.4 $\pm$ 2.1
F1	9.6 $\pm$ 1.3	10.1 $\pm$ 1.2	10.1 $\pm$ 0.6	8.6 $\pm$ 0.9	7.4 $\pm$ 2.8
F2	8.6 $\pm$ 1.4	9.5 $\pm$ 2.1	10.7 $\pm$ 1.2	7.9 $\pm$ 1.7	6.0 $\pm$ 3.2
BcH	11.4 $\pm$ 1.0	10.3 $\pm$ 1.3	11.7 $\pm$ 0.9	10.8 $\pm$ 1.6	13.0 $\pm$ 2.1
BcL	7.6 $\pm$ 1.5	7.5 $\pm$ 2.4	8.9 $\pm$ 0.9	6.0 $\pm$ 1.4	5.2 $\pm$ 3.2
<i>a</i>	3.9	3.3	3.1	3.2	4.9
<i>d/a</i>	0.3	0.5	0.1	0.1	-0.4

a, additive effect; *d*, dominance deviation; *d/a*, dominance effect; SD, standard deviation.

found between H and L mice of selections III and PHA. The positive correlation between antibody responsiveness and lifespan was further analyzed in interline hybrids of selection II and found statistically significant in most of these mouse populations. Moreover, the lifespan of the last 30%, 20% and 10% survivors, which are scarcely affected by early disease-induced mortality and mainly influenced by genes acting on the rate of physiologic aging, appeared as a polygenic character regulated by 3–7 independent loci. Noteworthy, the long lifespan was incompletely dominant in the total population, but was longer in the last 30% and completely dominant in 20% and 10% survivors (Table 3). Thus, the results of this analysis suggest that antibody responsiveness and lifespan are polygenic traits regulated by a small number of the same or closely linked loci.

The incidence of spontaneous malignant lymphomas was markedly higher in L than in H mice of selections I, II and PHA, whereas no difference was found between L and H mice of selection III.

Improvement of the effect of selection was obtained by assortative breeding from two foundation populations, F0H and F0L, each of which was produced with balanced frequency of the gene pools from the H or L lines of selections I, II, III, IV and V. After 16 generations of selection for primary or secondary responses to all the antigens used in the original five selections the difference between H and L lines in antibody responsiveness was remarkably amplified and the multispecific effect of the selection was generalized to several antigens. These results, obtained in selection GP for general-primary and selection GS for general-secondary responses, suggest that more genes with upward effects had accumulated in H mice or, rather, more genes with downward effects had accumulated in L mice during both selections. The influence of immune responsiveness on lifespan and disease susceptibility was also investigated in H and L mice of the GS selection at the F16 generation. It was found that the cumulative mortality rate is remarkably higher in L than in H mice, the difference being accounted for mostly by

Table 3 Agglutinin titer ($\log_2$) and lifespan of H, L lines, and interline hybrids in selection II

Mice	Total population		Lifespan of subpopulations (days)		
	Agglutinin titer Mean $\pm$ SD	Lifespan (days) Mean $\pm$ SD	Last 30% survivors Mean $\pm$ SD	Last 20% survivors Mean $\pm$ SD	Last 10% survivors Mean $\pm$ SD
H	11.4 $\pm$ 0.8	612 $\pm$ 148	694 $\pm$ 88	752 $\pm$ 64	787 $\pm$ 45
L	5.2 $\pm$ 1.0	346 $\pm$ 110	407 $\pm$ 87	444 $\pm$ 74	490 $\pm$ 61
F1	9.4 $\pm$ 0.7	549 $\pm$ 186	670 $\pm$ 90	747 $\pm$ 62	785 $\pm$ 64
F2	9.8 $\pm$ 1.2	513 $\pm$ 183	639 $\pm$ 100	681 $\pm$ 85	724 $\pm$ 87
BcH	10.8 $\pm$ 0.9	530 $\pm$ 215	693 $\pm$ 101	748 $\pm$ 71	786 $\pm$ 64
BcL	7.5 $\pm$ 1.4	464 $\pm$ 158	531 $\pm$ 106	608 $\pm$ 100	670 $\pm$ 96
<i>a</i>	3.1	133	144	154	149
<i>d/a</i>	0.3	0.5	0.8	1.0	1.0

a, Additive effect; *d*, dominance deviation; *d/a*, dominance effect; SD, standard deviation.

malignant lymphomas that are the major cause of death in L mice. The interline difference in lifespan and lymphoma incidence in mice of the GS selection is much larger than that observed in mice of selections I and II.

The cellular modifications of the immune system brought about by selection are not completely understood. At present a major difference, between selections I and II on one hand and selections III and IV on the other hand, concerns macrophages. In selections I and II macrophages of L mice show a more active antigen catabolism and less efficient antigen presentation than macrophages of H mice. Conversely, no differences in macrophage activities were found between H and L mice of selections III and IV. Natural killer (NK) cell activity was found to be lower in L than in H mice of selection I but higher in L than in H mice of selections II and III. Expression of genes regulating antibody responsiveness in lymphocytes was mostly investigated in H and L mice of selection I. It was found that high or low effect genes accumulated in H or L mice were expressed in B cells (higher rate of cell proliferation and differentiation in H than in L mice) and at the level of all Ig isotypes but not in T cells involved in helper activity, skin graft rejection, graft-versus-host reaction, PHA mitotic response, and delayed-type hypersensitivity. However, a limiting dilution assay revealed higher numbers of T cells mediating delayed-type hypersensitivity in H than in L mice.

The significance of the humoral and cellular changes induced by selective breeding stems from studies on innate and acquired immunity to infections in selection I. The conclusion from these studies is that H mice are more resistant than L mice to extracellular pathogens such as *Pneumococcus*, *Klebsiella*, *Trypanosoma* and *Plasmodium*, which are sensitive to the consequence of antibody binding, whereas L mice are more resistant than H mice to

intracellular pathogens such as *Salmonella*, *Brucella*, *Yersinia*, *Mycobacterium* and *Leishmania*, which are sensitive to the bactericidal activity of macrophages.

The Biozzi experimental approach of bidirectional selective breeding has also been used to investigate the polygenic control of inflammation and chemical carcinogenesis.

The application of bidirectional selective breeding to acute inflammatory reaction (AIR) yielded two lines of mice, one giving the maximal (max) AIR and the other giving the minimal (min) AIR. Both max AIR and min AIR were triggered by subcutaneous injection of polyacrylamide microbeads and measured by the leukocyte and serum protein accumulation in the exudate. Selection was started from a genetically heterogeneous foundation population produced by the intercrossing of eight inbred strains of mice and carried out by assortative mating of extreme phenotypes. The response to selection in 11 consecutive generations was highly asymmetrical, as AIR markedly increased in the max line whereas it remained unchanged in the min line. The h^2 mean value in the AIR max line was 0.26 for leukocyte and 0.18 for protein concentrations. The response to selection and the large interline difference resulted from the interaction of 7–9 independent loci with additive effects. The difference between max AIR and min AIR lines is not limited to inflammatory responsiveness as max AIR mice are also more inclined to produce IgE and are more resistant to *Salmonella typhimurium* and to chemical carcinogenesis than are min AIR mice.

The polygenic control of chemical carcinogenesis has been studied by bidirectional selective breeding for susceptibility and resistance to two-stage skin carcinogenesis, initiated with 9,10-dimethyl-1,2-benzanthracene (DMBA) and promoted with 12-O-tetradecanoyl-phorbol-13-acetate (TPA). The selection was started from a heterogeneous population,

Table 4 Carcinogenesis induced in Car-S and Car-R mice at F10, and in their F1 hybrids after 49 days of promotion

Line	No. of mice	DMBA (μg per mouse)	TPA (μg per mouse)	Incidence (%)	Mean number of papillomas $\pm$ SE per treated mouse
Car-S	40	25	5	100	9.07 $\pm$ 0.70
	36	2.5	1	100	6.16 $\pm$ 0.69
	33	1.0	0.5	98	3.51 $\pm$ 0.42
	33	0.5	0.25	41	0.94 $\pm$ 0.23
Car-R	40	50	20	18	0.25 $\pm$ 0.10
	40	50	10	5	0.05 $\pm$ 0.03
	40	25	5	0	0.00 $\pm$ 0.00
F1	36	50	10	89	3.72 $\pm$ 0.64
	36	25	5	75	3.50 $\pm$ 0.56
	36	2.5	1	23	0.47 $\pm$ 0.24

SE, standard error.

as produced by the intercrossing of eight inbred strains of mice, and the character chosen for the assortative mating was the number of papillomas at the end of the promotion period. Susceptible (Car-S) and resistant (Car-R) lines of mice separated by ten consecutive generations of bidirectional selective breeding display a very large difference in responsiveness to carcinogenesis (Table 4). The dominance effect (d/a) = 0.38) measured in (Car-S $\times$ Car-R)F1 hybrids indicates that the susceptibility of papilloma induction is an incompletely dominant character. The difference between Car-S and Car-R lines is restricted to the skin, implying that the selected genes produce a tissue-specific effect.

See also: Immune response (Ir) genes.

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BLOOD TRANSFUSION REACTIONS

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In addition to the transmission of infectious diseases by donor blood, immunological reactions between antibodies in the blood of the patient, and sometimes in that of the donor, and the antigens against which

they are directed, may cause more or less dangerous transfusion reactions. The antibodies involved are alloantibodies against products of allelomorphic genes, but immunoglobulin E (IgE) antibodies

against nonhuman antigens also play a role. The various relevant antibodies and the reactions they may cause will be briefly discussed.

Alloantibodies against red cells

These include 'naturally occurring' antibodies, particularly anti-A and -B, and immune antibodies induced by previous transfusions and/or pregnancies. Except in exceptional cases (see below) blood transfusion reactions due to red cell antibodies should not occur. The correct determination of the ABO blood group of donor and recipient, correct antibody detection and cross-matching and correct patient identification should prevent such reactions. Because donor red cells are destroyed when a reaction is caused by red cell antibodies, such reactions are called hemolytic.

The signs and symptoms of the reaction are due on one hand to the liberation of hemoglobin and thromboplastic substances in stroma, and on the other to the activation of complement, leading to the liberation of C3a and C5a which increase vascular permeability, stimulate mast cells to liberate vasoactive substances such as histamine, and macrophages to liberate cytokines such as interleukin-1 (IL-1).

When ABO incompatible red cells are transfused the reaction is usually severe: the patient becomes restless and complains of a feeling of oppression, which is often accompanied by substernal pain. The patient may have abdominal pain and may vomit.

In serious cases renal function is seriously impaired and there may be uncontrollable bleeding. There is jaundice and hemoglobinuria. Reactions due to antibodies of other specificities are usually less severe, fever being the most common symptom, but jaundice and hemoglobinuria also quite frequently occur.

Exceptional cases

Delayed-type hemolytic transfusion reaction

Whereas the above hemolytic transfusion reactions begin during or shortly after a transfusion, a hemolytic reaction may also occur 3–5 days after a transfusion. In these cases the patient has been immunized in the past against a blood group antigen, but before the present transfusion is given the antibodies are either very weak or even undetectable. The transfused red cells induce a secondary immune response against the antigen and the antibody concentration rises so quickly that after 3–5 days there is enough antibody to destroy the donor cells. If the responsible antibodies are undetectable before the transfusion, the reaction cannot be prevented unless it is known that

antibodies were detected in the patient's serum on a previous occasion, in which case compatible blood must be given. In some cases the direct antiglobulin test may remain positive long after the donor red cells have been eliminated, due to the formation of cross-reacting or autoantibodies secondary to the alloimmune response.

Hemolytic transfusion reaction without detectable antibodies

Rarely a typical hemolytic transfusion reaction occurs while no antibodies are detectable at any time. It has been shown that in these cases the reaction must be due to alloimmunity against a particular red cell antigen. For example only the survival of C-positive red cells is shortened in a C-negative patient. Why anti-C is not detectable in the serum of such a patient and what the mechanism is which is responsible for the sometimes rapid destruction of the donor cells is unknown. Obviously, such a reaction cannot be prevented by routine laboratory investigations.

Red cell alloantibodies in the serum of a donor may be responsible for destruction of red cells of the patient, but this has only been described for potent Anti-A and/or B.

Alloantibodies against platelets

These are of two categories: HLA class I antibodies and platelet-specific ones. Whether transfusion reactions may develop as a result of the interaction of donor platelets and HLA antibodies has not been established. Antibodies against platelet-specific antigens may cause the following reactions.

Febrile reactions

These reactions follow a typical pattern. However, such reactions, in the vast majority of cases are caused by a reaction of antibodies with granulocytes and will therefore be described below.

Post-transfusion purpura

In this serious and, from an immunological point of view, interesting reaction, severe thrombocytopenia develops 5–7 days after a transfusion. The reaction occurs in patients, nearly always women, who have become sensitized to a platelet-specific alloantigen, usually as a result of pregnancies but occasionally by blood transfusion. The reaction is thus due to platelet-specific antibodies, anti-HPA-1a in the great majority of cases, but antibodies against other HPA antigens have also been involved. In this reaction, the patient's platelets, which are of course negative for

the alloantigen involved, are destroyed. The mechanism responsible for this destruction has not yet been definitely established. It may be due to the fixation of immune complexes consisting of alloantibodies and the antigen against which they are directed. These complexes may be formed with free platelet antigens present in the donor plasma or may be liberated after the destruction of donor platelets, or both. Possibly the attraction of different platelet glycoproteins for each other, e.g. GPIIb for GPIIIa, may be the basis of the fixation of such immune complexes to the patient's platelets. Another possibility is that during the secondary immune response against the alloantigen, cross-reacting or autoantibodies are formed which react with the patient's own platelets. Post-transfusion purpura can be prevented by selecting compatible platelets, which is often difficult because of the very high frequency of most of the antigens involved.

Rarely thrombocytopenia is induced by platelet-specific antibodies in the donor plasma. In contrast to post-transfusion purpura, thrombocytopenia occurs immediately after the transfusion in these cases.

Alloantibodies against granulocytes

Granulocyte-reactive alloantibodies, mainly HLA class I and granulocyte-specific ones, are the main cause of febrile transfusion reactions. Typically in this reaction the temperature begins to rise sharply a few hours after the transfusion which is often accompanied by chills. The temperature begins to decline after about 8 h. The reactions are due to antibody-antigen complexes liberated after the destruction of leukocytes. These complexes bind to and activate monocytes, which, after a delay, release cytokines with pyrogenic properties. These reactions can be prevented by removing most of the leukocytes from red cell and platelet concentrates, e.g. by filtration.

A much more serious reaction, transfusion-related acute lung injury (TRALI) is characterized by chills, fever, a nonproductive cough, dyspnea, and infiltration and edema of, particularly, the lower lung fields. The reaction, which may be fatal, is nearly always due to the presence of leukocyte antibodies in the donor plasma, although rarely it has been caused by antibodies in the recipient. Donors whose blood induced the TRALI should be removed from the donor panel.

Febrile reactions induced by cytokines in the transfused blood

Febrile reactions may be caused by cytokines derived from leukocytes, which accumulate due to active synthesis by leukocytes in platelet concentrates during storage. There is a correlation between the frequency of febrile reactions and the number of leukocytes in the platelet concentrate and the time of storage. Pre-storage removal of leukocytes from the platelet concentrate prevents these reactions.

Antibodies against plasma proteins

Most important are antibodies against IgA, which mainly occur in IgA-deficient subjects. Most of the antibodies are directed against nonpolymorphic epitopes on the $\alpha 1$ or $\alpha 2$ chains but anti-allotypic (A2m) antibodies may also occur. The interaction of these antibodies with donor IgA, even if very small quantities are transfused, can cause a severe anaphylactic reaction which may be fatal. Patients with such antibodies can be transfused with red cells which have been washed six times. For other blood components it is necessary to select IgA-deficient donors. Also, A2m antibodies may occur in poly-transfused patients. The reaction caused by them is much less severe, urticaria usually being the only symptom. The extent to which antibodies against other plasma proteins may cause transfusion reactions is controversial.

IgE Antibodies against nonhuman antigens

In patients who are allergic to an allergen, an allergic reaction occurs if the donor plasma contains the allergen. This may particularly occur if it concerns food allergens, which may be present in the donor plasma after consumption of the food involved. The patient develops urticaria. The reactions can be prevented, in the case of red cell concentrates, by washing the cells three times. Very rarely a similar reaction is induced by donor IgE antibodies against an allergen which at the time of the transfusion is present in the patient.

See also: ABO blood group system; Alloantigens; Erythrocytes; Neutrophils; Plasma; Platelets; Rh antigens; Transfusion.

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B LYMPHOCYTE ACTIVATION

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B lymphocytes, precursors of antibody-secreting cells, originate from pluripotent hematopoietic stem cells and differentiate in the fetal liver and, in adult life, principally in the bone marrow. During differentiation the diversity of antigen specificity among B cells is generated in an antigen-independent manner through the rearrangement of gene segments encoding parts of the variable region of the B cell receptor (BCR) for antigen, that is, immunoglobulin (Ig). The newly generated, surface Ig-expressing mature B cells leave the bone marrow and circulate into peripheral lymphoid organs in which they can be activated to proliferate and to differentiate into antibody-secreting cells by encounter with antigens that bear epitopes complementary to their surface Ig.

B cell responses to antigens can be classified into T cell dependent and T cell independent. Some antigens such as bacterial polysaccharides can induce vigorous B cell stimulation independently of T cells by strong cross-linking of the BCR. In contrast, efficient B cell responses to many protein antigens need an intimate interaction of B cells with 'helper' T cells. Stimulation of BCR by antigen leads to endocytosis and degradation of the antigen captured by the BCR. Peptides derived from degraded antigen are bound to major histocompatibility complex (MHC) class II molecules and transported to the cell surface to be presented to T cells. T cells expressing a particular T cell receptor (TCR) that recognizes the peptide–MHC complex presented on the B cells then become activated. The activated T cells provide help to B cells in two forms: soluble mediators (cytokines) such as interleukin-4 (IL-4), IL-5 and IL-6, and membrane-bound stimulatory molecules including CD40 ligand.

B cell activation through BCR

The BCR is a complex with a hetero-oligomeric structure in which antigen recognition and signal transduction are compartmentalized into distinct subunits. The antigen recognition subunit of the BCR is a clonally-distributed membrane-bound Ig, a

tetrameric complex of heavy and light chains, whereas the signal transduction subunit is a disulfide-linked heterodimer composed of Ig α (CD79a), a *mb-1* gene product, and Ig β (CD79b), a *B29* gene product (Figure 1). Both Ig α and Ig β chains contain within their cytoplasmic domains a sequence motif (immunoreceptor tyrosine-based activation motif, ITAM), D/E-X7-D/E-X2-Y-X2-L/I-X7-Y-X2-L/I, which is also found in the cytoplasmic tails of the signal transducers of the TCR and of Fc receptors (Figure 2).

Stimulation of B cells through the BCR results in rapid increases in tyrosine phosphorylation on a number of proteins and induces an increase of phosphatidylinositol and mobilization of cytoplasmic free calcium. Though none of the BCR subunits contains intrinsic protein tyrosine kinase (PTK) activity, the BCR associates with two types of cytoplasmic PTKs: Src family PTKs, including Lyn, Fyn, Blk and Lck, and the more distantly related PTK, Syk (Figure 1). Activation of these PTKs through ligation of the BCR by antigen leads to the phosphorylation of the two tyrosine residues within the ITAM in Ig α and Ig β , which in turn leads to recruitment and activation of additional PTKs. The activated PTKs including Btk appear to phosphorylate numerous cellular proteins involved in intracellular signaling pathways, such as the adaptor protein Shc in the Ras pathway, phospholipase C (PLC)- γ 2, GTPase-activating protein (GAP), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and Vav as well as HS-1 (Figure 1). The tyrosine phosphorylation of PLC- γ 2 increases its activity to convert phosphatidylinositol 4,5-bisphosphate into the two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates protein kinase C while IP3 causes Ca²⁺ release from intracellular stores (Figure 1).

The regulated signal transduction through the BCR can be achieved by coordinated actions of PTKs and protein tyrosine phosphatases (PTPs). CD45 is a transmembrane PTP expressed on hematopoietic

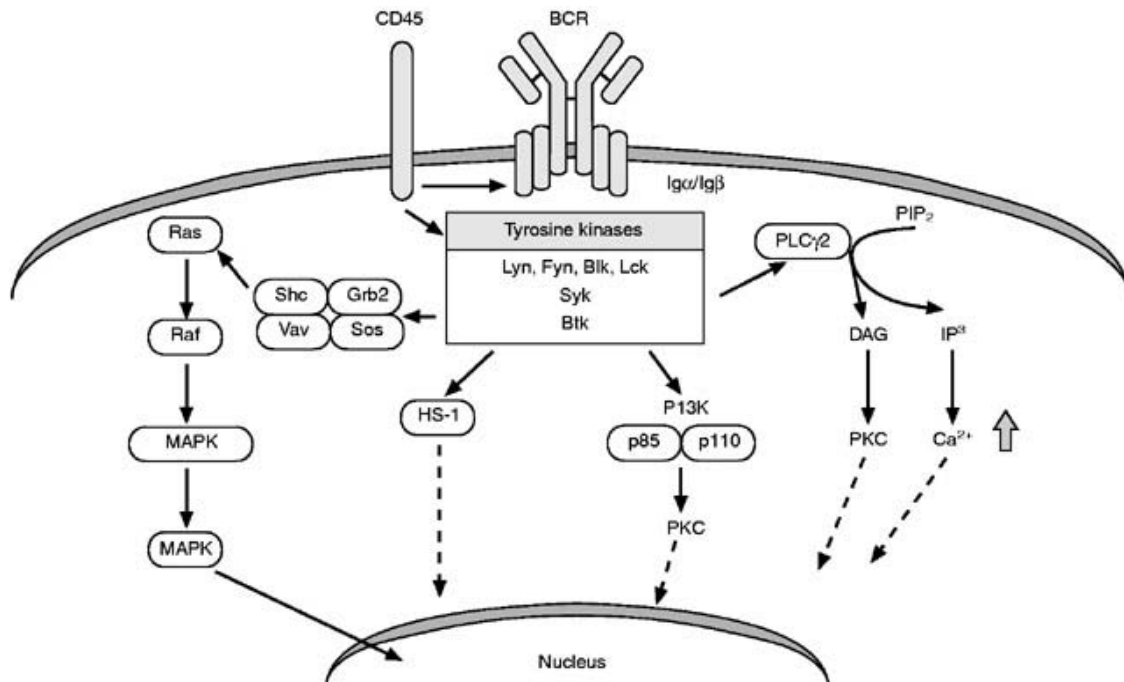


Figure 1 Signaling pathway of BCR.

cells and plays a critical role in B cell activation following ligation of the BCR (Figure 1). The regulation of Src family PTKs by CD45 appears to be one of the bases for requirement of CD45 in antigen-induced BCR signaling. Since Src family PTKs contain a negative regulatory tyrosine phosphorylation site at their carboxyl termini, dephosphorylation of this site by CD45 results in an increase of their kinase activity. The phosphorylated tyrosine residues within ITAM of Ig α and Ig β also appear to be substrates for CD45. The importance of CD45 in BCR signaling has been confirmed by generating CD45-deficient mice in which B cells are completely refractory to proliferation stimulated by anti-IgM antibody.

Cognate T cell help for B cell activation

The CD40 ligand (CD40L) expressed by activated T cells is a 33 kDa type II membrane glycoprotein with sequence homology to tumor necrosis factor. T cells activated through TCR ligation transiently express on their surface CD40L, which interacts with CD40 expressed on B cells to deliver signals for B cell activation and proliferation. Signaling through CD40 in combination with T cell-derived cytokines such as IL-4 and IL-5 in mice leads to B cell differentiation accompanied by antibody secretion and Ig isotype switching. The CD40–CD40L interaction is also important for other B cell functions including the formation of germinal centers and the induction of

memory B cells. The physiological significance of the CD40–CD40L interaction *in vivo* has become evident from the observation of patients with X-linked hyper-IgM syndrome, who have mutations in the gene encoding CD40L and are therefore unable to express functional CD40L on their T cells. These patients fail to produce Ig of isotypes other than IgM in response to antigen challenge and also lack germinal centers in their secondary lymphoid organs.

The cognate interaction of B and T cells triggers the engagement of many other accessory molecules on both cells, which serve to stabilize the B–T conjugation and to coordinate the responses of the interacting cells. They include B7-1 (CD80), B7-2 (CD86), CTLA-4, LFA-1 (CD11a/18), LFA-2 (CD2), LFA-3 (CD58), ICAM-1 (CD54), VCAM (CD106) and VLA-4 (CD49d/29). The B7-1/B7-2 expressed on B cells interacts with the CD28 expressed on T cells to deliver a costimulatory signal to TCR-stimulated T cells, which then become fully activated to produce cytokines. T cell-derived cytokines such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and interferon γ (IFN γ) have been shown to enhance B cell activation and proliferation and to be essential costimuli for subsequent B cell differentiation and Ig production.

Modulation of BCR signaling

Several antigen-independent accessory molecules play an important role in the regulation and modu-

lation of B cell activation mediated by the BCR (Figure 2). Key molecules include the CD19/CD21/CD81/Leu13 complex, CD22, CD38 and the Fc receptor Fc γ RIIB1, in addition to CD45 and CD40 discussed above.

CD19, CD21 (complement receptor 2), CD81 (TAPA-1) and Leu13 form a complex on the surface of B cells. This complex functions as a coreceptor which substantially lowers the threshold for cellular activation through the BCR. Ligation of the BCR induces the phosphorylation of tyrosine residues in the cytoplasmic tail of CD19, to which PI3K binds. The coligation of CD19 and the BCR reduces by two orders of magnitude the number of antigen receptors that must be ligated to induce B cell proliferation. Therefore, B cells that encounter antigens in the form of immune complexes containing complement components may be hyperstimulated by virtue of the coligation of the BCR and the CD19/CD21 complex. Since CD21 has been shown to also interact with CD23, antigens presented on CD23⁺ cells such as follicular dendritic cells in germinal centers may also hyperstimulate B cells. Indeed, CD19-deficient mice showed the severe impairment of B cell responses to T-dependent antigens and lacked germinal center formation.

CD38 is a 42 kDa membrane glycoprotein expressed in many cell types, including B cells, and has been shown to associate with the BCR in humans. Ligation of CD38 with specific antibodies induces a potent proliferative signal to B cells in the presence of other growth cofactors such as IL-4, IL-5 or lipopolysaccharide (LPS). Engagement of CD38 also rescues germinal center B cells from apoptosis. CD38 stimulation activates Btk tyrosine kinase, and

it has been found that CD38 signaling is abolished in *xid* mice which carry a mutation in the *btk* gene and show a global B cell abnormality.

Immune complexes consisting of antigen and IgG antibodies are potent inhibitors of B cell responses. The coligation of the BCR and Fc γ RIIB1 through immune complexes leads to abortive BCR signaling (Figure 2). It has been shown that Fc γ RIIB1, when coligated with the BCR, recruits and activates a cytosolic protein tyrosine phosphatase, SHP1, through the interaction of a phosphorylated tyrosine residue within the sequence motif (immunoreceptor tyrosine-based inhibitory motif, ITIM) in the cytoplasmic domain of Fc γ RIIB1 and the SH2 domain of SHP1. The important role of SHP1 in negative regulation of BCR signaling has been clarified by the identification of mutated SHP1 genes as causative of the systemic autoimmune and immunodeficiency syndrome observed in *motheaten* (*me*) and *motheaten viable* (*me^v*) mutant mice. These SHP1-deficient mice are characterized by hypergammaglobulinemia and the production of multiple autoantibodies besides abnormalities of other hematopoietic cells, suggesting that SHP1 normally functions as a negative regulator of BCR signaling. Though the mechanism by which SHP1 modulates signaling through BCR has not yet been fully elucidated, SHP1 appears to be involved in Fc γ RIIB1-mediated negative regulation. Inhibitory signaling mediated by Fc γ RIIB1 is deficient in *motheaten* mice, indicating that SHP1 is an effector of the negative regulation of BCR signaling.

CD22, a 135 kDa B cell restricted glycoprotein, has also the sequence motif ITIM on its cytoplasmic domain, to which SHP1 is recruited after BCR ligation (Figure 2). B cells from CD22-deficient mice

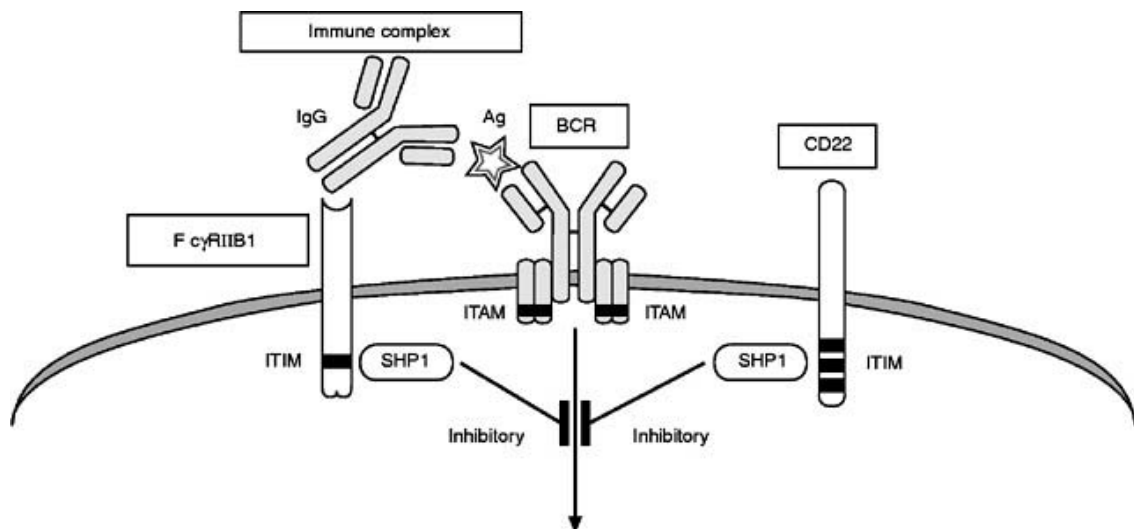


Figure 2 Negative regulation of BCR signaling.

(ITAM: immunoreceptor tyrosine-based activation motif) (ITIM: immunoreceptor tyrosine-based inhibitory motif).

show hyperresponsiveness to BCR signaling, suggesting that CD22 serves as a negative regulator of BCR signaling.

See also: Affinity maturation; Antibodies, synthesis; Antigens, T dependent and independent; B lymphocyte, antigen processing and presentation; B lymphocyte differentiation; B lymphocytes; CD22; CD45 (the leukocyte common antigen); Helper T lymphocytes; Cytokines; Fc receptors; Germinal center; Immunoglobulin, cell surface; Interleukin 4; Interleukin 5 and its receptor; Interleukin 6; Protein kinases.

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B LYMPHOCYTE ANTIGEN PROCESSING AND PRESENTATION

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Antigen presentation by B cells: a historical view

Two different cellular populations are required for the production of antibodies, as early studies have suggested. These are thymus (T cell)- and bone marrow (B cell)-derived cells. T cells help B cells to differentiate into antibody-producing cells. *In vitro* studies have revealed that the cell populations are further subdivided. Cells from the spleen are fractionated by their ability to adhere to plastic, the adherent and nonadherent cells. The adherent cells mainly consist of macrophages, whereas the nonadherent cells are comprised of T and B cells. Further studies established that T and B cells are required for primary and secondary antibody responses, and that macrophages can also act as accessory cells involved in T cell activation.

Initially, macrophages were considered to be the critical accessory cells. Subsequent experiments established the capacity of other cells to function as accessory cells, or antigen-presenting cells (APCs). These include dendritic cells (DCs), Langerhans cells, endothelial cells, Schwann cells and Kupffer cells. The expression of major histocompatibility complex (MHC) class II molecules is essential for APCs. Therefore, it is quite logical to ask whether B cells could act as APCs, as B cells express moderately high levels of the molecule. The use of rabbit antimouse immunoglobulin (RAMIg) as an antigen demonstrated that rabbit Ig-specific T cells were able to respond to B cells and macrophages with similar efficiencies in the antibody response. Macrophages presented normal rabbit Ig (NRIg) to T cells as effectively as RAMIg; however, B cells failed to present

NR1g to T cells at the same level, suggesting that antigen-specific T–B collaboration is responsible for the efficient antigen presentation by B cells. Similar experiments were conducted using a soluble antigen, keyhole limpet hemocyanin (KLH)-specific B cells. Normal resting (small) B cells from unimmunized mice were poor APCs, whereas lipopolysaccharide (LPS)-activated B cells could present KLH to KLH-specific normal T cells. Thus, it was demonstrated that normal resting B cells from unprimed mice were poor at antigen presentation.

Studies using hapten carrier systems demonstrated that antigens are recognized by two distinct cells in order to produce a hapten-specific antibody. These experiments clearly demonstrated that B cells recognize the haptenic determinant, whereas T cells recognize the carrier determinant. Based on these results an antigen bridge model was proposed, in which B cells and T cells are bridged by native antigens (hapten carrier) bound to membrane immunoglobulin (mIg) and T cell receptor (TCR). However, two important discoveries virtually destroyed the antigen bridge model. These were: 1) MHC restriction, and 2) antigen processing. The TCR makes contact with B cells via the antigen-MHC complex, thus mIg is not required for interaction with the TCR. Only processed peptides are presented to the TCR. The antigen bridge model was challenged by utilizing antigen-specific B cell and T cell clones. These experiments clearly demonstrated that antigens bound by antigen-specific mIg molecules were presented to T cells with 1000–2000-fold greater efficiency than nonspecific presentation. The antigen taken up by the B cells is processed within acidic cellular compartments and the peptides generated bind to MHC class II molecules for presentation to the TCR. Thus, the functional role of mIg on B cells for efficient antigen presentation has been firmly established. These are *in vitro* results; the role of B cells as APCs *in vivo* remains to be determined.

Antigen presentation by B cells *in vivo*

Experiments addressing this question have been carried out using mice chronically treated from birth with anti- μ antibody (anti- μ mice), a procedure which depletes Ig⁺ B cells. These mice fail to mount a secondary T cell proliferative response following immunization with hapten carrier, but do so if injected with hapten-coupled normal splenocytes, suggesting that the presence of B cells in lymph nodes is required for T cell priming. The antigen presentation capacity of unfractionated lymph node APCs was much stronger, however, than that of unfractionated spleen APCs.

The importance of B cells to T cell priming *in vivo* was further demonstrated by inoculating purified syngeneic normal resting B cells before priming them with antigen. B cell-depleted mice primed with antigen in the presence of exogenous B cells were shown to respond to the antigen, whereas control mice without inoculation of exogenous B cells could not respond. Anti- μ -treated mice reconstituted with fluorescein isothiocyanate (FITC)-specific B cells responded markedly better to FITC-ovalbumin than to trinitrophenyl-ovalbumin. These results suggest that B cells are involved in the generation of primary T cell responses. However, several groups have presented contradictory results to these early experiments, as described below.

Investigators agree that activated B cells are able to stimulate primed T cells. However, several experiments seem to suggest that even activated B cells are poor at stimulating naive T cells compared to DCs. The activation of antigen-specific T cells requires two signaling events which must be provided by APCs. The first signaling event consists of a cognate APC–T interaction between TCR and antigen peptide/MHC class II molecules. The second signal is provided by an interaction between costimulatory signaling molecules such as B7 molecules (B7-1 and B7-2) on APCs and CD28/CTLA-4 expressed on T cells. Deficiency in the second signaling in the APC–T interaction is thought to result in T cell tolerance. It has been demonstrated that B cells expressing high levels of CTLA-4/CD28 ligand fail to provide T cell-stimulating signals as strong as DCs. It is still possible that as yet unidentified costimulatory molecules are involved in this interaction. The form in which antigens are introduced into animals may additionally play a role in T cell activation. Transgenic mice expressing I-E molecules in different cell populations (i.e. B cells or DCs) were utilized to investigate the effect of different forms of antigen (peptide versus native protein). Peptide antigens are more efficiently presented to T cells by DCs. However, DCs show poor activity for the presentation of native protein antigens. In contrast, B cells are efficient APCs for protein antigens, but not peptides. Further, these experiments suggest that as little as 1% contamination of peptides in protein antigen preparations is sufficient for T cell activation by DCs. Previous experiments suggesting a poor ability of resting B cells for T cell priming should be re-examined in light of these new data.

The functional role of B cells as APCs was recently re-examined by using mice genetically lacking B cells. These mice (μ MT knockouts) were established by depleting the μ MT (membrane) region by gene targeting, which may be less abrasive than anti- μ treat-

ment. The μ MT knockouts had impaired T cell priming to protein antigens but not to peptides. Interestingly, however, KLH demonstrated an unusual ability to prime naive T cells in the B cell-depleted mice. The antigen is very potent and widely used for *in vivo* priming experiments. Investigators should pay extra attention to the experimental design when KLH is used. Antigen-specific B cells pulsed *in vivo* revealed an impressive upregulation of B7 molecules. Antigen stimulation may induce positive signaling mediated via mIg molecules; these activated B cells can then become competent APCs for the activation of naive T cells *in vivo*. Contradictory results have, however, been reported using μ MT mice. T cell responses to several antigens including not only KLH, but also H-Y (male antigen), MHC and *Shistosoma mansoni* eggs have been described. However, antigens such as H-Y and MHC are expressed on the membrane surface of APCs. Concentration of these antigens by mIg molecules and subsequent internalization are not required for presentation. One can speculate, therefore, that this critical step in B cell activation is not required for presentation of these antigens. The functional role of B cells as APCs *in vivo* has yet to be firmly established; however, investigators believe B cells must be initially activated to have the capacity of APCs for T cell priming.

Processing

MHC class II molecules are transported from the endoplasmic reticulum (ER) via the Golgi to endosomal compartments as a complex with invariant chain (Ii). The Ii prevents endogenous peptides from binding to the groove of MHC class II molecules. After

removal of Ii in the acidic endosomal compartments, peptides are able to bind to the MHC groove. Peptide-loaded MHC class II molecules are then transported to the membrane surface for antigen presentation. The amino acid residues 81–104 of Ii, dubbed class II-associated invariant-chain peptides (CLIP), remain bound to the class II antigen-binding grooves. In endosomal compartments, MHC class II-like DM molecules promote the dissociation of CLIP from the MHC class II complex. Because cells can utilize a different mechanism for antigen presentation from other APCs, namely mIg-mediated antigen internalization, the antigen peptide loading of MHC class II in B lymphocytes is expected to take place in a compartment accessible to mIg. The process may occur in early and late endosomes and lysosomes. However, investigators have found compartments which are distinct from the conventional vesicles described above. These compartments for peptide loading (CPLs) meet several requirements for antigen loading vesicles in B cells (Figure 1). mIgG and mIgM efficiently enter the compartments, and transiently accumulated MHC class II molecules are evident. MHC class II molecules in these compartments are not associated with the Ii chain. Some of the MHC class II molecules are loaded with antigen peptides. Most newly synthesized MHC class II molecules are transported through CPLs on their way to the plasma membrane. These compartments are not accessible to the transferrin receptor. Morphologically, these compartments in B cells demonstrate a physical structure with an internal vesicle membrane similar to that seen in the multivesicular bodies, an intermediate in the conventional endosomal pathway. Antigen-presenting B cells may therefore be

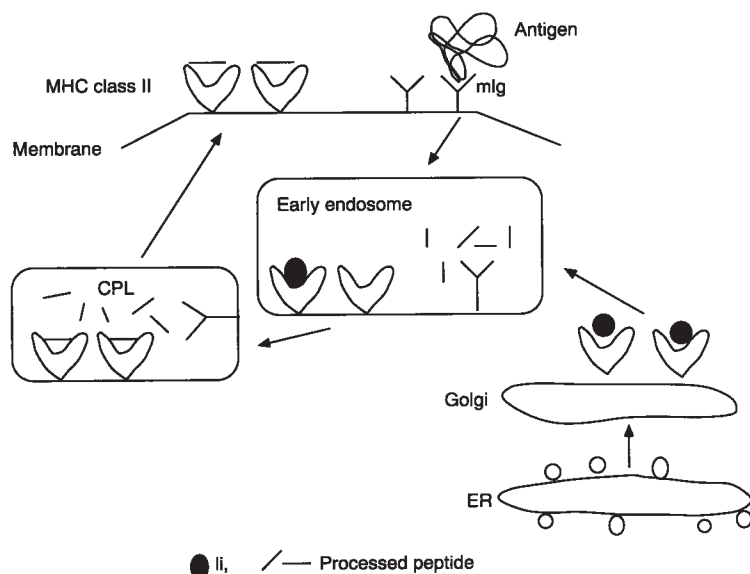


Figure 1 Antigen processing and presentation in B lymphocytes.

able to selectively target internalized antigen and increase the efficiency of antigen presentation by utilizing the CPL-mediated pathway.

See also: Antigen-presenting cells; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; B lymphocytes; Immunoglobulin, cell surface; MHC restriction.

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B LYMPHOCYTE DIFFERENTIATION

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B cell differentiation broadly construed encompasses both the generation of B lymphocytes from precursor or stem cells (i.e. B cell development), as well as their maturation into plasma or memory cells. B cell lymphopoiesis begins in the embryonic yolk sac, switches to the fetal liver, and shortly after birth becomes established in the bone marrow. While B-2 (conventional) B cells are continuously generated in the adult bone marrow, B-1 (CD5⁺) B cells are predominantly produced early in ontogeny in the fetal liver. Early B cell differentiation has been divided into steps or stages based on the status of immunoglobulin gene rearrangements, the expression of cell surface molecules, and the *in vitro* growth requirements of the developing B cells. One commonly used scheme based on the expression of the cell surface markers B220, CD43, BP-1 and HSA is shown in Table 1. Included in the table are the expression

levels of other important B cell markers along with the status of immunoglobulin gene rearrangements in the cells at various stages.

As B cell progenitors mature, they pass through checkpoints that assess the status of their antigen receptors (see below). Those developing B cells that fail to pass die by apoptosis. Having acquired a mature phenotype, B cells enter the secondary lymphoid tissues such as spleen or lymph nodes, where they may encounter foreign antigens. Depending upon the type of antigen and the accompanying signals the B cells receive, they enter one of several different differentiation pathways that lead to the generation of plasma and/or memory B cells. B cell differentiation is a hazardous duty and relatively few cells pass all the checkpoints.

Genetic experiments in mice, both gene targeting experiments and the transgenic expression of B cell

Table 1 B lymphocyte developmental stages in mice^a

Phenotype	Pro-B				Pre-B	Immature B	Mature B
	A	B	C	C'	D	E	F
B220	++	++	++	++	+++	+++	++++
CD43	++	+	+	+	—	—	—
HSA	—	+	+	+++	++	+	+/-
BP-1	—	—	++	++	+	—	—
sIgH	—	—	—	—	-/+	(μ+)	(μ-δ+)
sIgL	—	—	—	—	—	+	+
IgH locus	GL ^b	DJ,VDJ ^c	DJ,VDJ	VDJ	VDJ	VDJ	VDJ
IgL locus	GL	GL	GL	GL	VJ	VJ	VJ
λ5	—	+	+	+/-	+/-	—	—
VpreB	—	+	+	+/-	—	—	—
Mb-1	—	+	+	+	+	+	+
RAG-1	+++	+++	++	+	++	+/-	— ^d
TdT ^e	—	+	+	—	—	—	—
c-kit	+	++	++	—	—	—	—
IL-7R	—	+	+	+	+	—	—
CD19	-/+	++	++	++	+++	+++	+++
CD22	—	—	+	+	+	++	++
CD40	—	—	—	—	+	+	+

^a See Loffert *et al* (1994) for more details.^b Germline.^c Stage D has GL as well as DJ and VDJ joints.^d RAG-1 is re-expressed in germinal center B cells, the functional significance unknown.^e TdT is not expressed in fetal liver B cells, hence their Ig genes lack N-region additions.

antigen receptors of defined specificity, have greatly deepened our understanding of the molecular and biologic processes involved in B cell differentiation. Because of the importance of these experiments they will be referred to when appropriate and the phenotypes of mice that have had molecules important in B cell differentiation altered by gene targeting have been summarized in Table 2. While there are some minor differences between human and mouse B cell differentiation, the overall scheme is remarkably similar.

Early B cell differentiation

Within the bone marrow microenvironment B cell development is divided into two main physiologic compartments. The small B220⁺, CD43⁺ population that contains many rapidly proliferating cells and the larger, but mostly resting, CD43⁻ pre-B cell compartment, which derives from CD43⁺ cells. The stromal cell-derived growth factors interleukin 7 (IL-7) and kit ligand help to maintain the proliferation of the CD43⁺ cells. The recombinase machinery necessary for immunoglobulin gene rearrangements predominantly acts on the IgH loci in the CD43⁺ compartment. The Ig rearrangements are catalyzed by a protein complex that contains two lymphoid-specific components, RAG-1 and RAG-2, that are involved in recognizing the recombination sequences and cutting the DNA. DNA joining requires the DNA-

dependent protein kinase complex. Once a B cell progenitor has successfully rearranged one heavy chain gene it terminates the rearrangement process on the other allele, a process termed allelic exclusion. Expression of μ heavy chain in conjunction with the surrogate light chains λ5 and Vpre-B results in the assembly of the pre-B cell receptor. This is a major checkpoint in B cell development. The failure to express this receptor dramatically reduces the transition of developing B from the CD43⁺ into the CD43⁻ compartment, stage C' to D. Whether the outside environment delivers a signal through the pre-B cell receptor or its simple expression provides the signal remains unclear although it is likely that the tyrosine kinase p72syk is required for signal transmission. The successful progression of a progenitor B cell into the CD43⁻ compartment results in a cessation of IgH rearrangements and the completion of light chain rearrangements. The choice of κ versus λ light chain appears to be a stochastic event which in the mouse is an approximately 10:1 preference of κ over λ. A successful κ or λ rearrangement terminates the rearrangement process and the cell expresses surface IgM with either κ or λ light chains, its antigen-binding receptor. Such a cell, termed an immature B cell, must pass additional checkpoints before entering the mature B cell pool. One of those checkpoints was revealed by the analysis of mice that lack a functional mb-1 protein. Their immature B cells fail to enter the peripheral B cell pool, implying

a checkpoint that assesses the competence of the antigen receptor. Evidence for another checkpoint at this stage came from the analysis of mice transgenically expressing an autoreactive antigen receptor.

Immature B cells in these mice are either tolerized or deleted depending upon the nature of the autoreactive antigen. However, a mechanism may have evolved to rescue autoreactive B cells from this fate.

Table 2 Gene targeting of molecules involved in murine B cell development and differentiation

Targeted gene	Phenotype
<i>Cell surface proteins</i>	
BP-1	No obvious effect on B lymphocyte development and differentiation
CD19	B cell development normal, reduced responsiveness to thymus-dependent (TD) antigen, a lack of germinal centers, and failure of B1 cell development
CD21	Normal B cell development although reduced B1 cells, decreased responsiveness to TD antigens and germinal center size, increased threshold for B cell activation
CD22	Reduced number of IgM ⁺ , IgD ⁺ cells, increased Ca ²⁺ response to sIg signaling, and decreased thymus-independent (TI) response, expanded B1 population
CD23	No obvious effect on B lymphocyte development and differentiation
CD40	Impaired isotype switching, poor TD responses, and absent germinal centers
CD45	A decreased threshold for antigen-induced signaling resulting in the accumulation of potentially autoreactive B cells
HSA	Modest inhibition of early B cell development
Ig- α tail	A small impairment in B cell development, but a marked reduction in mature B (mb-1) cells, implying a checkpoint for the expression of the antigen receptor
Ig- β	Arrest in B cell development at CD43 ⁺ stage, normal DJ joining but reduced (B29) VDJ joints
Ig κ	A limited B cell repertoire, reduced number of B cells
IgD	Normal development, delayed affinity maturation of the antibody response
IL-7R	A severe block in early B cell development, failure of CD43 ⁺ cells to expand
Invariant chain	Decreased response to TI antigens, development block at immature B cell stage
TNFR1	Required for germinal center formation in the spleen
$\lambda 5$	Impaired expansion of pro-B cells to stage D and a lack of allelic exclusion
$\mu\mu$ membrane exons	An early failure of B cell development and a lack of allelic exclusion
<i>Transcription factors</i>	
Bcl-6	Normal B cell development, failure to generate germinal centers
BSAP	A failure of B cell development with an arrest at stage B. DJ but no VDJ rearrangements
E2A	An early failure of B cell development probably occurs prior to BSAP expression as no DJ rearrangements are detected
EBF	A failure of B cell development with only very early B cell progenitors present. No Ig rearrangements
Ets-1	A marked expansion of IgM plasma cells
Ikaros	A complete lack of B cell progenitors
Oca-B	Reduction in the number of mature recirculating B cells. Poor response to TD and TI antigens, a lack of germinal center formation
Oct-2	Normal development, impaired response to LPS, and impaired Ig secretion
p50	B cell development normal, impaired response to LPS and decreased serum Ig levels and impaired switching to IgG3, IgE, and IgA
PU.1	A lack of B cell progenitors, transcription and rearrangement of Ig blocked
RelB	Normal B cell development, mild reduction in B220 ⁺ cells in spleen, no germinal center formation during TD responses
Sox-4	Partial block in B cell development, Stages A–C intact, reduced number of C' and stage D cells in the bone marrow
<i>Protein kinases</i>	
BTK	Reduction in B2 cells and a severe deficiency in B1 cells, decreased IgM and IgG3, defective response to TI-2 antigens
c-Abl	Reduction in the number of mature B cells, reduction in LPS response and Ig response following SRBC immunization
Lyn	Reduced number of recirculating B cells, elevated levels of serum IgM, presence of autoantibodies implicating lyn in establishing B cell tolerance
Syk	Markedly impaired B cell development likely due to an impaired signaling from the pre-B cell receptor, thereby preventing clonal expansion
<i>Cell survival proteins</i>	
Bcl-2	Normal B cell development, collapse of peripheral immune system by massive apoptosis
Bcl-x	Markedly impaired B cell development, absence of mature B cells
Bax	B cell hyperplasia

Table 2 Continued

Targeted gene	Phenotype
<i>Miscellaneous</i>	
Ig κ intronic	Greatly impaired VJ rearrangements, normal levels of κ expression, κ : λ enhancer ratio of 1:1 rather than 1:10
Ig κ 3' enhancer	Reduction in the number of κ -bearing B cells, reduced number of rearrangements at the κ locus
J μ	Mice cannot assemble a μ heavy chain, they lack slg μ ⁺ B cells and B cell development is blocked at large, CD43 ⁺ stage (C)
RAG-1	No Ig rearrangements and an arrest of B cell development at stage B, C
SDF-1	Failure of B cell development, reduced precursors in fetal liver and bone marrow
TdT	Lack of N-terminal additions in the adult repertoire, no obvious B cell phenotype
Vav	Normal B2 B cell development but a lack of B1 cells. Mature B cells hyporeactive to antigen signaling

They may reactivate the recombinase machinery and rearrange another light chain, generating a new antigen receptor with a different specificity, a process termed receptor editing. Finally, an immature B cell acquires IgD and passes into the periphery.

B cell differentiation in peripheral lymphoid tissues

B cell differentiation in the peripheral lymphoid tissues is predominantly antigen driven. B cell antigens can be broadly divided into thymus dependent (TD) and thymus independent (TI). TD antigens are usually soluble protein antigens that require major histocompatibility complex (MHC) class II-restricted T cell help for antibody production while TI antigens do not require such help, and are often multivalent and poorly degraded *in vivo*. Good examples of TI antigens are bacterial polysaccharides. In general, TI responses generate poor immunological memory, induce minimal germinal center formation, and trigger IgG2 secretion. The B cells responding to TI antigens have a distinct phenotype (IgM⁺, IgD⁻, CD21^{high}) and localize in the marginal zone in the spleen. The dependence upon these cells for responses to TI antigens may account for the poor responses to polysaccharide antigens seen in splenectomized individuals and human infants (the marginal zone B cells don't mature until ~2 years of age). Coupling polysaccharides to a carrier protein results in an effective infant vaccine since the conjugate triggers a TD response.

Exposure to a TD antigen triggers two pathways of B cell differentiation, the extrafollicular pathway, which leads to early antibody production, and the germinal center pathway, which leads to germinal center formation, immunological memory, and the generation of plasma cell precursors. In the spleen, the antigen-activated B cells migrate to T cell-rich zones in the periarteriolar lymphoid sheath searching for T cell help. Failure to find it likely results in sub-

sequent unresponsiveness or anergy, however, successful T-B cell collaboration produces short-lived oligoclonal proliferative foci (each derived from several B cells). Many of the B cells in these foci secrete IgM and undergo isotype switching (a DNA splicing event that exchanges IgH constant region). These events depend upon costimulatory signals, CD40 and CD40L interactions (see below), and cytokines such as IL-2, IL-4, IL-6, IL-10 and transforming growth factor β (TGF β). Some B cells migrate from these foci into primary follicles to enter the germinal center pathway. Within a primary follicle an oligoclonal expansion of B cells forms the dark zone of the germinal center. There somatic mutations are introduced into the variable regions of Ig genes that may alter the B cell antigen receptor specificity or affinity. Eventually these cells migrate into the light zone of the germinal center region where they interact with helper T cells and follicular dendritic cells that have trapped antigen localized on their surfaces. Here, B cells with high-affinity antigen receptors are selected to survive, while those that lack such receptors die or perhaps re-enter the dark zone for another round of somatic mutations. B cells that acquire an autoreactive antigen receptor are also eliminated. Rescue signals such as the engagement of CD40 (a member of the TNF α receptor family expressed on B cells) induce Bcl-x and Bcl-2, resulting in the survival of selected cells. CD40, CD40L (a member of the TNF α family expressed on activated T cells), B7-1, CD21, TNF α R and CD19 all have critical roles in germinal center formation. Individuals with the X-linked hyper-IgM syndrome underscore the importance of CD40L, as they do not express functional CD40L following T cell activation and do not form germinal centers.

Passage through the germinal center leads to the formation of memory B cells or plasma cell precursors (there are few antibody-secreting cells within the germinal center). Cytokines and CD40L influence this decision. IL-2, IL-10 and IL-6 promote differen-

tiation toward plasma cells while CD40/CD40L interactions promote memory cell formation and inhibit plasma cell generation. Cytokines also contribute to isotype switching. IL-4 enhances switching to IgE and IgG4; IL-10 toward IgG1, IgG3, and IgA; and TGF β toward IgA. While plasma cells secrete large amounts of Ig, they are short-lived cells that need to be replenished to sustain high antibody levels. Plasma cells lose their membrane Ig and many of the markers used to identify B cells although they express high levels of CD38. Memory B cells are long-lived cells that contain somatically mutated V genes and are morphologically distinct from naive B cells. They can be rapidly restimulated to generate a secondary antibody response. Together the extra follicular and germinal center pathways of B cell differentiation lead to a coordinated humoral response providing the very rapid production of low-affinity antibodies, the subsequent production of high-affinity antibodies, and the potential for a rapid amnestic response. The failure of B cell differentiation at any of the many steps may result in a crippled immune system incapable of protecting an individual against many potentially pathogenic organisms or in an autoreactive immune system capable of inflicting damage upon self tissues.

See also: Antigen, T dependent and independent; B

lymphocyte activation; B lymphocyte repertoire; B lymphocytes; Bone marrow and hematopoiesis; Bursa of Fabricius; CD22; CD40 and its ligand; CD45 (the leukocyte common antigen); Cytokines; Diversity, generation of; Humoral immunity; Immunoglobulin, cell surface; Immunoglobulin class switching; Lymphocyte trafficking; Lymphocytes; Microenvironment; Ontogeny of the immune response; Somatic mutation.

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B LYMPHOCYTE REPERTOIRE

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Over the years two experimental approaches have been used to assess antigen-specific B cell populations at the clonal level. These use antigenic or polyclonal mitogenic stimulation of B cells in limiting dilution. Since polyclonal stimulation encompasses a broad range of B cell responsiveness, assays for antibody production discriminate between the various antigen reactivities. In contrast, antigen stimulation is more selective in the B cells that respond, thus enabling analyses for positive antibody production. The advent of hybridoma technology not only allowed for ease in experimental execution but also made feasible the analyses of the molecular events involved. With advances in molecular biology technology, e.g. polymerase chain reaction (PCR) and reverse transcriptase-PCR, sequences could be obtained from single cells without any form of selection.

Among the early major findings are that B cell

antigen receptors are clonally distributed, each B cell expresses a single immunoglobulin specificity and each stimulated primary B cell gives rise to a clone of antibody-forming cells (AFCs) whose antibody replicates the variable region clonotype of the antigen receptor of the original stimulated B cell. Although the primary B cell clonotype repertoire is extremely diverse, individuals of the same murine strain tend to express similar repertoires. Many studies have led to the conclusion that the B cell clonotype repertoire of a given mouse strain as well as the individual mice is highly restricted during late fetal and early neonatal development, and is acquired in a highly patterned and reproducible fashion. Among the more recent findings are the elucidation of the molecular events involved in the diversity of B cell repertoire expression, the mechanisms by which each B cell obtains a single specificity, and the mechanisms that underlie the gradual

acquisition of B cell repertoire during neonatal development.

Molecular basis for repertoire expression

Diversity in the primary B cell repertoire can be generated at three levels: 1) combinatorial association of V_H , D_{H1} and J_{H1} and of V_L and J_L gene segments; 2) junctional diversity due to imprecise joining, P- or N-nucleotide additions; and 3) combination of heavy and light chains. Given all these sources of diversity, the potential for variable region diversity in the primary B cell repertoire of mice can be estimated to be at least 10^7 – 10^8 specificities. Although antibody specificity of a given B cell is determined by the genome of that cell, random somatic events as well as evolutionarily selective forces contribute to V region expression. Among those processes that exemplify stochastic somatic events is somatic mutation. Somatic mutations can occur throughout the V region but antigen selection appears to favor those mutations leading to amino acid replacements in the hypervariable regions. The studies of Berek, Milstein, Manser, Rajewsky and Siekevitz suggest that although the process of somatic mutation which occurs during the generation of a secondary immune response is responsible for an almost limitless number of new selectable specificities, it plays no major role in the diversification of the primary B cell repertoire.

Junctional diversity appears to be less random than originally thought. This is supported by the reproducible occurrence of major clonotypes in all mice of a given strain, such as the cross-reactive idiotype (CRI) found in the A strain of mice after immunization with arsonate (ARS). In addition, junctional diversity may be greatly influenced by particular D region haplotypes.

V gene segment combinations and selection are not simply a random assortment of functional V gene elements. For example, the occurrence of predominant clonotypes, such as T15, is due to a disproportionate selection of variable region gene segments from within the V region pool. In addition, Press, Fernandez, Cancro, Denis, Fung, Kearney, Klinman and Teale have shown that repertoire expression during late fetal and early neonatal B cell development is severely restricted, representing less than 1% of the total potential repertoire of an adult individual. Repertoire expression in neonates is also reproducibly and sequentially expressed among individuals of the same strain, such that the V_H region gene segments utilized during early development are clustered toward the D proximal end of the V_{H1} gene segment

complex. Reproducible differences in repertoire are found not only in neonates versus adults but, as Zharhary, Riley and others have shown, also in aged individuals. Together these findings may be interpreted as the consequence of an evolutionarily predetermined repertoire acquisition. A V_H gene may be over-represented due to a number of possibilities, including chromosomal location, copy number and accessibility to recombinases, as well as through environmental influences such as positive or negative selection.

Environmental influences on repertoire expression

Clonal selection begins to exert its influence on the B cell at the time of surface heavy chain acquisition in B cell development. Recently, several stages of pro- and pre-B cell development have been defined and in some of these stages cell division and potentially selection may occur. Following precedents in the T cell lineage, Schwartz and Stollar have suggested that the immature μ^+/κ^- or μ^+/λ^- B cell dies if its surface μ variable region binds with high affinity to a self antigen. At the μ^+/κ^+ or μ^+/λ^+ stage of differentiation, it is possible that a B cell with a high-affinity antiself receptor could avoid deletion by undergoing a second V_L (or V_H) gene rearrangement ('receptor editing'). The ability of unmutated natural autoantibodies to bind both to autoantigens and bacterial antigens raises the possibility that, in adults, pre-B cells could be selected positively or negatively by either self antigens or microbial antigens.

The expressed B cell repertoire appears to arise from the interplay of environmental influences acting upon an extremely diverse repertoire of clonally distributed specificities that are determined by the aforementioned molecular events. The environment may up- or downregulate the pre-existing specificities. To assess the effects of environmental regulation on B cell repertoire expression, the repertoire expressed in the mature B cell population is compared to that in cells directly derived from the slg^- population of bone marrow cells because these developing B cells cannot be influenced by antigenic or anti-idiotypic mechanisms requiring slg interactions. Upon comparison, the frequency of newly generated bone marrow B cell responses to various antigens (such as BALB/c responses to 2,4-dinitrophenyl (DNP), (3-hydroxy-4-nitrophenyl) acetyl (NP), phosphorylcholine (PC), α 1,3 dextran (Dex), and influenza A/PR/8 hemagglutinin (HA)) appears to be the same as that of mature B cell responses. Importantly, examination of B cell responsiveness to HA variants revealed similar reactivity patterns in the two B cell populations.

Thus, the environment does not appear to impact upon the diversification of the primary B cell repertoire.

Similarly, environmental influences appear to play little or no role in the establishment of the predominant clonotypes or clonotype families expressed in the primary B cell repertoire, e.g. the T15⁺ clonotype in PC responses, the λ -bearing antibodies to α 1,3-Dex in Igh^a mice and the λ NP^b responses. The frequency with which these clonotypes are expressed in the spleen is entirely reflective of the frequency with which they emanate from the generative cell pool. Therefore, environmental upregulation cannot explain the occurrence of dominant clonotypes.

The major environmental influences that affect primary B cell repertoire expression are through clonotype elimination during B cell maturation. During development, B cells pass through a phase during which they are susceptible to inactivation. Although the mechanisms for the downregulation of particular clonotypes are believed to involve tolerance induction and anti-idiotypic regulation, formal proof of the latter's existence *in vivo* awaits. Nonetheless, substantive evidence exists for the downregulation of the expression of certain B cell clonotypes. Examples of downregulation include responses to antigens such as murine cytochrome *c* (cyt *c*), PC, NP and α 1,3-Dex.

Experimentally induced anti-idiotypic suppression has been shown by Kearney, Kelsoe, Kohler, Accolla, Cerny and others to affect the expression of the primary B cell repertoire. Among the more convincing data for anti-idiotypic immunoregulation is the finding by Froscher and Klinman examining the λ -bearing, α 1,3-Dex specific response. The representation of this phenotype in Igh^b mice is low compared with that in Igh^a mice, and the responsiveness in the precursor versus mature B cell pool appears normal. Interestingly, if the Igh^b sIg⁻ B cell precursors are allowed to mature and respond in an allogeneic environment, the frequency of responsiveness is significantly increased. Because of the Igh haplotype specificity of this phenomenon, the elimination of the λ -bearing, α 1,3-Dex-specific B cells in Igh^b mice is thought to be the result of naturally occurring idiotype specific downregulation.

In some of the aforementioned antigen systems, clonotype elimination during B cell development is presumed to occur by tolerance induction, although the responsible self antigen has not been definitively identified. Teale, Klinman, Metcalf, Riley, Nossal, Chiller and others have experimentally induced B cell tolerance. Recently, using immunoglobulin transgenic mice, Nemazee, Weigert, Goodnow and others have demonstrated B cell tolerance *in vivo*. The toler-

ance trigger has been demonstrated as an active process requiring metabolic energy and protein synthesis by the B cell as well as surface receptor interlinkage in the form of multivalent antigen or multivalent antigen presentation. In addition, the affinity threshold to tolerize B cells as they mature through the tolerance gauntlet is higher than that required to trigger these cells once they have fully matured. Thus, B cells whose affinity for self antigen are low may escape tolerance induction; once fully mature their affinity could be sufficient to permit triggering. This phenomenon could serve to preserve repertoire diversity but at the expense of the persistence of B cells in the primary repertoire with low affinity antiself reactivities.

Repertoire expression in B cell subpopulations

The secondary B cell repertoire can be divided into three broad categories. The first category contains those secondary responses whose predominant clonotype or clonotype families are the same as those found in the primary response (i.e. total overlap in V gene usage), although the secondary antibodies have accumulated somatic mutations. Examples of these responses include the T15 clonotype, the λ -bearing antibodies to α 1,3-Dex and to some extent the λ NP^b clonotype. In the last example, the secondary response to NP in Igh^b mice is ultimately dominated by other clonotype families. The second category includes those responses whose specificities constitute a minority of the primary response but dominate the secondary response. Examples include the response to PR8-HA and the CRI in response to ARS in strain A mice. This phenotype can be explained by the preferential selection by antigen of those somatic mutants which could increase affinity. Finally, responses to 2-phenyl-5-oxazalone (phOX) by BALB/c mice, NP by C57BL/6, horse cyt *c* and *Salmonella typhimurium* by BALB/c are examples which display almost total disparities between primary and secondary responses. One possible explanation is that secondary B cells and their progenitors express variable region gene combinations that are not used by primary B cells and their precursors.

The secondary B cell repertoire, unlike the primary B cell repertoire, appears to be highly dependent upon environmental influences for its generation and regulation. Since antigenic stimulation and continued antigen selection is necessary for the establishment of secondary B cells, the secondary repertoire of individual mice within a strain can vary considerably, as seen with responses to PR8-HA and tobacco mosaic

virus protein. Although still controversial, Linton and Klinman have shown the splenic progenitors to memory B cells to be separable from the precursors to primary AFCs. These secondary B cell progenitors are stimulated by lower affinity interactions than those that are required for stimulation of precursors to primary AFCs. Moreover, as shown by Linton and Klinman *in vitro*, and later by Nossal and Goodnow *in vivo*, the progenitors to memory B cells pass through a tolerance susceptible phase. *In toto*, secondary B cell specificity appears to be derived from a broad spectrum of potentially responsive cells which are stimulated in a much less specific fashion. These specificities can somatically mutate and continue to be selected upon positively by antigen or negatively through tolerance induction of antiself reactivities.

Another small subpopulation of murine B cells has been defined by their expression of the cell surface marker Ly-1 (CD5 in humans). Because the names applied to this subset are misleading (since CD5 clearly can be induced on 'conventional' B cells and there is a subset of cells in mice that shares many features with Ly-1 B cells (i.e. 'sister' population)), these cells are now referred to as B1 cells. The B1 cells show a variety of novel features: a novel anatomical localization (enrichment in the peritoneal cavity); early appearance in ontogeny; secretion of particular autoreactive antibodies (notably antibody to a cryptic determinant on mouse red blood cells revealed by proteolytic treatment with the enzyme bromelain (BrMRBC)); extensive capacity for self-renewal; increased frequency in autoimmune mouse strains; repetitive usage of particular germline immunoglobulin variable genes; and an apparent predisposition for unregulated growth.

Since B1 cells represent a sizable portion of neonatal splenic B cells, and fetal/neonatal-derived B cells have been shown to lack non-templated nucleotide (N) region additions found at the VDJ junctions, it was thought that all B1 cells lacked N additions. This restriction in repertoire size has been attributed to terminal deoxynucleotidyl transferase, TdT, the enzyme which adds N nucleotides and becomes active during the first week after birth. A comparison of heavy chain sequences from B1 cells isolated at different stages of development revealed some N additions in the B1 cells of the adult population. The increased N-region diversity is found in the 'sister' population of B1 cells.

Since B1 cells develop early in ontogeny, repertoire differences may, at least in part, reflect selection by different endogenous antigens. For instance, the enrichment of B1 cells with N additions in adults

may be the consequence of a selective advantage. In addition, analysis of cells responding to BrMRBC has shown that the antigen receptor is encoded by novel V_H genes (V_H11 and V_H12), and the significant increased frequency of V_H11 usage in B1 cells is largely restricted to V_H11-J_H1 rearrangements. There appears to be no general overuse of particular V genes, rather such genes are used in distinctive V_H-V_L combinations to encode selected autoreactive antibodies. Moreover, B1 cells show a preference for using J_H1 gene segments which is not observed in the conventional B cell population, and while most murine pre-B and B cells preferentially use the DSP and DFL D_H gene segments in a given reading frame (RF1), cells in the 'sister' population frequently express D_H genes in another reading frame (RF2).

See also: Affinity maturation; Antibodies, specificity; B lymphocyte differentiation; CD5; Clonal selection; Diversity, generation of; Hybridomas, B cell; Idiotype network; Immunoglobulin genes; Maturation of immune responses; Memory, immunological; Neonatal immune response; Polyclonal activators; Somatic mutation; Tolerance, peripheral.

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B LYMPHOCYTES

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B lymphocytes are the antibody-forming cells of the immune system. B cells were so named in 1965, when they were shown to develop in the bursa of Fabricius (an outpouching of the gut) of chickens and were distinguished from T cells, which develop in the thymus. Physical separation allowed identification of separate functions of B cells (antibody production) and T cells (cell-mediated responses and regulatory processes). Mammals do not have a bursa of Fabricius, but their B cells arise in the bone marrow, so the original initial-based name is still applicable.

B lymphocytes are found in blood, lymph nodes, spleen and tonsil and other mucosal tissues. About 5–25% of all human blood lymphocytes, which number 1000–2000 cells mm³, are B lymphocytes. B cells comprise a majority of the bone marrow lymphocytes, one-third to one-half of lymph node and spleen lymphocytes, but less than 1% of those in thymus. Nonactivated B cells circulate through lymph nodes and spleen, in which they are clustered in follicles and marginal zones around the follicles. Circulating B cells may interact with and be activated by T cells at extrafollicular sites, where T cells occur around small blood vessels in association with antigen-presenting dendritic cells. Appropriately activated B cells enter the follicles, where they proliferate and displace resting cells, forming germinal centers. There they differentiate into plasma cells specialized for antibody production and long-lived memory B cells.

Each B cell produces many copies of its unique antibody molecule, which it may anchor on its cell membrane or secrete into tissue fluid and blood. The membrane antibodies form a major part of antigen-binding receptors, which may initiate cell proliferation and antibody production or processing of antigen for presentation to T cells. Secreted antibodies bind antigen in blood or tissues and initiate reactions that lead to elimination of antigen from the body. Thus B lymphocytes function both in afferent and effector phases of antibody-producing immune responses.

Intracellular features of B cells

A defining feature of B lymphocytes is that they are the only cells that rearrange chromosomal DNA in the loci that code for antibodies (immunoglobulins). Other intracellular features help to identify B cells

but are not as exclusively characteristic as is gene rearrangement. Proteins associated with the rearrangement include the RAG-1 and RAG-2 recombination proteins and the enzyme terminal deoxynucleotidyl transferase (TdT). Proteins related to immunoglobulin gene expression include the transcription factors Oct-2, dimeric E2A, Bob-1 and Pu.1. Certain protein kinases that mediate signals from the antigen receptor are also characteristic of B cells.

Cell surface features of B cells

The unique B cell surface feature is the antigen receptor, formed in part by membrane immunoglobulin. Receptor assemblies of newly formed mature B cells contain immunoglobulin M (IgM) and/or IgD. B cells appropriately stimulated by interaction with T helper cells and their interleukin products may switch to production of other Ig classes, and the B cell antigen receptors then contain IgG, IgA or IgE, but not IgM or IgD. The H chain of receptor immunoglobulin is anchored in the membrane, in association with proteins Ig- α (CD79a) and Ig- β (CD79b). The intracellular domains of Ig- α and Ig- β participate in generating a receptor-mediated signal within the cell, transmitted through activation of protein kinases. Lyn (Src family) and Syk (Syk/Zap70 family) are protein kinases that associate with the resting B cell receptor and, along with additional kinases, participate in B cell activation pathways.

Other B cell membrane proteins regulate the intensity of responses that follow antigen binding (Table 1). Cross-linking of a B cell-specific complement-binding assembly (proteins CD19, CD21, CD81 and Leu-13) to the antigen receptor dramatically lowers the antigen concentration required for B cell activation. CD45, a positive regulator (perhaps an initiator) of signaling, is a transmembrane protein with a cytoplasmic tail that has essential phosphatase activity. Other phosphatases (e.g. SHP-1) are attenuating, and some membrane molecules (e.g. CD22, Fc γ R) increase the effect of attenuating phosphatases.

Additional B cell surface molecules have diverse functions, serving to present processed antigen fragments and costimulatory signals to T cells, mediate adhesion between B and T cells, or as receptors for cytokines produced by T cells (Table 2). B cell activation and class switching can be stimulated through

Table 1 B cell receptors and associated membrane proteins

<i>Receptor complex</i>	<i>Protein</i>	<i>Family</i>	<i>Function</i>
B cell antigen receptor (BCR)	IgM, IgD, IgG, IgA or IgE	Ig	Receptor-mediated signaling
	Ig- α (CD79a)	Ig	Receptor-mediated signaling
	Ig- β , Ig- γ (CD79b)	Ig	Receptor-mediated signaling
Pre-B cell receptor	Ig- μ H chain	Ig	Signaling for maturation progression
	Vpre-B	Ig	Component of surrogate L chain
	14.1 (human) λ 5 (mouse)	Ig Ig	Component of surrogate L chain
Complement receptor complex	CD19	Ig	Costimulates BCR signaling when cross-linked to BCR. Forms a complex with CD21, TAPA-1 and Leu-13
	CD21	Selectin, C-R	EBV receptor gp350/220; complement receptor, binds iC3b, CD23, IFN α
	TAPA-1 (CD81) Leu-13	Tetraspan 16 kDa membrane protein	
Modulators of receptor signaling	CD45	Tyr phosphatase	Increases BCR signaling
	CD22 (CR2)	Ig	Inhibits BCR signaling; mediates adhesion; binds to SHP-1, Syk, PLC γ 1
	Fc γ RII	Ig	Low-affinity IgG receptor
	CD23	Lectin	Inhibits BCR signaling Low-affinity Fc ϵ R2; regulates IgE production

B cell CD40, a receptor for a ligand, CD40L (also called gp39), produced by T cells. B cell apoptosis follows engagement of the cell surface protein Fas (CD95) by specific Fas ligand, which is produced by activated T cells, dendritic cells and cells of many tissues in which apoptosis occurs. Some of these surface proteins, such as CD19, are nearly unique to B cells; others, such as major histocompatibility complex (MHC) class II molecules and Fas, occur on cells of several tissues.

B cell development

B cells are generated from hematopoietic stem cells. In birds, new B cells are produced in the bursa of Fabricius in both embryonic and adult life. In mammals, B lymphocytes are produced mainly in liver and spleen in fetal life, but in bone marrow after birth. Multipotential hematopoietic stem cells can develop into lymphoid cells or myeloid cells (granulocytes, erythrocytes and platelets). Along the lymphocyte differentiation pathway there is an intermediate lymphoid precursor for both B and T cells. The earliest precursors committed only to B cell development, pro-B cells, have not yet rearranged their V region genes, but can be identified by their

ability to grow in culture in the presence of bone marrow stromal cells, kit-ligand and IL-7; their cell surface CD19 and CD43; and their intracellular terminal deoxynucleotidyl transferase, RAG-1 and RAG-2. Somewhat more differentiated (pre-B I) cells have begun to rearrange H chain V region genes but do not synthesize H chain protein. When rearrangement of H chain but not L chain gene segments is completed, developing cells synthesize and express μ H chain on the cell surface in association with a surrogate L chain (Table 1). Membrane expression of this complex is essential for progression from the pre-B I to the pre-B II stage, at which L chain rearrangement begins. L chain synthesis and Ig assembly occur in immature B cells. The unmodified term 'B cell' is applied to small lymphocytes that have completed V gene rearrangements, synthesize both chains and express cell surface Ig. Most B cells express only one rearranged H and one L chain; this process, known as allelic exclusion, ensures that a B cell will make only one antibody. The progressive stages of V gene rearrangement are accompanied by characteristic expression of cell surface proteins that are also used as markers of these stages.

At birth, before exposure to foreign antigens, new IgM⁺IgD⁺ B cells comprise a 'primary repertoire' of

Table 2 B cell membrane proteins that interact with T lymphocytes and cytokines produced by T lymphocytes

<i>B cell protein</i>	<i>Nature/family</i>	<i>Interacting T cell surface molecule</i>	<i>Function</i>
MHC class II	Ig	TCR, CD4	Presentation of processed antigen to TCR
B7-1 (CD80) and B7-2 (CD86)	Ig Ig	CD28, CTL4	Costimulatory signal in antigen presentation to TCR
CD40	TNFR	CD40L (gp39)	Germinal follicle formation, class switching
ICAM-1	Ig	LFA-1	Adhesion
LFA-1	Integrin	ICAM-1	Adhesion
LFA-3	Ig	CD2	Adhesion
Fas (CD95)	TNFR	FasL	Apoptosis signal
		<i>Soluble T cell product</i>	<i>Effect on B cells</i>
IL-2R	HCR type I	IL-2	Growth, activation, Ig secretion
TNF α R	TNFR family	TNF α	Costimulation of proliferation, Ig secretion
IFN γ R	HCR type II	IFN γ	Class switch to IgG2a; enhanced MHC class II expression
IL-4R	HCR type I	IL-4	Class switch to IgE, IgG1; stimulation of MHC class II and CD23 expression
IL-5R	HCR type I	IL-5	Enhanced growth, differentiation, Ig secretion by activated B cells
IL-6R	HCR type I	IL-6	Growth, differentiation, Ig secretion
IL-10R	Related to IFN receptors	IL-10	Increased MHC class II expression (mouse); proliferation, differentiation, Ig secretion (human)
TGF β R	Protein kinase	TGF β	Inhibition of growth; class switch to IgA, IgG2b

HCR, hematopoietic cytokine receptor; ICAM, intercellular adhesion molecule; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LFA, leukocyte function antigen; MHC, major histocompatibility complex; TCR, T cell receptor; TGF, transforming growth factor; TNF, tumor necrosis factor.

cells producing diverse Ig products. Diversity of the primary repertoire is generated in various ways: rearrangement of numerous Ig V gene segments without somatic mutations (fetal mice); similar rearrangements plus the addition of untemplated nucleotides at the junction of H gene segments (humans, post-natal mice); gene conversion with a smaller number of functional genes (rabbit, chicken); or mutation of functional genes (sheep ileal Peyer's patches). By the time the B cells emerge from the bursa or bone marrow, or shortly thereafter, the great potential diversity has been narrowed by elimination of cells making strongly autoreactive antibodies and by the short lifespan of cells that encounter no antigen. About 1.5×10^7 B cells are produced in adult mouse bone

marrow daily; 10–15% of them survive to enter the total B cell pool, which totals about 5×10^8 cells. A significant fraction of neonatal IgM reacts both with bacterial antigens and, weakly, with autoantigens, as do IgM products of newly formed B cells through adult life. Normal regulatory processes, however, prevent the expansion of B cells that would produce large amounts of high-affinity autoantibodies of other Ig classes. When that regulation fails, autoantibody production is often associated with autoimmune disease.

In humans and mice a small subset of B cells expresses CD5 (Ly-1), a protein present on all T cells. CD5⁺ B cells are prominent in fetal and neonatal mice, in the peritoneum of adult mice, and

among human fetal B cells and adult B cells that make natural autoantibodies. CD5 expression may be a reflection of how antigen stimulation occurs or a marker for a particular B cell lineage.

B cell activation

When activated by either T-independent antigens or by interactions with T helper cells, small resting B cells are converted to large lymphoblasts and then either to plasma cells, specialized for antibody production, or long-lasting small memory cells. Within germinal centers, B cells are converted to large replicating centroblasts and then to nonreplicating centrocytes. Additional intermediate stages in the activation process are defined by cell surface protein expression and localization within the germinal center. Striking features of the germinal center activation process are the generation of frequent mutations in the Ig V regions and the switch from IgM to IgG, IgA or IgE production. Mutation greatly increases the diversity of antigen-binding sites. If mutation eliminates antigen binding, the cell has a short life, dying by apoptosis. The few cells in which mutation yields an Ig product with high affinity for the antigen are selected for survival. Antigen-selected cells may differentiate terminally into plasma cells, which then secrete a large amount of the antibody, or into small, long-lived, memory B cells that are distributed in blood and lymphoid tissues. These cells no longer express surface IgD.

B cell deficiency diseases

The physiological importance of B cell function is revealed by diseases that result from selective B cell deficiencies and consequent lack of antibodies (agammaglobulinemia). Bruton's agammaglobulinemia (XLA) is an X-linked defect in B cell maturation in humans, with arrest at the Pre-B I stage and a resulting deficiency in all immunoglobulin classes. A corresponding B cell maturation defect, the Xid mutation, also occurs in CBA/N mice. In male children with XLA, the maturation block results from deficiency of a B cell-specific protein tyrosine kinase, *btk*. With a profound lack of mature B cells but normal T cells, these children are particularly susceptible to infections by bacteria, mycoplasma, hepatitis virus and enteroviruses. They have recurrent middle ear infection, pneumonia, sinusitis and tonsillitis caused by *Pneumococcus*, *Streptococcus* and *Hemophilus*. Problems with infection begin several months after birth, when the pool of protective maternal antibody decreases. XLA children are not unusually susceptible to most virus or fungal infections, for which T

cell responses provide sufficient protection. In contrast, subjects with profound defects of both B cells and T cells (severe combined immunodeficiency, SCID), are threatened by all infectious agents; they survive only in a germ-free environment unless the deficiency can be treated.

Immunodeficiency with elevated IgM but very low IgG or IgA levels results from a failure in the signaling that instructs B cells to switch from production of IgM to other classes of Ig. The signals come in part from T cells, and in most subjects the basic defect is in the T cell even though it is expressed through B cell dysfunction. An X-linked type of this disease is due to a mutation in the gene for CD40L, the T cell ligand for B cell CD40. High levels of IgM in this disease are associated with autoimmunity. A non-X-linked immunodeficiency, in which there is a selective lack of IgA-producing B cells, leads to bacterial infections primarily in the respiratory, gastrointestinal and urogenital tracts.

B cell malignancies

B cells may undergo malignant transformation at various stages of development or activation. Some human acute lymphocytic leukemia cells express features of early B cells, such as expression of TdT or only cytoplasmic H chain. Infection of mouse B cell precursors with Abelson virus generates a variety of leukemic B cells frozen in development at the pre-B cell stage. Epstein-Barr virus (EBV) activates human B cells to become lymphoblasts and, occasionally, to undergo transformation to malignancy. Mature B cells occur in some acute lymphocytic leukemias and in B cell lymphomas, hairy cell leukemia, and chronic lymphocytic leukemia. Some leukemic B cells produce IgM with properties of natural autoantibodies.

Multiple myeloma is a malignant clonal expansion of a differentiated plasma cell, with production of a large amount of a single Ig molecule or Ig chain (myeloma protein). Some spontaneous myeloma proteins have identifiable antigen-binding activity, and analyses of myeloma proteins provided important keys to understanding antibody structure. Mutated myeloma cells of mice also serve as partners for fusion with specific antibody-producing cells, forming hybridomas that produce monoclonal antibodies. Overproducing cells that lack malignant properties but yield a large amount of Ig product occur in Waldenström's macroglobulinemia and primary amyloidosis.

See also: Antibodies, synthesis; Antigen presentation via MHC class II molecules; Autoimmunity; B lymphocyte activation; B lymphocyte, antigen processing

and presentation; B lymphocyte repertoire; B lymphocyte differentiation; Bruton's agammaglobulinemia; CD antigens; CD5; CD22; CD40 and its ligand; CD45 (the leukocyte common antigen); Cell surface molecules, immunoprecipitation of; Clonal selection; Cytokines; Dendritic cells; Diversity, generation of; Epstein-Barr virus, infection and immunity; Fas (CD95) and fas ligand; Fc receptors; Gene conversion; Germinal center; Heavy chain diseases; Humoral immunity; Hybridomas, B cell; Hypergammaglobulinemia; Hyper-IgM syndrome; Immunodeficiency, primary; Immunoglobulin genes; Leukemia; Lymphocytes; Mucosal immunity; Natural antibodies; Polyclonal activators; Second signals for lymphocyte activation; Somatic mutation; Tolerance, peripheral.

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β_2 -MICROGLOBULIN

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β_2 -Microglobulin (β_2 M) is a protein of approximately 12 kDa and is found in serum and on the surface of most mammalian cells. It was first isolated from the urine of patients whose renal tubules were damaged by chronic cadmium poisoning. The protein is referred to as β_2 -microglobulin because of its low molecular weight and its localization in the β_2 region of globulins when blood plasma is subject to moving-boundary electrophoresis.

β_2 M is noncovalently associated with several transmembrane proteins, including the heavy chain of class I major histocompatibility complex (MHC) proteins encoded by the mouse H-2 K, D and L and Qa/Tla loci and the human HLA-A, -B, and -C loci. β_2 M is also found in noncovalent association with an immunoglobulin G (IgG) receptor (Fc receptor) of neonatal gut cells and with CD1 glycoproteins. β_2 M is encoded by a single gene on human chromosome 15 and mouse chromosome 2.

Biochemical nature and evolution

β_2 M consists of a single, nonglycosylated, 99 amino acid polypeptide chain. Homologs of β_2 M have been described and sequenced for a number of mammalian species. Comparison of amino acid sequences indicates a high degree of evolutionary conservation of this protein. The overall sequence identity among the various mammalian β_2 Ms is approximately 70%, except for mouse and rat β_2 M, which, as might be expected, show greater identity (86%). The high degree of structural conservation of β_2 M among species is further indicated by the observation that β_2 M of different species can functionally substitute for each other. Since β_2 M is located entirely outside the cell with no direct attachment to the cell membrane, and since the association between β_2 M and the class I heavy chain is noncovalent, β_2 M on the cell surface can exchange with free β_2 M present in serum used to grow cultured cells. Mammalian β_2 M

contains two cysteine residues at positions 25 and 80, and these are involved in a disulfide bond. Because of sequence similarity of β_2 M to Ig molecules and because of the presence of a disulfide loop with a predicted tertiary structure similar to that of Ig domains, β_2 M has been considered a member of the Ig gene superfamily. The primary structural identity between β_2 M and the Ig constant region domain is about 30%. The similarity between them is more significant considering matches of specific key amino acids. The placement of β_2 M in the Ig gene superfamily has been confirmed by crystal structure studies discussed below. The heavy chain of class I MHC proteins is also a member of Ig superfamily.

Polymorphism

Within a species β_2 M is virtually invariant. Although a few variants have been found in many species, only in the mouse could the difference be attributed to a change in an encoded amino acid. In guinea pig, the structural difference between two variants has been shown to involve the presence or absence of a lysine residue at the C-terminus of the molecule, and it is postulated that this variation results from post-translational modification, but not from genetic polymorphism. Three alleles of β_2 M have been defined among laboratory strains of mice. Amino acid sequence analysis of the β_2 M protein obtained from Balb/c (β_2 M^a) and C57BL/6 (β_2 M^b) mouse strains revealed a substitution of aspartic acid for alanine at position 85 as the only difference between the two forms. A third variant has been identified by sequence analysis of cDNA from the mouse strain B10.pa-H-3^a/Sn. All three alleles among laboratory strains differ by a single base substitution at exactly the same base position: the second nucleotide of codon 85. β_2 M^a (e.g. Balb/c) has GCC (alanine), β_2 M^b (C57BL/6) GAC (aspartic acid) and β_2 M^c (B10.pa-H-3^a/Sn) GTC (valine).

Structure of β_2 M

Primary sequence comparisons and physicochemical data suggested that β_2 M strongly resembles Ig constant domains in polypeptide chain folding and overall tertiary structure. The structure of β_2 M in the class I heterodimeric protein was also shown to be very similar to that determined for the free β_2 M monomer from bovine serum. The crystal structure of the human class I MHC protein HLA-A2 was determined, and these data confirmed the expectation that β_2 M is very similar in tertiary structure to the $\alpha 3$ domain of class I heavy chains and to Ig

constant domains (Figure 1). Both the $\alpha 3$ and β_2 M domains in the class I MHC molecule are β -sandwich structures composed of two antiparallel β -pleated sheets, one with four β strands and one with three β strands, connected by a disulfide bond. The residues in contact across the $\alpha 3$ - β_2 M interface are in the four-stranded β sheets. The crystal structure also shows how β_2 M binds to the heavy chain of class I protein; β_2 M is positioned upward apparently supporting the $\alpha 1/\alpha 2$ peptide-binding platform (Figure 1A). This interaction results in minimal contact between the heavy chain $\alpha 3$ domain and $\alpha 1/\alpha 2$ domains, while β_2 M contacts all three domains of the heavy chain. The interaction between β_2 M and the $\alpha 1/\alpha 2$ domains extends across the entire β -pleated platform and one long stretch of β_2 M fits into a pleat on the underside of the $\alpha 1/\alpha 2$ β -pleated sheet (Figure 1C). These interactions appear to stabilize the $\alpha 1/\alpha 2$ conformation and therefore the peptide binding site on the class I protein.

Expression of β_2 M

Mouse β_2 M is encoded by a single gene per haploid genome, indicating that the same β_2 M gene encodes the light chain of the various class I proteins, CD1 and intestinal Fc receptors. The β_2 M gene consists of four exons, with the majority of the protein-coding sequence (amino acids 3–95), within a single exon (exon II). All types of interferon (IFN) (α , β and γ), of which IFN γ is usually the most potent, and tumor necrosis factor α (TNF α) have been shown to induce or increase expression of both chains of class I molecules in a wide variety of cell types. For β_2 M this is thought to occur by both transcriptional and post-transcriptional events. The expression of the β_2 M gene has been thought to be related to that of the class I heavy chain by sharing common regulatory mechanisms. However, the expression of these genes follows a different time course during embryogenesis; the synthesis of β_2 M is first detectable at the two-cell stage, while class I heavy chain mRNA is detectable after the midsomite stage on gestation day 9 in the mouse embryo. This suggests that expression of these genes is not always coordinately regulated. It has been shown in some embryonic carcinoma cell lines that expression of class I MHC proteins can be induced by driving the cells from an undifferentiated state to a differentiated state, and that transcription of both the β_2 M and class I heavy chain genes is activated by this induction. A transcriptional enhancer activity has been found in the 5' flanking sequence of the mouse β_2 M gene. This enhancer may have at least two important regions, one of which shows sequence similarity to the H-2 enhancer (class I regu-

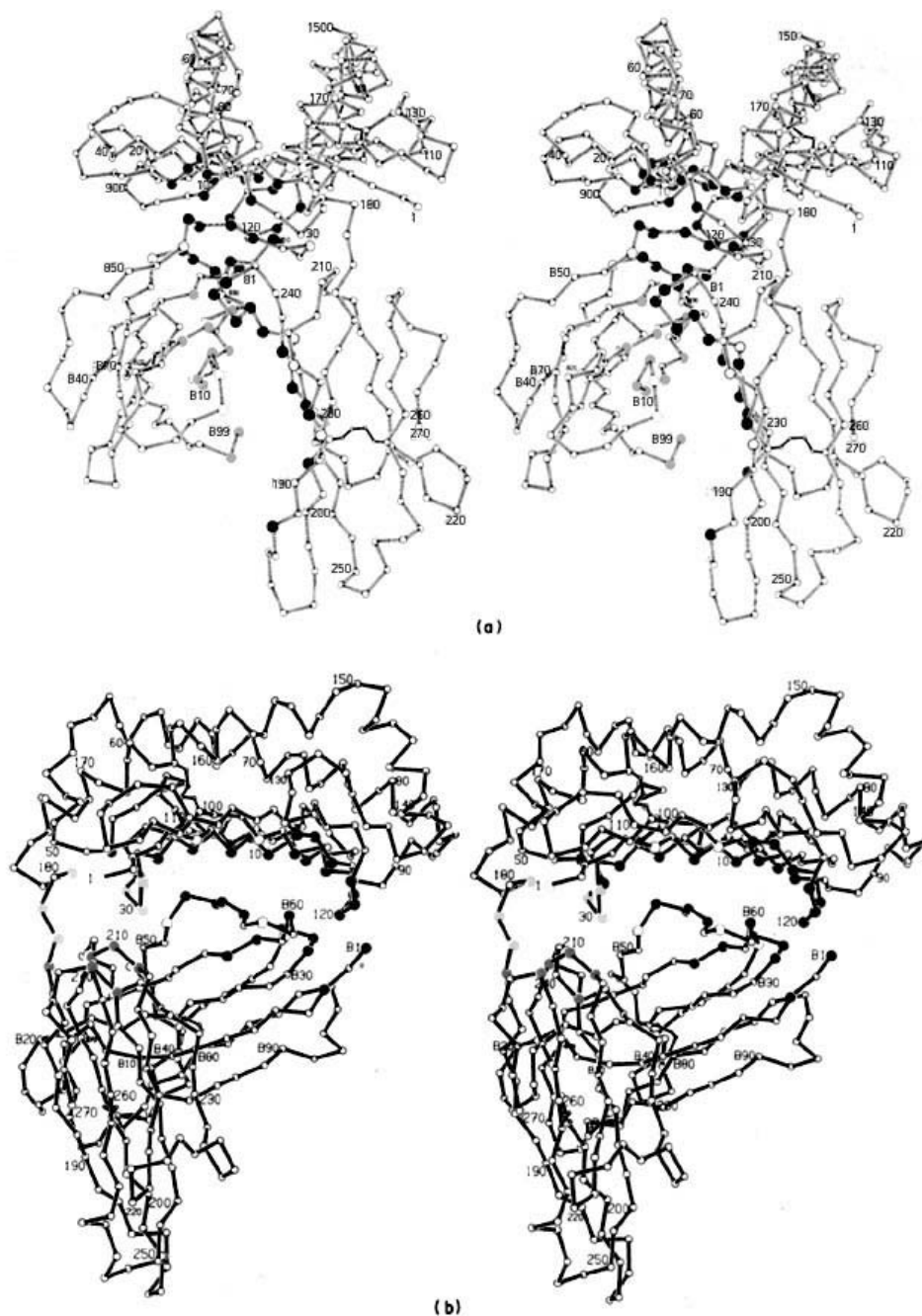


Figure 1 The residues forming the domain interfaces are highlighted in color on the C α backbone stereogram. Filled and colored C α atoms make contacts ≤ 4 Å. Red, α_1 , α_2 residues in interface with β_2 M; green, β_2 M residues in interface with $\alpha_1\alpha_2$; blue α_3 residues in interface with β_2 M; yellow, β_2 M residues in interface α_3 ; pink, $\alpha_1\alpha_2$ residues in interface with α_3 ; orange, α_3 residues in interface with $\alpha_1\alpha_2$. (A) A view perpendicular to $\alpha_1\alpha_2$ pseudodyad with the binding cleft viewed end-on ($\alpha_1\alpha_2$ - α_3 interface not shown). (B) A side view with the molecule rotated 90° about the pseudodyad (α_3 - β_2 M interface not shown). (C) A drawing of the β_2 M residues (blue) interacting underneath the $\alpha_1\alpha_2$ β sheet. $\alpha_1\alpha_2$ Residues in β_2 M interface are in red; those in α_3 interface with small green labels. Hatched region highlights the pleat under which β_2 M side-chains Phe-B56 and Trp-B60 make van der Waals' contacts with $\alpha_1\alpha_2$. (Reprinted with permission from Saper MA, Bjorkman PJ and Wiley DC (1991) Refined structure of the human histocompatibility antigen HIA-A2 at 2.6 Å resolution. *Journal of Molecular Biology* 219: 277–319.) (See also color Plate 9.)

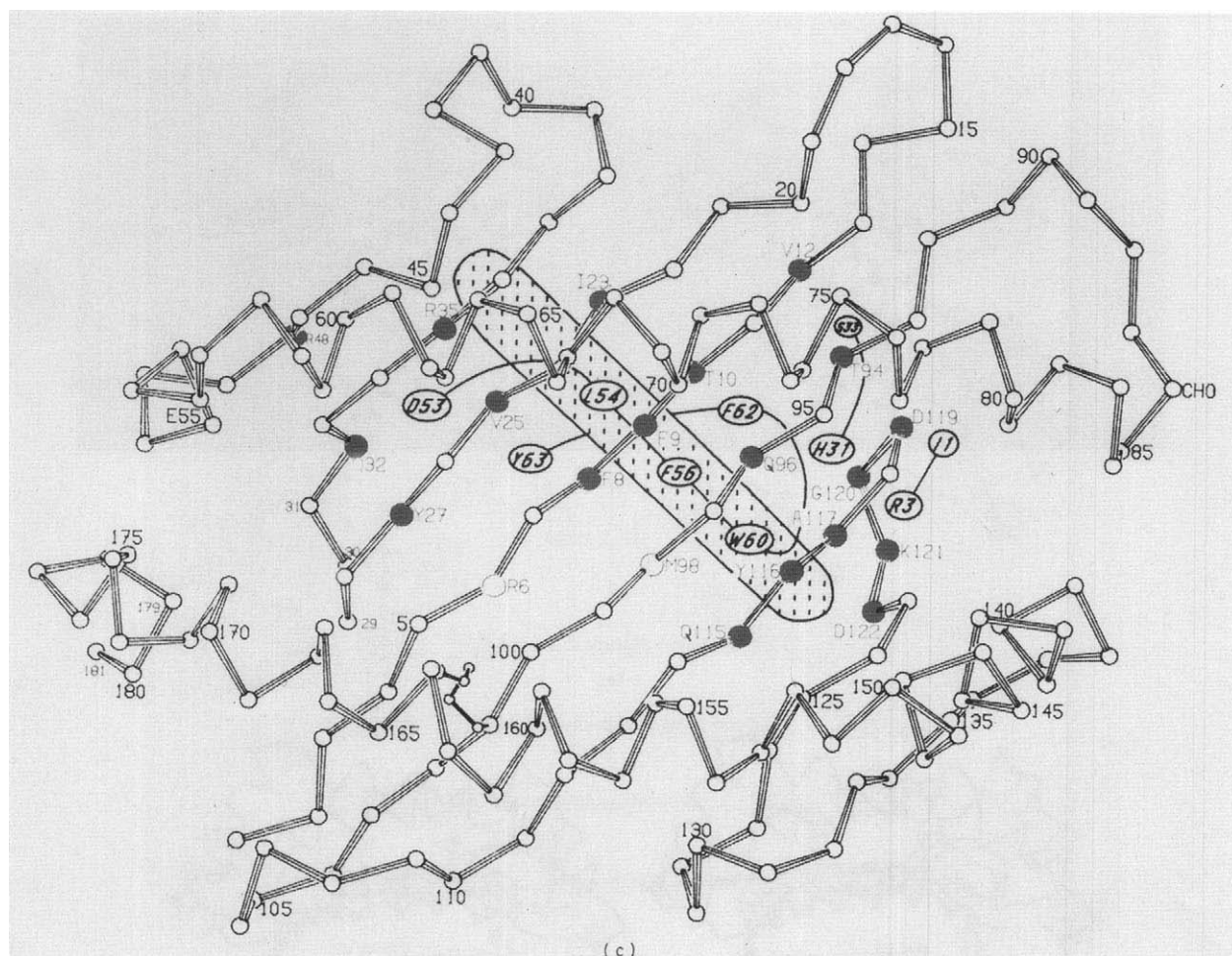


Figure 1 Continued.

latory element, CRE) region 1. Both this β_2 M sequence and region I of the H-2 enhancer bind the nuclear factors KBF1 and H2TF1.

Function of β_2 M

Although the precise role of β_2 M is not known, a number of studies suggest possible roles for it. It serves to stabilize the tertiary structure of class I heavy chains. Circular dichroism studies show that isolated heavy chains lose some of their β -pleated structure and assume more of a random coil configuration in the absence of β_2 M. Other studies have shown that when β_2 M is dissociated from the HLA heavy chain, HLA alloantisera are less able to recognize the protein, and antigenic activity can be restored if the heavy chain- β_2 M complex is carefully renatured. Cytoplasmic HLA molecules lack β_2 M in Daudi cells and are not recognized by alloantisera, but can be recognized by xenoantisera raised against denatured HLA heavy chains. In addition to stabiliz-

ing the tertiary structure of class I heavy chains, β_2 M seems to be required for the expression of these chains on the cell surface. This conclusion has been based on studies of the human Daudi cell line and of mutants of the mouse R1 cell line. In both systems mutations in the β_2 M gene are accompanied by lack of expression of β_2 M protein and lack of cell surface class I molecules. Cell surface expression of class I proteins was shown to be restored in each of these mutant cell lines after fusion to cells that express normal β_2 M protein. Furthermore, transfection of the β_2 M gene into these cell lines rescued cell surface expression of class I proteins. It has been shown that peptide binding is also required for the cell surface expression of class I proteins. Using a mutant cell line deficient in β_2 M which transports low amounts of nonpeptide-associated heavy chains to the cell surface, it has also been shown that only after association with β_2 M could heavy chain stably bind peptide. An approach using purified class I proteins also strongly suggested that peptide binding to yield

active antigenic class I MHC complexes can occur after β_2 M binding to heavy chain occurred. Thus, β_2 M association with the class I heavy chain is required for optimal peptide ligand binding to form the antigenic complex recognized by T cells. β_2 M-deficient mice were produced by disrupting the β_2 M gene by homologous recombination. In these mice, cell surface expression and function of the entire array of class I molecules were eliminated. Neonatal β_2 M-deficient mice also lacked the intestinal cell transepithelial transport of IgG from mother's milk by the β_2 M-associated Fc receptor, suggesting that the Fc receptor heavy chain in mouse must associate with β_2 M for functional expression on the cell surface. Furthermore, most CD4⁺CD8⁺ cytotoxic T cells failed to develop in these mice because of the lack of cell surface class I MHC proteins. Interestingly, the fact that these mice were born and appeared normal (except for the cellular alterations mentioned above and their consequences) demonstrates that no β_2 M-associated proteins are critical during mouse development.

See also: CD1; Fc receptors; H2 class I; HLA class I; immunoglobulin gene superfamily; Interferon α ; Interferon β ; Interferon γ ; MHC restriction; MHC, evolution of; MHC, functions of.

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bm MUTANTS

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Background

The H-2 complex (the major histocompatibility complex of the mouse) consists of a series of genes which are intimately involved in regulation of many functions of the immune system. Of particular relevance here are the class I and class II genes within the H-2 complex. The products of these highly polymorphic genes are particularly crucial to the various activities carried out by lymphocytes, particularly T lymphocytes. Mutations of these loci have been extremely

useful in dissecting the relationships between the fine structure and function of these genes/gene products. H-2 gene mutations have been recovered from many different genetic strains of mice, but the best known and most widely utilized have been those which occurred in the *H-2^b* haplotype (the set of H-2 genes found in strains such as C57BL/6 and C57BL/10). The mutants were typically identified because they resulted in unexpected graft rejections between animals presumed to be genetically identical, and therefore expected to be completely histocompatible.

H-2 mutants are designated by the letter of the haplotype from which they were derived (in this case, 'b'), the letter 'm' (for 'mutant') and an arabic numeral which designates the chronological sequence in which the mutants were found – thus the mutations of *H-2^b* are collectively known as the '*bm*' mutants. Prior to recent methods developed for site-directed mutagenesis, exon shuffling and transgene insertion, H-2 mutants provided the only available means for evaluating the effects of such small discrete changes in the class I and II genes expressed in the *H-2^b* haplotype. The current listing of mapped *bm* mutants is shown in Table 1.

Frequency and sources of *bm* mutants

The fact that *bm* mutants are more frequent than those derived from other haplotypes is not due to a more intensive screening of mice bearing *H-2^b*, but to the fact that one of its genes (*K^b*, the *H-2^b* allele at the *H-2K* locus) mutates spontaneously at the unusually high rate of approximately 2×10^{-4} per locus per gamete. As a result, over 80% of all H-2 mutants recovered are derived from the *K^b* gene.

One of the most interesting features of class I and class II H-2 genes is their high polymorphism, i.e. a large number of alleles per locus in the population, and it is assumed that this unusually high diversity is an important part of their biological role(s). The *bm* mutants display three characteristics which bear on the source of this variability: 1) the unusually high mutation rates, 1–2 orders of magnitude above those for average mammalian genes; 2) the recurrence of identical amino acid changes in several independently arising mutations; and 3) multiple (but not necessarily adjacent) amino acid changes resulting from single mutational events. It has been shown that these genes engage in exchanges of DNA sequences between homologous, but non-allelic, genes. This process (referred to as gene conversion, microconversion or microrecombination) can occur during either meiosis or mitosis, and the details of its mechanism are still unknown. Analysis of H-2 mutations derived from the *H-2^d*, *H-2^f* and *H-2^k* haplotypes indicate that these mutations can also be attributed to microrecombination, although their frequency is far below that of *K^b*.

Functional consequences of *bm* mutations

Class I H-2 molecules are crucial in directing the function of CD8⁺ cytotoxic T lymphocytes. As they progress toward the cell surface during their synthesis, they bind to peptide fragments, which they

ultimately 'display' on the cell surface. They thus present to CD8⁺ T lymphocytes a sampling of intracellular peptides, including those derived from infectious organisms growing within the cell.

The T cell receptors of each CD8⁺ T lymphocyte

Table 1 Known and mapped *bm* mutations

Locus	MHC class	Mutation	Type	Amino acid change ^a
H-2K	I	<i>H-2^{bm1}</i>	GL	152 Glu → Ala 154 Arg → Tyr 156 Leu → Tyr
		<i>H-2^{bm2}</i>	GL	Unknown
		<i>H-2^{bm3}</i>	GL	77 Asp → Ser 89 Lys → Glu
		<i>H-2^{bm4}</i>	GL	173 Lys → Glu 174 Asn → Leu
		<i>H-2^{bm5}</i>	GL	116 Tyr → Phe
		<i>H-2^{bm6}</i>	GL	116 Tyr → Phe 121 Cys → Arg
		<i>H-2^{bm7}</i>	GL	116 Tyr → Phe 121 Cys → Arg
		<i>H-2^{bm8}</i>	GL	22 Thr → Phe 23 Met → Ile 24 Glu → Ser 30 Asp → Asn
		<i>H-2^{bm9}</i>	GL	116 Tyr → Phe 121 Cys → Arg
		<i>H-2^{bm10}</i>	GL	163 Thr → Phe 165 Val → Met 173 Lys → Glu 174 Arg → Leu
		<i>H-2^{bm11}</i>	GL	77 Asp → Ser 80 Thr → Asn
		<i>H-2^{bm15}</i>	L	Unknown
		<i>H-2^{bm16}</i>	GL	116 Tyr → Phe
		<i>H-2^{bm17}</i>	GL	116 Tyr → Phe 121 Cys → Arg
		<i>H-2^{bm18}</i>	GL	Unknown
		<i>H-2^{bm19}</i>	GL	Unknown
		<i>H-2^{bm20}</i>	GL	Unknown
		<i>H-2^{bm21}</i>	GL	Unknown
		<i>H-2^{bm22}</i>	GL	Unknown
		<i>H-2^{bm23}</i>	GL	75 Arg → His 77 Asp → Ser
		<i>H-2^{bm29}</i>	GL	89 Lys → Arg
		<i>KB-98</i>	GL	116 Tyr → Phe
I-A _B	II	<i>H-2^{bm12}</i>	GL	67 Ile → Phe 70 Phe → Tyr 71 Thr → Lys
H-2D	I	<i>H-2^{bm13}</i>	GL	114 Leu → Gln 116 Phe → Tyr 118 Silent 119 Glu → Asp
		<i>H-2^{bm14}</i>	GL	70 Gln → His

Table 1 Continued

Locus	MHC class	Mutation	Type	Amino acid change ^a
I		H-2 ^{bm24}	GL	63 Silent 70 Gln → Asn 73 Trp → Ser 77 Ser → Asp 80 Asn → Thr
I		H-2 ^{bm28}	GL	97 Gln → Trp 99 Ser → Tyr 103 Silent

GL, simultaneous gain and loss of antigenic determinants; L, loss of antigenic determinants.

^aReferences: McIntyre KR and Seidman JG (1984) *Nature* **308**: 551–553; Nathenson SG, Geliebter J, Pfaffenbach GM and Zeff RA (1986) *Annual Review of Immunology* **4**: 471–502; Hemmi S, Geliebter J, Zeff RA, Melvold RW and Nathenson SG (1988) *Journal of Experimental Medicine* **168**: 2319–2335; Pfaffenbach GM, Melvold RW and Nathenson SG (1990) *Biochemical Genetics* **28**: 433–411; Pease LR, Horton RM, Pullen JK and Cai Z (1991) *Critical Reviews in Immunology* **11**: 1–32; Horton RM, Loveland BE, Parwani A, Pease LR and Fischer Lindahl K (1991) *Journal of Immunology* **147**: 3180–3184; Pease LR, Horton RM, Pullen JK and Yun TJ (1993) *Molecular and Cellular Biology* **13**: 4374–4381; Yun TJ, Melvold RW and Pease LR (in press; for *bm28*).

are specialized so that they bind only to a specific combination of [antigen + class I molecule] on a cell surface. The amino acid sequence of the peptide-binding region(s) of a major histocompatibility complex (MHC) class I molecule imparts an affinity for preferential binding of particular peptides. Thus changes in the amino acid sequence of a class I molecule can alter the subset of peptides which are displayed on the cell surface, and this in turn determines which subset(s) of CD8⁺ T cells are able to bind and become activated. Therefore, changes in MHC class I molecules can alter the immune repertoire. The *bm* mutations, by altering only one or a few amino acids at various sites in the *H-2K* and *H-2D* gene products have been crucial in identifying which portions of these molecules are important sites for the associations between class I molecule and peptide and between class I molecule and T cell receptor. Small changes, even only a single amino acid, can completely alter the ability of some individuals to respond to particular antigens. While all of the class I *bm* mutants affect cell-mediated functions, their effects on serologic functions are somewhat less consistent and dramatic, and many of them fail to stimulate antibody production when mutant and parental mice are immunized against one another.

Only a single spontaneous *in vivo* mutation of an H-2 class II gene has been recovered, and it too is a *bm* mutant (*H-2^{bm12}*). CD4⁺ T lymphocytes recognize combinations of [peptide + class II molecule] on cell surfaces. The *bm12* mutation, which involves a change in only three amino acids, has profound

effects on the immunobiology of the mutant animal by altering the way in which the class II molecule interacts with antigenic peptide or with T cell receptors on CD4⁺ lymphocytes. For example, the mutant is unable to respond to some antigens to which the parental strain responds well. On the other hand, the mutant has gained the ability to respond to certain antigens to which the parental strain is unable to respond. The mutation is also able to confer resistance to the development of an autoimmune disease, experimental myasthenia gravis, to which the parental strain is susceptible.

In summary, the *bm* mutants have provided exquisitely subtle genetic tools for the fine-detail analysis of H-2 gene structure and the relationship of that structure to the function of the resultant gene products, and are particularly valuable where whole, intact animals are required to study a particular process. They have been instrumental in the assignment of diverse biological and immunologic functions to single H-2 genes, and have even been shown to influence mating preferences, in that mice can distinguish parental animals from those carrying *bm* mutations by urine-associated odors. The *bm* mutations, and those derived from other haplotypes as well, will undoubtedly continue to demonstrate their value in immunogenetic research.

See also: Cell-mediated immunity; Congenic mice; Cytotoxic T lymphocytes; Gene conversion; H2 class I; H2 class II; Immune response (Ir) genes; Inbred strains; Mouse inbred strains.

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BONE MARROW AND HEMATOPOIESIS

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Ontogeny

During human development hematopoiesis passes through several distinct phases. In the embryo, large megaloblastoid nucleated red cells are produced in the extraembryonic membranes, from where they enter circulation. These cells synthesize the embryonic hemoglobins Hb Gower 1, Hb Gower 2 and the relatively minor hemoglobin Hb Portland. From 6 weeks gestation, erythropoiesis transfers to the fetal liver. At this time there is a switch in Hb production from the embryonic Hbs to fetal Hb ($\alpha_2\gamma_2$). This switch is time rather than site dependent, with residual erythroid cells in the extra-amniotic membranes at this stage also producing fetal Hb. Bone marrow hematopoiesis is established between the 11th and 22nd week of gestation and probably arises by stem cell migration into appropriate marrow stroma. In mid-gestation there are high levels of circulating hematopoietic progenitor cells which decline progressively until birth. At about 40 weeks gestation the synthesis of fetal Hb is replaced by adult Hb ($\alpha_2\beta_2$) and this event is related not to birth but to the time of gestation. The Hb switching involves the serial expression of the α -like globin chains on chromosome 16 and the β -like globin chains on chromosome 11 in a 5' to 3' direction. The precise mechanism of the switch is not understood.

The fetal liver contains both myeloid and erythroid progenitor cells and yet few phagocytes are produced until the end of gestation. This indicates that in aseptic fetal life there is inadequate environmental stimulus to induce the proliferation and differentiation of myeloid progenitor cells. Around the time of birth there is a pronounced rise in the peripheral blood neutrophil and monocyte levels. Lymphocytes appear in the blood by 8 weeks gestation and by the second trimester the T cells have a mature but generally naive phenotype. Full functional competence is not gained until after birth. The fetal blood contains relatively high numbers of circulating stem and progenitor cells and although these levels fall throughout gestation, cord blood levels are still sufficiently high to allow the use of cord blood for allogeneic hematopoietic stem cell transplantation.

Stem cell origin of hematopoiesis

There is definitive evidence that multipotential stem cells give rise to cells of all the myeloid and lymphoid elements. In chronic myeloid leukemia the clonal expansion involves not only the red cell, platelet and phagocyte series but also B lymphocytes. Transplantation experiments in mice using stem cells marked by retroviral insertions indicate that T cells are also derived from a common lymphohematopoietic stem cell.

In the adult the large majority of hematopoietic stem cells are noncycling. These cells can be assessed in mice by competitive repopulation assays into irradiated recipients. The enumeration of cells capable of forming day 12 spleen colonies (CFU-S) in irradiated mice (assay developed by Till and McCulloch in the early 1960s) allows a simpler quantitation of primitive cells, but the most primitive repopulating stem cells are at an earlier stage in the developmental pathway than the average CFU-S. There is obviously no direct human equivalent of the CFU-S assay but stem cells can be grafted into immunodeficient mice, and such studies are providing important information about human stem cell biology. Assays of cells capable of forming 'cobblestone areas' on preformed stroma also provide an assessment of very primitive cells. When appropriately stimulated these cells proliferate and either undergo a process of self-renewal or differentiate ultimately to mature cells. A single stem cell can almost totally repopulate a mouse, although in the steady state a very large number of stem cells are available. The morphological appearance of the most primitive stem cells is probably similar to a small lymphocyte. As it proliferates and differentiates it assumes a blastic appearance. The majority of cell divisions arise in this blast cell pool, and the recognizable precursor cells in the bone marrow are the outcome of only the terminal six or seven divisions. As stem cells pass down the differentiation pathway, there is progressive commitment to a given lineage and the stem cell progeny lose the potential for self-renewal. The process of commitment has a stochastic (random) element but is sensitive to environmental modulation. Cells intermediate between the stem cells and the more mature precursor cells are known as progenitor cells and can be quantitated by *in vitro* colony forming assays. These assays were developed

in the murine system by Pluznik and Sachs and by Bradley and Metcalf in the mid-1960s. In these assays, immobilized suspensions of hematopoietic cells are induced to form colonies in the presence of 'colony stimulating factors'. The cells giving rise to colonies are capable of many cell divisions and may have either restricted or multiple lineage potential. They are not, however, capable of self-renewal. The colonies and cells giving rise to them are named after the mature progeny that they produce: G-CFC, M-CFC, GM-CFC, Eo-CFC and Mega-CFC give rise to granulocytes, monocytes, both granulocytes and monocytes (and also eosinophils), eosinophils and megakaryocytes, respectively. Relatively late erythroid colony forming cells are referred to as colony forming units – erythroid (CFU-E), and the early erythroid progenitor cells as burst forming units – erythroid (BFU-E) because of the multiple cluster appearance of these colonies. CFC-mix give rise to cells of multiple lineages. There is some evidence that T cells may be derived from these CFC-mix progenitors, but this has not been widely confirmed.

Myelopoiesis

Hematopoietic growth factors

Myelopoiesis is regulated at least in part by a series of glycoproteins referred to as hematopoietic growth factors (HGFs). Erythropoietin, produced in response to hypoxia by the peritubular adventitial cells of the kidney, is a true hormone, and G-CSF is similar in so far as the circulating levels are inversely related to the neutrophil count. Interleukin 6 (IL-6) levels also rise in neutropenic and infective states. Thrombopoietin, which regulates megakaryocyte production, is of particular interest as the serum levels are largely regulated by end-organ (platelet) sequestration, and more thrombopoietin becomes available when the platelet count falls. Several other HGFs such as GM-CSF and IL-3 cannot be readily measured in the plasma and probably act in a paracrine manner.

From *in vitro* and *in vivo* studies, the HGFs can be grouped into three broad categories. Firstly, there are the late-acting factors which are relatively lineage

restricted and stimulate the terminal divisions of the maturation pathway with associated differentiation to mature cells (Table 1). These include erythropoietin, G-CSF, M-CSF, Eo-CSF, known as IL-5, and thrombopoietin (TPO). The proliferation of more primitive cells in the pathway is regulated by the multi-CSFs IL-3 and GM-CSF. Both of these factors stimulate the early divisions of several cell lineages, including granulocytes, monocytes, eosinophils, megakaryocytes and red cells; IL-3 also stimulates mast cell progenitors and some early lymphoid cells. It is probably active at an earlier stage in the myeloid differentiation pathway than GM-CSF but probably has little effect on the most primitive stem cells. More primitive stem cells are influenced by a range of factors including stem cell factor (SCF), Flt-3 ligand, IL-6, IL-11, and leukemia inhibitory factor (LIF). These factors render very primitive cells susceptible to the later-acting factors and are thus often referred to as 'synergistic factors'. The categorization of the HGFs into three groups is undoubtedly an oversimplification and G-CSF and M-CSF, for instance, have some effects on primitive progenitor cells.

The most primitive of all stem cells appear not to be responsive to known soluble factors and the additional signals required for proliferation of these cells is poorly understood.

Finally, the activity of the HGFs is not restricted to immature cells. G-CSF enhances many of the functional activities of mature neutrophils; GM-CSF enhances neutrophil, monocyte and eosinophil function; and M-CSF and IL-3 prime some monocyte functions – the latter also modulating eosinophil and mast cell function.

The central importance of erythropoietin in red cell production is apparent from the profound anemia that occurs in anephric individuals. Similarly, mice with knockouts of the G-CSF or thrombopoietin genes have severe neutropenia and thrombocytopenia, respectively. Knockout mice without a functional GM-CSF gene have normal blood counts but do develop a pulmonary condition secondary to poor alveolar macrophage function, in line with the view that many of the earlier-acting factors exhibit considerable redundancy with regard to hematopoiesis and that a major role for GM-CSF is as an inflammatory mediator. The Steel mouse however, which is unable to produce SCF, develops a macrocytic anemia, mast cell deficiency and albinism which in some strains can be lethal.

Hematopoietic growth factor receptors

Each HGF has a specific receptor or receptors, these being expressed at low levels, generally in the range

Table 1 Growth factors in hematopoiesis

Stem cells		Mature myeloid cells
SCF	IL-3	Erythropoietin
Flt-3 ligand	GM-CSF	Thrombopoietin
IL-6		G-CSF
IL-11		IL-5
LIF		(M-CSF)

of 10^2 – 10^3 receptors per cell. These receptors can be considered as two categories. Firstly the M-CSFR, which is the cellular homolog of v-fms and the SCFR which is the cellular homolog of v-kit are tyrosine kinase receptors similar to the PDGF receptor. Ligand occupation causes autophosphorylation and dimerization of the receptor chains followed by a cascade of signaling events mediated largely by cytoplasmic enzymes binding to phosphorylated tyrosine residues in the intracytoplasmic domains of the receptor. The EpoR, G-CSFR, GM-CSFR, TPOR, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-11R, IL-13R and IL-15R are all members of the cytokine receptor family. The EpoRs, TPORs and G-CSFRs appear to consist of a single chain which dimerizes on activation by ligand. Other members of the family appear to be composed of two components. The GM-CSFR has an α chain which binds to GM-CSF with low affinity and a β chain which, although unable to bind GM-CSF when expressed alone, when complexed to an α chain produces a high affinity GM-CSF receptor. This β chain is common to the IL-3R and the IL-5R, and is largely responsible for signal transduction, which accounts in part for the similarity of action of these factors in cells which express more than one of the relevant α chains. Other receptors are even more complex, with at least three heterologous components: α , β and γ . The receptors for IL-2, IL-4, IL-7, IL-13 and IL-15 all share a common γ chain. The cytokine receptor chains do not contain a tyrosine kinase but they are able to bind and activate members of the Janus family of tyrosine kinases (JAKs). Activation of these kinases leads to activation of a family of transcription factors known as STATS, the c-myc pathway and the ras-MAP kinase pathway. The latter pathway is also stimulated by the shc binding directly to the activated receptor with subsequent activation of ras.

Bone marrow microenvironment

The specialized environment provided by the bone marrow stromal tissue is essential for effective hematopoiesis, particularly for the survival, self-renewal and proliferation of stem cells. The microenvironment is often considered to provide a 'stem cell niche', but this terminology is not particularly illuminating and, although it is now over 15 years since Dexter and colleagues established bone marrow stromal cultures which could support stem cell and thus long-term hematopoiesis *in vitro*, a limited amount is known about the precise function of the stromal tissue. Adhesion of certain categories of stem cells to the stroma is probably essential for proliferation and it is also likely that the stroma can provide negative regulatory signals to other stem cells, helping to

maintain them in the relatively protected G_0 state. A large range of adhesion molecules has been described on primitive hematopoietic cells, including the integrins VLA-2, -4, -5 and LFA-1, members of the immunoglobulin superfamily such as ICAM-1 and -3, PECAM-1, Thy-1 and LFA-3, the selectin L-selectin, the ligands for P-selectin and L-selectin and a number of sialomucins and other ligands, including CD6, CD34, CD43 and CD44.

The bone marrow stromal cells undoubtedly produce SCF. SCF contains a highly hydrophobic region so that, unlike the other HGFs, a large amount remains cell associated rather than being secreted as a soluble factor. It is likely that there are other stromal associated factors of this type. M-CSF, IL-6 and IL-7 are also produced constitutively but it has proved difficult to show that stromal cells produce other HGFs. This may be because only very small quantities are required in the context of intimate cell-to-cell contact. Furthermore, it has been shown that components of the stromal matrix avidly bind a range of HGFs, and this may serve to present a local stimulus to a stem cell or progenitor cell without releasing HGF into the surrounding milieu.

Lymphopoiesis

Primitive hematopoietic stem cells migrate from the bone marrow to the specialized environment of the thymus which is the site of T cell development. B cells by contrast, or at least immature B cells, are produced in the bone marrow, with only the later stages of B cell maturation and selection occurring in the peripheral lymphoid tissues. The bone marrow stages of B cell development have been designated as stem cell, early pro-B cell, late pro-B cell, pre-B cell and immature B cell on the basis of sequential immunoglobulin gene rearrangements and the expression of surface immunoglobulin. The earliest B cell precursors appear to be dependent on adhesion to the bone marrow stroma and VCAM-1, VLA-4 and CD44 have been particularly implicated in B cell development. The multipotent stem cells can be regulated by a number of early-acting cytokines as discussed above, but at the late pro-B cell stage a requirement develops for the presence of IL-7, which is produced in small quantities by the stromal cells.

See also: Colony stimulating factors; Granulocyte colony stimulating factor (G-CSF); Granulocyte-macrophage colony stimulating factor (GM-CSF); Interleukin 1 and its receptors; Interleukin 3; Interleukin 4; Interleukin 5 and its receptor; Interleukin 6; Macrophage colony stimulating factor (CSF-1); Microenvironment.

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BORDETELLA, INFECTION AND IMMUNITY

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Bordetella pertussis is a strict human pathogen which causes the respiratory disease whooping cough or pertussis. The other *Bordetella* species (*B. parapertussis*, *B. bronchiseptica*, *B. avium* and *B. hinzii*) are primarily animal pathogens but can, at times, cause disease in humans.

It is estimated that over 60 million cases of pertussis occur worldwide each year, resulting in 700 000 deaths. Clinically, pertussis has three phases. The disease begins with the catarrhal phase which may last from 7 to 21 days and is typified by symptoms similar to a common cold. The paroxysmal phase begins with severe, violent coughing spells and an inspiratory 'whoop' typical of the disease. This intense coughing lasts 2–4 weeks and can result in cyanosis, vomiting, severe exhaustion and death in infants. During the final or convalescent phase, cough paroxysms gradually decrease over 3–4 weeks. Although pertussis is considered a childhood disease, recent studies have demonstrated a high incidence of pertussis in adults.

Characteristics of the organism and its antigens

B. pertussis is a strictly aerobic, gram-negative coccobacillus which oxidizes amino acids as energy and carbon sources. Diagnosis of pertussis involves culture of the organism from nasopharyngeal swabs on Bordet–Gengou or Regan–Lowe agar (charcoal agar) and specific identification by direct immunofluorescence. Growth of *B. pertussis* on all media (at 35–37°C) is slow, requiring 2–6 days for the appearance of tiny, pearl-like colonies.

B. pertussis produces a large number of antigens, including adhesins and toxins that play a role in disease. The bacterium adheres specifically to the ciliated respiratory epithelium and remains localized in

the airways. Attachment factors include filamentous hemagglutinin (FHA), fimbriae and pertactin (PRN). FHA is a secreted and surface-associated filament-like protein composed of numerous monomeric 220 kDa subunits which agglutinate erythrocytes from several different species *in vivo*. *B. pertussis* produces two serologically distinct fimbriae (types 2 and 3) which appear to facilitate adherence to the upper respiratory tract. PRN is a nonfimbrial, 69 kDa outer membrane protein that also plays a role in attachment. Both PRN and FHA contain the amino acid sequence Arg-Gly-Asp (RGD) that mediates attachment to mammalian cells.

B. pertussis produces several toxins including pertussis toxin (PT), adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), and dermonecrotic toxin (DNT). Pertussis toxin (PT) is the most studied and has many biological activities *in vivo* including leukocytosis, histamine sensitization, stimulation of insulin secretion, and mitogenicity. Pertussis toxin is a classic A-B type toxin. After binding a specific host cell receptor via the B subunit, the toxin is internalized, and the A subunit catalyzes the adenosine 5'-diphosphate ribosylation of a group of guanine nucleotide regulatory proteins. This leads to increased host cell adenylate cyclase activity.

Host cell cAMP levels are also increased by the action of ACT. ACT enters mammalian cells where it is activated by calmodulin and converts host ATP to cAMP. As a consequence of its ability to insert into eukaryotic membranes, ACT is also responsible for the organisms's hemolytic activity on blood-containing culture media. ACT is a member of the RTX family of bacterial toxins which have in common a series of nonameric amino acid repeats in the C-terminus of the protein.

TCT is a 1,6-anhydromuramic acid-containing disaccharide-tetrapeptide fragment of peptidoglycan

released by growing bordetellae. In humans, TCT causes extrusion of ciliated cells, increases the frequency of sparsely ciliated cells and is toxic for other cells at epithelial surfaces. These changes cause a profound disruption of mucociliary clearance, leaving coughing as the only means to clear mucus and debris from the airways.

DNT is a heat-labile toxin that produces vasoconstriction and subsequent necrosis when injected subdermally in mice. Its specific role in disease progression is not understood.

B. pertussis lipopolysaccharide (LPS) is highly immunogenic with the properties typical of an endotoxin. It has a lipid A moiety linked via keto-deoxyoctulosonic acid to a branched-chain oligosaccharide domain containing heptoses and hexoses. The role of LPS in the pathogenesis of pertussis has not been well studied. The recent cloning and sequencing of the LPS operon should allow a specific determination of its contribution to virulence.

All *B. pertussis* virulence factors (except TCT and LPS) are regulated by the BvgAS system which belongs to the two-component family of bacterial transcriptional regulators. BvgS senses changes in the environment and phosphorylates BvgA; phosphorylated BvgA then activates transcription of the virulence factor genes. While the *in vivo* signals are not known, the Bvg system can be activated *in vitro* by increasing the temperature from 25°C to 37°C or by decreasing the concentration of MgSO₄ and nicotinic acid in the culture medium. This system presumably prevents wasteful synthesis of virulence factors not needed until the organism encounters a suitable host. The Bvg⁺ phase is required for full virulence of *B. pertussis*. The Bvg⁻ phase may be important in establishment of infection, persistence, transmission and/or survival in an unidentified environmental reservoir.

Immune response of the host

Humoral and cell-mediated immune (CMI) responses to pertussis are poorly understood. Neutralizing antibodies are considered to be a major protective mechanism against infection with *B. pertussis*. Recent vaccine trials demonstrated that antibody levels to *B. pertussis* antigens including PT, FHA, PRN and fimbriae correlate with protection against pertussis. In addition, a nonlethal pertussis infection provides long-term immunity to subsequent pertussis, and recovered patients produce anti-*B. pertussis* immunoglobulin A (IgA) in serum and saliva, suggesting the importance of mucosal antibodies.

However, CMI may also be important since *B. pertussis* specific T_H1 cells are found in humans fol-

lowing vaccination or infection. In mice, high anti-*B. pertussis* IgG correlates with rapid clearance of the bacteria in a respiratory infection model but a pertussis-specific CMI response is required for complete elimination of the organisms from lungs. *B. pertussis* survives in human monocytes and other mammalian cells *in vitro*, and a CMI response may be necessary for eliminating this potential intracellular reservoir of bacteria. In humans, natural immunity induced by infection may prevent respiratory colonization whereas immunity generated by vaccination may protect against toxin-mediated disease.

Evasive strategies by the organism

Many of the antigens produced and secreted by *B. pertussis* have been demonstrated to suppress the normal responses of the host to pertussis *in vitro* and *in vivo*. TCT, by destruction of ciliated epithelial cells, impedes clearance of bacteria, mucus and debris from airways. Mucociliary clearance is an important first line of defense and its destruction predisposes the patient to the secondary bacterial infections that commonly occur in pertussis.

Both ACT and PT have been demonstrated to inhibit immune cell function via induction of increased intracellular cAMP levels. PT and ACT reduce the migratory, chemotactic and phagocytic capabilities of polymorphonuclear leukocytes, blood monocytes, alveolar macrophages and lymphocytes. A synergistic effect between ACT and PT may occur locally in the respiratory tract to suppress the immune response to the infection and allow the organism to survive and persist in the host.

Variation of fimbrial type occurs readily in pertussis infections. *B. pertussis* may produce one, both or neither type of fimbriae and this may represent a means of avoiding fimbrial type-specific immune responses.

Vaccines

A whole-cell vaccine was in extensive use by the 1950s but, while the vaccine is effective at reducing the incidence of pertussis in vaccinees, it is reactogenic, fails to confer lasting immunity and is poor at protecting infants less than 6 months old. The whole-cell vaccine is composed of killed whole virulent *B. pertussis* combined with diphtheria and tetanus toxoids (DT) in the DTP vaccine. In the USA, infants are immunized beginning at 2 months of age and given three primary injections, followed by two boosters at 4–6 years of age. The US whole-cell vaccine is efficacious when all doses are given, with 80–95% protection levels reported. However, adverse

effects including local pain, redness, swelling and fever occur in 30–70% of vaccine recipients. Inconsolable crying, excessive somnolence and febrile seizures have been reported in rare cases with no permanent neurological damage.

Concerns about adverse effects associated with the vaccine led to loss of public confidence in the vaccine and decreased use, with a resultant increase in pertussis cases. This prompted efforts to produce an acellular vaccine, which was first developed in Japan and shown to be safe and effective. Various acellular pertussis vaccines contain 2–5 purified components which may include pertussis toxoid, FHA, PRN, and fimbriae types 2 and 3. The results of initial field trials led to the approval in the US of an acellular vaccine for boosters (fourth or fifth dose) when the whole-cell preparation is used for primary immunization.

Recent studies in Sweden and Italy compared DT alone and DT combined with either acellular or whole-cell pertussis vaccines for primary immunization. In both trials, the acellular preparations produced fewer and milder side-effects than the whole-cell vaccine and were more effective at inducing serum antibody responses and protection from pertussis. In 1996, acellular pertussis vaccines were approved for use in primary immunization against pertussis by the US government.

See also: Adjuvants; Toxins; Vaccines; Vaccines, adverse reactions to.

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BORRELIA, INFECTION AND IMMUNITY

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Although the clinical presentation of borreliosis, especially relapsing fever, has fascinated physicians for centuries, the mainstream of immunologic research bypassed the disease for many years. The advent of penicillin and DDT discouraged researchers anxious to study the immunology of borreliosis. After World War II, except for a few devotees whose laboratories continued to work on some problems related to the microbiology and immunology of relapsing fever, research on *Borreliae* was ignored. Scientific publications plunged from 50 a year in the 1950s to approximately 10 a year in the 1980s.

The increased recognition of Lyme disease

renewed interest in the *Borreliae*. Lyme disease was first recognized in Europe during the early 1900s. However, this illness was not reported in the USA until 1969, when a grouse hunter from Wisconsin contracted the first reported case of Lyme disease in North America. Subsequently, additional cases of Lyme disease were reported from the upper Atlantic Coastal states and the upper midwest of the USA. Human cases have since become increasingly recognized throughout the world. Lyme disease is the most common tick-associated illness in the USA.

During the 1980s, the spirochetal bacterium *Borrelia burgdorferi* was implicated as the causative agent of Lyme disease. Since that time, molecular

characterization studies have demonstrated at least several *Borrelia* subsp. capable of causing Lyme disease. The three most common are *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*. To date, only *B. burgdorferi* sensu stricto spirochetes have been detected in the USA, while these three genospecies are found throughout Europe.

Borreliae are entirely host-associated. Spirochetes have been found only in arthropods or host vertebrates that the arthropods have fed upon. *B. burgdorferi* sensu lato spirochetes are transmitted to human hosts by ixodid ticks of the *Ixodes ricinus* complex. Lyme disease is a multisystem disorder which usually begins with localized infection of the skin manifested by an expanding skin lesion, erythema migrans (EM), and constitutional symptoms such as fatigue, headache, mild stiff neck, arthralgia, myalgia and fever. Subsequent dissemination of the spirochete can cause more severe clinical manifestations, including secondary annular skin lesions, meningitis, Bell's palsy, radiculoneuritis and atrio-ventricular heart blockage. Arthritis or nervous system manifestations are hallmarks of chronic Lyme disease. An additional late manifestation, acrodermatitis chronica atrophicans, is seen primarily in Europe. Differences among the genospecies of *B. burgdorferi* sensu lato may account for geographic and regional variations in the clinical presentations.

In contrast to Lyme disease, relapsing fever caused by *B. hermsii* confirms that this illness is primarily a result of infection in the bloodstream. The clinical presentation is characterized by periods of fever lasting several days, separated by week-long intervals of normal temperatures. Diseases caused by *Borreliae* are curable with antimicrobial agents and are rarely fatal.

Spirochetes are not closely related to either gram-negative or gram-positive bacteria. *B. burgdorferi* sensu lato spirochetes are 0.2–0.3 by 20–30 µm. These organisms are highly flexible, left-handed (rotate in counterclockwise direction) helical cells, composed of 3–10 loose coils. They are susceptible to drying, but can survive for several months in medium at 4°C. The Lyme spirochete is composed of an outer cell membrane (outer sheath), protoplasmic cylinder and numerous flagella.

Some *Borreliae* are also surrounded *in vivo* by an amorphous slime layer. The slime layer, likely composed of host components, is weakly attached to the spirochete and is lost upon washing. The presence of host proteins in the slime layer may explain the inability of humans or animals to eliminate the Lyme disease spirochete despite a vigorous immune response. Thus, the spirochete may evade direct cell killing, antibody-dependent phagocytosis or anti-

body-mediated lysis with a coating of protective 'self molecules' that prevent immune recognition.

A similar outer envelope exists on *Treponema pallidum*. Treatment of treponemes with detergents to remove this outer envelope is required to achieve high reactivity in immunologic assays. The polysaccharide 'capsular' material appears to prevent phagocytosis. Similarly, there is evidence that carbohydrates are present in the slime layer of *B. burgdorferi* sensu lato, which may also influence the destruction of spirochetes by host factors.

The outer cytoplasmic membrane of Lyme disease spirochetes has a trilaminar organization of 45–62% protein, 23–50% lipid and 3–4% carbohydrate. The fluid membrane can be easily separated from the underlying protoplasmic cylinder with dilute solutions of sodium dodecyl sulfate or nonionic detergents. The outer membrane can move to one end of the spirochete by a phenomenon called 'patching' or 'capping'. The outer envelope layers also form blebs when *B. burgdorferi* sensu lato are incubated with specific antibody and complement. Bleb formation is a prelude to cell death.

The major surface proteins are located in the outer cell membrane of *Borreliae*. To date, outer surface proteins (Osp), designated A, B, C, D, E and F, have been demonstrated in *B. burgdorferi* sensu lato organisms; however, their functions remain unknown. These proteins are heterogeneous, especially among European isolates. Initial investigations demonstrated that the Osp A of USA isolates was homogeneous; however, more recent investigations have confirmed significant heterogeneity. The genes for Osp A and Osp B are located on a 40 kb double-stranded linear plasmid. Linear plasmids also encode the variable major proteins (VMP) of *B. hermsii*, which undergo antigenic variation. These antigenic changes allow survival of spirochetes in the human host for extended periods of time. Lyme disease spirochetes also appear to upregulate or down-regulate individual Osps, especially Osp A and Osp C, during infection of humans.

A variety of other antigens can also be detected in the outer membrane of *B. burgdorferi* sensu lato. These include proteins with molecular weights of 16, 27, 55, 60, 66 and 83 kDa. Antibodies against these proteins are readily detectable in chronic and complicated cases of Lyme disease. However, their relationship to the chronic nature of the disease and development of autoimmune immunologic reactions has not been elucidated. No single or combination of polypeptides has been directly associated with the different clinical manifestations of Lyme disease. The development of persistent or chronic Lyme arthritis that is not responsive to antimicrobial therapy has

been associated with an immune response to Osp A and Osp B and class II major histocompatibility complex molecules HLA-DR4 and HLA-DR2.

The flagella, responsible for motility, are located within the outer membrane and are generally not exposed to the surface. Between 7 and 11 flagella are inserted subterminally and bipolarly to the protoplasmic cylinder of *B. burgdorferi* sensu lato. Numbers of flagella vary among other *Borreliae*. The flagella run parallel to the long axis and overlap in the middle of the spirochete. Flagellin, with a molecular weight of 41 kDa, is the predominant flagellar protein. An antibody response to flagellin is a consistent feature in all stages of Lyme disease. Despite the presence of high concentrations of flagellar antibodies, Lyme disease continues to progress, suggesting that flagellin does not induce a protective immune response.

The structure and importance of the protoplasmic cylinder remains undefined. The cell wall contains muramic acid and ornithine as part of the peptidoglycan. Another component may be lipopolysaccharide. The Jarisch–Herxheimer reaction (characterized by a transient high fever), which can occur in Lyme disease patients after treatment with antibiotics, may be due to the sudden release of lipopolysaccharide from lysed organisms. Many patients have experienced a Jarisch–Herxheimer reaction following antimicrobial therapy.

Borreliae can be cultivated in artificial medium; however, the bacteria multiply slowly. Generation times range from 8 to 15 h (*B. burgdorferi*) to 26 h (*B. recurrentis*). Continuous passage of *Borreliae* in artificial medium alters the antigenic structure and renders the bacteria noninfectious.

The clinical presentation alone is usually sufficient to diagnose relapsing fever. In contrast, diagnosis of Lyme disease is often difficult because of the variety of symptoms that can develop. Visualization of *B. burgdorferi* sensu lato in blood has not been successful because Lyme disease spirochetes rapidly disseminate into tissues. Silver staining has been used to demonstrate spirochetal forms in both biopsy and autopsy tissues; however, spirochetes cannot be positively identified.

Lyme disease spirochetes have been successfully cultured *in vitro* from blood, cerebrospinal fluid and synovial fluid but only a small number of these cultures have yielded organisms. In contrast, recovery of *B. burgdorferi* sensu lato from EM skin lesions has been more successful. Polymerase chain reaction (PCR) technology has also been used to directly detect Lyme disease spirochetes in patients. To date, PCR testing appears to be useful for recovery of *B. burgdorferi* sensu lato DNA from atypical EM

lesions, cerebrospinal fluid and synovial fluid, provided the laboratory has sufficient expertise with the technology.

In many cases, serologic evidence of infection with Lyme disease spirochetes is the only option available to clinicians. Unfortunately, gross inaccuracies caused by the lack of specificity of conventional diagnostic assays and their subjectivity have made misdiagnosis and overdiagnosis common. These factors have contributed to confuse the general public and make many clinicians distrust serologic testing. During the past several years, however, diagnostic assays have been greatly improved. Unfortunately, there is currently no single assay that offers sufficient sensitivity and specificity to become the 'gold standard' of Lyme disease testing, although detection of borreliaecidal antibodies in Lyme disease patients offers promise. A flow cytometric borreliaecidal antibody test that measures the killing of live spirochetes after they were inoculated in the patient's serum and complement showed a sensitivity of 72% and more than 98% specificity. Regardless, quality laboratory procedures are available, provided results are obtained from experienced laboratories and interpreted correctly and the clinician understands the advantages and disadvantages of each laboratory procedure.

In early studies of the pathogenesis of Lyme disease, a major obstacle was the lack of suitable animal models. Rabbits and guinea pigs infected with *B. burgdorferi* develop skin lesions which histologically resemble human EM. These lesions, however, are not consistently induced and are the only clinical manifestations detected. Disseminated infection occurs in mature hamsters, rats and mice but clinical manifestations similar to those seen in human Lyme disease do not occur, despite persistence of spirochetes in the tissues. The hallmark of chronic *B. burgdorferi* sensu lato infection in humans, Lyme arthritis, appears to be observable in rodent models only when the immune system is immature or compromised. Rhesus monkeys have been shown to develop clinical signs and symptoms similar to localized and disseminated human infection; however, development of clinical arthritis only occurred in a small percentage of infected animals. In addition, the high costs associated with these animals has precluded widespread use of this Lyme disease model.

Despite these imperfections, animals models have been used extensively to elucidate pathogenic mechanisms of *B. burgdorferi* sensu lato. Lyme disease spirochetes have been shown to stimulate various inflammatory cytokines, including interleukin 1 (IL-1), IL-6 and tumor necrosis factor α (TNF α), and

several autoimmune mechanisms which appear to play a role in pathogenesis.

Animal models also continue to play a vital role in efforts to develop an effective Lyme disease vaccine. The role of cell-mediated immunity remains largely unknown; however, an important role for antibody-mediated immunity after vaccination has been established. The induction of antibodies, termed borreliacidal, that can specifically kill *B. burgdorferi* sensu lato are often responsible for antibody-mediated protection. Vaccination of animals with several individual Osps, including Osp A, Osp B, Osp C, and a 39 kDa protein have provided protection against Lyme disease spirochetes. Concomitantly, borreliacidal antibodies against Osp A, Osp B and the 39 kDa protein have been detected *in vitro*.

Osp A has emerged as the leading Lyme disease vaccine candidate and the efficacy of several recombinant Osp A vaccines is being investigated in animals and humans. Clinical trials have demonstrated safety in human volunteers; however, several significant obstacles must be overcome before induction of long-term comprehensive protection can be demonstrated. Most importantly, the success of Osp A vaccination appears to be dependent on the induction and long-term maintenance of high concentrations of borreliacidal antibodies. The present formulations of the Osp A vaccine have not maintained and sustained high concentrations of borreliacidal antibodies.

An anamnestic response will likely occur too slowly to prevent infection. Shortly after infection with *B. burgdorferi* sensu lato, the spirochetes become refractory to killing by borreliacidal antibodies. High levels of borreliacidal antibodies are detectable in sera from humans with all stages of Lyme disease; however, spirochetes are not eliminated. A recently developed flow cytometric test for detecting these highly specific borreliacidal antibodies is useful for confirming a Lyme disease diagnosis, and widespread availability of this test should greatly improve Lyme disease testing. However, induction of protective levels of anti-Osp A borreliacidal antibodies for extended periods of time has been difficult, even after vaccination of animals with

high concentrations of Osp A in combination with adjuvants.

In addition, experimental Osp A vaccines comprise a single protein, despite the fact that Osp A is antigenically polymorphic. Thus, vaccination provides little or no protection against heterologous *B. burgdorferi* sensu lato isolates. Significant frequencies of anti-Osp A escape mutants have also been observed in animals after vaccination with a single Osp A protein. More recently, researchers demonstrated down-regulation of Osp A expression and upregulation of other Osps shortly after infection of the vertebrate host. Thus, the incorporation of multiple Osp A proteins and other *B. burgdorferi* sensu lato Osps, especially Osp C, appears necessary for comprehensive protection against the Lyme disease spirochete.

See also: Antigenic variation; Bacterial cell walls; Endotoxin (lipopolysaccharide (LPS)); Rheumatological disorders.

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BRUCELLA, INFECTION AND IMMUNITY

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Brucellae are gram-negative coccobacilli, which lack capsules or flagellae. They establish persistent intracellular infections in mammals (Table 1). In animals, brucellosis typically affects the reproductive organs, and abortion is usually the only outward sign of the disorder. Human brucellosis is either an acute febrile disease or a chronic disease with a wide variety of symptoms, often difficult to diagnose. It is not associated with abortion. It is a true zoonosis, in that virtually all human infections are acquired from animals (Figure 1). Typically, brucellae may infect handlers of infected livestock or carcasses, and people consuming unpasteurized milk products. Aerosol transmission is a hazard in abattoirs. Diagnosis of brucellosis often rests on serology, but the protective immune response is cell mediated.

Characteristics of the organisms and their antigens

Species of *Brucella* were originally classified according to the reservoir hosts (Table 1). The species are differentiated in the laboratory by colony morphology, growth requirements, various biochemical tests and lysis by bacteriophages. Nevertheless, DNA hybridization studies suggest they are so closely related that they should be classified as a single species.

Fresh isolates of most *Brucella* spp. are smooth,

but dissociation occurs readily into rough or mucoid colonies. *B. ovis* and *B. canis* are normally rough or mucoid and lack the O chains of lipopolysaccharide (LPS). *Brucella* LPS is less toxic, less pyrogenic and less effective as a polyclonal activator than that of most gram negatives. Nevertheless, it appears to be the main virulence factor of *Brucella* species.

Two different O chains occur on *Brucella* LPS: A, generally associated with *B. abortus*, and M, with *B. melitensis*. However, some *B. abortus* biovars carry M antigens, and some common *B. melitensis* carry A antigen. Both are linear polymers of an unusual amino sugar, perosamine (4,6-dideoxy-4-formamido-D-mannopyranose). They differ in that the A chain sugar molecules are always linked 2–1, whereas the M chain has a 3–1 linkage at every fifth junction. They can be distinguished by monoclonal antibodies, or by polyclonal serum rendered specific by cross-absorption. Cross-absorption is needed because the core structure of LPS is common to all species.

Other cell envelope antigens include the outer membrane proteins (MPS) which fall into two molecular weight ranges, 25–27 kDa and 36–38 kDa. The latter act as porins. In addition there are more than 30 internal antigens identified by western blotting or immunoelectrophoresis.

Table 1 Host ranges of the genus *Brucella*

Species	Colony type ^a	Predominant hosts	Occasional hosts ^b
<i>B. melitensis</i> (3 biovars)	S	Sheep, goats	Cattle, humans
<i>B. abortus</i> (7 biovars)	S	Cattle	Sheep, goats, horses, other domestic and wild ungulates, dogs, humans
<i>B. suis</i> (5 biovars)	S	Biovar 1: pigs Biovar 2: hares Biovar 3: pigs Biovar 4: reindeer Biovar 5: wild rodents	Humans Pigs Humans Humans
<i>B. neotomac</i>	S	Desert wood rat ^c	Not reported
<i>B. ovis</i>	R	Rams	Ewes
<i>B. canis</i>	R	Dogs	Humans

^a Wild-type on isolation. S, smooth; R, rough. Avirulent R mutants of S strains are readily obtained on laboratory media.

^b Not a complete list – examples only.

^c *Neotoma lepida*.

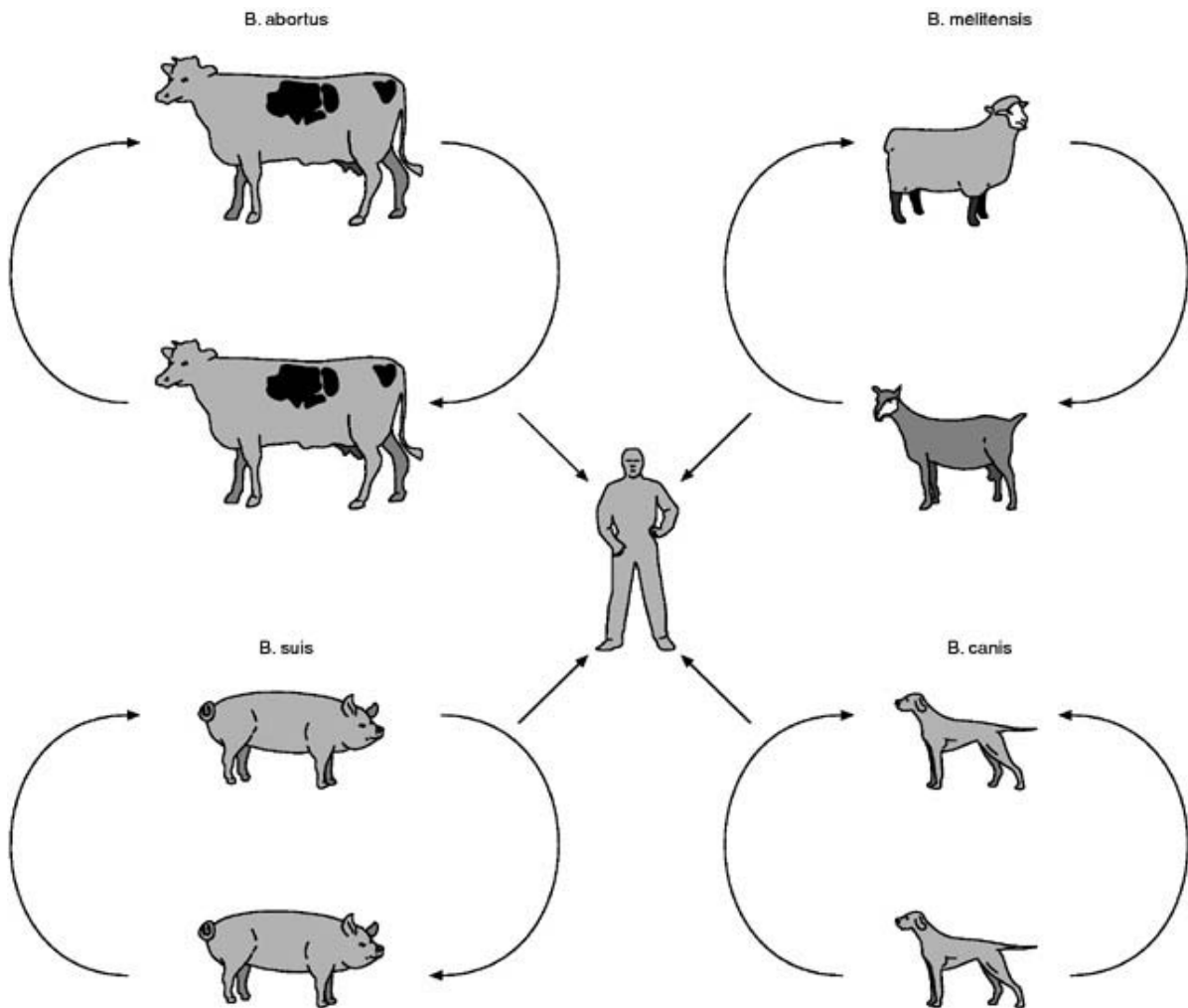


Figure 1 Brucellosis is a true zoonosis with different bacterial species infecting different animal species, and only incidentally infecting humans, often as a result of occupational hazard or drinking unpasteurised milk.

Immune responses of the host

Protective immunity

The primary antibody response to *Brucella* infection in humans involves early immunoglobulin M (IgM) production followed by a switch to IgG and IgA. IgE can also be detected by radioimmunoassay. IgM persists for an unusually long period into the late stages of the disease and in subclinical infection. It is interesting to speculate that this might relate to the T cell-independent nature of the major antigen, LPS, acting on B cells without causing class switching. Notwithstanding the importance of antibody in serodiagnosis of brucellosis, the role of antibody in protection is probably confined to pre-existing mucosal antibody reducing the likelihood of initial infection.

As facultative intracellular bacteria, brucellae are controlled by the activation of macrophages and the formation of granulomas to localize the infection. In

experimental infection of mice, it has been shown that both CD4⁺ and CD8⁺ T lymphocytes are involved. The function of the CD8⁺ T cells is apparently to lyse ineffective macrophages and non-professional phagocytes to release the bacteria for phagocytosis by activated macrophages. The function of CD4⁺ T cells is to produce interferon γ (IFN γ) and other cytokines to activate and attract macrophages.

Depletion of IFN γ from infected mice by injection of monoclonal antibody specific for IFN γ increases bacterial numbers and suppresses the attraction of macrophages to the infected tissues. Even more dramatic are the effects of depleting interleukin 12 (IL-12), the cytokine which controls the differentiation of T cells and natural killer (NK) cells to produce IFN γ . Injection of antibody to IL-12 prevents IFN γ production by the T cells, prevents the influx of cells which causes splenomegaly, and grossly exacerbates

bacterial numbers over a prolonged period of time. Other cytokines produced during experimental infection include IL-1, tumor necrosis factor α (TNF α), IL-6, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF). TNF α is important early in infection, either directly activating macrophages or stimulating the NK cells to produce IFN γ . Its depletion also increases early bacterial numbers and suppresses splenomegaly but, in contrast to the effect of IL-12 depletion, the mice develop IFN γ -producing T cells and recover.

In humans, the tissue lesions produced by *Brucella* sp. consist of minute granulomata, comprising epithelioid cells, neutrophils, mononuclear leukocytes and some giant cells. Necrosis is not common, and abscesses do not form, except in *B. suis* infection. Hepatosplenomegaly is a common clinical manifestation, a symptom shared by experimental mice. As with experimentally and naturally infected animals, human patients develop delayed-type hypersensitivity (DTH), a correlate of the cell-mediated resistance to infection, but also a cause for immunopathology.

Immunopathology

Immunopathology is a major factor in the disease. The granulomata which form at the foci of infection provide the main histopathologic signs. A pathogenic role for immune complexes has been suggested but there is no correlation between their levels and severity of disease. In people who have been sensitized by previous infection, albeit subclinical, needlestick injury can be followed within 24–48 h by a severe, generalized DTH response which resembles many aspects of the infection itself. The ability of components of brucellae to induce polyclonal activation of B lymphocytes could explain the high incidence of autoantibodies in acute infection. Alternatively, the presence of stress proteins which have been conserved across species and cross-react with mammalian proteins have been implicated in autoimmune sequelae of other infections, and may be important in brucellosis also.

Serology

Because of the nonspecific nature of the symptoms of human brucellosis, and the difficulty of isolating the causative organisms, diagnosis often must be made serologically. Most of the antibody detected is directed towards the LPS, with some specific for protein antigens. A variety of tests have been used, including agglutination, complement fixation, precipitation, radioimmunoassay, indirect fluorescence antibody assays and ELISA. To date, the agglutination assay remains the most widely used,

although this is likely to be replaced by ELISA when better standardization is achieved. A feature of the agglutination assay has been the prozone, seen in high-titer sera, and blocking antibodies. The majority of studies suggest that these are IgA, although some have found blocking antibody amongst IgG. The problems of prozone or blocking antibody can be overcome by further dilution of the serum or the use of an indirect agglutination assay. Where it is necessary to distinguish IgM and IgG antibodies, 2-mercaptoethanol is used to inactivate IgM agglutination, leaving 2-mercaptoethanol-resistant IgG.

In domestic animals intradermal testing for DTH is the most convenient way of establishing the presence of infection in the herd. Nevertheless serology is the best way of detecting infected individual animals.

Evasive strategies by the organism

These bacteria can survive within the phagocytic cells of the host. Extracts of *B. abortus* have been reported to inhibit phagosome–lysosome fusion in macrophages, degranulation of neutrophils and the oxygen-dependent antibacterial system of bovine neutrophils. The organisms may spread from the initial site of infection either within these cells or free in the bloodstream. They localize in certain target organs, such as lymph nodes, spleen, liver, bone marrow and, especially in animals, reproductive organs. The presence of mesoerythritol in the testes and placenta of ruminants and pigs stimulates enormous multiplication of brucellae in those tissues, leading to infertility and abortion. However, erythritol is absent in humans.

Vaccines

While LPS is the target of antibodies, there is a spectrum of proteins which can stimulate T cells. A number have been identified. Some were found to be stress proteins. Nevertheless, a defined vaccine is some time away, since, as with other intracellular bacteria, live attenuated vaccines are the only ones so far shown to be effective in the induction of cell-mediated resistance.

B. abortus strain 19 is used to immunize cattle, while *B. melitensis* strain Rev 1 is used in sheep and goats. However, vaccination of humans has not been widely used outside Russia due to the questionable safety of these strains. Accidental infection with vaccines is a hazard for veterinarians exposed to needlestick injuries or splashes on to the conjunctiva.

The preferred approach to prevention of human infection is the pasteurization of milk products and

the ultimate eradication of infected herds. Once infection in domestic animals is reduced by vaccination, vaccination is replaced by testing followed by slaughter of infected animals. At this stage, vaccination of the herds only leads to confusion, since neither the serological tests used nor DTH screening can distinguish between infected and vaccinated animals. Although vaccination of very young animals usually results in antibody dropping to undetectable levels by the time surveillance of the herd is undertaken, remaining positives lead to confusion as to whether or not they are infected.

See also: Cell-mediated immunity; Cytotoxic T lymphocytes; Delayed-type hypersensitivity; Granuloma; Immunoglobulin class switching; Interferon γ ; Interleukin 12 and its receptor; *Listeria*, infection and immunity; Macrophage activation; *Mycobacteria*, infection and immunity; T cell vaccination; Tumor necrosis factor α .

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BRUTON'S AGAMMAGLOBULINEMIA

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Historical background

In 1952 Colonel Ogden C Bruton reported the case of an 8-year-old boy with a 4 year history of recurrent bacterial sepsis, osteomyelitis and otitis. This child failed to make antibodies to pneumococcus after repeated antigenic challenge. In a seminal observation, Bruton noted that the patient's serum lacked the gamma globulin fraction by electrophoretic analysis but was otherwise normal. This was the first case in which an abnormal result in a laboratory study explained the clinical problems and dictated the therapy for an immunodeficient patient. The patient was treated with gamma globulin and had a marked decrease in the incidence of infection. In the next 5 years many similar patients were reported.

Most of these patients could be divided into one of two groups. The first group consisted of adults who had the onset of recurrent or persistent infections after early childhood. Males and females appeared to be equally affected and the disorder could not be accounted for by a single gene defect by mendelian inheritance. This disorder came to be called acquired hypogammaglobulinemia or common variable immunodeficiency. The patients in the second group were boys who had the onset of their disease in the first 2 years of life. Many of these children had similarly affected brothers, maternal uncles or cousins. This disorder is now referred to as X-linked agammaglobulinemia (XLA), congenital agammaglobulinemia or Bruton's agammaglobulinemia.

Studies performed in the 1970s showed that patients with XLA had markedly reduced numbers

of B cells in the peripheral circulation; however, pre-B cells could be found in their bone marrow, suggesting that stem cells could enter the B cell lineage but they could not progress through normal B cell differentiation. In 1986, preliminary linkage studies mapped the XLA defect to the midportion of the long arm of the X chromosome. The same year, X chromosome inactivation studies performed on the B cells of carriers of XLA, the mothers of affected boys, showed that the XLA gene product was expressed in B cells and it was not transportable between cells. In 1993, two groups, using positional cloning or analysis of a candidate gene, independently showed that XLA was due to mutations in the gene encoding a cytoplasmic tyrosine kinase which is now called Btk (Bruton tyrosine kinase) (Table 1).

Clinical characteristics

Most patients with XLA are healthy at birth and remain well for the first few months of life. Recurrent infections, particularly otitis and pneumonia, usually develop sometime during the second 6 months of life, after the loss of maternally acquired antibody. The majority of patients with XLA are evaluated for immunodeficiency before 3 years of age. However, 20–30% of patients present later; some of these patients have higher concentrations of serum immunoglobulins and/or more B cells than are typical for XLA. A helpful clinical clue to the diagnosis of XLA is unusually small peripheral lymph nodes and tonsils. Germinal follicles, which constitute a considerable proportion of the volume of lymph nodes, are largely made up of B cells. In the absence of B cells, lymph nodes are quite small. Except for signs of past infection, there are no other striking physical findings. By the time of diagnosis approximately 80% of patients have experienced recurrent upper and/or lower respiratory tract infections. Diarrhea and pyoderma are also frequently seen before diagnosis and initiation of therapy. Between

20 and 30% of patients have experienced sepsis, meningitis, arthritis or failure to thrive. Profound neutropenia is present at the time of diagnosis in about 25% of patients. Overwhelming bacterial sepsis with rapid demise is not unusual. The most frequent infecting organisms in patients with XLA, and in all other patients with hypogammaglobulinemia, are *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus aureus*.

After diagnosis and initiation of gamma globulin replacement therapy, upper and lower respiratory tract infections may continue to be a problem, particularly if the patient has developed chronic pulmonary changes prior to diagnosis. Diarrhea due to Giardia is not uncommon in patients with XLA but is usually easily treated. Mycoplasma or ureoplasma infections can cause persistent arthritis, pneumonitis or urologic problems. Between 5 and 10% of patients with XLA have chronic enteroviral infections resulting in meningitis/encephalitis, arthritis or a dermatomyositis-like syndrome. These infections are often slowly progressive and fatal. Although echovirus is the most common organism, vaccine-associated polio and Coxsackie have also been isolated.

High-dose gamma globulin therapy is expected to decrease the incidence of chronic lung disease and enteroviral or mycoplasma infections in patients with XLA; however, it has been the source of hepatitis C infection in some patients. At the present time, the majority of adolescents with XLA have intermittent or chronic mild sinusitis or bronchitis but are otherwise completely well with normal growth and development.

Laboratory characteristics

The hallmarks of XLA are markedly decreased concentrations of serum immunoglobulins and an almost complete absence of B cells in the peripheral circulation. The serum immunoglobulin G (IgG) is usually less than 2 mg ml⁻¹, and the IgM and IgA are less than 0.1 mg ml⁻¹. B cells, which constitute 5–18% of the peripheral blood lymphocytes in the normal individual, cannot be detected using standard techniques; however, a small number (0.005–0.1% of peripheral blood lymphocytes) are uniformly present. As evaluated with antibodies to surface markers, these B cells have an immature phenotype that is similar to that seen in B cells from X-linked immunodeficient (xid) mice. Epstein-Barr virus (EBV)-transformed B cell lines, produced from the blood of patients with XLA, make normal immunoglobulins with correct rearrangements of V, D and J gene segments. A disproportionate number of the

Table 1 Characteristics of Bruton's agammaglobulinemia

X-linked recessive (occurs only in males)
Onset in the first 3 years of life
Recurrent infections with encapsulated bacteria
Sinusitis, otitis, bronchitis and pneumonia
Increased susceptibility to mycoplasma, enterovirus and hepatitis C
Marked decrease in serum IgG, IgM and IgA
Profoundly reduced numbers of B cells
Pre-B cells present in the bone marrow
Treatment with gamma globulin and antibiotics
Mutations in the gene for Bruton's tyrosine kinase (Btk)

pre-B cells found in the bone marrow of patients with XLA are at the earliest stage of development and a reduced percentage of them are in the S phase of the cell cycle.

Abnormalities in cell lineages other than B cells have been reported; however, these abnormalities may be secondary to the marked reduction in B cell numbers or the hypogammaglobulinemia rather than a direct effect of the genetic defect.

Bruton tyrosine kinase

Bruton tyrosine kinase (Btk) is a 659 amino acid member of a recently identified subfamily of src-related cytoplasmic tyrosine kinases (Figure 1). It is expressed throughout B cell and myeloid development but it is not expressed in nonhematopoietic cells. Like src, Btk has a carboxy-terminal catalytic domain adjacent to SH2 and SH3 (src homology 2 and 3) domains. However, unlike src but similar to the other members of its subfamily, which include Tec, Itk and Bmx, Btk has an amino-terminal PH (pleckstrin homology) domain followed by a proline-rich region. Although the substrates phosphorylated by Btk have not yet been identified, like other tyrosine kinases, Btk is thought to function in signal transduction. This view is supported by the observation that the protein-protein interaction domains of Btk bind to other molecules known to be involved in signal transduction, including src family members, the $\beta\gamma$ subunit of G proteins, protein kinase C and cbl.

Btk is phosphorylated and its kinase activity is increased by stimulation of a variety of cell surface receptors, including, and perhaps most importantly, the B cell receptor complex (BCR). After cross-linking of cell surface IgM, src family members phosphorylate Btk, which then increases its catalytic activity by autophosphorylation. This process is associated with movement of Btk to the inner surface of the cell membrane.

The well-characterized murine immunodeficiency,

xid, is caused by an amino acid substitution in the PH domain of Btk. The phenotype of the xid defect appears to be milder than the XLA defect. Mice with either the spontaneous Btk mutation or a null mutation in Btk induced by homologous recombination have reduced concentrations of serum IgM and IgG3 and they lack a mature population of B cells; however, they do have an antibody response to T cell-dependent antigens and they have relatively normal concentrations of serum IgG1, IgG2a and IgG2b. The phenotype of the xid mice suggests that Btk is required not only at the transition from pre-B cell to B cell but also at later stages of differentiation.

Genetics

Bruton's agammaglobulinemia occurs in all racial groups, with a prevalence of between 2 and 8 per 1 000 000. As is true with all X-linked disorders that are lethal without medical intervention, the disorder is maintained in the population by the occurrence of new mutations. This has two important consequences. First, approximately half of the patients with the clinical and laboratory characteristics of XLA have no family history of the disorder because they are the first manifestation in their family of a new mutation. Second, the mutations in Btk that cause XLA are independently derived and highly variable.

The gene for Btk consists of 19 exons spread over 37 kb at Xq22. Human Btk has over 95% homology to murine Btk and 58% homology to a drosophila kinase, Dsrc28C. Over 100 different mutations in Btk have been identified. Although deletions that are detectable by Southern blot analysis do occur, they constitute less than 10% of the mutations in this gene. About one-third of the mutations result in amino acid substitutions, the majority of which are in the kinase domain; however, amino acid substitutions in the PH domain and the SH2 domain have also been seen. The remaining mutations are almost equally divided between single base pair substi-

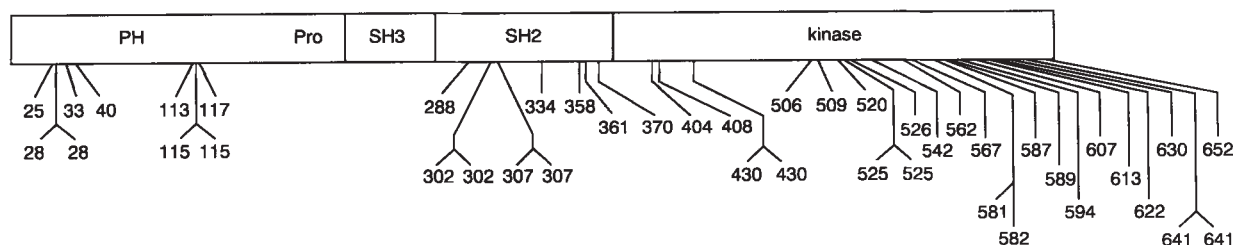


Figure 1 Structure of the 659 amino acid cytoplasmic tyrosine kinase, Btk. The amino-terminal pleckstrin homology domain (PH) is followed by a proline rich region (Pro), and SH2 and SH3 domains and the catalytic domain. The mutations causing amino acid substitutions in patients with XLA and the codons affected by those mutations are shown. Some codons have been altered by two different mutations; xid mice have an amino acid substitution in codon 28 in the PH domain.

tutions that cause premature stop codons, small insertions or deletions that result in frameshift mutations and splice defects. These mutations are spread almost evenly throughout the Btk gene. Studies done on EBV-transformed cell lines or myeloid cells from patients with XLA indicate that this last group of mutations (stop codons, frameshifts and splice defects) usually result in poor accumulation of the Btk mRNA in the cytoplasm. Thus, these mutations are functionally equivalent.

Attempts to correlate the severity of the clinical manifestations of XLA with specific mutations in Btk suggest that the site or type of mutation may influence the phenotype. Amino acid substitutions in the SH2 domain or noncritical regions of the catalytic domain are sometimes seen in patients with milder disease. However, additional factors, including other genes, past infections and therapy, may also influence the phenotype of patients with XLA.

Differential diagnosis

X-linked agammaglobulinemia should be distinguished from two other X-linked disorders that result in hypogammaglobulinemia in infant males. X-linked severe combined immunodeficiency (SCID), which is due to mutations in the common γ chain of cytokine receptors, is associated with the onset of persistent fungal and viral, as well as bacterial, infections in the first 6 months of life. Laboratory studies show normal or increased proportions of B cells but markedly decreased numbers of T cells, and absent proliferation in response to T cell mitogens. Mutations in the gene for CD40 ligand cause X-linked hyper-IgM syndrome, a disorder in which serum concentrations of IgG and IgA are markedly decreased but the level of IgM, while not always strikingly elevated in infancy, is higher than that usually seen in XLA. Patients with X-linked hyper-IgM syndrome have normal numbers of peripheral blood B cells and T cells. Patients with common variable immunodeficiency can be distinguished from patients with XLA in that they usually have somewhat higher concentrations of serum immunoglobulins and normal or near normal numbers of B cells in the peripheral circulation. T cell number and function may be abnormal in patients with common variable immunodeficiency. A small number of girls in whom both the clinical and laboratory findings are indistinguishable from XLA have been reported, suggesting that there is one or more autosomal recessive disorder(s) phenotypically identical to XLA.

Therapy

Patients with XLA are treated with gamma globulin replacement therapy and liberal use of antibiotics. All live viral vaccines should be avoided. Although the identification of Btk as the defective gene in XLA sets the stage for gene therapy, a better understanding of the regulation and function of Btk, as well as improved technology of gene transfer, will be required before this goal can be achieved.

See also: B lymphocyte differentiation; CD40 and its ligand; Common variable immunodeficiency; Gammaglobulin; Humoral immunity; Immunodeficiency, animal models; Immunodeficiency, primary; Immunoglobulin, cell surface; Protein kinases; Severe combined immunodeficiency.

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BUNYAVIRIDAE, INFECTION AND IMMUNITY

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In 1943 a virus was isolated from a pool of wild-caught *Aedes* mosquitoes collected in an uninhabited area of Semliki Forest in western Uganda known as Bunyamwera. The virus was unrelated to yellow fever virus, or to any of the 20 or so arthropod-borne viruses or 'arboviruses' then known, and it was named Bunyamwera virus. Although antibodies against Bunyamwera virus were detected in some residents of nearby villages, no disease association was apparent, and the virus appeared to be little more than a virological curiosity. By 1960 about a dozen arboviruses isolated in North and South America, Europe, Africa and Asia had been shown to be serologically related to Bunyamwera virus, and this number increased over the next decades. More than 50 years after its isolation, Bunyamwera virus remains an unimportant virus in terms of human disease, causing at the most a mild fever with a rash, but it has been intensively studied as the type species both of the genus *Bunyavirus* and of the large and diverse family Bunyaviridae which now contains more than 300 members placed in five different genera, *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* (Table 1).

Although the majority of Bunyaviridae resemble the type species in being of minor medical importance, the family does contain a number of major pathogens of humans and/or animals. Most bunyaviruses are transmitted by mosquitoes or by *Culicoides*, most phleboviruses by *Phlebotomines*, and all nairoviruses by ticks, whereas hantaviruses are not arthropod-borne, but are zoonotic agents capable of infecting humans after close contact with infected animals, mainly rodents and voles. Tospoviruses are thrip-transmitted plant viruses such as tomato spotted wilt and related viruses. They are of no medical or veterinary interest, but are included in the Bunyaviridae on the basis of their close physical, biochemi-

cal and genetic similarities to viruses in the other genera. They illustrate the great genetic diversity shown by members of this substantial family.

Characteristics of the Bunyaviridae

Bunyaviridae are enveloped RNA viruses about 100 nm in diameter containing a tripartite, single-stranded RNA genome of negative polarity (Figure 1). Virions contain three main proteins, namely the envelope glycoproteins G1 and G2, and the nucleocapsid protein N, together with a minor large protein L. The three viral RNA species are designated as small (S), medium (M) and large (L), of which the S RNA encodes the N protein, the M RNA encodes the two envelope glycoproteins G1 and G2 and the L RNA encodes the large, virion-associated polymerase protein L. Three different expression strategies are used to generate the nonstructural proteins of the S

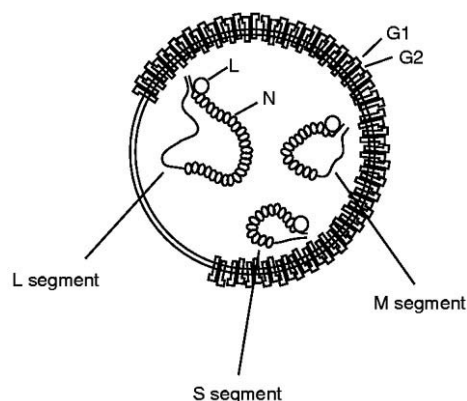


Figure 1 Schematic representation of a bunyavirus particle, showing the two virion spike glycoproteins, G1 and G2, and the three RNA genome segments L, M and S encapsidated by the N (nucleocapsid) protein and associated with the L (RNA-dependent RNA polymerase) protein. From J.F. Simons, PhD Thesis, Stockholm, 1992.

Table 1 Pathogenic Bunyaviridae

Genus	Vector	Serogroup	Pathogenic viruses
<i>Bunyavirus</i> (over 150)	Mosquito	Anopheles A (12)	Tacaiuma
		Anopheles B (2)	—
		Bakau (5)	—
		Bunyamwera (32)	Bunyamwera, Cache Valley ^a , Calovo, Germiston, Guaroa, Ilesha, Maguari, Tensaw, Wyeomyia
		Bwamba (2)	Bwamba
		C group (14)	Apeu, Caraparu, Itaqui, Madrid, Marituba, Murutucu, Oriboca, Ossa, Restan
		California (14)	<i>California encephalitis</i> , La Crosse, Inkoo, Jamestown Canyon, snowshoe hare, Tahyna, trivittatus
		Capim (10)	—
		Gamboa (8)	—
		Guama (12)	Catu, Guama
		Koongol (2)	—
		Minatitlan (2)	—
		Nyando (2)	—
		Olifanstsvlei (5)	—
		Patois (7)	—
		Simbu (24)	Aino ^a , Akabane ^a , <i>Oropouche</i> , Shuni
		Tete (6)	—
		Turlock (5)	—
		Ungrouped (4)	—
<i>Hantavirus</i> (>8)	None	Hantaan (>8)	<i>Bayou, Black Creek Canal, Dobrava, Hantaan, Muerto Canyon, Puumala, Seoul, Sin Nombre</i>
<i>Nairovirus</i> (34)	Tick	Crimean–Congo (3)	<i>Crimean–Congo hemorrhagic fever</i> , Hazara
		Dera Ghazi Khan (6)	—
		Hughes (10)	Soldado
		Nairobi SD (3)	Dugbe, Ganjam ^a , Nairobi Sheep Disease ^a
		Qalyub (3)	—
		Sakhalin (7)	Avalon
		Thiafora (2)	—
<i>Phlebovirus</i> (57)	Sandfly	Phlebotomus (44)	Alenquer, Candiru, Chagres, <i>Sandfly fever, Naples & Sicilian</i> , Punta Toro, <i>Rift Valley Fever</i> ^b , Toscana
	Tick	Uukuniemi (13)	Uukuniemi, Zalev-Terpeniya
<i>Unassigned</i> (53)	Mosquito		Bangui, Kasokero, Tataguine
	Tick		Bhanja, Keterah, Tamdy, Wanowrie
<i>Tospovirus</i> (>3)	Thrip	Tomato spotted wilt	

The approximate number of viruses in the genus or serogroup is given in parentheses.

^aViruses causing important animal diseases.

^bMosquito vector for Rift Valley fever virus.

Viruses in italics are responsible for important human diseases.

segment, and two different strategies are found for the M segment. Viral replication occurs in the cytoplasm of infected cells, using a Golgi-associated morphogenesis with budding into intracytoplasmic vacuoles.

The most important bunyavirus disease of man is probably California encephalitis, which occurs in North America and affects mainly children. Most infections are due to La Crosse, snowshoe hare or Keystone viruses rather than to California encephalitis virus itself. These viruses are maintained in nature in small rodents and are transmitted to man by mosquito bites, *Aedes triseriatus* being the principal vector. Aino and Akabane are two Simbu serogroup

bunyaviruses which are causes of congenital malformations (arthrogryposis, hydranecephaly and anencephaly) in sheep, goats and cattle in Japan, Australia, East and South Africa, Cyprus and Israel. Oropouche virus is another Simbu serogroup bunyavirus which has caused thousands of human infections in Brazil, many involving the central nervous system, although fatalities are very rare.

Hantaan virus and related hantaviruses cause Korean hemorrhagic fever, otherwise known as hemorrhagic fever with renal syndrome (HFRS), a severe and often fatal infection that occurs across Asia from China into Europe. In Scandinavia, Puumala and related viruses cause a much milder clinical

syndrome known as nephropathia epidemica. In 1993 a new and severe illness known as Hantavirus pulmonary syndrome (HPS) was recognized in the southwestern USA, and by 1996 similar infections had been identified in Canada, Brazil, Paraguay and Argentina, associated with three different hantaviruses, Sin Nombre, Black Creek Canal and Bayou viruses. The overall mortality in 128 confirmed cases of HPS in the USA to February 1996 was 50%, with a peak incidence in June and July. Most cases have been in rural residents. Rodent association with human disease is indicated in Table 2.

Crimean–Congo hemorrhagic fever, which occurs in countries of the former USSR, the Middle East and Africa, as far south as the Cape, is the most important *Nairovirus* infection, having a mortality of around 20%. Deaths have occurred in hospital staff following surgical intervention in patients who have presented with severe intestinal bleeding.

Phlebotomus fever occurs in the Mediterranean and Middle East regions as a mild, but troublesome erythematous fever, but the most important *Phle-*

bovirus is Rift Valley fever virus, transmitted by mosquitoes rather than phlebotomines. Rift Valley fever is enzootic in sub-Saharan Africa and produced a major human epidemic with some 600 deaths in Egypt in 1977; a decade later another epidemic occurred in Mauritania, and there have been many smaller outbreaks in humans and animals.

Immune responses of the host

Bunyaviridae are highly immunogenic, inducing neutralizing antibodies which are type specific but show limited cross-reactivity within the genus. Recovery is normally followed by solid protection against reinfection. In some infections the immune response occurs too late to prevent invasion of the central nervous system or the liver. Thus animal infection with Rift Valley fever virus can produce overwhelming infection, with liver necrosis and high mortality. Some American hantaviruses target the lungs, rather than the liver, kidneys or central nervous system, resulting in hantavirus pulmonary syndrome. The intense inflammatory response in these infections leads to the release of mediators followed by increased vascular permeability throughout the lungs, and consequent clinical disease.

Antibodies against the G1 glycoprotein neutralize viral infectivity, and inhibit hemagglutination. The nucleocapsid protein is the dominant antigen in complement fixation tests. There have been few studies of cell-mediated immunity, but mice infected with Tahyna *Bunyavirus*, which normally recover following a subclinical infection, die when immunosuppressed with cyclophosphamide. Circulating immune complexes have been reported in nephropathia epidemica.

Evasive strategies by the organism

The possession of a segmented genome allows the possibility of genetic reassortment, which has been demonstrated in the laboratory and also occurs in nature.

Vaccines

Formalin-inactivated veterinary vaccines against Rift Valley fever and Nairobi sheep disease have been available for a number of years, but vaccines for use in humans are still experimental. Laboratory workers immunized with inactivated Rift Valley fever virus vaccines develop high levels of neutralizing antibodies and remain free from infection. Attenuated strains of Rift Valley fever virus have been developed with a view to their possible use as live viral vaccines. Hantavirus vaccines are being developed: one prepared using brains of newborn

Table 2 Taxonomic relationship between rodents and their related hantaviruses (associated human diseases in parentheses)

Order Rodentia	
Suborder Sciurognathi	Genus <i>Hantavirus</i> (family Bunyaviridae)
Family Muridae	
Subfamily Arvicolinae	
Genus <i>Clethrionomys</i>	
Species <i>glareolus</i>	Puumala (Nephropathia epidemica/HFRS)
<i>Microtus</i>	
<i>pennsylvanicus</i>	Prospect Hill
<i>californicus</i>	Isla Vista
Sigmodontinae	
<i>Peromyscus</i>	
<i>maniculatus</i>	Sin Nombre (HPS)
<i>leucopus</i>	New York 1 (HPS)
<i>Oryzomys</i>	
<i>palustris</i>	Bayou (HPS)
<i>Reithrodontomys</i>	
<i>megalotis</i>	El Moro Canyon virus
<i>mexicanus</i>	Rio Segundo virus
<i>Sigmodon</i>	
<i>hispidus</i>	Black Creek Canal (HPS)
<i>Oligoryzomys</i>	
<i>microtis</i>	Rio Mamore virus
Murinae	
<i>Apodemus</i>	
<i>agrarius</i>	Hantaan (HFRS)
<i>flavicollis</i>	Dobrava/Belgrade (HFRS)
<i>Rattus</i>	
<i>norvegicus</i>	Seoul (HFRS)

mice has been used in South Korea with no significant adverse reactions.

See also: Attenuated organisms as vaccines; Vaccines; Viruses, immunity to.

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BURSA OF FABRICIUS

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The bursa of Fabricius is a sac-like lymphatic organ present only in birds and situated dorsal to the cloaca. It was first described by Hieronymus Fabricius, Professor of Anatomy and Surgery at Padua University in 1565–1610. Fabricius erroneously proposed the bursa to be a female organ into which the cockbird delivers semen, so that it may be stored. It was William Harvey, the most famous of the many students of Fabricius, who pointed out that the organ was present in both sexes and could not serve the function assumed by Fabricius. Thereafter it took more than three centuries until a serendipitous experiment by Bruce Glick in 1955 revealed the central role of the bursa of Fabricius in antibody production. Glick was involved in a demonstration for students, of antibody production against *Salmonella*. The only animals available were from an experiment where the birds had had the bursa of Fabricius removed soon after hatching. These birds failed to produce the antibody.

Development and structure

The bursal anlage is first observed in the chicken embryo at 4 days of incubation as an epithelial bud in the cloacal region. While enlarging, the bud becomes increasingly vacuolized, the coalescence of the vacuoles giving rise to the bursal lumen. Epithelial longitudinal folds are formed on the inner sur-

face of the sac, and the columnal epithelium, covering the plicae, proliferates and forms outgrowths into the underlying lamina propria. Formation of these epithelial buds is dependent on interaction between the mesenchymal and epithelial tissues. Each epithelial bud is colonized by one or two prebursal stem cells. They proliferate vigorously, leading to formation of the medulla of the bursal follicles, which start to appear on day 12 of incubation in the chicken embryo. Prebursal stem cells originate in the intra-embryonic mesenchyme. On arrival at the bursa they have the germ-line immunoglobulin (Ig) genes rearranged, being already committed to development along the B cell lineage. The period of prebursal stem cell arrival in the chicken bursa extends from day 8 to day 14 of incubation. Cells bearing IgM on the surface appear in the bursa on day 12 of embryogenesis. At the time of hatching, about 90% of the bursal cells carry surface IgM and <1% IgG or IgA.

The epithelial anlage of the bursa of Fabricius is decisive in providing differentiation signals to the arriving prebursal stem cells and to their descendants, the bursal stem cells. How the signals are mediated remains unknown, as no definitive evidence, e.g. for existence of a specific bursal hormone, is available.

Epithelial cells within the medulla form a loose cellular network with desmosome connections, and

they are secretory in morphological structure. In addition to epithelial cells, the fully developed bursal medulla contains small lymphocytes, plasma cells and phagocytic histiocytic cells. Less than 1% of the bursal cells are T cells. They are mostly located in a diffusely infiltrated area of lymphoid cells, dorsal to the bursal duct opening to the cloaca. At the time of hatching lymphoid cells also appear in the mesenchymal tissue surrounding each follicular medulla, and a follicular cortex develops. The medulla and cortex are separated by a basement membrane.

At the plical surface against the bursal lumen the epithelial cells differentiate into a follicle-associated

epithelium, with a specific function to phagocytose and transport antigenic material from the bursal lumen into the follicular medulla. The epithelial cells in the follicle-associated epithelium and within the bursal medulla are different in their phagocytic capacity and enzyme activities. Structural organization of the bursa is presented in Figure 1.

At the time of hatching the chick bursa of Fabricius weight 30–40 mg and reaches a maximum of 3–4 g at the age of 2–4 months. Thereafter, with the beginning of sexual maturation, it involutes, and both the lymphoid and epithelial structures have completely disappeared at the age of 6–12 months.

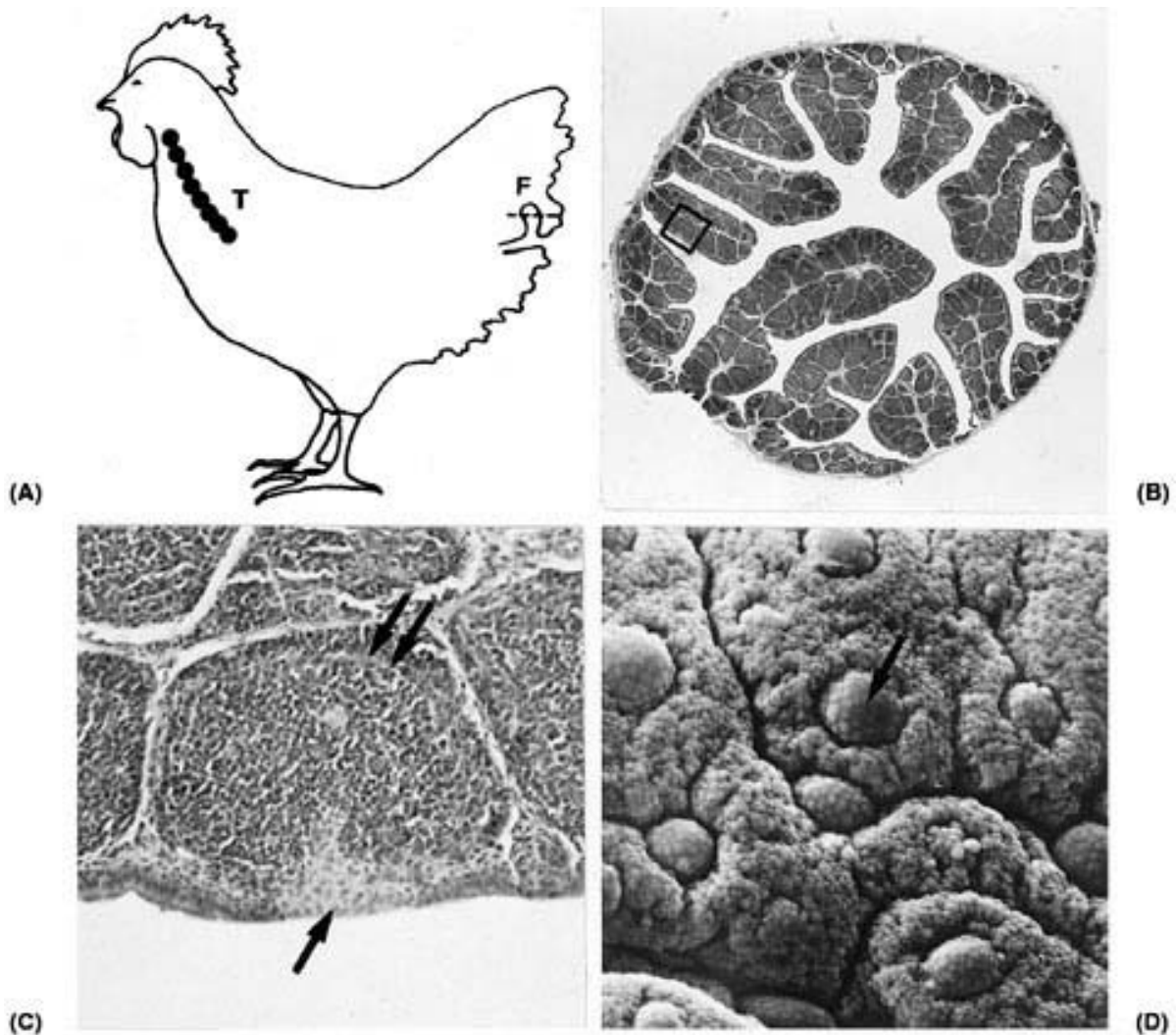


Figure 1 (A) Location of bursa of Fabricius (F) and thymus (T). (B) Cross-section of bursa of Fabricius at the level indicated by a broken line in Figure 1A. The plicae are longitudinal in dorsoventral direction, with a bursal lumen opening ventrally to the cloaca; 4-day-old chick. H & E; magnification $\times 10$. (C) A bursal follicle from the area marked with a square in Figure 1B. The cortex and medulla are separated by a basement membrane (double arrows). The interfollicular epithelium against the bursal lumen is broken by the follicle-associated epithelium (arrow). H & E; magnification $\times 250$. (D) Scanning electron micrograph of plical surface against the bursal lumen. The phagocytosing follicle-associated epithelium appears as clearly distinguishable areas (arrow). Magnification $\times 200$.

Bursectomy

Experiments on birds with the bursal tissue removed have proved crucial in revealing the function of the bursa. Surgical bursectomy in the newly hatched period is not sufficient to prevent migration of B cells into the periphery because the cellular outflow starts during the embryonic period. The same holds true if the surgical bursectomy is carried out during late embryogenesis. More complete results have been obtained when the surgical bursectomy has been combined with *in ovo* treatment with antibodies against the heavy chain of IgM. Surgical removal of the bursal anlage can also be performed before the bursa is populated by prebursal stem cells. However, even in such animals, operated on days 2–5 of incubation, some plasma cells and antibodies with extremely limited antibody repertoire are found, indicating restricted extrabursal B cell maturation.

Other methods of bursectomy include treatment with testosterone or cyclophosphamide. Testosterone given *in ovo* also destroys the epithelial compartment of the bursa, whereas cyclophosphamide leaves it

intact, allowing experimental repopulation by prebursal or bursal stem cells.

Central function

The bursa of Fabricius functions as a central lymphoid organ, required for development of the antigen-specific B cell repertoire. It is necessary for the differentiation of prebursal stem cells into bursal stem cells present in the bursa until the 5th week after hatching. These cells are capable of reconstituting the bursal morphology and specific antibody production when transferred into birds with the bursal lymphoid structures destroyed with cyclophosphamide in the newly hatched period. They comprise a clearly defined stage in the stepwise maturation of the avian antibody-producing cells.

The bursa of Fabricius is necessary for the development of the postbursal B cell population, including the mature antigen-specific B cells and the self-renewing postbursal stem cells. Neither of these populations is capable of homing, in contrast to the

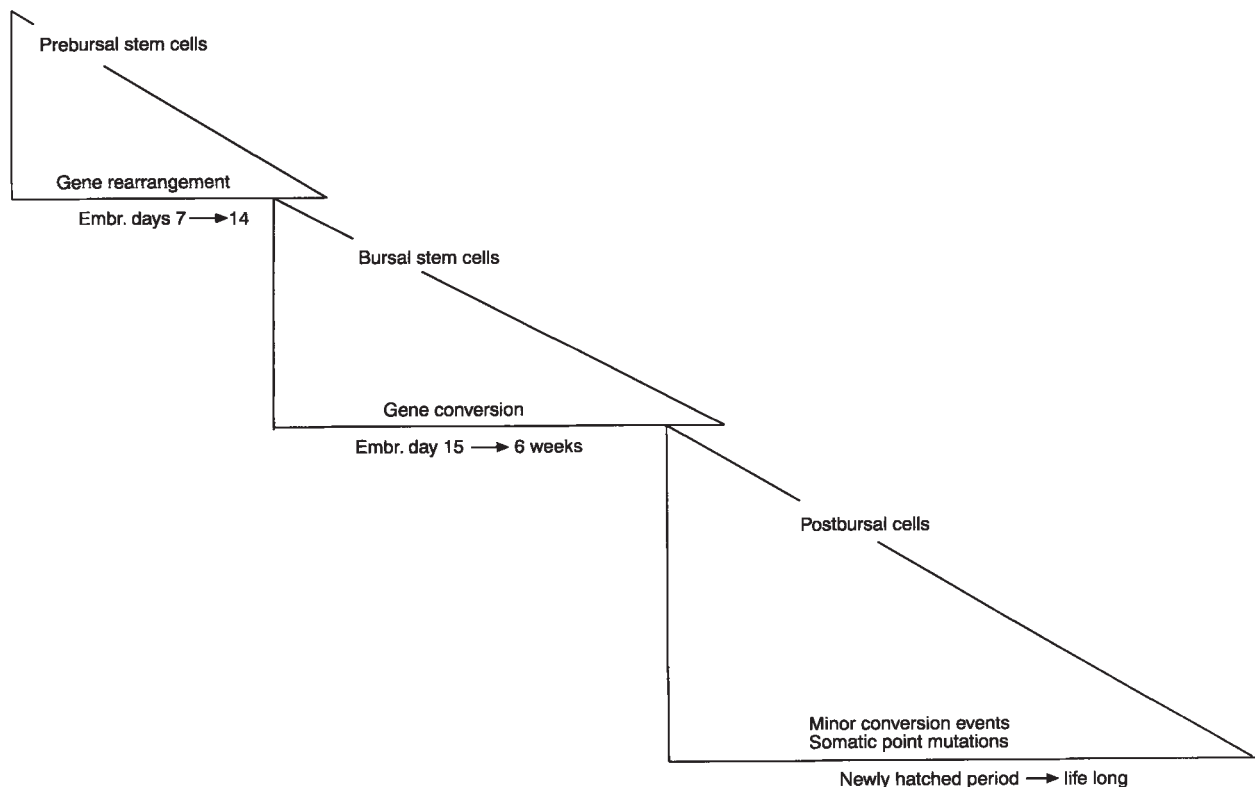


Figure 2 The bursa of Fabricius is necessary for the development of the antigen-specific B cell repertoire. Prebursal stem cells with germ line immunoglobulin genes rearranged are found in the intraembryonic mesenchyme from embryonic (Embr.) day 7 onwards. After arrival in the bursa of Fabricius they become bursal stem cells undergoing extensive, high rate gene conversion, which leads to diversification of the germ line repertoire; these cells need the bursal microenvironment for further maturation. Postbursal cells, present both in the bursa and periphery, include mature antigen-specific B cells and self-renewing postbursal stem cells, capable of further maturation without bursal influence.

prebursal or bursal stem cells, to the cyclophosphamide-treated bursal reticulum, even though they are capable of reconstituting antibody production. In other words, the postbursal cells have passed the stage of bursal development once and for all.

The bursa of Fabricius is necessary for the diversification of the antibody repertoire by providing a site for extensive, high rate gene conversion, which occurs between only one functional V gene (V_{H1} or V_{L1}) and a group of V pseudogenes. The bursal microenvironment selects and promotes for further differentiation bursal stem cells undergoing productive gene conversions, whereas all the others (>90%), probably with unproductive conversion events, die within the bursa. Only minor gene conversion events and somatic point mutations may occur outside the bursa. Molecular events in avian B cell development, as related to the central function of the bursa of Fabricius, are summarized in Figure 2.

Peripheral function

At the time of hatching the bursal lumen opens to the cloaca, allowing environmental antigens to enter the bursal space. The antigens arrive in the cloaca after digestion in the gastrointestinal tract and, with the help of anal sucking movements in the newly hatched period, also through the caudal end of the cloaca. The bursal follicles in turn have contact with the bursal lumen through the phagocytosing follicle-associated epithelium.

A peripheral function of the bursa of Fabricius in the uptake of environmental antigens has an important role in enforcing the expansion of specifically reacting B cell clones which lead to formation of plasma cells and memory cells.

Mammalian equivalent

In the mammalian species the basic, preimmune repertoire of antibody diversity is created in the fetal

liver and bone marrow; the role of intestinal lymphoid tissue is negligible, as it is only weakly developed during the fetal period. In the postnatal expansion and selection of the antibody repertoire the intestinal lymphoid tissue also has an important function in the mammalian species. Peyer's patches play a key role here and have a similar follicular structure as in the bursa of Fabricius. In sheep, Peyer's patches may even have a central function similar to that of the bursa of Fabricius.

Mammalian species generate the antibody repertoire by use of multiple functional V (V_{H1} and V_{L1}) genes when compared to the few functional ones in the avian species. Therefore, by relying on gene rearrangement and other types of somatic diversification, mammals are able to create an extensive antibody repertoire without having an organ particularly specialized for this purpose.

See also: Avian immune system; B lymphocyte differentiation; Diversity, generation of; Gene conversion; Humoral immunity; Immunodeficiency, animal models.

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BYSTANDER EFFECTS

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The fine specificity of effector cells and molecules for an eliciting antigen is usually considered the most important feature of an immune response. However, each immune response can in fact be viewed as being composed of two parts: 1) direct, antigen-specific interactions between cells, resulting in the specific

response towards the antigen; and 2) indirect, non-antigen-specific phenomena, termed 'bystander' effects, resulting in polyclonal responses. Both aspects of the response occur simultaneously and account for the overall response, which comprises both specific and nonspecific events.

Bystander effects, as opposed to antigen-specific interactions, can therefore be defined as the result of cellular interactions occurring without antigen recognition or under conditions where antigen and receptors for antigen are not involved. However, bystander effects are described as phenomena linked to the specific immune response, in that they do not happen on their own but only in connection with a specific response. Cells that are not directly involved in the antigen-specific responses are then 'trans-stimulated' or 'carried along' in the response.

Experimental evidence for bystander effects

The existence of bystander phenomena involving T cell as well as B cell responses has been demonstrated in several *in vitro* experimental models.

T helper cell responses

Specific T cell proliferative responses are elicited by recognition through the T cell antigen receptor of the specific antigenic complex presented by specialized cells. Antigen-specific primed T cells proliferate when challenged *in vitro* with their specific antigen. However, in addition to antigen-specific T cells, the proliferative response can also affect neighboring T lymphocytes with unrelated T cell receptors. Proliferation of bystander T cells occurs independently of antigen recognition on antigen-presenting cells but simultaneously with a specific T cell proliferative response.

B cell responses

One of the most studied bystander phenomena concerns the T cell-dependent B cell response. B cell proliferation and antibody production in response to most soluble proteins requires the participation of T helper cells. This antigen specific T-B cooperation involves linked recognition of the antigen: the epitopes recognized by T and B cells need not be identical but need to be linked. This effect is known as the hapten-carrier effect. The interaction between T and B cells is termed cognate interaction and leads to the secretion by B cells of specific antibodies directed towards the eliciting antigen.

However, several *in vitro* experimental situations suggest that B cell responses can also be elicited under conditions of 'unlinked' recognition, where the antigens recognized by T helper cells and B cells are not covalently linked (bystander help).

When cultured in the presence of naive B cells and antigen, preactivated T cells, or T cell lines specific for a given antigen, elicit a specific B cell response

and antibody production (linked or cognate response). In addition, if another, unrelated antigen is added to the culture, antibody production against this unrelated antigen can also be observed in the culture (unlinked or bystander response). For example, sheep red blood cell (SRBC)-specific T cells induce SRBC-specific antibody production by B cells if SRBC but no horse red blood cells (HRBC) are present in the culture (specific response). However, when SRBC and HRBC are added together, simultaneous production of antibodies to SRBC and to HRBC is observed (specific and bystander response).

Other systems involving the bystander proliferation of B cells in response to specific T cells and antigens have been described. In response to cognate T-B cell interaction syngeneic B cells proliferate but proliferation of allogeneic B cell can also be observed in the culture. In this case, allogeneic B cells do not bear the appropriate major histocompatibility complex (MHC) class II antigens to be recognized by the T cell receptor but they are stimulated by nonspecific signals and/or interactions.

CTL responses

Cytotoxic T cells (CTLs) are differentiated effector T lymphocytes that specifically kill target cells bearing an appropriate antigenic complex (peptide-MHC) recognized by their T cell receptor. However, during this process, nonspecific lysis of unrelated bystander target cells can be observed in the culture. The unrelated target cells do not exhibit the appropriate antigenic complex on their surface and therefore cannot be recognized by the T cell receptor. This phenomenon can be detected *in vitro* using cytotoxic T cell lines of defined specificity and mixing together target cells bearing the appropriate or unrelated antigenic complex.

Possible mechanisms controlling bystander effects

Trans-stimulation of spectator T or B lymphocytes and killing of unrelated target cells imply that a signal can be transmitted to these cells independently of the structures involved in specific antigen recognition. Several explanations have been proposed but the exact mechanism controlling this phenomenon is still a matter of debate. In fact, whether there is an essential difference between specific and bystander effects is not clear and, apart from the process of antigen recognition, mechanisms involved in specific cellular responses could also be responsible for bystander responses.

Cytokines

The most accepted explanation for the existence of such nonspecific effects is the secretion of soluble factors – cytokines – by activated T lymphocytes. During the course of an antigen-specific T cell response, activated T lymphocytes release various lymphokines and, in particular, interleukin 2 (IL-2), IL-4, interferon γ (IFN γ) and tumor necrosis factor β (TNF β ; lymphotoxin). These lymphokines exhibit nonantigen specific, pleiotropic effects. They could act on any other cell present at the site of the immune response. These cells could be T or B lymphocytes, macrophages or target cells for CTLs, provided they are competent to respond to lymphokines (i.e. they express functional surface receptors for these factors). Local effects of such nonspecific factors on neighboring cells not directly involved in the antigen-specific response could explain the involvement of these cells in the overall response: for example, IL-2 could induce the proliferation of T cells and CTLs; IL-4 could transmit activation signals to naive B cells; and IFN γ and TNF β could prepare target cells for CTL lysis.

Stage of lymphocyte activation

Bystander responses could be restricted to particular subpopulations of T or B lymphocytes that would be more susceptible to nonspecific signals. Of particular interest is the question of whether bystander effects involve cells in a particular stage of activation. Since cytokines are likely to be responsible for bystander effects, expression of cytokine receptors by the reacting cell may be an important key in this phenomenon.

Cell surface antigens

Cell-to-cell contact through specific and nonspecific cell surface molecules is obviously an important feature of antigen-specific responses. Similarly, nonantigen-specific cell-to-cell contact could occur during bystander responses. This could involve nonpolymorphic surface molecules, conserved regions of polymorphic cell surface molecules (including MHC molecules), or molecules induced after stimulation.

Polyclonal B lymphocytes can be stimulated in cultures containing T cells and a T cell mitogen or polyclonal activator (such as anti-CD3 or anti-T cell receptor monoclonal antibodies). Under these conditions, polyclonal B cell proliferation and antibody production are observed. Therefore, when efficient

T cell stimulation is obtained, induction of a B cell response without any added antigen can be observed. In this case, bystander B cell stimulation is thought to be dependent upon T–B cell contact and probably involves recognition of B cell surface molecules by T cells (e.g. CD28/B7 interactions).

Similarly for CTL responses nonspecific attachment of any target cell to a specific cytotoxic T lymphocyte by means of lectins causes the lysis of the target cell independently of antigen recognition.

In vivo significance of bystander effects

Although bystander antibody production has been observed for primary responses in the mouse, bystander effects are more readily observed *in vitro* than *in vivo* and their physiological significance has been questioned. Nevertheless, it is known that a polyclonal, nonspecific response frequently accompanies any specific immune response *in vivo*. In particular, B cell responses to a given antigen generally consist of antibodies recognizing the initial antigen, together with a significant production of nonspecific immunoglobulins.

Bystander effects have been evoked to explain the onset of autoimmune responses, as self antigens could be present at the site of the immune response elicited by a foreign antigen. *In vitro* bystander responses can be obtained towards self antigens or normally tolerated antigens.

Finally, bystander phenomena might not have a precise role in the effector phase of the immune response and might simply reflect the existence of a network of connections between the different cellular elements of the immune system. These connections could be maintained by the cytokine network or cell surface molecule interactions. Altogether these interactions may participate in the homeostasis of the immune system.

See also: Cooperation, mechanisms of cellular; Cytokines; Enhancement, immunological; Proliferation, lymphocyte; Specificity.

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C

CALICIVIRUS, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

General features

The caliciviruses (Table 1) comprise a monogenic family of 34 nm, nonenveloped viruses (Figure 1) with characteristic morphology; the surface is covered with cup-shaped depressions (*L. calix*, cup). In some caliciviruses (insect, rabbit) smooth particles are formed, possibly by partial proteolysis. These lack surface projections and are composed of smaller proteins. The calicivirus genome is single-stranded, positive-sense RNA and genes are arranged in two basic patterns.

Table 1 Probable and confirmed caliciviruses

Virus	Serotypes	Infection
San Miguel sea lion virus	16 or more	Vesicular
Vesicular exanthema of swine	13	FMDV-like
Feline calicivirus	1	URTI, stomatitis, limping syndrome
Rabbit hemorrhagic disease virus	1	Hemorrhagic hepatitis
European brown hare syndrome virus	1	Hemorrhagic hepatitis
Human calicivirus	5 or more 'typical' HuCV; 4–9 SRSV	Enteric infections
Hepatitis E virus	1	Hepatitis
Canine calicivirus	At least 2	1 Enteric; 1 vesicular
Infectious stunting calicivirus of chickens	?	Enteric infections
Newbury agent of cattle	2?	Enteric infections
Porcine enteric calicivirus	?	Enteric infections

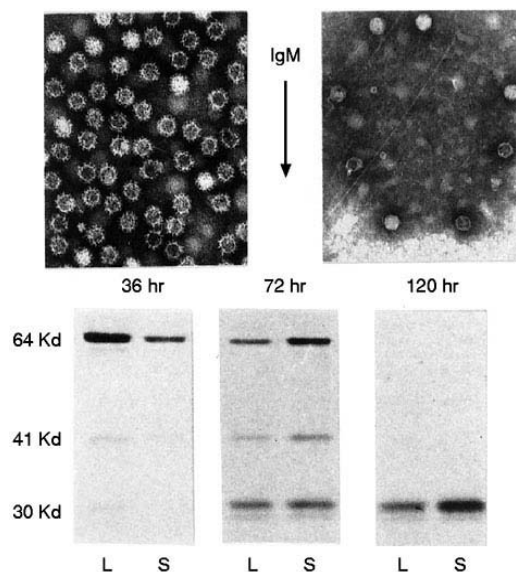


Figure 1 Particle morphology. Calicivirus particles (34 nm) are built from a single species of capsid protein which gives a characteristic morphology, unique among animal viruses; the structural proteins are linked by arch-like protuberances which ring the points of five- and threefold symmetry, giving the impression of cup-like depressions on the surface of the virus. Partial proteolysis occurring around 72hr pi may degrade the capsid protein forming smooth particles. Figures courtesy of Dr Lorenzo, Istituto Zooprofilattico Sperimentale, Brescia, Italy.

Feline calicivirus, some human caliciviruses, vesicular exanthema and San Miguel sea lion virus possess a genome that contains three sequential open reading frames (ORFs). ORF 1 encodes nonstructural proteins and ORF 2 the capsid protein. ORF 3 is of unknown function but known to be used. This basic structure appears to be flexible and in some viruses ORFs 1 and 2 are fused (e.g. rabbit hemorrhagic disease virus, European brown hare syndrome virus and some human caliciviruses). All viruses appear to synthesize two separate mRNAs, the first to direct the manufacture of ORF 1 proteins, and the second for proteins from both ORFs 2 and 3. Additional open reading frames have been

reported in some of these viruses but there is no evidence that these are functional.

Hepatitis E virus (HEV), also possesses three ORFs but there are significant differences in the sequence of the genes. There is no protein equivalent of ORF 3 in the former viruses; the third ORF of HEV differs in position, sequence and probably in manner of expression. These differences have led to calls for the subdivision of the family. Multiple genera will certainly be required to account for these differences and HEV may be removed to a subfamily or possibly a different family entirely.

Best characterized members

HEV is endemic in parts of eastern Europe, Asia, Africa and South America. Symptoms and transmission are similar to hepatitis A and both infections are more severe in adults. Pregnant women are particularly vulnerable and can develop fulminant hepatitis (20–40% mortality).

Human caliciviruses were recognised as a cause of gastric illness in the 1970s; symptoms include diarrhea, vomiting, pyrexia, anorexia, abdominal pains, aching joints and nausea. Historically, human caliciviruses have been divided according to the clarity of their morphology: those with obvious calicivirus appearance were designated 'typical caliciviruses' (HuCV), e.g. HuCV/Sapporo; those in which the cup structure was indistinct were termed 'small round structured viruses' (SRSV), e.g. Norwalk virus (NV) and Hawaii virus (HV). However, underlying particle architecture and genome organization were similar in both cases and all are now termed human caliciviruses (HuCV); any subdivisions are best made according to RNA sequence. HuCV infect all age groups, although reportedly some occur more often in children (e.g. HuCV/Sapporo) and others in older individuals (NV, HV), but age distribution of infection varies geographically. When infections occur in hosts of the same age the clinical features are indistinguishable. Spread is through contaminated water or food, and especially consumption of shellfish from contaminated waters.

San Miguel sea lion virus (SMSV) is a common cause of death in neonatal pinnipeds and causes abortions in California sea lions. Vesicles or wart-like lesions occur on the tail and flippers.

Vesicular exanthema of swine virus (VESV) was identified in 1932 and is clinically indistinguishable from foot-and-mouth disease in pigs. High fever is often accompanied by severe diarrhea; vesicular lesions form on the tongue, lips and snout and between the toes. Strains vary in severity and some carry a high risk of piglet mortality. VESV is closely related to SMSV and may have originated as a cross-

species infection, transmitted to pigs through uncooked sea lion meat in swill.

Feline calicivirus (FCV) is a major cause of upper respiratory tract infection (URTI) in Felidae but symptoms also include conjunctivitis, stomatitis and sometimes diarrhea. Some strains also cause polyarthritis (limping syndrome). Virulence is variable; mortality may reach 33% in kittens. Recovery is commonly accompanied by virus clearance, but persistent infections can be established in which apparently healthy animals continue to shed virus.

Rabbit hemorrhagic disease virus (RHDV) was described in China (1984), and is now widespread. Adult mortality is 40–90%, but younger animals (<2 months) often survive; this is not due to maternal antibody. RHDV is of major economic and ecologic importance, killing 64 million farmed rabbits in Italy in 1989, and drastically diminishing wild populations. Infection causes high fever and hemorrhagic viremia, and virus reaches high titer in many organs. Retrospective serology shows that a related (presumably nonpathogenic) virus predated the emergence of this condition.

European brown hare syndrome virus (EBHSV) is related to RHDV and has occurred throughout Europe since the 1980s. Clinical signs are similar, with high adult mortality (90–100%); however, the viruses do not cross host species and spread independently.

Antigen structure of the viruses

Many caliciviruses (especially human viruses) cannot be grown in culture, and conventional serotyping is impossible. Noncultivable viruses have been typed by ELISA, immunoelectron microscopy (IEM) or solid phase IEM (SPIEM) and both type-specific and common epitopes exist. However, reagents have been limited and independent classification schemes have been devised. These should be unified now that recombinant antigens are available.

FCV and RHDV/EBHSV are the best understood. The single structural protein is the major antigen for all viruses. In FCV neutralizing antibodies bind to a highly variable region of the capsid protein in the C-terminal half of the protein, which is presumably exposed on the virus surface. This region is bisected by a conserved sequence of 20 amino acids which may contribute to the cell-binding site, and antibodies binding here may prevent attachment; variability in this region could allow FCV to evade immune control. Nonneutralizing antibodies bind elsewhere. RHDV and EBHSV strains are well conserved and so potentially variable regions cannot be identified. However, most monoclonal antibodies (including those which are protective) also recognize

the C terminal half of the protein. Such antibodies tend to be specific for RHDV or EBHSV and react poorly with smooth particles which lack the surface projections. Conversely, antibodies recognizing smooth particles tend not to be protective and react with both RHDV and EBHSV. Thus the antigenically distinctive surface structure of the virus is probably formed by the C-terminal of the protein, while underlying structures are similar.

Immune responses of the host

Feline calicivirus

FCV infection causes transient lymphopenia and neutrophilia, but cats may also be infected with feline immunodeficiency virus (FIV), which also decreases lymphocyte number and immune response. Thus coinfection with FCV and FIV increases the clinical severity of infection. However, FIV has no effect on the period of FCV shedding and is not important in the establishment of persistent infections.

Persistently infected cats mount both cell-mediated and humoral immune responses but fail to clear the virus promptly. The reasons for this are unknown. The tonsils are the major site of persistence but tonsillectomy does not cure the infection and other lymphoid tissue may be involved; feline T lymphoblastoid cell lines have been persistently infected with FCV. Persistence may be lifelong but some studies imply a half-life of approximately 75 days, with animals spontaneously eliminating the virus.

Human caliciviruses

Seroprevalence studies still reflect the previous distinction between the typical caliciviruses and SRSVs (above). Exposure to one of these forms (SRSVs in particular) may induce antibody which cross-reacts with other viruses of the same form (i.e. other SRSVs). These cross-reactions may be one-way (presumably because the immunogenicity of certain epitopes is sometimes functionally masked). Thus a fourfold rise in antibody titer is not sufficient for diagnostic purposes, and an eightfold rise may be preferable; alternatively IgA is more type specific.

Reinfection by NV is believed to be common; surprisingly, pre-existing antibody (secretory or serum) has been correlated with susceptibility to illness. Recovery was associated with an antibody rise, but many volunteers were soon susceptible, becoming ill again on rechallenge. Thus adults may differ in susceptibility to NV – sensitive individuals being protected only by high titers acquired after several infections; however, other studies have not confirmed this correlation.

In Scandinavian countries, infection occurs early in childhood but adults are protected. Thus age at first exposure could be relevant to protection in adults. Swedish children soon develop immunoglobulin G1 (IgG1) responses to NV, but persons over 21 years old also showed NV-specific immunoglobulin G4 (IgG4), indicating repeated exposure, but it is not known how often this was associated with illness. Immunity to these agents thus requires more investigation.

In contrast, antibody to the morphologically typical HuCV is protective and mainly type specific. Antibodies to HuCV/Sapporo were detected in 65% of Japanese children aged 2–5 years and in 90% of those aged 6–12 years. A second virus, UK1, also circulates in Japan and 69% of children aged 6–12 years had antibodies to both viruses. Both viruses are probably distributed worldwide.

Evasive strategies of the virus

The FCV capsid protein is known to be variable (above). FCV comprises a single serotype but variation is such that sequential infection by antigenic variants is possible. During infection (persistent or acute), the antigenic profile of the virus may drift; virus isolated later in infection may be more vaccine resistant. This drift may allow the virus to resist herd immunity. It may also affect pathology by influencing host tissue range; for example, enteric forms of FCV resist bile salts, while respiratory forms do not. However, bile-resistant variants can be selected from bile-sensitive populations. Monoclonal antibodies may differentiate between strains associated with different clinical presentations, implying that some determinants of pathology may be close to antigenic regions.

Vaccines

Vaccines are available for FCV and RHDV. The FCV vaccine is based on a live virus, using a single strain (F9), chosen for low pathogenicity and cross-reactivity; it induces neutralizing antibody to approximately 50% of field strains. Protection is probably wider, as non-neutralizing antibody can protect *in vivo*. Recently FCV field strains have changed; this may reflect natural variation, but the effects of widespread vaccination with a strain of restricted antigenicity cannot be ruled out. These changes have not yet undermined the usefulness of F9 as a vaccine. Vaccine 'failure' is probably due to inadvertent vaccination of cats already incubating the virus after natural infection. Isolates obtained from postvaccination complications are not normally vaccine

related, but are usually wild-type strains, susceptible to neutralization by vaccine-induced antibody. Multivalent vaccines may be required in future because low levels of antibodies to different strains give better neutralization than high levels of antibody to just one. Vaccination neither prevents nor cures a persistent infection.

The RHDV vaccine is made from virulent virus grown in rabbits and inactivated; it is effective but difficult to manufacture. The recently characterized nonpathogenic form of RHDV may be a suitable live vaccine if cultivation problems can be solved. Recently, recombinant virus-like particles (VLPs) have been developed for RHDV and are effective immunogens. VLPs have also been made for some of the human caliciviruses (e.g. NV and HV) but these have yet to be proven as vaccines.

See also: Antigenic variation; Epitopes; Fascioliasis; Gastrointestinal tract infections; Immunoassays; Vaccines; Viruses, immunity to; Viruses, infection of immune cells by.

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CAMPATH-1 ANTIGEN (CD52)

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The CD52 antigen of human lymphocytes was first defined with monoclonal antibodies isolated in the Department of Pathology, University of Cambridge. It is a lipid-anchored glycoprotein, unusual in having an extremely small peptide component. CD52 antibodies are remarkably lytic, killing cells *in vitro* with human complement, and also *in vivo*, probably by

other mechanisms. They have many possible applications for therapy, for example in treatment of leukemia, bone marrow transplantation, organ transplantation, rheumatoid arthritis, vasculitis and multiple sclerosis.

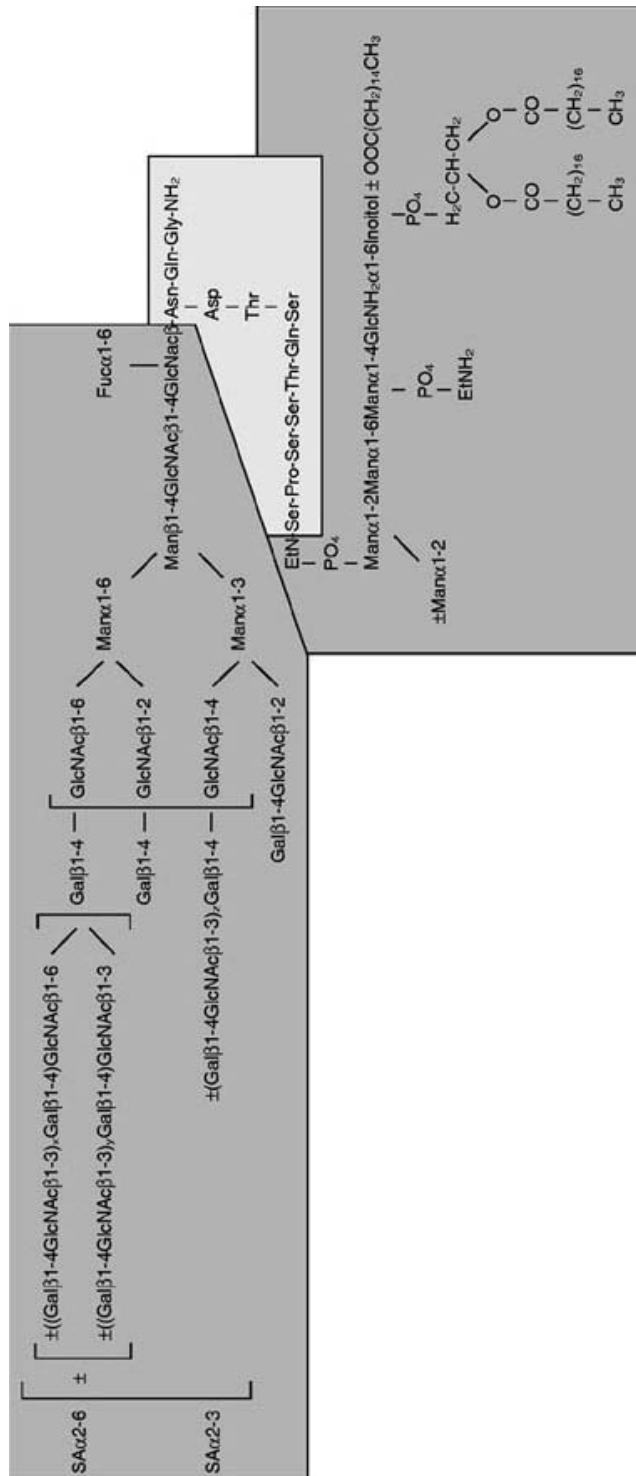


Figure 1 Primary structure of the CD52 antigen, showing the large N-linked carbohydrate, the small peptide and the glycosylphosphatidylinositol anchor. (Reproduced with permission from Treumann *et al.* (1995).)

Monoclonal antibodies

Many CD52 antibodies have been generated including different rat isotypes (e.g. CAMPATH-1M, rat immunoglobulin M (IgM) and CAMPATH-1G, rat IgG2b), matched sets of chimeric human/rat IgG and a variety of mutants. These have been used to demonstrate the role of IgG isotype for complement activation and Fc receptor binding, showing that rat IgG2b and human IgG1 are among the best subclasses for activating human effector systems. However, even human IgG4 (previously thought to be inert) was able to deplete human lymphocytes *in vivo*. CAMPATH-1H (human IgG1) was the first fully humanized monoclonal antibody. It was created by transplantation of the complementarity-determining regions of CAMPATH-1G into human heavy and light chain genes. Initial binding affinity was diminished, but this was corrected by small modifications to framework residues. Administration of the humanized antibody to patients has greatly reduced the possible antiglobulin response, compared with the original rat antibody. Following this experience, most other therapeutic antibodies are likely to be humanized.

Antigen structure

The CD52 antigen is well defined at a genetic and structural level, but its function is currently unknown. Encoded by a single gene on chromosome 1, it is a remarkably small glycoprotein, having only 12 amino acids (Figure 1). Attached to Asn3 is a complex carbohydrate consisting of sialylated polylactosamine units with a tetra-antennary fucosylated mannose core. At the C-terminus is a glycosylphosphatidylinositol (GPI) anchor. The CAMPATH-1 epitope includes the C-terminal amino acids and part of the GPI anchor. This implies that the antibodies bind close to the cell membrane, which explains why they are so good for cell lysis. Other antigens with a similar structure to CD52 include CD24 and its mouse homologue J5 (heat-stable antigen).



(A)



(B)

Figure 3 Treatment of polymphocytic leukemia with CAMPATH-1H. Complete clearance of tumor cells from blood and bone marrow was achieved, together with substantial reduction in the mass of tumor in the spleen, as shown by these computed tomograms taken (A) before and (B) after therapy. In total it was estimated that approx 4 kg of tumor was cleared by 126 mg of antibody. (Reproduced with permission from Hale *et al.* (1988).)

Tissue distribution

The distribution of the CD52 antigen is one of its most distinctive features. In humans the antigen is abundantly expressed on virtually all lymphocytes at most stages of differentiation (except plasma cells)

Human	GQ-----NDTS-----QTSSPS
Monkey	SQ-----NATS-----QSSPS
Mouse	GQATTAASGTINKNSFS-----TKKTP LKS
Rat	GQ-----NSTAVTTPANKAATTAAATTCAAATTATKTTTAVRKT PGK
Dog	G-----NST-----PRMTTKKVK SATPA

Figure 2 Comparison of CD52 protein sequences from different species showing the diversity of the mature sequences. Signal peptides (not shown) are much more similar. The human peptide has a single *N*-linked oligosaccharide. The rat peptide (SMemG) probably has, in addition, up to 14 *O*-linked oligosaccharides attached to the various Thr residues.

and also on monocytes, macrophages and eosinophils. T cells have approximately 450 000 molecules per cell. The antigen is not found on any other tissues except the male reproductive tract, where it is expressed very strongly on epithelial cells lining the epididymis, vas deferens and seminal vesicle. Antigen is shed from these cells in large quantities into seminal plasma, where it exists in small micellar particles and in larger membrane-bound structures called prostasomes. From these, it can be transferred to other cells, particularly spermatozoa. The CD52 antigen, along with other GPI-anchored antigens such as CD55 and CD59, is a major membrane protein of sperm, acquired during maturation. It is a matter of speculation whether transfer of GPI-anchored molecules from one cell to another is of more general physiological significance. Genes homologous to human CD52 have been described in monkey, dog, rat and mouse. They are all expressed in the male reproductive tissues and (where tested) in lymphocytes. Although the genes have substantial homology in the nontranslated regions and signal peptides, the short mature peptides are quite different (Figure 2).

Antibodies in therapy

CAMPATH-1 antibodies have been used in a variety of clinical trials. The IgM antibody, CAMPATH-1M, was developed for prevention of graft-versus-host disease by depletion of donor T cells from bone marrow. This was very effective, but resulted in an increased risk of graft rejection and of leukemia relapse. Now CAMPATH-1G or CAMPATH-1H are being tested for simultaneous control of both donor and host T cells, with a proportion of donor T cells being kept for later administration in the event of leukemia recurrence. CAMPATH-1H has been used for treatment of lymphoid malignancies and was very effective in patients with certain leukemias (T cell prolymphocytic leukemia and B chronic lymphocytic leukemia), but less so in patients with solid tumor masses (Figure 3). It has also been used as an immunosuppressive agent for treatment of transplant rejection and autoimmune diseases, including rheumatoid arthritis, vasculitis and multiple sclerosis (Figure 4). Treatment resulted in longlasting lymphopenia, particularly of CD4⁺ cells, which may not

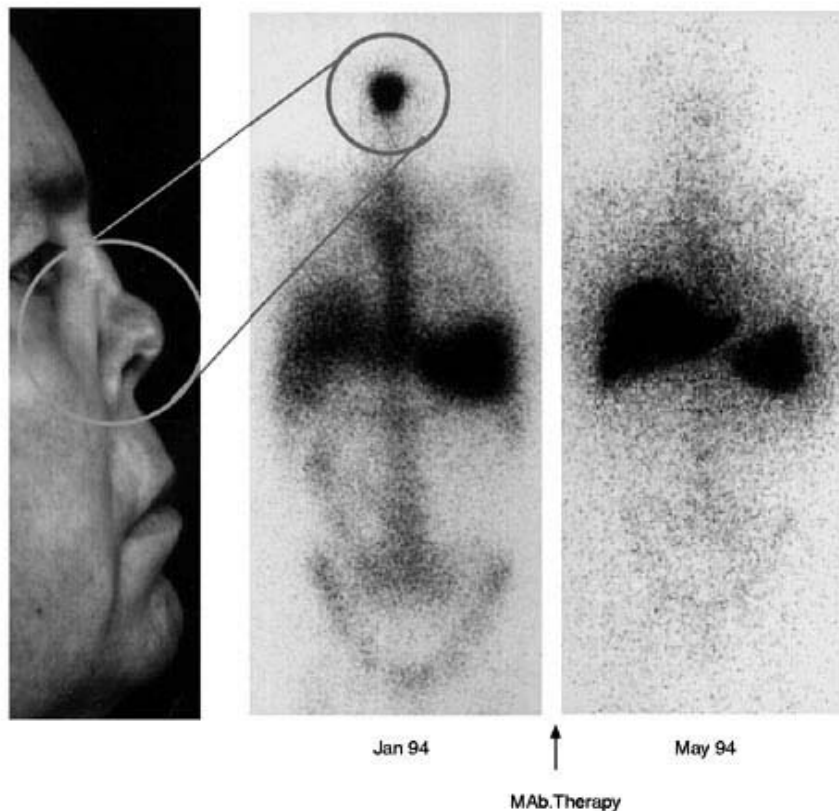


Figure 4 Treatment of Wegener's granulomatosis with CAMPATH-1H. This patient suffered from severe erosion of sinus passages due to leukocyte infiltration, as shown by whole body scanning after injection of ¹¹¹In-labeled leukocytes (area circled). Antibody treatment depleted the T cells which were driving the inflammatory process and this resulted in a normal leukocyte scan and disease stabilization for at least 12 months. (Reproduced with permission from Reuter *et al.* (1995).)

return to normal blood levels for several years. The immunological significance of this is not obvious as few, if any, of the patients appear to suffer any long-term immunodeficiency. The majority of patients experienced an improvement in symptoms (in the case of multiple sclerosis, a decline in the frequency of new lesions) which lasted from 2 months to over a year. In the event of relapse, retreatment was possible and only infrequently limited by an anti-idiotypic response.

In a minority of patients, the first lymphocytes to regenerate lacked GPI-anchored proteins. This is characteristic of cells in paroxysmal nocturnal hemoglobinuria (PNH) and probably results from a somatic mutation in the X-linked PIG-A gene which codes for a critical step in GPI biosynthesis. Considering the frequency and time course of appearance of these cells, it seems unlikely that a mutation was induced by therapy, rather that it selected GPI-deficient cells already present at low numbers. So far, these cells appear to have normal functions and may even mediate graft rejection. Patients do not appear to suffer any adverse effect and in due course they are replaced by normal lymphocytes.

Conclusion

The CD52 antigen is an unusual molecule which provides a model for studying the role of carbohydrate and GPI anchors in immune and reproductive systems. The lytic ability of CD52 antibodies *in vivo* raises many possibilities for therapy and for study of the role of lymphocytes in different diseases.

See also: Antilymphocyte serum; Autoimmune diseases; Complement, classical pathway; Glycosylation of immune system molecules; Graft-versus-host reaction; Idiotype; Immunosuppression; Leukemia; Monoclonal antibodies (mAbs); Multiple sclerosis; Rheumatoid arthritis, human; Transplantation.

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CAMPYLOBACTER, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

The *Campylobacter* genus comprises several species of gram-negative, highly motile, bacteria with an S-shaped morphology and microaerobic growth requirements. Clinically the most important *Campylobacter* species are the thermophilic organisms, *C. jejuni* and *C. coli*, which colonize and invade the small intestine and colon and are the most common cause of human acute bacterial enteritis. Asymptomatic infection is common in underdeveloped countries but in susceptible patients diarrhea occurs, usually 3 days after infection, and lasts 2–3 days, although excretion may continue for several weeks. The mechanisms by which these campylobacters cause enteritis are, as yet, unknown but may involve adherence, invasion and/or toxin production. Several other *Campylobacter* species, including *C. lari*, *C. hyointestinalis*, *C. concisus* and *C. upsaliensis* have also been implicated as causative agents of enteritis. All these enteric campylobacters may also cause blood and systemic infections. *C. fetus* subsp. *fetus* usually causes opportunistic septicemic infections in humans. Campylobacteriosis is generally self-limiting but arthritis and glomerulonephritis are not uncommon reactive complications and about 50% of cases of Guillain-Barré syndrome are thought to be associated with a prior *C. jejuni* infection.

In the following text the term 'campylobacter' will refer to the *C. jejuni/coli* group of organisms unless otherwise stated. The strains in this group are diverse and two serotyping schemes have been developed for use in epidemiologic tracing. The Lior serotyping scheme is based on heat-labile antigens and utilizes slide agglutination of live bacteria with serotype-specific rabbit antisera preabsorbed with homologous heated and heterologous unheated cross-reactive antigens. Flagellin is considered to be one of the antigens involved in this serotyping scheme. The Penner serotyping scheme is based on heat-stable antigens and involves the passive hemagglutination of erythrocytes, sensitized with boiled saline bacterial extracts, by serotype-specific rabbit sera. Lipopolysaccharides (LPSs) are thought to be the dominant heat-stable antigen involved in this scheme.

Several surface protein antigens of the campylobacters have been described. The most well charac-

terized antigens are the flagellins. The campylobacters have single, bipolar flagella. In animal models using isogenic mutants fully active flagella are essential virulence factors. There are two flagellin genes (*flaA* and *flaB*) in tandem in all strains, although only one of these genes may be expressed. The flagellins have a molecular weight of approximately 62 kDa and are the major protein surface antigen. The antigenicity of campylobacter flagellin is complex and epitopic analysis indicates that common as well as serotype-specific linear epitopes are expressed. The common epitopes are antigenically cross-reactive with flagellins from most *Campylobacter* species, including *C. fetus fetus*, *C. lari* and *Helicobacter pylori* (formerly *C. pylori*). Antigenic variations occur between these flagellins, and a microheterogeneity in charge properties due to post-translational modification, probably glycosylation, also demonstrates some antigenic variation. Additionally there is a reversible transition between flagellated and aflagellated variants.

There are several other conserved groups of protein antigens. The variable molecular weight (42–47 kDa) major outer membrane protein (MOMP) is surface exposed. The antigenicity of this protein mainly relies on its native conformation and it is antigenically cross-reactive with similar proteins in *C. coli* and *C. lari* but not other *Campylobacter* species. A group of surface extractable antigens (26–32 kDa), called the PEB proteins (PEB 1–4), have been isolated and partly characterized. PEB 1 has been cloned and sequenced and may have adherence properties. Some of the campylobacters produce detectable toxins, one of which has some antigenic cross-reactivity with cholera toxin, but is structurally different, comprising a 66 kDa polypeptide, probably a holotoxin, and a series of antigenically related subunits. Recently the expression of pili on *C. jejuni* has been described but the role of these structures in pathogenicity has yet to be determined.

Immune response of the host

Patients with campylobacter enteritis produce a rapid circulating antibody response. These antibodies have complement fixing, bactericidal and agglutinating properties. The initial specific immunoglobulin M (IgM) response is produced by the 8th

day of infection and is relatively short lived. This is followed by a rapid serum polymeric and monomeric IgA response, which peaks at about 2 weeks after onset of symptoms but disappears by the 5th week. The IgG antibodies are detectable within 10 days of the illness and persist for several months. The short illness generally associated with campylobacter enteritis suggests that these antibodies are effective at limiting infection. The antibody responses form the basis of a serologic test for the retrospective diagnosis of infection using ELISA, with an acid-extractable surface antigen which primarily comprises flagellin.

Analysis of the antigenic specificity of the host immune responses shows that the immunodominant surface antigen is flagellin. Antibodies directed against this polypeptide are produced early in infection and may be protective. The MOMP is also immunogenic, during infection, in most patients. When present all these antibodies react against homologous and heterologous strains. LPSs appear to induce inconsistent antibody responses, which when present may be serotype specific.

Guillain-Barré syndrome is characterized by acute motor neuropathy, which appears to be a sequela of campylobacter enteritis in about 50% of cases. Many patients with this disease have increased circulating IgG and IgM antibodies binding to GM1 and GD1 ganglioside epitopes and which cross-react with LPS of certain serotypes of *C. jejuni*, especially types O:19 and O:4.

To date, T cell responses to the campylobacters have been poorly investigated.

Evasive strategies by the organism

No mechanism by which *C. jejuni/coli* evade the host immune response has been identified; however, in some experimental models virulence is associated with persistence in the bloodstream, survival in extraintestinal organs and resistance to macrophage ingestion. One potential mechanism for bacterial avoidance of host immune responses could be antigenic variation of the flagellin proteins.

C. fetus fetus has a well-defined microcapsule, comprising surface array proteins (S-layer proteins), which is responsible for resistance to the complement-mediated bactericidal activity of normal human serum and phagocytosis by polymorphonuclear cells. These S-layer proteins, which form a crystalline subunit structure on the outer surface of the bacterium, have a range of molecular weights (97–149 kDa) and are encoded by about eight homo-

logs of the *sapA* gene. The S-layer proteins are highly immunogenic during infection. There is significant antigenic variation between each of these proteins, and antigenic switching occurs throughout infection, apparently enabling the persistence of this organism in host tissues.

Vaccines

The evidence suggesting that protective immune responses can be produced after campylobacter infection includes: a progressive decrease in the illness:infection ratio in children from underdeveloped countries with endemic infections; high antibody levels and asymptomatic infections after multiple exposures to campylobacter-contaminated food-stuffs; and the development of protective immunity to rechallenge in experimentally infected volunteers and nonhuman primates. Passive immunization may also be effective as campylobacter-specific sIgA antibodies in breast milk apparently protects infants from disease. These sIgA antibodies are primarily directed against flagellin. Epidemiologic evidence suggests that protective immunity controls the symptoms of disease but does not necessarily prevent intestinal colonization.

Vaccines against campylobacter enteritis are currently under development. A killed whole-cell vaccine induces protection in a mouse model using the heat-labile toxin of *Escherichia coli* (LT) as the mucosal adjuvant. The same vaccine has been successfully tested for safety and immunogenicity in nonhuman primates. Potential candidates for subunit vaccines include the flagellins and other surface acid-extractable proteins.

See also: Bacteria, immunity to; Bacterial cell walls; Endotoxin (lipopolysaccharide (LPS)); Neurological autoimmune diseases; Opportunistic infections; Toxins.

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CANDIDA, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Infections due to organisms in the genus *Candida* are relatively common, with a variety of different manifestations of disease in both humans and animals. These organisms all grow as yeast and most of the individual species also have filamentous forms, which are either pseudohyphae or true hyphae. There are over 150 species of *Candida*, but only 10 are significant pathogens for humans. These species include: *C. albicans*, *C. glabrata* (formerly *Torulopsis glabrata*), *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. Stellatoidea* (now considered *C. albicans*), *C. tropicalis*, *C. pseudotropicalis*, *C. lusitaniae* and *C. rugosa*. Of these, *C. albicans* is the primary pathogen for humans.

Candida can produce a wide variety of human infections that can be generally divided into superficial and deep infections. Superficial *Candida* infections involve skin, nails or mucous membranes of the mouth and vagina. Deep *Candida* infections include candidemia, localized infections of various deep tissues, and disseminated candidiasis in immunosuppressed patients. This classification is significant because the host defense mechanisms involved in the two groups of infections may be different. In particular, cell-mediated immunity is a primary host defense in superficial candidiasis, while neutrophils (and possibly macrophages) are important in deep candidiasis. Hence, patients with the acquired immune deficiency syndrome (AIDS) and its prominent T cell immune defect commonly have oral thrush, but usually do not have deep *Candida* infections. On the other hand, patients with hematologic and other malignancies who become neutropenic during chemotherapy are more at risk for deep *Candida* infections.

The antigens of these organisms are complex, and infected patients often respond to a number of cell wall, cell membrane or cytoplasmic components. The major cell surface antigen of *Candida* is mannan, a part of the cell wall. *C. albicans* isolates have been serotyped into two groups, A and B, by antigenic differences in their mannins. Cytoplasmic antigens of this organism have also been extensively studied for use in the diagnosis of *Candida* infections. Humoral immunity to a cytoplasmic antigen of approximately 50 kDa in patients with deep candidiasis has been described by a number of investigators. Unfortu-

nately, immunosuppressed patients at the highest risk of serious disease may not have an appropriate antibody response, and often have false-negative antibody levels. Also, because some patients with superficial *Candida* infections may develop a significant antibody response, the assays for humoral immunity to *Candida* antigens have not proven to be useful diagnostically. However, because an early clinical diagnosis of systemic *Candida* infection is frequently difficult, there is interest in other serologic tests. Testing for the presence of *Candida* cytoplasmic antigens has been described by multiple investigators. Unfortunately, this test has a high incidence of false negative results. It has been suggested that the antigen does not circulate or may not circulate in a detectable form. Other diagnostic tests more recently studied include amplification of *Candida* DNA by polymerase chain reaction (PCR). This assay can detect as few as 15 cells of *Candida* in clinical specimens, but its clinical utility remains to be determined.

Immune responses of the host

Candida species are found commonly in the environment, and can be recovered from the soil. They are normal flora in the human gastrointestinal tract, and can often be found at other mucosal sites and on diseased skin. The initial host defenses are the non-specific barriers including intact skin and mucosal epithelium, as well as the indigenous bacterial flora that competes for binding sites. Breakdown of these barrier functions, (e.g. from burns or indwelling intravascular devices) allows for invasive *Candida* infections. Once the organisms invade past the non-specific defenses, the major cellular mechanism against *Candida* infections is through neutrophils. These cells are capable of phagocytosing *Candida* spores as well as damaging the pseudohyphae. Intracellular killing appears to be primarily through oxidative mechanisms. Other cells of the immune system (monocytes and eosinophils) are also able to phagocytose and kill *Candida* organisms. Even so, the initial response in the tissues is predominantly by neutrophils, frequently with the formation of microabscesses in infected tissues. This response may be reduced in patients with significant impairment of host defenses, particularly in neutropenic patients or those on pharmacologic doses of corticosteroids. With more chronic infections the inflammatory

response usually becomes mononuclear. In cutaneous candidiasis, the organisms are generally confined to the stratum corneum; the resulting inflammatory response may be an epidermal accumulation of neutrophils in acute infections, or a dermal mononuclear cell response in more chronic infections. Both yeast and pseudohyphae are seen in infected tissues of both deep and superficial sites, although the filamentous forms are generally felt to be more characteristic of tissue invasion.

Because *C. albicans* is part of the normal gastrointestinal flora, the majority of normal human subjects will show evidence of prior sensitization to the antigens of this organism. Most people will have low levels of antibodies to *Candida* mannan. In infected patients, elevated levels of *Candida*-specific antibodies have been found, representing the IgG, IgM and IgA immunoglobulin classes. Elevated salivary IgA levels have also been demonstrated in response to *Candida* stomatitis, and IgA levels against this organism have been found in vaginal secretions. In addition, patients with either allergic respiratory diseases or the hyper IgE syndrome have been demonstrated to have elevated levels of IgE antibodies specific for *Candida*. Whereas antibody titers to *Candida* are usually elevated in infected patients, the humoral immune response does not appear to play a major role in host defense against *Candida*. It has been shown, for instance, that serum and plasma alone (containing antibodies and complement) are not able to kill *Candida* organisms.

In addition to making antibodies, most people will also demonstrate delayed hypersensitivity to *Candida* antigens when subjected to skin testing with extracts of this organism. As described above, patients with defects in cell-mediated immunity are at higher risk for superficial *Candida* infections. This finding is especially apparent in conditions such as AIDS and in the condition known as chronic mucocutaneous candidiasis. AIDS patients are commonly afflicted with recurrent oral *Candida* infections. Patients with chronic mucocutaneous candidiasis have long-lasting and often disfiguring superficial candidiasis without obvious predisposing causes. Endocrinopathies, thymomas, vitiligo, alopecia, and a tendency to develop other types of infections are found in some of these patients. A variety of immunologic abnormalities have been described in this condition, but of these, defective cell-mediated immunity to *Candida* antigens, as manifested by negative delayed hypersensitivity skin tests and abnormal *in vitro* assays of lymphocyte function, have been found most consistently. In contrast, patients at risk for deep candidiasis usually have other types of predisposing factors such as neutro-

penia, steroid therapy, indwelling vascular devices, broad-spectrum antibiotic therapy, and the like. Some of these factors may also enhance the patient's susceptibility to superficial candidiasis as well.

Evasive strategies by the organism

Adhesion is an important initial step in infection of epithelial or mucosal sites. In this regard, *Candida* organisms have been shown to express three types of adhesion molecules. After adhesion of the organisms, other potentially important virulence factors of *Candida* which may assist in promoting invasive disease include lytic enzymes, hyphal formation, and contact sensing. One lytic enzyme, an aspartyl proteinase, may assist penetration into the stratum corneum. Another enzyme, a keratinase, allows invading organisms to use keratin as a source of nutrients. Hyphal formation appears to be useful both in establishing colonization of surfaces and in tissue invasion but may not be absolutely essential for invasion. Contact sensing (also called thigmotropism), may also be a possible mechanism by which *Candida* organisms distribute and align themselves in order to penetrate surfaces.

Infection of superficial sites such as the skin, mucous membranes and nails may also be a method by which *Candida* organisms can avoid a vigorous inflammatory response. In acute infection, neutrophils do seem to be able to reach the site in the stratum corneum where the organisms reside; however, in chronic mucocutaneous candidiasis this infiltrate is generally not present, and the mononuclear infiltrate which does occur is usually confined to the dermis and is therefore not directly in contact with the organisms.

As discussed above, neutrophils appear to be a very important component of the host's defense against *Candida* invasion into deep tissues. These cells are capable of ingesting and killing *C. albicans* yeast cells *in vitro*; however, unkilld yeast cells have also been observed to germinate within neutrophils and perforate through the cell membrane to the outside of the cell. More virulent strains of *C. albicans* have been demonstrated in one study to be relatively resistant to intracellular killing by human or mouse neutrophils, perhaps because they form germ tubes more rapidly than do less virulent strains. Therefore, germination to the filamentous hyphal or pseudohyphal forms may be a mechanism by which this organism can avoid killing by phagocytic cells. In addition, the filamentous forms of *C. albicans* have been shown to release a soluble product which inhibits chemotaxis, adherence, iodination of ingested particles, and superoxide production.

Vaccines

Studies with experimental animals have shown that vaccination with *C. albicans* preparations containing cell surface antigens can be protective when the animals were challenged with *Candida* organisms. However, as described above, most normal human subjects show both humoral and cell-mediated immunity to *Candida* antigens in the absence of preceding vaccination or known infection. Because lack of exposure to the organism does not appear to be involved in the susceptibility of humans to *Candida* infections (i.e. *Candida* is an opportunistic pathogen) vaccination is unlikely to be an effective method for preventing these infections.

See also: Acquired immune deficiency syndrome (AIDS); Fungi, immunity to; Macrophage migration inhibitory factor (MIF); Polyendocrine autoimmunity.

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CANINE IMMUNE SYSTEM

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The canine immune system is just as impressive in its complexity and diversity as are the more thoroughly studied murine and human immune systems. Relative to most species, a large body of knowledge has accumulated about the canine immune system. This knowledge has arisen largely because of the importance of the dog as both a companion animal and transplantation model. Although this entry will primarily concentrate on the differences between the canine immune system and the immune systems of the more thoroughly studied species, it is important to note that the overall structural and functional organization of the canine immune system closely resembles its murine and human counterparts and that there are more similarities than there are differences.

Ontogeny

The gestation period of the dog is approximately 63 days. As with other animals, the stem cells of the immune system are believed to originate in the yolk sac. After a few weeks of gestation, the fetal liver and later the bone marrow become the primary source of these undifferentiated cells. The fetal thymus is apparent by the 28th day of gestation. However, it is not until 35 days postconception that precursor T cells are found in this organ. The ability to reject skin allografts first develops on day 40 of gestation, whereas antibody responses to sheep red blood cells cannot be elicited until the 48th day. Splenic lymphocytes first respond to phytohemagglutinin (PHA) on day 45. However, thymocytes do not develop this ability until gestation day 48. It is not until the second day after birth that peripheral blood lymphocytes respond effectively to PHA. Furthermore, this response does not peak until the puppy is at least 6

weeks old. Thus, the canine immune system develops late and is not fully mature at birth. This, in part, explains the importance of maternal immunity.

Maternal immunity

Maternal immunity, a form of passive immunity, plays a vital role for the neonate. It helps to protect the neonate during the critical transition from the protected uterine environment of the fetus to the hostile external environment of the newborn. Not only does this transition occur at a time when the neonate's immune system is not fully developed but also at a time when the neonate's immune system is naive to virtually all pathogens. Without the acquisition of maternal immunity, the survival of the neonate is greatly reduced. However, maternal immunity is not without its negative effects. It often interferes with active immunization, and is the most common cause of vaccine failure in the dog.

Rodents and primates obtain most of their maternal immunity by placental transfer, whereas dogs obtain the majority of their maternal immunity via colostrum transfer. Dogs have an endotheliochoral placenta. With this type of placenta, the fetal and maternal circulation are separated by four tissue layers: 1) the maternal capillary endothelium, 2) the chorionic epithelium, 3) the fetal connective tissue, and 4) the fetal capillary endothelium. In the hemochorial placenta, the placenta of primates and rodents, the maternal capillary endothelial layer is not present. With this latter type of placenta, the maternal blood actually bathes the fetal placental tissues. Because of the close approximation of the maternal and fetal circulation in the hemochorial placenta, maternal immunoglobulin G (IgG) is efficiently transferred to the fetus. Thus, approximately 75% of the primate and rodent maternal immunity is conveyed by placental transfer. In contrast, in the dog only 25% of the maternal immunity is transferred by this process, the additional placental layer being largely responsible for the decreased placental transfer of IgG.

The ingestion of colostrum during the first 24 h of life is responsible for the transfer of the remaining maternal immunity. Colostrum is the first secretion of the mammary gland after parturition and contains a high concentration of immunoglobulins. Within the first 24 h of life, the gastrointestinal system of the neonate is not fully functional. During this time, protein digestion does not occur. Thus, the immunoglobulins within the colostrum are not digested and remain intact as they pass through the digestive tract of the neonate. Additionally, the intestinal epithelial cells at this time are actively transporting proteins,

including the intact colostral immunoglobulins, through the intestinal wall of the neonate. Once the immunoglobulins are transported through the intestinal epithelium, they are taken up by the lymph system and eventually reach the general circulation. Colostral intake is particularly important to the dog, since 75% of its maternal immunity is derived via this route. The degree and duration of the dog's maternal immunity is largely dependent on the following factors: the amount of colostrum produced, immunoglobulin content of the colostrum, the amount of colostrum ingested, the age of the neonate at the time of colostral ingestion, and the size and growth rate of the neonate.

Natural resistance

The dog, like most multicellular organisms, has a vast array of defense mechanisms. Some of the most effective defense mechanisms are not part of the immune system but are classified as natural resistance. Natural resistance is defined as defense mechanisms which prevent or combat disease-causing agents by nonimmunologic means (without memory or specificity). Examples of canine natural resistance are barriers (skin, mucous membranes), commensal organisms (normal bacterial and viral flora), mechanical action (mucociliary tree, coughing, and desquamation), flushing action (urine, saliva, tears, milk and diarrhea), chemical and enzymatic action (gastric acid, digestive enzymes, lysozyme, and complement), nonspecific phagocytosis (macrophages and polymorphonuclear neutrophils), hormonal (α and β interferon), iron-chelating agents (lactoferrin), and natural killer cells. Although the dog does not have unique natural resistance mechanisms, it is important to understand the role of natural resistance in aiding the canine immune system. Natural resistance is the first line of defense against disease. Without natural resistance, the immune system would be overwhelmed and the dog or any other organism could not survive. It is only when natural resistance fails to prevent or eliminate disease-causing agents that the immune system has a role in disease control.

Humoral immunity

The immunoglobulin composition of the dog consists of the isotypes IgM, IgG, IgA, and IgE. Each of the canine isotypes serves the same basic function as the corresponding murine and human isotype. In a primary antibody response, IgM is the first and the most abundant immunoglobulin isotype produced. IgM is a pentameric molecule and, on a molar basis, is the

most efficient isotype in complement fixation, opsonization and agglutination reactions.

The immunoglobulin isotype with the highest concentration in blood is IgG, which has a higher affinity for antigen than does IgM. IgG, a monomeric molecule, can also participate in complement fixation, opsonization and agglutination reactions, although relative to IgM more molecules of IgG are required. Canine IgG has four subtypes, identified as IgG1, IgG2, IgG3, IgG4.

Usually found as a dimer, IgA is the most important isotype in canine mucosal immunity. IgA is often produced by submucosal lymphocytes and transported to mucosal surfaces by epithelial lining cells. As it is transported through the epithelial cell, the IgA molecule acquires a secretory piece. This secretory piece, which is produced by the epithelial cell, protects the IgA molecule from proteolytic enzymes that are frequently found on mucosal surfaces. Although IgA is the second most common isotype in human sera, it is only a minor component of canine sera. IgA agglutinates antigens but does not fix complement nor does it act as an opsonin. A major function of IgA is to prevent potential pathogens from adhering to or being absorbed by the various mucosal surfaces.

The canine serum concentration of free IgE is very low, the majority of IgE being bound to mast cells and basophils. Immediate hypersensitivity and systemic anaphylaxis reactions are mediated by vasoactive amines that are released when antigen cross-links mast cell-bound IgE. The most frequent form of immediate hypersensitivity in the dog is inhalant dermatitis, whereas immediate hypersensitivity usually involves the respiratory system in man. Another difference between the human and canine immune systems is the major anaphylaxis shock organ. In the dog, the shock organ is the liver; while in people it is the lung.

Cell-mediated immunity

Cell-mediated immunity in the dog, as in other species, is genetically restricted to class I and class II histocompatibility antigens. The major histocompatibility complex (MHC) of the dog, known as the dog leukocyte antigens (DLA), is less well characterized than the human or murine MHC. Three class I loci have thus far been identified in the dog: DLA-A (8 alleles), DLA-B (5 alleles), and DLA-C (4 alleles). For the canine class II loci, DRB, DQB and DPB genes have been identified. The class III MHC loci for the canine C4 has been identified. This locus codes for the fourth complement component of the dog.

Positive identification of the various canine T cell

subtypes has been hampered by the lack of appropriate reagents. However, recently two developments have helped to rectify this situation: 1) the production of monoclonal antibodies with reactivity to various canine T cell subtypes and 2) the recognition that some murine and human reagents cross-react with their canine homologs.

Canine cell-mediated immunity does not differ greatly from the murine and human systems. Antigen-processing cells, usually macrophage or dendritic cells, ingest the antigen, secrete interleukin 1 (IL-1) and present the various antigenic determinants on its surface in the contexts of the dog's MHC (class I or class II antigens). T helper cells, that have encountered IL-1 and recognize antigenic determinants complexed with the class II MHC, clonally expand and secrete various interleukins (e.g. IL-2, IL-4 and IL-5). Interleukins 2, 4 and 5 enable antigen-stimulated B cells to clonally expand and differentiate into plasma and memory cells. Additionally, IL-2 facilitates the differentiation and clonal expansion of cytotoxic T cells which have recognized antigenic determinants complexed primarily with class I MHC.

Natural killer (NK) cells, killer (K) cells, and T suppressor cells have also been identified in the dog. The NK cell of the dog is a large granular lymphocyte that directly kills viral-infected and cancerous cells in a nonimmune manner, without prior exposure to the antigen. In contrast, K cells kill their targets through antibody-dependent cellular cytotoxicity (ADCC). Macrophages, polymorphonuclear neutrophils, and some of the null (non-T non-B) lymphocytes can function as K cells. Genetically restricted T suppressor cells help to limit and control the canine immune response.

Pathologic alterations

There are numerous examples of inherited canine immunodeficiencies, which, not surprisingly, are often associated with a particular breed of dog. Canine cyclic hematopoiesis (gray collie syndrome) has been described in gray collies, pomeranians and cocker spaniels. With this condition, there is a cyclic fluctuation in the number of peripheral blood neutrophils, which, in turn, is associated with periodic bouts of bacterial infections. Although not a true immunodeficiency, Kartagener's syndrome, an absence of ciliary defense mechanisms, has been recognized in puppies with chronic respiratory disease. A selective IgA deficiency has been reported in beagles, doberman pinschers, German shepherd dogs, and shar-peis. A condition in weimaraners and bull terriers has been described, where the puppies

have an atrophied thymus, grow at a reduced rate and have a severely depressed lymphocyte response to mitogen. Severe combined immunodeficiency (SCID) in basset hounds and Cardigan Welsh corgis has been identified. Also, in Brittany spaniels, a C3 deficiency is associated with an increase in the number and severity of bacterial infections.

Acquired immunodeficiencies occur in a variety of conditions ranging from vitamin and mineral deficiencies (vitamin E and selenium) to viral and bacterial infections (neonatal canine distemper virus infection and severe staphylococcal pyoderma).

A variety of autoimmune conditions occur in the dog. These include but are not limited to autoimmune hemolytic anemia, thrombocytopenia, pemphigus, immune-complex glomerulonephritis, autoimmune thyroiditis, Sjögren's syndrome, rheumatoid arthritis and systemic lupus erythematosus. Anti-nuclear antibodies have been associated with the MHC gene DLA-12; and systemic lupus erythematosus is associated with MHC DLA-A7. An association with diabetes mellitus with DLA-B4 as well as DLA-A3, A7, and A10 has also been reported.

Evaluation of immunologic responsiveness

From the discussion of the canine immune system, it is clear that many similarities to the murine and human immune system are found. However, it is vital to realize that critical differences between these systems exist. It is tempting to directly apply a human or murine function test to assess the canine immune system. This direct application could yield erroneous results or interpretations. Often, all that is required is a minor alteration of the assay or its

interpretation to make the assay applicable to the canine immune system. Since it is beyond the scope of this entry to discuss each assay and their potential alterations, the reader is referred to the Further reading section for more information.

See also: Autoimmune disease, spontaneous experimental models; Innate immunity; Maternal antibodies; Ontogeny of the immune response.

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CAPPING, CLUSTERING, MEMBRANE MICRODOMAINS AND CELL SURFACE DYNAMICS

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The discovery of membrane component mobility and redistribution (capping and clustering), by immunologists, some 25 years ago was a major breakthrough in cell surface science. It has also been a major step in the development of our understanding

of how the cell surface may have evolved and how it may work to transmit messages which are more complex than those mediated by the simple binding of a ligand to a receptor molecule. By contrast, cell-to-cell recognition is a phenomenon involving a co-

ordinated sequence of interactions of several different ligands and recognition elements on each cell partner. To fully achieve antigen presentation by one cell and antigen recognition by the other cell, there is a need for a reciprocal recognition of two-dimensional glycoprotein *patterns* which can be created only by the precise *topographical* rearrangements of membrane components within given *microdomains* of the plasma membranes of both interacting cells.

Capping

Capping phenomena have been described for a variety of membrane components in a variety of cell types. The basic model remains the polar capping of membrane immunoglobulins (mIg) on B lymphocytes by anti-immunoglobulin antibody. When different membrane components are successively capped, they migrate to the same cell pole. This pole is assigned to the cell by the zone of contact of its membrane either with another cell or with a substratum. The general characteristics of the capping phenomenon, particularly the energy requirements, temperature dependence, inhibition or reversion by microfilament-directed drugs (cytochalasins), and the accumulation of microfilaments under the cap, have led to the suggestion that capping is probably an active cellular contractile phenomenon. Early cell adhesion studies also suggested that the expression, distribution and dynamics of ligand recognizing domains might be controlled by the microfilamentous network of the cell. Ligand recognition may be followed by cell motility changes and by increased adhesion to a substratum or to other cells. As early as 1973, expression of adhesive sites by lymphocytes was also found to be an active cellular process, its linkage to microfilament dynamics being shown later. It is unclear, however, whether adhesion and capping may be mechanistically related to each other.

For capping to occur, contractile microfilaments drag *anchored* patches of *aggregated* membrane components into an area of the cell where they can be endocytosed and digested, or shed from the surface. As microtubules do not appear to enhance microfilament mobility but rather to inhibit it, their role in the capping phenomenon is more complex. When microtubules are depolymerized by exposing the cells to specific drugs (e.g. colchicine, *Vinca* alkaloids) or to a chilling shock, the capping occurs on the uropod of the cell. Shedding or endocytosis may, however, occur on the whole cell surface when microtubules interfere with the polar redistribution of clustered ligands and membrane components. Capping of any membrane component would occur as a consequence of entrapment of microfilament-

associated membrane anchors within the lattices or clusters formed on the plasma membrane. These microfilament-driven anchors would then sweep any aggregated membrane components or adsorbed material toward the cap area, a zone of high endocytotic activity. Both the membrane sites involved in intercellular adhesion and the membrane anchors involved in capping might be proteins of the integrin and cadherin families.

Clustering

Monomeric antigens, monovalent lectins and monovalent antibody to mIg or any other membrane component do not cap on the cell surface, nor do they form large clusters (spots or patches). Like capping, clustering requires multivalent ligand binding, but unlike capping it is a passive redistribution process: spotting/patching does not require the cell to be alive or metabolically active. It is actually best to observe clustering on cells whose capping machinery is inhibited either by specific drugs or as a consequence of a general inhibition of cellular metabolism. In the context of the original postulate of Singer and Nicolson for cell membrane organization as a 'fluid mosaic of proteins and lipids', the clustering of membrane components may be viewed as an immunoprecipitin reaction or a lectin-mediated aggregation occurring in a two-dimensional pattern at the level of the plasma membrane. Clustering should therefore be affected not only by all the factors that commonly control such phenomena occurring in a three-dimensional fluid aqueous phase (e.g. 'antigen' or 'antibody' excess in a precipitin reaction), but also by the physicochemical properties of the plasma membrane. Besides factors intrinsic to the lipid-bilayered membrane itself, the outcome of the cluster formation should also be influenced by physiological interactions between the membrane proteins themselves. While intermediate filaments, actin filaments and microtubules constitute the main skeleton of the cell and control its overall 'long-range' shape, a variety of both integral and peripheral membrane components may constitute a distinct but interacting skeleton in the submembranous peripheral cell cortex which would sustain the plasma membrane and may cause a 'mid-range' modulation of membrane component topography.

Colocalization

A method of 'differential redistribution' of membrane components into patches and caps has been broadly used to probe the possible interactions between various plasma membrane components, or

between the cell membrane and cytoplasmic structures. If two components A and B were stably associated in the membrane, the clustering and capping of A should always copatch and cocap B. If on the contrary, A and B were never associated, the clustering of one should not affect the distribution of the other. Obviously, since mIg epitopes and major histocompatibility complex (MHC) epitopes were not cocapped, it could be concluded that these were different and independent molecules. Similarly the myosin involvement in the contractile processing and removal of the aggregates from the cell surface was supported by it being found concentrated in the cytoplasmic cortex of the cell just beneath patches of membrane components aggregated by an external ligand.

A number of membrane and cytosolic components were studied in this way and showed 'colocalization'. This led to the discovery of a host of membrane skeletal proteins which may create complex networks of interacting components on and between both faces of the membrane. Similarly, interactions of antigen receptor complexes with proximally recruited CD membrane components in the plasma membrane plane eventually lead to three-dimensional scaffolds of cytosolic protein tyrosine kinases and phosphatases and adaptor proteins as transducer units.

Such differential redistribution studies have a variety of potential pitfalls. Indeed, besides the two aforementioned, simple cases, there were a variety of alternate forms of incomplete and/or unidirectional interactions, three of which are suggested hereafter. 1) Although A and B are molecularly independent, ligand binding might cause conformational changes in A, which then allows binding of A to B, so that capping of A may lead to cocapping of B, whereas the interaction of B with its ligand would not bring A in the cap ('syn-capping'). 2) If there is a large excess of one membrane component over another, a massive aggregation and capping of the major component might entrap the minor one and bring it into the cap, even though the two types of components never showed any affinity for each other during this 'mass effect'. 3) Ligand binding might also trigger a complement-like 'cascade reaction' on the cell surface which may involve several membrane components, both integral proteins and peripheral membrane components in the glycocalyx and in the cytosolic membrane skeleton.

Although such colocalization studies may show that a variety of integral and peripheral membrane components do easily interact with each other, they may not always indicate whether there was *prior colocalization* or whether there has been *recruitment*. They say little about the natural status of distri-

bution of such components in a 'resting' cell. To evaluate membrane component interactions, e.g. to determine whether various membrane components constitute homodimers, heteropolymers or large complexes of several different molecules, instantaneous ('pulse') nearest-neighbor-type biochemical studies of the membrane of cells, both resting and in different activated status, are more suitable than patching and capping.

In addition to these restrictions, the possible isomeric forms of some membrane proteins should also be taken into account; the most representative cases are those of proteins which may exist as two widely different membrane forms, either with a classical transmembrane polypeptide anchor ('TM proteins') or with a glycosylphosphatidylinositol anchor (GPI proteins'), and which may show different physicochemical and/or physiological relationships with other molecular partners in *cis* on the membrane. Moreover, the hypothesis has been formulated that not only ligand-clustered components but rather whole *microdomains* of plasma membrane (including unliganded components) might be brought into the cap. This concept puts severe restrictions on the use of capping as a means of *selective* removal of given membrane components from the plasma membrane.

Finally, it is unclear whether any membrane component may be capped by clustering ligands, and there is also no evidence that all types of membrane component redistributions reported as 'caps' actually proceed through a unique mechanism. Even though the caps were made on the same cell pole and mediated by actin filaments, capping may involve anchorage through integrins for some membrane components, but through cadherins for some others, whereas some 'caps' may actually represent a polar redistribution of microvilli, or a passive recruitment of all clusters into a single one. If whole membrane microdomains rather than individual components show polar redistribution, cocapping may simply reflect the fact that the capped components belong to the same type of domain, whereas independent capping would show they are in different domains, and further conclusions on physical interactions of components with each other are probably speculative.

Capping as a sign of leukocyte polarization?

A misinterpretation of the Singer-Nicolson model of biological membranes would be that the entire cell surface was organized as a continuous fluid mosaic of proteins and lipids without any short-, mid- or long-range order. Obviously, this could not hold true

for highly polarized cells. The epithelial cell represents the most straightforward case for a *segregation* of the whole cell surface as large domains, the apical membrane and the basolateral membranes not only showing widely different protein, lipid and carbohydrate compositions but also being sustained by different types of submembranous skeletons and controlled by different sets of deeper cytoskeletal networks.

Of greater concern in immunology are the domains with the ligand recognition-coupled transduction signals. Would the plasma membrane of the round and resting leukocyte also show segregation of its components as multiple microdomains? Would their polarized redistribution on the leukocyte, when it develops a prominent uropod, possibly mimic the epithelial cell model? Effective triggering through ligand-recognizing domains is usually coupled to changes in cell motility. Moreover, one of the most prominent responses of lymphocytes to recognition of their ligands is an increased adhesion, through tangles of microvilli, either distributed all over the cell surface or redistributed to the uropod, such as observed when various types of killer cells bind to their targets, when lymphocytes interact with macrophages, when leukocytes bind to vascular endothelium for migrating into tissues, when T cells form rosettes with red cells, and when lymphocytes form aggregates either spontaneous or induced by ligands or mitogens.

Membrane component freedom

Most *functional antigen-receptor units* on the lymphoid cell surface are 'receptor complexes' which are constituted by several interacting subunits, some endowed with a recognition function (ligand binding), some responsible for effector function (cytoplasmic signaling) and some having a conveying function (linker, transducer). Thus, in 'resting', i.e. non-activated conditions, a cell surface may show the existence of discrete microdomains, with a short- to mid-range order organization of selected membrane components. Most such receptor complexes might not be under the 'permanently assembled' form, however – nature would not have missed such a key regulatory mechanism and a good occasion to create a host of variants. Rather, components of receptor complexes must be in a continuous equilibrium of association and dissociation, being regulated both by the intracellular, physiological status of the cell and by its extracellular, environmental context. Such regulation of receptor complex assembly needs not to be exclusively occurring at the level of the integral elements which are directly anchored within the lipid

bilayer itself; it may also be mediated by peripheral membrane elements which belong to the outer cell surface, such as components of the glycocalix, and to the inner cell surface, such as components of the membrane skeleton.

If they do not permanently form covalent or non-covalent complexes, the dissociated elements of the receptor complexes must find each other. Membrane fluidity would be a disadvantage if such elements which need to interact reversibly with each other could diffuse on the whole cell surface. Therefore, lateral diffusion of the membrane proteins should be restricted in fluid membranes in order to allow the coupling in a two-dimensional space of spatially organized receptor units whose function requires vectorial interactions.

Membrane microdomains

So long as spatial order of the fluid membrane is maintained, fluidity will actually increase the rate of coupling of independent molecules. It may be worth recalling here that the surface representation of antigen receptors on B and T cells would be very low, were such B and T cell receptors uniformly distributed over the entire cell surface, and that restriction of their distribution to selected domains of the lymphocyte surface might favor both their clustering by polymeric antigens and their recognition by membranous and cytosolic adaptor and transducer molecules. For instance, assuming it is a smooth sphere, a small B lymphocyte with a mean diameter of 8 μm would have a surface of about 200 μm^2 , but there must be at least equivalent (if not much higher) amount of surface membrane stored (e.g. for cell division) under the form of various membrane ruffles and pseudopodia or microprojections. Thus, assuming a near-maximal expression of 40 000 mIg molecules for at least 400 μm^2 B cell surface, one reaches the fairly low surface density of ≤ 100 mIg molecules per μm^2 . While this is a maximal estimate, it is interesting to consider that this corresponds to only one single mIg molecule per patch of 100 nm $\times$ 100 nm, which is about the size reported for some membrane microdomains! For comparison, the diameter of the membranous IgM stem (stalk) is in the range of 4 nm only, and the distance between its two Fv domains is about 12 nm. Because of such a low surface density of antigen receptors, cross-linking of two individual IgM molecules by an antigen would require much mobility, were they distributed at random.

Similarly, there would be a rather poor efficacy of coupling between recognition by the extracellular antigen/ligand-binding elements, and transduction

and signaling by the CD3 complex(-like) elements of the membranous receptor complexes, were they randomly distributed over the whole cell surface. Moreover, the antigen receptor complexes themselves should be in close proximity to their stoichiometrically associated, membrane-anchored and function-controlling elements such as specific protein kinases and protein phosphatases, as well as to the variety of CD coreceptors which are regulating the signal levels. Therefore, there is a high probability for such receptor-operator units to function within specialized membrane microdomains. The latter might not exist as such in the resting cells, and the fully functional signaling unit would probably proceed through *recruitment* of some key elements of the receptor-operator unit. Possibly, only a few of these units are functional at any given time, but a first successful coupling of recognitive elements with effector elements might cause something I would call 'membrane stressing'. This might be acting like a catalyst for further unit assembly, e.g. the activation of a phospholipase whose primordial membrane effects would be to favor the further segregation of the membrane into microdomains, which would much enhance the chances of encounters of relevant elements.

Proximity and promiscuity

Besides the *covalent* interactions of membrane elements such as the various polypeptide chains which constitute the TCR, BCR and MHC antigen receptors, and a number of oligomeric CD coreceptor components which are involved in coupling ligand adhesion to signal transduction, two major mechanisms may favor the selective encounters of membrane components: proximity and promiscuity. The differences between these two terms may look subtle or semantic, but they may be large in terms of mechanisms and consequences.

Promiscuity would refer to membrane components, both integral and peripheral ones, which are loosely held close to each other by *low affinity interactions* such as those taking place between elements of the 20 nm thick glycocalix (e.g. weak lectin-like interactions in a microenvironment where local carbohydrate concentrations may be in 0.1 M range) and those occurring between the array of membrane-stabilizing proteins constituting the endoskeleton. Moreover, promiscuity may also be created within the membrane by lipid domains showing hydrophobicity features which may be more or less favorable for the preferential localization of integral proteins with a TM anchor (TM proteins) or with a GPI anchor (GPI proteins), of cytosolic proteins with a

myristoyl anchor, and also more or less suitable for the association of some glycolipids, such as gangliosides, endowed with an innate tendency for self-association and microdomain formation with the membrane phospholipids. Molecular promiscuity may have a coordinated biosynthetic origin, most interacting elements being synthesized and organized as a membrane patch before the membrane vesicle reaches the cell surface.

Proximity would rather refer to a higher occurrence of membrane components within a microdomain, which would only be favored by the existence of *molecular boundaries* in the membrane, in the absence of specific molecular interactions, even of low affinity, between the components of the microdomain. Such domain boundaries might be constituted by protein fences both within the lipid bilayer (integral proteins) and on both of its faces by peripheral proteins, some of which constitute a subplasmalemmal skeleton (membranous endoskeleton) and some others participating to the cell glycocalix (membranous exoskeleton). Within such boundaries of the microdomain, the membrane components would thus be kept in close proximity to each other.

Specialized membrane microdomains defined by submembranous protein networks

In addition to those which may be defined by TM protein- or glycocalix-based fences and others which may depend on particular lipid clusters (caveolae), other membrane microdomains may be defined by the subplasmalemmal assembly of various types of protein skeletons. These endoskeletal networks may recruit a selection of peripheral and cytosolic proteins, with the eventual connection of a membrane patch to the actin microfilaments. At least four types of microdomains may be defined, the first one being mostly an endoskeleton, the next two involving TM proteins with intercellular adhesion sites, and the last one concerning microvilli and possibly ligand recognition.

In erythrocytes, a spectrin-based filamentous network is apposed to the inner face of the lipid bilayer, conferring mechanical support to the membrane. Several members of the *spectrin family* occur as endoskeletal components of nonerythroid cells (ankyrin, fodrin, TW260/240, dystrophin, dystrophin-related protein, α -actinin). These proteins constitute membrane skeletons with a fixed stoichiometry but a flexible morphology (due to the properties of spectrin). They help to organize and stabilize microdomains which are enriched in some

integral membrane proteins. None of these skeletal proteins is a TM protein, i.e. they cannot be directly in touch with the cell outside, but one of them (often ankyrin) can link the assembly to a TM protein (often a channel or transporter). Moreover, most of these proteins bind to actin, providing specific attachment sites for cytoskeletal components; the latter may thus define the topographical distribution and cell surface dynamics of such microdomains whose occurrence in leukocytes remains unknown.

Integrin-mediated adhesion to the extracellular matrix reveals the colocalization of two submembranous skeleton proteins, α -actinin and talin; these actin-binding proteins connect the cytosolic domains of integrins to a matrix of actin microfilaments and other actin-binding proteins, including vinculin, paxillin, tensin and zyxin. *Cadherin-mediated adhesion* similarly results in the localized recruitment to their cytosolic domain of several cytosolic proteins (β -catenin, plakoglobin, p120) which bind to α -catenin (homologous to vinculin), itself binding to fodrin and actin, with α -actinin joining the complex. In both the integrin and the cadherin cases, the networks of interacting submembranous skeletal proteins strengthen cell adhesion and provide protein scaffolds for signaling networks, which involve kinases, phosphatases, GTP-binding proteins and adaptor proteins.

Another actin-based cytoskeleton characterizes the noncontacting surface of epithelial cells, i.e. its apical surface which expresses numerous *microvilli*. Each microvillus is sustained by a bundle of actin filaments, cross-linked by villin and fimbrin, coupled laterally to the membrane by myosin I, and anchored into the cortical actin filament network by fodrin. In the case of epithelial/endothelial borders, the microvilli are found on the apical surfaces of the cells, thus facing the 'not self' in the lumen. Would adhesion through the integrin- and cadherin-based domains be rather involved in recognition of self, whereas microvillus-based recognition would be devoted to the not self? In the case of lymphoid cells, microvilli tend to redistribute to the area of contact with the target cell, possibly showing their direct involvement as an early recognition step. Assuming the analogy to epithelial cells goes further, lymphocyte microvilli might not mediate adhesion, but only be involved in signaling ligand recognition.

Another case for specialized membrane microdomains is found on the apical surface of epithelial cells: these are the *caveolae* which are 50–100 nm invaginations found at the bases of the apical microvilli. These membrane protein–lipid microdomains are remarkably resistant to detergent (e.g. Triton-X100)-mediated solubilization. They are

coated with caveolin, a TM protein which is a v-Src substrate, and they contain G protein-coupled receptors; in caveolae, caveolin exists as a heterooligomeric complex with integral proteins and known cytosolic signaling molecules, suggesting the involvement of caveolae in transmembrane signaling. Moreover, caveolae would recruit and 'cluster' GPI proteins (in a 'promiscuity' sense), such as the folate-binding protein (showing their important physiological role). In T cells, GPI proteins (e.g. Thy-1 or DAF[CD55]) may play a role in signal transduction, particularly through an activation of submembranous protein tyrosine kinases (e.g. Fyn or Lck), which are proteins anchored to the inner leaflet of the lipid bilayer by N-terminal myristoylation. It is assumed that the connection (as found by coimmunoprecipitation) of the latter proteins to the extracellular GPI proteins (whose anchor is restricted to the outer leaflet of the lipid bilayer) might be done by the TM protein caveolin.

Finally, the topography of the various microdomains might be determined on the cell surface by anchorage of the various scaffolds to the *microtubular cytoskeleton*. As suggested for the epithelial cell model, microtubule organization might confer a polarity to the lymphocyte for microdomain redistribution to the cap/uropod area.

Adhesion or recognition, but not both?

Does the cell segregate adhesion molecules and recognition-coupled effector molecules into separate microdomains? An *ad hoc* argument was made that molecules endowed with recognition of the non self, such as antigen receptors, might be naturally expressed on microvilli. Microvilli were indeed reported to express mIg at higher cell surface density than the rest of the B cell surface. Whether this is true for the TCR was not examined, but one may also assume that T cell microvilli might have the function of recognizing target cell-presented antigens. A microvillus redistribution to the intercellular contact area would also bring the caveolae to the same pole, if these two microdomains are topographically associated on lymphocytes, as they are on the apical surface of epithelial cells.

Since microvilli are labile structures which may grow or retract quickly, whereas caveolae may be structurally stable structures occurring (and possibly forming boundaries) at the microvillus bases, the high expansion and contraction of microvillus membrane might be explained if the fluid lipid bilayers would percolate between the caveolae: the lipids may rapidly flow through the caveolae network and go

back and forward between the bulk of the plasma membrane and the microvillus membrane domains. Selected membrane components, including proteins, would cross the caveolae network boundaries much more slowly.

Once recognition has occurred, microvilli could retract, bringing the lymphocyte closer to the other cell. At the membrane level, a fast microvillus retraction after ligand encounter might thus markedly increase the surface density of selected components of the 'recognition domain'. For instance, assuming a 1 μm long microvillus with a 0.1 μm diameter, its 0.16 μm^2 surface would be converted into a flat 0.008 μ^2 patch on the cell membrane, possibly causing a 20-fold increased density of selective TM proteins, GPI proteins and myristoylated proteins and other microvillus membrane components whose diffusion was delayed by the caveolae network. This mechanism would thus provide a higher occurrence for interactions of the receptor, adaptor and effector elements of the recognition units displayed on the former microvillus membrane, as well as higher chances of interactions of such initiating complexes with other effector components of the caveolae at the boundaries.

Clustering for cell activation, but capping against it?

How ligand binding results in a signal to differentiate or to divide is a complex sequence of events. Although the actual consequences of capping for the physiology of the cell remain poorly understood, it seems that clustering rather than capping actually initiates cell triggering. It is generally admitted that the very first signal is the clustering of receptors by interaction with a cross-linking ligand (e.g. polyvalent antigen, lectin, antibody). Capping itself may not be perceived by the cell as a triggering signal, although it may be used as a *scavenging* mechanism for the efficient removal of bound ligands and engaged cellular receptors. One could, however, speculate that a more efficient capping of antigen-mIg complexes on a B cell surface might lead to an earlier and/or more efficient endocytosis, processing and antigen presentation.

There is tremendous support for the generalization that clustering of plasma membrane components is a prerequisite for triggering. In all cases where a ligand is mitogenic, it is found to be bi- or polyvalent, and its monovalent derivatives are no longer mitogenic. Even more conclusive is the fact that monovalent, nonmitogenic ligands can be made mitogenic by polymerization. Thus, the cross-linking of membrane sites is required for triggering. Yet

attempts to establish a closer link between clustering and triggering are not easy, as the clustering of antigen receptors by itself does not appear to trigger the whole activation cycle. This first signal is obviously necessary but not sufficient to activate the cell.

Whether lymphocyte activation may involve more subtle and specific consequences of antigen receptor clustering, such as increased frequency of encounters between given elements of the B cell or T cell receptor complexes, is a matter for ongoing research. The essential fact is that mIg clustering, like patching of any other membrane component by its cognate ligand, creates patches of membrane with a *selective* component composition which might initiate cellular activation but which are normally removed by capping. Thus, capping rather appears as a mechanism which limits the *duration* of the signaling period. By limiting the persistence of patches of selected membrane components on the cell surface, capping may regulate cell activation by up- or down-modulating the overall signal delivered by clustered receptor units. The early changes in the physicochemical properties of the membrane normally last for a few minutes only, as does the normal persistence of the mIg clusters on the B cell surface (at least *in vitro*).

Too rapid a removal of clustered mIg by capping may actually impair triggering, this being due to insufficient first signal hits delivered to the cell, and thus resulting in a failure to reach a hypothetical threshold level for stimulation. Conversely, in the case of very delayed capping, the long persistence of large clusters of receptors would deliver excess signals which might lead either to cell paralysis and inactivation or to terminal differentiation, both of which pathways ultimately result in deletion of the cell concerned. Thus, the rate of capping-mediated removal of clustered receptors from a cell surface may be crucial to position the signal levels within a triggering window where cell activation to mitosis and clonal expansion will occur, a window defined by a minimum threshold level below which the signals are not perceived as activating and a maximum threshold above which the signals are inactivating the cell, potentially leading to 'clonal deletion'. The microdomain concept brings a novel dimension to the problem: in T cells, for instance, it may be crucial to know whether TCR/CD3-containing receptor-effector complexes, which if stimulated alone would lead to anergy, occur in the same or different microdomains as the CD28-operated units, whose involvement is needed for stimulation.

Abnormalities of capping

Various alterations of capping are found in cases of immunodeficiency and/or autoimmunity, or following cell treatment with pharmacological agents known to show immunomodulatory properties. Various agents or conditions, which may be physiological, pharmacological or genetic factors and are known to modulate the immune responsiveness, are also found to alter the rate of capping. Thus, capping of B cell mIg occurs at different rates for mice of different strains and shows different susceptibilities to pharmacological agents; immature, fetal or neonatal B cells cap faster than mature, adult B cells; peripheral blood lymphocytes from elderly human beings show decreased capping rates in comparison with young adults; and cyclosporine-treated B cells show a faster capping.

Within identical genetic contexts, single mutations, which do not seem to directly target components of the capping machinery, can affect the capping rate found for B cell mIg, such as the *xid*, *nu*, *lpr*, *gld* and *me^v* mutations. Presumably, such mutations lead to the selective development of some particular B cell subsets which may be minor components of the wild mouse B cell population. However, some mutations may directly touch the capped or associated components. Thus in the mouse, the *bg* mutation (a mouse model of the Chédiak–Higashi syndrome) targets a gene encoding a coiled-coil phosphoprotein 'Lyst' which may act on the microtubular network as a relay integrating cellular signal response coupling; the *Lyst* deficiency accelerates mIg capping and even allows capping of some lectins in the absence of treatments which weaken the microtubular restraints on membrane mobility. Similarly, the *sph* mutation (α -spectrin deficiency causing hereditary hemolytic anemia) accelerates the lectin or antibody-induced capping of membrane glycoproteins on erythroleukemia cells, suggesting that spectrin may limit the lateral mobility of integral membrane proteins in cells with a fluid membrane (i.e. other than the erythrocytes). Similarly, in human beings, several cases of abnormalities of leukocyte function and of capping and/or adhesion have been reported, such as in Duchenne muscular dystrophy (an abnormality of

dystrophin, a member of the spectrin family of membrane skeleton proteins) and in leukocyte adhesion deficiency (deficiency of the β_2 -integrins).

See also: Adhesion molecules; Antigen-presenting cells; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Antigens, cell surface; B lymphocyte activation; B lymphocytes; CD antigens; Cytotoxicity, mechanisms of; Erythrocytes; High endothelial venules; Immunoglobulin, cell surface; Integrins; Lectins; Lymphocyte trafficking; Membrane-associated cytoskeleton: role in regulating immune cell function; Rosetting techniques; T lymphocyte activation.

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CARBOHYDRATE ANTIGENS

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Carbohydrates, containing n asymmetric carbon atoms, can exist in 2^n stereoisomeric forms. A common hexose possesses *a priori* four asymmetric carbon atoms (C-2, -3, -4 and -5) and thus can exist in 16 stereoisomeric forms (i.e. D-glucose, L-glucose, D-mannose, L-mannose, etc.). Each of these isomers can occur in either a six-membered (pyranose) or five-membered (furanose) ring form. When a hexose is in the ring form, the C-1 aldehyde function also becomes asymmetric, and the 1-hydroxyl group can assume either the α or the β configuration, thereby doubling the number of possible isomers. In total, a hexose can thus occur in an oligo- or polysaccharide in 64 different ways. This enormous capability for diversity makes carbohydrates very versatile as nature's alphabet for recognition in biological events, including immunology. Bacteria, viruses and mammalian cells nearly always have polysaccharides as capsular or cell wall material, and/or have glycolipids and glycoproteins on, or in, their walls, and these wall components or capsules often dictate the ensuing cascade of biological events in which they participate.

Structure of polysaccharides and the saccharides of glycoproteins and glycolipids

Polysaccharides, and the saccharide epitopes of glycoproteins and glycolipids, are secondary gene products. Thus, glycosyl transferases are encoded at the genetic level, and these in turn catalyze the formation of oligo- or polysaccharides or the glycosylation of proteins and lipids. One of the consequences for polysaccharides is that they have no uniform, sharply defined molecular weight but instead possess a molecular weight distribution. Linkages between monosaccharides in the polysaccharide chain are acetal linkages; they involve a carbon atom bearing two oxygen atoms: the ring oxygen and the intersaccharidic oxygen. Acetals are susceptible to acid-catalyzed hydrolytic cleavage, as well as enzymic cleavage, since both operate by the same chemistry.

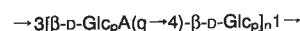
Sugar residues in an oligo- or polysaccharide chain frequently carry substituents such as (acyl)amino-, carboxyl-, acetoxyl, pyruvoyl-, phosphorylcholine-, or can be esterified with long-chain fatty acids. This additional diversification can profoundly affect the

immunogenicity or biological activity of the entire molecule.

Naturally occurring carbohydrate antigens can be divided into two main categories, which can again be subdivided:

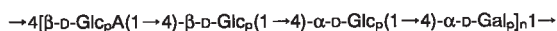
1. Polysaccharides
 - Bacterial, capsular polysaccharides
 - Bacterial cell wall polysaccharides
 - Bacterial lipopolysaccharides
 - Plant polysaccharides (e.g. cellulose, etc.)
 - Storage polysaccharides (e.g. glycogen, etc.).
 - The last two classes of polysaccharides will not be discussed here.
2. Carbohydrates borne on other (macro)molecules
 - Glycoproteins
 - Glycolipids.

Concerning (1), capsular polysaccharides of bacteria exist on the outside of the cell wall. The loss of capsular material does not lead to autolysis, but it does often lead to loss of virulence. By and large, bacterial capsular polysaccharides are made up of small, linear or branched units (of from one to several sugar residues). These are repeated with great regularity to make up the extended, polymeric chain. Most of these polysaccharides possessed by bacteria within a given species (e.g. within the gram-positive species *Streptococcus pneumoniae* there are more than 80 types) show pronounced immunologic specificity. However, if capsular polysaccharides share certain features between them that are structurally akin, they may cross-react with each other's immune sera. If the structural similarity is extensive, infection with one could even confer cross-reactive protection towards the other. For instance, the capsular polysaccharide from *S. pneumoniae* type III is a high molecular weight, linear heteroglycan (= a polysaccharide containing differing sugars) made up of D-glucuronic acid (in the six-membered ring, or pyranose form: Glc₆A) whose acetal carbon-1 is linked to the 4-hydroxyl group of D-glucose (also in the pyranose form) by a β linkage, and these disaccharide units are repeated by being linked to each other by $\beta(1\rightarrow3)$ linkages to make up the extended polymeric chain (I).



I

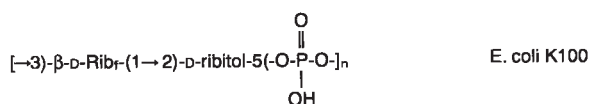
The high molecular weight capsular polysaccharide from *S. pneumoniae* type VIII has a tetrasaccharide repeating unit, the disaccharide part of which is identical to the one above. In addition it has a 4-linked glucose that is linked $\alpha(1\rightarrow4)$ to a galactose residue. The tetrasaccharide repeating units are connected by $\alpha(1\rightarrow4)$ linkages (II).



II

These two polysaccharide antigens – having 50% of their structure in common – show extensive cross-reaction with one another's serum antibodies.

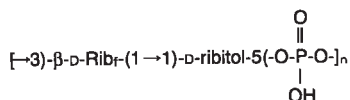
Several gram-negative bacteria can produce extracellular polysaccharides. When these surround the organism, i.e. when they become a capsular polysaccharide, they also dictate the immunologic specificity of the organism. Those polysaccharides are often acidic. They can have intracatenary phosphoric diester groups, substituents such as pyruvic acid acetalated with certain sugar residues, or they can carry simple uronic acids. An example is the capsular antigen of *Escherichia coli* K100, possessing intracatenary diester groups (III).



(where Rib_r stands for ribofuranosyl).

III

Differing species can show cross-reactivity as well, and these can be biologically significant. For instance the antigen of *Hemophilus influenzae* type b (Hib) shows near structural identity (IV) with the capsule of *E. coli* K100 (see above) and protective antibodies towards Hib are believed to result from the presence of that *E. coli*.



H. influenzae type b (Hib)

IV

Cryptococcus neoformans is the only fungal species of unarguable pathogenicity that possesses capsular polysaccharides, whose most abundant components are glucurono-xylo-mannans. There also, the capsule may be a factor in virulence. Returning to bacteria: compromise of the underlying bacterial cell wall, for instance through interference of the biosynthesis of the wall's peptidoglycan (see below)

by penicillin, causes autolysis, as the wall's integrity arises mostly from a high molecular weight, cross-linked and 'net-like' peptidoglycan polymer. That substance is composed of two components. The first is a polysaccharide of alternating *N*-acetyl-D-glucosamine (D-GlcNAc), linked $\beta(1\rightarrow4)$ to *N*-acetyl muramic acid (NAM = D-GlcNAc carrying a lactic acid substituent at its O-3). The carboxyl group of the lactic acid in NAM is attached to a short peptide chain, which, in *Staphylococcus aureus* consists of L-alanine, D- γ -glutamic acid, L-lysine and D-alanine. The D-alanine's carboxyl end is connected by a pentaglycine to the ϵ -amino group of the lysine of another tetrapeptide. Further bridging is probably achieved to make this giant molecule three dimensional. The peptides vary in differing bacterial species, but the intricate, resulting network of cross-linked polysaccharide/polypeptide is very similar (V).

Teichoic acids, composed of sugar residues, some of which are linked by phosphodiester- rather than acetal-linkages, may be attached to some of the GlcNAc residues by a phosphate linkage. Peptidoglycans occur in both gram-positive and gram-negative organisms, where they have similar, albeit not identical, structures.

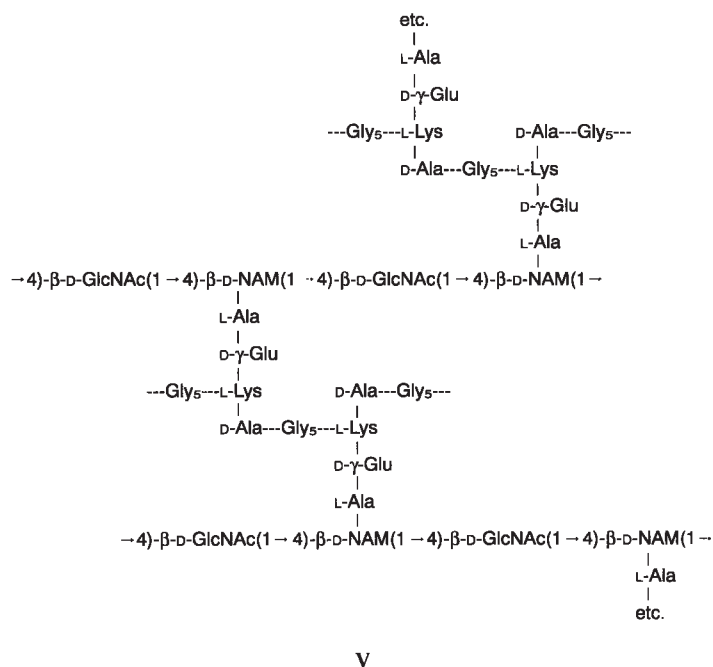
Gram-negative bacteria possess a lipopolysaccharide (LPS), also called endotoxin. It is made up of three distinct segments.

First, the *lipid A*, which consists in most cases of two residues of *N*-acyl D-glucosamine, linked $\beta(1\rightarrow6)$. This disaccharide fragment is phosphorylated at positions 1 and 4', and each residue is extensively esterified, as well as *N*-acylated, with long-chain fatty acids. The lethal toxicity the LPS can exhibit in animals (and humans) resides in the lipid A segment.

Second, the so-called *core region* is covalently attached to the 6' position of lipid A. It is a fairly short oligosaccharidic segment (usually less than 15 sugars) consisting of one or more residues of keto-deoxy-octulosonic acid (forming the linkage with the lipid A), heptoses, various sugars, ethanolamine (EtAm) and phosphate (P) substituents. It is similarly but not identically structured in most Gram-negative bacteria.

Third, the so called *O-polysaccharide* (O-PS) can possess up to roughly 100 sugar residues, made up of distinct repeating units, linear or branched, that can involve (from one to) several sugars. It is covalently linked to the core region.

The lipid A segment is inserted – and anchored through the hydrophobic interaction of its fatty acid chains with the cell wall lipids – into the fluid lipid bilayer of the bacterium's wall. The entire LPS is oriented so that its O-polysaccharide projects out

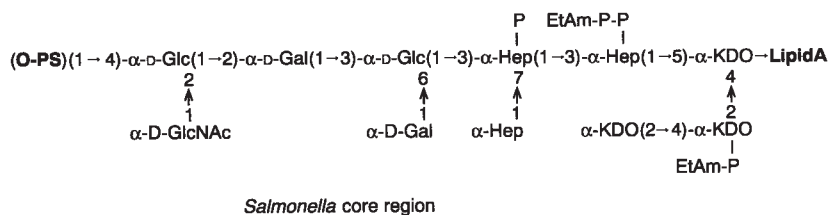


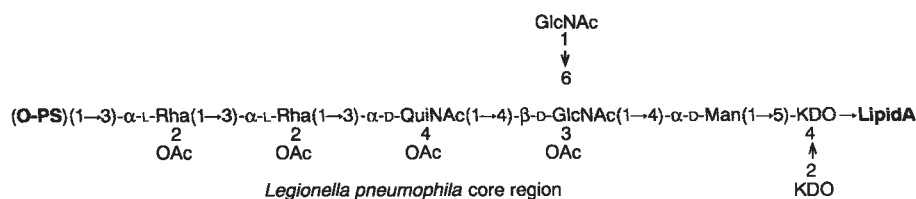
from the bacterial wall into the surroundings. Consequently, the O-polysaccharide often dictates the immunodeterminant behaviour of the organism, and it differs for each type within a species. Most wild-type bacteria carry O-polysaccharide and are then said to be in the smooth- or S-form. Bacteria not carrying O-polysaccharide, but only possessing the lipid A/core combination are said to be of the rough- or R-form. These terms are descriptive of their respective colony morphology. The presence of O-polysaccharide cannot be regarded as a necessary factor for pathogenicity, since R-type LPS is observed in some wild-type bacteria that are highly pathogenic in humans (e.g. *Bordetella pertussis*, *Neisseria gonorrhoeae*, and others). Below are two examples of an LPS. The first is for *Salmonella*, the second for *Legionella pneumophila*. Note that although the core regions are quite different, both are short saccharidic stretches situated between the lipid A and the O-chain. Note also that KDO is the sugar residue linked to the lipid A portion (VI):

where GlcNAc = N-acetyl D-glucosamine, Gal = galactose, Hep = Heptose, KDO = keto-deoxy-octulosonic acid, EtAm = 2-aminoethyl, P = phosphate, FE = fatty acid ester. The lipid A is indicated on the right, while the O-polysaccharide is indicated on the left. The location of the phosphates or 2-amino-phosphates, or the branch-terminating KDO, remains tentative (VII).

In the case of systemic infection by gram-negative organisms, the overwhelming amount of solubilized endotoxin produced by bacterial lysis can cause shock and even death. It is interesting to note that affinity-purified rabbit anti-LPS immunoglobulin M (IgM) and IgG protects rats lethally challenged with *Pseudomonas aeruginosa*.

Concerning (2), in addition to the polysaccharide antigens, such as discussed above, many mammalian or viral cell walls carry glycoproteins and/or glycolipids on or in them, and these can define their antigenicity. Glycoproteins are proteins that are naturally substituted by short and/or medium length





VII

oligosaccharide substituents, whose internal linkages are purely of the acetal type. The linkage between protein and carbohydrate can be of the O-linkage type (C-1 of the sugar is glycosidically linked to the hydroxyl of a serine or threonine residue, thus forming a regular glycosidic linkage), or of the N-linkage type (C-1 of the sugar is linked to the NH_2 of an asparagine residue of the protein, forming an N-glycosidic linkage). Two examples are given below, one is human gastric mucin and the other is one of the many glycoforms of an oligosaccharide chain that occurs on human IgG at asparagine (Asn)-297 of the heavy chain of the immunoglobulin (Ga1NAc stands for N-acetyl galactosamine) as in VIII.

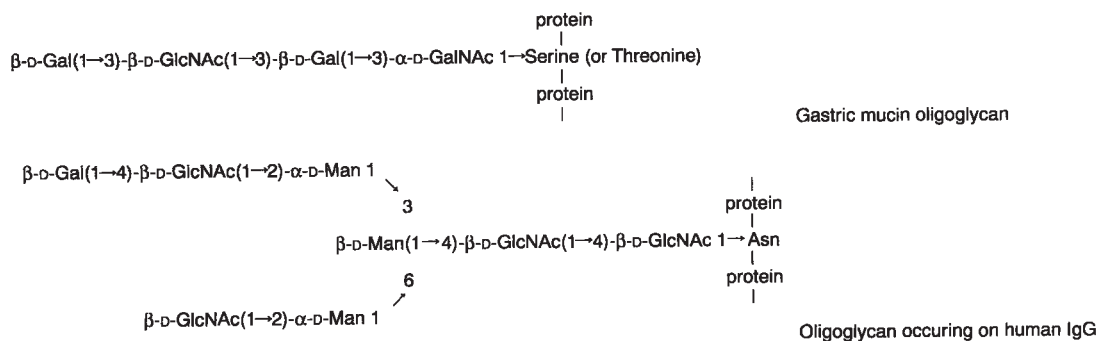
The term 'neoglycoprotein' indicates a glycoprotein that arises through synthetic glycosylation of a protein. Several 'conjugate' vaccines are in fact neoglycoproteins where the saccharide part is the desired immunodeterminant that is linked synthetically to a carrier protein.

Glycolipids consist of an oligosaccharidic fragment linked to, for instance, ceramide. They are frequently of fairly low molecular weight, and the carbohydrate moiety is the immunodeterminant. Below is shown a typical glycolipid (GM1 ganglioside) where NeuAcid stands for neuraminic (= sialic) acid (IX).

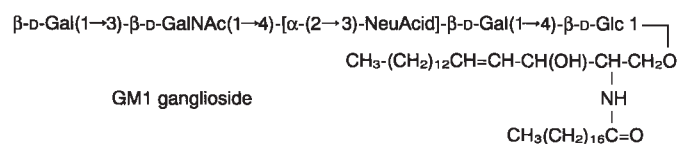
Hakmori and associates have shown that glycolipids can seat their lipid moiety in cell wall membranes, projecting their carbohydrate antigenic grouping into

the environment outside the cell. Human blood-group specificity is a function of oligosaccharidic determinants carried on erythrocyte surfaces as glycolipids or glycoproteins. Transfusion into individuals possessing differing blood groups causes an antibody response in the host due to the immunogenicity of the carbohydrate determinant, or causes the transfused antigen to cross-react with pre-existing antibodies already present in the host. Both of these substances, glycoproteins and glycolipids, can occur as oncodevelopmental antigens. These are antigens which appear and disappear during successive stages of embryogenesis. They may reappear in adult life as tumor antigens, but are not necessarily unique to cancer cells *per se*, as they can occur at low levels in most normal adult cells.

Viruses, including the human immunodeficiency virus (HIV), are usually not capable of encoding for glycosylation enzymes. This means that the glycoprotein-120 on the surface of the HIV virus has its carbohydrates (making up some 50% of the gp120) attached by enzymes and substrates defined by the cells the virus has infected. The various glycosyl transferases or substrates may be modified by the infection, and thus the expressed virus can possess differing glycan structures. The diversity amongst N-glycans in gp120 is very great – there is more diversity than there are glycosylation sites – indicating structural heterogeneity. Since the glycans are attached by the mechanism of the host cell, they may



VIII



IX

present an image of 'self' to the host, and thus escape immune surveillance. Nevertheless, some monoclonal antibodies showing specificity for rare carbohydrate structures can neutralize a broad range of HIV-1 and -2 isolates, as well as block syncytia, even by virus taken directly from patients with the acquired immune deficiency syndrome (AIDS).

Conformation in solution

Polysaccharides as well as the oligosaccharide components of glycoproteins and glycopeptides and glycolipids may be linear or branched, homo- or heteropolymer, neutral or charged. These oligo- and polysaccharides can assume tertiary (globular) structure, and in some cases the particular spacial presentation of a carbohydrate becomes critical in dictating the immunologic specificity. For instance, Jennings and associates have evidence that the capsular polysaccharide of group B streptococcus assumes a conformation in which short, sialic acid-terminated side-chains each form a hydrogen bond with an intrachain GlcNAc nearby. That results in an immunologically specific conformation. This, even though the sialic acid itself is not a (contact) immunodeterminant, and apparently only functions as the vector in creating the correct conformational rigidity. However, it must be kept in mind that for carbohydrates, conformational changes on the polymeric level are easier to achieve (i.e. have lower energy barriers) than they are for proteins. Indeed, various conformational possibilities are probably populated in proportion to the energy differences between them. Critically important requirements such as in the example above do not (yet?) appear ubiquitous for carbohydrate antigens. It should be realized that, for proteins, loss of tertiary structure (i.e. denaturation), once it occurs, is often hard to reverse, even when conditions are changed, while denaturation of polysaccharides is unknown, or rare at best. Polysaccharides are quite heat stable – although autohydrolysis can occur if they are acidic polysaccharides – and their solution structure is less influenced by changes in electrolyte concentration unless they carry charged groupings.

Immune response to carbohydrates

The early discovery that the 'soluble-specific substance', the capsular material of *Streptococcus pneumoniae*, is immunogenic in humans prompted immunizations with purified polysaccharide in the early 1940s. The contemporary and subsequent great success of the treatment of pneumonia with antibiotics rapidly diminished the initial interest in vaccines. However, the continued frequency of serious diseases such as pneumococcal meningitis, and the appearance of bacterial strains showing resistance to antibiotics, including *S. pneumoniae*, has rekindled an intense interest in immunization with polysaccharide-based vaccines. Unfortunately, polysaccharides themselves are often poor immunogens, particularly in infants and in immunocompromised individuals. In addition, for pneumococci, the existence of 84 distinct serotypes has complicated the issue. For this organism, penicillin resistance is concentrated amongst the (pediatric) serotypes 6, 14, 15, 19 and 23, while types 1, 2, 3 and 4 are still largely penicillin sensitive at this time. Polysaccharides are conveniently termed 'T cell independent' (TI) immunogens, and they do not induce immunologic memory, which is required for booster responses. Often only a limited number of B cell clones are activated, resulting in restricted – albeit polyclonal – heterogeneity. Most polysaccharides can induce a state of unresponsiveness, or tolerance, and also do not elicit delayed-type hypersensitivity. In addition, the immunogenicity of polysaccharides increases with molecular weight, and when that is less than 50 kDa the polysaccharide is nonimmunogenic. For all these reasons, immunization with purified polysaccharides has been only partially successful. Frequently, covalent linkage of the carbohydrate determinant epitope, or the entire polysaccharide, to a protein carrier to give a so-called 'conjugate vaccine' allows enhancement of immunogenicity in animals and humans, and instates immunologic memory. The resulting antibodies are protective, and show binding to the parent capsular polysaccharide from which they were derived. Some such immunogens, i.e. Hib polysaccharide linked to tetanus toxoid, have been licensed as human vaccines, and have become very successful in

the immunization of infants. The length of an oligosaccharide, to be attached to proteins to form a suitable conjugate vaccine, must vary. In cases where there is a distinct tertiary structure (see above) of the saccharidic immunodeterminant in the natural, bacterial or viral product, that tertiary structure must undoubtedly be mimicked by the (synthetic or derived) saccharide to be conjugated. The resulting antibodies generated by the immune response would then be able to recognize readily the natural immunogen.

Interaction of carbohydrate antigens with antibodies

In the 1960s Kabat showed that polyclonal anti-dextran antibodies bound maximally to about five sequentially linked sugar residues of the antigen. Modern work on monoclonal antibodies and sharply defined carbohydrate immunodeterminants have since shown that the determinant in an antigenic polysaccharide can be 2–4 sugars long. Affinity constants for the entire immunodeterminant can range from 10^3 to 10^6 M^{-1} . These values suffice for the initiation of an immune response. Antibodies elicited by polysaccharides can be directed against either the interior segments or the terminal segment of the antigenic chain, and the antibody can bind the polysaccharide chain on its surface, or inside cavities. In the case of a monoclonal antibody to $\alpha(1\rightarrow6)$ -dextran it was shown that one group of monoclonal antibodies could bind the dextran all along its chain. Another set of monoclonal antibodies could bind only to a stretch of residues of the dextran that had to include the single chain-end glucose residue of the dextran. The latter was held inside a pronounced cavity, while its three sequential sugars were held near the surface of the immunoglobulin. It was found that an antibody combining area was thus made up of a set of subsites, and these could be mapped. Each one could

accommodate a single sugar residue of the polysaccharide. All together these subsites can bind the entire immunodeterminant of four sugar residues. Generally, the major subsite shows an affinity of 10^2 – 10^3 M^{-1} for its single carbohydrate residue, while the other subsites often possess far less affinity for their particular sugar residues. In the case of a comprehensively studied monoclonal antibody to a homopolysaccharide (galactan), it was found that the immunoglobulin could also maximally bind four sugar residues. Here the monoclonal immunoglobulin could access and bind these repetitively occurring four galactosyl residues in the interior of the polysaccharide chain. All four subsites of the immunoglobulin were on the protein surface, and the subsite with the major affinity was located *within* the sequence of four. In yet another study, a monoclonal anti-*Shigella dysenteriae* type 1 antibody could also bind interiorly located repeating determinants of the bacterium's O-specific heteropolysaccharide.

See also: ABO blood group system; Affinity; Antibody-antigen intermolecular forces; Antigen-binding site; Antigens; Bacterial cell walls; Lectins; Tumor antigens; Vaccines.

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CARBOHYDRATES, IMMUNE STIMULATING

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Recent developments in glycobiology have shown that carbohydrates play an important part in diverse immunological processes such as opsonization and phagocytosis of microorganisms, and cell activation and differentiation (Table 1). Carbohydrates exert

their functions through interacting with carbohydrate-binding proteins or lectins which are widely distributed in mammalian tissues including those of the immune system. Those with immune-stimulating effects are usually large polymers of glucose (glucans

Table 1 Immunostimulating carbohydrates and their structures

Carbohydrates	Structures
β -Glucans	$\beta 1 \rightarrow 3$ Glucose
Lentinan	$\beta 1 \rightarrow 3$ Glucose with $\beta 1,6$ -glucose branches
Mannan (plant)	$\beta 1 \rightarrow 4$ Mannose
Mannan (yeast)	$\alpha 2 \rightarrow 6$ Mannose with $\alpha 1,3$ -mannose branches
Acemannan	$\beta 1 \rightarrow 4$ Acetylated mannose
Pectin	Mainly polygalactose
Inulin	$\beta 2 \rightarrow 1$ Fructofuranosyl- α -glucose
Levan	$\beta 2 \rightarrow 6$ Fructose
Xylan	$\beta 1 \rightarrow 4$ Xylose
Lichenan	$\beta 1 \rightarrow 4$ and $\beta 1 \rightarrow 3$ Glucose

and lentinans), mannose (mannans), xylose (hemicelluloses), fructose (levans) or mixtures of these sugars.

Immune stimulation

Complex carbohydrates act as immune stimulators through two distinct mechanisms. Some, such as the fungal glycans, activate macrophages while others, such as lentinan, are T cell stimulators. Other carbohydrates, such as acemannan, derived from the pulp of the *Aloe vera* plant, act on both macrophages and T cells, and as a result, may influence both cellular and humoral immunity.

Intravenous injection of purified glucan in rodents results in an increase in liver and spleen weight attributed to increased macrophage activity. Clearance of colloidal carbon is significantly enhanced in glucan-treated animals. Macrophages from glucan-treated mice are larger than controls and attach and spread more rapidly on glass. These same macrophages show augmentation of their chemotactic activity. Spleen cells from glucan-treated mice elicit a much more severe graft-versus-host reaction in recipient animals than do cells from normal mice. Administration of glucan to irradiated mice prior to bone marrow transplantation has no effect on syngeneic bone marrow grafts but may prevent acceptance of allogeneic or xenogeneic bone marrow. Depending on the injection protocol, glucan administration to mice may either enhance or depress the *in vitro* response of isolated spleen lymphocytes to concanavalin A and *Escherichia coli* endotoxin.

Lentinan has an adjuvant effect on the response of mice to sheep red cells having a greater effect on the immunoglobulin G (IgG) response than on the IgM response and it enhances antibody-dependent cellular cytotoxicity (ADCC) reactions. Inulin, a poly-

saccharide consisting of fructose and glucose, also has adjuvant properties. It increases the mouse IgG response to keyhole limpet hemocyanin as well as delayed-type hypersensitivity (DTH) reactions to sheep red blood cells. Levan causes macrophage spreading and swelling and stimulates their acid phosphatase and peroxidase activities. β -Glucan and inulin exist in insoluble or microparticulate forms. The microparticulate carbohydrates are better adjuvants because of enhancement of antigen phagocytosis. Targeting antigens into the phagocytic pathway is required for inducing a strong cell-mediated response, a key element for viral and tumor vaccines.

Interaction with lectins on immune cells and the consequent changes in cellular functions is most likely an important underlying mechanism for the observed immunostimulation and adjuvant effect of these polysaccharides. Macrophages play a central role in initiating the immune response. There are several different lectins on macrophages, including the mannose receptor. The receptor on macrophages for β -glucan is Mac-1 (CD11b/CD18).

Antimicrobial effects

Because glucans activate macrophages and stimulate their lysosomal and phagocytic activity, they stimulate immunity to bacterial diseases. Thus, glucan has a protective effect against experimental footpad inoculation of *Mycobacterium leprae*. It reduces the concentration of both viable and dead bacilli in the lesion. Mannan, in contrast, has only a brief protective effect while lentinan is inactive in this system. Intraperitoneal glucan therapy also enhances survival in mice challenged intraperitoneally with virulent *E. coli*. This is probably due to enhanced macrophage phagocytosis and accelerated blood clearance of bacteria.

Attachment to cell or tissue surfaces is often the first step in microbial invasion. This process is mediated by adhesins on the surface of bacteria, many of which are lectins. Thus, blockage of the interaction between bacterial lectin-like adhesins and cell surface oligosaccharides can be a mechanism for the antimicrobial effect of these polysaccharides. That is, these polysaccharides act as structural analogs of the cell surface carbohydrate receptors, binding to bacterial adhesins and therefore preventing them from attaching to cell surfaces. Sugars or oligosaccharides that are known to interact with lectin-like adhesins of a specific bacteria have been shown to inhibit its infection in animal models.

Effects on tumors

The practical importance of complex carbohydrate therapy may rest on their use as immune stimulants in humans or animals suffering from neoplastic conditions.

Glucans

Although common polysaccharides such as starch ($\alpha 1 \rightarrow 6$ glucan), and inulin (fructan) do not have antitumor activity, there is abundant evidence that some mannans and glucans are very potent anticancer agents. The factors that determine this are unclear. The soluble D-glucans have antitumor activity if they are mainly linear, without excessively long branches, and provided they are relatively resistant to degradation by glucanases. Thus glycogen, starch and dextrans are inactive while compounds with long stretches of $\beta 1 \rightarrow 3$ linkages are effective. Dextrans may be made active if derivatized with diethylaminoethyl groups. Studies on synthetic, $\beta 1 \rightarrow 6$ linked celluloses suggest that antitumor activity against sarcoma-180 in mice is greatest in molecules with a high degree of polymerization and homogeneous distribution of side-chains.

Intravenous glucan inhibits the growth of a murine allogenic adenocarcinoma and increases tumor macrophage populations. The glucan decreases the number of hepatic metastases as well as the size of the primary tumor. Enhanced Kupffer cell antitumor activity is correlated with this increased resistance to hepatic metastases. Intravenous administration of soluble glucans also results in a significant reduction in the growth of syngeneic murine mammary carcinoma and melanoma B16. Glucan from *Saccharomyces cerevisiae* has been reported to reduce the size of malignant melanomas and adenocarcinomas in patients with advanced neoplastic disease when administered in and around selected lesions. This reduction in size is associated with necrosis, abscessation and liquefaction of the tumor and a monocytic infiltrate. Water-soluble glucan administered in combination with lymphokine-activated killer (LAK) cells to mice, significantly suppresses the growth and metastasis of a reticulum cell sarcoma. This therapy increased splenic natural killer (NK) cell activity as well as Kupffer cell tumoricidal activity.

Other investigators have failed to substantiate the antitumor activity of glucans in syngeneic systems. Thus when *Saccharomyces* glucan was tested in a guinea pig hepatoma, two murine fibrosarcomas, a murine melanoma and a murine adenocarcinoma and compared with BCG vaccine, no effect was noted. Intralesional, intraperitoneal or intravenous administration of glucan was also found to be inef-

fective in the murine system. The glucan had no significant effect on tumor size, tumor incidence or host survival. It was concluded that the effects of glucan, although impressive in allogeneic systems (such as the sarcoma-180 system), are much less effective in syngeneic tumor systems.

Saccharomyces glucan probably exerts its anti-tumor effects by activating macrophages. Its activity appears not to be T cell mediated since administration of glucan severely inhibits the growth of melanoma B16 cells in nude mice. This antitumor activity in nude mice was associated with enhanced macrophage phagocytosis. The glucan probably stimulates macrophages to produce cytokines such as interleukin 1 and tumor necrosis factors. Thus macrophages from glucan-treated animals have been shown to release factors cytotoxic for tumor cells. Glucan also increases serum lysozyme levels, suggesting increased macrophage function and stimulated *in vivo* and *in vitro* secretion of interleukin 1 (IL-1). IL-2 production by splenic lymphocytes is enhanced 6 h after glucan administration and remains elevated for 9 days, presumably in response to the IL-1. Peak plasma IL-1 and IL-2 activities are found 9 and 12 days, respectively, following glucan administration. Given the very short half-life of these interleukins, this sustained elevation of IL-1 and IL-2 suggests that there is a very high rate of production.

Lentinans

Lentinan is a glucan derived from *Lentinus edodes*, a common edible mushroom. It has been shown to be active against several different allogeneic and syngeneic tumors. Lentinan, like other glucans has no direct cytotoxicity on tumor cells. Nevertheless it shows an optimal dose for antitumor action. The antitumor activity of lentinan varies between mouse strains which may be classified as either high or low responders. In strong responder mice, lentinan will completely regress 3-methylcholanthrene-induced transplantable fibrosarcomas. Lentinan is very effective against mouse methylcholanthrene-induced primary tumors in combination with cyclophosphamide. When administered to cancer patients in phase I and II trials, encouraging results have been obtained.

The antitumor activity of lentinan, unlike that of glucan, does not occur in neonatally thymectomized mice or in mice treated with antilymphocytic serum or by whole-body irradiation. It does not increase phagocytosis but it can stimulate macrophage cytotoxic activity *in vivo*. As a result, the antitumor effect of lentinan may be blocked by antimacrophage agents such as carrageenan or silica. Lentinan does not accelerate antibody formation, nor does it cause

an increase in blood lymphocytes, accelerate allograft rejection or influence delayed-hypersensitivity reactions.

Mannans

Mannans with significant antitumor activity have been isolated from several species of yeast. They have been tested for activity against sarcoma-180 in Swiss albino mice. There appears to be no relationship between the amount of glucose in these mannans and their activity. The mannan from *C. utilis* that contains an α -linked glucose is poorly inhibitory while the mannan from *C. albicans* that contains a β -glucan component is highly active. Mannans from *S. cerevisiae* and from *Candida utilis* inhibit the growth, not only of sarcoma-180 but also of 3-methylcholanthrene-induced tumors, Ehrlich carcinoma and NF sarcoma. None of these mannans appear to be active against the ascites form of sarcoma-180.

When mannoglucan prepared from *Magnaporthe grisea* is administered intravenously to C3H mice bearing the solid MH134 hepatoma, the blood flow to the tumor is inhibited within 6 h and tumor growth retarded within 3 days. If the tumor mass is excised and extracted at various intervals after administration of mannan, a soluble cytotoxic factor is detectable in the tumor homogenate. This factor is probably a form of tumor necrosis factor (TNF) since its activity is inhibited by anti-TNF serum, it has the same molecular weight range as TNF (70–80 kDa) and the dose dependencies of the cytotoxin and TNF are similar.

Acemannan (polyacetylated mannan) obtained from *Aloe vera* stimulated a tenfold release of ^{51}Cr from labeled tumor cells in the presence of macrophages. When injected intraperitoneally into female CFW mice subcutaneously implanted with murine sarcoma cells, acemannan reduces mortality from 100% of the control animals to 60–65% mortality in treated animals. Acemannan-treated animals exhibit characteristics of *in vivo* tumor necrosis, including development of concave liquefied areas on the tumor mass, and show visible evidence of necrotic toxemia. Necrotic tumors are found to exhibit central necrotic foci with hemorrhage and peripheral fibrosis. Similar effects have been observed in cats and dogs with spontaneous fibrosarcomas treated with acemannan.

Levans

The antitumor activity of levans, unlike that of the other polysaccharides described above, is a result of its ability to stimulate not only macrophages, but also B cells and T cells. For example, levan is a B

cell mitogen and can therefore stimulate a polyclonal B cell response. Inhibition of growth of Lewis lung carcinoma (3LL) by levan in mice is probably due primarily to macrophage activation. The site and timing of treatment with high molecular weight levan (2×10^4 kDa) significantly affects its activity against transplanted AKR lymphoma. Thus tumor growth is best inhibited by inoculation of levan directly into the primary tumor soon after tumor inoculation. It is ineffective if administered more than 2 days later. However, metastases are most effectively inhibited by intraperitoneal inoculation, suggesting that macrophage activation may be required for antitumor activity.

Pectin

Pectin, a galactose-rich carbohydrate abundant in citrus, is also known to have an antitumor effect. Inhibition by pectin of the metastasis of prostate cancer cells has been shown in a rat model. It has been suggested that pectin blocks galectin-3, a galactose-specific lectin, on cancer cells so that they cannot interact with the cellular matrix and therefore cannot emigrate.

In conclusion, it is clear that many complex carbohydrates have immunostimulating activity which may be of use for the treatment of malignancies. However, all complex carbohydrates clearly do not have the same mode of action, but act on different components of the immune system. Further studies are clearly required to determine the optimal conditions for their use in humans and animals. Recent developments in understanding carbohydrate–lectin interactions in the mammalian system will certainly add to these efforts.

See also: Carbohydrate antigens; Immunopotential; Tumors, immune response to.

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CARDIAC DISEASE, AUTOIMMUNE

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In contrast to the extensive experimental and clinical knowledge that has accumulated concerning immunological and autoimmune mechanisms in the pathogenesis of tissue injury and dysfunction, notably the kidney, the concept that autoimmunity might play an important role in cardiac disease is just beginning to emerge. The slow progress in this area is, in part, due to the lack of good experimental models of autoimmune heart disease and also perhaps because of the difficulty of direct accessibility to the target organ itself. With the advent of the endocardial biopsy and other technological advances in cardiology, the ability to study cardiac tissue '*in situ*' has improved considerably and should provide the investigator with reasonable amounts of material for study similar to that seen since the advent of the renal biopsy.

These drawbacks notwithstanding, knowledge supporting the idea of autoimmune mechanisms in human heart disease has evolved greatly over the last two decades.

Microbe-induced cardiac autoimmunity

Rheumatic fever

The 'grand-daddy' of all microbial-induced autoimmune heart disease is rheumatic fever (RF). The concept of autoimmunity playing a role in this disease was introduced over 50 years ago by a number of investigators when antibodies to heart were noted in the sera of patients with acute rheumatic fever (ARF) and/or rheumatic heart disease (RHD). The origin of these antibodies was better defined when Kaplan and colleagues noted that immunization of rabbits with group A streptococci induced antibodies that bound to human heart in a manner strikingly similar to that observed with human patient sera. Further experiments revealed that the cross-reacting

antigen being identified was similar to (but not identical with) M protein and was also present in a limited number of group A streptococcal strains. Fischetti and colleagues identified the complete chemical structure of the M protein and demonstrated a close homology of the M protein moiety with cardiac cytoskeletal proteins. Further studies by Sargent and coworkers have clearly demonstrated that the M protein moiety contains numerous epitopes cross-reactive with certain cardiac proteins (e.g. tropomyosin and myosin) including those unrelated to the cytoskeletal proteins.

Similar studies in our own laboratory demonstrated the presence of a second cross-reactive antigen residing in the streptococcal membrane unrelated to M protein. This antigen was present in all streptococcal throat stains tested, and has been purified to a series of four peptides ranging in molecular weight from 23 to 22 kDa. An important feature of this protein was the demonstration by van de Rijn that the heart-reactive staining pattern of ARF sera was completely abolished by preabsorption of the sera with these closely spaced peptides. Further studies on lymphocytes by Zabriskie from ARF patients revealed enhanced cellular reactivity to this membrane antigen but not the cell wall antigen. This reactivity was confined to membrane preparations from streptococcal throat strains and not strains isolated from skin-associated streptococcal infections.

Autoimmune mechanisms are also thought to play a role in another major clinical manifestation of ARF, namely rheumatic chorea. Through the work of Husby and Williams, it was clear that the sera of patients with active chorea contained an antibody which bound to the cytoplasm of the cells of the caudate nuclei and was specific for these cells. The presence of the antibody correlated with the clinical activity of the disease and could be absorbed by streptococcal antigens. Further, the antibody was

detected in the cerebrospinal fluid of five patients with active chorea.

The nature and reactivity of lymphocytes from the RF valvular lesions is an area of active investigation in our laboratory. For example, using a panel of monoclonal antibodies specific for various cell surface markers, Dr Kemeny demonstrated that valvular lesions contained equal numbers of macrophages and T cells. Helper T cells predominated in the more active cardiac lesions; the T helper/T suppressor ratio in chronic carditis closely resembled that seen in normal peripheral blood. Recently, Dr Yoshinaga extended these experiments one step further and was able to isolate and clone T cell lines from these valvular specimens. When these T cell clones were stimulated with various streptococcal antigens, only streptococcal membrane antigens isolated from rheumatic fever-associated strains elicited a reaction in proliferation assays. Antigens isolated from nephritogenic strains or antigens from other groups of streptococci were nonreactive. One unanticipated and therefore surprising finding was that none of these T cell clones reacted against cardiac antigens or a variety of purified M protein antigens. In contrast to these findings Guilherme and colleagues have isolated individual T cell clones from RF valvular specimens which reacted both with streptococcal antigens and mammalian cytoskeletal proteins. The exact nature of these reactive antigens is under investigation.

The question of whether or not these heart-reactive antibodies or activated T cells isolated from patients are actually cytotoxic for the relevant tissue antigens has been difficult to prove. The experiments of Yang and colleagues clearly demonstrated that guinea pigs sensitized to streptococcal membranes produced activated T cells specifically cytotoxic for guinea pig neonatal myofibers but not for other tissues, whereas immunization with cell walls did not. However, the addition of heart-reactive antibodies obtained from the sera of these animals did not enhance the cytotoxicity, indicating that the observed cytotoxicity was antibody independent. The few experiments performed to date with cells obtained from 'inactive' rheumatic fever patients also show increased cytotoxicity for human atrial myofibers. Once again, the addition of heart-reactive antibody from the sera of these patients did not enhance the cytotoxicity. These results would suggest that cellular autoreactivity by activated T cells may play a more important role in the rheumatic cardiac lesion than previously thought.

In this context, recent observations by Cunningham and her coworkers are worth mentioning and may correlate with observations made by Rose and colleagues in an experimental model of cardio-

myopathy (see below). Using streptococcal-induced monoclonal antibodies that cross-react with M protein and human cardiac myosin, they could neutralize coxsackieviruses B3 and B4. The viral-neutralizing antibodies were also cytotoxic for heart and fibroblast cell lines and reacted with viral capsid proteins on western blots.

Given the evidence of an abnormal host immune response to streptococcal antigens cross-reactive with relevant target antigens, the tantalizing and as yet unresolved question is whether there is a genetic basis for this response in rheumatic fever patients. Beginning with Cheadle's work 100 years ago, numerous investigators have presented evidence for and against this concept. Recent articles have suggested that susceptibility to ARF following streptococcal infection might be related to alleles of the human major histocompatibility complex (MHC) but the association with a given phenotype has been either controversial or inconclusive.

To date the strongest association of a cell marker with rheumatic fever has been the identification of a B cell marker apparently unrelated to the MHC by Patarroyo in Colombia and our group in New York. The relative risk of contracting the disease if one bears the marker was 12.9. Because of the limited supply of the original alloantiserum used to identify the marker, we embarked on a series of studies attempting to reproduce this marker using hybridoma technology and immunization of mice with B cells isolated from rheumatics. Of a number of monoclonal antibodies, we have recently isolated one called D8/17 which identifies all rheumatics tested in a number of widely differing geographic areas of the world. The antibody is also not related to the MHC, and family studies indicate this marker is inherited in an autosomal recessive fashion. The exact nature of the antigen is as yet unknown but our experiments suggest it is not present on other lymphoid cells. The antigen is, however, also expressed on other tissues, most notably the heart and smooth muscle cells. What role this antigen plays in the disease process is unknown at present but its expression on B cells from all rheumatics strongly suggests its direct involvement in the etiopathogenesis of rheumatic fever.

Chagas disease

Although rarely seen in more temperate climates this disease is endemic in many parts of the world, most notably in South America where an estimated 11 million people or more are infected with *Trypanosoma cruzi*. The acute disease is the result of multiplication of the organism in the reticuloendothelial system followed by direct invasion of muscle tissue, liver

and occasionally the meninges. In contrast, in chronic Chagas' cardiomyopathy, the organism is absent but there is a progressive inflammatory cardiac myofibrillar degeneration with necrosis. An important clue to the etiology of the chronic form of disease was provided by Cossio and coworkers who noted that the sera from a high percentage of these patients contained antibodies reactive against components of cardiac sarcolemma as well as with the valvular endothelial cells. A causal role for this antibody in disease pathogenesis was suggested when cardiac biopsies from Chagas patients demonstrated immunoglobulin bound to cardiac myofibers. Eluted antibody from this tissue bound to myofibers of unaffected heart tissue in a pattern similar to that seen in the patient.

In addition to the presence of immunoglobulin in these biopsies, numerous investigators have also commented on the presence of chronic lymphocytic infiltrates in these tissues, suggesting that abnormalities in cellular reactivity might also be involved in the initiation and/or perpetuation of target organ damage. Santos-Buch and colleagues examined cellular immunity in a mouse model of Chagas' disease and noted that sensitized cells from these animals exhibited cytotoxic activity for mouse heart cells in culture. As with rheumatic fever (see above) the addition of heart-reactive antibody from these animals did not enhance the cytotoxicity, again suggesting that cellular autoimmunity to heart antigens might play the primary role in this disease.

As with chorea of rheumatic fever, a new chapter is being written on the relationship of the *T. cruzi* antigens to structures of the parasympathetic nervous system causing the neurological manifestations of the disease. Several investigators have now reported antigenic cross-reactions between *T. cruzi* and neuronal and glial antigens. Using cloning techniques and recombinant technology, Voorhis and Eigen have shown that a 160 kDa *T. cruzi* flagellar protein cross-reacts with a 48 kDa human neuronal antigen. Immunoperoxidase studies of brain sections revealed that at least part of the reaction is related to a neuronal protein of the myenteric plexus (also found in the neurons in the ileum) perhaps explaining the observed megacolon and megaesophagus so often seen in Chagas patients. Interestingly, this cross-reaction was also localized to the Purkinje fibers of the brain.

In summary, autoimmune phenomena have been reported and implicated in the etiology of progressive cardiac damage in both experimentally infected animals and chronic Chagas disease in humans (Cossio and colleagues and Gazzinelli and colleagues). It has been suggested that both changes in the T cell reper-

toire (Miniprio and coworkers) and also molecular mimicry (Van Voorhis and colleagues) might be implicated in the pathogenesis of the autoimmune phenomena seen following *T. cruzi* infection. Yet another intriguing possibility is a chronic immune response to local parasites that may persist in cardiac tissue during the chronic stage of the disease (Jones and coworkers). D'Imperio Lima and colleagues have described persistent B cell activation and immunoglobulin production in the murine model of *T. cruzi* infection, even when injected parasites are no longer detected in the blood. Finnegan and colleagues have reported that autoreactive CD4⁺ T cell clones are capable of polyclonal activation of immunoglobulin production of B cells in both MHC-restricted and non-cognate fashions.

Recently, Freire-de-Lima and colleagues have reported that splenic CD4⁺ T cells from chronically infected mice have markedly enhanced autoreactivity towards normal syngeneic but not allogeneic resting B cells. This suggests that enhanced CD4⁺ T cell autoreactivity can be a potential cause of persistent B cell activation and autoantibody production in chronic Chagas' disease.

Cardiomyopathies

It has long been known that numerous microbes, parasites and viruses can cause acute myocarditis. In these clinical syndromes the organism can be readily demonstrated in the tissues of the individual. In contrast, there is an ill-defined group of cardiomyopathies in which the offending organism that caused the acute inflammation is no longer present, yet the cardiomyopathy continues to progress. These cases are distinguished by the presence of lymphocytic infiltrations and, in a majority of cases, increased titers of heart-reactive antibodies. Among the agents considered to be important causal agents in this group of cardiomyopathies have been the group B coxsackieviruses. These agents frequently cause acute cardiac inflammation in humans, and 10–12% of patients go on to develop a more chronic phase of the disease. Because chronic and progressive myocarditis is restricted to a small number of patients, it has also been suggested that there might be a genetic basis for the disease.

This concept has been greatly strengthened by the elegant work of Rose and his colleagues, working with an animal model of coxsackie B3 virally induced myocarditis. Using genetically different strains of mice, they could induce a chronic myocarditis primarily in H-2 congenic strains bearing an A background (i.e. ACA, ASW) whereas C57BL/10 H-2 mice were resistant. A key finding in these studies was that only those mice with heart-reactive anti-

bodies in their sera went on to develop chronic cardiomyopathy; these antibodies were primarily directed against the cardiac isoform of myosin. A causal role for myosin in the etiology of this cardiomyopathy was shown when injection of myosin alone was able to induce severe immunologically mediated myocarditis in certain genetic strains of mice.

Hoping to demonstrate that myosin and the coxsackie B virus shared common antigen determinants, Rose and colleagues were disappointed in their inability to show that heart-reactive antibodies in their coxsackie B3-induced cardiomyopathy cross-reacted with viral antigens. Saegusa and coworkers however, in preparing a series of different monoclonal antibodies against coxsackie B4 virus identified one which both neutralized the coxsackie B4 virus and also bound to rabbit and mouse heart tissue. An important observation was that this antibody did not bind to human heart tissue, suggesting that both the strain of virus and the myosin of a given species may be crucial for the induction of cardiac disease. Whether or not viruses other than coxsackie B are involved in chronic human cardiomyopathies and whether this progression is on a genetic basis, though unknown at present, is a particularly fertile field for future study.

Postpericardiotomy syndrome

The possible pathogenetic role of heart-reactive antibodies in the postpericardiotomy syndrome (PPS) has been investigated over a number of years by Dr Engle and her colleagues. In both adults and children the syndrome appears 10–14 days after surgery, and is characterized clinically by fever, chest pain, pericardial and plural effusions. Long thought to be related to rheumatic fever, it is clearly a separate syndrome. One common feature, however, is the presence of high-titer heart-reactive antibodies in the sera of patients with these conditions.

An interesting and as yet not entirely explained observation for the induction of postpericardiotomy syndrome is that while the syndrome can be induced by damage to cardiac muscle and the surrounding pericardium, it is almost never seen in operations such as those involving repair of coarctation of the aorta or closure of a patent ductus. Furthermore, the degree of trauma to cardiac muscle often defines the severity of the syndrome. For example, excision of ventricular muscle (such as repair of tetralogy of Fallot) results in the highest incidence of the syndrome.

The question of whether or not a microbe is responsible for the induction of the autoimmunity in this syndrome is unresolved. Engle noted that

approximately 70% of her PPS patients had a rise in antiviral titers to one or more of eight common viruses tested (e.g. coxsackie, megalovirus, adenovirus, etc.). The heightened antibody response to a given virus was not constant however, and seemed to vary with viruses common in the community that year. Preliminary experiments attempting to identify viral presence or isolate whole virus from the excised tissues of these patients have been uniformly unrewarding.

In vitro cytotoxic studies do, however, suggest that the heart-specific antibody in the sera of these patients does play a direct role in the disease pathogenesis. The addition of heart-reactive antibody from these patients to cultured beating heart cells *in vitro* was not cytotoxic, yet caused a marked decrease in contractility. The questions of whether or not susceptibility may be mediated via alleles of the human MHC and the role of sensitized lymphocytes in disease pathogenesis have not been answered.

Significance

It is now clear that many microbes share antigenic determinants not only with other microbes but also with a variety of human tissues. Cross-reactions between streptococci and heart tissue, *E. coli* antigens and colonic antigens, HLA-B27 and shigella antigens, gram-negative bacteria and blood groups are but a few examples of these cross-reactions seen in nature. The majority of these cross-reactions are generally harmless and may serve the purpose of protecting humans against a wide variety of microbes which might be pathogenic. However the combination of the right microbe infecting the genetically susceptible host may result in serious autoimmune consequences.

As indicated above, perhaps the two best examples of microbially induced autoimmune cardiomyopathies are RF and Chagas disease. Here the cross-reactions between host and relevant target organs have been well defined; there is an abnormal host cellular and humoral response to tissue and microbial antigens. Further, in RF, there appears to be a genetic predisposition to the disease. Whether this genetic predisposition allows for breakage of tolerance on the part of the host, an abnormal response to the specific cross-reactive determinant or production of anti-idiotypic antibodies bearing the cross-reactive epitopes is unknown and will require further investigation.

In terms of the immune response of the host, it is becoming increasingly apparent that the cellular response to cross-reactive antigens may play a more significant role in the observed pathological damage

then previously thought. In both rheumatic fever and Chagas disease, T cells are specifically cytotoxic for the target organ and the addition of antibody does not enhance the cytotoxicity, suggesting a more crucial role for cellular immunity in the disease process. Similar studies of experimental models of autoimmune heart disease point to the same conclusion. To date, these studies have not been done in either the PPS syndrome of the idiopathic cardiomyopathies.

In more general terms, what is the significance of heart-reactive antibodies in these disease states? It is well-known that organ-specific antibodies increase with age; appear in a variety of other disease states (e.g. systemic lupus erythematosus, RA) and in many instances do not appear to cause damage. Even for rheumatic fever and Chagas disease, is it a question of the 'chicken or the egg'?

In other words, are these cross-reactive antibodies directly involved in the disease process or are they merely a reflection of prior heart damage and a fortuitous cross-reaction with a given microbe? The evidence in rheumatic fever suggests the latter concept is not true since these heart-reactive antibodies are absorbed by both streptococcal and cardiac antigens while heart-reactive antibodies in PPS are absorbed only by cardiac tissue. Thus in RF the heart-specific antibody appears to relate to the infection whereas in PPS the antibody appears to relate to tissue damage.

Whether or not these heart-reactive antibodies (from whatever origin) play a direct role in the disease process is difficult to ascertain with certainty. They do correlate with the disease state and disappear during convalescence; they are seen in the pathological tissue specimens but they do not apparently kill target organ cells or enhance specific cellular cytotoxicity *in vitro*. Perhaps complement activation and/or other factors are needed *in situ* to produce the observed immunoglobulin staining seen in the lesions of several cardiomyopathies. To our knowledge, these experiments have not been attempted *in vitro*.

What is becoming increasingly clear, not only in cardiac autoimmunity but also in other autoimmune diseases such as rheumatic arthritis, is that cellular activation specific for a given target tissue appears to be important in the pathogenesis of disease. Cytotoxic T cells specific for cardiac myofibers are seen in both rheumatic fever and Chagas' disease, and it is our impression they may be important in the pathogenesis of other cardiomyopathies as well. Obviously, it will be important to define the exact nature of the cross-reactive epitopes recognized by these active cells and their role in causing cytotoxicity to human tissue.

Finally, the specific mechanisms operating in the interaction between host genetic factors and environment resulting in the induction of autoimmune disease of the heart is still not clear. In rheumatic fever there appears to be a marker called D8/17 which is inherited in an autosomal fashion and is preferentially increased in the B cells of rheumatic fever patients. However it is well-known that not all strains of group A streptococci cause rheumatic fever, even in the genetically susceptible individual. The most plausible explanation for this discrepancy is that only certain strains exhibit the epitopes that activate the immune system to produce tissue cross-reactive antibodies and activated T cells. Then, only in the context of the immune response (a heightened response, break in tolerance, etc.) will the disease occur. A similar mechanism can be postulated for the other cardiomyopathies, as it appears that only select individuals will develop progressive autoimmune disease following active infection. Future studies of each of the cardiopathies mentioned above should concentrate not only on the immune response of the host to a given cross-reactive antigen (either in the microbe or host) but should examine more carefully whether or not a particular microbe exhibits a specific epitope.

Obviously the field is ripe for applying the modern tools of molecular biology to the study of these intriguing host-microbial relationships in cardiac disease.

See also: Autoimmune diseases; Autoimmunity; Chagas' disease; Circulatory system infections; Molecular mimicry; *Streptococcus*, infection and immunity; Trypanosomiasis, African.

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CARRIER

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Our current understanding of antibody production by B lymphocytes, and the underlying cooperation between T and B lymphocytes, owes much to the early use of hapten-carrier conjugates in immunologic studies. It had been known from the work of Landsteiner and of Pauling in the 1940s that haptens, small antigenic determinants such as dinitrophenol (DNP), were insufficient by themselves to elicit specific antibody, but would bind to antibody once it was produced. The problem of how to generate hapten-specific antibodies was resolved by physical coupling of the hapten to a larger 'carrier' molecule. Proteins that have proved to be suitable carriers include bovine serum albumin, bovine gammaglobulin and ovalbumin.

We now understand that an efficient carrier is an immunogenic antigen that elicits a response from helper T lymphocytes. These cells will provide the necessary signals to enable B lymphocytes to generate hapten-specific antibodies. The following recapitulates the evidence for this. The first evidence that the carrier molecule itself was also recognized during an immune response to the hapten was provided by Ovary and Benacerraf in 1963. Animals primed with hapten A coupled to carrier B developed a secondary,

anamnestic antihapten (and anticarrier) antibody response after boosting with the original A–B conjugate. However, no antibodies to A were seen if the mice were challenged with hapten A coupled to an unrelated carrier such as carrier C. This curious phenomenon was termed the 'carrier effect'.

The mid-1960s was an exciting period that saw the emergence of the small circulating lymphocyte, hitherto regarded as uninteresting, as a primary component of the immune system. This apparently homogeneous population of cells was soon split into two functionally different, but cooperative subsets. The pioneering work of Gowans, in which the immune system could be severely compromised by depletion of circulating lymphocytes, demonstrated the fundamental importance of this cell. A functional dichotomy was revealed by extirpation experiments by both Glick and Cooper, using chickens, and in mice by Miller. Their findings, that depletion of B lymphocytes by bursectomy abolished antibody production without affecting cell-mediated immunity, and that depletion of T lymphocytes by neonatal thymectomy ablated both antibody and cellular immune responses, laid the foundations of modern cellular immunology. A series of influential studies then fol-

lowed (Claman; Davies; Mitchell and Miller) which dealt with establishing that an antibody response required both T and B lymphocyte subsets. In essence, B cells which were shown to be responsible for the generation of antibodies, and T cells which helped by providing regulatory signals, both had to interact with each other before a specific antibody response could be elicited.

The next problem was to explain how the antigen fitted into this interaction between helper T cells and B cells. Hapten-carrier conjugates provided a powerful tool in addressing this question. It was already known that some proteins were better carriers than others. Immunization with hapten-carrier conjugates only induced hapten-specific antibody responses when the carrier to which the hapten was attached was itself 'immunogenic', that is if the carrier was a T cell (or thymus) dependent antigen. Nonimmunogenic molecules served as poor carriers for haptens. This, and the 'carrier effect', indicated that separate recognition systems existed for the hapten and carrier, presumably mediated by B lymphocytes and T lymphocytes, respectively. The first direct evidence that cooperating T and B lymphocytes responded to different determinants on the same antigen molecule was provided by Mitchison (1971) using hapten-carrier conjugates. An adoptive transfer system, originally developed by Playfair and colleagues was used, in which lymphocytes were transferred from donor mice to a syngeneic recipient which had been rendered immunoincompetent by sublethal irradiation. T and B lymphocytes primed previously to different determinants were tested for their ability to cooperate within the recipient host (as manifested by the production of hapten-specific antibodies) in response to challenge with the determinants complexed on the same molecule. Mitchison observed that adoptively transferred spleen cells primed to hapten A conjugated to carrier B responded to challenge with A-B but not A-C (the carrier effect). However, if spleen cells from A-B primed mice were transferred together with cells primed to carrier C, an excellent response to challenge with A-C occurred. Moreover, hapten A and carrier C had to be physically linked.

These data, which have been confirmed in many experimental systems since, established that recognition of hapten and carrier is performed by different populations of cells that can cooperate to produce hapten-specific antibody, provided that the hapten and carrier are part of the same molecule. Formal proof that T lymphocytes recognized carrier determinants came from Raff in 1970. Employing the adoptive transfer system, depletion of Thy-1⁺ lymphocytes (T cells) from the carrier-primed population

using Thy-1-specific antibody plus complement abolished the adoptive antihapten response. Elimination of T lymphocytes from the hapten-primed population had no effect.

These experiments using defined B lymphocyte determinants coupled to carrier proteins revealed the importance of cognate T and B lymphocyte interactions in antibody synthesis and the recognition of spatially disparate determinants within the antigen by the cells participating in the response. A macromolecular antigen can thus be regarded as a complex of carrier and haptenic determinants, or, more precisely, T helper and B lymphocyte epitopes.

Recent interest in carriers has been revived by the potential use of synthetic polypeptides which contain antigenic determinants for use as vaccines. The efficient generation of antibody responses to peptides usually requires the peptide to be coupled to an immunogenic carrier as a source of helper T cell determinants. In order that an appropriate anamnestic response is mounted upon subsequent pathogenic infection, the carrier must possess helper T cell determinants derived from the same pathogen as those recognized by the antibodies, thereby ensuring that T and B cells capable of cognate interaction in response to the pathogen will be present in the memory pool. Studies in inbred animals have shown that the helper T cell determinants above, in the form of synthetic peptides, can be used in place of the carrier molecule - thereby raising the prospect of vaccines comprising no more than arrays of contiguous T and B cell epitopes.

See also: CD40 and its ligand; B7 (CD80 & CD86); Cooperation, mechanisms of cellular; Cytokines; Epitopes; Hapten; Helper T lymphocytes; Vaccines

Further reading

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CATALYTIC ANTIBODIES

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The concept of inducing antibodies that would possess, in addition to their exquisite ligand specificity, catalytic potential has its roots in the seminal contributions of Pauling. His proposal, that the ability of an enzyme to speed up a chemical reaction stemmed from the 'complementarity of the enzyme's active site structure to the activated complex', shifted the research focus from concerns about substrate–enzyme fit to a means for defining the structural requirements for binding the transition state. Since a transition state by definition has a negligible lifetime, evidence was sought for transition state stabilization in the tighter binding of inhibitors whose structures mimicked those of the presumed transition state relative to the weaker binding of substrate. Many examples of such high-affinity transition state inhibitors are now documented. However, stabilization of the transition state alone is necessary but not sufficient to give catalysis, which requires differential binding of substrate and transition state. The use of transition state analogs to induce catalytic antibodies successfully stems not only from this differential binding but other factors such as the introduction of catalytic residues. Nevertheless, it was apparent that such inhibitors would furnish a convenient starting point for creating catalytic antibodies which would owe their catalytic properties to their ability to bind the transition state of the reaction. Earlier attempts, however, were thwarted by the use of polyclonal rather than monoclonal antibodies, and by the need for better transition state mimics. In some cases, optimum haptens cannot be obtained owing to difficulties in the synthesis of precise mimics.

Reaction types catalyzed by antibodies

There are now approximately 100 reactions that have been catalyzed by antibodies. Representative examples include: pericyclic processes (Diels–Alder condensation, Claisen rearrangement, oxy-Cope rearrangement); elimination reactions (E2 elimination of benisoxazole, syn elimination of hydrogen fluoride from fluoroketones, dehydration, decarboxylation); various hydrolyses (carbonate esters, amides, esters, lactones, enol ethers); several types of bond-forming reactions (lactonization, peptide synthesis, cationic cyclization, aldol condensation) and redox processes (sulfide oxidation, reduction of ketones, epoxidation). Specific cases are given in Figures 1–

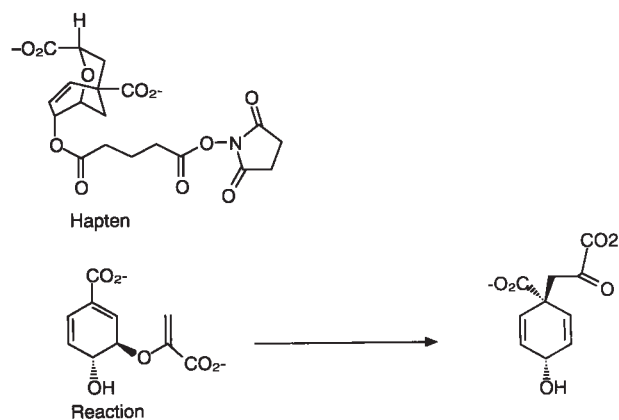


Figure 1 Claisen rearrangement. (Reproduced with permission from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29–54, © 1992, by Annual Reviews Inc.)

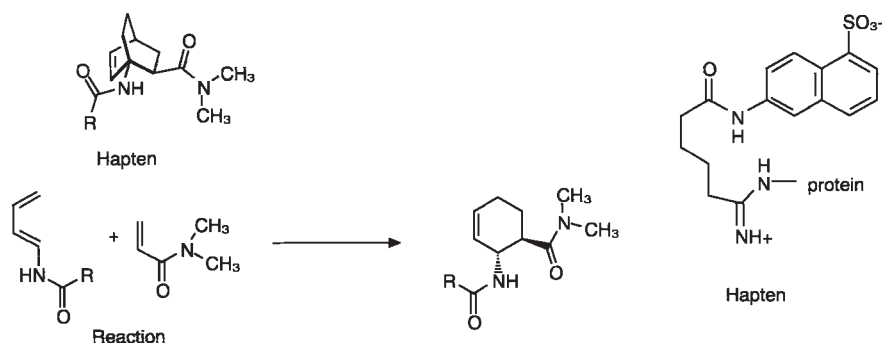


Figure 2 Disfavored exo Diels-Alder reaction. (Modified from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29-54, © 1992, by Annual Reviews Inc.)

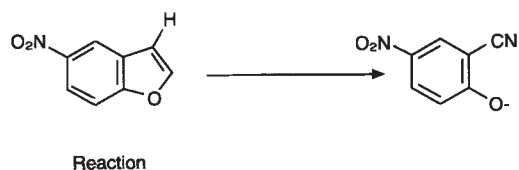
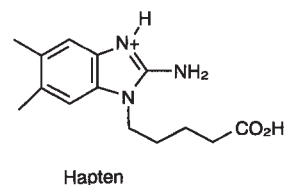


Figure 3 E2 Elimination. (Modified from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29-54, © 1992, by Annual Reviews Inc.)

11. The chosen reactions illustrate the more significant features of antibody catalysis. The majority of antibody-catalyzed reactions are highly stereospecific; the Diels-Alder condensation (Figure 2) is an example of enantioselectivity in which the normally disfavored exo product is generated over the inherent

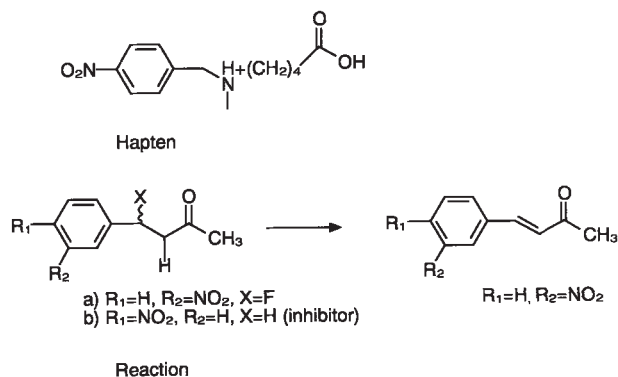


Figure 4 β -Elimination. (Reproduced with permission from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29-54, © 1992, by Annual Reviews Inc.)

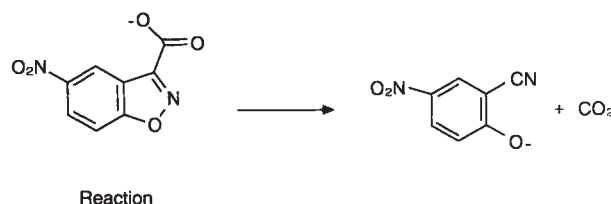


Figure 5 Decarboxylation. (Reproduced with permission from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29-54, © 1992, by Annual Reviews Inc.)

endo specificity of the Diels-Alder reaction. Similarly the antibody catalyzed cyclization (Figure 7), in which racemic starting material is resolved kinetically, represents a case in which one major product is generated under solvolytic conditions that generally yield a mixture. The stereo- and regiospecificity of antibody catalysis, particularly for transformations for which there is no comparable enzymic process, has the potential to be useful in commercial synthetic and medical applications such as the formation of pharmaceuticals and prodrug activation.

For efficient catalysis, strategies are needed for the introduction of catalytic functions within the antibody-combining site correctly juxtaposed to the substrate. Charge complementarity to induce a catalytic base for promoting the elimination of hydrogen fluoride is shown in Figure 4. A catalytic base also is operative in the E2 elimination illustrated in Figure 3. The use of a reactive diketone hapten (Figure 11) to elicit antibodies with a combining-site amine provided catalysts capable of forming an active-site enamine. The latter forms an aldol product (the α -hydroxyketone) upon reaction with a substrate aldehyde followed by hydrolysis. Alternatively, the reactive reagent could be provided externally, as in the case of the periodate (Figure 10).

Nature of the combining site

There is a small repertoire of main-chain conformations, 'canonical structures', for at least five of the six hypervariable regions of antibodies whose

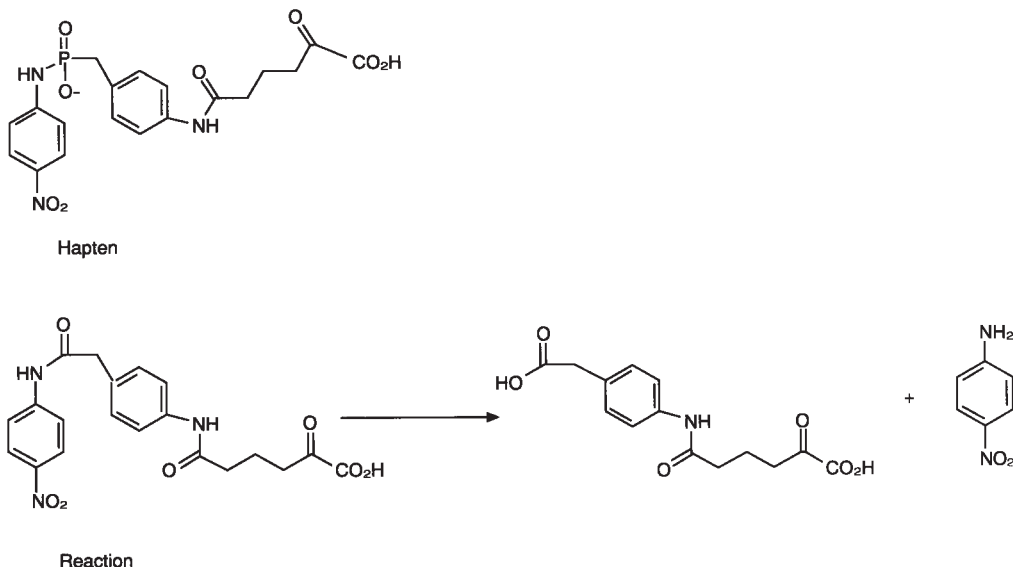


Figure 6 Amide hydrolysis. (Reproduced with permission from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29–54. © 1992, by Annual Reviews Inc.)

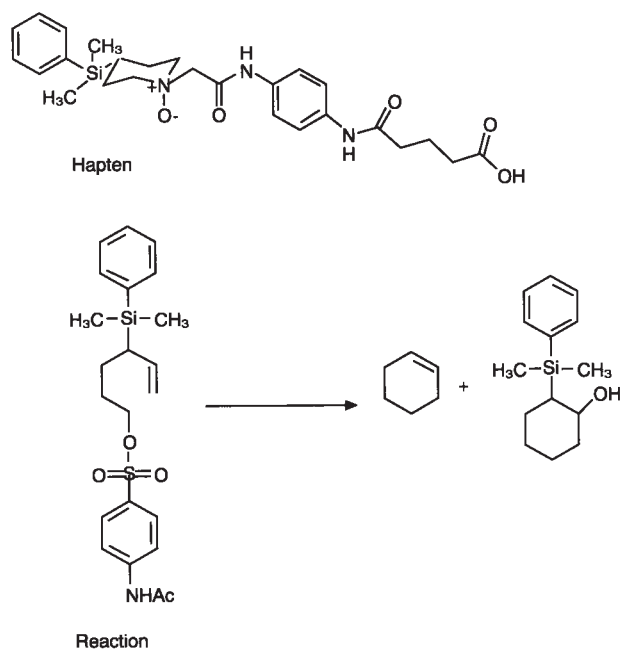


Figure 7 Cationic cyclization. (Modified from Schultz PG and Lerner RA (1995) Molecular diversity to catalysis: lessons from the immune system. *Science* **269**: 1835–1842.)

conformation is determined by a few key residues. The area of interaction between the antigen and antibody may be relatively flat and extensive for protein antigen binding to an antibody (700–750 Å²), whereas in the case of small organic molecules the binding may occur by way of clefts whose volumes

are in excess of $600\text{\AA}^3$. For small organic molecules the dissociation constant of the antibody-antigen complex ranges from 10^{-4} to 10^{-10} M, which if totally coupled to drive a chemical transformation would provide a free energy change up to 15 kcal M^{-1} , sufficient to promote most reactions in aqueous solution. The binding of antigen does not result in a global conformational change in the antibody. Rather, the union is accommodated by conformational adjustments in the specific amino acid side-chains that improve the initial binding interactions of hydrogen bonds, van der Waals and electrostatic forces. For a series of catalytic antibodies that were induced by phosphonate haptens (Figure 6) and hydrolyze esters, the active site structures are surprisingly congruent, suggesting the immunological response despite its diversity is tightly programmed by the chemical nature of the hapten. In general the crystallographic studies of catalytic antibodies show the bound hapten has induced antibodies that provide a three-dimensional arrangement of critical residues which resemble that found in enzymes of analogous activity. Although specific transition state binding is a feature of any enzyme or catalytic antibody, there is no assurance that the combining site has side residues suitable for efficient catalysis. From crystallographic studies of both the catalytic antibody and enzyme that catalyze the Claisen rearrangement of chorismate to prephenate, the active sites of both provide environments that complement the bound transition state analog (Figure 12). The 10^4 difference in catalytic efficiency may be more a matter of degree, as the enzyme apparently provides

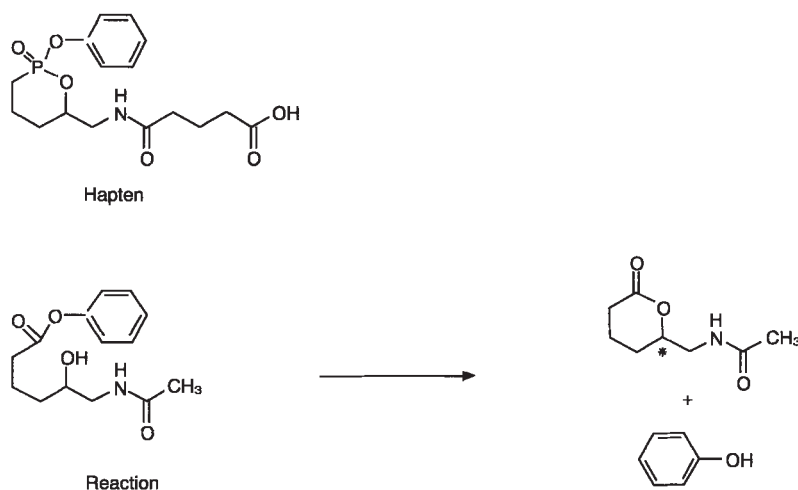


Figure 8 Lactonization. (Reproduced with permission from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29–54, © 1992, by Annual Reviews Inc.)

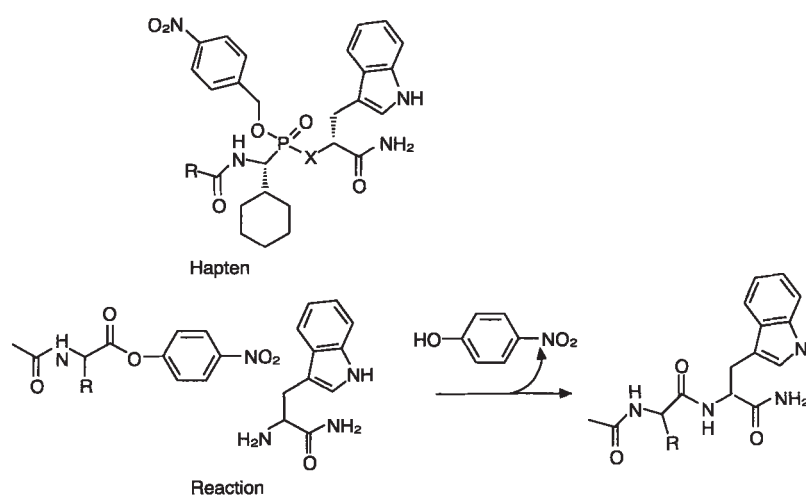


Figure 9 Peptide synthesis. (Reproduced with permission from Hirschmann R *et al* (1994) Peptide synthesis catalyzed by an antibody containing a binding site for variable amino acids. *Science* 265: 234–237.)

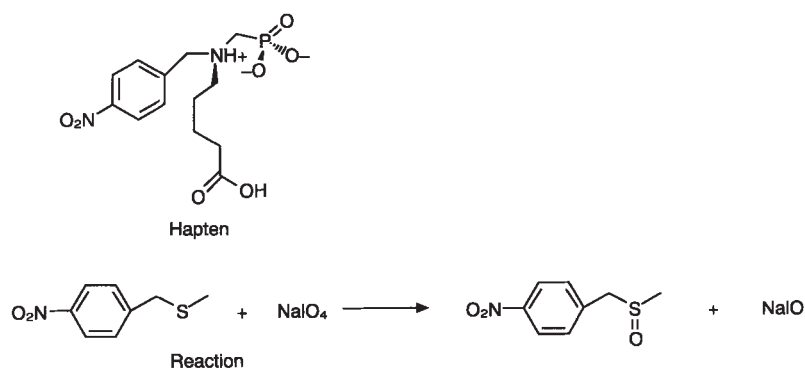


Figure 10 Periodate-dependent oxidation. (Modified with permission from Benkovic SJ. Catalytic antibodies. *Annual Review of Biochemistry* 61: 29–54, © 1992, by Annual Reviews Inc.)

more extensive electrostatic and hydrogen binding interactions than the antibody (Figure 12).

Nature of catalysis

Catalytic antibodies (abzymes) like enzymes process their substrates through a Michaelis complex in

which the chemical transformation occurs, followed by product dissociation. There are two general indices based on steady-state kinetic analyses that are used to assess the catalytic efficiency of an antibody: $k_{\text{cat}}/k_{\text{uncat}}$ and k_{cat}/K_M . The steady-state kinetics for all abzymes obey the Michaelis–Menten rate expression for both K_M (the concentration of sub-

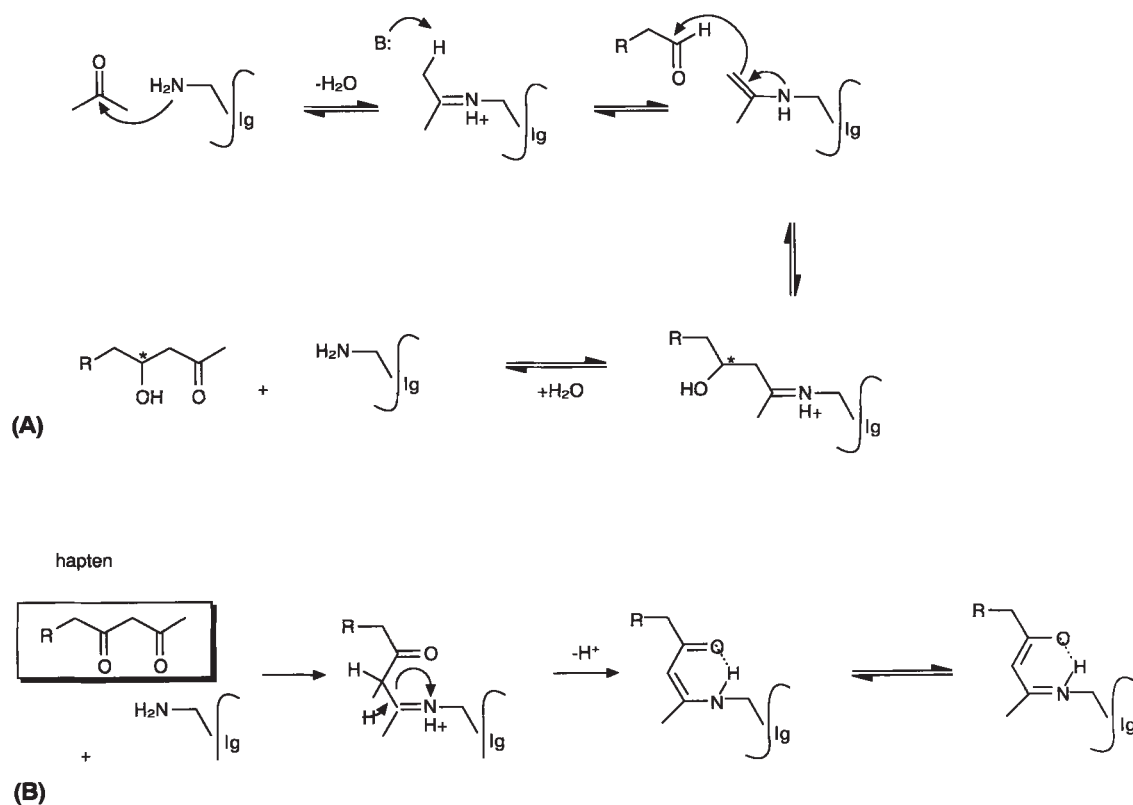


Figure 11 (A) Mechanism-base screen for an active site lysine residue. (B) Antibody-catalyzed aldol condensation via Schiff-base formation. (Reproduced with permission from Jacobsen JR and Schultz PG (1995) The scope of Antibody Catalysis. *Current Opinion in Structural Biology* 5: 818–824.)

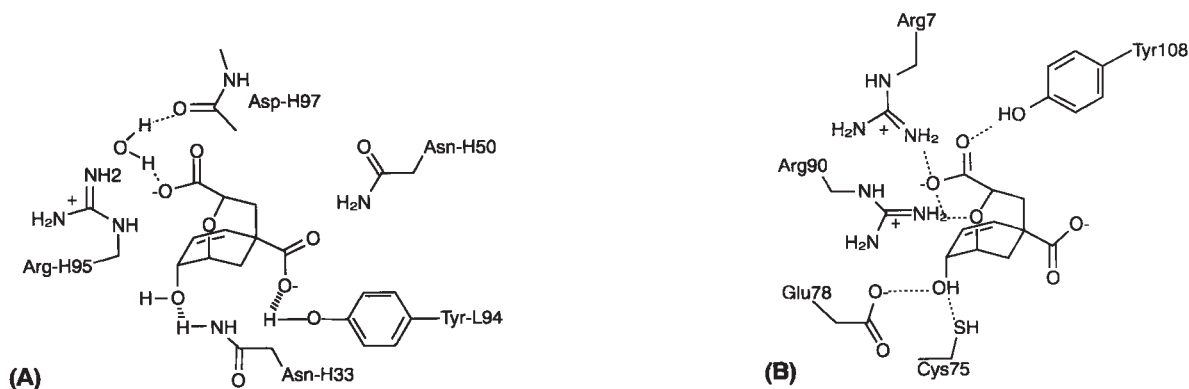


Figure 12 Comparison of the transition state analog binding between antibody 1F7 (A) and *Bacillus subtilis* chorismate mutase (B). (Reproduced with permission from Haynes MR *et al* (1994) Routes to catalysis: structure of a catalytic antibody and comparison with its natural counterpart. *Science* 263: 646–652.)

strate that produces one-half the maximal catalytic rate) and k_{cat} (the rate constant for product formation under conditions when the antibody is saturated with substrate). Note that the K_M parameter also represents an approximate measure for the dissociation of the abzyme–substrate complex. The meaning of the first index $k_{\text{cat}}/k_{\text{uncat}}$, where k_{uncat} is the rate constant for the same chemical process in

the absence of antibody, is obvious and for enzymes often exceeds 10^{10} . (For hydrolytic reactions both k_{cat} and k_{uncat} have the same units, as the activity of water is set equal to unity.) The second index of efficiency, the ratio k_{cat}/K_M , represents a measure of the kinetic barrier encountered, commencing with the combination of antibody and substrate and proceeding along the reaction coordinate to the trans-

ition state of the highest energy. This ratio has a limit of approximately $10^7 \text{ M}^{-1} \text{ s}^{-1}$ when the reaction is limited by diffusion together of the substrate and antibody.

The values of $k_{\text{cat}}/k_{\text{uncat}}$ for catalytic antibodies generally span a range from 10 to 10^6 . Likewise, the values of $k_{\text{cat}}/K_{\text{M}}$ are below that of a diffusion-controlled process. Both lines of evidence are consistent with the chemical step of a particular transformation as being rate limiting. For the majority of abzyme-catalyzed reactions there is a fair proportionality of the $k_{\text{cat}}/k_{\text{uncat}}$ ratio with the affinity of the antibody for its inducing hapten, although the coupling is generally not complete.

This observation is in accord with a view that antibodies catalyze their reactions primarily through restrictions which the active site cavity imposes on the translational and rotational movements of the bound substrate and to a lesser extent through active

site acid/base or nucleophilic catalysis. The latter apparently are not optimized like the situation with enzymes.

The few, unequivocal examples of covalent catalysis (in Figure 13 the reaction proceeds through an acyl histidine) rank near the upper end of the kinetic indices, although overall flux here may be limited by the buildup of product inhibition. The energy profile of this antibody-catalyzed reaction recapitulates a number of characteristics expected of an enzyme whose later evolution reduces the free energy barriers for the chemical and product release step. Therefore this catalytic antibody can be viewed as a primitive form of its enzyme counterpart. At present the success of this technology is greatly influenced by the serendipitous selection and placement of functional catalytic active site residues, particularly if the reaction is mechanistically complex.

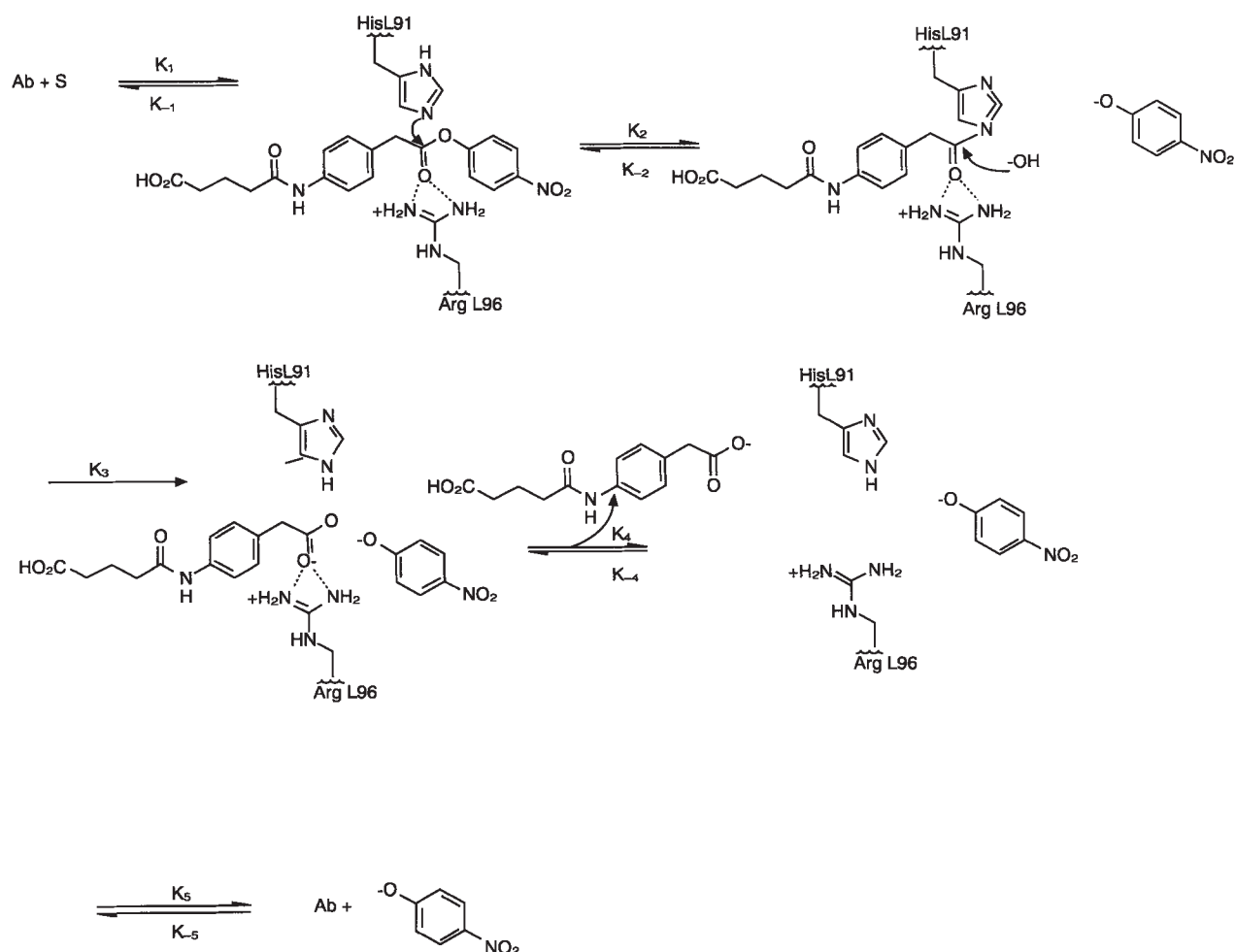


Figure 13 Covalent catalysis exhibited by antibody 43C9. (Reproduced with permission from Stewart JD *et al* (1994) Site-directed mutagenesis of a catalytic antibody: an arginine and histidine residue play key roles. *Biochemistry* 33: 1994–2003.)

Screening for catalysis

To date the majority of catalytic antibodies were derived from monoclonals, in most cases fewer than 100 were screened for activity. Recent developments include the adaptation of phage expression systems displaying single chain or Fab antibody fragments, permitting screening of larger numbers ($>10^6$) for catalytic activity. Given the often low activity of catalytic antibodies, several methods have been developed to identify an active fragment. Cat ELISA is based on screening for catalytic activity by using an amplification system in which the expected product is specifically bound by another antibody which recognizes the product as antigen. A second method depends on the catalytic release of a reactive species that can be trapped via covalent modification of bacteriophage-displayed antibody fragments, thus permitting the capture of the parent phage and the encoding DNA for the active abzyme. A third procedure is one of selection: antibodies that complement yeast or bacterial auxotrophs are identified by sustaining growth of the organism under minimal reaction conditions. The host organism, however, may also impose an addition selection on the catalytic antibody that can be recovered from an antibody library, as the host generally will not survive expressing an antibody with high toxicity. The latter two methods are particularly powerful because they reveal directly active species in large libraries. It is evident that screening large libraries resulting from primarily immunization or secondarily from a shuffled library of heavy and light chain fragments is the next critical step in the evolution of this technology for practical applications, which often require higher catalytic flux than generally found by limited screening.

See also: Affinity; Antibodies, specificity; Antibody-antigen intermolecular forces; Antigen-binding site; Phage display of antibodies; Antibody-antigen complexes, three-dimensional structures.

CD ANTIGENS

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Recognition of signals from outside and mediation of appropriate responses are key functions of immune cells. In particular, these processes are mediated by cell surface molecules which have the capacity to receive and transmit signals from the

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environment and to execute effector functions. Therefore, a better knowledge and understanding of immune cell surface molecules has been a dream of immunologists since the formulation of Ehrlich's side-chain theory.

For several decades, however, progress in this field has been hampered by technical difficulties. The turning point was clearly the introduction of the monoclonal antibody (mAb)-producing hybridoma technology by George Köhler and César Milstein in 1975. This technique allowed workers for the first time to reproducibly demonstrate, purify and characterize minute amounts of distinct molecules on the surface of immune cells, the leukocytes, by specific mAbs.

The introduction of this technology into leukocyte biology quickly led to the identification of a vast array of single mAb-defined, but otherwise ill-characterized cell surface molecules by individual laboratories. In 1981, at the leukemia marker conference held in Vienna many of these new mAb-defined molecules were presented and discussed. There it became clear that intense international collaboration and gathering of the expertise available in the various laboratories is a prerequisite for optimal progress in this field.

Laurence Boumsell and Alain Bernard were the pioneers to initiate this international collaboration and to gather a group of scientists (J. Dausset, R.L. Evans, J.A. Hansen, B.F. Haynes, W. Knapp, A.J. McMichael, C. Milstein, E.L. Reinherz, S.F. Schlossman) willing to collaborate in this approach. The still valid basic rules of this cooperation were set up by this council, and Laurence Boumsell and Alain Bernard then organized in 1981/82 the First International Workshop on Human Leukocyte Differentiation Antigens with the final conference being held in Paris in 1982. Everybody working in the field was invited to join and to submit mAbs for evaluation and/or to offer analytical skills for characterizing these mAbs.

The specific reactivity of an mAb with certain cell types and/or differentiation stages and the molecular weight of the recognized antigen (molecule) were at that time the main features which allowed distinction of different and clustering of similar mAb specificity. This explains the now historical terms used in the nomenclature (CD for cluster of differentiation) and in the title of the workshop and conference series (Leukocyte Differentiation Antigens rather than Leukocyte Surface Molecules).

So far, six international workshops and conferences have been held. Each workshop collects data over a period of approximately 2 years and meets in a conference which results in a well-documented proceedings volume: Ist Conference – Paris November 1982, IInd Conference – Boston September 1984, IIIrd Conference – Oxford September 1986, IVth Conference – Vienna February 1989, Vth Conference – Boston November 1993, VIth Conference –

Kobe November 1996. The organization of the VIIth Workshop is led by David Mason (Oxford), and the Conference is planned for June 2000 in Harrogate, UK, <http://phoenix.jr2.ox.ac.uk/HLDA7>

The principal organization of the workshops has not changed dramatically since the Ist Workshop in Paris. However, new technologies have been integrated into the evaluation process rapidly, and have profoundly influenced the overall design and philosophy of the series. This has not only maintained the nomenclature authority of the workshop series but also strengthened its role as a creative international instrument with a substantial impact on modern immunological research.

In essence, international investigators are invited to submit interesting mAbs to the workshop organizers, who group the mAbs in coded or open panels on the basis of the information provided. The panels are then distributed to investigators who can offer informative and innovative studies on these reagents. The generated data have to be returned to the organizers who are responsible for joint evaluation and publication. For example, at the VIth Workshop, within 2 years, 1152 mAbs directed to more than 200 leukocyte cell surface molecules were evaluated by more than 500 laboratories. This resulted in 42 new CD entities, the redefinition of several previously established, mainly provisional clusters, and a considerable advancement in understanding the structure and function of leukocyte surface molecules in general. The database of the VIth Workshop including the individual reaction patterns of the mAbs is available at the Internet address: <http://immunol.mcb.osaka-u.ac.jp/>.

Originally and still as a rule, the CD designation describes a cluster of mAbs (e.g. CD2 mAbs) all of which show identical cellular reaction patterns and identify the same molecular species. Consequently, the term 'anti-CD...' would mean anti-idiotypes and, therefore, should not be used to name CD mAbs. Later, the CD designation was used to describe the recognized molecule, but it had to be clarified by using the terms antigen or molecule (e.g. CD2 molecule). Meanwhile, the CD nomenclature without the additive is used by most people to designate the leukocyte surface molecules, the characterization for which the CD mAbs have been established.

So far, 202 CD entities have been assigned to the 166 main CDs delineated in Table 1. Provisional clusters are designated as CDw. Further information on the CDs is available in the proceedings of the individual workshops and the Protein Reviews on the Web (PROW) database. PROW has been established based on a concept developed by Stephen Shaw and aims to organize and synthesize information on all

Table 1 Human leukocyte CD molecules

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD1a	T6	Thy, LHC, DC	Noncovalently associated with β_2 M. Structural similarity with MHC-class I molecules (see CD1b and CD1c)	49
CD1b		Thy, DC	Noncovalently associated with β_2 M (see also CD1a). Potential lipid antigen-presenting molecule	45
CD1c		Thy, LHC, DC, B sub	Noncovalently associated with β_2 M (see also CD1a). Potential lipid antigen-presenting molecule	43
CD2	T11; Tp50; sheep red blood cell (SRBC) receptor; LFA-2	T, NK	Cytoadhesion molecule binding to CD58 molecule (LFA-3), CD48 molecule and probably also to CD59 molecule	50
CD2R	CD2 epitopes restricted to activated T cells	T act	Same molecule as CD2 molecule, CD2R antibodies recognize CD2 epitopes expressed on activated T cells	50
CD3	CD3 complex	T	Multigene cell surface complex that is part of the T cell antigen receptor	16, 20, 22, 25, 28
CD4	T4	T sub	Coreceptor of the T cell antigen receptor; binds to MHC class II molecules. Also, receptor for interleukin 16, and for human immunodeficiency virus	55–59
CD5	Tp67	T, B sub	Member of the scavenger receptor family (see CD6). Possible CD72 counter-receptor	67
CD6	T12	T, B sub	Member of the scavenger receptor family. Counter-receptor of CD166	110
CD7		T sub	Type I integral membrane glycoprotein	40
CD8	T8	T sub	Two-chain complex expressed as disulfide-linked α/α homodimers or α/β (see CD8beta) heterodimers. Coreceptor of the T cell antigen receptor; binds to MHC class I molecules	32–38
CD8beta	T8	T sub	β chain of CD8	30–34
CD9	p24	Pre-B, T act, Plt, Eo, EC	Member of the tetraspans gene molecules that contain four transmembrane domains and have both the N- and C-termini on the cytoplasmic side (see CD37, CD53, CD63, CD81, CD82, CD151)	24
CD10	Neutral endopeptidase; gp100; common acute lymphatic leukemia antigen (CALLA)	Lymphoid Prog, cALL, Germ Ctr. B, G	Type II transmembrane glycoprotein, which is a neutral endopeptidase (enkephalinase)	100
CD11a	α chain of: leukocyte function associated antigen 1 (LFA-1), gp180/95	Leukocytes broad	β_2 -integrin member (see CD11b, CD11c, CD18). The CD11a/CD18 (LFA-1) complex has binding capacity to CD54 molecules (ICAM-1), CD102 molecules (ICAM-2), CD50 molecules (ICAM-3), Laisolsteiner-Wiener antigen (ICAM-4), telencephalin (ICAM-5)	180
CD11b	α chain of: C3bi receptor, complement receptor type 3 (CR3), gp155/95, Mac-1, Mo1	M, G, NK, T sub	β_2 -integrin member (see CD11a, CD11c, CD18). The CD11b/CD18 complex (CR3) binds C3bi, lipid IVa in LPS, kininogen, coagulation factor X, CD54 molecules, the neutrophil inhibition factor of the hookworm <i>Ancylostoma caninum</i> , oligonucleotides	170
CD11c	α chain of: complement receptor type 4 (CR4); gp150/95	M, G, NK, B sub, T sub	β_2 -integrin member (see CD11a, CD11b, CD18). The CD11c/CD18 complex (CR4) binds C3bi, fibrinogen and CD54 molecules	150
CDw12		M, G, Plt		90–120
CD13	Aminopeptidase N; gp150;	M, G	Type II transmembrane glycoprotein, identical to the proteolytic enzyme aminopeptidase N	150

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD14	gp55; lipopolysaccharide (LPS) receptor	M, G	Receptor for the LPS/LPS binding protein (LBP) complex	55
CD15	Lewis ^x (Le ^x); 3-fucosyl-N-acetyllactosamine (3-FAL); X-Hapten; lacto-N-fucopentaose III; SSEA	G, some antibodies also M	CD15 antibodies recognize the trisaccharide 3-FAL (Gal1-4 (Fuc1-3) GlcNAc) which is also recognized by CD62P molecules	shared by a variety of proteins and lipids
CD15s	Sialyl Lewis ^x (sLe ^x)	Broad	Sialylated form of CD15. It is recognized by CD62E, CD62L and CD62P molecules	shared by a variety of proteins and lipids
CD16	Fc γ receptor type IIIa (Fc γ RIIIa); gp50-65	NK, G, Mac	Low-affinity receptor for complexed IgG	50-65
CD16b	Fc γ receptor type IIIb (Fc γ RIIIb);	G	Glycosylphosphatidylinositol-anchored form of the CD16 molecule expressed on granulocytes	48-60
CDw17	Lactosylceramide	G, M, Plt	Lactosylceramide (Gal-Glc-Cer type 2 chain)	
CD18	Integrin β_2 β chain	Leukocytes broad	Noncovalently linked to either CD11a, CD11b or CD11c molecules (α chains) Defect in CD18 results in leukocyte adhesion deficiency (LAD)	95
CD19	Bgp95, B4	B	Part of the B cell antigen receptor complex	95
CD20	B1; Bgp35	B	Phosphoprotein expressed on B cells in three forms (37 and 35 and 33 kDa) as a result of different phosphorylation. This molecule has four membrane-spanning regions	33, 35, 37
CD21	C3d receptor (CR2); gp140	B sub	Member of the regulator of complement activation (RCA) gene family (see CD35, CD46, CD55). Molecule functions as C3d receptor (CR2) and serves also as receptor for Epstein-Barr virus	140
CD22	Bgp135; BL-CAM	Cytoplasm: pan B; surface: B sub	Functions as an adhesion molecule with a sialic acid binding capacity for carbohydrates which were found e.g. on CD45RO, CDw75 and CDw76 molecules	135, 140
CD23	Low-affinity Fc ϵ -receptor; Fc ϵ RII; gp50-45; Blast-2	B, M	The extracellular region contains a C-type lectin domain (see CD69, CD72, CD94, CD161)	45-50
CD24	Heat stable antigen homolog; BA-1	B, G, EPC	Glycophosphoinositol-linked, extremely glycosylated protein. Possible counter-receptor for CD62P	42
CD25	Interleukin (IL)-2 receptor α chain; Tac-antigen	T act, B act, M act	Has low-affinity IL-2 binding capacity. By noncovalently associating with the CDw122 molecule and the CD132 (common to CD124 and CD127) molecule, it forms the high-affinity IL-2 receptor complex	60
CD26	Dipeptidylpeptidase IV; gp120; Ta1	T act, B, Mac, NK	Has also collagen binding properties. Associated with adenosine deaminase and CD45 molecules	110
CD27	T14	T sub	Member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family (see CD30, CD40, CD95, CD120a, CD120b, CD134, CDw137). Counter-receptor for CD70	55
CD28	Tp44;	T sub	Counterreceptor for CD80, CD86 and B7-3 molecules	44
CD29	Integrin β_1 chain; platelet GPIIa; VLA (CD49) β chain	Broad	β chain of the VLA protein family (CD49 molecule family), however, can also associate with other integrin α chains (see CD51)	130
CD30	Ki-1 antigen	T act, B act, Reed Sternberg cells, Hodgkin cells	Member of the TNF/NGF receptor family (see CD27, CD40, CD95, CD120a, CD120b, CD134, CDw137). Counter-receptor for CD153	105

Table 1 Continued

	Other name (s)	Main reactivity of mAbs	Short characteristic of the CD molecule	Molecular weight reduced (kDa)
CD31	PECAM-1; platelet GPIIa ¹ ; endocam	Plt, M, G, B, T sub	Adhesion molecule involved in migration of leukocytes and regulation of the T cell response	140
CD32	Fc γ receptor type II (Fc γ RII), gp40	M, G, B, Eo	Six isoforms are known which originate from alternative splicing of three genes on chromosome 1 (Fc γ RIIA, -B and -C)	40
CD33	My9	M, normal and malignant myeloid progenitor cells	Related to myelin-associated glycoprotein and CD22 molecules	67
CD34	My10	Prog, EC	Sialomucin-like (see CD43, CD68, CD164) transmembrane glycoprophosphoprotein. Potential cytoadhesion molecule probably interacting with CD62E and CD62L molecules	105–120
CD35	C3b/C4b receptor; complement receptor type 1 (CR1)	G, M, B, some T/NK	Has binding capacity for C3b, iC3b, C3c and C4b complement fragments. Mediates erythrocyte clearance of immune complexes, involved in inhibition of complement activation by blocking C3 convertase formation	190, 220, 250, 280
CD36	Platelet GPIV, GPIIb, OKM-5 antigen	M, Plt, (B)	Functions as receptor for <i>Plasmodium falciparum</i> -infected erythrocytes. Binding affinity for thrombospondin (thrombospondin receptor), collagen I and IV and oxidized low-density lipoprotein has been shown	90
CD37	gp40-52	B, (T), (M), (G)	Member of the tetraspans family (see CD9)	36–52
CD38	T10; gp45	PC, Thy, T act	Has NAD ⁺ glycohydrolase, ADP ribosylcyclase and cyclic ADP-ribose hydrolase activity	45
CD39	gp80	B sub		80
CD40		B, DC	Member of the TNF/NGF receptor family (see CD27, CD30, CD95, CD120a, CD120b, CD134, CDw137). Counter-receptor of CD154	44, 48
CD41	Platelet glycoprotein GPIIb	Plt	Heterodimeric 140 kDa transmembrane glycoprotein (posttranslationally cleaved into a disulfide linked 120 kDa α chain and a 22 kDa β chain) also known as platelet glycoprotein GPIIb, which forms a calcium-dependent complex with platelet glycoprotein GPIIIa (CD61 molecule)	120, 22 (GPIIb), 110 (GPIIIa)
CD42a	Platelet glycoprotein GPIX	Plt	Forms a noncovalent complex (CD42 complex) with platelet glycoprotein GPIb and GPV (see CD42b, CD42c and CD42d). Complex functions as receptor for von Willebrand factor and as von Willebrand factor-dependent adhesion receptor	23
CD42b	Platelet glycoprotein GPIb- α	Plt	135 kDa protein (α chain) that forms with the CD42c molecule (β chain) a disulfide-linked 160 kDa membrane protein known as platelet glycoprotein GPIb (subunit of the CD42 complex, see CD42a)	135(α), 22(β)
CD42c	Platelet glycoprotein GPIb- β	Plt	See CD42b	135(α), 22(β)
CD42d	Platelet glycoprotein GPV	Plt	See CD42a	85
CD43	Leukosialin; gp95; sialophorin; leukocyte sialoglycoprotein; gp115	T, G, M, NK, Plt	Member of the 'cell surface mucin' family (see CD34, CD68, CD164). Suggested to function as adhesion molecule interacting with MHC class I, CD54 and CD62P molecules, or (controversial) as antiadhesion protein	95–135

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD44	Pgp-1; gp80-95; In (Lu)-related p80, Hermes antigen, ECMR-III and HUTCH-I	T, B, G, M	Receptor for hyaluronate and involved in lymphocyte homing	80–95
CD44R	CD44 variant; CD44v9	T act, carcinoma cells	CD44R (CD44v9) may be a marker for cell transformation	130, 190, 250, 300
CD45	T200; leukocyte common antigen (LCA); EC3.1.3.4	Leukocytes	Transmembrane glycoprotein consisting of 4 isoforms, which result from differential splicing. The CD45 molecule has intrinsic cytoplasmic protein tyrosine phosphatase activity	220, 205, 190, 180
CD45R0	Restricted T200; gp180	T sub, G, M	Isoform of the CD45 molecule family, this isoform does not contain exon A, B, or C sequences. Interacts via carbohydrate residues with the CD22 molecule	180
CD45RA	Restricted T200; gp220; isoform of leukocyte common antigen	T sub, B, G sub, M	Isoform determinant of CD45 sharing exon A sequences. Expressed on resting/naive T cells	220
CD45RB	Restricted T200; isoform of leukocyte common antigen	T sub, B, G, M	Isoform determinant of CD45 sharing exon B sequences	220, 205, 190
CD45RC	Restricted T200; isoform of leukocyte common antigen	T sub, B, NK	Isoform determinant of CD45 sharing exon C sequences within CD4 cells expressed on long living memory cells	220, 205
CD46	Membrane cofactor protein (MCP); gp45-70	Leukocytes broad	Member of the RCA gene family (see CD21, CD35, CD55). Inhibits complement activation by inhibition of C3 convertase formation. Receptor for measles virus	51–58/59–68
CD47	Integrin associated protein; OA3; 1D8	Extremely broad	Physically and functionally associated with the vitronectin receptor (CD51/CD61)	47–52
CD48	gp41; BLAST-1	Leukocytes	Glycophosphatidylinositol-linked glycoprotein. Counter-receptor for CD2	41
CD49a	VLA-1 α chain	T cultured	Associates with the CD29 molecule to form the VLA-1 (integrin $\alpha_1\beta_1$) complex. VLA-1 mediates cell adhesion to collagens and laminin	210
CD49b	VLA-2 α chain platelet glycoprotein GPIa	Plt, T cultured	Associates with CD29 molecules to form the VLA-2 (integrin $\alpha_2\beta_1$) complex. Functions as collagen receptor or collagen/laminin receptor, receptor for echovirus 1	170
CD49c	VLA-3 α chain	Adherent cell lines	Associates with CD29 molecule to form the VLA-3 (integrin $\alpha_3\beta_1$) complex. VLA-3 mediates cell adhesion to fibrinogen, laminin, collagens and epiligrin/kalinin, VLA-2	125, 30
CD49d	VLA-4 α chain	M, T, B, (LHC), Thy	Associates with CD29 molecules in VLA-4 (integrin $\alpha_4\beta_1$) which binds fibronectin, thrombospondin, invasin and CD106 molecules. Upon noncovalent association with the integrin β_7 subunit the integrin $\alpha_4\beta_7$ complex is formed, which binds fibronectin, MadCAM and CD106 molecules	150
CD49e	VLA-5 α chain	T, M, Plt	Associates with CD29 molecules to form the VLA-5 (integrin $\alpha_5\beta_1$) complex, which is a receptor for fibronectin	135, 25
CD49f	VLA-6 α chain	Plt (T)	Associates with CD29 molecules in VLA-6 (integrin $\alpha_6\beta_1$). This complex has been shown to be a laminin receptor. The CD49f molecule can also associate with CD104 molecules to form the $\alpha_6\beta_4$ laminin receptor	120, 130
CD50	Intercellular adhesion molecule 3 (ICAM-3)	Broad, not on EC	Functions as counter-receptor for LFA-1 (CD11a/CD18)	130

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD51	Vitronectin receptor (VNR) α chain; integrin α_v	(Plt), EC, Fibroblasts	Can noncovalently associate with β_1 (CD29), β_3 (CD61), β_5 , β_6 or β_8 integrins. The $\alpha_v\beta_1$ complex was found to bind vitronectin, fibronectin and collagen I. The $\alpha_v\beta_3$ complex binds vitronectin, fibronectin, fibrinogen, von Willebrand factor, thrombospondin, osteopontin and collagen, $\alpha_v\beta_5$ binds to vitronectin, and $\alpha_v\beta_6$ to fibronectin	125, 25
CD52	Campath-1; gp21–28	Leukocytes	Good target for complement-mediated cell lysis	21–28
CD53		Exclusively leukocytes	Member of the tetraspans family (see CD9)	32–40
CD54	Intercellular adhesion molecule 1 (ICAM-1)	Broad	Counter-receptor of LFA-1 (CD11a/CD18) and CR3 (CD11b/CD18). Also serves as major group rhinovirus receptor	90
CD55	Decay-accelerating factor (DAF)	Broad	Glycophosphatidylinositol-linked membrane glycoprotein. Molecule prevents the formation and causes dissociation of C4b2a and C3bBb, the amplification convertases of the complement cascade. Counter-receptor of the CD97 molecule, receptor for echoviruses and enterovirus	80
CD56	NKH1; isoform of neuronal cell adhesion molecule (N-CAM)	NK, T act	Homotypic adhesion molecule	175–185
CD57	HNK1; gp110	NK, T, B sub, brain	Carbohydrate structure, probably attached to several proteins and lipids	
CD58	Leukocyte function-associated antigen 3 (LFA-3)	Leukocytes, EC, EPC, fibroblasts	Counterreceptor of CD2	40–65
CD59	gp18-20; Ly6 analogous; homologous restriction factor 20 (HRF-20); Protectin	Broad	Glycophosphatidylinositol-linked glycoprotein. Mediates inhibition of the complement membrane attack complex by binding to C8 on C5b678 and thereby preventing accumulated binding of C9	18–20
CDw60		T sub, Plt	Acetylated disialosyl group (NeuAc-NeuAc-Gal) predominantly found on ganglioside GD3	
CD61	Integrin β_3 chain, platelet glycoprotein GPIIIa	Plt	Forms a complex with CD41. The CD61 molecule can also associate with integrin α_v (CD51 molecule) forming the $\alpha_v\beta_3$ vitronectin receptor	90
CD62E	E-selectin; ELAM-1; LECAM-2	EC act	Related to CD62L and CD62P molecules (selectin family). Recognizes carbohydrate ligands (e.g. CD15s) on various molecules including GlyCAM1, CD34, CD162, ESL1	115
CD62L	L-selectin; LAM-1; Leu-8; TQ1; LECAM-1	G, M, T, B sub, NK	Recognizes carbohydrate ligands (e.g. CD15s) on various molecules including GlyCAM-1, MadCAM, CD162, CD34 as well as sulfated glycolipids	75
CD62P	P-selectin; gmp140; PADGEM	Plt act, EC act	Recognizes carbohydrate ligands (e.g. CD15 and CD15s) on various molecules including CD162	150
CD63	Platelet 53 kDa activation antigen; LIMP; ME491	Plt act, M, (G, T, B)	Member of the tetraspans family (see CD9)	30–60
CD64	Fc γ receptor type I (Fc γ RI); high-affinity Fc-IgG receptor	M, G act	Encoded by three genes. Associates with the γ chain of Fc ϵ receptor type I	75

Table 1 Continued

	Other name (s)	Main reactivity of mAbs	Short characteristic of the CD molecule	Molecular weight reduced (kDa)
CD65	Desialylated form of CD65s	G	Fucoganglioside (Gal-GlcNAc-Gal-GlcNAc(Fuc)-Gal-Gl cNAc-Gal-GlcNAc-Gal-Glc-Cer) based on a type II chain. Desialylated form of CD65	
CD65s	Ceramide-dodecasaccharide 4c, VIM2 Antigen	M, G, AML blasts	Fucoganglioside (NeuAc-Gal-GlcNAc-Gal-GlcNAc(Fuc)-Gal-Gl cNAc-Gal-GlcNAc-Gal-Glc-Cer). Carried by various glycolipids and glycoproteins. Potential ligand for CD62E and CD62L	
CD66a	Biliary glycoprotein (BGP); nonspecific cross-reacting antigen (NCA) 160	G, EPC	Member of the carcinoembryonic antigen (CEA) gene family. Adhesion molecule, has bacterial binding property, in particular, for Opa-proteins of pathogenic <i>Neisseria</i>	160–180
CD66abce		G	Antibodies recognizing CD66a, CD66b, CD66c, CD66e molecules	
CD66acd		G	Antibodies recognizing CD66a, CD66c, CD66d molecules	
CD66acde		G	Antibodies recognizing CD66a, CD66c, CD66d, CD66e molecules	
CD66ace		G	Antibodies recognizing CD66a, CD66c, CD66e molecules	
CD66ade		G	Antibodies recognizing CD66a, CD66d, CD66e molecules	
CD66ae		G	Antibodies recognizing CD66a, CD66e molecules	
CD66b	CD67; p100; CGM6; nonspecific cross-reacting antigen (NCA) 95	G	Glycophosphatidylinositol-anchored glycoprotein. Member of the carcinoembryonic antigen (CEA) gene family. Adhesion molecule	95–100
CD66be		G	Antibodies recognizing CD66b, CD66e molecules	
CD66c	nonspecific cross-reacting antigen (NCA) 50, NCA 90 or TEX	G	Glycophosphatidylinositol-anchored glycoprotein. Member of the carcinoembryonic antigen (CEA) gene family. Recognizes type 1 fimbriae from <i>E. coli</i> and Opa-proteins of pathogenic <i>Neisseria</i>	90–95
CD66ce		G	Antibodies recognizing CD66c, CD66e molecules	
CD66d	CGM1	G	Member of the carcinoembryonic antigen (CEA) gene family. Recognizes Opa-proteins of pathogenic <i>Neisseria</i>	30
CD66de		G	Antibodies recognizing CD66d, CD66e molecules	
CD66e	Carcinoembryonic antigen (CEA)	EPC	Prototype of the carcinoembryonic antigen (CEA) gene family. Glycophosphatidylinositol-anchored. Adhesion molecule, binds to CD66a and CD66c molecules and Opa-proteins of pathogenic <i>Neisseria</i>	180–200
CD66f	Pregnancy-specific glycoprotein (PSG), SP-1	Myeloid cells, placenta, fetal liver	Secreted molecule. Member of the carcinoembryonic antigen (CEA) gene family. Low blood level predicts spontaneous abortion	54–72
CD67	Deleted, now CD66b			
CD68	gp110, Macrosialin	M, Mac	Mucin-like molecule (other members CD34, CD43, CD164, GlyCAM-1) and lysosomal/plasma membrane shuttling protein (see CD107a, CD107b). Recognizes oxidized low-density lipoprotein	110
CD69	Activation inducer molecule (AIM); EA1; MLR; Leu23; BL-AC/p26	B and T early act, Mac act	Member of the Ca ²⁺ -dependent (C-type) lectin superfamily of type II transmembrane receptors (see CD23, CD72, CD94, CD161)	28, 34
CD70	Ki-24	T, B-EBV, pre-BLL act	Member of the TNF family (see CD153, CD154, CD95L). Counter-receptor for CD27	50
CD71	Transferrin receptor; T9 antigen	T and B act, Mac, proliferating cells	Mediates cellular iron uptake via internalization and recycling of transferrin	95

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD72	Lyb-2	B	Member of the Ca ²⁺ -dependent (C-type) lectin superfamily of type II transmembrane receptors (see CD23, CD69, CD94, CD161). Possible counter-receptor for the CD5 molecule	39, 43
CD73	ecto-5'-nucleotidase (ecto-5'-NT), EC.3.1.3.5.	B sub, T sub, EC	Glycophosphatidylinositol-linked membrane glycoprotein. Catalyses the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside	69
CD74	MHC class II-associated invariant chain (Ii)	B, M, T act, EC act, EPC act	Involved in intracellular transport and surface expression of MHC class II proteins	33, 35, 41, 42
CDw75		Mature B	Carbohydrate, suggested to be recognized by CD22 molecules	
CDw76		B, T sub, EC	Carbohydrate of the sialylated type 2 glycosphingolipids. May be recognized by CD22 molecules	
CD77	Globotriaosylceramide (Gb3); P ^k blood group antigen; Burkitts lymphoma-associated antigen (BLA), ceramide trihexoside (CTH)	B sub	Neutral glycosphingolipid of the globo series (Gal α 1-4 Gal β 1-4 Glc1-1 Cer). Interacts with CD19 molecules on B cells and seems to be involved in CD19 signaling	
CDw78	Ba	B	Not fully characterized	
CD79a	mb-1; Ig α	B	Forms with the CD79b molecule a disulfide-linked heterodimer which functions as a coupling module of the B cell antigen receptor	47
CD79b	B29; Ig β	B	Forms with the CD79a molecule a disulfide-linked heterodimer which functions as a coupling module of the B cell antigen receptor	37
CD80	B7-1; BB1	B, B act, M, DC, T act	Counter-receptor of the T cell accessory molecules CD28 and CD152	60
CD81	Target of an antiproliferative antibody (TAPA-1); M38	Broad	Member of the tetraspans family (see CD9)	26
CD82	R2; 4F9; C33; IA4	M, B, B act, T act, LGL	Member of the tetraspans family (see CD9)	40-60
CD83	HB15	LHC, DC, reticulum cells (T and B act)	Dendritic cell marker	45
CD84	p75	B, M, Plt, Mac.	Not fully characterized	74
CD85	VMP-55; GH1/75	PC, Mac, hairy cells	Not fully characterized	83
CD86	B7-2; B70	B act, M, DC	Structurally related to the CD80 molecule, and functions as counter-receptor for the T cell accessory molecules CD28 and CD152	75
CD87	Urokinase plasminogen activator-receptor (uPA-R)	M, G, EC	Glycophosphatidylinositol-anchored glycoprotein involved in cell migration. Serves also as receptor for vitronectin	45-55
CD88	C5 α receptor	G, M, DC, smooth muscle cells	Member of the rhodopsin superfamily that contain seven transmembrane domains	
CD89	Fc α receptor	G, M, B sub, T sub	Binds serum and secretory IgA1 or IgA2	55-70
CD90	Thy-1	Prog sub, EC, neurons	Glycophosphatidylinositol-anchored protein	25-35
CD91	α_2 -macroglobulin receptor; low-density lipoprotein receptor-related protein	M	Receptor for a variety of ligands including α_2 -macroglobulin-proteinase complex, plasminogen activators (urokinase and tissue) in complex with type 1 inhibitors, chylomicron remnants, lipoprotein lipase, <i>Pseudomonas</i> exotoxin A	85, 515
CDw92	p70	G, M		70

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD93	p120	M, EC		120
CD94		NK, T sub	Member of the Ca^{2+} -dependent (C-type) lectin superfamily of type II transmembrane receptors (see CD23, CD69, CD72, CD161). The CD94 molecule forms a disulfide linked heterodimer with NKG2A. This complex functions as receptor for certain HLA-A, B, C alleles and HLA-G, and is involved in the downregulation of NK cell activity. In contrary, upon interaction with a less-defined 39 kDa protein, CD94 seems to form an activatory NK cell receptor	30
CD95	Apo-1; Fas	T act, M act, B act	Member of the TNF/NGF receptor family (see CD27, CD30, CD40, CD120a, CD120b, CD134, CDw137)	43
CD96	Tactile	T, T act		160
CD97	p74/80/89	G, M, T act, B act	Seven-span transmembrane protein belonging to the secretin receptor superfamily. Counter-receptor for the CD55 molecule	74, 80, 89
CD98	4F2	T, B, Plt	Disulfide-linked heterodimer	40, 80
CD99	E2; MIC2	Broad	The extracellular domain contains five G-X-Y repeats that are found in collagen and collagen-like proteins	32
CD99R	Restricted CD99	T sub	CD99 epitopes, the expression of which is restricted to T cell subpopulations	
CD100	p150	Broad	The extracellular region consists of a semaphorin domain and an Ig-like domain. Modifies CD40-CD40L B cell signaling by augmenting B cell aggregation and survival	150
CD101	P140; V7	G, M, T sub, DC	The extracellular domain consists of 7 IgV-like domains. Some mAb modify T cell activation	140
CD102	Intercellular adhesion molecule 2 (ICAM-2)	M, Plt	Functions as counter-receptor for the CD11a/CD18 (LFA-1) complex	55–65
CD103	α chain of HML-1, $\alpha_E\beta_7$ integrin; integrin α_E	Intestinal intraepithelial lymphocytes, hairy cells	Post-translationally cleaved into two disulfide-linked units of 25 kDa and 150 kDa. Noncovalently associates with the 105 kDa integrin β_7 chain to form the human mucosal lymphocyte 1 (HML-1) antigen	25, 150
CD104	Integrin β_4 chain		Associates with CD49f molecule (integrin α_6 chain) to form the integrin $\alpha_6\beta_4$ laminin receptor. This complex also binds epiligrin	220
CD105	Endoglin	EC, M act	Binds in association with TGF β receptor I and II, TGF β_1 , and TGF β_3	95
CD106	Vascular cell adhesion molecule 1 (VCAM-1); INCAM110	EC act	Binds to CD49d/CD29 (VLA-4, integrin $\alpha_4\beta_1$) and CD49d/integrin β_7 (integrin $\alpha_4\beta_7$) complexes	95, 100
CD107a	Lysosomal-associated membrane protein (LAMP)-1	Plt act, EC act, G act	Together with the 60% homologs CD107b molecule, the major sialoglycoprotein of lysosomal membranes	110
CD107b	Lysosomal-associated membrane protein (LAMP)-2	Plt act, EC act, G act	Together with the 60% homologous CD107a molecule, the major sialoglycoprotein of lysosomal membranes	120
CDw108	GPI-gp80, JMH blood group antigen	HPB-ALL (T cell line)	Glycosylphosphatidylinositol-anchored cell membrane glycoprotein	75
CD109		EC, T act, Plt, stromal cells	Glycosylphosphatidylinositol-anchored cell membrane glycoprotein. Sialomucin	120–165, 170
CD114	G-CSF receptor	G, M	Member of the cytokine receptor family	130

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD115	Colony-stimulating factor (CSF)-1 receptor; macrophage CSF receptor, <i>c-fms</i> proto-oncogene	M, Mac	Belongs to the Ig and receptor tyrosine kinase family (see CD117, CD135, CD140a, CD140b). The cytoplasmic region contains a protein tyrosine kinase domain	150
CD116	Granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor α chain	M, G	Member of the cytokine receptor (hematopoietin) superfamily. Upon association with CDw131 (common to CDw123 and CDw125), it forms the high-affinity GM-CSF receptor	75–85
CD117	Stem cell factor receptor; steel factor receptor; <i>c-kit</i>	Mast cells, myeloid Prog	Belongs to the Ig and receptor tyrosine kinase family (see CD115, CD135, CD140a, CD140b)	145
CDw119	Interferon γ receptor	M, G	Related to the interferon α/β receptor and the CD142 molecule	90–100
CD120a	55 kDa tumor necrosis factor receptor	(M)	Member of the TNF/NGF receptor family (see CD27, CD30, CD40, CD95, CD120b, CD134, CDw137). It binds both TNF α and β with high affinity	50–60
CD120b	75 kDa tumor necrosis factor receptor	M, G	Member of the TNF/NGF receptor family (see CD27, CD30, CD40, CD95, CD120a, CD134, CDw137). It binds both TNF α and β with high affinity	75–85
CD121a	Interleukin 1 receptor type I	T, B, M, G, NK, EC	Binds both IL-1 α and IL-1 β with high affinity, as well as the IL-1 receptor antagonist	80
CDw121b	Interleukin 1 receptor type II	G, T, B, M	Binds both IL-1 α and IL-1 β with high affinity, as well as the IL-1 receptor antagonist	60–70
CDw122	Interleukin 2 receptor β chain	T, NK, B, M	Member of the cytokine receptor (hematopoietin) superfamily. Binds IL-2 with low affinity. Noncovalently associates with the CD25 molecule and the CD132 molecule (common to CD124 and CD127 molecules) to the high-affinity IL-2 receptor complex. Also part of the IL-15 receptor complex	75
CDw123	Interleukin 3 receptor α chain	M	Associates with CDw131 (common to CD116 and CDw125) to form the functional IL-3 receptor	70
CD124	Interleukin 4 receptor	(Broad)	Receptor subunit for IL-4 and IL-13 by noncovalently associating with the CD132 molecule or the IL-13 receptor, respectively	140
CDw125	Interleukin 5 receptor α chain	Eo, B act, basophils	Associates with CDw131 (common to CD116 and CDw123) to form the functional IL-5 receptor	60
CD126	Interleukin 6 receptor	T, B act, M	Binds IL-6 with low affinity. Noncovalently associates with the CD130 molecule to form the functional high-affinity IL-6 receptor	80
CD127	Interleukin 7 receptor	T	Associates with the CD132 molecule, which results in augmentation of binding affinity and internalization of IL-7	75
CDw128	Interleukin 8 receptor	G	The two identified forms are members of the rhodopsin superfamily that contain seven transmembrane domains and couple to GTP-binding proteins	58–67
CD130	gp130	(Broad)	Binds oncostatin M (OSM) with low affinity. Noncovalently associates with the CD126 molecule to form the high-affinity receptor for IL-6. Signal transducing component of the IL-11 receptor. Upon association with the leukemia inhibitory factor (LIF) receptor, the high-affinity receptor for LIF, OSM and cardiotrophin-1 is generated. This functional LIF/OSM receptor is probably converted to the ciliary neurotrophic factor (CNTF) receptor upon association with the CNTF binding protein	130

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CDw131	Common β chain	Myeloid cells, pre B	Associates either with the CDw123, the CDw125, or the CD16 molecule to form the appropriate functional receptor	120
CD132	Common γ chain	Broad	γ chain of the receptors for IL-2 (see CD25 and CDw122), IL-4 (see CD124), IL-7 (see CD127), IL-9, and IL-15. Mutation causes x-linked severe combined immunodeficiency	64
CD134	OX40	T act sub	Member of the TNF/NGF receptor family (see CD27, CD30, CD40, CD95, CD120a, CD120b, CDw137). Costimulatory molecule of T cells. Involved in T-B and T-EC interactions by binding to the OX40 ligand	48–50
CD135	flt3/flk2; STK-1	Prog sub, pre B	Belongs to the Ig and receptor tyrosine kinase family (see CD115, CD117, CD140a, CD140b). Receptor for flt3 ligand. Important receptor/ligand system for hematopoietic cell proliferation and differentiation	155
CDw136	Macrophage-stimulating protein receptor (MSPR), RON	M, G, EPC	Member of the receptor tyrosine kinase family. The mature protein is organized as a disulfide-linked α/β dimer, which is derived from a single chain pro RON. Involved in the regulation of production of blood cells, and in the development of epithelial tissue	150, 35
CDw137	4-1BB	T act	Member of the TNF/NGF receptor family (see CD27, CD30, CD40, CD95, CD120a, CD120b, CD134). Receptor for 4-1BB ligand. Seems to be important for T cell survival	30
CD138	Syndecan-1, B-B4	PC, EPC	The extracellular domain bears heparan sulfate glycosaminoglycans through which it binds both growth factors and extracellular matrix constituents. The cytoplasmic domain interacts with cytoskeletal components. Proposed to have roles in growth factor action, extracellular matrix adhesion and cytoskeletal organization that controls cell morphology	80–160
CD139		B, M, G	Not fully characterized	220+250
CD140a	α -platelet-derived growth factor (PDGF) receptor	Undetectably expressed	Belongs to the Ig supergene and receptor tyrosine kinase family (see CD115, CD117, CD135, CD140b). PDGF binding induces either homodimerization or heterodimerization with the CD140b molecule. Binds both PDGF-A and PDGF-B chains. Induces a mitogenic response	180
CD140b	β -platelet-derived growth factor (PDGF) receptor	Focal EC, stromal cell lines	Belongs to the Ig supergene and receptor tyrosine kinase family (see CD115, CD117, CD135, CD140a). PDGF binding induces either homodimerization or heterodimerization with the CD140a molecule. Binds PDGF-B chains. Induces a mitogenic response, actin reorganization and chemotaxis	180
CD141	Thrombomodulin	Myeloid cells, EC, Plt	Receptor for thrombin. Involved in regulation of coagulation	100
CD142	Tissue factor, thromboplastin, coagulation factor III	M act, EC act, EPC	Related to the IFN α receptor and the CDw119 molecule. High-affinity receptor for factor VII, and it is the essential cofactor for factor VII. Forms a ternary complex with factor VII and X to induce the extrinsic pathway of the coagulation cascade	45

Table 1 Continued

	<i>Other name (s)</i>	<i>Main reactivity of mAbs</i>	<i>Short characteristic of the CD molecule</i>	<i>Molecular weight reduced (kDa)</i>
CD143	Angiotensin-converting enzyme (ACE), peptidyl dipeptidase A, EC3.4.15.1	EC, EPC	Somatic form of ACE. Zinc metallopeptidase with specificity for the vasoactive peptides angiotensin I and bradykinin. Differentially expressed along the vascular tree. EC of arterioles, small arteries, capillaries in organs such as lung are positive. EC of large arteries and veins are mostly negative	170
CD144	Vascular endothelial cell cadherin (VE-cadherin), cadherin 5, 7B4	EC	Ca ²⁺ dependent homophilic cell adhesion molecule. It interacts with catenins which link CD144 to the cytoskeleton; this complex is crucial for the regulation of cell adhesion	130
CDw145		EC	Defined by the mAbs 7E9 and P7A5. Not fully characterized	25
CD146	Muc18/S-endo, MCAM, Mel-CAM	EC, T act sub, smooth muscle cells, melanoma cells	Member of the Ig supergene family. Potential cell adhesion molecule	130
CD147	Neurothelin, basigin, M6, EMMPRIN	Leukocytes broad, EC, Plt, red blood cells	Member of the Ig supergene family. Potential cell adhesion molecule	50–60
CD148	p260, HPTP η /DEP1	Leukocytes broad, Plt	Density enhanced protein tyrosine phosphatase-1 (DEP-1). Type III protein tyrosine phosphatase	200–260
CDw149	MEM-133	Lymph, M	Not fully characterized. Defined by mAbs MEM-120 and MEM-133	120
CDw150	Signaling lymphocyte activation molecule (SLAM), IPO-3	T sub, B sub, Thy	Member of the Ig supergene family. It is a homotypic adhesion molecule involved in T/T, T/B, B/B interaction, and thereby in the regulation of T cell and B cell activation	70
CD151	Platelet-endothelial tetra-span antigen (PETA)-3	Plt, EC, stromal cells	Member of the tetraspans family (see CD9)	27
CD152	Cytotoxic T lymphocyte antigen (CTLA)-4	T act	Receptor for CD80 and CD86. Functions as a regulator of the T cell activation	44
CD153	CD30 ligand	T act	Member of the TNF family (see CD70, CD95L, CD154). Counter-receptor for CD30	40
CD154	gp39; TRAP-1; CD40 ligand	T act, mast cells	Member of the TNF family (see CD70, CD95L, CD153). Essential for germinal center formation and for Ig class switching	39
CD155	Poliovirus receptor	M, CD34 ⁺ cells	Member of the Ig supergene family	80–90
CD156	MS2, ADAM-8	M, G	Belongs to the ADAM (a disintegrin and metalloproteinase domain) family. The extracellular region consists of a disintegrin-, metalloproteinase-, cysteine rich and EGF domain. Probably involved in loosening of cell–cell, cell–matrix contacts	69
CD157	Bst-1, Mo5	M, G, bone marrow stromal	Glycosylphosphatidylinositol-linked, CD38-like protein having both ADP-ribosyl cyclase activity and cyclic ADP-ribose (cADPR) hydrolase activity	42–50
CD158a	p58.1/p50.1	NK Sub, T sub	Receptor for HLA CW2, CW4, CW5, CW6. Killer cell inhibitory receptor (KIR) family member. p58.1 is the inhibiting form, p50.1 is the activating form; the difference is in the truncation of the cytoplasmic domain in p50.1	58.50

Table 1 Continued

	Other name (s)	Main reactivity of mAbs	Short characteristic of the CD molecule	Molecular weight reduced (kDa)
CD158b	p58.2/p50.2	NK	Receptor for HLA CW1, CW3, CW7, CW8. Killer cell inhibitory receptor (KIR) family member p58.2 is the inhibiting form, p50.2 is the activating form; the difference is in the truncation of the cytoplasmic domain in p50.2	58.50
CD161	NKR-P1A	NK, M, T sub, Thy precursors	Member of the Ca^{2+} -dependent (C-type) lectin superfamily of type II transmembrane receptors (see CD23, CD69, CD72, CD94)	30
CD162	PSGL-1, CD62 ligand	M, G, T	Counter-receptor of CD62E, CD62L and CD62P. Involved in leukocyte rolling	110
CD163	KiM8, M130, GHI/61	M	The CD163 molecule is a type I transmembrane molecule. The extracellular domain consists of nine scavenger-like repeating elements	130
CD164	MGC-24, MUC-24	T, M, G, epithelial cells, stroma cells, bone marrow cells	Sialomucin-like glycoprotein (see CD34, CD43, CD68). Involved in adhesion of hematopoietic cells to stroma cells	80
CD165	AD2/GP37	Plt, NK sub, T sub	Not fully characterized. Defined by mAbs AD2 and SN2	42
CD166	Activated leukocyte-cell adhesion molecule (ALCAM), CD6 ligand	M act, T act, Thy epithelium, neurons	Counter-receptor for CD6	100

Abbreviations: B, B-cells; act, activated; DC, dendritic cells; EC, endothelial cells; Eo, eosinophils; EPC, epithelial cells; G, granulocytes; LHC, epidermal Langerhans cells; M, monocytes; Mac, macrophages; NK, natural killer cells; PC, plasma cells; Plt, platelets; Prog, progenitor cells; sub, subpopulation; T, T cells; Thy, thymocytes. Cell types in brackets indicate weak reactivity or heterogeneous expression.

human proteins/genes. Within this database the CDs form a subgroup. The individual CD guides of PROW are written and reviewed by different experts and will be updated permanently. Links to other resources including Entrez, MEDLINE and sequence databases are available. There is free access to PROW via the Internet address <<http://www.ncbi.nlm.nih.gov/prow/>>.

See also: Adhesion molecules; Campath-1 antigen (CD52); CD1; CD2; CD3; CD4; CD5; CD8; CD22; CD28; CD40 and its ligand; CD44; CD45 (the leukocyte common antigen); CD46 (membrane cofactor protein, MCP); CD59; Cell surface molecules, immunoprecipitation of; Lewis^x/sialyl-Lewis^x (CD15/CD15s); Complement receptors; Cytokine receptors; Decay-accelerating factor (CD55); Fas (CD95) and fas ligand; Fc receptors; Ig α/β (CD79a/CD79b); Integrins; Inter-cellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Interferon γ receptor; Invariant chain (II); Lymphocyte function-associated antigen 1 (LFA-1); Lymphocyte function-associated antigen 3 (LFA-3); Selectins (CD62-E/L/P); Thy-1; Transferring receptor (CD71); TNF receptors; Cell surface receptors and adhesion molecules, three-dimensional structures

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CD1

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The CD1 family of cell surface glycoproteins is a well-defined group of non-major histocompatibility complex (MHC) encoded molecules. This nonpolymorphic cluster is the best understood of the MHC-unlinked antigen-presenting molecules. Five CD1 genes (CD1a, b, c, d and e) have been identified in humans and two genes (mCD1.1 and mCD1.2) in the mouse. However, CD1 genes and their products are evolutionarily conserved in mammals as they were identified in rats, rabbits, guinea pigs, cows and sheep.

Apparently, CD1 molecules represent a third and distinct lineage of antigen-presenting molecules that differ from MHC class I and class II molecules. Whereas CD1 molecules are structurally similar to MHC class I molecules and are associated with β_2 -microglobulin (β_2m), their expression at the cell surface requires an endocytic compartment similar to MHC class II molecules.

CD1a was fortuitously discovered in 1979 as the first human leukocyte antigen (HLA) recognized by a monoclonal antibody. Nevertheless, the most remarkable discovery regarding these molecules was reported only a few years ago when human CD1b was shown to present a nonpeptide antigen to T cells. Indeed, CD1 proteins are capable of presenting nonpeptide antigens such as microbial lipids and glycolipids as well as peptide antigens. Such novel bio-

chemical properties may reflect a specialized role for CD1 in the immune system and the potential to exploit it in vaccine development.

CD1 genes and CD1 protein structure

CD1 genes are MHC-unlinked, i.e. the human CD1 genes are encoded on chromosome 1, while HLA genes are encoded on chromosome 6. Similarly, the mouse CD1 genes are encoded on chromosome 3, whereas MHC genes are mapped to chromosome 17. The intron-exon structure of CD1 genes is similar to that of the MHC class I genes (Figure 1).

At least four human CD1 proteins (the CD1a, b c and d isoforms) are expressed on the surface of cells in association with β_2m . The protein products of these genes have been loosely classified into two groups according to their structure and tissue distribution (Table 1). The first group includes human CD1a, CD1b and CD1c, which are abundantly expressed on professional antigen-presenting cells such as Langerhans cells, dermal and lymph node cells, dendritic cells, mantle zone B cells and cytokine-activated monocytes. The second group includes human CD1d, and mouse CD1.1 and CD1.1, which are strongly expressed on the intestinal epithelium.

Amino acid homology of CD1 proteins and MHC class I and II molecules is minimal in the $\alpha 1$ domain

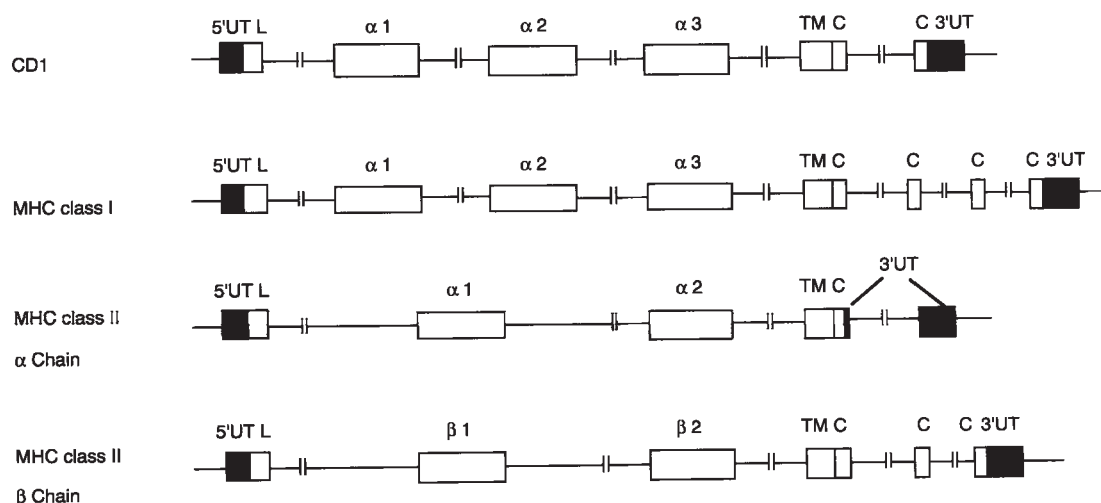


Figure 1 Intron-exon organization of CD1 and MHC class I and II genes. Open boxes are coding for exons. 5' UT, 5' untranslated region; L, leader sequence; TM, transmembrane domain; C, cytoplasmic domain; 3' UT, 3' untranslated region. (Reproduced with permission from Porcelli (1995).)

Table 1 CD1 molecules are classified into two groups according to their structure and tissue distribution

CD1 group	Known members	Tissue distribution
Group 1	Human CD1a, CD1b, CD1c and CD1e Sheep CD1b	Professional antigen-presenting cells such as Langerhans cells, dendritic cells, dermal and lymph node cells, mantle zone B cells and cytokine-activated monocytes
Group 2	Human CD1d Mouse CD1.1 and CD1.2 Rabbit CD1d	Intestinal epithelium, cortical and medullary thymocytes, circulating and splenic B cells, and hepatocytes

and is very limited in the $\alpha 2$ domain (especially with respect to class I proteins) (Figure 2). In the $\alpha 3$ domain, detectable homology is seen between CD1 and both MHC class I and class II proteins. The highest similarity scores in this region are with HLA-F (34%), HLA-A2 (27.8%), and HLA-DR β (25.8%). Indeed, the $\alpha 3$ domain of CD1 proteins falls within the C1 group of immunoglobulin (Ig) superfamily members.

Antigen processing and presentation

The majority of antigens presented by MHC class I molecules are generated in the cytosol after proteolysis of endogenous proteins by proteasomes. Short peptides are then transferred into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), and after binding to newly assembled MHC class I molecules, are transported

to the plasma membrane. Similar to MHC class I, class II molecules assemble in the ER. However, class II molecules bind antigenic peptides in endosomal structures. MHC class II antigen processing and presentation can be blocked by reagents that raise endosomal pH (such as ammonium chloride, concanamycin A or chloroquine). In the ER, MHC class II molecules associate with the invariant chain (Ii); this association is required for efficient release of MHC class II from the ER. The signals that target the class II-Ii complex to the endosomal compartment are located in the cytoplasmic tail of Ii. Upon arrival in the endosomes, Ii is degraded, allowing the peptide-binding groove of MHC class II to be exposed for loading of the antigenic peptide. Two MHC class II-like structures, HLA-DMA and HLA-DMB, play an essential supporting role in processing of antigens presented by MHC class II molecules. Class II molecules on cells with specific deletions in HLA-DM

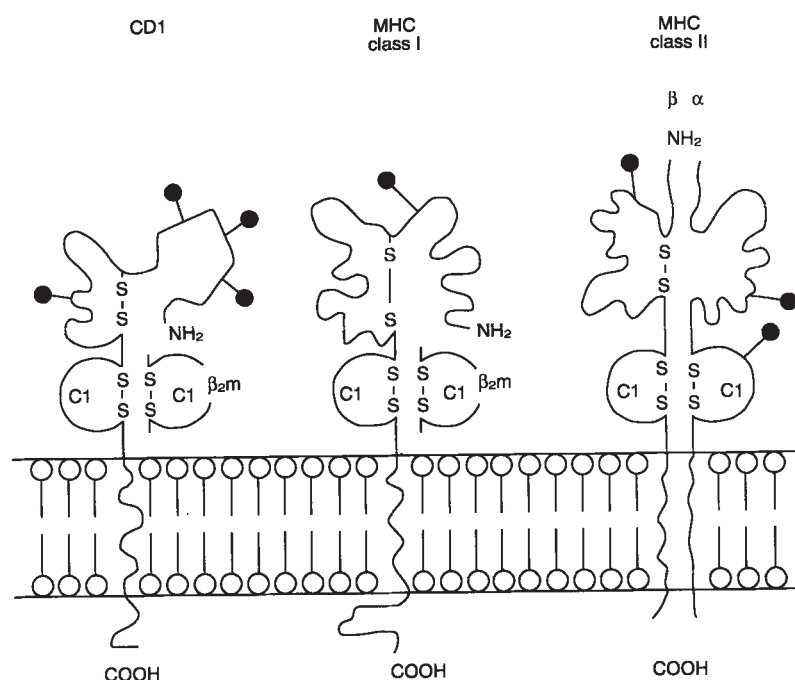


Figure 2 A proposed domain organization of CD1 proteins in comparison to MHC class I and II proteins. ●—, N-linked glycosylation; C1, immunoglobulin C1-like domain; S-S, intradomain disulfide bonds. (Reproduced with permission from Porcelli (1995).)

genes fail to present most but not all antigens and their MHC molecules contained mostly an Ii-derived fragment designated CLIP (class II-associated Ii peptide). Recently it has been demonstrated that HLA-DM molecules facilitate the release of CLIP and the subsequent binding of other peptides to MHC class II molecules. Although not all MHC class II molecules depend on HLA-DM for antigenic loading, HLA-DM proteins appear to function as chaperones that help in the last step of MHC class II folding and antigenic peptide loading.

Despite association with β_2m , the antigen processing pathway employed by CD1 molecules is closer to that of MHC class II than to that of class I. CD1 antigen presentation is independent of TAP-1 and TAP-2. Using cells from TAP-deficient patients (TAP^{-/-}), recent studies demonstrated that CD1a and CD1c are normally expressed on the surface of epidermal Langerhans cells. In addition, normal expression of CD1a, b and c was seen on dendritic cells differentiated *in vitro* from monocytes from the same patients. Furthermore, these studies showed that CD1a molecules on the surface of the TAP^{-/-} dendritic cells were functional. Thus, this implies that either cell surface CD1 molecules are empty, or that CD1 molecules present TAP-independent antigens.

Although antigen presentation by CD1 does not require the MHC class II-like dimer, HLA-DMA/B, it is inhibitable by chloroquine and concanamycin A. In addition, recent studies demonstrate that nonpeptide antigens are processed in endocytic compartments. CD1b molecules appear to arrive at the endosomal compartment through at least two distinct routes: 1) a major recycling pathway, where CD1b

molecules are endocytosed at the plasma membrane and subsequently delivered to the MHC class II compartment (also called MIIC); this pathway is mediated by the cytoplasmic domain of CD1b molecules; and 2) a pathway comparable to MHC class II-Ii targeting to the endosomes; however, the signals that target CD1 molecules to MIIC appear to be a tyrosine-based motif in its cytoplasmic tail.

CD1-restricted antigens

Among the most unique features of the CD1 family members is the ability of certain CD1 molecules to present nonpeptide antigens to T cells. At least three classes of antigens have been shown to be presented by CD1 molecules, i.e. a lipid moiety, a lipoglycan and peptide antigens were recognized by CD1-restricted T cells.

In 1994, a group of scientists at Harvard University described the first CD1-restricted antigen as a protease-resistant moiety that copurified with the lipid fraction of the mycobacterial extract. Biochemical analysis of this antigen showed that it is a member of the mycobacterial family of mycolic acids (Figure 3). The fact that mycolic acid is found in the cell wall of several bacterial species such as *Nocardia* and *Corynebacterium* spp. suggests that CD1 molecules may present antigens derived from bacterial pathogens other than mycobacteria. A year later, these findings were extended by describing two *Mycobacterium leprae*-specific CD1b-restricted human T cell lines that recognize lipoarabinomannan (LAM), a glycolipid from the mycobacterial cell wall, and do not cross-react with mycolic acid. This molecule belongs to the lipoglycan family and is com-

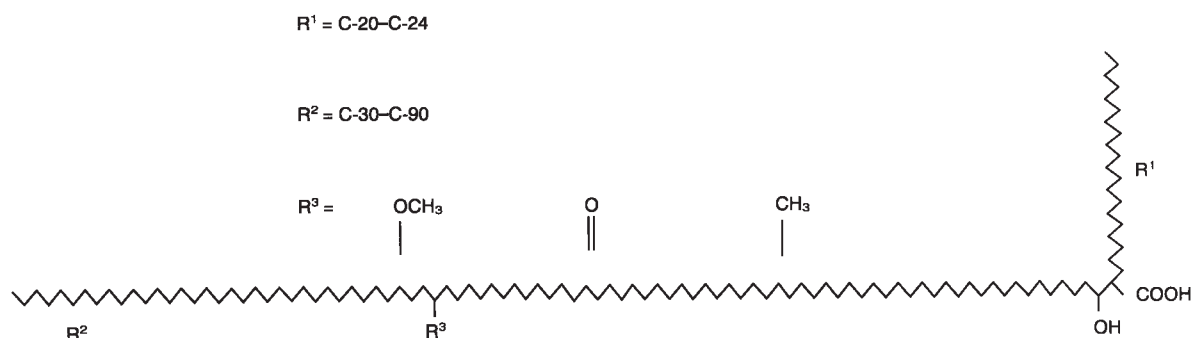


Figure 3 Mycolic acids are high molecular weight α -branched, β -hydroxy fatty acids that are components of the cell wall of all mycobacteria. All known mycolic acids have the basic structure $R^2\text{CH(OH)CHR}^1\text{COOH}$ where R^1 is a C-20–C-24 linear alkane and R^2 is a more complex structure of 30–90 carbon atoms that may contain various numbers of carbon–carbon double bonds and/or cyclosporane rings, methyl branches and oxygen function groups such as $\text{C}=\text{O}$, $\text{CH}_3\text{OCH}=\text{COOH}$. Three principal categories of mycolic acid are known, i.e. corynomycolic acids (C-28–C-40), nocardomycolic acid (C-40–C-60) and mycobacterial mycolic acids (C-60–C-90), produced by strains of *Corynebacterium*, *Nocardia* and *Mycobacterium*, respectively.

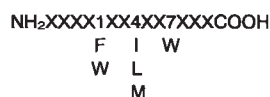


Figure 4 Mouse CD1 binds peptides with a sequence motif comprising aromatic, bulky and hydrophobic amino acids with an overhanging NH₂-terminus. The majority of these long peptides include aromatic residues at positions 1 and 7 and an aliphatic residue in position 4. F, Phenylalanine; W, tryptophan; L, leucine; I, isoleucine; M, methionine; X, any amino acid.

posed of a hydrophobic lipid-containing phosphatidyl inositol group attached to a large and complex hydrophilic heteropolysaccharide. Further studies using chemically modified LAM and related compounds showed that both carbohydrate and lipid components of the antigen were required for presentation and/or T cell recognition. In addition, these two T cell lines differed in their ability to recognize LAM purified from different species of mycobacteria, suggesting a significant level of specificity in CD1-restricted T cell responses. This distinct recognition may arise from subtle variations in the lipid and carbohydrate moieties of LAM.

The previous examples suggest that CD1 proteins could have evolved to present nonpeptide lipid antigens to T cells. However, using random peptide phage displayed libraries, mouse CD1 was shown to bind peptides with a sequence motif comprising aromatic, bulky and hydrophobic amino acids with an overhanging NH₂-terminus (Figure 4). The study identified several dozen peptides which appear to be of immunological relevance as they can elicit specific CD1-restricted CD8⁺ cytotoxic T lymphocytes (CTLs). The length of these peptide antigens was not critical; however, the majority of these peptides

include aromatic residues at positions 1 and 7 and an aliphatic residue in position 4. The characteristics of the mouse CD1-peptide interactions are similar to the interactions of MHC class II molecules with its ligands. For example, in common with class II ligands, mouse CD1 appears to prefer long peptides with hydrophobic and bulky amino acids at certain positions. In addition, the affinity of interaction of these peptides is similar to that of naturally processed peptides copurified with class II molecules.

Although mouse CD1 has been shown to bind peptides, it is not known if mycolic acid and the other lipoglycans directly bind to CD1b. In addition, it is not yet known whether CD1b presents one or more members of the mycolic acid families to T cells as a processed or intact molecule, or as part of a modified epitope.

Interestingly, the amino acid sequence of all known CD1 molecules from various mammalian species, as well as another MHC-unlinked molecule, neonatal Fc-receptor (FcRn), share a proline at position 162 in the $\alpha 2$ helix. Recent crystallographic studies of FcRn showed that Proline 162 induces a kink that closes the peptide-binding groove, thus preventing peptide binding. If this kink is found in CD1 molecules, then it is possible that the interaction between CD1 molecules and their ligands is different from that known for conventional MHC class I molecules. Also, it may resemble that of MHC class II-superantigen association. In addition, the full range of antigens presented by the CD1 molecules is not yet known and the natural ligands remain to be defined. However, studies from several laboratories support the hypothesis that CD1 molecules contain a hydrophobic pocket between the $\alpha 1$ and $\alpha 2$ domains capable of accommodating lipids, glycolipids or

Table 2 Comparison between CD1, MHC class I and MHC class II molecules

	CD1 Molecules	MHC class I	MHC class II
Antigen processing and presentation			
TAP-1/TAP-2 requirement	No	Yes for the majority	No
HLA-DMA/B requirement	No	No	Yes for the majority
Chloroquine sensitivity	Yes	No	Yes
Invariant chain (Ii) requirement	No	No	Yes
Endosomal compartment passage	Yes	No	Yes
ER to endosome targeting	Signal in the cytoplasmic tail	Not applicable	Signal in the Ii
$\beta_2\text{m}$ association	Yes	Yes	No
T cell recognition	CD4 ⁻ /CD8 ⁻ , CD8 ⁺ , CD4 ⁺	Mostly CD8 ⁺	CD4 ⁺
Nature of antigens presented	Lipid, glycolipid and long hydrophobic peptides	Mostly peptides, 8–10 amino acids long	Mostly peptides, 8–20 amino acids long

hydrophobic peptides. It is clear that further biochemical studies and the determination of the crystal structures of both mouse and human CD1 are needed to elucidate these issues.

Role of CD1 in the immune system

The initial reports implicating CD1 in T cell function demonstrated that human T cell clones expressing either α/β or γ/δ T cell receptors (TCRs) recognized tumor cells expressing specific isoforms of human CD1. These CD1-restricted T cells were phenotypically CD4⁺CD8⁻ or double negative (DN) T cells. CD1b-restricted proliferative and cytotoxic responses of human DN- α/β ⁺ and DN- γ/δ ⁺ lymphocytes specific for *M. tuberculosis* and *M. leprae* were seen in cells isolated from blood of normal humans. Also, CD1-restricted T cells specific for mycobacterial antigens have been found among CD8⁺ TCR- $\alpha\beta$ ⁺ T cells. In addition, mouse NK1⁺ TCR- $\alpha\beta$ ⁺ T cells, which appear to be entirely CD1 restricted, were isolated recently. These T cells were either DN or CD4⁺. Thus, it is now accepted that CD1 recognition is broadly distributed among T cell subsets. The different biochemical features of the various members of the CD1 family and the wide spectrum of recognition by T cell subsets indicate that CD1 molecules may subserve several specialized functions in the immune system, such as presentation of hydrophobic ligands (lipids or peptides) and/or regulation of the classes of immune responses.

The mouse MHC class I molecule, H2-M3, was the first definitive example of a specialized molecule in antigen presentation. H2-M3 preferentially binds hydrophobic N-formylated peptides, which make it suited to the presentation of antigens derived from intracellular pathogens. CD1 and H2-M3 molecules share a common feature, i.e. both bind and present hydrophobic antigens of bacterial origin. While both CD1 and H2-M3 may have evolved to combat microbes, each of these molecules seem to use different antimicrobial strategies. The antigens presented by H2-M3 are from the NH₂-terminus of prokaryotic proteins and appear to be intracellular in nature. In contrast, CD1 molecules seem to bind bacterial cell wall lipids that come from the extracellular milieu. Interestingly, H2-M3 has not been found in species other than rodents (including humans). Thus, human group I CD1 molecules, may accomplish the role assigned to H2-M3 in the mouse.

Autoreactive CD1-restricted T cells (NK1⁺) have been identified in normal unimmunized mice. Although this rare T cell population includes the DN phenotype, the majority are CD4⁺ cells. A prominent subset of these CD1-specific cells uses an invariant

TCR α chain in conjunction with a restricted set of TCR V β families. During their differentiation in the thymus, these cells acquire several unique properties, i.e. they express natural killer cell receptors, and they display unusual cytokine secretion functions. In particular, the CD4⁺ subset secretes large amounts of interleukin 4 (IL-4) upon primary stimulation, within an hour of TCR engagement. Several studies indicated that NK1⁺ T cells could potentially influence the TH1/TH2 profile of the immune response, and thus could act as natural suppressors of graft-versus-host disease, mediate acute graft rejection, or regulate autoimmune symptoms in lupus and diabetes. As yet, however, there is no physiological phenomenon that these T cell subsets are associated with.

CD1b is abundantly expressed in the lesions of patients with the self-healing form of leprosy (where interferon γ secreting TH1 CD4⁺ cells are recruited) but not with the lepromatous form (where TH2 CD4⁺ cells predominate). Thus, it is suggested that CD1b expression may determine the TH1/TH2 set of specialized cytokines produced in response to *M. leprae*. Mycobacterial lipids are an ideal foreign target; indeed, mycolic acid is an active component of one of the most powerful adjuvants (Freund's complete adjuvant). Therefore, it is postulated that early production of cytokines by CD1b-restricted, mycobacterial-specific T cells may jump-start the immune response or may determine its TH1 differentiation. The possible roles of CD1-expressing cells and NK1⁺ T cells in influencing the TH1/TH2 differentiation and development pathway remains to be fully understood.

The oligomorphic CD1 proteins are a third and distinct group of antigen-presenting molecules (Table 2) that are conserved in all mammalian species tested. Monomorphic and oligomorphic antigen-presenting molecules may be more effective in inducing a protective immune response, and for potential vaccine design, than their polymorphic counterparts. Whereas the latter group might bind tens of antigens with different sequences, the former binds a single or very limited number of antigens. A single peptide or a microbial lipid, in the case of CD1 proteins, might be sufficient to induce this protective response in all recipients. Thus, CD1 proteins are definitive candidates for antigen-presenting molecules in vaccination strategies intended to induce a universal CTL response.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; β_2 -microglobulin; Effector lymphocytes; Fc receptors; MHC, functions of; *Mycobacteria*, infection and immunity.

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CD2

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CD2 is a glycoprotein of 50 kDa expressed by the vast majority of non-B lymphocytes in humans. In the mouse, unlike in humans, CD2 is also present on B lymphocytes and, in the rat, on splenic macrophages. CD2 appears at the earliest level of T lineage ontogeny and demonstrates a remarkable structural conservation among species. Several differentially glycosylated forms have been found on the cell surface of human non-B lymphocytes. In the mouse, two distinct isotypic forms of CD2 exist in different strains.

Structure

The CD2 gene maps to the human chromosome 1. Analysis of CD2 cDNA clones indicates a cleaved signal peptide of 24 amino acids, an extracellular segment of 185 residues, a transmembrane domain of 26 residues and a cytoplasmic region of 116 residues. Restriction analysis and sequencing of genomic DNA shows that the gene is divided into five exons, one encoding the signal peptide, two encoding the two extracellular domains, one encoding the transmembrane domain, and one encoding the intracellular domain. On the basis of significant primary

sequence homology, a relationship of CD2 to the Ig superfamily has been proposed. Secondary structure predictions reveal a mix of α helical and β sheet structures.

Ligands

Employing transfection of cells with mutagenized plasmids or human CD2 chimeric and mutant proteins, distinct CD2 epitopes are defined by monoclonal antibodies and mapped to one of the Ig-like domains of the CD2 ectodomain. CD2 binds to the ubiquitously expressed cell surface glycoprotein, CD58, which is present on many human cell types including antigen-presenting cells and endothelial cells. A sheep homolog of CD58 exists which binds to human CD2, providing the molecular explanation for the fortuitous observation that human T lymphocytes and sheep blood cells spontaneously form rosettes. A second structurally related ligand, CD48, binds to CD2 but with an affinity two orders of magnitude weaker than that of the CD2/CD58 interaction. CD59 is also thought to bind to CD2.

Interactions of CD2 with its ligands are mediated through the N-terminal membrane distal Ig-like

domain. The precise role of the second or membrane-proximal Ig domain has not been elucidated in depth, although activation of T cells in response to paired mitogenic combinations of CD2-specific monoclonal antibodies requires that one monoclonal antibody is directed against an epitope (CD2R) within or closely related to this region.

Role of CD2

Expression of CD2 on resting versus activated human T cells not only differs from a quantitative point of view (CD2 on resting T cells: approximately 30 000 molecules per cell; CD2 on activated T cells: approximately 200 000 molecules per cell) but also with regard to expression of the CD2R epitope, which is only found on activated T cells and exposed as a consequence of ligand-induced conformational changes within the CD2 ectodomain.

The involvement of CD2 in T lymphocyte adhesion and activation is well documented. In addition to providing accessory signals for T cell receptor-mediated activation of lymphocytes, *in vitro* activation in the absence of direct stimulation through the T cell receptor using mitogenic combinations of CD2-specific monoclonal antibodies has been described. CD2 has been shown to play a role in potentiating T cell responses to the cytokines interleukin 1 (IL-1), IL-6 and IL-12 and to be involved in the regulation of T cell anergy. In this regard, perturbation of particular regions of CD2 can inhibit T cells and this inhibition is independent of the disruption of the adhesion function of CD2 with its ligands. Rather, polyclonal T cell hyporesponsiveness which persists long after the recovery of CD2 cell surface expression may be due to an inhibitory signal which is delivered to T cells through CD2. T cell anergy correlates with a loss of the CD2R epitope expression but not CD2 expression. Reversal of anergy following culture with IL-2 is induced when challenged with antigen and CD58 and this coincides with the reappearance of the CD2R epitope. Among the accessory T cell molecules examined so far, CD2 appears to be unique in its ability to participate in the reversal of anergy.

Signaling

CD2 itself has no enzymatic activity but transmits signals in part via the T cell receptor/CD3/ ζ complex through its interaction with the ζ chain and CD3 ϵ and also through other unknown pathways as suggested by the tyrosine phosphorylation of distinct

intracellular substrates and the serine dephosphorylation and nuclear translocation of cofilin, a cyclosporine-insensitive event. Of particular interest is the interaction of CD2 with the tyrosine phosphatase CD45. CD2 also interacts with the tyrosine kinases p56^{lck}, p59^{fyn} and the ζ subunit of the T cell receptor/CD3/ ζ complex, each of which have been shown to be targets for CD45 phosphatase activity. Indeed it is possible that CD2 regulates T cell activation by bringing CD45 into the proximity of these molecules.

Perturbation of CD2 and/or its associated signaling molecules provides a means for positively and negatively regulating T cell responses and may turn out to represent a central component of therapeutic efforts to regulate tolerance.

See also: Adhesion molecules; CD45 (the leukocyte common antigen); Cell-mediated lysis; Lymphocyte function-associated antigen 3 (LFA-3); T lymphocyte activation; T lymphocyte differentiation.

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CD3

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Originally known as T3, CD3 was identified by Kung and colleagues in 1979 and was one of the first groups of human T lymphocyte surface antigens identified using monoclonal antibodies. It was subsequently shown that antibodies against CD3 could, depending on the conditions used, either stimulate T cells to divide or inhibit the development of effector functions such as cytotoxicity. Therefore, it was apparent early on that CD3 had an important role in T cell function. Initially, CD3 was thought to consist of a single polypeptide, but later analysis demonstrated its three-chain nature (CD3 γ , δ and ϵ) and its close association with T cell antigen receptor (TCR) heterodimers. This established the CD3 molecules, together with other invariant subunits (ζ and η), as part of the core TCR. Although the TCR $\alpha\beta$ or $\gamma\delta$ heterodimers govern the antigen recognition capability of the TCR, signal transduction and the regulation of TCR function are mediated through the invariant subunits.

Structure and assembly of TCR invariant subunits

Investigation of the structure of the TCR has been an area of intense activity, since such studies should provide insight into the molecular details of receptor function, but much remains to be learned. The current picture is of an elaborate structure comprising at least four invariant polypeptides forming a complex with the antigen-binding heterodimer. The most common form of the $\alpha\beta$ -type core receptor is likely to have the stoichiometry $\alpha\beta\gamma\delta\epsilon_2\zeta_2$, where the γ , δ and ϵ chains are the CD3 subunits proper, being specific to the TCR, and ζ is the most prevalent member

of a family of similar molecules which include η and the γ chain of some Fc receptors. The structural characteristics of the most important of these molecules are given in Table 1.

The cDNAs for the three CD3 molecules were cloned in the mid-1980s and examination of the predicted amino acid sequences suggested that they form a small family of homologous integral membrane proteins, with γ and δ showing a particularly high degree of relatedness to each other. Close linkage of the CD3 genes is consistent with their evolution from a common ancestral gene. The extracellular domain of each chain possesses some sequence similarity with immunoglobulin (Ig) and the potential to arrange stretches of β sheet as a partial Ig fold. The CD3 proteins, therefore, like TCR $\alpha\beta$ and TCR $\gamma\delta$, are members of the Ig superfamily. The transmembrane regions of the CD3 molecules each contain an acidic amino acid within the stretch of hydrophobic residues, an unusual feature which is probably significant given the presence of corresponding basic residues in the transmembrane regions of TCR heterodimers. Although it is unlikely that full ionization of acidic and basic side-chains takes place in the hydrophobic environment of the plasma membrane, interactions between the transmembrane sequences of heterodimers and the CD3 subunits may help to stabilize the TCR complex. The sequences of the cytoplasmic domains of the CD3 subunits show little relatedness to other known proteins, but all contain the tyrosine-containing Reth motif implicated in signal transduction, which is also found three times in the ζ cytoplasmic domain and in other receptor-associated polypeptides including the surface IgM-associated Ig- α and Ig- β proteins, and the β and γ subunits of Fc ϵ RI.

Table 1 Structural data on TCR invariant subunits

Subunit	Molecular weight (kDa)		Ig superfamily?	Glycosylated?
	Human	Mouse		
CD3 γ	25	21	Yes	Yes
CD3 δ	20	28	Yes	Yes
CD3 ϵ	19	25	Yes	No
ζ	16	16	No	No
η	Not encoded	22	No	No

In mouse, the vast majority (80–90%) of the ζ chain exists as a disulfide-linked homodimer, but can also form heterodimers with the related η , γ and (possibly) θ chains. This leads to the formation of several isoforms of the mature TCR complex within a single T cell, with the potential for functional variation between forms. Cloning and sequencing predict a structure for ζ which is markedly different from that of the CD3 proteins. One feature which ζ does share with CD3 subunits is the acidic residue in its transmembrane region. Otherwise, it has a minimal extracellular domain, consisting of only nine amino acids including the cysteine responsible for disulfide linkage, and an intracellular domain with a consensus sequence for ATP binding and multiple (six in mice; seven in humans) tyrosines, whose phosphorylation results in a dramatic apparent increase in M_r to 21 kDa. Intriguingly, η and θ derive from the same gene as ζ , by alternative mRNA processing, although γ is encoded by a separate, though linked, gene. The significance of the η , γ and θ chains is unclear, however, given that η has been demonstrated to be absent from human T cells.

Study of the assembly of a multisubunit complex such as the TCR is hindered by a number of problems. First, the receptor is built up of several transmembrane proteins and requires translocation of subunits across a membrane for its formation. Therefore, it is difficult to study interactions of purified proteins in solution. Furthermore, all TCR components (excepting η) may need to be present for efficient complex formation. However, the combination of several approaches including the examination of immature T cells and T cell lines, together with the analysis of partial receptor complexes, has provided considerable information.

Thymocytes express CD3 γ , δ and ϵ at an early stage in their development, before the TCR α and β genes have completed their rearrangements: between days 14 and 16 of mouse fetal thymus development, and from week 10.5 in human fetal thymus. In humans, there is also evidence for expression of the

CD3 genes from week 7 in fetal liver, prior to colonization of the thymus. Thus, the CD3 genes are among the earliest T cell lineage genes to be expressed. Without the antigen-binding heterodimer proteins, immature thymocytes do not express significant levels of surface CD3. Cell lines lacking either TCR α or β also fail to deliver CD3 to the cell surface, or do so inefficiently. Mutant cell lines defective in ζ production express only 5% of normal cell surface receptor levels. Cumulatively, these data indicate that a ζ_2 -type receptor requires most, and possibly all, subunits for efficient transport to the plasma membrane.

During assembly of the TCR complex, the individual components form partial complexes, a situation which can be mimicked experimentally when genes for the individual subunits are transfected in various combinations into heterologous cells. Such partial complexes are largely unstable and most are rapidly degraded within the cell before reaching the mid-Golgi, but they can be studied to reveal likely subunit interactions within the mature TCR complex. Thus, observed isolated pairwise combinations are CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$, but not CD3 $\gamma\delta$, which is interpreted as evidence against close contact between the CD3 γ and δ chains. Intracellular TCR complex assembly may be aided by the molecular chaperone proteins TRAP and calnexin, which are thought to mediate correct subunit interactions. Whether the TCR α and β chains dimerize before association with CD3 molecules may depend on which cells are examined, but in immature double-positive thymocytes this is thought not to occur. Instead, trimeric complexes, $\alpha\delta\epsilon$ and $\beta\gamma\epsilon$, form first which then combine via $\alpha\beta$ dimerization and finally associate with the ζ_2 dimer giving a stoichiometry of $\alpha\beta\gamma\delta\epsilon_2\zeta_2$. This is supported by transgenic mouse experiments which indicate one TCR α , one TCR β and two CD3 ϵ chains per complex. A model structure for the core receptor based on these data is shown in Figure 1.

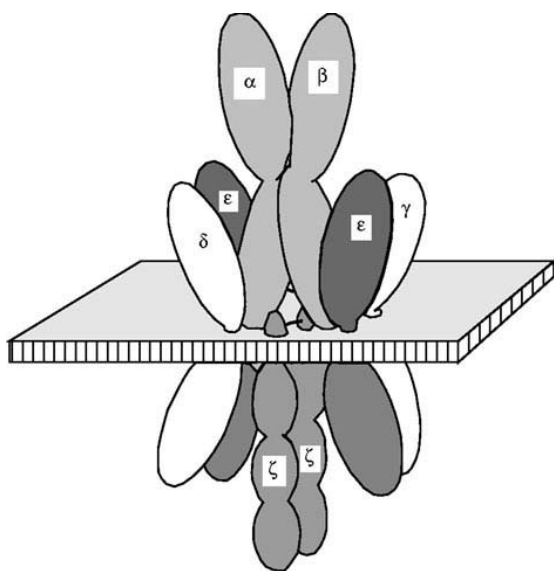


Figure 1 A model of the T cell antigen receptor ($\alpha\beta\gamma\delta\epsilon\zeta_2$ -type) showing an arrangement of subunits compatible with current data. A transverse section through the plasma membrane is shown. Disulfide bonds between α and β , and between ζ subunits, are shown as solid lines.

Function of TCR invariant subunits

A critical early finding was that monoclonal antibodies to CD3, most of which recognize the CD3 ϵ chain, were able to activate resting T cells in the presence of monocytes bearing appropriate Fc receptors. Antibodies directed to the $\alpha\beta$ heterodimer can behave similarly. Thus, a T cell bridged to an antigen-presenting cell via TCR/mAb/FcR reacts in a manner analogous to the physiological situation, where the link is TCR/Ag/MHC (major histocompatibility complex). For signaling through the TCR, therefore, some form of cross-linking or partial immobilization of the receptor is necessary, which need not be dependent on specific antigen recognition. This signaling process takes place through the invariant subunits of the TCR, since the heterodimeric antigen-binding chains themselves have very small cytoplasmic domains which are unlikely to have more than an anchoring function. In peripheral T cells, full activation also requires signaling through the costimulatory CD28 molecule by the B7-1/B7-2 antigen on B cells and macrophages. Signaling through the TCR alone results in the functionally unresponsive state known as anergy.

T lymphocyte activation involves multiple intracellular events, all leading to transition from the quiescent G_0 phase of the cell cycle into G_1 and induction of interleukin 2 (IL-2) secretion and IL-2 receptor expression. T cell growth, involving pro-

gression from G_1 to S phase and mitosis, is then controlled by IL-2 binding to the IL-2 receptor. At least two signaling pathways are thought to result from TCR occupancy, the first routing through the G protein p21^{ras}, and the second through phospholipase C. Both pathways act synergistically with CD28-derived signals to effect action of the transcription factor NF-AT1 (nuclear factor of activated T cells) on, for example, the IL-2 gene.

During antigen recognition by the TCR on a CD4⁺ helper T cell, the coreceptor molecule CD4 is recruited into the receptor complex, having associated with monomorphic sites on the MHC class II molecule of the antigen-presenting cell. The Src family protein tyrosine kinase p56^{lck} associates with CD4 and, in one model of the molecular consequences of antigen binding, p56^{lck} phosphorylates the CD3 and ζ chains on the tyrosine residues within their Reth activation domains. This promotes association of the TCR with the T cell-specific protein tyrosine kinase ZAP-70 through its SH2 domains, when ZAP-70 itself becomes phosphorylated and provides a signal to both p21^{ras} and phospholipase C pathways via intermediate protein species.

The structure of the TCR thus reflects its two major functions of antigen recognition and signal transduction, with the latter role being governed by the invariant chains. The CD3 proteins, together with the ζ chain, must interact with other cell surface molecules, including CD4 and CD8, and several intracellular proteins such as p56^{lck} and ZAP-70, as well as other tyrosine and serine/threonine kinases. It is now apparent that the complexity of the TCR is not unique, with other receptors governing cellular activation, such as the B cell antigen receptor (surface IgM), the Fc receptors and the IL-2 receptor, also having oligomeric structures commensurate with their complex function.

See also: CD4; CD8; CD28; CD45 (the leukocyte common antigen); Fc receptors; IgM; Interleukin 2; Interleukin 2 receptor; Protein kinases; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T cell receptor, recognition by; T lymphocyte activation; T lymphocyte differentiation.

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CD4

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The cell surface glycoprotein CD4 is expressed on a subpopulation of mature T lymphocytes, where its presence is tightly correlated with recognition of major histocompatibility complex (MHC) class II antigens. It is also present on various other cell types.

Structure

The CD4 molecule is an elongated (125 Å × 25–30 Å) cell surface glycoprotein that is conserved among various primates and rodents, e.g. 79% amino acid identity between mouse and human (Table 1). It is made up of four extracellular domains (D1–D4). The N-terminal, outermost domain (D1) consists of nine β strands disposed in the topology of variable immunoglobulin domains. Crystallography of the D1–D2 fragment has confirmed that the last strand of D1 runs directly into the first strand of D2, which brings both domains in close contact and confers rigidity to this part of the molecule (Figure 1). Crystallographic and proteolytic studies have shown that a short, hinge-like region bridges the D1–D2 fragment of CD4 to the D3–D4 part. The membrane proximal fragment, comprising the D3–D4 domains, is referred to as a connecting peptide. It is made up of two additional, degenerate, v-like domains that also make up a rigid structure. Recently, the four extracellular domains of the human CD4 molecule have been crystallized. This work has confirmed the presence of the hinge region

between D2 and D3. More interestingly, in all crystals obtained, the CD4 appears as a dimer (H. Wu and W. Hendryckson, personal communication). The highly basic cytoplasmic tail is the most conserved domain in the molecule. Three serine residues may serve as regulatory phosphorylation sites. The D1 domain is encoded by two exons, while D2–D4 are encoded by individual exons. The human gene is made up of nine exons and eight introns and spans 33 kb, while the murine gene includes ten exons covering 26 kb.

Expression

The full length 3.7 kb CD4 transcript, along with the expression of the protein on the cell surface, was identified initially in thymocytes and mature T lymphocytes in mice and humans. However, CD4 is also seen on human macrophages, Langerhans cells, neuronal and glial cells and some Epstein–Barr virus-transformed human B cell lines. CD4 is found as a monomer on the cell surface. The presence of a smaller 2.7 kb transcript has been described in the mouse forebrain, cortex and striatum. The predicted protein from this smaller mRNA would be a truncated form of mouse CD4 beginning at amino acid 214 (within the connecting peptide) and continuing through the normal cytoplasmic tail. This smaller transcript has also been seen in human brain tissue. The significance and function of this truncated CD4

Table 1 Comparison between human and murine CD4 genes and molecules

	MW (kDa)	Protein (AA)	Leader (AA)	Extracellular (AA)	Transmembrane (AA)	Cytoplasmic (AA)	Cysteines	Glycosylation (sites)	Chromosome	Gene size (kb)
Mouse	54	457	26	368	25	38	13	4	6	26
Human	55	458	25	372	23	38	12	2	12	33

AA, amino acids; MW, molecular weight.

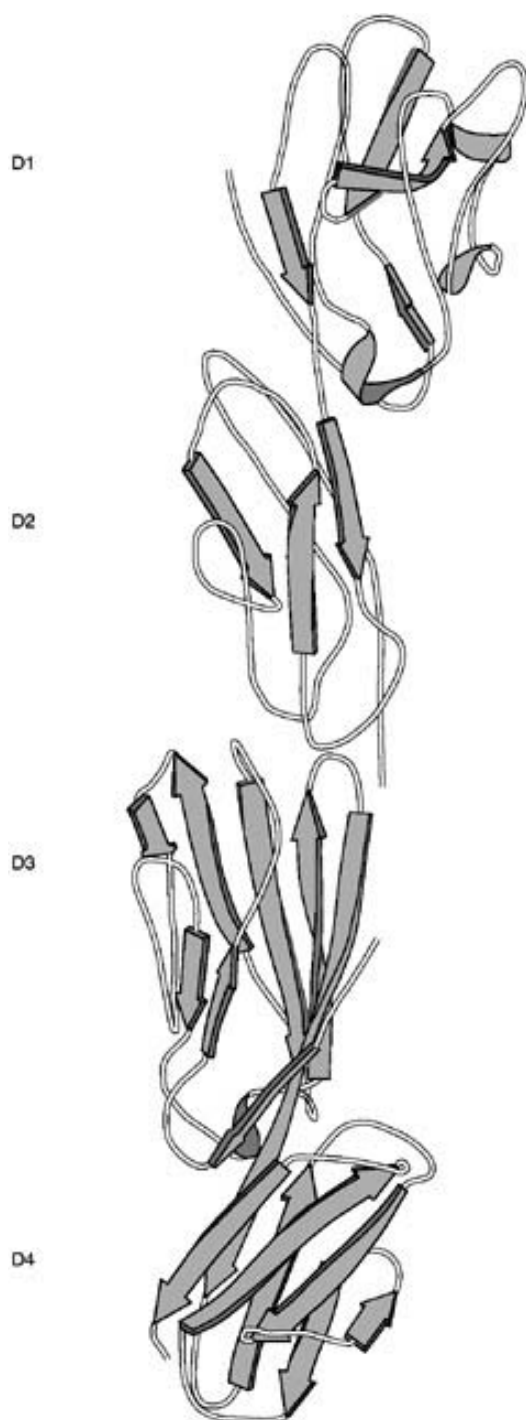


Figure 1 Crystal structure of CD4. The figure was generated using Molscript (Kraulis PJ (1991) *Journal of Crystallography* **24**: 946–950) according to X-ray diffraction coordinates published by Rye SE *et al* (1994) *Structure* **2**: 59–74 for D1 and D2, and by Brady RL *et al* (1993) *Science* **260**: 979–983 for D3 and D4. Domains 1 and 2 are from the human molecule while domains 3 and 4 are from the rat molecule. The hinge region is not shown.

mRNA are not known. Littman and coworkers have identified and characterized a T cell-specific enhancer located 13 kb upstream of the transcriptional initiation site of the mouse and human CD4 genes. They also have demonstrated the existence of an intronic regulatory region located between the first and second exons of the murine gene. The element is orientation and position independent. This negative regulatory region functions in combination with heterologous *cis*-acting regulatory elements, indicating that it is a developmentally regulated transcriptional silencer.

Interactions

Early studies suggested that CD4 might have signal transduction as well as ligand-binding capabilities. The advent of transgenic and knockout animals has confirmed and significantly extended these results, showing that the CD4 molecule is involved in a number of crucial steps during the lifespan of T cells. It is obvious that to play such a central role in the choices made by T cells, the CD4 molecule must interact with many other molecules.

MHC class II molecule

Doyle and Strominger provided the first strong evidence for physical binding between CD4 and class II MHC molecules. This was done with the use of transfected cells, which also indicated that this adhesion can take place in the absence of the T cell receptor (TCR). By using a class I (D^d) specific, CD4⁺ T cell hybridoma, Gay and coworkers investigated the role of CD4 in T cell activation. These experiments correlated the interaction of CD4 and class II MHC with an enhanced T cell activation. More recently, mutagenesis studies indicated CD4 affinity changes when residues 110, 137, 140, 141 and 142 of the solvent-exposed loop of the β 2 domain of class II were altered. Moreover, peptides with the sequence of the β 2 domain residues 134–148 (less so for 138–152) can bind CD4. From these studies and the fact that no natural polymorphic variant of MHC class II proteins have been identified that fail to bind CD4, it has been assumed that CD4 interacts with a relatively invariant part of the β 2 domain of class II. This idea has recently been challenged by Fleury and coworkers, who associated crystallographic and functional data obtained from class II molecules with varying affinities for CD4. They mapped polymorphic residues between positions β 180 and β 189 that can exert a dramatic influence on the interaction with CD4; this could contribute to the predominance of certain DR alleles or isotypes in antigen-restricted responses.

Moreover, König and coworkers have also added to our view of the interaction between CD4 and MHC class II by demonstrating that a second surface-exposed segment in the $\alpha 2$ domain of class II is also critical for the function of CD4. Both these reports are in accordance with the crystallographic data and suggest that in order for the CD4 molecule to contribute efficiently to signal transduction, it has to dimerize or oligomerize.

On the other hand, the class II contact residues on the CD4 remain controversial and will only be solved by cocrystallization of CD4 and class II. Different regions throughout D1, D2 and D3 domains have been suggested. A recent study making use of alanine-scan mutants showed that residues from both lateral faces of D1 and from one loop of D2 are involved in the interaction. This suggests an extended region of contact with the membrane-proximal lateral face of the class II molecule. These results are also consistent with an oligomerization event during the interaction.

The efficacy of interspecies interactions of human and murine CD4 with murine or human class II MHC molecules has also been a matter of debate. Sequence comparisons among human (hCD4), murine (mCD4) and rat CD4 show that residues that are implicated in the overall conformation are conserved, suggesting they will adopt similar three-dimensional structures. It was found that hCD4 and mCD4 can function to a comparable extent with at least five different class II MHC and four different mouse class II MHC molecules. Moreover, this work indicates that the structural features of this cross-species interaction are strongly conserved. These data confirm previous experiments which used transgenic animals that express various HLA class II proteins and showed that the interaction between HLA class II and mouse CD4 molecules is sufficient in provoking peptide-specific, HLA class II restricted T cell responses.

T cell receptor

Various approaches have led to the conclusion that the CD4 molecule can also physically associate with the TCR. The first evidence came from the observation that under certain circumstances, CD4-specific antibody can inhibit T cell responses. Other groups have shown that cocapping and comodulation of CD4 and TCR/CD3 at the cell surface can be induced with CD3-specific antibodies. Energy transfer experiments suggest that one TCR molecule associates with more than one CD4 in a single receptor complex. This association results in an enhanced avidity of the TCR complex for recognition of antigen-MHC class II complex and increased T cell

responsiveness. However, the molecular nature of the TCR-CD4 interaction remains poorly understood, although it is presumed to involve the membrane-proximal D3 and D4 parts of CD4.

p56^{lck}

The availability of monoclonal antibodies directed against CD4 and the TCR/CD3 complex provided strong evidence for a role of these molecules in the T cell antigen recognition and effector function. While the TCR itself does not have intrinsic protein tyrosine kinase (PTK) or phosphatase functions, an src-related PTK, Lck, is physically associated with a distinct sequence in the cytoplasmic domain of CD4. A region towards the amino-terminal end of p56^{lck} containing a stretch rich in negatively charged amino acids is believed to form ionic bonds with basic residues found within a conserved consensus sequence in the cytoplasmic domain of CD4. Moreover, p56^{lck} gains kinase activity when aggregated on the cell surface. p56^{lck} is anchored to the cytoplasmic face of the plasma membrane through a myristylated amino-terminal glycine. In lymph node cells, 75–95% of cellular Lck is associated with CD4. Conversely, 30–40% of membrane CD4 is bound to Lck. This kinase is activated by receptor cross-linking, which in turn induces phosphorylation of numerous targets. p56^{lck} serves to phosphorylate TCR ζ and possibly CD3 γ, δ, ϵ chains. By acting on TCR ζ , p56^{lck} initiates an activation cascade by enabling the receptor to recruit downstream molecules. Also, it has been proposed that CD4-associated p56^{lck} anchors CD4 and the stimulated TCR/CD3/ ζ complex to the same MHC molecule.

HIV-1 proteins

The CD4 molecule also plays an important functional role in the course of human immunodeficiency virus 1 (HIV-1) infection. Recent reports have demonstrated that the CD4 protein is not only the cellular receptor for HIV-1 but also participates actively in postbinding events important for infection and cell fusion. Mutagenesis and monoclonal antibody studies have shown that the region spanning amino acids 40–60 of the D1 domain of CD4 is the part of the molecule directly in contact with the viral gp120 for all isolates examined, with residues Phe43 and Arg59 being particularly important. However, the gp120-CD4 combining site is not universally conserved in its molecular details. A 15–100-fold range in affinities for CD4 have been reported. This reflects a complex situation that can depend on glycosylation, oligomerization of receptors, sequence variations and/or involvement of other cellular components, which ultimately contributes to viral tropism and

cytopathicity. It has also been shown that mutants in D3 can abrogate the binding of gp120. Moreover, Healey and colleagues reported that by blocking viral access to Gln425 and Gln428 they could block steps subsequent to the viral binding without affecting binding of the virus. It has been speculated that the hinge region in CD4 may be necessary to bring gp41 close to the target membrane. To test this hypothesis, a panel of point mutations and small deletions spanning the hinge region of CD4 were generated, transferred into a CD4⁺, HIV-1 susceptible T cell line, and their individual capacity to render this cell line infectable by HIV-1 assessed. Syncytia formation was also examined. This study clearly demonstrated that the hinge region of CD4 is necessary for HIV-1-induced cell fusion but not for infection. This confirmed the data from a number of groups that movement of this region is required for fusion. A region of the D1 domain of human CD4 has also been shown to participate in these events. This stems from the observation that although they share the same affinity for gp120 and susceptibility to HIV-1 infection, unlike their human homolog, the CD4 molecules from chimpanzee and rhesus monkey do not support syncytia formation. Complementary sequence exchange mapped the fusion determinant to Glu87 of the human CD4. At least three other HIV-1 proteins interact with the CD4 present at the cell surface and have been shown to be involved in its downmodulation. In HIV-1 infected cells, newly synthesized CD4 molecules are retained in the endoplasmic reticulum by a phenomenon termed receptor interference. This is the result of the formation of a complex between new CD4 and the viral gp160-env protein in the endoplasmic reticulum. The retained molecules of CD4 are degraded in this compartment by a mechanism induced by another HIV-1 protein, vpu. Another viral protein interacting with CD4 is nef. HIV-1-nef is a 206 amino acid, cytoplasmic protein that is nonessential for viral replication and associates with the plasma membrane through an N-terminal myristic acid. It is responsible for the loss of CD4 from the cell surface by inducing its endocytosis and ultimately resulting in its lysosomal degradation. This phenomenon was shown to occur early in the course of the infection, before gp160-induced receptor interference. It is mediated by a dileucine motif similar to endocytosis and lysosomal degradation signals, contained in the membrane-proximal 20, residues of the CD4 cytoplasmic tail.

Function

It is believed that the encounter between a TCR and its antigen involves a low-affinity interaction. T cells

have circumvented this problem by making use of coreceptors. In the case of a T cell that expresses a TCR recognizing antigenic peptides in the groove of a class II MHC molecule, this coreceptor function is fulfilled by CD4. If the TCR recognizes a class I MHC molecule, the coreceptor is CD8. By acting synergistically with the TCR, the coreceptors induce signal transduction events at low levels of receptor occupancy. T cells that go through maturation steps in the thymus are bipotential and can differentiate either along the CD4⁺ (helper) pathway or the CD8⁺ (cytotoxic) pathway.

Early work addressing the role of the CD4 coreceptor in thymocyte differentiation made use of monoclonal antibodies specifically directed towards CD4. Although most of these experiments were instrumental in describing the coreceptor and/or cosignaling functions of CD4, they are at odds with more contemporary data using mouse lines containing either disrupted genes or transgenes. Indeed, the latter models have generated good evidence that thymocyte commitment is a stochastic event. It follows that no distinct TCR-mediated signals are required in this choice. This is consistent with experiments in which a mutated molecule of CD4 (tailless), incapable of binding p56^{lck}, can rescue T helper development in a CD4^{-/-} mouse line. Therefore, in the context of thymic selection, the role of CD4 in the coreceptor-mediated TCR signaling is one of stabilization of the TCR-MHC interaction. In mice deficient for CD4 coreceptor expression, 90% of peripheral $\alpha\beta$ TCR⁺ T cells are CD8⁺. These CD4^{-/-} mice have been used to show that the human CD4 molecule can substitute for its murine counterpart. Interestingly, the requirement for CD4 in superantigen-mediated negative selection of specific V β seems to be differential and inversely proportional to the avidity of some V β for Mls. That is, Mls1^A-CD4^{-/-} animals, thymocytes expressing V β 6, V β 8.1 and V β 9 are deleted as in normal animals, while V β 7⁺ thymocytes are not, reflecting the low avidity of V β 7 for Mls1^A. These cells might require CD4 coreceptor-mediated stabilization of binding or signaling for clonal deletion. T helper activity and most class II-restricted T cell responses are also virtually absent in the periphery of these animals. For example, antibody titers are diminished 100-fold when these animals are immunized with T cell-dependent antigens. Moreover, these animals fail to mount an allo-response.

See also: CD3; CD8; CD45 (the leukocyte common antigen); Thymic epithelium: potential role in regulatory T cell tolerance; H2 class II; HLA class II; T cell

receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T lymphocyte activation; T lymphocyte differentiation.

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CD5

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CD5 was initially detected as an alloantigen called Ly-1 on murine T cells and together with the alloantigen Ly-2,3 (now CD8) they were the first 'markers' to be used to identify functional T cell subpopulations. Ly-1 identified a helper T cell population while Ly-2,3 was present on a cytotoxic-suppressor subset. With more sensitive analysis it was shown that the Ly-2,3⁺ T cells also expressed low levels of surface Ly-1 in their membranes, thus indicating that the majority, if not all, T cells expressed Ly-1. A few years later, a pan-T cell marker of similar molecular mass was identified on human lymphocytes by a variety of monoclonal antibodies and termed CD5. Subsequent studies showed that this molecule was homologous at the DNA level with Ly-1.

Structure of the molecule

CD5 is a type 1 transmembrane glycoprotein with a molecular mass of 67 kDa. It comprises a single polypeptide of around 470 amino acids as deduced from the nucleotide sequence. The first 25 amino

acids form the signal peptide. The external portion of the molecule contains 22 cysteines. The outer of the three external domains shows the greatest homology with the immunoglobulin supergene family of molecules with an intrachain disulfide bridge. All three outer domains show homology with a protein, Pap D, which is a chaperone protein mediating assembly of P pili of *E. coli*. In addition, there is strong homology of the extracellular domain(s) with the scavenger receptor on macrophages. CD5 has a transmembrane sequence of 29 residues and an intracytoplasmic tail of 92 amino acids with four phosphorylation sites – consistent with the function of this molecule being one of signal transduction (see later). Approximately 25% of the CD5 molecule is composed of N- and O-linked oligosaccharides.

Cellular distribution of CD5

CD5 is expressed on the surface of all T cells bearing the $\alpha\beta$ heterodimer T cell receptor (around

50 000 molecules per cell) but is absent or at lower density on the $\gamma\delta$ T cell subset.

It is present but at lower density (2–10 000 molecules per cell) on a minor population of murine and human B cells. More sensitive techniques are therefore required to detect CD5 on B than on T cells. CD5⁺ B cells, now termed B1 cells, represent a significant proportion of murine thymic and peritoneal B cells. They are the first B cells to colonize lymphoid tissues during early development in humans and are present at very high frequency in the blood of newborns, diminishing through childhood, adulthood and up to old age. Interestingly, all B cells in mature rabbits are CD5 positive.

CD5 has been found on many murine B cell lymphomas, some human B cell lymphomas and on the majority of human chronic lymphocytic leukemia (CLL) cells.

Other cells which have been shown to express CD5 are endothelial cells of blood vessels in the pregnant sheep uterus but the significance of this isolated example of nonlymphoid cell expression of CD5 is at present unknown.

Ligands for CD5

To date, three ligands for CD5 have been identified: 1) CD72 on B cells (mouse and human) is thought to act as a costimulatory molecule during cognate interaction with T cells (see later); 2) immunoglobulin V_H α 2 framework sequences in the rabbit bind to CD5; and 3) a molecule of around 32 kDa on activated murine spleen cells binds to CD5. The significance of the latter two ligands is at present unclear but might be involved in selection processes.

Role of CD5 on T cells

As indicated above, CD5 is found on thymocytes and the majority of peripheral T cells. Increased expression of CD5 by a number of protein kinase C activators has suggested that this molecule is important in the activation process of T cells (and probably B1 cells). CD5 is associated with the TCR ζ chain and the cytoplasmic domain of CD5 is phosphorylated on tyrosines on activation via the TCR. This is thought to bind SH2 domain-containing proteins. Studies on peripheral T cell activation *in vitro* have shown a costimulatory role for CD5 on TCR-mediated proliferative responses. Thus, cross-linking of CD5 on T cells by monoclonal antibodies results in a rise in intracellular Ca²⁺ derived from an extracellular source (via Ca²⁺ channels) and generation of

cyclic GMP. Furthermore, solid-phase immobilization of CD5 antibodies alone provides a stimulus for interleukin 2 (IL-2) receptor induction and responsiveness to IL-2. It is thought that, along with other costimulatory interactions of molecules on T and B cells, CD5 on T cells binds to CD72 on B cells which enhances T cell and B cell activation. In contrast, in CD5 knockout mice, unlike peripheral T cells, thymocytes are hyper-responsive to stimulation through the TCR. In addition, selection of T cells expressing transgenic TCRs were abnormal in these mice indicating that CD5 plays a role as a negative regulator of TCR mediated activation during T cell development.

B cells and CD5

Unlike T cells, CD5 is present on only some B cells and there is now strong evidence that this is a marker which identifies a normal B cell subpopulation (B1 cells), the size of which is genetically regulated. This population is different in origin, and can be distinguished from, conventional B cells responsible for most of the antibodies specific for exogenous antigens. In mice, B1 cells are self-renewing.

Both murine and human studies have indicated that B1 cells have minimal mutation of their rearranged immunoglobulin genes. They are responsible for the production of polyreactive and autoreactive antibodies and give rise to the so-called 'natural antibodies' present in normal serum. In this regard, elevated levels of CD5⁺ B cells have been described in a plethora of autoimmune diseases in man including rheumatoid arthritis, primary Sjögren's syndrome, systemic lupus erythematosus, Graves' disease, diabetes and many more. The significance of this is at present unclear. The presence of CD5 on human CLL cells and the polyreactive nature of antibodies programmed by these cells indicates that these malignant B cell tumors, are derived from this particular B cell subpopulation.

It is likely that normal B1 cells can act as 'antigen-presenting cells', using their polyreactive receptors to take in antigens for processing and presentation with surface major histocompatibility complex (MHC) class II molecules.

Identification of CD5 as a lineage marker has recently been challenged in experiments with murine and human cells. Expression of CD5 can be induced on murine splenic B cells (mostly B2 cells) following activation. In humans, B cells activated by phorbol ester treatment express CD5, and IL-4 has been shown to downregulate this molecule on the surface of these cells.

Taking these data together, it is possible that classical B1 cells prominent in early life are antigen-presenting cells and are a separate lineage representing germline encoded antibodies, many of which are activated through recognition of self antigen *in vivo*. Other B cells (B2 cells) appear to be capable of being induced to express CD5. The function of CD5 on these two B cell types may be different. The function of CD5 molecules on B cells has recently been addressed and there is now substantial evidence for its role in regulating B cell activity. CD5 is part of the B cell receptor complex as shown by capping and co-capping studies. Cross-linking of CD5 on B cells by monoclonal antibodies has a profound effect on stimulation of B cells through the antigen-specific immunoglobulin M (IgM) receptor. In mice, stimulation of peritoneal B1 cells with anti-IgM results in apoptosis. This is inhibited in CD5-deficient (CD5 knockout) mice. Cross-linking of CD5 in normal wild-type mice rescues the cells from anti-IgM-induced apoptosis and leads to proliferation of these cells. A similar effect is seen with human tonsillar CD5⁺ B cells in the presence of IL-2. Direct stimulation of CD5 on human tonsillar B cells alone can also stimulate apoptosis but different biochemical pathways appear to be used compared with induction of apoptosis via anti-IgM. The physiological relevance of these latter observations is currently unknown.

Recent data have suggested that in mice, one of the signal transduction activators of transcription (STAT3) is constitutively activated in B1 cells but not in unmanipulated B2 cells. This may play a role in B cell antigen-specific signaling and its constitutive activation, associated with the intrinsic proliferative nature of this population.

CD5 as a target for therapy

Since CD5 is a pan-T cell marker it is used as a target for depletion of normal T cells in the clinical setting for transplantation and human therapy. It has an advantage over anti-CD3 treatment since it does not result in activation of cytokine production which gives rise to transient 'flu-like' symptoms after injection. Monoclonal antibodies coupled to ricin (immunotoxin) are used in the treatment, *ex vivo*, of allogeneic bone marrow for prevention of graft-versus-host disease following ablation therapy for transplantation. These antibodies have also been used successfully with additional therapy in the treatment of CLL tumors.

See also: Autoimmune diseases; Autoimmunity; B lymphocyte repertoire; B lymphocytes; IgM; Leuke-

mia; Natural antibodies; Neonatal immune response; Nude (athymic) mice.

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CD8

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Definition of T cell subsets

CD8 represents the first cell surface glycoprotein used to define T cell subsets. Studies into the cellular basis of the immune response had shown that T lymphocytes mediated a wide variety of immunological functions. These cells generated cytotoxic responses to alloantigens, exerted helper and suppressor effects on the production of antibodies by B cells and initiated both delayed-type inflammatory responses and graft-versus-host responses. These findings could be explained by one of two alternative hypotheses. A single mature T lymphocyte, after activation by antigen, could give rise to cells capable of mediating the complete range of T cell responses. Alternatively, each T cell function might be mediated by a subset of T cells programmed during differentiation to express a limited range of immunological functions.

A direct approach to this question made use of alloantibodies that defined cell surface differentiation antigens, called Ly antigens, found on mouse thymocytes and a fraction of peripheral lymphocytes. Since these alloantigens had not yet been detected on the surface of nonlymphoid cells, it was hoped that they might represent components expressed exclusively on the surface of lymphocytes undergoing thymus-dependent differentiation. Lyt-2, since renamed CD8, and Lyt-3 represent cell surface antigens encoded by two closely linked genes. Lyt-2⁺3⁺ cells accounted for approximately half of peripheral T cells and included those cells required for generation of cytotoxic activity and excluded T cells that mediated helper activity. Additional studies of the major histocompatibility complex (MHC) specificity of CD8⁺ and CD8[−] subclasses of T cells revealed that thymus-dependent differentiation gave rise to at least two separate sublines of mature T cells. The CD8[−] subset was programmed to amplify a number of immune responses and was preferentially activated by class II MHC products in association with

antigenic peptides. The CD8⁺ subset was programmed for the development of cytotoxic T lymphocyte (CTL) activity and responded preferentially to class I MHC products in association with antigenic peptides. These studies showed that thymus-dependent differentiation resulted in two lineages of T cells, CD8⁺ and CD8[−], equipped to recognize and respond to products of the MHC that were foreign by virtue of polymorphic variation (alloantigen) or because they had been modified by association with nonself peptides. Selective engagement of either screening system by foreign or altered class I or class II MHC region products activated a series of immune reactions already programmed in one or another activated T cell subset.

Structure of CD8

CD8 exists as a disulfide-linked dimer of either α and β chain or two α chains. The crystal structure of the N-terminal 113 amino acids of human CD8 revealed homology to immunoglobulin (Ig) variable domains with nine β strands divided into two β sheets, one of four and the other of five strands. An Ig-like intersheet disulfide bridge from the B strand to the F strand is present in CD8 α . Another disulfide bond may be formed between cysteines in the B strand and the C strand. The hinge region connecting the amino-terminal Ig-like domain to the transmembrane segment consists of 50 residues in the α chain and 30 residues in the β chain, both of which are likely to be glycosylated and sialylated. The level of sialylation on the β chain hinge region varies with the activation state of the T cell and may have functional implications for CD8 (Figure 1).

Class I MHC recognition

It has become apparent that the CD8 molecule directly contributes to the development of cytotoxic activity by CD8⁺ cells. Antibodies to CD8 block the

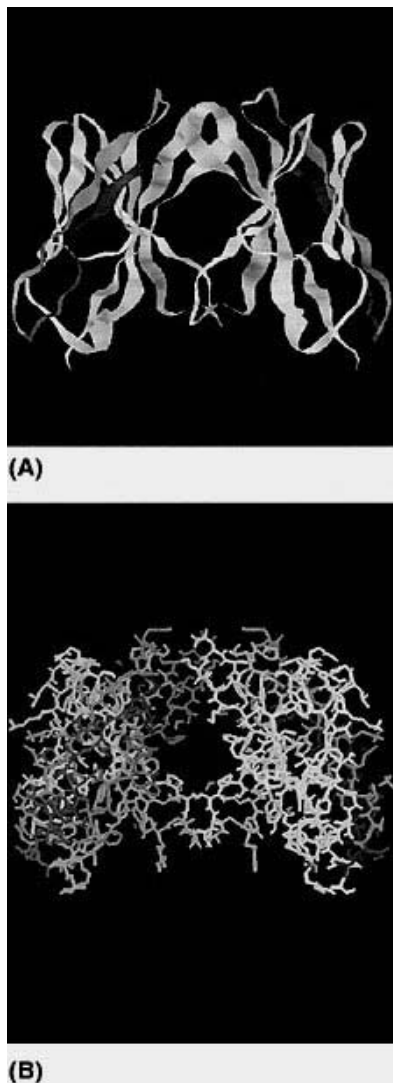


Figure 1 Three-dimensional molecular model of the N-terminal region of a CD8 homodimer consisting of two α chains; (A) ribbon presentation, (B) stick presentation. The structural data for the N-terminal 113 amino acids are based on crystallography (protein data base: 1CD8). The three-dimensional model for each monomer was generated with RasMol version 2.5 (Roger Sayle, Greenford, Middlesex, UK), and the two monomers were combined as described (Leahy *et al.* (1992) *Cell* 68: 1145). The color coding represents groups; blue corresponds to the CDR1-like loop, light blue to the CDR2-like loop, and lime to the CDR3-like loop. The monomers are distinguished in brightness of color. (See also color Plate 10.)

generation and effector phases of the CTL response specific for class I MHC antigens without regard to function. Functional and cell-binding studies have shown that the ligands for the CD8 receptor are encoded by MHC class I genes. A role for CD8 in the interaction with class I molecules was demonstrated from the ability of CHO cells transfected with the

CD8 gene to form conjugates with class I-positive but not class I-negative cells. Similar to the interaction between CD4 and MHC class II, CD8 binds with weak affinity to MHC class I and contributes to cell adhesion. CDR-like loops (homologous to the complementarity-determining region 2 loop of antibodies) in the N-terminal end of CD8 α are the most likely binding site for MHC class I as judged by mutational analyses, crystal structure and orientation in the cell membrane. In addition, an A/B strand surface may interact with a region on the $\alpha 2$ domain of MHC class I. The primary binding site on MHC class I is the $\alpha 3$ loop, a nonpolymorphic region in the membrane proximal domain and distant to the antigen-binding cleft. Furthermore, the structures of CD8 and MHC class I make it possible that one CD8 dimer could simultaneously bind two MHC class I molecules.

Biochemistry and genetics

Lyt-2 and Lyt-3 (murine CD8) are both transmembrane glycoproteins which are encoded by two closely linked genes on murine chromosome 6. Except for a subset of natural killer cells, the expression of murine Lyt-2/3 appears to be restricted to T cells. The molecule is a 70 kDa homo- or heterodimer composed of two covalently linked chains: an α chain (32–38 kDa), which expresses epitopes detected by Lyt-2-specific antibodies, and a β chain (30–34 kDa), which expresses Lyt-3 epitopes. The Lyt-2 chain is the homolog of the CD8 chain in humans, which consists of homodimers and multimers of this chain. Recently, human CD8 β genes have been identified that encode a CD8 monomer corresponding to murine Lyt-3.

The Lyt-2 gene contains five exons. The first encodes a signal peptide and an N-terminal region which shows close homology with IgV κ light chain regions, including a cysteine-mediated intrachain disulfide bond. Exons 2–5 encode the spacer region, the transmembrane region and two cytoplasmic regions, C1 and C2, respectively. Although there is only a single Lyt-2 gene segment there are two types of Lyt-2 chains, α and α' , of slightly different size. The 34 kDa α' chain, found only in mice and expressed preferentially in the thymus, is a truncated form of the α chain resulting from alternative splicing of exons encoding the C-terminal cytoplasmic domain including the p56^{lck} binding site.

There is uneven homology between human and murine CD8 molecules. Human CD8 α and β chains are encoded on chromosome 2p12. The most substantial degree of genetic conservation is found in the transmembrane and cytoplasmic domains, while the

least is in the external V gene-like domain. Since there is 70% identity of the class I MHC external domain residues between mouse and human, this degree of divergence in the putative binding regions of CD8 is somewhat surprising. On the other hand, conservation of the C-terminal domain may reflect a requirement for this region to interact with intracellular T cell proteins involved in transmembrane signaling. A comparison of rodent CD8 chains with the α and β chains of the T cell receptor (TCR) suggests evolution of these three receptor genes from a common ancestor. Divergence between them may be reflected in the acquisition of intervening sequences between V and J by the TCR genes, resulting eventually in the capacity to rearrange multiple V genes during development. The CD8 gene has lost regions encoding C-region domains but, like TCR, has acquired the capacity to recognize MHC molecules at some point in evolution (Figure 2).

T cell activation

Studies have shown that CD8 may function as a coreceptor with the α/β TCR in recognition of adjacent epitopes on the same class I molecule. These findings support the concept that T cell activation involves formation of a ternary complex composed of CD8 and TCR on the T cell and class I MHC molecules on the target cell. Indeed, ligation of CD8 with the TCR complex elicits much more vigorous T cell responses than ligation of either receptor molecule alone, indicating that CD8 plays a decisive role in augmenting TCR-dependent activation, either by increasing the avidity of binding to MHC class I or

by amplification of signal transduction. High-avidity interaction between T cells and antigen-presenting cells is heavily dependent on CD8 expression, which is more important for primary proliferative responses than for CTL activity. While stimulation of T cells by anti-TCR antibodies results in coordinate localization of TCR and CD8, it is not yet clear whether the TCR α/β chain directly contacts the CD8 molecule in the lipid bilayer of a cell membrane. However, the ability of the two cell surface molecules to interact with distinct but adjacent regions of class I MHC products places CD8 and the TCR in close proximity to one another.

A potential molecular basis for the role of CD8 in T cell activation comes from studies showing that CD8 is closely associated with the tyrosine kinase p56^{lck}, an src-related tyrosine kinase expressed mainly in T lymphocytes. p56^{lck} is a cytoplasmic protein that, unlike other growth factor receptors, is found on the inner leaflet of the plasma membrane where it associates with a highly conserved cytoplasmic domain of CD8. Site-directed mutagenesis of CD8 defines a motif of CD8 α which may bind to the N-terminal region of p56^{lck}: ++-x-cys-x-cys-(pro) where x denotes a nonconserved residue and + a basic residue. It is currently thought that engagement of CD8 leads to enhanced activity of the associated p56^{lck} enzyme which phosphorylates the ζ chain of the CD3 complex leading to signal transduction. In mature T cells, CD4 and CD8 may associate with equal amounts of p56^{lck}; however, kinase activity after cross-linking of CD8 is much lower than after cross-linking of CD4. On the other hand, protein kinase C appears to be activated much more strongly after ligation of CD8 than after ligation of CD4. The cytoplasmic segment of CD8 α also has two serine residues which are likely to be phosphorylated in signaling. T cells with targeted mutations of the cytoplasmic serine residues are insignificantly impaired in their response to antigen, whereas cells with substitutions in both cytoplasmic cysteine residues display deficient responses. Surprisingly, transgenic mice whose CD8 lacks the p56^{lck} binding site show no impairment of T cell function.

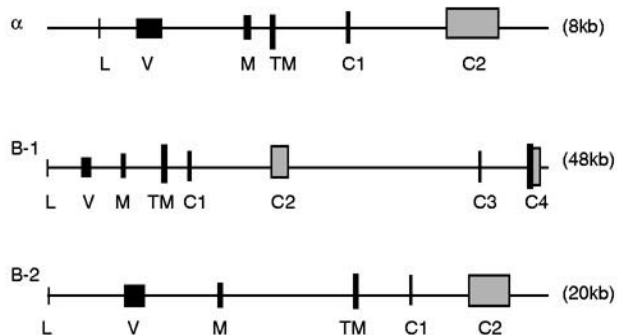


Figure 2 Exon-intron organization of the human CD8 genes. The CD8 α gene is comprised of six exons which span about 8 kb. There are two genes encoding CD8 β , CD8B-1 contains eight exons and is spread over approximately 48 kb, whereas the CD8B-2 gene consists of six exons and has a length of 20 kb. L = leader peptide, V = IgV-like sequence, M = membrane proximal region, TM = transmembrane domain, C = cytoplasmic domains.

T cell selection

The ability to trace T cell clones according to their TCR α/β chain expression has led to the demonstration of MHC-dependent negative and positive selection in the thymus. These studies also provide strong evidence for the involvement of the CD8 molecule in class I-mediated positive selection. Although the precise role of CD8 in this process is not known, there is good evidence that the generation of single-

positive CD8 cells from double-positive ($CD4^+8^+$) precursors in the thymus is associated with class I MHC recognition. Possibly, coordinate ligation of TCR and CD8 with class I MHC at the $CD4^+8^+$ precursor stage induces the cell to shut off CD4 expression. Alternatively, CD4 or CD8 might be eliminated on a random basis at the double-positive stage, allowing the formation of single-positive CD8 cells or CD4 cells. The former $CD8^+4^-$ population would be equipped to be selected by class I MHC, while the latter ($CD8^-4^+$) cells would display a TCR specific for class II and would die in the thymus. CD8 may also contribute to negative selection, as highly elevated levels of $CD8\alpha$ block thymocyte maturation. Immature $CD4^+CD8^+$ thymocytes express CD8 at levels fourfold higher than CD4; nevertheless, activation of $p56^{lck}$ is mostly seen after ligation of CD4 but not CD8, as a minority of surface CD8 molecules are associated with $p56^{lck}$. It has been shown that negative selection does not require increased CD8-dependent signaling through $p56^{lck}$. A critical question that remains to be answered involves the role of CD8 in the differentiation of cytotoxic cells. Possibly, a signal mediated by CD8 results in the differentiation of a thymic precursor cell to a prekiller and killer cell. An alternative explanation is that CD8 represents part of the mature cytolytic cell program. In either case, a critical feature of CD8 expression is restriction of cytolytic responses to T cells that recognize class I but not class II MHC products. This feature of the CD8 genetic program may prevent cytolytic cells from reacting to and eliminating class II-bearing B cells and macrophages that display relevant peptide antigens.

Analysis of CD8 by gene-targeting techniques

Models with manipulated CD8 genes have become available. Disruption of the $CD8\alpha$ gene prevents expression of CD8 protein on the cell surface and impedes generation of cells which express receptors specific for MHC class I. Disruption of the $CD8\beta$ gene, or removal of its cytoplasmic tail, has a less dramatic effect on MHC class I restricted cells. Mutant mice contain 20–30% of the normal number

of CD8 cells and those may have a restricted set of TCRs. These results are similar to the effects of eliminating the CD8 ligand: a deficiency of MHC class I gene products also prevents maturation of $CD4^+8^+$ cells. Introduction of a CD8 transgene with a mutated binding site for $p56^{lck}$ into $CD8\alpha^{-/-}$ mice leads to reduction of mature CD8 cells by half but no apparent restrictions of the TCR repertoire. In contrast, introduction of a tailless $CD8\alpha$ transgene into $CD8^{-/-}$ mice leads to a more dramatic reduction in CD8 cell numbers, reduced capacity to mount cytotoxic T lymphocyte responses, and restrictions in the TCR repertoire.

See also: CD4; Cytotoxic T lymphocytes; H2 class I; HLA class I; T lymphocyte activation; T lymphocyte differentiation; T lymphocytes.

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CD22

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CD22 expression and cDNA cloning

CD22 is a B cell lineage, restricted cell surface phosphoglycoprotein of 130–150 kDa which demonstrates a unique developmental pattern of expression closely paralleling that of immunoglobulin D (IgD). Cytoplasmic expression of CD22 can be detected in pro-B and pre-B cells, whereas surface expression is first identified in immature B cells. B cell activation results in a transient increase in surface CD22 expression, which is followed by progressive down-regulation as activated B cells differentiate into plasma cells.

Two cDNA clones encoding human CD22 have been isolated and shown to predict an integral membrane polypeptide and a member of the Ig superfamily. The smaller isoform, CD22 α , possesses an extracellular domain composed of five Ig-like subdomains, while the larger isoform, CD22 β , is identical to CD22 α with the exception of two additional Ig-like extracellular subdomains (subdomains 3 and 4) positioned between subdomains 2 and 3 of the smaller isoform. The predominant isoform of human CD22 expressed in B cells is CD22 β and is henceforth called CD22 unless otherwise specified. A murine CD22 cDNA clone has been isolated and displays 62% amino acid identity to the human form, with the closest relatedness being located between the seventh Ig domain and the cytoplasmic tail, and the greatest divergence observed within Ig domains 1 and 2. The extracellular domains of CD22 are highly related to several known adhesion molecules, which include myelin-associated glycoprotein (MAG), Schwann cell myelin protein (SMP), CD33, sialoadhesin, neural cell adhesion molecule (NCAM), and the vascular adhesion molecule VCAM/InCAM110. Interestingly, the first Ig-like domain of CD22, like CD33, MAG, SMP and sialoadhesin, is a V-set domain which contains an unusual intradomain disulfide bond between the predicted B and E β strands rather than the B and F β strands ordinarily found in immunoglobulins.

B cell adhesion molecule CD22 is a sialic acid-binding lectin

COS cells expressing both human CD22 isoforms promote sialic acid-dependent leukocyte rosetting,

but transfectants expressing CD22 β promote leukocyte rosetting more strongly than COS cells expressing CD22 α . The sialic acid binding domain of CD22 has been demonstrated to be situated on the GFCC'C' β sheet of the V-set Ig domain centered on an arginine residue in the F strand. Furthermore, the second N-linked glycosylation site within the V-set Ig domain of CD22 has been demonstrated to play an important role in the regulation of CD22-mediated adhesion. While the binding domain of CD22 is localized to the first Ig-like domain, the second and third Ig-like domains as well as the second N-linked glycosylation site, play an important role in generating the optimal binding conformation.

Several studies using a soluble recombinant CD22-Ig fusion protein, CD22Rg, have shown that CD22 binds a variety of cell surface and soluble sialoglycoproteins and that the observed adhesion is dependent upon the presence of ligand-associated N-linked oligosaccharides containing α 2,6-linked sialic acid. The role of sialic acid in CD22 recognition is further supported by the observation that mild periodate oxidation of B and T cells, which selectively removes the C-7–C-9 exocyclic side-chain of sialic acid, results in the abrogation of CD22-mediated binding. The minimal oligosaccharide motif specifically recognized by CD22 is Neu5Ac α 2–6Gal β 1–4Glc(NAc). The apparent affinity of this interaction is low ($K_d \sim 30 \mu\text{M}$), suggesting that in common with most lectins the functional avidity of CD22 may be attained through multivalent binding. Further detailed analysis of N-linked oligosaccharides isolated from CD22 glycoprotein ligands has demonstrated that high-affinity recognition by CD22 is determined by the number of sialic acid residues associated with a glycoprotein ligand and by appropriate sialic acid presentation by the underlying ligand polypeptide backbone. These observations demonstrate that although CD22 is structurally related to the Ig superfamily of adhesion molecules, it has the functional properties of a sialic acid-binding lectin. The structurally related molecules CD33, MAG, SMP and sialoadhesin have been demonstrated to mediate α 2,3-linked sialic acid-dependent adhesion and, together with CD22, constitute a family of mammalian lectins, designated I-type lectins.

Regulation of CD22-mediated adhesion

CD22-expressing COS cells adhere to multiple subsets of leukocytes *in vitro*, yet CD22-bearing B cells do not demonstrate appreciable cell-cell adhesion *in vivo*. This suggests that B cells which constitutively express CD22 might possess regulatory mechanisms to avert undesired CD22-mediated aggregation. Interestingly, CD22 expressed in B cells possesses N-linked oligosaccharides that are sialylated exclusively in an α 2,6 linkage. Since α 2,6-linked sialic acid provides a natural CD22 ligand, this observation raises the possibility that α 2,6-linked sialic acid on CD22 itself might play a role in regulating CD22-ligand interactions. Recent studies have shown that α 2,6-specific sialylation of CD22 itself abrogates CD22-mediated adhesion. Additional studies *in vivo* have demonstrated that regulation of CD22-mediated adhesion may also occur at the level of the glycoprotein ligands. More specifically, 9-O-acetylation of the sialic acid side-chain of splenic, thymic and lymph node lymphocytes and macrophages has been demonstrated to negatively regulate CD22-mediated adhesion. Thus, regulation of CD22-mediated adhesion occurs at the level of reciprocal α 2,6 sialylation and sialic acid side-chain modification of both receptor and ligand. CD22 is the first known example of an Ig superfamily member whose function is under the control of two specific glycosyltransferases, α 2,6 sialyltransferase and O-acetyltransferase.

Role of CD22 in lymphocyte signal transduction

Multiple T and B cell surface glycoproteins have been immunoprecipitated using a soluble recombinant CD22-Ig fusion protein, CD22Rg. Several of these putative T and B cell ligands appear to have an identical molecular mass, and may represent the same polypeptides expressed on both T and B cells. The first of the CD22 T cell ligands to be identified was CD45. Subsequent *in vitro* experiments demonstrated that cross-linking of CD3 and CD22 T cell ligands with anti-CD3 monoclonal antibody (mAb) and CD22Rg on the surface of the T cell line CEM results in the inhibition of calcium flux and tyrosine phosphorylation of phospholipase C γ 1 (PLC γ 1) generated by cross-linking CD3 alone. However, the presence of multiple unknown CD22-binding glycoproteins on the surface of T cells, in addition to CD45, precluded the conclusion that the observed modulation of early T cell activation events was due exclusively to CD22-CD45 interactions. Through the use of several Jurkat cell lines, including a CD45-

deficient variant which was reconstituted with a recombinant fusion protein composed of the intracellular domain of CD45 and the extracellular and transmembrane domains of major histocompatibility complex (MHC) class I molecule HLA-A2, it was demonstrated that a direct physical interaction between CD22 and CD45 resulted in modulation of early signal transduction events. These experiments suggest that the interaction of CD22 on the surface of B cells with CD45 on adjacent T cells may play a role in regulating an immune response during T cell-B cell interactions.

The pattern of CD22 cell surface expression, coinciding with the acquisition of B cell responsiveness to antigen, suggests that CD22 may also play a role in early B cell activation events. Two early studies supported this notion. First, cross-linking of surface CD22 with anti-CD22 mAbs resulted in augmentation of anti-IgM-induced B cell activation signals. Second, anti-Ig responsiveness was demonstrated to be restricted to B cells which express surface CD22. Subsequent work has further supported the notion that CD22 plays a role in B cell signal transduction. CD22 has been demonstrated to be associated with surface Ig (sIg) and to be rapidly phosphorylated upon cross-linking of sIg. Furthermore, these phosphorylated intracellular tyrosine residues have been demonstrated to be arranged in one or perhaps two intracellular tyrosine-based activation motifs (ITAMs), one of which associates with p72^{syk}, p53/56^{lyn} and phosphatidylinositol 3-kinase during B cell activation. Most recently, intracellular tyrosine phosphorylation of CD22 has been shown to bind and activate protein tyrosine phosphatase 1-C (PTP1-C), which negatively regulates signaling through sIg. This suggests that ligation of sIg induces tyrosine phosphorylation of CD22, which in turn localizes and activates PTP1-C at the intracellular site of the signaling complex and thereby results in an inhibitory feedback effect on the activation of B cells. Additional experiments have shown that engagement and sequestration of CD22 from sIg with anti-CD22 mAb-coated protein A-sepharose lowered the threshold of B cell activation in a phosphorylation-dependent manner. Thus, overall these studies suggest that the absence or presence of CD22 in the sIg complex may serve to upregulate or downregulate B cell activation, respectively.

The functional relationship between the extracellular and the intracellular domains of CD22 is relatively unexplored; however, at least one line of evidence suggests that engagement of the extracellular domain of CD22 may, in turn, influence the function of the intracellular domain. Previous studies with the CD22 mAbs HD6 and HD39 have shown

that these antibodies could costimulate B cell proliferation with anti-IgM, but they were unable to directly stimulate B cell proliferation. Engagement of CD22 with the anti-CD22 mAb, HB22.23, which blocks the binding of CD22 to its ligands, results in rapid phosphorylation of CD22, increased association with Src and non-Src family kinases, and proliferation of B cells. These results taken together suggest that engagement of different epitopes of the extracellular domain of CD22 has different functional sequelae. It is rather interesting to note that the anti-CD22 mAb which could stimulate B cells directly was also an antibody which blocks the interaction of CD22 with its ligands; this raises the possibility that this particular mAb may recognize the same structural element which is required for CD22-mediated adhesion. If this is in fact the case, then engagement of CD22 by one of its ligands may result in direct modulation of B cell signal transduction events independent of sIg.

See also: B lymphocyte activation; B lymphocyte differentiation; CD antigens; CD45 (the leukocyte common antigen); Lectins.

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CD28

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CD28 is a receptor on T cells that is activated by engagement with ligands CD80 (B7-1) and CD86 (B7-2) that are expressed on the surface of activated antigen-presenting cells (APCs). CD28 delivers signals to the T cell that increase expression of the high-affinity interleukin 2 (IL-2) receptor (CD25) and enhance the synthesis of multiple cytokines, including IL-2. Thus CD28 regulates T cell responsiveness to antigen during T cell contact with APCs. CD28 is referred to as a costimulatory receptor because the signals it provides are synergistic with signals provided by the T cell antigen receptor (TCR/CD3) in driving T cell activation and proliferation. In the absence of a signal from TCR/CD3, signals from CD28 can stimulate only minimal proliferation of T cells, and may result in T cell unresponsiveness. Although CD28 and TCR/CD3 work together, CD28 signals are at least partly independent of TCR/CD3, as, unlike TCR/CD3, CD28 signals are resistant to inhibition by cyclosporine.

CD28 Structure and expression

CD28 was first detected and characterized with a monoclonal antibody (mAb) (9.3) made by Paul Martin and John Hansen at the Fred Hutchinson Cancer Research Center in 1981. Studies with this mAb showed that CD28 is a differentiation antigen expressed on thymocytes and most mature T cells, including all CD4 T cells and CD8 T cells with cytolytic activity. A subpopulation of CD8 T cells with suppressor activity are CD28 negative. The CD28 molecule was shown to be a homodimeric glycoprotein composed of a disulfide-bonded 44 kDa subunit. The cloning and expression of a CD28 cDNA by Aruffo and Seed showed that each CD28 monomer contains 134 extracellular amino acids with a single transmembrane domain and a short cytoplasmic tail. The extracellular domain of CD28 shows homology with immunoglobulin variable region domains. The CD28 gene contains four exons, with each exon defining a functional domain of the protein. CD28 shares amino acid sequence homology with CTLA-4, a receptor expressed by activated T cells. CD28 and CTLA-4 genes are closely linked on human chromosome 2, bands q33-q34 (Figure 1).

CD28 Receptor function

The potent costimulatory activity of anti-CD28 mAbs was first recognized by Gmunder and Lesslauer and was later confirmed and studied by many others. Identification of CD80 and CD86 molecules as CD28 ligands has allowed a comparison of CD28 signaling through anti-CD28 mAbs versus the natural ligands. These studies show that CD28 clustering on the cell surface during cell-cell adhesion results in optimal stimulation, whereas soluble CD80-Ig or CD86-Ig fusion proteins are not effective. CD28 mAbs have activity in solution when they are bivalent or multivalent, but are less effective than CD80- or CD86-positive cells in activating the CD28 receptor. Optimal stimulation of the CD28 receptor therefore requires a high degree of receptor cross-linking or oligomerization.

CD28 signals depend on the activation of intracellular protein tyrosine kinases, including *lck* and *itk*, resulting in direct phosphorylation of CD28 on tyrosine residues. After activation, CD28 associates with phosphatidylinositol 3-kinase (PI3K) by an SH₂ interaction with phosphorylated Tyr170. However, the association with PI3K is not essential for CD28 costimulatory function, and Tyr188 plays a more important role. However, it is not yet clear how the CD28 cytoplasmic tail delivers costimulatory signals.

CD28-deficient mice have been generated and found to have an impaired immune system, with reduced responses to T cell mitogens and reduced ability to mount antibody responses to T-dependent antigens. In contrast, CTLA-4 deficient mice die at an early age of lymphoproliferative disease, demonstrating that CTLA-4 delivers inhibitory signals for T cell activation. CTLA-4 has been reported to associate with a tyrosine phosphatase, SYP, that is thought to inhibit the activation of tyrosine kinases or tyrosine kinase substrates during CD28 cross-linking.

CTLA4-Ig

Soluble CD28-Ig and CTLA-4-Ig fusion proteins have been expressed and found to bind their shared ligands with different affinities. CTLA-4-Ig binds more than 100-fold better than CD28-Ig to both CD80 and CD86, and CTLA-4-Ig inhibits the ability of CD80 or CD86 to stimulate CD28 responses.

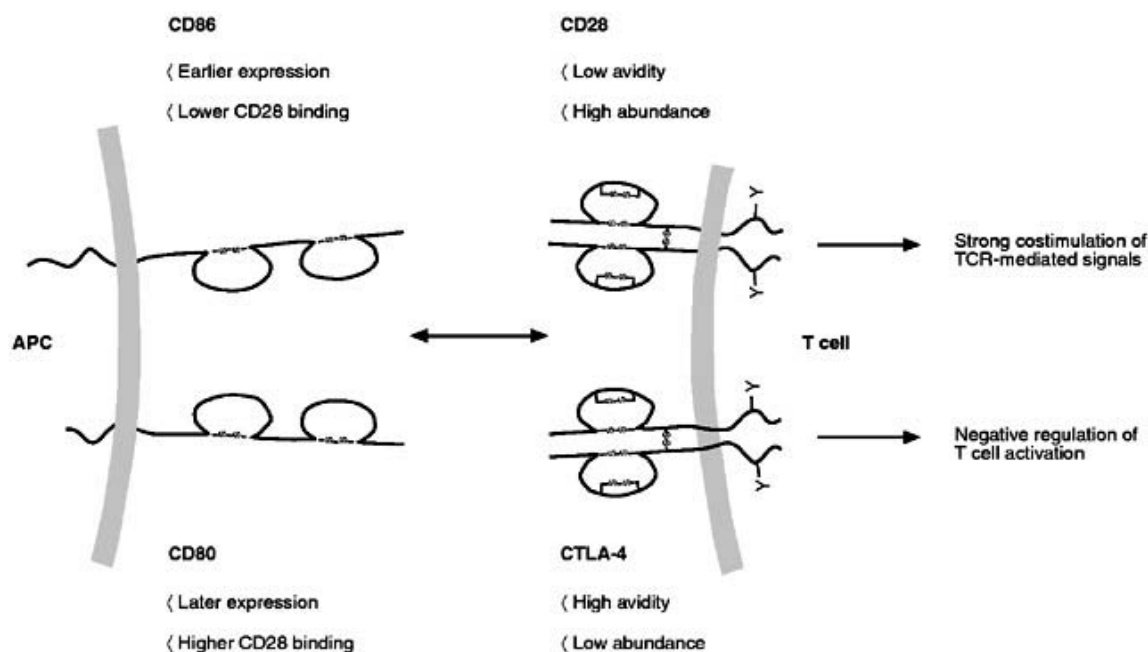


Figure 1 The CD28 receptor system. CD28 and CTLA-4 are stimulated by CD80 and CD86 ligands on activated APCs. Signals from CD28 are required for T cell activation and cytokine secretion, while signals from CTLA-4 inhibit T cell activation.

CTLA-4-Ig has been tested in numerous animal models of autoimmune disease and transplantation, and is currently in a phase I clinical trial in patients with refractory psoriasis. CTLA-4-Ig offers significant promise as a selective, nontoxic molecule for improved immunosuppressive therapy.

See also: CD antigens; B7 (CD80 & CD86); Cyclosporine; Cytokine genes, regulation of; Immunotherapy of tumors; Interleukin 2; Second signals for lymphocyte activation; T lymphocyte activation; T lymphocytes; Tolerance, peripheral; Tumors, immune response to.

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CD40 AND ITS LIGAND

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The CD40 antigen was identified in the mid-1980s from monoclonal antibodies that react with carcinomas and B cells and show costimulatory effects for B lymphocyte proliferation. In the early 1990s, the CD40 ligand (CD40-L) was identified on activated T cells and its genetic alteration was found to lead to the X-linked immunodeficiency referred to as the hyper IgM syndrome.

Structure and expression of CD40 and its ligand

The CD40 antigen, a 45–50 kDa glycoprotein of 277 amino acids, is a member of the tumor necrosis factor receptor family. The 193 amino acid extracellular domain is composed of four imperfect repeats of approximately 40 residues, anchored by a superimposable pattern of six cysteines. This organization is found in the other members of the superfamily including: the p75 low-affinity nerve growth factor receptor; the p55/CD120a and p75/CD120b receptors for tumor necrosis factor; the receptor (TNF-R_{rp}) for the lymphotoxin α /lymphotoxin β membrane complex; CD27 and CD30; OX40; 4-1-BB/ILA; Fas/CD95; and two viral homologs of the TNF receptors. The X-ray crystal structure of the complex formed by soluble binding domains of p55 TNF-R and a lymphotoxin α trimer suggests that CD40 looks like a slightly bent rod. Three CD40 subunits are likely to engage the CD40-L trimer and scrupulously avoid contact between each other. The mouse CD40 gene, composed of nine exons that span 16.3 kb of genomic DNA, is located on the distal region of chromosome 2, which is syntenic to human chromosome 20q11–q13, where the human CD40 gene is located. CD40 is expressed by multiple cell types. In the hematopoietic system, it is expressed on CD34⁺ hematopoietic progenitors, B cell progenitors, mature B lymphocytes, plasma cells, monocytes, dendritic cells and on some T lymphocytes. CD40 is also expressed on nonhematopoietic cells such as endothelial cells, fibroblasts and epithelial cells. In 1992, expression cloning using a CD40-Fc fusion protein allowed the isolation of a CD40-L from activated T cells. The human CD40-L is a polypeptide of 261 amino acids, including a 215 amino acid extracellular domain with five cysteines. CD40-L is a member of the tumor necrosis factor (TNF) family

that includes TNF α , LT α , LT β , CD27-L/CD70, CD30-L, 4-1BB-L, OX40-L, Fas-L and TRAIL. The gene for CD40-L is located on the X-chromosome position q26.3–q23.1. It spans over 12–13 kb and consists of five exons. CD40-L is readily expressed on CD4⁺ and CD8⁺ T cells after activation through pathways that are inhibited by cyclosporine. Basophils, eosinophils and activated B lymphocytes have also been reported as expressing CD40-L.

CD40 signal transduction

While the TNF-R family members display structural homologies in their extracellular ligand-binding domains, no obvious homologies can be detected in their intracellular domains. In particular, CD40 has no kinase domain and no known consensus sequence for binding to kinases. Yet, CD40 ligation activates protein-tyrosine kinases, including *lyn* and *syk* and induces the tyrosine phosphorylation of multiple substrates including phosphatidylinositol 3-kinase and phospholipase C γ 2. The phosphorylation of the latter is consistent with the anti-CD40 induced IP₃ production. The inhibitory effects of protein-tyrosine kinase inhibitors and of CD45 cross-linking demonstrate the functional relevance of the CD40-induced tyrosine phosphorylation. CD40 ligation also appears to activate the serine-threonine protein kinases and, most particularly, the stress-activated protein kinases (SAPK, also known as JNK for c-jun NH₂-terminal kinase). Finally, these different activation pathways result in the activation of various transcription factors including NF- κ B, NF- κ B-like molecules (such as p50, p65 (rel A), c-Rel), c-jun and NF-AT.

Through their intracytoplasmic domains, the members of the TNF-R family bind to a family of intracellular molecules, the TRAF proteins for (TNF-R)-associated factors. CRAF₁/TRAF₃, a 62 kDa intracellular protein, binds to the cytoplasmic domain of CD40 and to the EBV (Epstein-Barr virus)-transforming gene product LMP-1 (latent infection membrane protein 1) but not to TNF-R_I, TNF-R_{II} or Fas. CRAF₁/TRAF₃ appears to be essential for CD40 signaling inasmuch as a mutant CD40 with a point mutation Thr-234 to Ala does not bind CRAF₁/TRAF₃ and is functionally inactive. In addition to recruiting TRAF₃, CD40 utilized TRAF₂,

a 56 kDa intracellular protein that is also used by the 75 kDa TNF-R.

Functions of CD40 *in vitro* (Figure 1)

B lymphocytes

CD40 ligation activates resting B cells as shown by increase in size and expression of new surface molecules involved in homotypic and heterotypic aggregation (CD23, VLA-4), T cell costimulation (CD80/CD86) as well as cell death (Fas). Importantly, dual triggering of resting B lymphocytes through their CD40 and antigen receptor induces a phenotype characteristic of cells from germinal centers, the anatomical site where B cells become memory cells. Furthermore, CD40-activated B cells secrete a panel of cytokines which may act as autocrine and paracrine growth and differentiation factors. CD40-activated B cells also enter into proliferation which is further stimulated by addition of cytokines such as interleukin 4 (IL-4), IL-13 or IL-10 and their combination. Cytokines can also induce CD40-activated B cells to secrete immunoglobulins, with IL-10 inducing the secretion of large quantities of immunoglobulins (Igs) as a consequence of

induced plasma cell differentiation. In addition to being a differentiation factor, IL-10 acts as a factor inducing switch towards IgG1 and IgG3, as well as IgA when combined with transforming growth factor β (TGF β). IL-4/IL-13 induce CD40-activated B cells to switch towards IgG4 and IgE. Prolonged triggering of CD40 skews the maturation of B cells into memory cells, while interruption of CD40 signaling allows plasma cell differentiation.

While progenitor B lymphocytes proliferate in response to CD40 engagement, plasma cells appear unresponsive. Regarding B cell malignancies, chronic lymphocytic leukemias, acute lymphoblastic leukemias, B cell lymphomas, hairy cell leukemias and multiple myelomas proliferate in response to CD40 engagement. In contrast, CD40 ligation appears to inhibit the proliferation of diffuse B cell lymphomas, thus illustrating a possible negative role of CD40 on cell proliferation.

Monocytes and dendritic cells

The low spontaneous expression of CD40 on monocytes can be upregulated by cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and interferon γ (IFN γ). In contrast,

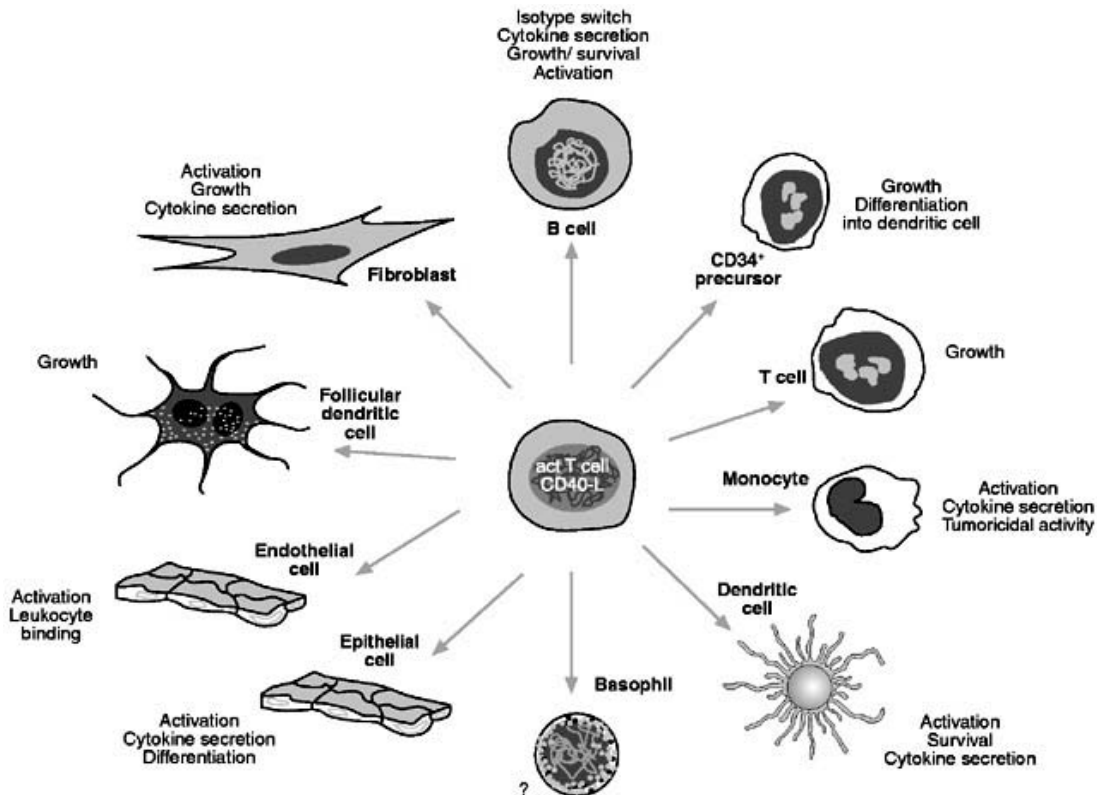


Figure 1 Functional consequences of CD40 triggering on different cell types. Activation includes increase in cell size and alteration of phenotype. (Reproduced with permission from van Kooten C and Banchereau J (1996) CD40 and its ligand. *Advances in Immunology* 61: 1–77.)

CD40 is expressed at high levels on dendritic cells isolated from different tissues or generated *in vitro* by culturing hematopoietic progenitors. CD40 ligation of monocytes and dendritic cells results in the secretion of multiple cytokines, including IL-1, IL-6, IL-8, IL-10, IL-12, TNF α and MiP1 α . Importantly, the secretion of IL-12 allows a skewing of T cell maturation towards the T_H1 pathway. CD40 ligation considerably alters the phenotype of these antigen-presenting cells (APCs) by upregulating the expression of costimulatory molecules such as CD54 (ICAM-1), CD58(LFA-3), CD80(B7-1), CD86(B7-2). Interrupting CD40/CD40-L interactions during T cell/dendritic cell cocultures results in reduced T cell proliferation. This observation is likely to be the consequence of both altered CD40 signaling to the APCs (reduced expression of costimulatory membrane molecules and cytokines) and altered CD40-L signaling to the T cells. Indeed, it was clearly demonstrated that CD40-L-dependent activation of T cells occurs through signaling of CD40 in the antigen-presenting cell to enhance requisite costimulatory pathways including B7. CD40 ligation turns on monocyte tumoricidal activity as well as nitric oxide synthesis. In response to CD40 ligation, CD34⁺ hematopoietic progenitors proliferate and differentiate into cells with prominent dendritic cell attributes. Such dendritic cells are not only capable of inducing primary T cell activation but also interact with activated B cells enhancing their proliferation and differentiation.

Endothelial cells, epithelial cells and fibroblasts

Immunohistology performed on various tissue sections shows that CD40-L-specific antibodies stain vascular endothelium, epidermal basal membrane, scattered fibroblasts, thymic epithelium and follicular dendritic cells. Consistently, primary lines of endothelial cells, thymic and kidney tubular epithelial cells, keratinocytes, skin and synovial fibroblasts as well as follicular dendritic cells express CD40 at relatively low density. Yet, these adherent cells express a functional CD40 whose ligation induces phenotypic alterations, cytokine secretion as well as stimulation or inhibition of proliferation. In particular, endothelial cells display marked upregulation of CD54(ICAM-1), CD106(VCAM-1) and CD62E(E-selectin), thus resulting in increased ability to bind leukocytes. Interestingly, while fibroblasts and follicular dendritic cell lines are induced to proliferate following CD40 ligation, keratinocyte lines cease proliferating to terminally differentiate.

***In vivo* functions of CD40(CD40-L) interactions**

Hyper IgM syndrome in humans

The first demonstration of the critical role of CD40(CD40-L) interactions *in vivo* came from the discovery that the hyper IgM syndrome, an X-linked immunodeficiency, is due to a genetic alteration of the CD40-L. This disease is characterized by a severe impairment of T cell dependent antibody responses with no B cell memory, no circulating IgG, IgA and IgE and no somatic hypermutation, as a consequence of the lack of germinal centers within secondary lymphoid organs. Enhanced susceptibility of patients to opportunistic infections, such as *Pneumocystis carinii* pneumonia and *Cryptosporidium* diarrhea, also indicates a role for CD40-CD40-L interactions in cell-mediated immune responses. Interruption of CD40 (CD40-L) interactions *in vivo* in mice impairs B cell memory as well as T cell priming. Initial experiments performed with antagonists to CD40(CD40-L) interactions, such as antibodies to CD40-L or CD40-Fc fusion proteins, demonstrated impairment of B cell memory. The generation of CD40 and CD40-L knockout (KO) mice confirmed and further extended these observations and revealed a phenotype comparable to that of the patients suffering from the hyper IgM syndrome. In particular, these mice display decreased IgM responses to thymus-dependent antigens, no antigen-specific IgG1, IgA and IgE and no germinal centers but normal T and B lymphocyte numbers. As expected, these mice respond normally to thymus-independent antigens with increased IgM and IgG3 levels. CD40-L KO mice display a considerable impairment of antigen-specific T cell priming and appear particularly susceptible to *Leishmania* infection. This is the result of a defective T_H1 response which is related to an impaired production of IL-12 by antigen-presenting cells. As observed in patients, CD40 KO mice show decreased reactive granulopoiesis in response to infections. Studies with a CD40-Fc fusion protein have highlighted the important role of T cell signaling through CD40 ligand in the development of helper function. In particular, administration of soluble CD40 *in vivo* to CD40 KO mice initiates germinal center formation, and T cells primed in the absence of CD40 are unable to help normal B cells to class switch and to form germinal centers. In this context, ligating CD40 ligand of human activated T cells considerably enhances their production of IL-4.

Interruption of CD40-CD40-L interactions as a treatment of autoimmunity

Administration of antibodies to CD40-L has been

shown to prevent the establishment of autoimmune symptoms in various murine models including: 1) collagen type II-induced arthritis, a model for human rheumatoid arthritis; 2) lupus nephritis in lupus-prone mice that represent models of systemic lupus erythematosus; 3) proteolipoprotein-induced experimental encephalomyelitis, a model of human multiple sclerosis. Importantly, in this last case, the antibody could induce a substantial reduction of the disease even when administered after onset. Consistently, activated helper T cells expressing CD40-L surface protein are detected in multiple sclerosis patient brain sections where CD40-bearing APCs can be found.

Interruption of CD40-CD40-L interactions as a way of inducing transplantation tolerance

Administration of CD40-L-specific antibodies prevents the development of graft-versus-host disease (GVHD) that occurs as a major complication of allogeneic bone marrow transplantation. This treatment affects both the acute GVHD, essentially mediated by cytotoxic T cells, and the chronic GVHD, mainly due to the polyclonal B cell activation and the production of self reactive antibodies. Antibodies against CD40-L markedly prolong the survival of cardiac allografts in both naive and sensitized hosts when administered at the time of transplantation. Finally, a combination of allogeneic B cells and CD40-L-specific antibody considerably decreases host reactivity of both CD4⁺ and CD8⁺ T lymphocytes, thereby allowing efficient transplantation of allogeneic pancreatic β islet cells. The addition of CD40-L-specific antibody appears to considerably enhance the tolerogenic effect of B cells.

Thus, multiple pathologic states appear to be improved by interruption of CD40-CD40-L interactions. While this is presently accomplished by preventing the association of the receptor with its ligand, using antibodies or soluble receptor, small synthetic chemical agents preventing this interaction may eventually be identified. However, it is likely that pharmacologic agents may be found that will

block the intracellular pathways turned on after ligation of either CD40 or CD40-L.

See also: B lymphocyte activation; Dendritic cells; Germinal center; Hyper-IgM syndrome; TNF receptors.

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CD44

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CD44 is a ubiquitous multistructural and multifunctional cell surface glycoprotein involved in adhesive cell–cell and cell–matrix interactions, as well as in cell migration and cell homing. Its principal ligand is hyaluronic acid (HA, hyaluronate, hyaluronan). The glycoprotein was accorded various functional names which merged to an established single name only after molecular sequencing. The CD44 cluster assigned by the Third International Workshop on Leukocyte Typing includes the following earlier synonyms: GP90^{Hermes}, extracellular matrix receptor III (ECMR^{III}), homing cell adhesion molecule (HCAM), phagocytic glycoprotein 1 (Pgp-1), glycoprotein 85 (gp85), Ly-24, hyaluronate receptor, HUTCH-1 and ln (Lu)-related-p80 glycoprotein.

Structure

CD44 is encoded by a single gene located on human chromosome 11 and mouse chromosome 2. The genomic organization of the CD44 gene involves 20 exons in both mouse and humans (Figure 1b). The first five exons coding for the extracellular domain are constant, whereas the next ten exons are subjected to differential alternative splicing, resulting in the generation of the variable region. Note that exon 6 (or V1) is not expressed in humans. Constant exons 16 and 17, together with part of exon 15, encode the membrane-proximal region; constant exon 18 encodes the transmembrane domain. Differential utilization of exons 19 and 20 generates the short version (3 amino acids) and the long version (70 amino acids) of the cytoplasmic tail, respectively.

Standard CD44 (CD44s), which lacks the entire variable region (Figure 1c), is preferentially expressed on hematopoietic cells, and is therefore also designated CD44H. Northern blot analysis of RNA isolated from CD44 of different hematopoietic cells revealed three major transcripts in humans (~1.6, 2.2 and 4.8 kb) and mice (~1.6, 3.3 and 4.6 kb). Human CD44s mRNA is translated into 361 (mouse 363) amino acids. The predicted size of the core protein is 37–38 kDa. Post-translational modification doubles the molecular size of human and mouse primary protein, bringing it to 80–95 kDa. The mature CD44s is a single-chain molecule composed of a distal extracellular domain, a membrane-proximal region (together containing 248 amino acids in humans and 250 amino acids in the mouse),

a transmembrane-spanning domain (23 amino acids), and a short (3 amino acids) or longer (70 amino acids), and much more abundant, cytoplasmic tail. The N-terminal domain contains six cysteine residues, which are possibly used to form one or more globular domains. This region, which includes the ligand (HA) binding sites of the molecule, displays ~30% homology with cartilage link protein and proteoglycan core protein, which also both bind HA. The N-terminus, transmembrane domain and cytoplasmic tail, but not the membrane-proximal region, exhibit high (~80–90%) interspecies homology. The cytoplasmic tail contains several optional phosphorylation sites (Figure 1a).

Differential utilization of the variable region exons yields at least 20 different CD44 isoforms; those expressing alternatively spliced exons are designated CD44v (v stands for variant) (Figure 1c). The CD44s and CD44v repertoire is further enriched by N- and O-glycosylation and glycosaminoglycanation (by heparan sulfate and chondroitin sulfate). The multistructural nature of CD44 (its molecular weight ranging from 85 to 230 kDa) may extend the ligand inventory of this molecule and further increase its optional functions.

Distribution

Standard CD44 (Figure 1) is a ubiquitous molecule expressed on many types of cells of lymphohematopoietic origin, including erythrocytes, T and B lymphocytes, natural killer cells, macrophages, Kupffer cells, dendritic cells and granulocytes. However, CD44s is also expressed on other cells, such as fibroblasts and cells of the central nervous system. After immunologic activation, T lymphocytes and other leukocytes transiently upregulate CD44v, particularly those containing V6 exon products. A CD44 variant, expressing exons V8 to V10 (CD44V8–10, also known as epithelial CD44 or CD44E), is preferentially expressed on epithelial cells. The longest CD44 isoform, CD44V3–10, was detected in keratinocytes (Figure 1c).

Function

Hyaluronic acid, the principal extracellular matrix (ECM) ligand of CD44, is a ubiquitous polysaccharide (glycosaminoglycan) with a high molecular mass

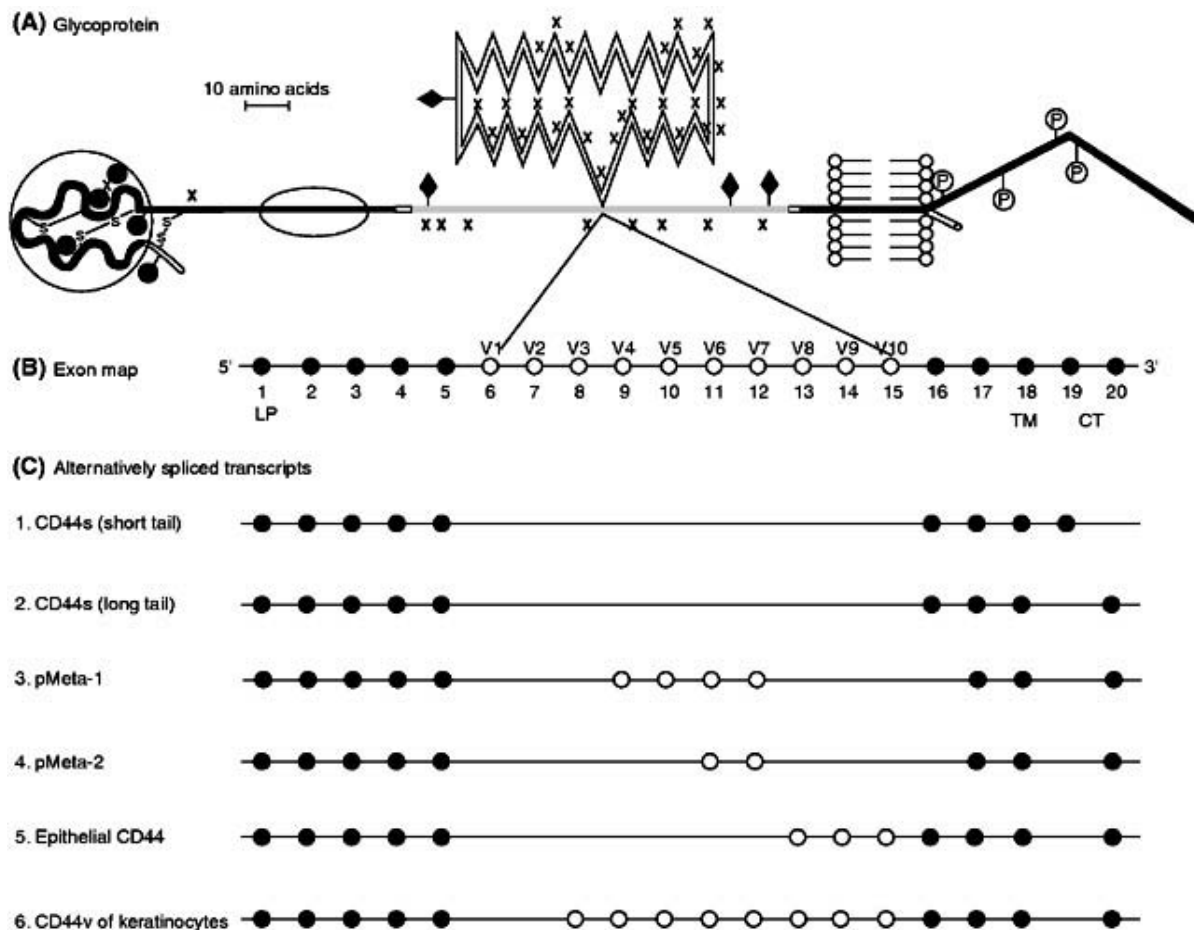


Figure 1 The CD44 glycoprotein, its exon map and examples of six alternatively spliced transcripts. (A) *Protein structure*. Using disulfide bonds, the amino terminus of the molecule forms a globular domain, or three globular subdomains. The circle and the 'downstream' ellipse represent areas that influence hyaluronate binding. The black track inside the circle refers to a region displaying ~30% homology with cartilage link protein and proteoglycan core protein, both showing HA-binding ability. The black track at the amino terminus (inside and outside the circle), transmembrane-spanning domain and cytoplasmic tail represent regions with ~80–90% interspecies homology. The alternatively spliced short cytoplasmic domain is nonproportionately represented by a small bar. The hatched track in the center indicates the nonconserved membrane-proximal region with ~35–45% interspecies homology. The variable region, containing various combinations of alternatively spliced exon products (see Part C), is inserted between amino acids 201 and 202 (mature protein) and marked by a zigzag track. ●, Potential *N*-linked glycosylation sites (only those of standard CD44 are shown); X, areas rich in serine/threonine, possible sites for *O*-linked glycosylation (those of the variable region are depicted arbitrarily); ◆, potential sites for glycosaminoglycans (chondroitin sulfate or heparan sulfate) incorporation; ⊕, potential sites for phosphorylation (only part of the sites are described). The symbols on the standard part of the molecule mostly refer to mouse CD44, whereas those of the variable region are based on information taken from both mouse and humans. (B) *Exon map*. The filled circles represent the exons of the constant regions. Empty circles represent exons that can be inserted by alternative splicing, resulting in the generation of the variable region. Note that exon V1 is not expressed in the human CD44. LP, leader peptide-encoding exon; TM, transmembrane-encoding exon; CT, cytoplasmic tail-encoding exons. (C) *Examples of alternatively spliced transcripts*. 1 and 2, standard CD44 with short and long cytoplasmic tails, respectively, which lack the entire variable region. 3, pMeta-1 (CD44V4–7). Exons V4, V5, V6 and V7 are inserted in tandem between exons 5 and 17. 4, pMeta-2 (CD44V6,7). Exons V6 and V7 are inserted between exons 5 and 17. pMeta-1 and pMeta-2 are known as 'metastatic' CD44, as their cDNA confers, upon transfection, metastatic potential on nonmetastatic rat tumor cells. Note that exon 16 is not expressed in both pMeta-1 and pMeta-2. 5, epithelial CD44 (CD44V8–10), expressed preferentially on epithelial cells. Exons V8, V9 and V10 are inserted between exons 5 and 16. 6, keratinocyte CD44 (CD44V3–10), one of the largest CD44 molecules known. Exons V3 through V10 are inserted between exons 5 and 16. (Reproduced from Naor *et al* (1997). © Academic Press.)

(10^6 – 10^7 Da), consisting of a linear polymer of the disaccharide (D-glucuronic acid [1-β-3] *N*-acetyl-D-glucosamine [1-β-4])_n. However, CD44 (or its chondroitin-sulfated version) also interacts with other

ligands of the ECM, such as collagen, fibronectin and laminin. Mucosal addressin, serglycin, osteopontin and class II invariant chain (Ii) are additional, ECM-unrelated, ligands of CD44. In many, but not in all,

cases CD44 does not bind HA unless it is stimulated by phorbol esters, activated by agonistic CD44-specific monoclonal antibody (mAb) or deglycosylated (e.g. by tunicamycin). Apparently, cytoskeleton-dependent receptor aggregation is required for HA binding, although cytoskeletal involvement can be bypassed by external cross-linking of cell surface CD44 molecules (e.g. with agonistic CD44-specific mAb or immobilized HA).

CD44 is a multifunctional receptor involved in cell-cell and cell-ECM interactions, cell traffic on endothelium or ECM, lymph node homing via binding to high endothelial venules, presentation of chemokines or growth factors to migrating cells, and transmission of growth signals, either independently or in association with other cell surface molecules. CD44 also participates in the uptake and intracellular degradation of HA, as well as in the transmission of signals mediating lymphohematopoiesis and prevention of apoptosis. Marked accumulation of CD44 (both standard and variant), and in some cases also of HA, is detected in areas of intensive cell migration and proliferation, as in wound healing, tissue remodeling, morphogenesis, inflammation and carcinogenesis.

Pathological aspects

Many cancer cell types, as well as their metastases, express high levels of CD44. Whereas some tumors, such as gliomas, exclusively display CD44s, other neoplasms, including brain metastases, gastrointestinal cancer, pancreatic adenocarcinoma, bladder cancer, uterine cervical cancer, breast cancer and non-Hodgkin's lymphomas, additionally and sometimes preferentially express CD44 variants. Hence, CD44, particularly its variants, may be used as diagnostic or prognostic markers of at least some human malignant diseases. Furthermore, it has been demonstrated in animal models that upon transfection with CD44s, or CD44v cDNA, nonmetastatic tumor cells acquire metastatic potential. In addition, injection of reagents interfering with CD44-ligand interaction (e.g. CD44s- or CD44v-specific mAb) inhibit local tumor growth and metastatic spread in murine species. In this context, CD44 may confer a growth advantage on some neoplastic cells, and consequently could be used as a target for cancer therapy. The functional significance of CD44 in malignant, autoimmune and infectious diseases, as well as in allergy, transplant rejection and graft-versus-host reaction, is yet to be established.

See also: Adhesion molecules; Glycosylation of immune system molecules; High endothelial venules;

Immunotherapy of tumors; Lymphocyte trafficking; Motility of immune cells.

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CD45

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CD45 is an abundant transmembrane protein-tyrosine phosphatase (PTPase) expressed uniquely by all nucleated cells of hematopoietic origin. With regard to lymphocytes, CD45 functions to activate Src-family members involved in antigen receptor signaling. Recent studies have demonstrated that several transmembrane PTPases, including CD45, are involved in regulating adhesion and this may be a common theme to their function.

Structure

CD45 contains a large extracellular domain, a single transmembrane region, and two cytoplasmic PTPase domains (Figure 1). cDNAs have been isolated from human, rat, mouse, chicken and shark and comparison of the predicted protein sequences indicates a conservation of domain structure. The extracellular domain of CD45 consists of three distinguishable regions: an O-linked carbohydrate domain, a cysteine-containing domain and a domain containing three fibronectin type III repeats (which also contain several cysteines). Electron microscopy analysis of the extracellular domain demonstrates that the O-linked carbohydrate region which is most membrane distal has an extended rod conformation. Multiple isoforms of CD45 exist, ranging in molecular weight from 180 to 220 kDa. The basis for the different isoforms is alternative splicing of exons that encode part of the O-linked carbohydrate region. Since this region has an extended conformation, the length of the extracellular domain is dramatically altered (28 nm for the low molecular weight isoform and 51 nm for the high molecular weight isoform) (Figure 2). The O-linked carbohydrate region is encoded by exons 3–8. cDNA cloning shows that exons 4, 5 and 6 (also named A, B and C) are spliced in a variety of combinations to give rise to eight distinct protein products. In addition, a ninth isoform is predicted to exist that is the product of a splice between exons 3 and 8. The proposed nomenclature for the different CD45 isoforms is shown in Table 1.

The extracellular domain contains numerous sites for N-linked carbohydrate addition. Indeed, CD45 is 25% carbohydrate by weight. Owing to the high level of expression on lymphocytes, 100 000 molecules per cell, and its large size, CD45 occupies approximately 10% of the lymphocyte cell surface and bears much of the carbohydrate of these cells. Further diversification of CD45 species is generated with the addition of carbohydrate groups by cell type-specific and developmentally regulated glycosyltransferases. The existence of specific modifications on CD45 has been demonstrated using monoclonal antibodies that recognize distinct carbohydrate groups.

The cytoplasmic domain of CD45 contains two tandemly arranged tyrosine phosphatase domains. Mutational analysis suggests the first, membrane proximal, domain is both necessary and sufficient for biological responses and contains most if not all the catalytic activity. This is consistent with the observation that the sequence of the second domain contains changes at critical amino acids in the active site signature motif 'HCSAGXGRTG'. Nonetheless, it is possible that the second domain does possess intrinsic phosphatase activity. Alternatively, the second domain may function to regulate substrate availability.

Expression patterns of CD45 isoforms

CD45 isoform expression is dynamically controlled in both leukocyte differentiation and cellular activation. Isoform expression by a given population has been characterized by both amplification of this region of the mRNA by reverse transcription-polymerase chain reaction analysis and by a large assortment of monoclonal antibodies that recognize epitopes affected by the use of the alternative exons. The antibodies that recognize epitopes encoded by the alternatively spliced exons 4, 5 and 6 have been termed CD45RA, B and C, respectively. For humans, an antibody termed CD45RO exists that recognizes

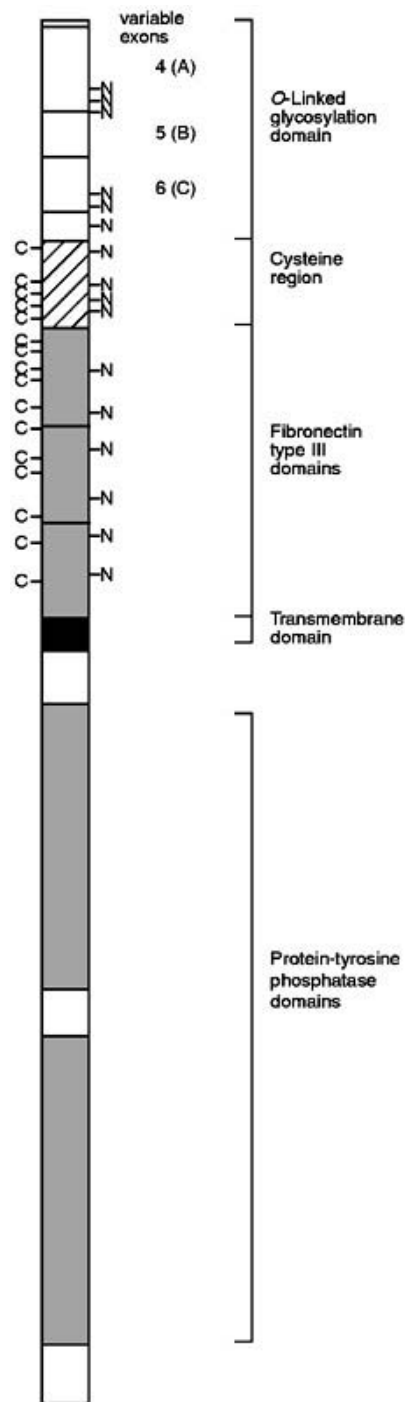


Figure 1 Human CD45. The variable exons 4, 5 and 6 are indicated. N represents site of potential N-linked glycosylation. C represents the location of cysteine residues in the extracellular domain.

the junction between exon 3 and 7; this is specific for the CD45 θ isoform. A similar reagent does not exist for mouse or rat CD45. Unlike CD45R0, however, the remaining CD45R antibodies are all capable of recognizing multiple isoforms (Table 1). This

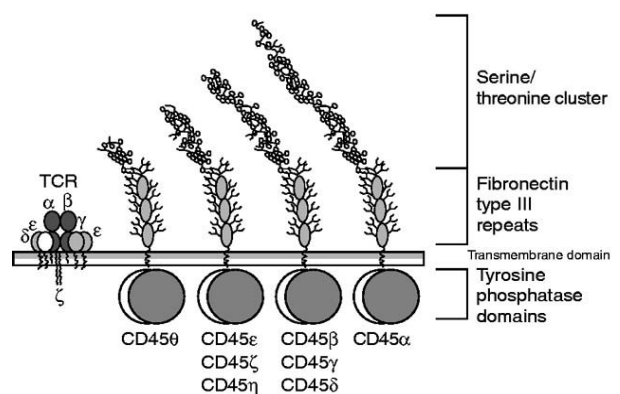


Figure 2 Representation of the sizes of different CD45 isoforms compared to the T cell receptor (TCR). Estimates are based on either electromicroscopy data or inferred from crystal structures.

complicates the characterization of isoform expression with these reagents. In addition, many of the CD45R antibodies are sensitive to carbohydrate modifications and some are specific for carbohydrate epitopes, not all of which are dependent on the presence of the alternatively spliced exons. While the CD45R antibodies provide only a limited insight into the specific patterns of isoform expression, they have proved a powerful tool in demonstrating that CD45 isoform expression is exquisitely regulated by different leukocyte populations.

T cell lineages express distinct CD45 isoforms depending on compartment and activation state. Most thymocytes express the CD45 θ isoform, however there is increased expression of the higher molecular weight isoforms during thymocyte development. Of interest, very early thymocyte precursors that do not express TCR, CD4 or CD8 also stain for CD45R isoforms. This may indicate that thymocyte maturation is accompanied by cyclical expression of

Table 1 CD45 isoforms

Isoform	CD45R antibody recognition	Alternative exon usage						
CD45 α	A, B, C	3	4	5	6	7	8	
CD45 β	A, B	3	4	5		7	8	
CD45 γ	A, C	3	4		6	7	8	
CD45 δ	B, C	3		5	6	7	8	
CD45 ϵ	A	3	4			7	8	
CD45 ζ	B	3		5		7	8	
CD45 η	C	3			6	7	8	
CD45 θ	O	3				7	8	
CD45 τ		3					8	

CD45 splice isoforms depending on maturation stage.

Naive mature T cells express CD45R epitopes, and activation shifts isoform usage towards the CD450 form. Furthermore, functionally distinct T cell subsets have characteristic patterns of CD45 isoforms. CD8⁺ cytolytic T cells express higher levels of CD45RA, and distinct CD45R staining patterns on T helper subsets are observed. Carbohydrate specific monoclonals also reveal distinct compartmental and activation state epitopes on T cell CD45. Therefore, T cell CD45 isoform usage is dynamic and likely reflects a complex regulatory program.

B cells normally express the high molecular weight CD45 α isoform (also termed B220). However, cytokine treatment and viral transformation induce a change in staining pattern of the CD45R antibodies, including an increase in the expression of lower molecular weight isoforms. B cells also express carbohydrate epitopes on CD45 distinct from other cell lineages.

Monocytes express primarily the CD450 isoform. Similar to the effects in T and B lineages, activation events induce the appearance of CD45RA epitopes, suggesting the expression of higher molecular weight isoforms.

Thus, the polypeptide and carbohydrate composition of the CD45 extracellular domain is highly regulated in a cell type-specific, developmentally regulated and activation state-dependent manner.

CD45 ligands

As yet it is not clear how the extracellular domain regulates the defined biology of CD45. While a number of candidate ligands have emerged, it has been difficult to identify specific interactions that modulate the biological function of CD45. Due to its extensive modification with carbohydrate, it is likely that lectins will be important CD45 ligands.

CD22 is a cell surface cation-independent lectin with a specificity for α 2,6-linked sialic acid moieties and is expressed specifically on mature naive B cells. It is a member of the immunoglobulin (Ig) superfamily, with two alternatively spliced isoforms that contain five or seven Ig domains, respectively. Upon activation, CD22 is tyrosine phosphorylated and associates with the protein-tyrosine kinase p72^{syk} and the protein-tyrosine phosphatase SHP-1. Initially studied as a cell-cell adhesion molecule, recombinant CD22 interacts with a number of T cell surface proteins, among them CD45. However, the interaction is dependent upon the expression of α 2,6-sialyltransferase, which is only expressed by some leukocytes. CD45 cross-linking with CD22 augments early

T cell activation events. Nonetheless, it is unclear whether in an immune response CD22 and CD45 represent a cognate pair in which binding results in a change in signal transduction through either protein.

Galectin 1 is a widely expressed β -galactoside binding protein family member. It is a dimer of 14 kDa subunits, each with a separate carbohydrate binding site. A number of primary T cell populations and T leukemic cell lines undergo apoptosis upon treatment with galectin 1. This effect requires CD45 N-linked carbohydrates, although it may be negatively regulated by increased O-linked glycosylation on higher molecular weight isoforms of CD45. Interestingly, in the thymus, galectin 1 expression occurs in areas where apoptosis is thought to occur. The very high level of CD45 protein expression in the thymus makes it difficult to conclude that interaction of CD45 with galectin 1 results in thymic apoptosis, although the development of galectin 1-deficient mice may clarify this issue.

Other carbohydrate-binding proteins may serve as CD45 ligands. Serum mannan-binding protein (S-MBP) is reported to interact specifically with the CD450 isoform. S-MBP is a cation-dependent lectin that binds mannose and N-acetylglucosamine. Originally studied as a serum opsonin, S-MBP inhibits antigen receptor-induced T cell apoptosis. Although these results remain to be examined in greater detail, they suggest the extracellular domain of CD45 will have a role in regulating its biological function.

CD45 function

Genetic analysis using CD45-deficient cell lines and mice has been instrumental in delineating CD45 functions. CD45 is an important regulator of T and B lymphocyte antigen receptors and natural killer cell activation. With regard to T cells, CD45 functions to dephosphorylate and activate *src*-family member protein tyrosine kinases p56^{lck} and p59^{fyn}, a necessary first step for signaling through the T cell antigen receptor.

CD45 is expressed by leukocytes that do not express antigen receptors. Although analysis is not complete, this may be explained by a role for CD45 in cell-cell adhesion events and coordinate regulation of the cytoskeleton. Monoclonal antibodies to CD45 induce or inhibit aggregation of primary and transformed lymphocyte cell lines. This latter inhibition is also correlated with reduced levels of tyrosine phosphorylation on ICAM-3 and LFA-1. Immobilized CD45-specific monoclonal antibodies promote the adherence to plastic of T cell lines in conjunction with an increase of cellular phosphotyrosine. Cell adhesion events involve the cytoskeleton, and CD45

interacts with the cytoskeletal component fodrin (non-erythroid spectrin). Association with the cytoskeleton via fodrin or other molecules may control the ability of CD45 to interact with its substrates. Whether there are distinct mechanisms by which CD45 regulates antigen receptor signaling and adhesion is not clear. However, it is of interest to note that in lymphocytes CD45 associates with the lymphocyte-specific protein termed CD45-associated protein (CD45AP). CD45AP contains a short extracellular domain, a single transmembrane region, and a 146 amino acid cytoplasmic domain that contains a putative WW domain (a protein-binding module containing two conserved tryptophans [W]) and an acidic domain. CD45 associates with CD45AP through the transmembrane domain, and thus the WW domain and the acidic domain may associate with other proteins. The possibility exists that CD45AP may affect the substrates that CD45 interacts with and, as such, may regulate the ability of CD45 to function in antigen receptor signaling and/or adhesion.

The ability of the CD45 extracellular domain to regulate function has been pursued either by expressing in CD45-deficient cells chimeric proteins containing a different extracellular domain or by expressing distinct CD45 isoforms. Reconstitution of CD45-deficient cells, in which the extracellular domain has been deleted or exchanged, partially reconstitutes antigen receptor signaling. Interestingly, while expression of a chimeric protein with the epidermal growth factor (EGF) receptor extracellular and transmembrane domains and the CD45 cytoplasmic domain does reconstitute some antigen receptor signals, the addition of EGF ablates the response immediately, presumably as a result of receptor dimerization. This is consistent with the inhibitory effects on T cell activation seen with some monoclonal antibodies against CD45. However, it is not known whether these experimental systems mimic a physiologically relevant mode of CD45 regulation.

Expression of distinct CD45 isoforms in either transgenic mice or CD45-deficient cell lines suggests that the extracellular domain is important in regulating function. Specific expression of CD45 α but not CD45 β in transgenic animals results in an augmented proliferative response. Furthermore, it has been suggested that the lower molecular weight CD45 β isoform more readily associates with the TCR complex to increase signaling capacity. Therefore, the extracellular domain appears to have the capacity to regulate CD45 function, although the

mechanism is unknown. Characterization of CD45 ligands and their ability to modulate CD45 function will help clarify this issue.

See also: CD antigens; CD22; Membrane-associated cytoskeleton: role in regulating immune cell function; Memory, immunological; T lymphocyte activation.

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CD46 (MEMBRANE COFACTOR PROTEIN, MCP)

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The complement system consists of a group of sequentially interacting proteins that promote the inflammatory response and facilitate the destruction of microorganisms. The system must be tightly regulated to prevent activation in the fluid phase (i.e. no target) and on host tissue (i.e. inappropriate target). Nearly half of the complement proteins serve in regulation. One such inhibitory protein is CD46, membrane cofactor protein (MCP). This regulator is a type one transmembrane protein that serves as a cofactor for the factor I-mediated proteolytic cleavage and inactivation of C3b and C4b deposited on self tissue. CD46 performs this role intrinsically in that it protects the cell on which it is expressed, not neighboring cells. CD46 is also the measles virus receptor and is involved in the adherence of *Streptococcus pyogenes* (group A streptococcus) to epithelial cells. CD46 belongs to a group of structurally, functionally and genetically related proteins called the regulators of complement activation (RCA) that includes decay-accelerating factor (DAF; CD55), complement receptors 1 (CR1; CD35) and 2 (CR2; CD21), and the plasma proteins factor H and C4-binding protein.

Structural characteristics

CD46 possesses an unusual electrophoretic pattern in that it consists of two rather heterogeneous protein species of 51–58 kDa ('lower' band) and 59–68 kDa ('upper' band). This variability is inherited as three phenotypic patterns consisting of upper band predominance in 65% of the population, equivalence of both bands in 29% and predominance of the lower band in 6%. This heterogeneity represents a size polymorphism and relates to CD46 being expressed as a family of at least four isoforms that arise by alternative splicing of a single gene (Figure 1). A dominating characteristic of CD46 is the presence of four of the repeating motifs called complement control protein repeats (CCPRs) (also called short consensus repeats). These are the building blocks of RCA proteins that contain the binding sites for C3b and C4b. Sites for N-linked glycosylation are found in CCPR-1, -2 and -4. Following the CCPRs is an area enriched in serines, threonines and prolines (STP) that is extensively O-glycosylated.

This domain consists of 14 or 29 amino acids depending on whether the STP exon B (15 amino acids) is spliced out. The presence of STP-B produces the higher molecular weight protein isoforms (possessing more sialic acid) while its absence generates the lower molecular weight forms. Following this is a tract of 12 amino acids of unknown significance, a transmembrane region, intracytoplasmic anchor and one of two alternatively spliced cytoplasmic tails (CYT-1 of 16 amino acids or CYT-2 of 23 amino acids). The CD46 gene consists of 14 exons and 13 introns with a minimum length of 43 kb and resides in the RCA cluster on the long arm of chromosome one (1q3.2). A partial duplicate of CD46 is also present in this cluster but no mRNA for this species has been identified.

Functional role

Host cells must be protected from inadvertent complement activation at sites of inflammation and from the continuous, natural 'tickover' of C3. A biochemical mechanism to regulate C3b/C4b is termed 'cofactor' activity. In this process, CD46 interacts with C3b or C4b to promote their limited enzymatic cleavage by factor I, a plasma serine protease. CD46 possesses 'intrinsic' cofactor activity, i.e. it protects the cell on which it is anchored, not bystander cells.

The reason for the expression of four isoforms in a given cell or tissue is only partially understood. For example, the cytoplasmic tails of CD46 mediate the differential processing of CD46 precursors. Isoforms bearing tail 1 (CYT-1) processed into their mature forms four times faster than isoforms with tail 2 (CYT-2). Additionally, the BC isoforms provide enhanced cytoprotection against the classical pathway as compared to C isoforms. The presence of distinct consensus phosphorylation signals on the cytoplasmic tails suggests roles in cell signaling.

The CCPRs are the sites of functional activity for CD46 and other RCA proteins. C4b-binding and cofactor activity map to CCPRs 2, 3 and 4. To regulate C3b, the third and fourth CCPRs are necessary for binding while 2, 3 and 4 are needed for cofactor activity.

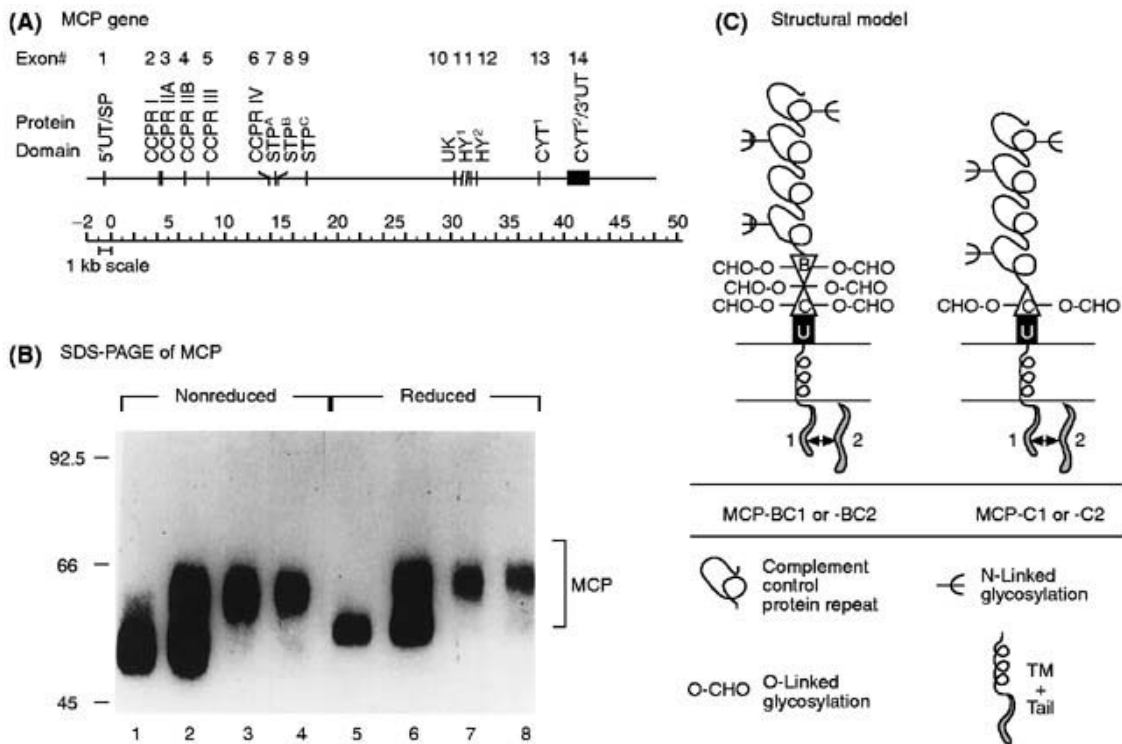


Figure 1 Genetics and structure of membrane cofactor protein (MCP; CD46). (A) the genomic organization of the alternatively spliced CD46 reveals 14 exons and 13 introns for a minimum length of 43 kb. 5'UT/SP, 5' untranslated region/signal peptide; CCPR, complement control protein repeat; STP^A, STP^B, STP^C are the segments enriched in serines, threonines and prolines; UK, region of unknown functional significance; HY¹ and HY² are hydrophobic regions 1 and 2; CYT¹, cytoplasmic tail 1; CYT²/3'UT, cytoplasmic tail 2 and 3' untranslated region. (B) The electrophoretic profile of the protein shows three phenotypic patterns: lower band predominant, equal banding, and upper band predominant. The apparent molecular weight of all species increases with reduction. (C) Structural model shows the four primary isoforms of CD46—note the alternatively spliced STP and cytoplasmic tail regions.

Tissue distribution

CD46 is widely expressed on host tissue, a logical consequence of the critical role it plays in self-protection. Although most cells express CD44, erythrocytes are an interesting exception. Soluble forms are present in low concentrations in plasma, tears, saliva and seminal fluid and are increased in plasma in the presence of certain malignancies.

As mentioned, CD46 is expressed as a family of isoforms. While most cells in a given individual express the same ratio of the four isoforms, a predominance of certain species has been noted in brain, salivary gland, kidney and fetal heart. Different molecular weights resulting from variable glycosylation have been noted on granulocytes and spermatozoa. Tissues including kidney, skin, reproductive, eye, thyroid, liver and synovium differ in the relative expression and distribution pattern of CD46. Additionally, malignant and transformed cells tend to express high amounts of CD46.

Homologous proteins of other species

CD46 has been identified in primates, pigs, guinea pigs and rabbits. A functional homolog has been characterized in the mouse (Crry/p65) and rat (5I2 antigen). However, these resemble the human complement receptor CR1 in that they possess both cofactor activity and decay-accelerating activity. However, human CR1 has limited tissue distribution.

CD46 in other biological areas

Since the identification of CD46 as a C3b-binding protein more than a decade ago, its biologic and pathophysiologic role has expanded. CD46 is the receptor for measles virus. Additionally, CD46 is involved in the adherence of *Streptococcus pyogenes* (group A streptococcus). Recently, it was found that human immunodeficiency virus 1 (HIV-1) can incorporate complement control proteins CD46, CD55

and CD59 as common surface constituents in order to promote resistance against complement.

CD46 also is of interest in transplant biology. Acute rejection of the organs of other species is in part mediated by the complement system. To overcome this, complement regulators are being engineered to produce transgenic pigs whose organs will be used in xenotransplantation.

In reproductive biology, CD46 is abundantly expressed at the maternal–fetal interface, on fetal tissue and on the inner acrosomal membrane of spermatozoa. Alterations in function or expression of CD46 may play a role in habitual abortion or infertility. The surprising acrosomal site for CD46 expression may be part of the egg–sperm attachment mechanism and/or protect against C3b deposition during penetration by spermatozoa.

See also: Complement, alternative pathway; Complement, classical pathway; Complement deficiencies; Complement fixation test; Complement, genetics; Complement, membrane attack pathway; Complement receptors; Decay-accelerating factor (CD55); Paramyxoviruses, infection and immunity; Streptococcus, infection and immunity; Xenotransplantation.

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CD59

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CD59 is an 18 kDa membrane glycoprotein whose primary function is to regulate the activity of the complement membrane attack complex (MAC). Alternative names for CD59 include protectin, membrane inhibitor of reactive lysis (MIRL), homologous restriction factor 20 (HRF20) and membrane attack complex inhibitory factor (MACIF). CD59 is attached to the outer layer of the membrane by a glycosylphosphatidylinositol (GPI) anchor and is very widely distributed (Table 1). It has also been detected in a number of body fluids, but often in association with lipoproteins or small membrane vesicles, and has probably been shed from cell surfaces

with its anchor intact or released enzymatically (Table 1). There is no evidence for a secreted form of the protein. The molecule is now extremely well-characterized and species homologs are known in a variety of primates as well as the rat, sheep, pig and mouse. Functionally analogous proteins have also been described in some microorganisms.

CD59 inhibits the formation of the MAC by binding to sites on C8 and C9, thus blocking the uptake and incorporation of multiple C9 molecules into the complex. CD59 from any given species interacts with C8 and C9 from heterologous species with variable efficiency, working best with homologous proteins.

Table 1 Cells and tissues expressing CD59

<i>Liver</i>	<i>Pancreas</i>
Bile canaliculi	Ductal epithelium
Bile duct	<i>Peripheral nervous system</i>
<i>Lung</i>	Schwann cells
Bronchi and bronchioles	Endothelial cells
<i>Kidney</i>	Neurons
Glomerular capillaries	<i>Eye</i>
Glomerular epithelial cells	Lacrimal gland
Distal tubules	Acinar cells
Collecting ducts	Retinal pigment epithelium
<i>Central nervous system</i>	Cornea and conjunctiva
Myelin	<i>Body fluids</i>
Microglia	Serum (low levels)
Neurons	Urine
Oligodendrocytes	Milk
Astrocytes	Seminal plasma
<i>Neoplastic cells</i>	Cerebrospinal fluid
Melanoma	Saliva
Malignant glioma	<i>Other</i>
Colonic adenocarcinoma	Hematopoietic cells
Thyroid carcinomas	Vascular endothelium
<i>Reproductive system</i>	Prostatic epithelium
Unfertilized oocytes	Nasal epithelium
Preimplantation embryos	Chondrocytes
Amniotic epithelium	Salivary duct
Syncytiotrophoblast	Thyroid follicular cells

This forms the basis for the phenomenon of 'homologous restriction' of lysis, a term used to describe the relative inefficiency with which complement lyses homologous cells. The physiological importance of the complement inhibitory role of CD59 is demonstrated in the acquired hemolytic disease paroxysmal nocturnal hemoglobinuria (PNH; see below), in which affected cells are deficient in CD59 and other complement regulatory proteins and are extremely sensitive to lysis. Roles for CD59 in T cell adhesion and activation have also been proposed but not firmly established.

Protein and gene structure

cDNAs for CD59 have been isolated by several independent groups. The nucleotide sequence encodes a precursor polypeptide of 128 amino acids in length, the first 25 residues constituting a signal peptide. The sequence contains one site for N-linked glycosylation at Asn18, and the carbohydrate attached here is a common biantennary structure accounting for approximately 25% of the molecular weight of CD59. At the C-terminus there is a hydrophobic stretch of residues characteristic of proteins which are processed to carry a GPI anchor. The presence of such an anchor in the case of CD59 was originally

confirmed by release of the protein from various cell surfaces by phosphatidylinositol-specific phospholipase C. Subsequently, protein sequencing of tryptic fragments and nuclear magnetic resonance (NMR) studies of the protein purified from human urine have indicated that the GPI anchor is attached to Asn77. The protein contains a total of 10 cysteine residues which form disulfide bonds as follows: Cys3-26, Cys6-13, Cys19-39, Cys45-63, Cys64-69.

CD59 is unique in the complement control protein superfamily in that it bears no structural or immunologic relationship with other member proteins, although these are distinctly related to one another and encoded by genes which are situated together in the RCA (regulators of complement activation) region on chromosome 1. The gene for CD59 is in contrast located on the short arm of chromosome 11, distributed throughout over 27 kb of genomic DNA. Although initial studies identified four exons, a fifth alternately spliced exon has recently been discovered between what were originally considered to be exons 1 and 2. The transcriptional start site for both forms of mRNA is the same. Since the newly identified exon occurs within the 5' untranslated region of the CD59 transcript there is still no evidence as yet to suggest that there are alternative forms of the mature protein. Both types of transcript are expressed concordantly in all the cell types analyzed so far, and their expression appears to be regulated similarly. The purpose of the alternately spliced exon is therefore not known.

The structure of the CD59 gene is similar to that of the murine Ly-6 antigens and of the human urokinase plasminogen activator receptor (HUPAR). These and other proteins such as the mouse thymocyte and B cell antigen ThB, and the squid glycoprotein SGP2, constitute a separate family of proteins which share some sequence homology (in particular, conserved cysteine residues) and therefore some structural similarity. CD59 has on occasion been proposed as the human homolog of the murine Ly-6 antigens, despite only 25% sequence identity between the two, and differences in the pattern and regulation of gene expression. However, the recent cloning of mouse CD59 clearly demonstrates that CD59 and Ly-6 are distinct proteins.

Inhibition of complement lysis

Mechanism of action

CD59 is the major cell surface inhibitor of the complement membrane attack complex (MAC), a multi-molecular assembly of complement components C5 to C9 which forms as a consequence of complement activation. At the C5b-7 stage of assembly, a labile

binding site is exposed on C7 which allows the growing MAC to bind cell membranes; then as C8 and multiple copies of C9 are incorporated, the complex inserts further into the membrane and finally forms a functional pore through the bilayer, leading to cell lysis. CD59 prevents lysis by interfering with the assembly of a fully functional MAC, binding to C8 in the C5b-8 complex and thereby blocking the uptake and polymerization of C9 (Figure 1). Binding sites for CD59 are located on the α subunit of C8 and on the C9b fragment (the putative membrane-binding domain) of C9. However, the regions on CD59 which are involved in this interaction have not yet been fully elucidated. Studies using proteolytic fragments of the protein implicate sites in the N-terminal region. Some early observations that deglycosylated CD59 did not inhibit cell lysis suggested that the carbohydrate moiety was also important for function, although paradoxically the deglycosylated CD59 retained its ability to bind to isolated C8 and C9 immobilized on plastic. One possible explanation for this is that the presence of the carbohydrate moiety somehow influenced the orientation of CD59 on the cell surface and thereby influenced its interaction with the MAC, without being directly involved in the binding of C8 and C9. Subsequent studies in which the site of N-linked glycosylation was removed from the CD59 cDNA by site-directed mutagenesis have demonstrated that the carbohydrate is not necessary for complement inhibitory activity.

CD59 deficiency and paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic disease characterized by bouts of intravascular hemolysis. The disease is caused by somatic mutation in a pluripotent stem cell of the hematopoietic system, leading to the appearance in the circulation of a clone of cells exhibiting increased sensitivity to complement lysis. Depending upon the point in the lineage at which mutation occurs, erythrocytes, granulocytes, monocytes, platelets and lymphocytes may all be affected. Cells in the affected clone are abnormally sensitive to complement because they fail to express GPI-anchored proteins on their surfaces and therefore lack CD59 and decay accelerating factor (DAF), another complement regulatory protein. The defect in PNH arises from mutation of the PIG-A gene whose product is an enzyme involved in the biosynthesis of the GPI anchor. Various mutations in PIG-A have been identified, and since PIG-A is carried on the X chromosome, loss of function in the single active allele is sufficient for disease to be manifest. There is an isolated case of CD59 deficiency due to a mutation in

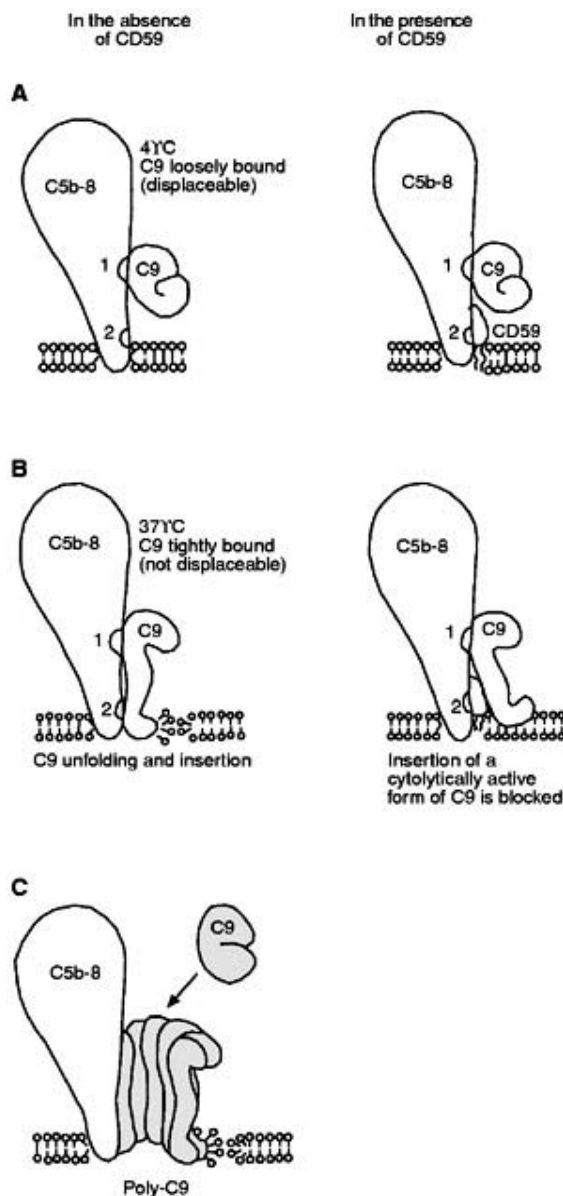


Figure 1 Model for the mechanism of action of CD59. Two putative binding sites on C5b-8 for C9 are shown. C9 binds loosely in its globular form at site 1 and at this stage may be displaced by other C9 molecules; this may be demonstrated experimentally by holding the temperature at 4°C (A). However, at 37°C C9 normally unfolds and interacts with site 2 on the C5b-8 complex (B), begins to penetrate the cell membrane, and exposes sites for interaction with additional C9 molecules which polymerise to form a transmembrane pore (C). In the presence of CD59, the initial interaction of C9 with C5b-8 occurs, but the binding of CD59 to C8 prevents the C5b-8 catalyzed unfolding and insertion of multiple C9 molecules into the membrane, and thereby blocks cell lysis. (Reproduced with permission of Blackwell Science Ltd, from Meri S, Morgan BP, Davies A *et al* (1990) Human protection (CD59), an 18,000–20,000 MW complement lysis restricting factor, inhibits C5b-8 catalyzed insertion of C9 into lipid layers. *Immunology* 71: 1–9).

the CD59 gene. Despite having all the other GPI-anchored proteins, including DAF, the patient with CD59 deficiency suffers from a PNH-like condition characterized by hemolysis. This underlines the importance of CD59 as the major factor for regulation of complement lysis on erythrocytes.

Role in T cell activation

Antibody cross-linking of CD59 on the surface of human T cells induces a rise in intracellular calcium, inositol phosphate production, release of cytokines and cell proliferation. These phenomena occur when any GPI-anchored proteins are cross-linked in the membrane, and the mechanisms by which their assembly in the *outer* layer of the membrane can transmit signals to the *interior* of the cell are currently under investigation. Whether or not the observed effects of experimentally cross-linking CD59 using antibody have any real significance depends upon there being a physiological ligand for CD59. There have been several claims that like CD58 (LFA-3) and CD48, CD59 is a ligand for CD2, a molecule involved in T cell activation, but an equal number of reports dispute this. As yet the role of CD59 in the processes of T cell activation remains equivocal.

Microbial counterparts of CD59

It has been to the advantage of many microbial pathogens to acquire mechanisms for immune evasion. For example, an open reading frame encoding a sequence highly homologous to that of human and various monkey species of CD59 has been identified in the genome of herpesvirus saimiri (HVS), whose natural host is the squirrel monkey. It appears highly likely that the virus has captured this sequence from its host during the course of evolution. Preliminary studies indicate that the sequence does indeed encode a functional protein, so expression of this sequence by HVS could be considered as a virulence factor in aiding the virus to evade the detrimental effects of complement activation. Complement-inhibiting proteins that appear to have some antigenic relationship to CD59 have also been described in *Entamoeba histolytica* and *Schistosoma mansoni*. The *E. histolytica* protein is an integrin-like molecule consisting of two subunits, one of which shares very limited sequence homology with CD59. A cDNA for the *S. mansoni* protein has not yet been isolated. Since many pathogens are adept at avoiding destruction by the complement system, it is highly likely that other

microbial proteins will emerge that are functionally, if not structurally, related to CD59 or the other human complement control proteins.

CD59 transgenics and xenotransplantation

The shortage of suitable donor organs for transplant surgery has led to the proposal that animal organs be used instead. A major problem with this is that of hyperacute rejection, whereby discordant xenografts come under attack within minutes, and are destroyed by complement that has been activated by naturally occurring antibodies in the host that recognize carbohydrate epitopes in the xenograft. A major strategy now being researched to overcome this is the production of transgenic animals (mainly pigs) that carry the genes for human complement control proteins, including CD59. These human proteins are expressed in a wide range of tissues in the transgenic animals, sometimes at very high levels, and have been shown to confer increased protection upon endothelial and other cell types against human complement. Although these results seem very promising, a major drawback to the use of xenotransplantation is the possibility that xenografts may harbor viruses or other microbes that could be extremely harmful to a human host, and this is currently the main objection to proceeding with clinical trials.

See also: CD antigens; CD2; Complement, membrane attack pathway; Decay-accelerating factor (CD55); Xenotransplantation.

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CELL-MEDIATED IMMUNITY

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Cell-mediated immunity is mediated by T lymphocytes; humoral immunity, by antibodies. These distinctions are based on observations that delayed-type hypersensitivity (DTH) and other manifestations of cell-mediated immunity can be adoptively transferred by lymphoid cells but not by serum from sensitized animals, while other manifestations of immunity can be passively transferred by serum from immune animals. Cell-mediated and humoral immunity can exist in isolation: some chemicals induce contact sensitivity, a manifestation of cell-mediated immunity, but do not induce production of antibodies, and some antigens can stimulate antibody production in the apparent absence of T lymphocytes. However, cell-mediated and humoral immunity usually develop concurrently *in vivo*, and the two responses often act synergistically. For example, although DTH can be adoptively transferred with T lymphocytes, the intensity of the reaction is usually greater if antibody is also passively administered. There also are conceptual difficulties inherent in these definitions: antibodies are produced by cells, and cells participate in many of the protective reactions mediated by antibodies. Also, T lymphocytes carry out many of their functions through soluble products which they secrete following stimulation with antigen.

In the early 1880s, Elie Metchnikoff proposed that inflammation had a protective role in responses to pathogens, and that phagocytic cells, rather than being harmful, constituted the primary line of defense through their ability to ingest and digest invading organisms. However, the contrary view, that humoral antibody played the dominant role, was furthered by the demonstration of bactericidal properties of immune serum and the protective effects of antibodies against bacterial exotoxins. Although in the early 1900s the phenomenon of DTH was shown to be unrelated to circulating antibody, it proved difficult to determine with certainty the relative contributions of humoral and cell-mediated immunity until the central involvement of lymphocytes in immune responses was established finally in the late 1950s. In the early 1960s, several serendipitous observations led to the identification of two separate lymphocyte lineages, based on their development pathways: B lymphocytes, which originate in the bursa of Fabricius in birds and in the

bone marrow of mammals; and T lymphocytes, which develop within the thymus from precursors originating initially in the yolk sac and later in the bone marrow.

These two developmentally distinct sets of lymphocytes were soon shown to have different functions: B cells display and secrete antibody molecules which react with intact antigen molecules; T cells react with processed antigen fragments which are associated with molecules of the major histocompatibility complex (MHC). T lymphocytes carry out some functions, such as the lysis of target cells that express the appropriate antigens, through direct cell-to-cell contact, but they exert many of their effector functions through secreted hormone-like proteins, called cytokines, which modulate the activities of various cells, including lymphocytes and phagocytes.

Although the classical manifestations of cell-mediated immunity are phenomena which can be observed only *in vivo*, it is difficult to dissect the cellular and biochemical processes involved in these reactions in animals. Therefore, a variety of responses by antigen-stimulated T lymphocytes have been used as surrogate cell-mediated responses *in vitro*. It is generally assumed that these responses – which include T lymphocyte proliferation, cytolytic activity, ‘helper’ functions, and/or secretion of cytokines – relate to manifestations of cell-mediated immunity observed *in vivo*. However, none of the phenomena of cell-mediated immunity observed in sensitized animals have been characterized fully in cellular and biochemical terms.

Recognition of antigens by T lymphocytes

T and B lymphocytes recognize antigens in fundamentally different ways. Both T and B lymphocytes have extensive antigen-specific receptor repertoires and can react with a very large number of different antigens, each being able to distinguish among more than 10^7 distinct antigenic determinants. However, entirely different genes which are located on different chromosomes are used to construct their cell surface receptors for specific antigens. Nevertheless, they use similar mechanisms to produce their antigen receptors, employing a limited number of gene segments which are combined in many different ways to construct a very large number of conformationally

distinct polypeptides. The T cell receptor (TCR) for antigen consists of a heterodimeric structure, which in most T cells consists of α and β chains. However, a minority of peripheral T cells (approximately 5%) employ a TCR composed of structurally related but distinct γ and δ polypeptides. Such T cells are the dominant population in most surface epithelia in mice. There is generally allelic exclusion in the expression of genes encoding TCR peptides so that a given T lymphocyte expresses only one functional α and one β gene or one γ and one δ gene. The use of α and β or γ and δ chain polypeptides in various heterodimeric combinations contributes to the very large repertoire. Somatic mutation, which contributes to the affinity maturation of antibodies, does not appear to take place in T lymphocytes.

T lymphocytes react with antigen fragments associated with molecules of the MHC expressed by the individual in which the T lymphocytes arose. During the course of maturation in the thymus, T lymphocytes appear to be positively selected for the ability to recognize self antigens, but those T lymphocytes which react too strongly with self antigens appear to be negatively selected and deleted. The vast majority of cells which enter the thymus fail to survive these selection processes and die; probably less than 5% of thymocytes find their way to peripheral lymphoid tissues as mature T lymphocytes.

In addition to rearrangements of genes encoding the TCR and subsequent selection events, maturation of T lymphocytes in the thymus also involves sequential expression of a number of lineage-specific molecules, including CD4 and CD8 which distinguish different T lymphocyte subsets. The most immature cells in the thymus express neither of these cell surface structures and have not rearranged their TCR genes. The TCR is expressed first in cells which express both CD4 and CD8; CD4⁺ and CD8⁺ subsets of T lymphocytes develop from this 'double-positive' population. Expression of CD4 is associated conventionally with 'helper' cell functions and CD8 expression with cytolytic activity, but these distinctions are by no means absolute. Many CD4⁺ T cells have cytolytic capabilities, and at least some CD8⁺ T cells can carry out helper functions. The mutually exclusive expression of CD4 or CD8 correlates better with the class of MHC antigen which serves as the restriction element for antigen recognition than it does with function: CD4⁺ T cells usually recognize antigenic peptides associated with class II MHC molecules, while CD8⁺ T cells are usually restricted to recognition of peptides associated with class I MHC molecules. Both CD4 and CD8 have 'coreceptor' function, probably by bringing the protein tyrosine kinase *lck* into the TCR-antigen complex.

Full activation of CD4⁺ T cells requires two signals: one delivered through TCR, and a second 'costimulatory' signal which is not antigen specific. Stimulation of the TCR alone induces anergy, defined as the long-lived inability to secrete interleukin 2 (IL-2) and proliferate in response to antigenic stimulation. CD28 has been identified as the dominant costimulatory molecule expressed by T cells. While stimulation through CD28 alone has little effect, interaction of CD28 with B7 on antigen-presenting cells (APCs) in conjunction with TCR engagement augments significantly the secretion of multiple cytokines. A full understanding of the phenomenon of costimulation is complicated by the fact that these counterreceptor families have multiple members: CTLA-4, which is structurally similar to CD28, also binds to B7-1 and B7-2, which also are related structurally. These B7 family members are not expressed concordantly on all cell types, and their expression is differentially regulated. While most resting T cells express CD28, CTLA-4 is expressed only on activated T cells. An attractive model proposes that interactions between CD28 and B7-2 provide costimulation for T cell activation, while interactions between CTLA-4 and B7-1, both of which are expressed later in immune responses, terminate ongoing responses.

Several other cell surface molecules are involved in the interaction between T lymphocytes and APCs. CD2, expressed on all human thymocytes and peripheral T cells, interacts with LFA-3, a broadly distributed glycoprotein. The T lymphocyte integrin, LFA-1, interacts with ICAM-1, a heavily glycosylated and widely expressed molecule, and with ICAM-2. There are probably additional cell surface structures which are important in interactions of T lymphocytes with other cells. While these structures enhance cell-to-cell contact, many of them also appear to be involved in cell activation, either participating directly in signaling events or indirectly by modulating, either positively or negatively, signals induced through the TCR.

Manifestations of cell-mediated immunity *in vivo*

Allograft rejection, DTH, and graft-versus-host disease are manifestations of T cell-mediated immunity *in vivo*. In addition, much of the protective immunity to viral and some bacterial infections is also dependent upon cell-mediated immunity. A subset of murine CD4⁺ T cells designated T_H1 appears to mediate DTH, while the T_H2 subset does not. Since these subsets differ in the array of cytokines they secrete, it is likely that DTH is mediated by parti-

cular cytokines. The pathophysiological processes that lead to organ allograft rejection are not well characterized, but it is likely that cytokines play a major role here as well. Mechanisms of allograft rejection depend on the nature of the antigens being recognized as well as the type of graft involved. Antibodies also are produced in response to allografts, and antibody-mediated vascular lesions often are prominent. T lymphocytes presumably can injure allografts of dispersed cells (bone marrow or lymphoid cells) by direct effector mechanisms such as cytotoxicity. T cells can also act indirectly through secreted cytokines (lymphotoxin or tumor necrosis factor) which can themselves directly injure target cells or through effects of cytokines (interferon γ , IFN γ) on macrophages to increase their killing and other activities. Vascularized allografts (kidney, heart and liver) can be rejected through effects on blood vessels rather than through primary injury to the parenchymal cells in these organs. Other tissue grafts can be rejected by mechanisms which interfere with revascularization. Although it is clear that T lymphocytes mediate these reactions, details of the cellular and biochemical processes that are involved remain obscure.

Manifestations of cell-mediated immunity *in vitro*

A number of model systems have been studied *in vitro* to evaluate the molecular mechanisms controlling T cell responses; these include proliferation, induction of cytotoxic activity, expression of helper functions for antibody production, secretion of cytokines and biochemical signaling events. Clonal populations of T lymphocytes which retain normal phenotypic characteristics and monoclonal antibodies reactive with different T cell surface structures have greatly facilitated the detailed analysis of these correlates of cell-mediated immunity *in vitro*. Monoclonal antibodies, which react with the TCRs, can serve as surrogate antigens, making it possible to distinguish the effects initiated by stimulation of the TCR from those involving other cell surface structures.

Many of the biological effects of T cells are mediated by secreted cytokines which affect the activities of different kinds of target cells. Different arrays of cytokines are produced by different T cell subsets following stimulation, and regulatory mechanisms that control which cytokines a particular T cell will secrete are beginning to be defined. It is clear that the array of cytokines secreted is of major functional significance. More than 15 different cytokines have been identified and their genes cloned. Their properties have been defined unequivocally, using

products obtained by recombinant DNA technology. Most cytokines act on several different cell types.

Most if not all T lymphocyte proliferation is due to growth factors secreted by T cells. T lymphocytes stimulated with antigen are induced to express receptors for transferrin and for T cell growth factors, and they undergo cell division in response to cytokines having T cell growth factor activity. IL-2 was first identified on the basis of its ability to stimulate proliferation of T cells, and was thought initially to be the only T cell growth factor. Subsequently, however, IL-2 was found to stimulate proliferation of B cells as well. IL-4 is an additional T cell growth factor; it was first identified as a stimulatory factor for B cells. Both IL-2 and IL-4 serve as autocrine growth factors for the T cells which secrete them, and as paracrine growth factors for T cells which do not. IL-4 favors the development of cells that produce IL-4, IL-15, produced preferentially by nonlymphoid cells, also stimulates T cell growth. Uncontrolled growth of T cells that would occur through the autocrine pathway is prevented by regulatory processes which include desensitization of the T cell receptor for antigen, limitation of the period of time that cytokines are secreted, and modulation of the level of expression of growth factor receptors.

Much of the 'help' provided by T lymphocytes for antibody production is mediated by secreted cytokines. However, resting B cells require interactions with cell surface molecules expressed by T cells. The interaction of CD40, expressed constitutively by B cells, with CD40 ligand, expressed by T cells only upon activation, renders B cells responsive to IL-4 and other cytokines. This interaction stimulates the T cell as well. The most efficient help for B cells is provided by the T cell subset designated T_H2 , which secretes IL-4 and IL-5. These cytokines provide optimal B cell proliferation and differentiation. The T_H1 subset, which secretes IL-2 and IFN- γ but not IL-4 or IL-5, also may be able to provide help for B cells. Cytokines influence which antibody isotype is produced: IL-4 is required for immunoglobulin E (IgE) responses, and IFN- γ and perhaps other factors from T_H1 cells favor IgG2a production. In addition to their effects on antibody production by B cells, cytokines secreted by CD4⁺ helper T cells enhance generation of CD8⁺ cytotoxic T cells. Thus, both T_H1 and T_H2 cells appear to be able to serve as helper cells, although they may function in different situations and promote qualitatively different immune responses.

In addition to the antiviral effects that led to its designation, IFN- γ causes increased expression of both class I and class II MHC antigens by macrophages and activation of macrophages for greater

killing of ingested microorganisms. However, IFN- γ also inhibits the effects of IL-4 on B cells and inhibits proliferation of bone marrow cells induced by several other T cell cytokines. IL-6, which is secreted by some nonlymphoid cells, acts as a B cell stimulation and differentiation factor and stimulates the release of acute-phase reactants from liver cells; IL-3 stimulates pluripotential bone marrow stem cells and mast cells. IL-10 inhibits the ability of macrophages to stimulate T_H1 cells. IL-12 increases production of IFN- γ by naive T cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates granulocyte and monocyte precursors. Tumor necrosis factor α (TNF- α) is produced by macrophages as well as T cells; it causes leukocytosis, fever, weight loss and necrosis of some tumors. Lymphotoxin also kills target cells; its gene is closely linked to that for TNF- α , and there is 30% homology in the sequence of amino acids for these two proteins. Most major cytokines have probably been identified. These and other interactions among cytokines are important in the regulation of immune responses.

Although cytolytic activity has usually been associated with CD8⁺ T cells, many CD4⁺ T cells also have cytolytic potential. There are several distinct cytolytic mechanisms. Perforin, a complement-like molecule, is found in intracellular granules in cytolytic T cells and natural killer (NK) cells. Interaction between surface-bound Fas ligand, expressed by activated T cells, with Fas, expressed by some target cells, can also mediate cytotoxicity. Cytokines, such as lymphotoxin or TNF, can kill some target cells. The killing process is unidirectional, and the T cell is not injured by the cytolytic events. NK cells appear to use similar killing mechanisms as CD8⁺ cytolytic T cells. Most NK cells, at least in the human, appear not to be of the T cell lineage; the receptors that NK

cells use to interact with target cells have not been well characterized. Also, NK cells can mediate antibody-dependent cytotoxicity (ADCC); this involves interactions of Fc receptors on NK cells with antibody bound to target cells.

Conclusion

Different T cell subsets have different roles in the various features of cell-mediated immunity, although these roles have not been fully characterized. In several experimental infections, the patterns of disease indicate preferential involvement of one or another T cell subset. Factors which influence the development of the several T lymphocyte subsets are beginning to be understood, and the mechanisms that selectively regulate their responses have been partially defined.

See also: Antibody-dependent cellular cytotoxicity; Helper T lymphocytes; Contrasuppression; Cytokines; Cytotoxic T lymphocytes; Delayed-type hypersensitivity; Effector lymphocytes; Graft rejection; Graft-versus-host reaction; Mononuclear phagocyte system; Natural killer (NK) cells; Perforin; Suppressor T lymphocytes; T lymphocytes.

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CELL-MEDIATED LYSIS

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Cell-mediated lysis (CML) of antigen-bearing target cells was first demonstrated in 1960, when it was shown that thoracic lymphocytes sensitized *in vivo* towards allogeneic kidney cells could, *in vitro*, lyse target cells expressing the appropriate alloantigens. This lytic activity of lymphoid cells from sensitized individuals is believed to be the correlate *in vitro* of corresponding effector functions of lymphoid cells *in vivo*. The destructive potential of lymphocytes from

immunized animals was confirmed subsequently by other investigators. Close cell-to-cell contact was found to be required between the lymphocytes and the target cells. Lysis was shown to be a rapid event which progressed as a linear function of time and depended on the concentration of effector cells. The observations suggested that a 'single hit' was required for inactivation of target cells, i.e. a single effector cell was able to destroy a target cell. These

remarkable early observations were made using model systems that involved relatively difficult and time-consuming methods of surveying target cell death.

The quantification of the lytic potential of lymphocytes was greatly facilitated by the development of convenient and sensitive assays for the measurement of target cell death, using radioactive isotopes to label target cells. One such well-defined system involved the measurement of release of radioactivity from ^{51}Cr -labeled cells lysed by immune lymphocytes. Improved culture systems made possible the detailed investigation of CML. By 1970, it was possible to generate cytotoxic murine and human lymphocytes in unidirectional mixed lymphocyte reaction (MLR). This was achieved by culturing normal lymphocytes with allogeneic lymphoid cells that had been rendered unable to divide by treatment with X-rays or mitomycin C. The reproducible populations of effector cells generated in MLR greatly facilitated the analysis of the genetic basis of CML and the investigation of the mechanism(s) of lysis. In addition, the MLR provided the fundamental techniques that enabled the derivation of clonal populations of effector lymphocytes. Homogeneous populations of lymphocytes allow specific functions to be associated with a particular cell type, providing the means to study the molecular basis for CML specificity, cell adhesion events, activation, and lytic mechanisms.

Effector cells capable of mediating lysis

With reproducible methods of generating effector cell populations and assessing target cell death, thymus-derived (T) lymphocytes were identified as the principal effector cells involved in antigen-specific CML. T lymphocytes have been divided functionally into helper T lymphocytes (T_H) or cytolytic T lymphocytes (CTLs) based on the apparent predominant function of these cells. However, recent studies indicate that there is considerable functional overlap among these populations. The capacity to demonstrate the lytic potential of a given lymphocyte population depends in part on the nature of the target cells used. Other T cell populations, whose recognition mechanisms are less well characterized than those of $CD4^+ T_H$ and $CD8^+$ CTL, are also capable of mediating lysis. These include the $CD4/CD8$ double negative $\alpha\beta$ TCR-positive T cells and the $\gamma\delta$ TCR-positive T cells.

Lymphoid cells other than T lymphocytes can also display lytic activity against certain target cells. Natural killer (NK) cells are lymphoid cells that have the capacity to lyse certain transformed target cells

such as K562 (human) or YAC-1 (murine) by a process that does not involve MHC restriction. NK activity occurs in cells having the morphology of large granular lymphocytes (LGLs); these cells express distinctive cell surface markers but they are not well defined with respect to their lineage. Natural killer lysis involves events similar to those that have been identified in antigen-specific lysis by CTLs, and may involve similar mechanisms. NK cells bear receptors for the Fc portion of immunoglobulin G (IgG); these receptors allow NK cells to lyse antibody-coated target cells by a process referred to as antibody-dependent cellular cytotoxicity (ADCC). Cells having properties that are distinguishable from those of NK cells and CTLs, termed killer (K) cells, also function as effector cells in ADCC. Finally, lytic activity can be induced in lymphoid cells in culture by exposure to supraoptimal concentrations of interleukin 2 (IL-2), which leads to the induction of lymphokine-activated killer (LAK) cells.

Properties of cell-mediated lysis

The process of CML involves the close interaction of cytotoxic lymphocytes with appropriate 'target' cells in a way that results in the unidirectional lysis of the target cell. It has been divided into several discrete stages: 1) recognition of the target cell by the effector cell, and the formation of a magnesium-dependent strong adhesion between the effector and target cells; 2) the 'programming for lysis' or 'delivery of the lethal hit'; 3) target cell lysis which does not require the continued presence of the effector cell; and 4) recycling of the effector cell.

CML is best characterized in systems involving antigen-specific T lymphocytes and, to a lesser extent, NK or ADCC effector cells. Initial cell contact between lymphocytes and target cells appears to be a random event. Once this has occurred, effector cells can form a close association or conjugate with target cells. Several cell surface structures have been implicated as mediating or contributing to cell adhesion, based on the capacity of specific antibodies to inhibit effector-target conjugation or lysis. $CD11a/CD18$ (LFA-1) and $CD2$ (LFA-2), which are expressed on lymphocytes, and $CD54$ (ICAM-1) and $CD58$ (LFA-3), which are expressed on target cells, represent adhesion molecules involved in interactions between immune cells and their targets. Other structures, including $CD8$ and $CD4$, may contribute to cell adhesion between T cells and target cells bearing class I or class II major histocompatibility complex (MHC) molecules, respectively. However, $CD4$, $CD8$ and $CD11a/CD18$ also may be involved in the regulation of signaling through the T cell receptor (TCR).

A similar role has been postulated for CD2 which reacts with CD58 expressed on target cells. Other receptor–ligand interactions are involved in the generation of CTLs from nonlytic precursors. Interactions between CD28 or the related CTLA-4 molecule on small resting T cells with CD80 (B7 or BB1) or CD86 (B70 or B7-2) on the antigen-presenting cell provide costimulatory signals for activation through the antigen receptor complex. However, CD28 costimulation does not appear to be essential for the effector phase of CTL function.

The recognitive events associated with CTL–target cell interaction is mediated through the clonally distributed TCR. In both the human and the mouse, the predominant TCR structure is that of an $\alpha\beta$ heterodimer, which has variable regions similar to but distinct from those of Ig molecules. Cells expressing the alternative, $\gamma\delta$ TCR structure have also been shown to function as lytic effector cells. The precise mechanism by which the lethal hit is triggered is not known. The TCR is physically associated on the cell surface with the CD3 complex, which has been identified as the primary structure involved in transmembrane signaling. The role of the TCR–CD3 complex in triggering the lethal hit comes from studies showing that soluble anti-TCR–CD3 monoclonal antibodies (mAbs) can inhibit lysis, while anti-TCR–CD3 mAb immobilized on the surface of target cells can induce lysis of those targets. The combination of calcium ionophore and phorbol esters (capable of activating protein kinase C) can mimic the activation via the TCR–CD3 complex. This treatment induces the release of CTL granule-associated molecules and triggers lysis of target cells nonspecifically.

Mechanisms of target cell destruction

No single mechanism seems to account for all of the events associated with target cell destruction observed in short term cell-mediated lytic assays. Membrane damage, through a process which requires extracellular calcium, has been implicated as an effector mechanism in nearly every form of CML, but the lysis of nucleated target cells also involves apoptosis. Two cell-mediated cytolytic pathways have been identified and molecularly defined. One pathway involves membrane damage inflicted on the target cell by the regulated release of pore-forming proteins by the killer lymphocyte, leading directly to the death of the target cell. A second pathway involves interaction of membrane proteins on the effector cell and the target cell which provides a signal leading to apoptosis in the target cell.

Membrane damage (perforin/exocytosis) pathway of lysis

Evidence that membrane damage may play a role in the lysis of target cells came initially from morphological studies which indicated that target cells swell and burst after contact with lymphoid cells. These observations led to the proposal that death resulted from ‘colloid osmotic’ forces due to the influx of water through the lesions. Direct evidence for the formation of ‘pores’ was provided by electron microscopy of erythrocyte ghosts following lysis by lymphocytes in an ADCC reaction, and these findings were extended to cloned CTL and NK cells. Enucleated target cells are susceptible to CML events including the release of ^{51}Cr , and anucleate erythrocytes coated with anti-TCR–CD3 mAb can be lysed by conventional CTL. Collectively, these and other data suggest that membrane damage can occur as a result of the interaction between at least some cytotoxic lymphocytes and target cells during CML. A complement component C9-like molecule, perforin, is present in the granules of CTL and NK cells. Stimuli that trigger lysis by CTL also trigger the exocytosis of granule-associated serine esterase activity. A model has been proposed in which TCR cross-linking induces the activation of protein kinase C and increased intracellular levels of calcium, which in turn leads to CTL granule exocytosis. Calcium appears to be required for exocytosis and is absolutely required for the perforin pathway. However, certain target cells can be lysed by CTL in the apparent absence of extracellular calcium or exocytosis of granular material. These findings suggested that the perforin/exocytosis pathway was not the sole mechanism of CML.

Internal disintegration (Fas) pathway of lysis

CTLs induce rapid DNA damage in target cells that is not observed in complement-mediated or perforin-mediated lysis of cells. Based on this observation, an ‘internal disintegration’ model has been proposed in which CTLs induce target cell endonucleases which digest DNA, yielding 150–180 base pair units. This process can occur in the absence of extracellular calcium.

Perforin-deficient mice have been generated using gene targeting techniques. They provide a direct assessment of the role of perforin *in vivo*. Lymphocyte development is unaffected in mice lacking perforin. Perforin-dependant cytotoxicity appears to be required to clear at least some pathogens *in vivo*. Perforin-deficient mice are unable to clear lymphocytic choriomeningitis virus, but were able to control vaccinia virus infection, although both induce strong

primary CTL responses. CTLs generated *in vivo* or *in vitro* in response to these stimuli are highly lytic toward antigen-bearing target cells that express the Fas antigen (CD95), but not against target cells which do not express Fas antigen. Studies using perforin-deficient mice have provided direct evidence for Fas-mediated pathway of lysis, which requires the expression of Fas antigen on the target cell, and involves DNA damage in target cells. The Fas ligand (FasL), is expressed on CTLs. It is currently thought that Fas-dependent cell-mediated cytotoxicity requires the interaction of the FasL on the effector cell and Fas on the target cell (perhaps inducing Fas trimerization) which triggers an, as yet poorly defined, cell death signal pathway. T cell activation can induce FasL expression, but the regulation of FasL expression is incompletely understood.

CD4⁺ as well as CD8⁺ T cells can lyse target cells bearing relevant antigen; however, not all T cells use the same lytic mechanisms. The perforin/exocytosis pathway is generally expressed by CD8⁺ CTLs and some CD4⁺ T_H2 cells, but not by CD4⁺ T cells having properties of T_H1 cells. The Fas pathway of lysis appears to be expressed by both CD4⁺ and CD8⁺ T cells.

Although perforin/exocytosis and Fas pathways seem to account for most, if not all, CML observed in short-term assays, lymphocytes produce several factors that are cytotoxic. Tumor necrosis factor α (TNF α), lymphotoxin (LT) and TNF α /LT-like molecules such as NK cytotoxic factor (NKCF) are potential effector molecules. In susceptible target cells, LT-containing supernatants produced by CTLs can induce DNA fragmentation, and LT-like molecules have been found to be associated with granules; LT-like molecules are produced by CTLs and NK, T_H and mast cells. Proteolytic enzymes have also

been implicated in the lytic activity of CTLs and NK cells and in ADCC, based on the observation that various protease inhibitors inhibit lytic activity. CTLs from mutant mice lacking the neutral serine protease, granzyme B, are able to induce ⁵¹Cr release from target cells with reduced efficiency, but are profoundly deficient in their ability to induce rapid DNA fragmentation. The role of these enzymes in lysis is not clear; however, they may be cosecreted with other granule-related molecules to induce or enhance DNA fragmentation.

Conclusion

Cell-mediated lysis is clearly a complex phenomenon that involves distinct mediators, which are either released through the process of exocytosis or are expressed on the cell surface. Those mediators which are critical to a particular form of lymphocyte-mediated lysis may depend on the type of effector cell involved and also on properties of the target cell. The identification of specific molecular mechanisms of cell mediated lysis *in vitro* should facilitate the assessment of their *in vivo* relevance.

See also: Adhesion molecules; Antibody-dependent cellular cytotoxicity; Cytotoxic T lymphocytes; Cytotoxicity, assays for; Cytotoxicity, mechanisms of; Lymphokine-activated killer (LAK) cells; Mixed lymphocyte reaction (MLR); Natural killer (NK) cells; Perforin; Tumor necrosis factor α ; Lymphotoxin.

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CELL SEPARATION TECHNIQUES

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The past three decades have witnessed major progress in the development of methods allowing the identification and separation of various cell populations. This has had a major impact upon the precise dissection of the immune system and the definition of the cellular basis of the immune response. The combined use of newly identified cell surface markers and cell separation techniques has been fundamental

for the precise assignment of given functional properties to well-defined cell subsets.

The first cell separation techniques, which were developed in the early 1960s, were based on physical differences such as cell size and density. The limitation of these methods is mostly related to the low degree of physical heterogeneity existing among lymphocytes. However, this approach may be parti-

cularly useful when combined with techniques which make use of phenotypic markers to tag the cells so that they can be separated by physical means (e.g. rosetting techniques). Major advances in cell separation techniques were made possible by the identification of membrane receptors or surface antigens which are differently expressed by lymphocyte subsets. For example, the identification of immunoglobulin (Ig) molecules on the surface of B lymphocytes and of receptors for sheep erythrocytes on human T cells allowed the identification and the separation of B and T lymphocytes, respectively. More recently, the development of monoclonal antibody (mAb) technology has provided a potent tool to precisely and reproducibly identify lymphocyte subsets expressing given antigens. Most of the mAb-defined surface leukocyte antigens have been classified into a still growing list of 'cluster of differentiation' (CD) antigens. Therefore, at present, the most widely applied lymphocyte separation techniques are represented by immunoselection procedures based on the use of mAbs. Among immunoselection techniques, the use of mAbs and complement (immunotoxicity) allows the selective depletion ('negative selection') of cells expressing a given surface antigen. Other immunoselection techniques, however, allow both 'negative' and 'positive' selection. Most of these techniques are based on the attachment of antibodies to different substrates or carriers, including plastic surfaces, erythrocytes or magnetic beads. Cells that are recognized by these mAbs and thus bind to these substrates can be subsequently separated by adherence, density gradients or magnetic fields, respectively. There is little doubt that the most precise and objective tool for the separation of cells stained with fluorochrome-labeled mAbs is represented by the fluorescence-activated cell sorter (FACS). A major advantage of FACS fractionation over the other immunoselection procedures is represented by the possibility of precisely defining the density of a given surface antigen and, therefore, the threshold values for cell separation. In addition, the FACS allows simultaneously analysis of several parameters and the separation of cells accordingly. In spite of these major advantages, a limitation of the FACS is related to the relatively low numbers of cells that can be processed per unit of time.

When approaching a problem of lymphoid cell separation, the choice of the technique to be applied should take into account a number of parameters, including cell numbers, purity and yield. In most instances, cell purity is inversely related to the yield. Less purity should also be expected when the cells of interest represent a minor fraction of the initial population. In addition, the possible consequences of

cell manipulations on sterility, cell viability and function should be carefully considered. In some instances it may be convenient to combine two methods, for example a rosetting technique (which allows the processing of large numbers of cells) followed by FACS sorting (to obtain a high degree of purity).

The principal methods of lymphoid cell separation are listed in Table 1 and are described below.

Techniques based on physical properties of the cells

The two procedures that have been most widely applied for lymphocyte separation based on physical differences are sedimentation separation and density gradient separation.

In *sedimentation separation* (or velocity sedimentation), cell suspensions are stratified in a thin layer on top of a fluid column contained in appropriate sedimentation chambers. Cells are allowed to sediment through the fluid under the action of gravitational forces (1g sedimentation). Alternatively, to speed up the procedure, larger forces can be applied by the use of centrifugation. To this end, a special device has been developed, the centrifugal elutriator, which allows the purification of larger numbers of cells within a short time. In sedimentation separation, cells are fractionated primarily on the basis of differences in size, while the cell density has a minor influence on the sedimentation rate. After appropriate time intervals cell fractions can be collected through the bottom of the sedimentation chambers. Clearly, cells collected first are those of a larger size.

Techniques based on *density gradient separation* are widely employed and many different procedures have been developed. These techniques are based on the preparation of gradients of appropriate density which may be either discontinuous or continuous. In principle, the material composing the gradient should be nontoxic for the cells. In the case of continuous gradients, cells are usually layered on top of the gradient (in some instances, however, it may be convenient to load cells on the bottom, or disperse them within the gradient). The range of densities of the gradient should cover the densities expected for the cells of interest. The gradient is centrifuged for a time sufficient to allow cells to reach an equilibrium in the portion of the gradient corresponding to their density. Cells of density greater than that of the gradient will sediment at the bottom of the tube. For lymphoid cell separation it should be noted that the differences in density among cell subsets are small, ranging between 1.05 and 1.10 g ml⁻¹. The materials originally employed for continuous density gradients

Table 1 Principal methods of cell separation

<i>Principal</i>	<i>Method</i>	<i>Applications</i>
Physical differences	Sedimentation Density gradient centrifugation	Cell separation according to size Cell separation according to density
Functional properties	Adhesion to plastic Adhesion on nylon wool columns Phagocytosis of iron particles 'Hot' thymidine pulse Selective cell stimulation and growth	Positive or negative selection of macrophages Isolation of T lymphocytes Depletion of macrophages Depletion of proliferating cells Enrichment of proliferating cells
Expression of surface receptors	E (erythrocyte) rosettes EA (erythrocyte-antibody) rosettes EAC (erythrocyte-antibody-complement) rosettes	Positive or negative selection of T cells: Fc γ -receptor ⁺ cells Complement receptor ⁺ cells
Expression of surface antigens (immunoselection)	Immunotoxicity (complement depletion) Immunoaffinity columns Immunoadherence (panning) Rosetting with antibody-coated erythrocytes Immunomagnetic beads Immunofluorescence and FACS fractionation	Depletion of cells expressing a given surface antigen Negative or positive selection of cells expressing a given surface antigen

included bovine serum albumin (BSA) and, more recently, colloidal silica. In discontinuous density gradients, which may be either single or multiple step, cell suspensions are layered on the top of the gradient and centrifuged until equilibrium is reached. Cells will not move into the portion of the gradient having a higher density than that of the cells and will thus accumulate at the interface. The most widely used materials for discontinuous gradients are Ficoll-Hypaque, BSA or colloidal silica. The Ficoll-Hypaque solution used for human lymphocyte separation is an isotonic solution of sodium metrizoate and Ficoll with a density of 1.077 g ml^{-1} . Ficoll-Hypaque gradients are primarily used for the isolation of mononuclear cells (lymphocytes and monocytes) from blood and for the isolation of lymphocytes forming rosettes with erythrocytes. Diluted blood (1:2–1:4) is layered on the top of Ficoll-Hypaque and then spun on a centrifuge. After a suitable time interval, mononuclear cells can be collected at the interface, while erythrocytes and neutrophils are found at the bottom of the tube. Percoll is a commercially available solution composed of colloidal silica coated with polyvinylpyrrolidone in order to render the material nontoxic for cells. Percoll is characterized by high density (1.130) and low osmolality; as a consequence, for the preparation of Percoll density gradients for cell separation, the osmolality is adjusted to physiological values by mixing 9 parts of Percoll solution with 1 part of $10\times$ saline. Percoll solutions with different densities can be easily obtained by appropriate dilutions in isotonic

medium, thus allowing the preparation of multiple step discontinuous gradients; these are particularly useful for the isolation of different subsets of lymphocytes characterized by density differences (e.g. isolation of 'large granular lymphocytes' (LGL) from a lymphoid suspension or isolation of activated blasts from resting lymphocytes).

Techniques based on functional properties of the cells

Adhesive or phagocytic properties

Certain mononuclear cell types have the property of adhering to plastic surfaces and/or to nylon wool. Thus, cells of the monocyte/macrophage lineage rapidly (30–60 min) adhere to plastic when incubated at 37°C . This property has been widely exploited for both positive and negative selection of monocytes from peripheral blood-derived mononuclear cells. The preferential ability of monocytes and B lymphocytes to adhere to nylon wool columns has been frequently applied to remove these cells from mononuclear cell suspensions and, thus, to enrich for T lymphocytes. The demonstration that subsets of interleukin 2 (IL-2)-activated effector cells capable of lysing fresh tumor cells (i.e. lymphokine-activated killer (LAK) cells) display the ability to adhere to plastic (adherent LAK) has provided a useful tool for enriching for these cells. Another method frequently employed for removal of monocytes is based on the fact that monocytes display phagocytic properties:

cells that have ingested iron particles (carbonyl iron) are removed by application of a magnetic field.

Selective depletion of cells undergoing proliferation

Lymphocytes undergoing proliferation induced by antigens or mitogens actively incorporate ^3H -thymidine [^3H]TdR and their DNA. Beside the conventional measurement of cell proliferation, [^3H]TdR with a higher specific activity can be used to induce selective killing of proliferating cells. If the amount of [^3H]TdR incorporated in the cDNA is sufficiently high, intranuclear irradiation due to β -particle emission will result in cell death. The addition of cold TdR will prevent further incorporation of [^3H]TdR. This technique allows, for example, the selective elimination of T cells proliferating in response to soluble antigens or alloantigens. An alternative procedure is based on the use of 5-bromodeoxyuridine (BrdU) followed by exposure of cells to ultraviolet (UV) light. When activated by UV light the BrdU incorporated into the DNA of proliferating cells will cause breaks in the DNA, resulting in cell death.

Selective cell stimulation and growth

Another approach to the selection of given lymphocyte populations is based on the ability of these cells to respond selectively to appropriate mitogenic stimuli in culture. For example, superantigens or mAbs specific for a given TCR- V_β will induce a selective activation and proliferation of T cells expressing the appropriate V_β . In addition, the use of recombinant cytokines acting as growth/differentiation factors may induce the preferential expansion of given subsets. For example, IL-4 will induce a preferential growth/differentiation towards T_H2 cells. On the other hand, peripheral mononuclear cells, in the presence of both IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) will lead to proliferation and differentiation of immature dendritic cells, which can be induced to a more mature phenotype by subsequent addition of, for example, tumor necrosis factor α .

Rosetting techniques

Rosetting techniques are widely employed in immunology for the detection or purification of cells expressing a given surface 'receptor' of antigen. The principle of this separation technique is the formation of cell agglutinates (rosettes) with a consequent change in size or density of the rosetting cells with respect to nonrosetting cells. A common approach is the use of erythrocytes which spontaneously bind to

certain lymphocyte subsets (e.g. sheep erythrocytes binding to human T cells via CD2 surface molecules). Other erythrocytes which do not spontaneously bind to human lymphocytes can be coated with appropriate ligands or mAbs which allow binding to specific receptors or surface antigens. Whatever the rosetting technique employed, the cell suspension containing rosetting cells can be subjected to either sedimentation or, more frequently, density gradient separation. Rosettes formed with erythrocytes can be easily separated from nonrosetting cells by Ficoll-Hypaque gradients and recovered from the bottom of the gradient. Rosetting cells can be freed from erythrocytes by lysis in appropriate buffers containing ammonium chloride or using distilled water.

Immunoselection techniques

These techniques make use of antibodies (either polyclonal antisera or preferably mAbs) directed to surface antigens expressed by given lymphocyte subsets. Both direct and indirect immunoselection procedures have been applied. Direct methods require a single step (i.e. only the antibody directed to a relevant antigen is needed), while indirect methods require a second reagent (i.e. an antiserum directed to the first antibody). The advantages of direct methods include a greater speed and a better specificity, since possible reactions of the second (anti-Ig) reagent with cell surface antigens are avoided. However, indirect methods may be preferable due to their sensitivity (amplification given by the second antibody) and the use of less reagent (e.g. mAbs). In addition, indirect procedures may be more convenient when several different first-step antibodies requiring a common second reagent are used.

Immunotoxicity

In these procedures, antibodies are used as a means to induce selective cytolysis of cells expressing a given antigen at the cell surface. This can be achieved either by coupling mAbs to cytotoxic reagents (immunotoxins) or by the addition of complement. These procedures (particularly the complement depletion that has been widely applied in both basic and clinical immunology) have the major advantage that large numbers of cells can be processed. For example, complement depletion has been used in *in vitro* studies aimed at establishing whether a given function is associated with a phenotypically defined cell subset. It should be noted that the inhibition of a cell function following depletion of a lymphocyte subset may be consequent either to the elimination of cells directly responsible for that function or to removal of cells with a regulatory role on the func-

tion itself. An important clinical application of complement depletion has been the removal of T lymphocytes from bone marrow prior to allogeneic transplantation. Both polyclonal antisera and mAbs can be used as a source of antibody to be used in complement depletion. However, not all antibody isotypes are capable of complement fixation and this should be taken into account when using mAbs: only murine IgM, IgG2a and IgG2b work in this procedure. Regarding the source of complement, rabbit or guinea pig serum is routinely used, either fresh, lyophilized or stored at -80°C . It should be stressed that sera may contain 'natural' antibodies reacting with the cells of interest, thus leading to complement activation and cell lysis even in the absence of test antibody. This problem must be avoided by carefully selecting sera on the basis of low 'natural' antibody titers. In addition, natural antibodies (e.g. antihuman) can be removed by adsorbing sera with appropriate cells (e.g. human erythrocytes) at 4°C . The procedure for complement depletion involves a first step in which cell suspensions are incubated for 30–60 min at 4°C with an appropriate dilution of the relevant antibody. Subsequently, cells are washed and, after the addition of appropriate amounts of complement, they are incubated at 37°C for at least 1 h. The first step (binding of antibody to cells) must be carried out at low temperature in order to avoid antibody-mediated surface antigen capping and modulation, which would prevent optimal complement fixation and cell lysis. An approximate estimation of the efficiency of cell lysis can be obtained by dye-exclusion viability tests (e.g. trypan blue). Dead cells can be further removed by density gradient separation.

Immunoadhesion

In these procedures, antibodies directed to cell surface antigens are bound to a solid support, so that cells expressing the relevant antigen can be easily separated by adherence to the support itself. The solid support is represented by either plastic surfaces (e.g. Petri dishes) or beads. The latter have been used to prepare immunoaffinity columns for cell separation. These columns have been primarily employed for negative selection of cell populations, as the recovery of cells sticking to the columns is difficult and results in poor cell yield and viability. In contrast, immunoadherence to plastic surfaces (panning) allows both positive and negative cell selection. Attachment of the antibody to the solid support can be achieved either by a chemical covalent link or by physical adsorption on appropriate hydrophobic plastic surfaces. In the case of panning, the antibody ($5\text{--}50\text{ }\mu\text{g ml}^{-1}$) can be easily attached to untreated

polystyrene Petri dishes by adsorption using alkaline buffers (e.g. 50 mM TRIS pH 9.5). After overnight incubation the unbound antibody is washed out and free adsorption sites of the plastic are saturated by incubation with a protein solution (e.g. BSA or fetal calf serum, FCS). For lymphocyte fractionation, cell suspensions are layered in a small volume on Petri dishes and incubated at 4°C in the presence of low protein concentration (e.g. 2% FCS) for 1 h. Incubation at low temperature is required in order to prevent nonspecific binding of cells to the plastic. The occurrence of cell adherence to Petri dishes during the procedure can be conveniently checked by microscopic examination. At the end of the incubation period, the nonadherent cells are gently removed by pipette, followed by washing. The adherent cells, expressing the relevant antigen, are recovered after vigorous pipetting or by scraping with a 'rubber policeman' (a rubber-covered glass rod).

Immunomagnetic beads

In this immunoselection technique, antibodies are attached to iron-containing microspheres ($2\text{--}5\text{ }\mu\text{m}$ diameter). Polystyrene microspheres obtained by polymerization in the presence of colloidal iron are now widely employed. The antibody is attached to the beads either by physical adsorption or by chemical covalent links. Either antibodies directed to relevant cell surface antigens or anti-Ig (second) antibodies can be linked to the beads for direct or indirect separation techniques, respectively. In the first approach, the cell suspension is allowed to interact directly with the microspheres; in the indirect approach, lymphocytes are first stained with the relevant mAb and subsequently mixed with the anti-Ig covered beads. In both cases, antigen-positive cells will form rosettes with the beads. Rosetting cells can be easily separated from other cells by applying a magnetic field for a short time (1–2 min). Both negatively and positively selected cells can be utilized for further analysis or cell culture. In general the magnetic bead:lymphocyte ratio should be higher in the case of negative selection. Release of magnetic beads from rosetting cells can be achieved by 24–48 h incubation of cells at 37°C or by the addition of an excess of soluble antibody, displacing the beads-bound antibody. Free beads can then be removed by magnetic separation. Magnetic beads have been successfully used both for fractionation of lymphoid subsets and for 'purging' bone marrow to remove tumor cells or T lymphocytes prior to transplantation.

Magnetic cell sorting

Magnetic cell sorting represents an interesting evolution of immunomagnetic separation techniques. The

principle is based on the use of mAbs or other ligands (e.g. lectins) which specifically bind to surface antigen/receptor expressed by a given cell subset. These reagents are labeled with microbeads (5×10^2 -fold smaller than eukaryotic cells). The cell suspension treated with the magnetically-labeled mAbs are passed through a separation column which is placed in a potent magnetic field. Unlabeled cells pass through the column and can be collected at the bottom, while labeled cells are retained on the column. In order to recover the labeled subset of cells the column is removed from the magnetic field, thus permitting the labeled cells to be eluted from the column. Both direct or indirect labeling can be used with this technique and it is possible to separate a relatively large number of cells in a short time.

Separation by fluorescence-activated cell sorter (FACS)

Flow cytofluorimetry has provided a precise and objective means of quantifying both the number of cells expressing a given surface marker and the extent to which the marker is expressed. By combining this technology with mAbs directed to lymphocyte surface antigens it is now possible to define and purify lymphocyte subpopulations with a precision that was not possible using other methodologies.

Lymphocytes to be analyzed and/or separated by FACS must be labeled with fluorochrome-conjugated antibodies using either direct or indirect immunofluorescence techniques. The FACS allows purification of both antigen-positive/negative subsets and also allows the definition and separation of subsets on the basis of multiple parameters.

See also: Antigen-specific cells, enrichment and isolation; Hematopoietic stem cell transplantation; CD antigens; CD2; Complement, membrane attack pathway; Cytotoxicity, assays for; Flow cytometry; Immune response *in vitro*; Lymphocytes; Lymphokine-activated killer (LAK) cells; Monoclonal antibodies (mAbs); Phagocytosis; Proliferation, lympho-

cyte; Rosetting techniques; Viability, methods of assessing leukocyte.

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CELL SURFACE MOLECULES, IMMUNOPRECIPITATION OF

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Immunoprecipitation of cell surface molecules is a quick and reliable method that allows the analysis of cell surface expressed molecules recognized by monoclonal antibodies and by antisera. In addition, using proper solubilization techniques, interactions between cell surface expressed molecules can be defined that give insight into subunit interactions in multichain receptor complexes. Usually lysates of cell surface iodinated cells are used. The principle of immunoprecipitation is based on 1) solubilization of membrane proteins by the use of nonionic detergents, 2) subsequent interaction of specific antibody with the solubilized membrane antigen, and 3) recovery of the antibody-antigen complexes by binding to an insoluble support, thereby allowing washing procedures to remove unbound molecules. Analysis of such immunoprecipitates usually takes place by SDS-PAGE or isoelectric focusing (IEF).

Solubilization of membrane molecules

The most widely used detergents for membrane solubilization are the nonionic detergents Triton X-100 and NP40 that have almost identical properties. Typically, $1-10 \times 10^7$ cells are resuspended in 0.5–1 ml of the following buffer: 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM iodoacetamide, pH 8, containing either 0.5–1% Triton X-100 or NP40 and the following protease inhibitors: leupeptin, antipain, chymostatin, pepstatin (all $1 \mu\text{g ml}^{-1}$) and 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF), and lysed at 0°C for 30 min. Insoluble material is subsequently removed by centrifugation at 13 000 g for 15 min.

As an alternative to NP40 and Triton X-100 the nonionic detergents CHAPS (5 mM final concentration) or digitonin (1% final concentration) can be used in the lysis buffers. CHAPS and digitonin share the property that, in contrast to NP40 and Triton X-100, they do not, or hardly, disrupt subunit interactions within multichain receptor complexes like the T cell receptor, thereby allowing the recovery of more intact receptor complexes.

Immunoprecipitation

For immunoprecipitation, the antibodies used to detect the antigens must be bound to an insoluble ligand either before or after binding to their target antigen in order to facilitate washing procedures. For this purpose there are a number of alternatives: either the antibodies are covalently coupled to Sepharose beads or they are bound to Sepharose beads that have been coated with protein A or anti-immunoglobulin (Ig) antisera. It is also possible to use fixed *Staphylococcus aureus* bacteria, that express the protein A on their cell surface. Since at pH 8 protein A binds to the large majority of mouse, rabbit and human derived Ig types (not IgM), Sepharose protein A is a very convenient reagent to immobilize many antibodies used for immunoprecipitation. Before specific immunoprecipitations are carried out, the lysates are precleared to remove non-specifically binding material by adding 100 μl Sepharose protein A beads and 75 μl normal rabbit serum to 1 ml lysate, followed by gently shaking at room temperature for 2 h. After removal of the beads, specific immunoprecipitations are carried out by mixing 1–10 μl monoclonal antibody or antiserum and 100 μl lysate for 60 min, either at room temperature or at 4°C , followed by the addition of 10 μl Sepharose protein A beads. After an additional 60 min, the beads are spun down (30 s, 13 000 g) and washed four times with 0.5 ml lysis buffer before analysis. This methodology can be easily modified for the use of immobilizing reagents other than Ig.

Sequential immunoprecipitation

Many cell surface expressed antigens are multichain complexes in which the chains may or may not be held together by disulfide bridges. The question may arise as to which chain in such complexes is actually recognized by the antibody or antiserum studied. Depending on the presence or absence of a disulfide bridge, one of the following procedures may be used to answer this question. Absence of a disulfide bridge: a normal immunoprecipitate is resuspended in 0.5% SDS and boiled for 3 min. This treatment leads to the dissociation of the individual chains and to the destruction of the antibody used as immunoprecipitating reagent. Subsequently, the SDS is

neutralized by the addition of three volumes of lysis buffer containing 1.5% NP40. This preparation is now used for immunoprecipitation with the antibody of interest. Part of the preparation can be analyzed directly. Comparison of the bands present in the original and in the reprecipitated material indicates which molecule is actually recognized by the antibody. Presence of a disulfide bridge: the immunoprecipitate is resuspended in a 0.5% SDS solution containing 2 mM DTT and boiled for 3 min to reduce the disulfide bridge and dissociate the individual chains. Subsequently the material is alkylated by the addition of 10 mM iodoacetamide. The remaining procedure is as described above.

Cross-linking

Biochemical cross-linkers are available that are reactive with lysine residues and, as these cross-linkers have two reactive groups, they can generate covalent, thiol-cleavable bonds between cell surface molecules that are in close vicinity to each other ($<13 \text{ \AA}$). They can thus be used to study the spatial relationship between cell surface expressed molecules. The most commonly used cross-linker is DSP (dithiobis (succinimidylyl propionate)). For cross-linking, cells are resuspended in 0.5 ml PBS containing 1 mM MgCl_2 , 0.02% NaN_3 . Subsequently 50–100 μg DSP is added (5–10 μl of 10 mg DSP per ml DMSO) and the mixture is incubated on a shaker at room temperature for 30 min. Then the cells are washed twice with PBS and lysed. Subsequently immunoprecipitates can be obtained from these lysates as described above. Analysis of such immunoprecipitates under both reducing and nonreducing conditions by SDS-PAGE indicates which molecules are cross-linked by the DSP treatment.

Bulk purification of molecules of the major histocompatibility complex

Since the recent insight into the molecular basis of peptide presentation by molecules of the major histocompatibility complex (MHC), the bulk purification of peptide-MHC complexes has become established as a standard technique. It is now widely used in order to identify natural peptide ligands or peptide-binding characteristics of particular MHC specificities. In principle, this purification method can be considered as a large-scale immunoprecipitation procedure, which can be adapted for the bulk purification of any cell surface molecule.

The isolation of MHC-bound peptides

The MHC-peptide complexes are purified from cells of which pellets are collected at -70°C . A lysate (of 10^{10} cells or more) is made by thawing and resuspending the cell pellet at a concentration of 2×10^8 cells ml^{-1} in NP40-containing lysis buffer (described above). The lysate is precleared by incubation with Sepharose CL-4B beads (10 ml beads per 50 ml lysate; Pharmacia). This incubation can be done batch-wise in 50 ml tubes under quiet agitation for 2 h at room temperature. After the incubation the Sepharose beads are removed from the lysate using a glass filter that is placed over a vacuum. The precleared lysate is subsequently incubated batch-wise for 16 h at 4°C with CNBr-activated Sepharose CL-4B beads to which antibody specific for the MHC molecule of interest has been covalently coupled (according to manufacturer's guidelines; Pharmacia). Then the affinity beads are washed on a glass filter (placed over a vacuum), taking care not to disturb the homogenous gel bed on the filter or letting the bed run dry. First, the beads are washed with minimally 5 bed volumes of a buffer containing 50 mM TRIS, 150 mM NaCl, pH 8.0, to which 0.5% NP40 (v/v; HPLC grade) has been added and subsequently with minimally 10 bed volumes of the TRIS/NaCl buffer without NP40. Then washing proceeds with 10 bed volumes 10 mM TRIS buffer, pH 8.0, and the beads are transferred and packed into a disposable column. The MHC-peptide complexes are eluted from the affinity beads with 3 bed volumes 10% acetic acid or 0.1% trifluoroacetic acid. The high molecular weight chains of the MHC molecules are removed from the eluate by ultracentrifugation over a membrane filter with a 10 kDa cut-off (Centriprep 10, Amicon). The ultimate peptide sample is concentrated by evaporation under vacuum and is ready for further processing.

The isolation of MHC molecules

In order to isolate intact MHC molecules for the purpose of (peptide) binding analyses, the procedure to follow is identical to that described for the isolation of MHC-bound peptides, except for the use of different buffers in the washing procedure and the elution of MHC under basic rather than acidic conditions. Briefly, the affinity beads that have been incubated with the precleared lysate are washed on a glass filter using the following buffers: minimally 5 bed volumes of TRIS/NaCl buffer (see above) with 0.5% NP40, 10 bed volumes of TRIS/NaCl buffer without NP40 and minimally 10 bed volumes of TRIS/NaCl with 0.4% (w/v) *n*-octylglucoside. In the latter buffer the beads are transferred to a disposable column and the

MHC complexes are eluted with 3 bed volumes of 50 mM diethylamine, 150 mM NaCl, 0.4% (w/v) *n*-octylglucoside, pH 11.5. The eluate can be neutralized using a 2M glycine solution (pH 2.5) and the high molecular weight MHC complexes in the eluate can be retained in a small volume after ultracentrifugation over a membrane filter (10 kDa cut-off).

See also: Affinity chromatography; Antibodies, specificity; Isoelectric focusing; Radiolabeling; SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

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CELL SURFACE RECEPTORS AND ADHESION MOLECULES, THREE-DIMENSIONAL STRUCTURES

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The extracellular regions of immune cell surface proteins consist of one or more protein domains. These domains are often independent folding units which can be expressed in soluble form and subjected to structural analysis. Since 1990, more than 30 three-dimensional structures of immune cell surface proteins have been determined. These include single or multiple domains of adhesion molecules, receptors, and a few receptor–ligand complexes. The studies have provided structural prototypes of cell surface protein superfamilies. The structural basis of cell adhesion, specific receptor–ligand interactions, and ligand-induced receptor oligomerization is beginning to be understood.

Cell surface proteins of the immune system

In recent years, significant progress has been made in understanding the three-dimensional structures of proteins which are expressed on the surface of immune cells. Table 1 lists 35 structures of such proteins (major histocompatibility complex molecules are not discussed here). Smaller domains have often been studied using nuclear magnetic resonance (NMR) methods. The larger and more complex structures have been determined by X-ray crystallography.

A large number of immune cell surface proteins include a single transmembrane region, and their extracellular binding domains are relatively inde-

pendent from the membrane. Thus, soluble extracellular fragments suitable for structural studies can be obtained in many cases. This is in contrast to, for example, G protein-coupled receptors of the nervous system. These receptors traverse the membrane seven times, and the ligand binding sites are more intimately associated with transmembrane segments and difficult to isolate.

Modular arrangement of extracellular regions and protein superfamilies

The extracellular regions of immune cell surface proteins include distinct domains which are often connected to the transmembrane domain by a linker or stalk region. Only one or two domains may have specific binding functions. Others may trigger oligomerization or ensure that binding domains are sufficiently distant from the membrane. Many extracellular protein modules belong to superfamilies. By definition, members of protein superfamilies share limited sequence similarity, often 30% or less, but similar three-dimensional structures. Table 1 includes structural prototypes of superfamilies determined to date. Some small domains found in different cell surface proteins consist of β sheets or hairpins which may be arranged sandwich-like and stabilized by disulfide bonds.

Table 1 Three-dimensional structures of immune cell surface receptors and adhesion molecules

EGF ^{a,b}	Cooke <i>et al</i> (1987) <i>Nature</i> 327 : 339
FN type I ^{a,b}	Baron <i>et al</i> (1990) <i>Nature</i> 345 : 642
FN type II ^{a,b}	Constantine <i>et al</i> (1992) <i>J Mol Biol</i> 223 : 281
CD4 (domains 1 + 2) ^c	Rye <i>et al</i> (1990) <i>Nature</i> 348 : 419
	Wang <i>et al</i> (1990) <i>Nature</i> 348 : 411
CD4 (domains 3 + 4) ^c	Brady <i>et al</i> (1993) <i>Science</i> 260 : 979
CD8 α ^c	Leahy <i>et al</i> (1992) <i>Cell</i> 68 : 1145
CD2 (domain 1) ^c	Driscoll <i>et al</i> (1991) <i>Nature</i> 353 : 762
CD2 (domains 1 + 2) ^c	Jones <i>et al</i> (1992) <i>Nature</i> 360 : 232
CD2 (domain 1, glycosylated) ^a	Whitka <i>et al</i> (1993) <i>Structure</i> 1 : 69
FN type III ^{a,b,d}	Main <i>et al</i> (1992) <i>Cell</i> 71 : 671
FN type III	Leahy <i>et al</i> (1992) <i>Science</i> 258 : 987
FN type III (domains 7–10)	Leahy <i>et al</i> (1996) <i>Cell</i> 84 : 155
hGH/hGHR ^d complex	De Vos <i>et al</i> (1992) <i>Science</i> 255 : 306
hGH/hPRLR ^d complex	Somers <i>et al</i> (1994) <i>Nature</i> 372 : 478
CCP (two domains) ^{a,b}	Barlow <i>et al</i> (1993) <i>J Mol Biol</i> 232 : 268
TNFR/TNF β complex	Banner <i>et al</i> (1993) <i>Cell</i> 73 : 431
TNFR	Naismith <i>et al</i> (1995) <i>J Biol Chem</i> 270 : 13303
NFcR	Burmeister <i>et al</i> (1994) <i>Nature</i> 372 : 336
NFcR/Fc complex	Burmeister <i>et al</i> (1994) <i>Nature</i> 372 : 379
E-selectin (C-type lectin + EGF domains)	Graves <i>et al</i> (1994) <i>Nature</i> 367 : 532
CD59 (glycosylated) ^a	Fletcher <i>et al</i> (1994) <i>Structure</i> 2 : 185
Tissue factor ^d	Harlos <i>et al</i> (1994) <i>Nature</i> 370 : 662
Tissue factor/FVIIa complex	Banner <i>et al</i> (1996) <i>Nature</i> 380 : 41
VCAM-1 (domains 1 + 2) ^c	Jones <i>et al</i> (1995) <i>Nature</i> 373 : 539
Integrin CD11b/CD18 (A domain, Mg ²⁺ form)	Lee <i>et al</i> (1995) <i>Cell</i> 80 : 631
Integrin CD11b/CD18 (A domain, Mn ²⁺ form)	Lee <i>et al</i> (1995) <i>Structure</i> 3 : 1333
cbEGF ^{a,b}	Rao <i>et al</i> (1995) <i>Cell</i> 82 : 131
cbEGF (two domains)	Downing <i>et al</i> (1996) <i>Cell</i> 85 : 597
TCR (V β + C β domains) ^c	Bentley <i>et al</i> (1995) <i>Science</i> 267 : 1984
TCR (V α domain) ^c	Fields <i>et al</i> (1995) <i>Science</i> 270 : 1821
IFN γ /IFN γ R α complex ^d	Walter <i>et al</i> (1995) <i>Nature</i> 376 : 230
E-Cadherin (domain 1) ^{a,d}	Overduin <i>et al</i> (1995) <i>Science</i> 267 : 386
N-Cadherin (domain 1) ^d	Shapiro <i>et al</i> (1995) <i>Nature</i> 374 : 327
E-Cadherin (domains 1 + 2) ^d	Nagar <i>et al</i> (1996) <i>Nature</i> 380 : 360
LM-EGF (three domains) ^b	Stetefeld <i>et al</i> (1996) <i>J Mol Biol</i> 257 : 644
TCR/MHC class I complex ^c	Garcia <i>et al</i> (1996) <i>Science</i> 274 : 209
TCR/MHC class I complex ^c	Garboczi <i>et al</i> (1996) <i>Nature</i> 384 : 134
EPOR/peptide complex ^d	Livnah <i>et al</i> (1996) <i>Science</i> 273 : 464
Link domain ^a	Kohda <i>et al</i> (1996) <i>Cell</i> 86 : 767
NCAM (domain 1) ^{a,c}	Thomsen <i>et al</i> (1996) <i>Nature Struct Biol</i> 3 : 581
ICAM-2 (domains 1 + 2) ^c	Casanovas <i>et al</i> (1997) <i>Nature</i> 387 : 312
IL-1R/IL-1 β complex ^c	Vigers <i>et al</i> (1997) <i>Nature</i> 386 : 190
IL-1R/IL-1RA complex ^c	Schreuder <i>et al</i> (1997) <i>Nature</i> 386 : 194
CD4 (domains 1–4)	Wu <i>et al</i> (1997) <i>Nature</i> 387 : 527
CD8 α /MHC class I complex	Gao <i>et al</i> (1997) <i>Nature</i> 387 : 630

C, constant; CCP, complement control protein; C-type, calcium-dependent; EGF, epidermal growth factor; cbEGF, calcium-binding epidermal growth factor; EOPR, Erythropoietin receptor; FVIIa, blood coagulation factor VIIa; Fc, antibody constant fragment; FN, fibronectin; hGH, human growth hormone; hGHR, human growth hormone receptor; hPRLR, human prolactin receptor; ICAM, intercellular adhesion molecule; IL-1R, interleukin-1 receptor; IL-1RA, interleukin-1 receptor antagonist; IFN, interferon; IFN γ R α , high-affinity interferon γ receptor; LM-EGF, laminin-type EGF-like; NCAM, neural cell adhesion molecule; NFcR, neonatal Fc receptor; TCR, T cell receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; V, variable; VCAM-1, vascular cell adhesion molecule 1.

^aStructures determined by NMR.

^bSmall domains which occur in many cell surface or extracellular matrix proteins.

^cImmunoglobulin superfamily (IgSF) members.

^dStructurally similar to IgSF domains.

The immunoglobulin superfamily (IgSF): variations of a common fold

Many of the leukocyte-specific cell surface proteins characterized to date include immunoglobulin (Ig) domains or closely related structures. The Ig fold, as seen in antibody structures, is a tightly packed sandwich of two antiparallel β sheets of four and three (constant, C) or four and five strands (variable, V), respectively, which are connected following conserved topology. In V domains, the sheets are formed by strands ABED and GFCC'C'', respectively. In C domains, the C' and C'' strands are absent. A canonical disulfide bond covalently links the sheets. The N- and C-termini are on opposite sides of the domain, thereby permitting Ig domains to form linear arrays.

Structural studies on Ig-like domains have revealed variations of the Ig fold. For example, the length of β strands and the spatial positions of strands at the edges of the sheets vary. The N-terminal (A) strand is often split into A and A' strands which hydrogen bond to different sheets. Strands may completely switch from one sheet to the other and thereby change the topology of the structure. Furthermore, the length and conformations of loops connecting the sheets are variable. The canonical disulfide bond is absent in some structures and other disulfide bonds may be present. On the basis of sequence and structure comparison, IgSF domains have been classified as, for example, V, C1 or C2 (related by a strand switch), I (intermediate between V and C), or A (V variant where the C'' strand switches the sheet)

domains. V domains often include (part of) the binding site in IgSF cell surface proteins. Only a few examples of V domain-mediated interactions have been studied in detail. The (A')GFCC'C'' face appears to be the center of interactions studied so far, irrespective of the molecular nature of the ligand.

Structures of IgSF cell surface receptors and related proteins

Figure 1A shows examples of IgSF cell surface receptor structures. The extracellular region of CD8 includes a single V domain which dimerizes like the variable light and heavy chains of an antibody. The structure of a CD8/MHC class I complex shows that residues in Complementarity Determining Region (CDR)-like loops and β -strands of the CD8 homodimer contact the $\alpha 2$ -, $\alpha 3$ -, and $\beta 2$ -microglobulin domains of MHC class I. The extracellular regions of CD4 and CD2 are stable as monomers and include four and two IgSF domains, respectively. In both structures, an N-terminal V-like domain is followed by a C2 domain. In CD4, a continuous β strand connects the first and second domain which form an extensive and rigid interface. This structure is very similar to the structure including the third and fourth domain of CD4. Structures of CD4 including all four extracellular domains in different crystal lattices reveals that domains 2 and 3 are more flexibly connected. In CD2, the two domains are connected by a linker and the interface is less extensive. Despite

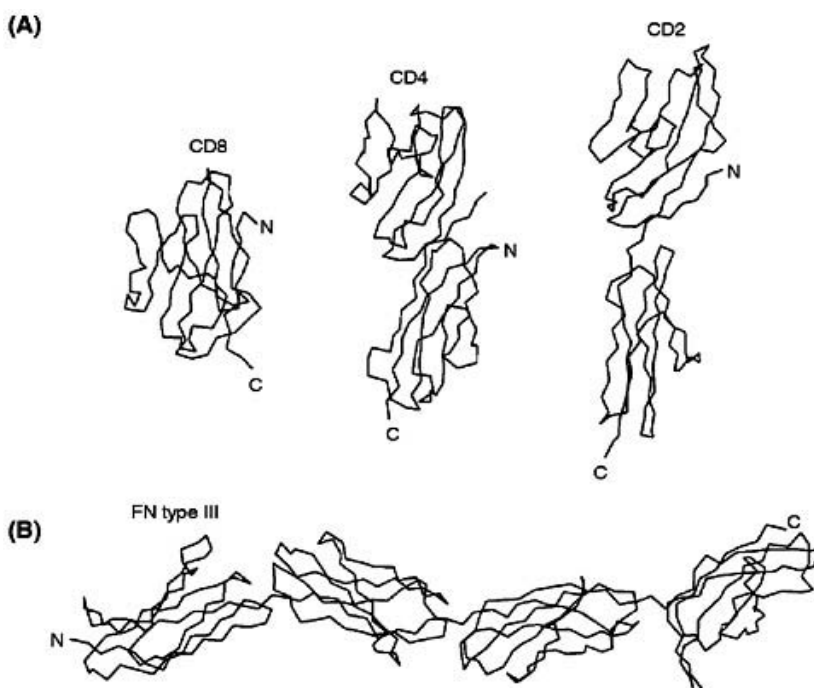


Figure 1 Cell surface proteins of the IgSF and related molecules. Structures are represented as α traces. N- and C-termini are labeled. (A) Three IgSF members: CD8, CD4 and CD2. (B) Structure of a four-domain fragment of fibronectin. The three-dimensional structure of FN type III is similar to IgSF C domains.

these differences, the relative orientation of the two domains is similar in CD4 and CD2.

Structures of a V_α domain and β chain of different TCRs, a complete mouse TCR (and its preliminary complex with MHC class I), and a human TCR/MHC class I complex have been reported. These structures confirm the structural similarity of antibodies and TCRs. In the V_α domain, which forms a tetramer of homodimers, the C' strand switches the sheet, a departure from V domain structure (see above). The β chain includes a V and a C domain which form an extensive interface (two to four times larger than in antibody structures). Overall, the mouse and human TCR structures are similar. In the mouse TCR structure, the $C\alpha$ domain, which is disordered in the human TCR, substantially departs from known IgSF structure types. In the structure of the complex with peptide-bound MHC class I, the TCR binds with its CDR-like loops diagonally across the composite MHC/peptide surface. The orientation is such that the $V\alpha$ CDR loop 1 contacts the N-terminal and the $V\beta$ CDR loop 3 the C-terminal region of the peptide.

In Figure 1B, a structure consisting of four fibronectin (FN) type III domains is shown. FN type III domains are structurally similar to C2 domains, but are not included in the IgSF. Their structure is a sandwich of a four-stranded and a three-stranded β sheet. The domain lacks the Ig disulfide bond, and the packing of its hydrophobic core is distinct from the IgSF. FN type III domains are found in extracellular matrix proteins and in cytokine receptors. Domain 10 of fibronectin includes an RGD sequence in a loop and is therefore an integrin ligand and

implicated in cell adhesion. Although the structures of single FN type III domains are very similar, their domain interfaces are not conserved. The two central domains display a relative orientation similar to CD4 and CD2.

Cadherins, also distantly related to the IgSF, are homophilic and calcium-dependent adhesion molecules. Like fibronectin, cadherins may include ten or more extracellular domains. The interfaces between domains are stabilized by calcium binding which also supports dimerization of cadherin domains. Crystal packing has suggested different ways in which cadherins interact. On the basis of crystal lattice interactions, models have been proposed for the formation of network-like multivalent cellular adhesion interfaces.

Structures of leukocyte and endothelial cell adhesion molecules

In the course of an inflammatory response, leukocytes are recruited to activated vascular endothelium by adhesion interactions of increasing strength. Selectins are responsible for the initial low avidity (protein/carbohydrate) interactions. Firm adhesion is achieved by high avidity (protein/protein) interactions between leukocyte integrins and their endothelial ligands. Structures of binding domains of key molecules have been determined and are shown in Figure 2.

The X-ray structure of E-selectin includes an N-terminal calcium-dependent (C-type) lectin domain which is followed by an EGF-like domain. The C-type lectin fold, first observed in the structure of rat

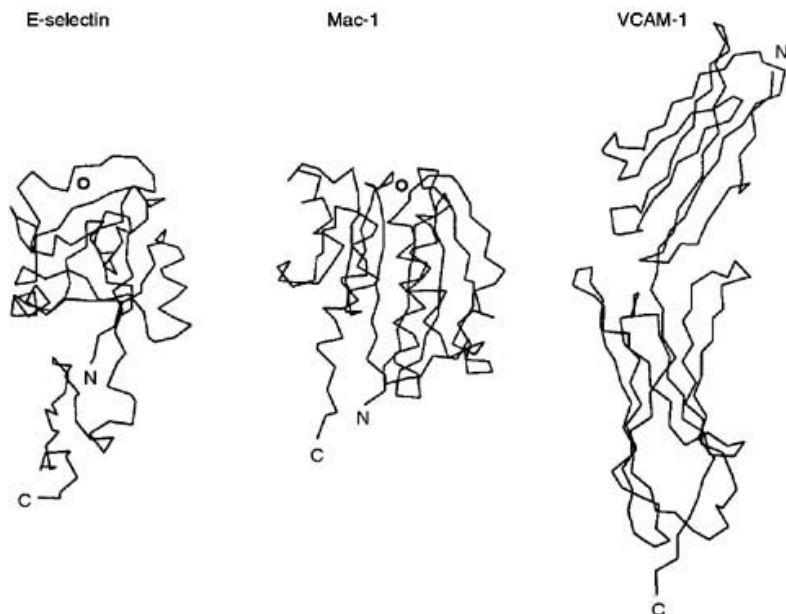


Figure 2 Binding domains of adhesion proteins. Structures are represented as α traces, and the N- and C-termini are labeled. E-Selectin includes two (C-type lectin and EGF-like) domains. The circle indicates the position of a conserved calcium ion. The I domain of Mac-1, a β_2 -integrin, is shown. The circle indicates the position of the MIDAS motif (see text). The structure of VCAM-1, a β_2 -integrin ligand, includes two Ig domains (see also Figure 1).

mannose-binding protein, includes extended regions of unusual secondary structure in the vicinity of a highly conserved calcium-binding site. In the mannose-binding protein, this calcium is directly involved in carbohydrate binding. A similar role has been proposed for the conserved calcium in E-selectin, but the structure of a complex between E-selectin and its carbohydrate ligand remains to be determined.

The ligand binding domain of the β_2 -integrin Mac-1 (CD11b/CD18) adopts a dinucleotide-binding (Rossmann) fold which consists of a hydrophobic parallel β sheet covered by amphipathic α helices on both sides. The Mac-1 structure includes an Mg^{2+} -binding site which topologically corresponds to the active site in dinucleotide cofactor-binding enzymes. The cation coordination sphere is open and, in the crystal, completed by a negatively charged residue from a neighboring molecule. This site has been termed the metal ion-dependent adhesion site (MIDAS motif) and is thought to be directly involved in ligand binding. Structural analysis of an Mn^{2+} -bound form of Mac-1 has revealed conformational changes, compared to the original structure.

The structure of ICAM-1, the Mac-1 ligand, is unknown, but the structure of the related VCAM-1 has been determined. This structure includes two of seven IgSF domains which form VCAM-1's extracellular region. A terminal IgSF I domain is followed by a C2 domain, and the domain interface is reminiscent of CD2. The conserved integrin-binding sequence (QIDSPL) resides in the CD loop of the I domain. The aspartic acid is thought to complement

the MIDAS motif. The interaction involves surface regions proximal to the shared cation-binding site.

Structures of cell surface receptor-ligand complexes

The structures of several receptor-ligand complexes have also been reported (see Table 1). For immune cell surface receptors, ligand-induced receptor oligomerization is a major determinant of signal transduction. Figure 3 compares the structures of the complexes involving human growth hormone (hGH) receptor (hGHR) and the 55 kDa tumor necrosis factor (TNF) receptor (TNFR).

The prototypic fold of the TNFR/nerve growth factor receptor superfamily (Figure 3A) consists of stacked elongated domains. These domains have a very limited hydrophobic core and are stabilized by conserved disulfide bonds in a ladder-like arrangement. TNFR includes four domains, but only domains 2 and 3, which contact ligand, are well ordered in the X-ray structure. In contrast, the TNF fold consists of an antiparallel β sandwich with jelly-roll topology. Three monomers form a threefold symmetrical trimer. Three symmetry-related receptor-binding sites are formed at the interfaces between the TNF monomers. The receptor binds roughly parallel to the threefold axis, and the bound receptors do not contact each other. Thus, ligand binding leads to TNFR trimerization. Structures of free TNF and TNFR have shown that neither ligand nor receptor undergo significant conformational changes upon binding. In the crystal of uncomplexed TNFR,

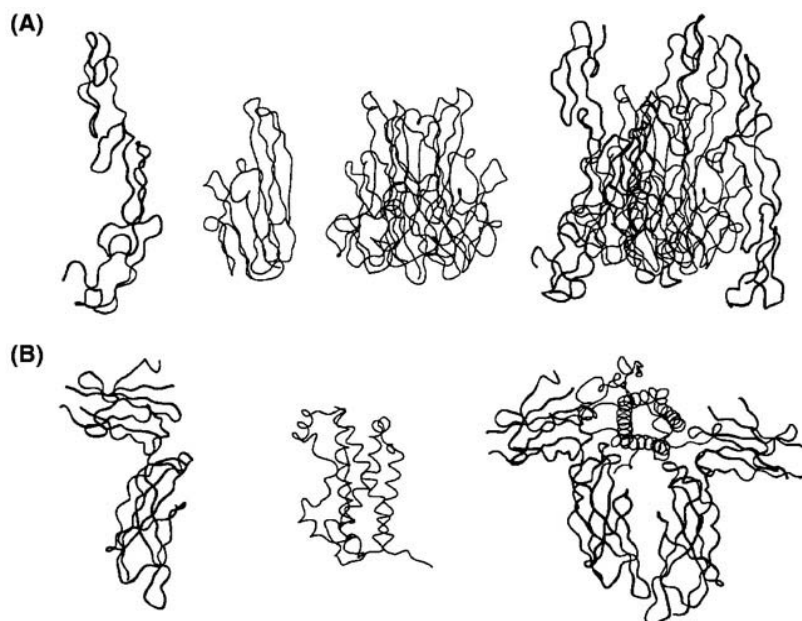


Figure 3 Cell surface receptor-ligand complexes. Structures are represented as backbone traces (receptors, thick lines; ligands, thin lines). (A) The TNF β /TNFR complex. From left to right: TNFR, a TNF subunit, trimeric TNF, and the TNF/TNFR complex. (B) hGH/hGHR. From left to right: hGHR, consisting of two FN type III-like domains (see also Figure 1), hGH, and the hGH/hGHR complex.

dimeric forms were observed which, if present on the cell surface, may stabilize an inactive state of TNFR in the absence of ligand.

Figure 3B shows the structure of the hGH/hGHR complex. The receptor consists of two FN type III domains, and the ligand displays a four helix bundle fold. The hGHR architecture is shared by other cytokine receptors including the human prolactin receptor (hPRLR), the interferon γ receptor (IFN γ R α) and tissue factor (TF), but the relative orientations of the FN type III domains differ. Binding of hGH to its receptor leads to receptor dimerization. In contrast to TNF/TNFR, the binding sites in the hGH/hGHR complex are not equivalent, and the bound receptors contact each other. The hGH/hGHR interaction is asymmetrical in the sense that the receptors bind to different sites of the ligand. The complex forms sequentially. First, a high affinity 1:1 complex is formed, then a second receptor is engaged in the interaction. The binding of the second receptor is stabilized by receptor-receptor interactions. hGH also binds to hPRLR, and the structure of a 1:1 (high-affinity) hGH/hPRLR complex has been determined. This interaction is made possible in part by changes in the relative domain orientation of hPRLR compared to hGHR.

The erythropoietin (EPO) receptor is also structurally similar to hGHR but dimerizes in a somewhat different orientation when complexed with an agonist peptide. In contrast to hGH, IFN γ is an intertwined helix bundle homodimer. Two equivalent receptor-binding sites are presented which correspond to the high-affinity site in hGH. In the structure of the complex, receptors are bound at twofold symmetry-related sites and are separated by ~ 27 Å. Tissue factor binds a structurally distinct ligand, blood coagulation factor VIIAa, in a 1:1 complex. The TF region involved in binding differs topologically from the binding sites in hGHR. The interleukin (IL)-1 receptor consists of three extracellular Ig-like domains and binds IL-1 β (and an antagonist) involving regions not corresponding to those in other cytokine receptors.

The structure of the rat neonatal Fc receptor (NFCR), responsible for binding maternal IgG, is very similar to MHC class I. However, the helices corresponding to the MHC peptide-binding site move together and essentially close the groove. A low-res-

olution structure of NFCR in complex with an Fc fragment has shown that the Fc-binding site does not correspond to the MHC peptide-binding site. Rather, the Fc fragment binds to the side of the receptor. The NFCR/Fc complex provides an instructive example of how similar structures can mediate distinct binding functions.

See also: Adhesion molecules; Antigens, cell surface; CD antigens; Cytokine receptors; Domains, immunoglobulin-type; Immunoglobulin gene superfamily; Specificity; Antibody-antigen complexes, three-dimensional structures.

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CHAGAS' DISEASE

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Characteristics of the organism and its antigens

Life cycle of *Trypanosoma cruzi*

T. cruzi, the causative agent of Chagas' disease in humans, infects 16–20 million people in Central and South America and constitutes a prominent health problem. The parasite is a kinetoplastid protozoan whose life cycle alternates between an insect belonging to the family Reduviidae, and a mammalian host. Transmission is initiated by insect vectors which after a blood meal defecate and release infective metacyclic trypomastigotes near the bite wound. These infective stages differentiate from epimastigotes, the noninfective replicative form that live in the insect gut. Both forms are flagellated and highly motile. In contrast to the African trypanosomes, the vertebrate stages of *T. cruzi* are obligate intracellular pathogens and must enter host cells to replicate. After disruption of the membrane-bounded vacuole, parasites escape to the cytoplasm and differentiate into amastigotes, less motile with a very short flagellum and spherical body (Figure 1). After a 24 h lag period, amastigotes start dividing by binary fission, which occurs 7–10 times. After 5–7 days, more than 500 parasites have accumulated in the cytoplasm. When the replicative period is completed, amastigotes differentiate into trypomastigotes, the host cell ruptures and parasites are released into the bloodstream; thus, infection disseminates to other tissues. These released forms, equivalent to the metacyclic trypomastigotes, are called bloodstream trypomastigotes and are able to invade nearly every kind of nucleated cell (Figure 2). The *T. cruzi* life cycle (Figure 3) is completed when trypomastigotes are ingested by blood-sucking insect vectors. Amastigotes released from ruptured cells are also able to invade new host cells, probably involving different receptors from those used by trypomastigotes.

Invasion of the mammalian cell

Several antigens from the trypomastigote as well as host cell receptors are involved in sequential processes leading to invasion. The ability of *T. cruzi* to invade almost every nucleated cell from any mammalian species indicates the presence of ubiquitous and conserved receptor(s) on the host cell. Among

the parasite molecules involved in binding to the host cell, a relevant role has been attributed to trypomastigote stage-specific molecules of a large family that includes trans-sialidases/sialidases. Sialic acid on the surface of host cells influences the invasion process. *T. cruzi* parasites do not synthesize sialic acid, but make use of transialidases that transfer mammalian sialic acid to highly glycosylated mucin-like acceptors (35–50 kDa) on the parasite surface. Treatment with antibodies to both trans-sialidase or the mucin acceptors, desialylation of parasites or treatment with tunicamycin (which blocks glycosylation) inhibit infection, and mutants defective in sialic acid are more resistant to infection. Moreover, trans-sialidase released inside the phagolysosome desialylates lysosomal proteins, allowing activity of a parasite-encoded acidic pore-forming molecule (Tc-TOX or

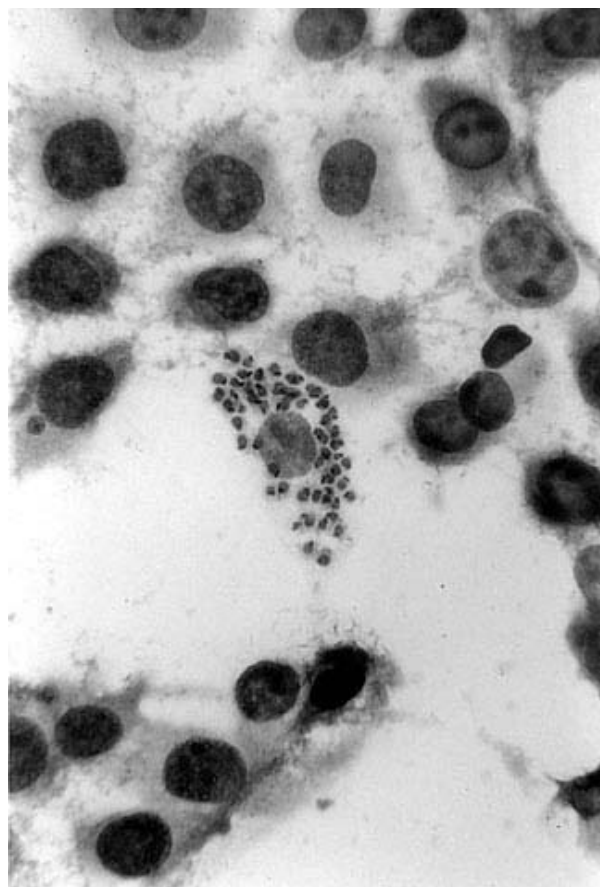


Figure 1 Photomicrograph of *T. cruzi* amastigotes in the cytoplasm of mouse peritoneal macrophages (316 × magnification).

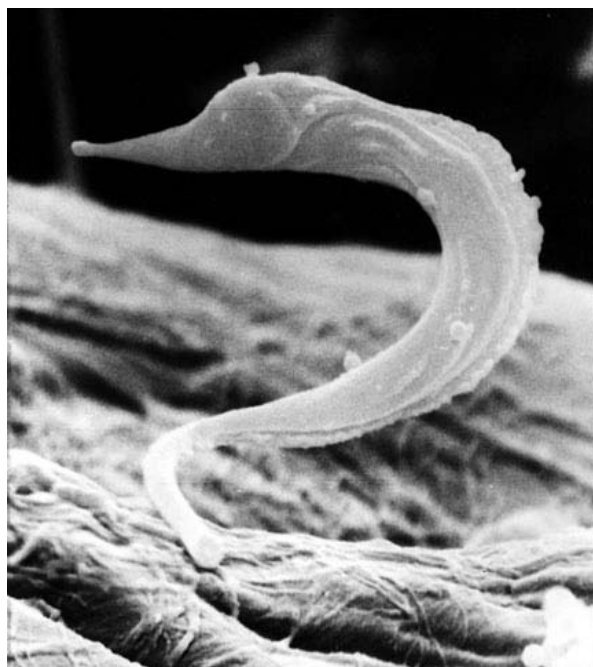


Figure 2 Scanning electron micrograph of a *T. cruzi* trypomastigote invading a fibroblast cell (L-929) (25000 × magnification). (Courtesy of Dr L Nilsson and D Sunnemark.)

hemolysin) immunologically related to the C9 component of complement, and probably involved in escape into the cytosol. Penetrin, a 60 kDa trypomastigote-specific heparin-binding protein, has also been implicated in attachment to host cells. On the host surface, receptors binding fibronectin (as β_1 -integrins) and collagen play a role in the invasion process.

T. cruzi does not bind well to the host plasma cell membranes at 4°C, suggesting the need for energy-dependent mechanisms. In particular, those depending on parasite energy appear to play a key role in invasion. *T. cruzi* trypomastigotes invade the mammalian cell through a unique mechanism, distinct from phagocytosis, and without the need of pseudopodia formation and actin polymerization. Clusters of lysosomes gather in close proximity to the host plasma cell membrane at regions in contact with trypomastigotes. The parasite slides gradually into the host cell and fusion of lysosomes probably provides the membrane required for the formation of a vacuole that surrounds the parasite (Figure 4). Such an interaction is accompanied by, and probably depends on, alterations of cellular homeostasis, involving signal transduction events such as an increase in the intracellular concentration of free Ca^{2+} transients, both in the host cell and the infecting parasite. Ca^{2+} elevation in the cell is mediated by a parasite soluble factor that mediates its activity

through activation of host cell phospholipase C, at least in some cellular populations. Buffering or depleting intracellular Ca^{2+} results in inhibition of *T. cruzi* entry, indicating a physiologic role for such ions in the infection. *T. cruzi* invasion also directly activates the transforming growth factor β (TGF β) signaling pathway which is in turn required for parasite entry into the mammalian cells. Cruzipain, a cysteine protease from *T. cruzi*, is also thought to be involved in differentiation between amastigotes and trypomastigotes.

Immune responses of the host

Outcome of infection with *T. cruzi* in vivo

Following initial infection, the acute phase of Chagas' disease ensues. This phase is characterized by high blood parasitemia and extensive tissue parasitism. In a high proportion of Chagas' cases the acute phase is asymptomatic but, especially in children, fever, hepatosplenomegaly, adenopathy and myocarditis may occur. Parasitemia subsides after 1–2 months, coincident with the development of a specific immune response, and the patients enter a chronic stage of infection, without showing symptoms of disease. The asymptomatic phase of infection lasts for an unspecified period (up to 10–30 years), and is followed in 30–40% of cases by a clinical chronic disease, usually focused in the heart or the digestive tract. Chronic chagasic cardiac pathology may include arrhythmia, conduction abnormalities, apical aneurysm, myositis and infarction with fibroadipose replacement of the myocardium. In a final stage the most significant clinical manifestations of Chagas' cardiomyopathy include congestive heart failure, thromboembolic phenomena, severe arrhythmias and sudden death. Less frequent abnormalities in the digestive system involve hypertrophy of the esophagus or colon.

All mammalian species are susceptible to infection; however, the relative susceptibility of animals varies not only between, but also within, species. The genetic basis for this susceptibility is unclear. Genes both within and outside the major histocompatibility complex (MHC) loci have been implicated, as well as the genetic background of different strains of the parasite.

Immune response

Polyclonal activation and immunosuppression *T. cruzi* infection elicits both specific immune responses and several non-specific alterations in the homeostasis of the immune system in humans and experimental animals. The immune response is both humo-

ral and cell mediated and has been associated both with protection and pathogenesis.

A polyclonal activation of B cells (in particular Ly-1^+ [CD5]), and CD4^+ and CD8^+ T cells, follows early after infection with *T. cruzi*. The majority of the responding clones are not directed against parasite antigens. Although the parasite does not seem to possess any T cell-specific superantigens, an octapeptide from an 85 kDa parasite antigen has been shown to generate nonspecific B cell polyclonal activation. Elevated levels of type I interferons, interleukin 12 (IL-12) and IL-2, are found early after infection. These cytokines might be responsible for elevated cytolytic activity and secretion of interferon γ ($\text{IFN}\gamma$) by natural killer (NK) cells during early infection, and are probably involved in protection. An immunodepression stage characterizes the infection later during the acute phase. This immunosuppression is transient, although partially depressed responses have also been documented during the chronic stage of human and experimental infections. It is currently thought that the immunodepression undermines the defences against the establishment

and dissemination of the invading parasite in the host. During the acute stage of infection, T and B cells have an impaired ability to proliferate in response to mitogens as well as to specific or heterologous antigens. The unresponsive state has been associated with an impaired capacity to produce IL-2 and a downregulation of IL-2 receptor expression, and to an apoptotic death of CD4^+ T cells. Additionally, the activation of suppressor T cells, macrophages secreting nitric oxide (NO) or prostaglandin E and the downregulation of CD28 on T cells have all been proposed to explain the unresponsive state. Parasite molecules or soluble factors released by *T. cruzi* (proteins as well as a glycosylinositol phospholipid) mediate different features of the immunodepression *in vitro* and *in vivo* in a parasite dose-dependent way. *In vivo*, the immunodepression phase is also accompanied by an extensive thymic destruction and splenomegaly, whereas lymph nodes appear not to be affected to similar levels. High levels of $\text{IFN}\gamma$, released by $\text{CD4}^+\text{CD8}^-$ T cells, and specific antibodies are detectable during this phase, indicating the presence of active immune responses during

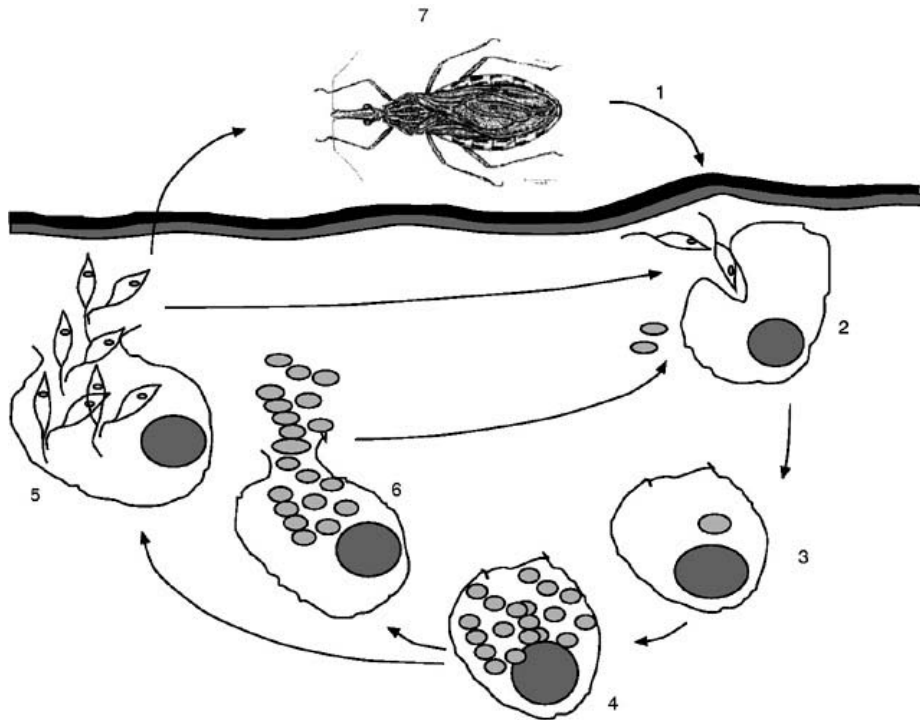


Figure 3 The life cycle of *T. cruzi*. (1) Metacyclic trypomastigotes are released in feces of the insect and enter the vertebrate host through the bite wound. (2) Trypomastigotes invade mammalian cells (see Figure 4). (3) After 30–120 min trypomastigotes escape from the vacuole and transform into replicative amastigotes. (4) Amastigotes multiply in the cytoplasm. (5) After a number of cycles amastigotes differentiate into trypomastigotes, which will rupture the host cell. (6) Amastigotes may also lyse infected cells before differentiation into trypomastigotes, and are thereby released into circulation, invade macrophages, survive, divide and further differentiate into trypomastigotes. (7) With a blood meal, the insect vector ingests bloodstream trypomastigotes, which in the insect midgut differentiate into noninfective and replicating epimastigotes. Epimastigotes further differentiate into metacyclic trypomastigotes in the hindgut of the vector, completing the life cycle.

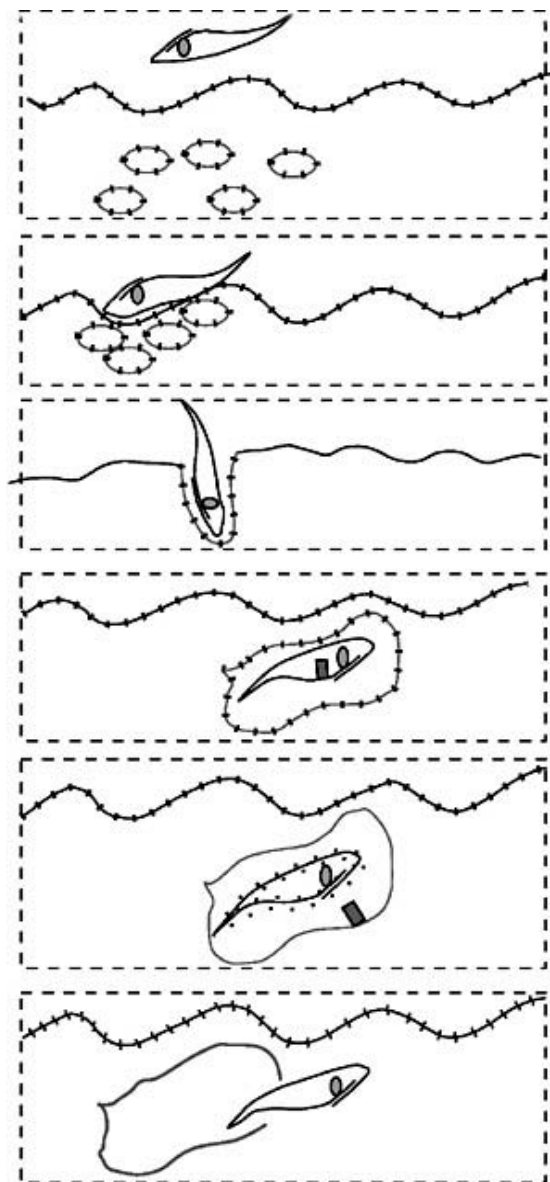


Figure 4 *T. cruzi* invasion into a mammalian cell. (1) *T. cruzi* trypomastigotes attach to the cell surface. (2) Host cell lysosomes (○) migrate to the attachment site. (3) Lysosomes fuse with the plasma membrane at the attachment site and start to form an intracellular vacuole around the parasite. (4) The intracellular vacuole is formed. (5) Lysosomal glycoproteins are desialylated by the *T. cruzi* trans-sialidase and sialic acid (black dots) is transferred to parasite acceptors. *T. cruzi* Tc-TOX (■) anchors in the lysosome membrane. (6) The vacuolar membrane is disrupted. The parasite escape into the cytoplasm, where they will differentiate into amastigotes.

a stage of general dysregulation. In summary, the relevance of both polyclonal activation and immunodepression in the biology of the infection and outcome of disease is unknown. It is believed that the immunomodulation events occurring in the acute phase influence alterations later during the infection

by modifying the quality of the specific immune responses.

Protective immune responses The immune response controls the high parasite load in the acute phase to produce virtually undetectable parasitemia in the chronic phase. However, sterile immunity and complete parasite clearance and cure are unknown in humans and in experimental models of infection. Rather than achieving a cure, the immune response maintains a host-parasite balance which lasts for the lifetime of the infected individual.

NK cells, macrophages, B cells and both CD4⁺ and CD8⁺ T cells play important roles in resistance of mice infected with *T. cruzi*. Such diversity of cellular populations is reflected in manifold humoral and cellular immune effector mechanisms of destruction of both intracellular amastigotes and bloodstream trypomastigotes. Antibodies can lyse the parasite through activation of the classical or alternative pathways of complement. Antibodies might also lyse trypomastigotes through antibody-dependent cell-mediated cytotoxicity. Eosinophils, neutrophils, mononuclear cells and platelets have been shown to lyse the trypomastigotes when coated with specific antibodies. T cells can regulate production of such antibodies, lyse infected target cells or release cytokines (Table 1) that modulate different trypanocidal mechanisms of phagocytes. Whether they influence the physiology of infection in nonphagocytic cells is not known. Macrophage activation by tumor necrosis factor α (TNF α) IL-3, IFN α , IFN β , IFN γ or granulocyte-macrophage colony-stimulating factor (GM-CSF) leads to inhibition of the replication or killing of the intracellular forms of *T. cruzi*. Of these cytokines, IFN γ has been most closely associated with host resistance. Conversely, cytokines such as IL-4, IL-10 and TGF β counteract the effects of IFN γ during *T. cruzi* infection *in vitro* and *in vivo*. However, *T. cruzi* infection appears not to be a model for T_H1/T_H2 dichotomy, as in infection with *Leishmania major*. Cytokines belonging to both T_H1 and T_H2 are simultaneously produced during infection in a variety of mice, but the balance between cytokines belonging to either pattern appears to influence the outcome of infection. Such cytokine balance regulates the transcriptional rate and mRNA stability of inducible nitric oxide synthase (iNOS) by macrophages, mediating high output of NO. Macrophages produce reactive oxygen species (ROS) and NO and show induction of iNOS during the course of *T. cruzi* infection *in vivo*. Both NO and ROS are toxic for the parasite *in vitro*, and some trypanocidal drugs, such as nifurtimox or crystal violet, act by generating such species. In addition, the superoxide anion and

Table 1 Role of cytokines in the outcome of infection with *T. cruzi* *in vivo* and *in vitro*

Cytokine	Effect on <i>T. cruzi</i> infection
IFN γ	Reduces parasitemia and mortality. Activates trypanocidal activity of macrophages, mainly through induction of inducible NO synthase. The peak of production coincides with the high parasite load during the acute phase of infection
TNF α	Inhibits intracellular multiplication. Synergizes with and mediates IFN γ trypanocidal activity. It has been shown both to aggravate and protect <i>in vivo</i> . Produced by macrophages after <i>in vitro</i> infection with parasites
TGF β	Inhibits IFN γ -mediated trypanocidal activation of macrophages. Is involved in parasite penetration and intracellular multiplication. Increases susceptibility to infection <i>in vivo</i>
IL-1	Released by infected macrophages and endothelial cells. Probably associated with microcirculatory alterations, and thereby heart dysfunction
IL-2	Spontaneously released early after infection, its production is markedly suppressed during the acute phase of infection
IL-3	Activates a trypanostatic activity of macrophages
IL-4	Inhibits IFN γ -mediated macrophage activation and NO release. Its levels are increased in the chronic phase of infection, especially in susceptible hosts. Increases susceptibility to infection with at least some <i>T. cruzi</i> strains
IL-6	Decreases cumulative mortality <i>in vivo</i> . Released by endothelial cells after <i>in vitro</i> infection with <i>T. cruzi</i>
IL-10	Inhibits IFN γ -mediated macrophage activation. Increases susceptibility to infection. Increased in susceptible as compared with resistant strains of mammalian hosts
IL-12	Reduces parasitemia and mortality, through IFN γ - and possibly TNF α -mediated mechanism of resistance

NO might react with each other to form peroxynitrite, a stronger trypanocidal molecule than its precursors. Whereas the relevance of ROS is still controversial, NO is necessary for control of the parasite load both in *in vitro* activated macrophages and during *in vivo* infection with *T. cruzi*.

Pathogenesis and immunopathology Different mechanisms have been proposed to participate in the pathogenesis of chagasic cardiopathy, and probably all have some degree of involvement in such a process. Microvascular changes, as manifested by platelet aggregates, increased levels of P-selectin, thrombus formation and histochemical evidence of myocardial hypoxia in humans and experimental models, might cause focal necrosis. Conversely, the

reduction of parasympathetic ganglion cells in the heart and gut of chronic Chagas' patients has prompted suggestions for a role for denervation in pathogenesis. Vagal and myenteric plexus denervation could induce an increased sympathetic tone that may have a direct effect in heart arrhythmogenesis or gut megasyndrome. There is by now convincing evidence that immune responses are involved in pathogenesis. Chronic inflammatory reactions depend on a persistent antigenic stimulation by parasite antigens. Although a chronically infected host displays undetectable parasitemia and very rarely tissue amastigotes, parasite antigens have been detected in inflammatory infiltrates and such lesions correlate with the severity of the disease. Autoantibodies or autoreactive cellular responses generated through polyclonal activation or by parasite antigens cross-react with mammalian neurons, lymphoid cells, laminin, ribosomal proteins, sarcolemma, myocardial β receptors and myosin. The levels of some of these antibodies correlate with the clinical status (myosin for example), and some antibodies mediate physiologic alterations in the target cells. For example, parasite cross-reactive antibodies trigger neurotransmitter-receptor interactions in myocytes and thereby alter the physiology of normal myocardium. Tissue destruction by inflammatory cells is probably facilitated by the presence of parasite antigens and ectopic expression of adhesion molecules and histocompatibility antigens on the surface of infected or noninfected cells during infection. CD8⁺ T cells have been shown to dominate the inflammatory infiltrate; however, the pathogenic role for CD8⁺ T cells is not clear. In contrast, CD4⁺ cells from chronically infected mice comprise only 5–10% of the inflammatory infiltrate but have been shown to play a role in pathogenesis, as they are involved in the rejection of grafted syngeneic heart tissue. Hypothetically, almost every immunologic mechanism described as participating in protection might also be involved in tissue pathology. Whatever the damaging mechanism, no data presented as yet can explain the prolonged latent period between the initial infection and the pathologic changes 5–40 years later in human infection.

Escape mechanisms

As with all microorganisms, *T. cruzi* has developed stage-specific strategies to circumvent host defence mechanisms before, during and after entry into host cells. The major evasion method employed by *T. cruzi* is its promiscuity, in other words the ability to invade all nucleated cells, thereby hiding from the immune response. Although antigenic variation of

surface antigens has not been described in *T. cruzi*, many of the surface genes including those coding for enzymes necessary for invasion (trans-sialidase for example) are expressed as multigenic families. Expression of such antigens might be modulated by the parasite to present a constantly changing face to the host immune system. As an alternative escape mechanism, some of the components of these multigenic families do not possess enzymatic activity but have extensive cross-reactivities with their enzymatic relatives. Moreover, the active sites of such enzymes are not immunodominant, and are thereby protected from neutralizing immune responses. As a protective back-up, *T. cruzi* displays several molecules involved in invasion, but none of them appears to be an absolute requirement for invasion and inactivation of any of them rarely renders a complete inhibition. Soluble molecules shed or secreted by *T. cruzi* bind non-infected cells, and if correctly presented to the immune response they will divert it. Moreover, this phenomenon, and the presence of cross-reactive antigens, can convert a potent and sterilizing immune response into a detrimental one as it might also harm noninfected tissue. Once inside the lysosome Tc-TOX and trans-sialidase cooperate in *T. cruzi* liberation into the cytosol, thereby avoiding the toxic milieu in the phagolysosome. Extracellular trypomastigotes have also developed evasion mechanisms and are protected from activation of the alternative complement pathway by complement regulatory molecules displaying genetic and functional similarities to decay-accelerating factor (DAF).

Vaccines

Vaccination studies have been undertaken in a number of different animal species by using a variety of different antigens, including whole lysed parasites, subcellular fractions and purified/recombinant antigens. The results generally show that many of these antigen preparations reduce parasitemias during the

acute phase of the disease and convert lethal to non-lethal infections. However, no vaccination study has achieved complete protection and the vaccinated animals become infected. Reduction of parasitemia might reduce the incidence and severity of the chronic phase of the disease, and might constitute a goal for immunoprophylactic studies. In any case, alternative measures, such as eradication of the insect vector, control of the parasite in blood banks and chemotherapy of congenital and acute cases, have rendered considerable positive results in the battle against Chagas' disease during the last 10–20 years.

See also: Cardiac disease, autoimmune; Contraception, immunological; Decay-accelerating factor (CD55); Parasites, immunity to; Trypanosomiasis, African.

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CHÉDIAK-HIGASHI SYNDROME

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Chédiak-Higashi syndrome (CHS) is an autosomal recessive disorder of humans that results in the formation of 'giant' intracellular vesicles. The formation of these large vesicles results in impaired function, which often compromises cellular activities. Many of

the granule types affected in this disorder are required for proper immune cell function, and hence cause a severe immunodeficiency in afflicted individuals.

This rare human disorder has numerous pheno-

typically similar disorders in other species including beige mice, Aleutian minks, cats, cattle, and even killer whales. The beige mouse has been used as a model organism for the study of CHS for many decades, and much of what is known about CHS has been obtained through the study of these mice. Somatic cell fusion studies have suggested that beige mice, Aleutian minks and CHS patients have defects within homologous genes, therefore providing genetic evidence which supports the hypothesis that beige and CHS are genetically similar disorders.

CHS/beige primarily affects four classes of intracellular vesicles: lysosomes, melanosomes, platelet-dense granules and cytolytic granules (Figure 1). It is thought that the formation of these 'giant' vesicles is due to a malregulation of either vesicle fusion or fission. The net result of this single gene defect is a reduction in the number of vesicles and an increase in their average size. The mouse beige gene has recently been identified and will be discussed below.

The clinical phenotypes in CHS/beige are the formation of giant vesicles, pigment dilution, a bleeding tendency and a severe immunodeficiency. Giant lysosomes are found in almost every cell of the body. This defect causes impaired lysosome function, which is tolerated by many cell types but which may ultimately kill others (e.g. neurons). The formation of giant melanosomes causes a pigment dilution that results in a near albino appearance of most CHS patients. In these individuals, the hair, eyes, skin, or any combination of the three, are the sites of pigment alterations. In mice and other mammals this defect

results in an alteration in coat color. Platelet-dense granules also form anomalous large structures that result in a platelet storage pool deficiency. Due to this deficiency, CHS patients and beige mice have decreased platelet aggregation and a bleeding tendency.

It is within the immune system that the CHS/beige defect exerts its most profound and deadly effects. The cell types known to show the CHS defect of enlarged granules include CD8⁺ T cells, eosinophils, basophils, neutrophils, mast cells, macrophages, megakaryocytes, B cells and natural killer (NK) cells. Each of these cell types contains large lysosomes as well as enlarged specialized granules; for example, neutrophils often contain large granules derived from the fusion of azurophilic and specific granules. Neutrophils from affected individuals also show defective chemotaxis; they have normal bacterial phagocytosis but impaired intracellular killing of bacteria. It has been demonstrated for neutrophils and macrophages that the newly formed phagosomes often fail to fuse with lysosomes, thereby allowing the internalized bacteria to survive longer and to multiply.

A second cell type profoundly affected by the CHS/beige defect is the NK cell. The numbers of NK cells and their target specificity and binding appear normal, but these cells are unable to kill their targets. The beige mouse is often used as a NK cell-deficient animal in order to study the role of NK cells in various immune responses. It is believed that defective NK cell activity is due to impaired exocytosis of cytolytic granules. A very similar defect is also observed

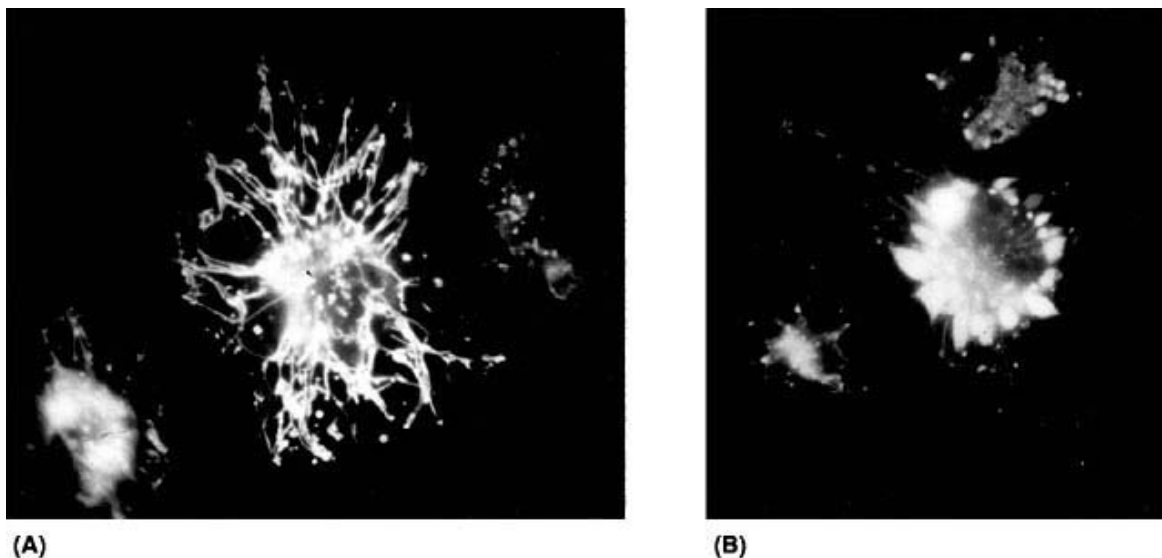


Figure 1 Bone marrow macrophages from (A) a C57BL/6J mouse and (B) a C57BL/6J-bg/bg mouse (100 × magnification). Note the 'giant' lysosomes clustered near the nucleus in (B). (Reproduced with permission of Company of Biologists Ltd from Perou CM and Kaplan J (1993) Chédiak-Higashi-syndrome is not due to a defect in microtubule-based lysosome mobility. *Journal of Cell Science* 106: 99–107.)

in CD8⁺ T cells. The decreased activity of these cell types can be overcome *in vitro* by chronic stimulation with exogenously added cytokines and longer incubation times. Unfortunately, these results cannot be replicated *in vivo*. Other cell types of the immune system show the CHS defect (e.g. B cells, eosinophils, basophils and mast cells), but their function does not appear to be significantly compromised. For example, CHS patients and beige mice are capable of making antibodies against invading organisms.

Until the advent of antibiotics, infections killed most CHS patients. Today, the most deadly effect of the CHS mutation is the 'accelerated phase'. The accelerated phase is a mononuclear lymphoma-like cellular infiltrate that invades the major organs of the body. This infiltrate, along with the bleeding tendency, causes major organ failure, hemorrhage and the death of the afflicted individual. It is thought that the accelerated phase is triggered by a viral infection but the precise virus or even the cellular origin of this lymphoma, is not known.

The only cure for CHS is to prevent the accelerated phase. This can be done by whole body irradiation to kill the endogenous bone marrow that gives rise to the lymphoma, followed by allogeneic bone marrow transplantation. This treatment has worked in many cases, and has prevented the accelerated phase from recurring for up to 13 years in one patient. As these CHS patients age, however, other problems may arise, such as neurologic defects due to the loss of neurons. CHS is a candidate for gene replacement in hematopoietic stem cells, which would cure the immunodeficiency and prevent the accelerated phase, while also circumventing the problem of graft-versus-host disease.

The mouse beige gene has recently been identified using a Yeast Artificial Chromosome complementation approach. The human homolog of the beige gene was then isolated and shown to contain mutations within CHS patients. These data demonstrate that beige and CHS are due to mutations within orthologous genes. The Beige/CHS protein is approximately 3800 amino acids in length and contains a protein-protein interaction motif (WD40 repeats) and a highly conserved domain of unknown function. This conserved domain identifies homologs in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, demonstrating that this protein is conserved throughout evolution. The overall sequence of the

Beige/CHS protein is novel and gives few clues as to its biochemical function.

See also: Exocytosis; Hematopoietic stem cell transplantation; Immunodeficiency, animal models; Natural killer (NK) cells; Phagocytosis.

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CHEMOKINES

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The salient feature in a number of inflammatory conditions, such as infection, hypersensitivity reactions or autoimmune diseases, is the presence of infiltrating leukocytes. The extravasating leukocytes are critical for host defense, leading to clearance of the inciting factors such as infectious agents and particulate antigens. However, it should be appreciated that leukocyte recruitment may also contribute to the pathogenesis of an underlying disease. The maintenance of leukocyte recruitment during inflammation requires communication between infiltrating leukocytes and the endothelium as well as various extravascular cells. These signals are mediated via the generation of early response cytokines, the expression of surface adhesion molecules and the production of chemotactic molecules. While many cytokines modulate cellular adhesion and promote leukocyte recruitment, few have been shown to directly affect integrin avidity. Recent studies have identified a superfamily of small, soluble, structurally-related cytokines called 'chemokines' (chemotactic cytokines) that appear to be excellent, physiologically relevant candidates for the rapid triggering of integrin-mediated adhesion and selectively induce the directional migration of various leukocyte subsets both *in vivo* and *in vitro* (Figure 1 and 2). These molecules and their receptors play critical roles in hematopoiesis, angiogenesis, cellular activation, cytotoxicity and wound healing, as well as in a variety of acute and chronic inflammatory disease states.

Characteristics of chemokines

Over 30 unique human and mouse cytokines have been identified as members of the chemokine superfamily (Table 1). Based on the presence or absence of an intervening amino acid residue located between the first two of the four conserved cysteine residues, the chemokine superfamily can be separated into two distinct subfamilies called the α (or C-X-C) and the β (or C-C) subfamilies. As the chemokines are secreted molecules, their cDNAs code for a precursor protein which contains a leader sequence (20–25 amino acids), which presumably enables the chemokines to

be produced, cleaved and secreted to yield a mature form at the cell membrane by a wide variety of cell types. Chemokines share many other similarities, including their high basic nature as well as their ability to bind heparin through heparin-binding domains. At the amino acid level these molecules exhibit between 24–80% homology. Chemokines in

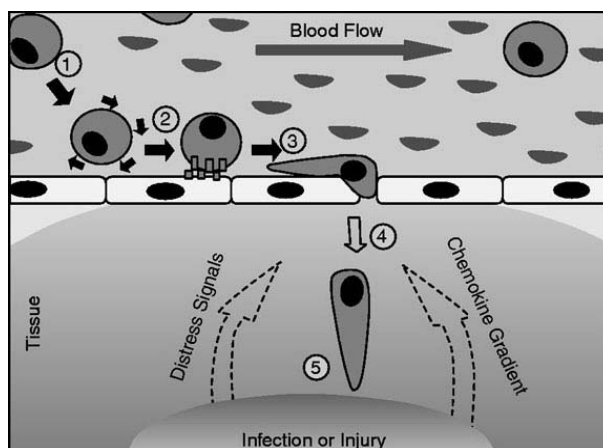


Figure 1 Schematic model of leukocyte trafficking into inflammatory tissues. Many of the models describing leukocyte entry into inflammatory sites depend on at least three distinct molecular interactions: selectin-carbohydrate, chemokine-receptor, and integrin-CAM. As leukocytes circulate in the blood and lymph (1), they constantly survey the surrounding endothelial barrier for the presence of distress signals alerting them to problem areas. Once alerted, a sequential series of signals is required to mediate leukocyte entry into the extravascular tissues. With the release of distress mediators such as IL-1, TNF, LPS, or histamine, various selectin ligands are rapidly expressed and mobilized to the membrane of the activated endothelial cells within the tissue environment. The selectins mediate the initial tethering and rolling of responding leukocytes to the blood vessel wall slowing their transit through the circulation (2). Upon interacting with proadhesive signals (3), most likely provided by heparan proteoglycan- or extracellular matrix-bound chemokines, the integrin molecules on the leukocyte surface are altered from a conformationally inactive complex to a high avidity state permitting leukocyte binding to the expressed cell adhesion molecules (such as ICAM-1, VCAM-1, etc.) present on inflamed endothelial cell surface (3). At this point, leukocyte rolling stops and firm adhesion begins. These adherent leukocytes are then permitted to spread out along the activated endothelial barrier and migrate (with the assistance of various proteinases) through the vascular endothelial barrier and basement membrane into the extravascular tissue space (5). Once through, soluble as well as extracellular matrix- and heparan proteoglycan-bound chemokines derived from the inflammatory lesion form a concentration gradient promoting the directional migration of leukocytes from areas of low chemokine levels toward the areas of chemokine production.

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<i>Subfamily</i>	α Chemokines		β Chemokines		γ Chemokines	
<i>Structure</i>	---C-X-C-----C---C---		---C-C-----C-C---		---C-----C---	
<i>Species</i>	Human	Mouse	Human	Mouse	Human	Mouse
<i>Chromosome</i>	4q12-q21	—	17q11-q21	11	—	1
<i>Members</i>	IL-8/NAP-1	—	MCP-1/MCAF	JE	Ltn	Ltn
	GCP-1					
	GRO α /MGSA	KC	MCP-2	HC14	—	ATAC
	GRO β /MIP-2 α	MIP-2	MCP-3	fic/MARC		
	GRO γ /MIP-2 β	—	RANTES	RANTES		
	ENA-78	ENA-78	MIP-1 α /LD78	MIP-1 α		
	NAP-2	—	MIP-1 β /ACT2 pAT744	MIP-1 β		
	—	KC	I-309	TCA-3/p500		
	NAP-4	—	—	C10/MRP-1		
	GCP-2	—	—	MRP-2		
	PF-4	—	HCC-1	—		
	IP-10	IP-10/C7 CRG-2	—	—		
	MIG	MIG	—	—		

their monomeric form range from 7 to 14 kDa and are active at concentrations ranging from 10^{-8} to 10^{-10} M. However, many of the chemokines form dimers, trimers and tetramers upon secretion. While chemokine dimer formation has been shown to be necessary for optimal ligand interaction with chemokine receptors by some investigators, this requirement is rather controversial as chemokine monomers have also been shown to be biologically active. Chemokine subfamily members are also coclustered on the same chromosomes. α Chemokines are clustered on human chromosome 4q12-q21, while β chemokines cluster on human chromosome 17q11-q21 and mouse chromosome 11. Lymphotactin (Ltn), a member of the 'C' subfamily (γ chemokines) lacking two of the typical four cysteine residues, is located on human chromosome 1. Many of the α chemokines possessing -ELR- residues at position 4-6 near the N-terminus have been shown to be potent mediators of neutrophil chemotaxis both *in vitro* and *in vivo* and have the capacity to bind the shared interleukin 8 (IL-8) receptor type II. All α chemokines which lack the -ELR- motif fail to chemoattract neutrophils or bind the shared IL-8 type II receptor. Chemokines are not typically expressed in

unstimulated cells, tissues or cell lines but are rapidly induced (usually within 1–2 h post stimulation) in response to either endogenous or exogenous stimuli. Typically, agents which enhance inflammation (e.g. lipopolysaccharide (LPS), IL-1, tumor necrosis factor α (TNF α), injurious stimuli and infectious agents) or immune activation (e.g. lectins, antigenic stimulation, mitogens, immunoglobulin E (IgE) antigen and phorbol esters) also promote chemokine production. Chemokine mRNA can comprise up to 1% of the total mRNA content of a stimulated cell. Chemokine production appears to be ubiquitous, in that almost every cell and tissue type in the body has been shown to produce chemokines upon stimulation. It is believed that chemokines produced within a given tissue, rather than remaining in solution, are preferentially immobilized through low-affinity binding to heparin-bearing proteoglycans (HP) on the vascular endothelial cell barrier or to extracellular matrix proteins within the extravascular tissues (Figure 1 and 2). As chemokines possess GAG-binding sites and bind heparin through their carboxyl-termini, this binding may be important in cytokine localization and immobilization on endothelial cells and matrix proteins as well as their biological activity.

Biological activities

The chemokine subfamilies can be distinguished from one another by their apparent leukocyte specificity in mediating cell migration and adhesion. Typically, ELR⁺ α chemokines induce neutrophil but not

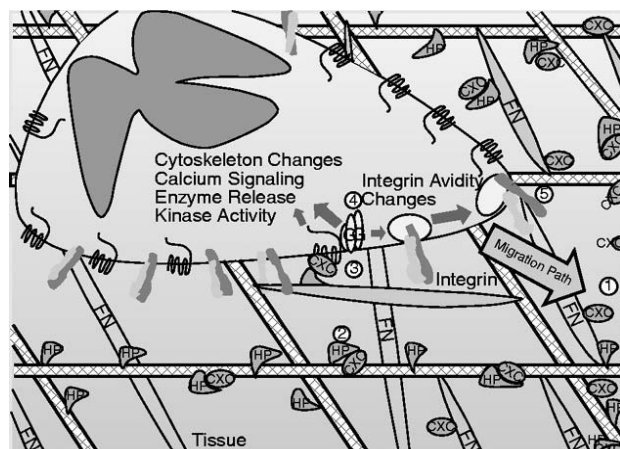


Figure 2 Leukocyte entry and migration within an inflammatory environment. Once across the vascular barrier, leukocytes directionally adhere and migrate along the various extracellular matrix proteins and CAMs within the tissue toward the sites of chemokine production (1). The chemotactic gradients are believed to be maintained via chemokine binding to extracellular matrix proteins (e.g. fibronectin (FN), collagen (CN), etc.) and heparan proteoglycans (HP) found within the tissues (2). The migrating leukocytes directionally move from regions of low levels of chemoattractants to regions containing higher concentrations. As leukocytes infiltrate sites of increased chemokine concentrations, chemokine receptor desensitization may inhibit further migration in response to a given chemokine gradient. In addition, it seems quite possible that the expression of various matrix- and proteoglycan-degrading enzymes may also serve to regulate leukocyte entry and departure from inflammatory sites by the cleavage of chemokine presenting molecules. The presentation of a chemokine to a specific cell surface chemokine receptor (CXCR or CCR) induces a complex series of intracellular signals that facilitate cell adhesion and migration (3, 4). As chemokine receptors are associated with a heterotrimeric G protein complex (with each receptor possessing its own unique G protein profile), signaling through these molecules is believed to transduce a signal(s) necessary to activate integrin adhesiveness (5). Both chemokine receptors and integrin molecules mobilize to the leading edge of the migrating leukocyte, optimizing ligand recognition and promoting directional migration. Signals induced by these receptors also induce an increase in integrin expression and the release of various metalloproteases/TIMPs may assist in transverseing the various layers of the basement membrane and tissue environment. Furthermore, it seems most likely that only certain integrin molecules are activated by chemokine receptor interactions (depending on the receptor and its G-protein association) possibly by regulating leukocyte adhesion to specific extracellular matrix proteins (5). A greater definition of the interplay between chemokines, adhesion molecules, and enzyme release may further elucidate the true mechanisms required for leukocyte transmigration through the vascular barrier and basement membrane.

monocyte migration, while β chemokines predominantly act on monocytes and macrophages with no activity on neutrophils. Besides neutrophils and monocytes, both α and β family members are active on a number of cell types (Table 2). In addition to the subtype of leukocyte being examined, the chemokine responsiveness of a cell may depend on its activation state. In addition, stimulation of certain cell populations with cytokines or activation stimuli appears to enhance their chemokine responsiveness. Chemokine activities are not only restricted to chemotaxis and adhesion, multiple other proinflammatory effects have been described. These include the induction of superoxide anion, nitric oxide, metalloproteinases, intracellular granules and cell adhesion molecules (Table 2). Many of these biological properties are believed to contribute not only to their immunological roles in inflammation but also to their physiological roles in tissue repair and cellular growth/differentiation.

Chemokine receptors

Chemokine receptors are members of the rhodopsin or serpentine receptor superfamily and have the characteristic G protein-coupled seven hydrophobic transmembrane spanning regions. These receptors also exhibit varying degrees of homology, particularly within their transmembrane spanning regions. Studies have revealed a number of human chemokine receptors with various chemokine binding characteristics (Table 3). Chemokine ligand-receptor interactions initiate a characteristic pattern of responses, including shape change, integrin activation, chemotaxis, degranulation, enzyme secretion and respiratory burst. Depending on the chemokine and leukocyte subtype, receptor ligation has been shown to activate a number of signaling pathways, including phosphoinositol hydrolysis, arachidonic acid metabolism, the activation of various kinases, and the rapid elevation of diacylglycerol and cytosolic Ca²⁺ levels. In addition, all chemokine receptors contain one or more of the consensus sequences for G_i protein activation which is believed to be essential for the high-affinity binding of chemokines and their adhesion/migration activities.

Pathological disease states

Many chemokines have been shown to be present in numerous inflammatory and noninflammatory disease states, including sepsis, atherosclerosis, arthritis, cystic fibrosis and asthma. In many of the inflamed lesions the presence of chemokines is highly correlated with the level of leukocyte infiltration within

Table 2 *In vitro* effects of α and β chemokine family members

Chemokine	Target cell	Biological effects on various target cells
<i>α Chemokines</i>		
IL-8, GRO α , GRO β , GRO γ , NAP-2	Neutrophils	Chemotaxis; shape change; increased degranulation; increased respiratory burst; increased cytosolic Ca ²⁺ ; increased adhesion to endothelial cells, fibrinogen and extracellular matrix proteins; increased <i>Candida albicans</i> growth inhibition; increased expression of CD11a, CD11b, CD11c and CD18; increased lysosomal enzyme release; decreased L-selectin expression
PF-4	Neutrophils	Chemotaxis; superoxide release
IL-8, GRO α	T cells	Chemotaxis; stimulated polyphosphoinositide hydrolysis
IP-10	T cells	Chemotaxis; increased adhesion to endothelial cells and extracellular matrix proteins
IL-8	B cells	Inhibits IL-4-induced IgE production; inhibits B cell proliferation
IP-10, IL-8	NK cells	Chemotaxis; increased killing of tumor targets
IP-10	Monocytes	Chemotaxis
IL-8	Basophils	Chemotaxis; inhibits histamine release; increased leukotriene release
IL-8, IP-10	Keratinocytes	Increased proliferation
IL-8	Endothelial cells	Increased blood vessel proliferation (angiogenic)
PF-4	Endothelial cells	Increased ICAM-1 expression; decreased blood vessel proliferation (angiostatic)
IL-8, GRO α	Melanomas	Increased adhesiveness and haptotactic response
IL-8	Smooth muscle	Chemotaxis
PF-4, CTAP-III, β -TG, NAP-2	Fibroblasts	Chemotaxis; increased proliferation
<i>β Chemokines</i>		
MCP-1, MCP-2, MCP-3, MIP-1 α , MIP-1 β , RANTES, C10	Monocytes	Chemotaxis; increased superoxide anion release; increased cytosolic Ca ²⁺ ; increased adhesion to endothelial cells and extracellular matrix proteins; increased <i>N</i> -acetyl- β -glucuronaminidase; increased cytostatic augmenting activity; increased intracellular calcium; induced arachidonic acid release
MCP-1, MIP-1 α , MIP-1 β , RANTES, MCP-3, C10	T cells	Chemotaxis; increased adhesion to extracellular matrix proteins and cytokine-activated endothelial cell monolayers; increased metalloprotease and TIMP release; increased CTL killing of tumor cell targets; degranulation; costimulation
MCP-1, MIP-1 α , RANTES	Mast cells	Chemotaxis; histamine release
MCP-1, MCP-3, MIP-1 α , RANTES	Basophils	Chemotaxis; increased histamine release; increased intracellular calcium; increased leukotriene release; increased adhesion
MIP-1 α , MCP-1, RANTES, MCP-3	B cells	Chemotaxis; increased proliferation; increased IgE and IgG4 production
MIP-1 α , MIP-1 β , RANTES, MCP-1	NK cells	Chemotaxis; increased killing of tumor targets; increased adhesion to extracellular matrix proteins
MIP-1 α , RANTES, eotaxin, MCP-3	Eosinophils	Chemotaxis; induces cationic protein release; increased intracellular calcium; increased cell surface integrins
MIP-1 α , MCP-3	Neutrophils	Weak increases in intracellular calcium; increased shape change

CTL, cytotoxic T lymphocyte; ICAM, intercellular cell adhesion molecule; NK, natural killer; TIMP, tissue inhibitor of metalloproteinase.

the diseased tissue. Under some conditions the chemokines facilitate leukocyte adhesion and transmigration into compromised tissues, resulting in the phagocytosis and killing of microbes or allogeneic cells (host resistance), while in other states aberrant chemokine production contributes to the induction and progression of disease pathology (Figure 1 and 2). Several chemokine receptors act as human immunodeficiency virus 1 (HIV-1) coreceptors. T cell tropism is associated with CXCR4 expression whereas macrophage-tropic strains bind to CCR5. A

greater understanding of the complexities of the chemokines that mediate cell adhesion and transmigration may assist in the orchestration, regulation and control of various pathological disease states.

See also: Acute inflammatory reaction; Adhesion molecules; Autoimmune diseases; Autoimmunity; Chemotaxis; Cytokines; Endothelium; Human immunodeficiency viruses; Hypersensitivity reactions; Integrins; Interleukin 8 and its receptor.

Table 3 Chemokine receptors

Chemokine receptor	Ligands
CXCR1 (IL-8RI)	IL-8, GCP-2
CXCR2 (IL-8RII)	IL-8, GCP-2, GRO- α , - β , - γ , ENA-78, NAP-2, LIX
CXCR3	IP-10, MIG, I-TAC
CXCR4	SDF-1
CCR1	MIP-1 α , RANTES, MCP-3, -4
CCR2	MCP-1, -2, -3, -4, -5
CCR3	Eotaxin-1, -2, RANTES, MCP-3, -4
CCR4	TARC
CCR5	MIP-1 α , MIP-1 β , RANTES
CCR6	MIP-3 α
CCR7	MIP-3 β
CCR8	I-309
Duffy receptor	All α and β chemokines except MIP-1 α , MIP-1 β and IP-10

See Table 1 for explanation of abbreviations.

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CHEMOTAXIS

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Chemotaxis, the directional locomotion of cells towards a source of a chemical gradient, was first described in bracken fern spermatozoa by Pfeffer in 1884. Shortly afterwards (1888) Leber described a similar phenomenon in mammalian leukocytes responding to a focus of injury. Since then, chemotaxis has been accepted as an important mechanism for mobilizing phagocytic and immune cells at sites of infection, tissue injury, and immune reactions. However, it is only recently that something has been learnt about the sensory mechanisms by which cells detect attractants and about the motor which provides the power for their locomotion, and knowledge of both is still very incomplete. Chemotaxis is simply a special form of locomotion, and a brief preliminary description of leukocyte locomotor behavior will help understand its mechanism.

Morphological events of leukocyte locomotion

Leukocytes which are unstimulated are spherical and immotile. This is their morphology both in the bloodstream and after careful *in vitro* preparation. Following stimulation with a chemoattractant, the cells change shape within a minute or so by extending a pseudopod, or lamellipodium, at one pole. Slow contraction waves may then pass antero-posteriorly down the cell from this point. The cell thus adopts a typical, tapered, locomotor morphology (Figure 1). The ruffled anterior lamellipodium is initially hyaline and is rich in actin and actin-associated proteins but lacking in organelles. Behind it is the organelle-rich cell body with a posterior tail (uropod) which forms after the complete passage of a contraction from front to back. All these changes occur whether the cell is in suspension or on a surface. Surfaces, either two-dimensional or three-

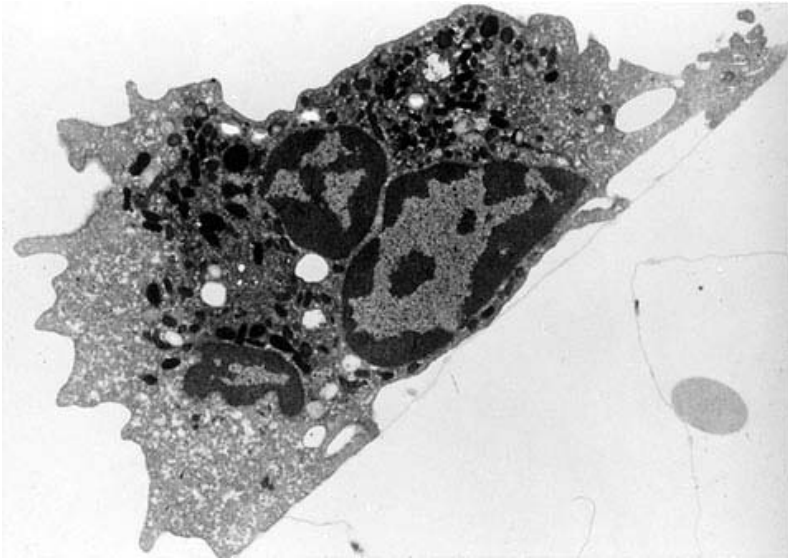


Figure 1 A neutrophil migrating on a surface. Note the broad, ruffled anterior lamellipodium which is free of organelles. Behind this is the cell body containing the nucleus and cytoplasmic organelles. The cell is 'waisted' about a third of the way down. Observation of moving cells shows that this is due to a constriction which passes from front to back as the cell moves forward. A posterior tail is also seen. Special techniques reveal that the anterior lamellipodium is rich in actin and actin-associated protein and that cell surface receptors are frequently concentrated at this pole (not shown in this picture). (Photography by courtesy of Dr Wendy Haston).

dimensional (such as collagen or fibrin gels) give purchase for locomotion. Similar changes are seen in neutrophils, macrophages, lymphocytes and eosinophils. They are initiated by binding of 'chemotactic' factors to cell surface receptors, and all of these cells show chemotaxis, though the neutrophil is the best studied. The term chemotactic factor is imprecise since such factors also activate chemokinesis (below) and other functions such as adhesion, oxidative metabolism, secretion, etc.

Definitions of locomotor reactions

Random and directional locomotion

The paths of moving cells may be directional or random. In random locomotion, the different cells of a population move in directions that are random in relation to one another and to the environment, but the individual cells may persist in their initial direction of locomotion for several minutes. This is called a 'persistent random walk'. When observed over long periods, the cells turn so that, over long periods (>5 min), mean square displacement is proportional to time. In directional locomotion the cells show preference for a particular direction, and their morphological axis is oriented in that direction.

Locomotor responses to physical or chemical cues

The environment of the cell may determine both directional and random locomotion. Chemical cues determine reactions as follows.

Chemokinesis Chemokinesis has two forms: orthokinesis and klinokinesis. In orthokinesis, the speed or frequency of locomotion is determined by the magnitude of the stimulus, i.e. the attractant concentration.

In klinokinesis the amount of turning the cell does is determined by the magnitude of the stimulus. Orthokinesis is thus a straightforward acceleration or deceleration of cells in response to stimuli. It is certainly important in leukocytes. Klinokinesis is of uncertain importance in leukocytes, though, in bacteria such as *Escherichia coli*, chemicals determine whether the organisms 'tumble' or show straight runs. In the rest of this article, the term 'chemokinesis' will be used synonymously with 'orthokinesis' as defined above.

Chemotaxis Chemotaxis is a reaction by which the direction of locomotion is determined by the direction and magnitude of a chemical stimulus. The stimulus is usually in the form of a concentration gradient. In leukocytes, chemotaxis is always positive, i.e. towards a gradient source. Negative chemotaxis away from a source may exist in other organisms.

Contact guidance Chemotaxis is not the only cue that causes cells to move directionally. Contact guidance is a reaction by which the direction of locomotion is determined by the shape or curvature of the substratum on which the cell moves, i.e. by physical rather than chemical properties of the environment. For example, in aligned matrices of collagen or fibrin, neutrophils and lymphocytes prefer to move in the axis of the aligned fibers rather than across them. Cells are equally free to move in both directions in that axis, i.e. this is not a unidirectional locomotion, like chemotaxis. It is likely to be an important determinant of the ability of leukocytes to accumulate, particularly in tissues with complex patterning.

Locomotor capacity Leukocytes only show the capacity for locomotion at certain stages of their

development. Thus myeloid precursors in bone marrow are nonmotile, and lack chemotaxis receptors, but they acquire these as they develop, so that virtually all blood neutrophils and monocytes have a capacity for locomotion and chemotaxis. T lymphocyte precursors may be attracted chemotactically into the thymus, though as they mature, they may lose locomotor capacity. Many lymphocytes in blood and peripheral lymphoid tissues are not motile in the G_0 stage of growth, but develop locomotor capacity when they enter the G_1 phase of cell cycle if cultured in the presence of mitogen or antigen. Obviously locomotor capacity is an essential prerequisite for chemotaxis, chemokinesis, etc.

Haptotaxis The term haptotaxis was introduced by Carter in 1967 to describe directional locomotion on a surface bearing a gradient of changing adhesiveness. It is now used in a different sense to describe chemotaxis on gradients of surface-bound attractants. This is postulated to be important for transmigration of leukocytes across vascular endothelium and through connective tissue matrices towards sites of inflammation. Leukocyte migration requires contact with a surface but is not always dependent on adhesion since all the morphological changes described above occur when chemoattractants are added to cells in suspension. Moreover cells are capable of migrating through three-dimensional matrices without adhering since they can gain purchase by using the meshwork of fibres like a climbing frame. Nevertheless on two-dimensional surfaces, migration must be dependent on adhesion and there is now much evidence that, in sites of inflammation, the adhesion of both leukocytes and vascular endothelial cells is greatly increased.

How do leukocytes perceive chemotactic gradients?

Various explanations have been proposed for how a cell exposed to a gradient knows that the concentration on one side is higher than on the other and how it translates this information into locomotion in the direction of the highest concentration. Leukocytes can detect differences of as little as 1% in concentration across their lengths. Various ideas are as follows.

Spatial detection of a gradient

Stationary leukocytes exposed to a gradient put out a pseudopod in the direction of the gradient source before the cell has moved, possibly because receptor occupancy by ligand on the side facing the gradient source is higher than receptor occupancy on the other side. The cell response occurs where receptor

occupancy is highest. There are difficulties with this idea. The difference in receptor occupancy between the two sides may be very small and there is no easily imagined biochemical mechanism for the cell to perform the complex computations involved in spatial sensing and converting them into a polarized response.

Temporal detection of a gradient

Bacteria are small and fast-moving and it is believed that they move from point to point, sample the attractant concentration as they move, and act on the information thus received. Thus they detect the gradient in time. This requires a simple memory, the biochemistry of which is simpler than for spatial sensing. However, leukocytes detect gradients without moving. It has therefore been suggested that temporal sensing is determined not by movement of the cell but by movement of receptors on the cell surface. Receptors moving forward on the front of the cell experience increased ligand binding, which would determine increased cytoskeletal activity and motion at this region. At the cell's rear the opposite would be the case.

Chemotaxis may be a stochastic process

Neither spatial nor temporal mechanisms explain why leukocytes assume a front-tail polarity (Figure 1) in uniform concentrations of attractant. Under these conditions the cells ought to respond uniformly all over if the response resulted from a simple ligand-receptor interaction linked to the cytoskeleton. One possibility is that the cellular response is determined by the position on the cell surface at which the first signal is received. This requires the attractant concentration to be low enough to allow a cell to distinguish a first signal from subsequent signals. At low attractant concentrations, cells appear to be able to do this, but at higher concentrations, they show irregular morphologies when stimulated and take a few minutes to adopt a head-tail polarity. Possibly one pole becomes dominant due to chance fluctuations in receptor occupancy or signal transduction in different parts of the cell. In gradients, most cells will polarize up-gradient because most cells will receive their first signals on the side facing the gradient source. Both in gradients and in uniform concentrations, polarization is stabilized by redistribution of receptors to the head of the cell so that subsequent ligand-receptor interactions are most likely to occur there. If the stochastic model is correct, the molecular mechanisms for chemotaxis and chemokinesis are identical, and the path of locomotion of the cell, random or directional, is determined by the topographical distribution of attractant molecules rather than

Table 1 Chemokine attractants for leukocytes

	Cell type				
	Neutrophils	Monocytes/ macrophages	T lymphocytes	Eosinophils	Basophils
<i>α Chemokines</i>					
IL-8	++	—	+		
NAP-2	+	—			
GRO	+	—			
IP-10	—	—	(+)		
ENA-78	+	—			
MIP-2	+	—			
GCP-2	+	—			
<i>β Chemokines</i>					
MIP-1α	—	++	(+)	+	
MIP-1β	—	++	(+)	—	+
MCP-1	—	++	+	—	+
MCP-2	—	++	+		
MCP-3	—	++	+	+	
MCP-4	—	++	+	+	
RANTES	—	++	±	+	+
Eotaxin	—	—		+	
<i>Other Chemokines</i>					
SDF-1	—	+	++		
<i>Receptors for chemokines</i>					
α Chemokines	CXCR1 (previously IL-8RA): specific for IL-8 CXCR2 (previously IL-8RB): broad specificity CXCR3: binds IP-10 CXCR4: binds SDF-1				
β Chemokines:	CCR1: binds MIP-1α and β, MCP-3, RANTES CCR2A: and B bind MCP-1 and 3 CCR3: binds eotaxin, RANTES, MCP-1 CCR4: binds MIP-1α, MCP-1, RANTES CCR5: binds MIP-1α and β, RANTES				

Note: The list of both chemokines and their receptors is incomplete. Parentheses indicate activity for a T cell subset. SDF-1 attracts resting T cells. Other chemokines attract only activated T cells.

by different cellular mechanisms for locomotion in gradients or in isotropic attractant concentrations.

Chemotactic factors

The term chemotactic factor is used of molecules which stimulate the directional locomotion of cells in gradients. In many cases a formal demonstration of directional locomotion has not been made and the less specific term chemoattractant is often used instead. There is a vast literature documenting such activity of many molecules for different classes of leukocyte. Recent interest has centered on the chemokine family of cytokines, different members of which are specific attractants for particular leukocytes, and which are believed to play a central role in inflammation. It is not possible to discuss all of these here but Table 1 lists some of the important chemokines and Table 2 lists some of the better-known nonchemokine attractants. Chemokines bind to serpentine seven membrane-spanning domain

receptors of the rhodopsin superfamily (type III transmembrane proteins) which are linked to trimeric GTP-binding proteins of the $G\alpha_1$ class. Many of the nonchemokine attractants bind to receptors of this family as well, e.g. formyl peptides, C5a, but other attractants, e.g. interleukin 2 (IL-2), IL-15, transforming growth factor β (TGF β), tumor necrosis factor α (TNF α), stimulate chemotaxis through other types of receptor, so no generalizations can be made about the structure of chemotaxis receptors. The responsiveness of lymphocytes to chemoattractants is more complex than these tables would suggest, since there are many classes of lymphocyte, and, for example, neither B cells nor natural killer (NK) cells are included in the tables although there is literature describing attractants for both.

Importance of chemotaxis and chemokinesis *in vivo*

These reactions have been defined in *in vitro* studies,

Table 2 Nonchemokine attractants for leukocytes

	Cell type				
	Neutrophils	Monocytes/ macrophages	T lymphocytes	Eosinophils	Basophils
Formyl peptides	++	++	—	±	
C5a; C5a des Arg	++	++	—	+	
Leukotriene B ₄	+	+	—		
PAF	±	±	—	+	
Thrombin		+			
<i>Cytokines (nonchemokine)</i>					
TGFβ		++			
TNFα	+	+			
GM-CSF	++	++	—		
M-CSF (CSF-1)		+			
IL-2	—	—	++		
IL-15	—	—	++		
IL-16			+		

Note: Partial list. A large number of other chemoattractants have been documented.

and are difficult to study with precision *in vivo*. Buckley, in 1963, made small burns in tissues with a microelectrode and observed leukocytes migrating directionally to the injured site. This is similar to the reaction of 'necrotaxis' described by Bessis in which individual red cells are damaged with a laser, and in which nearby neutrophils migrate in and ingest the damaged red cell, but only immediately after the burn. Chemotactic gradients *in vivo* may be easily disturbed. However, if chemotactic factors are also all chemokinetic factors, it is possible that leukocytes leaving a vessel will move around at speed even in the absence of a gradient. Those that get closest to the lesion, where gradients are likely to be sharpest, will then pick up the gradient and move directionally to the lesion site.

Methods

There are two basically different types of assay in use to study chemotaxis. The first is to film, with a camera or videotape, the detailed movements of cells and to measure speed, direction, etc. in different absolute concentrations and different concentration gradients of attractant. This gives precise information about the reactions defined above, but requires fairly specialized equipment. The second is to measure the distribution of a cell population at a fixed time-point after exposure to an attractant in uniform concentration, gradients, etc. This is the basis of the widely used filter assays, agarose assays, etc. and the methodology and measurement are simple. However, these assays only give indirect measures of the reactions defined above and it is difficult to dis-

tinguish chemotaxis from chemokinesis using them. This has resulted in much confusion, and, for example, many workers equate directional locomotion with chemotaxis, and random locomotion with chemokinesis. These are not synonymous as the definitions above make clear.

See also: Motility of immune cells; Phagocytosis; Chemotaxis of macrophages and monocytes; Chemotaxis of neutrophils; Chemotaxis of lymphocytes.

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CHEMOTAXIS OF LYMPHOCYTES

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Lymphocytes have long been known to be motile cells, but study of their locomotion has lagged behind that of monocytes and neutrophils, partly because of the heterogeneity of lymphocytes and their migratory behavior. Under normal circumstances there is continuous recirculation of both T and B cells between the blood and the lymphoid tissues. The recirculating population consists of naive small lymphocytes which are not in cell cycle. When lymphocytes recognize antigen, their migratory behavior alters. These cells enter the cell cycle and leave the recirculatory pool. Their adhesion phenotype changes, since they lose L-selectin and lose their affinity for the high endothelial venule (HEV) cells of lymphoid tissue. At the same time, they show increased expression and activity of many other adhesion molecules which allow them to attach to inflammatory endothelia, to cluster round antigen-presenting cells, and to bind to target cells for cytotoxicity. Thus the lymphocyte changes from a cell monitoring the environment for antigen to a cell that is focused on to effector functions. As would be expected, there are changes in locomotor properties between resting and activated lymphocytes which reflect these changes in function.

Small recirculating lymphocytes

Most of the lymphocytes in blood are recirculating cells. These cells give poor responses to most cytokines which have been reported to be lymphocyte chemotactic factors. It is difficult to induce more than 5–10% of blood lymphocytes to respond to any of these agents. However, small lymphocytes show a rapid change from a spherical to a polarized shape on contact with cultured HEV cell monolayers. Many of the cells transmigrate rapidly across to the undersurface of the monolayer where they continue to show active locomotion. It is likely that locomotion is activated by contact between specific surface molecules on the lymphocyte and the HEV. The chemokine, stromal cell-derived factor 1 (SDF-1) has recently been shown to attract resting T cells and is therefore likely to be a signal for lymphocyte recirculation.

Locomotion and chemotaxis of T cells: the role of activation

The capacity of lymphocytes from blood or lymphoid tissues to respond to chemoattractants is considerably enhanced by a period of 12–48 h of culture in the presence of activating agents. Polyclonal activators such as CD3-specific antibody, phytohemagglutinin (PHA), concanavalin A (con A), or even fetal calf serum will increase the proportion of locomotor cells from 10% to 30–70%. Antigens such as purified protein derivative of *Mycobacterium tuberculosis* (PPD) or superantigens such as staphylococcal enterotoxin B (SEB) have a similar effect. These are not direct chemoattractant effects. If the cultured cells are washed and resuspended in the presence of a fresh preparation of the activator, they may show no response, but if they are resuspended in their own supernatant medium, they do respond, suggesting that chemotactic cytokines are released during culture. Both lymphocyte activation and the release of attractants into the supernatant are dependent on the presence of accessory cells and highly purified T cells fail to become activated.

Following culture in the presence of accessory cells, the activated T lymphocytes can be purified and will then show chemotactic responses to pure cytokines (see tables 1 and 2 of the 'Chemotaxis' article). Interleukin 2 (IL-2) and IL-15 are both good chemotactic factors for these cells. The shared β chain (and probably also the γ chain) of the IL-2 and the IL-15 receptor are essential for this activity. The chemotactic activity of IL-15 probably plays a major role in recruitment of T cells into the lesions of rheumatoid arthritis. IL-16 also is a T cell attractant with a selective activity for CD4⁺ cells. Several chemokines also have activity. They include the α (C-X-C) chemokines, IL-8, and IP-10 (induced by IFN γ), and the β (C-C) chemokines, MIP-1 α and β , MCP-1, -2, -3 and -4, and RANTES. During activation with PPD, SEB or anti-CD3, as detailed above, sufficiently large quantities of IL-8 are released by monocytes to stimulate locomotion of T lymphocytes. β Chemokines have very little activity for T lymphocytes cultured for 24–48 h with the above-mentioned activators, but, during a week of culture with IL-2, the lymphocytes acquire chemokine receptors and show stronger locomotor responses to MIP-1 α and RANTES. The list of presently known attractant

cytokines is certainly incomplete. The profusion of lymphocyte attractant factors suggests that each may have specific roles in different types of lesion, but this is conjecture at present.

B cells

B cells, like T cells, recirculate and their first encounter with antigen may be in the T cell zone of lymphoid tissue where they meet antigen in clusters with interdigitating cells and T cells. Antigen-reactive B cells then migrate to the follicles to form a germinal center where a second encounter with antigen, in the form of immune complexes on the surfaces of follicular dendritic cells (FDCs), takes place. Further development to antibody-forming or memory cells also requires contact with CD40 ligand (CD40L)-positive germinal center T cells. All of these processes require active locomotion by B cells. Like T cells, B cells respond much better to attractants once they are activated.

Resting (G_0) B cells acquire locomotor activity during culture *in vitro* with T cell products, IL-4 and anti-CD40 (which mimics CD40L), and the motile cells are in the G_1 phase of growth. After a few hours of culture with these factors, the B cells are capable of locomotor and chemotactic responses to anti-immunoglobulin (anti-IgM and anti-IgD). Germinal center B cells die rapidly in culture unless they receive rescue signals, and anti-CD40 provides such a signal. Germinal center cells not only survive but become motile in culture in anti-CD40+IL-4. These cells, again, are attracted by anti-immunoglobulin and, since they have switched isotypes, they are capable of recognizing anti-IgA and anti-IgG as locomotor signals. This suggests that antigen may be a natural chemoattractant for B cells. It is possible, but has not been investigated, that germinal center cells migrate in antigen gradients towards FDCs. Immune complexes activate complement and it has been reported that germinal center B cells cultured with FDCs respond to C5a as a chemotactic factor. This could also form a gradient which would bring B cells into contact with FDCs.

Natural killer (NK) cells

There is little information on NK cell locomotion, though time-lapse films of these cells attached to target cells show vigorous locomotion of the attached cytotoxic cells, which then become released once the target cell dies. Reports suggest that NK cells,

especially after activation with IL-2, can respond to chemoattractants including the chemokines MIP-1 α , MCP-1, RANTES and IL-8, as well as IL-2 itself and C5a.

See also: Adhesion molecules; B lymphocytes; Chemokines; Chemotaxis; Endothelium; High endothelial venules; Interleukin 2; Interleukin 15 and its receptor; Lymphocyte trafficking; Lymphocytes; Natural killer (NK) cells; T lymphocytes.

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CHEMOTAXIS OF MACROPHAGES AND MONOCYTES

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The locomotor behavior of monocytes and macrophages, and their behavior in response to attractants, are as described in the article 'Chemotaxis'. Like neutrophils, they show chemotactic and chemokinetic responses under appropriate conditions and the direction of their locomotion also shows guidance in relation to aligned fibrous matrices. Monocytes and macrophages are quite strongly adherent cells and spread rapidly on substrata *in vitro*. As a consequence, their rate of locomotion on such substrata is slower than that of neutrophils, but is faster than that of fibroblasts which are even more adherent.

Locomotor capacity and macrophage heterogeneity

The locomotor capacity of mononuclear phagocytes is determined by their differentiation status. Marrow precursor cells (as those for neutrophils) are non-motile and motility is acquired as these cells develop into monocytes. This is illustrated by the behavior of monoblast lines such as U937 which develop locomotor capacity only on addition of agents such as dibutyryl cyclic AMP or cytokines which drive differentiation into monocyte morphology. These differentiated monocytes are polarized, motile, and have chemotactic receptors not found on their unstimulated precursors. Most blood monocytes are also motile and respond to a range of attractants (see below). Once monocytes leave the blood and become macrophages, they may differentiate into many forms depending on the nature and site of stimulation, and these heterogeneous macrophages vary widely in locomotor activity. This can be studied well using peritoneal exudate macrophages, e.g. from mice. Following an inflammatory stimulus such as thioglycollate, many highly motile macrophages enter the peritoneum, newly recruited from blood monocytes. However, following an immunological stimulus such as *Corynebacterium parvum* or BCG, a more mixed exudate is found with many lymphocytes as well as macrophages. These macrophages also are motile, but less so than those found in thioglycollate exudates. In immunologically stimulated exudates, the lymphocytes are very motile, and these activated lymphocytes show chemotactic responses.

Locomotion and chemotaxis contribute to inter-

actions between macrophages and lymphocytes (see below). Macrophages in culture may slowly lose their motile behavior, especially when they become adherent and sessile and resident peritoneal macrophages usually show little locomotor behavior and respond poorly to attractants. A similar state of affairs may exist in the pulmonary alveoli in which cells newly recruited in response to inflammation are more motile than the resident macrophage population. Macrophages in other tissues may form tight adhesions with neighboring tissue cells. Presumably, macrophages such as hepatic Kupffer cells have little requirement for locomotor activity, since agents needing removal are brought to them by blood flow, though this is difficult to study since methods for isolation may damage surface receptors on such cells.

Chemotactic responses

Like neutrophils, monocytes and macrophages show accurate chemotactic responses to microorganisms, and respond to factors such as formyl-Met-Leu-Phe, C5a, C5a des Arg, leukotriene B₄, platelet-activating factor, thrombin and denatured proteins or breakdown products of proteins such as elastin, and these responses are probably important both for the microbicidal role and the scavenger role of macrophages. In the latter role, alveolar macrophages are reported to be attracted to, and to ingest, surfactant lipoprotein, presumably in degraded form.

These factors are not monocyte or macrophage specific and may attract neutrophils or other leukocytes strongly. Monocytes also respond to many of the C-C (β) chemokines. These chemokines (MIP-1α and β, MCP-1, -2, -3 and -4, and RANTES) attract blood monocytes but not neutrophils. This allows selective attraction of monocytes into lesions containing these molecules, though the attraction is not completely specific, since the same chemokines may also attract T lymphocytes or eosinophils (see Table 1 of 'Chemotaxis' article). Cell specificity is determined by the possession by different cells of receptors for β chemokines (CCR1-8). Of these receptors CCR1 (which binds MIP-1α, MCP-3, MCP-4 and RANTES) is the most strongly represented on human monocytes. Other cytokines that attract mononuclear phagocytes are transforming growth factor β

(TGF β), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), tumor necrosis factor α (TNF α) and lymphotoxin (TNF β).

Attraction of other leukocytes by products of macrophages

Macrophages are important generators of attractants for other cell types and there is an extensive literature describing such factors. For example, many of the chemokines are made and secreted by monocytes or macrophages. These include neutrophil attractants such as interleukin 8 (IL-8). IL-15 is a molecule with functions similar to IL-2 but made by macrophages (and other cells) rather than by T cells. It is an effective attractant for T lymphocytes and is found in large amounts in the lesions of rheumatoid arthritis together with several of the chemokines. Murine macrophages from *C. parvum*-stimulated peritoneal exudates do not attract nearby lymphocytes unless they are given a phagocytic stimulus. However, immediately after phagocytosis, the macrophages release a brief pulse of an as yet unidentified attractant which causes nearby lymphocytes to migrate in by chemotaxis and to cluster round the macrophage. This may be a mechanism for focusing lymphocytes round a macrophage that is presenting newly processed antigen.

Clinical abnormalities

The most interesting of these is probably the observation that both patients with certain tumors (melanoma, certain carcinomas) and tumor-bearing experimental animals may have monocytes or macrophages which migrate poorly in chemotaxis assays. This is due to an inhibitor released by tumor cells and is reversed by removal of the tumor.

See also: Chemokines; Chemotaxis; Chemotaxis of neutrophils; Macrophage activation; Monocytes; Mononuclear phagocyte system; Neutrophils.

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CHEMOTAXIS OF NEUTROPHILS

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Neutrophil locomotion in inflammation

Neutrophils, the most numerous of the white blood cells, are the classical cells of acute inflammation and appear at inflammatory sites within a few hours of tissue injury. This mobilization from the bloodstream requires two events, each with its own molecular recognition mechanisms, the first being adhesion to vascular endothelium at the inflamed site, and the second, migration from the vessels in response to signals generated in the damaged tissue. These events occur in concert. In sites of inflammation, vascular endothelial cells rapidly become more adhesive due to rapid increased expression of P-selectin induced by thrombin or histamine, and later expression of E-selectin and ICAM-1 induced by lipopolysaccharide (LPS), interleukin 1 (IL-1),

tumor necrosis factor (TNF) and probably other stimuli. Chemotactic factors cause transient increases in adhesion on contact with neutrophils due to increased expression of β_2 -integrins, chiefly CD11b/CD18 (MAC-1). Since any chemotactic factors diffusing from the inflammatory site which cross the vascular endothelium and reach the blood are likely to be swept rapidly downstream, it is postulated that chemotactic factors must become attached to the luminal surface of endothelial cells at least transiently in order to do this. By these means, both surfaces, that of the endothelium and that of the neutrophil, increase their adherence to one another. Adherence is followed by transmigration across the endothelium and detachment from it by mechanisms which are still not entirely clear. This is followed by migration through the tissues to the site of injury by

a combination of chemotaxis, chemokinesis and contact guidance, as discussed in the article on chemotaxis. *In vitro*, neutrophils respond best (both by chemotaxis and chemokinesis) to many chemotactic factors at nanomolar concentrations or lower, but at higher (micromolar) concentrations locomotion is much slower. Thus cells at some distance from the injured site are fast moving and, as they reach the center of the gradient, they become slowed at the center of the lesion. Moreover, deposition of immune complexes, which bind to Fc receptors at the site of the lesion, also slows the cells by acting as an 'adhesive trap'. Also at micromolar concentrations of chemotactic factors, neutrophils are stimulated optimally to produce an oxidative metabolic burst, to secrete hydrolases and to ingest microorganisms. Thus the dose optima for locomotion and phagocytic events are arranged so that the cells perform these functions in sequence.

Chemotactic factors

Lists of chemotactic factors are given in Tables 1 and 2 under the Chemotaxis article, although there are many others which are not included. Many act on both neutrophils and monocytes/macrophages but not on lymphocytes. These include well-known factors such as C5a, formyl peptides and leukotriene B₄. More recently, studies of the chemokine family have shown that α -chemokines such as IL-8 are neutrophil specific, whereas β -chemokines are monocyte specific, thus providing a mechanism for the specificity of influx of inflammatory cells into different types of lesion.

It is important to emphasize that the term 'chemotactic factor' is inadequate to describe the actions of these factors. In locomotion assays their prime effect is always to stimulate locomotion, and the form the locomotion takes, i.e. whether it is directed or random, depends on whether a gradient is present or

not. But as well as effects on locomotion, these factors stimulate various metabolic and microbicidal activities of the cell (Table 1).

Formyl peptides are probably the most widely studied of all chemotactic factors. Formyl-met-leu-phe (FMLP) is the best-studied example but many related peptides have similar actions. They are putative representatives of release products from bacteria. Prokaryotic cells commence protein synthesis with a formyl-methionyl starter sequence which is later cleaved and may be recognized as a leukocyte attractant. There are probably many other prokaryotic chemoattractants which have not been defined, as most work has been concentrated on endogenously-derived attractants. The first of the latter to be defined was C5a, a peptide generated by complement activation which probably plays a major role in many inflammatory situations. Leukotriene B₄ (LTB₄) is generated by neutrophils themselves upon activation, and is likely to act as an amplifying device calling cells into sites of activation. Interest has centered recently on IL-8 and related chemokines, which are present at active concentrations in many inflammatory lesions, including those of rheumatoid arthritis and inflammatory lung disease, and which are produced by macrophages and other types of cells, including epithelial cells and vascular endothelial cells, usually following stimulation of these cells. Other cytokines such as TNF α and granulocyte-macrophage colony-stimulating factor (GM-CSF) also have chemoattractant activity for neutrophils. These are just as active as chemokines.

Chemotactic receptors and transduction systems

Receptors for formyl peptides, for C5a and for the chemokines all belong to the same family of serpentine, seven-membrane-spanning domain molecules, related to rhodopsin. These are linked to pertussis

Table 1

(a) Functions activated in neutrophils by chemotactic factors

Locomotion: chemotaxis; chemokinesis

Secretion: release of products of specific, azurophil, and other granules

Changes in cell adhesion: transient homotypic aggregation; longer term increase in adhesion to endothelium or to artificial surfaces

Increased microbicidal activity

(b) Effector biochemical events activated in neutrophils by chemotactic factors

Chemotaxis receptors: receptor redistribution to leading edge of cell; changes in receptor numbers or concentration-dependent changes in receptor affinity

Other events: increase in LFA-1 family surface receptors; increased actin polymerization; increased f-actin at leading edge; increased myosin-1 at leading edge; increased generation of superoxide (H₂O₂)

toxin-sensitive trimeric GTP-binding proteins containing a $G\alpha_1$ chain. Following ligand binding, these G proteins activate a phospholipase C, leading in turn to hydrolysis of phosphatidylinositol polyphosphates to form the second messengers, inositol triphosphate (IP_3) which, when released into the cytoplasm mobilizes calcium from internal stores, and diacylglycerol (DAG) which activates protein kinase C.

It is known that these are essential events for the activation of neutrophils by chemotactic factors, but a difficulty arises from the fact that these factors activate multiple functions in neutrophils. Most workers on signaling measure chemoattractant-induced metabolic activation or secretory events which are easier to quantify than locomotion, but which occur at higher ligand concentrations than those optimal for locomotion. Different signaling systems may be engaged at different ligand concentrations, and it seems probable that the different functions of neutrophils are signaled through different pathways. Locomotion can occur in the absence of calcium, either extracellular or intracytoplasmic, but this may simply reflect the possibility that there are back-up mechanisms for signaling. The essential requirements is to link the signal generated at the cell surface to actin polymerization so that a microfilament network can be formed which generates contractility and thus motility of the cell. It is likely that small G proteins such as p21 Rac as well as tyrosine kinases are involved downstream of the initial signal. It is also true that chemotaxis is signaled by cytokines which bind to receptors other than the serpentine receptors and these may use different signaling systems from the trimeric G proteins.

The polarity of the locomotor response should be emphasized. The response is polar in the sense that the cell does not behave as a unit but that different events are happening at the front of the cell, where recognition and response to chemotactic factors occurs, from those happening in the cell body. This is illustrated by experiments where the leading edge of a polarized neutrophil was detached from the cell body. These leading edge fragments contained cell surface receptors and actin filaments but lacked organelles or a nucleus. Such cell fragments continued to migrate and to detect chemotactic gradients. Conversely they lacked microbicidal activity, which depends on organelles which remain in the body of the cell. In neutrophils, polarization is initiated by events occurring at the leading edge of the cell, and many receptors, probably including chemotactic receptors, become selectively concen-

trated there. This edge of the cell is also rich in filamentous actin. As the cell moves forward, i.e. as a contraction wave moves from front to back, these events may sequentially involve the rest of the cell. A signaling system for an all-or-none event such as metabolic activation or secretion may be straightforward, but for locomotion over long distances in varying attractant concentrations it is probably more complex than we realize.

Clinical defects of neutrophil locomotion

Locomotor defects in neutrophils are rarely seen and poorly defined. The 'lazy leukocyte syndrome', described some years ago, is not a discrete clinical entity. Patients with leukocyte adhesion deficiency (LAD), due to a defect in the integrin β_2 chain and consequent defective adhesive function of β_2 -integrins, have neutrophils that show poor locomotion under circumstances where adhesion is required (e.g. on two-dimensional surfaces of in filters) but not where locomotion is adhesion independent (e.g. through collagen gels). LAD cells can polarize normally, which means that they recognize chemotactic factors normally. Neutrophils from Chédiak-Higashi syndrome have a chemotactic defect whose cause is unknown. A chemotaxis defect has also been reported in neutrophils with specific granule deficiency. In other reported abnormalities, the neutrophils are normal, but the patients have either circulating inhibitors of locomotion or are unable to generate endogenous chemotactic factors normally.

See also: Chemokines; Chemotaxis; Interleukin 8 and its receptor; Chemotaxis of macrophages and monocytes; Chemotaxis of lymphocytes.

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CHIMERISM, HEMATOPOIETIC

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The ancient Greek chimera, that awesome monster composed of different animals, has in modern times been tamed, and now represents a most desirable clinical status in recipients of organ transplants in general, and bone marrow transplants in particular. Thus, the term chimerism has come to represent a clinical status in which a graft of an organ from one individual is accepted and tolerated by the immune system of another. While acceptance of solid organs, such as kidney, heart or liver, is largely dependent on adequate suppression of the recipient's immune system, as well as on the degree of matching between donor and recipient histocompatibility antigens, bone marrow transplantation is unique in that, in most instances, incomplete or mixed hematopoietic chimeras are generated, and both host-type and donor-type blood cells can be detected in the recipient. Furthermore, while immune reactivity against donor type cells is an obstacle to bone marrow engraftment in all transplants, bone marrow transplantation is uniquely complicated by a second immune barrier, known as graft-versus-host disease (GVHD), mediated by donor type T cells reactive to host antigens.

A successful bone marrow transplant results in a state of hematological and/or immunological chimerism in which donor-type blood cells coexist permanently with host-type tissues, without manifesting alloreactivity to each other. If such a state of tolerance is achieved, subsequent organ grafts from the original bone marrow donor will be accepted without the need to further suppress the recipient's immune system. For this reason, investigation of the mechanisms of tolerance, as well as new and safer approaches for its induction, have challenged immunologists for over three decades.

Currently, bone marrow transplantation is mainly used as a rescue therapy following lethal radio-chemotherapy in patients with leukemia and other forms of cancer, but it can also be beneficial in the treatment of several nonmalignant hematological disorders and enzyme deficiencies.

The first demonstration that a bone marrow transplant could protect lethally irradiated animals from death came from the pioneering studies of Jacobson and colleagues and Lorenz and colleagues towards the beginning of the 1950s. However, it was only in 1956 that several laboratories independently confirmed that a cellular component of the transplanted

bone marrow protected recipient mice from the lethal effects of radiation, by its ability to generate new donor-type blood cells for prolonged periods of time.

A better understanding of the bone marrow cellular identity involved in radioprotection was achieved by Till and McCulloch, who discovered a correlation between the presence of spleen colonies in transplanted mice 8–12 days after bone marrow transplantation and the capacity of a given bone marrow cell suspension to protect lethally irradiated mice. This finding helped Trentin and Fohlberg to demonstrate that the entire repertoire of the hematopoietic system could be generated from a single spleen colony. By 1963, Becker and coworkers were able to use chromosome markers to demonstrate the clonal nature of spleen colonies. The existence of a pluripotent stem cell, and its importance in the protection of lethally irradiated mice, thus came to be postulated.

To this day, the characterization of the pluripotent hematopoietic stem cell has been the subject of intense research. With the identification of new cell surface markers, great progress has recently been made. However, since these cells have not been purified to a homogeneous state, they cannot be unequivocally identified in a bone marrow cell suspension.

Knowledge of the radioprotective properties associated with bone marrow transplantation found dramatic application in 1955, when five victims of the radiation accident in Vinca, Yugoslavia were flown to Paris and treated by Mathe and colleagues with bone marrow transplants. Due to technical problems in analyzing the origin of blood cells in these patients, interpretation of results was controversial, but it was agreed that, at best, some transient engraftment occurred, and could have helped the patients to survive, even though their own blood cells returned to normal levels. Fortunately for these patients, they did not suffer from GVHD, as the transplanted bone marrow did not engraft for a substantial period of time.

In the late 1960s, encouraged by the development of histocompatibility antigen (HLA) typing, it was felt that it might be possible to contain the severity of GVHD by transplanting bone marrow from matched sibling donors into patients with severe combined immune deficiency (investigated by Good and

colleagues) and leukemia (investigated by Thomas and colleagues). Both groups experienced many frustrating setbacks in their attempts to achieve stable chimerism, but it is primarily due to their perseverance that bone marrow transplantation has now become an integral part of the curative protocol used to treat such patients.

The duel between host-versus-graft activity and GVHD following bone marrow transplantation

The growing experience with transplantation of solid organs, such as kidney and heart, has clearly shown that it is possible to overcome graft rejection, and establish stable donor-type chimerism, when donors and recipients are appropriately matched for histocompatibility antigens and when adequate long-term immune suppression is maintained. Compared with other transplantable organs, however, cells in the bone marrow are highly antigenic, and recipients must first undergo a drastic immunosuppressive regimen of supralethal radiochemotherapy if the transplant is to succeed. In patients suffering from leukemia, as well as from other forms of cancer, this extreme conditioning serves to suppress adequately the recipient's immune system, as well as to eradicate pathological cells. However, despite the use of such intensive radiochemotherapy, administered in doses as high as the human body can tolerate, rejection of the bone marrow may still occur, in particular when poorly matched marrow is used for transplantation.

A second major immunological problem associated with bone marrow transplantation is the complication of GVHD, initiated by donor-type T cells capable of mounting an immune response against host-type antigens. In general, this reaction is directed against the skin, liver and gut, and is lethal in 10–15% of HLA-identical marrow recipients, as well as in over 40% of HLA-nonidentical marrow recipients.

During the 1970s, different laboratories demonstrated that removal of T cells from mouse or rat bone marrow by physical methods, by differential agglutination with lectins or by specific anti-T cell monoclonal antibodies, could effectively prevent the development of GVHD following transplantation of bone marrow allografts. In 1980, T cell-depleted mismatched parental bone marrow was successfully used for the first time to reconstitute the immune systems of infants suffering from severe combined immune deficiency (SCID). The growing international experience, with more than 100 such 'bubble children' to date, has shown that the use of this approach can allow successful bone marrow trans-

plantation across major histocompatibility barriers, thereby effectively curing this otherwise fatal disease. These cured patients have become 'split chimeras'. Their red cells, myeloid cells and platelets are generally of host origin, while their lymphocytes bear the donor type HLA phenotype. Moreover, since many SCID patients lack only T cells, the majority of B cells in these patients also remain of host origin. SCID patients are unique in that their immune response to donor cells is minimal. In these patients, therefore, the graft-versus-host (GVH) activity of T cell-depleted bone marrow can be studied with minimal interference from a host-versus-graft (HVG) reaction.

When T cell-depleted bone marrow was used to treat leukemia patients, however, results were disappointing, as residual immune cells and pathological cells which survived the intense conditioning regimen led to a markedly enhanced rate of graft rejection, as well as to a higher incidence of leukemia relapse in some types of the disease. Results from primate studies, later confirmed by analysis of peripheral blood lymphocytes (PBLs) from transplanted human leukemia patients, suggest that residual clonable T cells ($1-10 \times 10^6$ cells in the entire blood system) remain after completion of the supralethal conditioning protocol. Such cells more easily reject T cell-depleted bone marrow than unseparated bone marrow, as the latter contains about 1×10^9 donor-type T cells, an amount capable of outnumbering and destroying residual host T cells as well as leukemia cells. Thus, GVHD, which can be fatal, may also be beneficial, not only by paving the way for the engraftment of donor-type stem cells but also in its antileukemia effect (generally referred to as graft-versus-leukemia, GVL).

Altogether, although GVHD can be completely prevented in leukemia patients, the use of T cell-depleted bone marrow did not lead to an improvement in the overall long-term survival rate. Alternative methods, by which donor type T cells are partially suppressed by immunosuppressive drugs, such as cyclosporine A and methotrexate, seem to reduce the severity of GVHD and lower the risk of graft rejection and leukemia relapse. Thus, it may be possible to improve the therapeutic management of the 25–40% of patients for whom HLA-identical siblings are available. However, such methods cannot offer a solution for those patients who lack a matched sibling donor in the family.

Very recently, a novel approach to overcome the HLA barrier, employing megadose hematopoietic transplants, has been attempted successfully by Aversa and colleagues in mismatched leukemia patients. To achieve these megadose transplants, we

supplemented the conventional T cell-depleted bone marrow inoculum with CD34 peripheral blood progenitor cells obtained from the donor after the administration of recombinant human granulocyte colony-stimulating factor (G-CSF). The potent enhancement of engraftment which was experienced in these leukemia patients indicates that the massive number of infused stem cells may be capable of suppressing residual host cells which otherwise would mediate graft rejection. However, because only very miniscule numbers of T cells could be spared in these transplants, due to the marked GVHD risk associated with their HLA disparity, the T cell reconstitution in the engrafted patients is relatively slow.

Clearly, the question of whether the usefulness of T cells in promoting bone marrow engraftment and in conferring post-transplant immunity, as well as GVL effect, can be separated from their harmful GVH activity, has become a critical issue in bone marrow transplantation today.

Two independent studies have focused on this problem. The group of Tourain and our group showed that a transplant of non-GVHD-producing T cells from (donor $\times$ host) F1 mice could enhance long-term engraftment of allogeneic fetal liver cells or T cell-deficient 'nude' bone marrow cells, respectively. These results clearly suggest that T cells can enhance bone marrow engraftment in the absence of GVHD.

Although it is not yet known whether the T cell subpopulation responsible for this positive effect is separable from the T cell subpopulation which causes GVHD, recent studies have demonstrated that alloreactive T cells can be specifically contained *in vivo* by coblockade of the LFA-1:ICAM and CD28/CTLA-4:B7 pathways. Blazar and colleagues showed that hCTLA-4-Ig (inhibitor of the CD28/CTLA-4:B7 costimulatory pathway) plus LFA-1-specific antibody is highly effective in preventing GVHD induced lethality. Alternatively, Cavazzana and coworkers showed that specific elimination of interleukin 2 (IL-2) receptor-positive T cells, after allostimulation *in vitro*, can lead to attenuation of GVHD while maintaining a marked number of relatively nonalloreactive T cells. Both these promising approaches are being investigated in clinical trials.

Human-mouse hematopoietic chimeras

Successful xenogeneic bone marrow transplants between close species such as rat and mouse have in the past been demonstrated. Could human bone marrow cells be engrafted in lethally irradiated mice? This question is of particular relevance to acquired

immune deficiency syndrome (AIDS) research, as human immunodeficiency virus (HIV) does not cause AIDS in any other species except in humans, and it is believed that the presence of human CD4⁺ helper T cells is a prerequisite to infection. If such human-mouse chimeras could be created, they could provide a novel means by which to investigate the development of AIDS and leukemia, as well as for studies of human hematopoiesis.

The poor cross-reactivity between several human and mouse cytokines, as well as between other molecules involved in cellular recognition, was for many years believed to negate the possibility of producing human-mouse chimeras. In 1988, however, two independent studies involving the transplantation of human pluripotent stem cells into genetically determined immune deficient mice, such as the SCID mouse or the Bg/Nu/Xid mouse, documented for the first time the achievement of a transient split hematopoietic chimerism. Moreover, this xenogeneic chimerism was achieved without the apparent development of GVHD.

McCune and colleagues transplanted SCID mice with human fetal liver cells as a source of pluripotent stem cells void of GVHD, along with an additional grafting of human thymic epithelium under the kidney capsule. Thus, they were able to provide a human microenvironment sufficient for the development of human T lymphocytes bearing normal phenotypes. Such T cells remained in the peripheral blood of engrafted mice for 4-8 weeks, during which time they could be infected with HIV.

Kamel-Reid and Dick were able to engender transient myeloid engraftment in Bg/Nu/Xid immunodeficient mice by transplanting them with human bone marrow. Nine weeks after transplant, *in vitro* culture of bone marrow cells from these mice revealed the presence of human genetic material in early myeloid progenitors, suggesting that differentiation of human myeloid precursors can take place in such mice. Subsequently, this same murine model enabled the engraftment in Bg/Nu/Xid mice of pathological cells from human acute lymphocytic leukemia patients. More recently, Lapidot and colleagues identified a new human acute myeloid leukemia (AML)-initiating cell, which is less mature than colony-forming cells, by transplantation into SCID mice. These cells, which are CD34⁺ CD38⁻, home to the bone marrow and proliferate extensively in response to *in vivo* cytokine treatment.

Mosier's group further demonstrated that stable mixed lymphoid human-mouse chimerism could be established simply by intraperitoneal injection of human PBLs into SCID mice. Recently, they showed that donor T and B lymphocytes could be detected

in the peripheral blood of recipient mice several weeks later, without having caused GVHD. Such human-mouse chimeras could be infected with HIV. Several studies have shown that the engrafted human T cells in these chimera become anergic, unresponsive to stimulation with anti-CD3 or mitogens, and that the human T cell repertoire is limited to xeno-reactive clones.

Recently, Lubin and colleagues described a new approach enabling engraftment of human PBMC in normal strains of mice and rats, following split-dose lethal irradiation. Irradiated animals are initially converted into SCID-like animals by means of bone marrow transplantation from SCID donors and subsequently are infused with human PBMC. These chimeric animals allow an effective and rapid engraftment of human cells, enabling their functional study early after transplant. Moreover, a marked human primary and secondary humoral response, as well as vigorous antiallogeneic human cytotoxic T lymphocyte (CTL) response, could be generated in the human-BALB/c radiation chimera by immunizing them with foreign antigens and allogeneic cells, respectively. In addition, the marked immunodeficiency induced in these radiation chimera has recently enabled us to transplant human liver fragments under the kidney capsule in such mice and to induce hepatitis C viremia, so as to provide a new murine model for the study of hepatitis B and C infection.

More recently, engraftment of human cells was also enhanced in SCID mice within the first few weeks after transplant, by conditioning of the recipient mice and by using a large inoculum of human cells. Although these preparative regimens were associated with marked lethality, engrafted human lymphocytes exhibited human antibody production and T cell responses similar to those found in BALB/c radiation chimera.

Mixed lymphoid chimerism and organ transplantation

Mixed chimerism, in which both host and donor lymphocytes coexist in significant numbers, rarely develops following bone marrow transplantation. Generally, one cell type is overtly dominant, due to its increased potential to eliminate or to compete with the other cell type. However, stable, long-term mixed lymphoid chimerism can spontaneously occur in nature, as when stem cells from one of two dizygotic twins penetrate the bone marrow of the other twin by placental vascular anastomoses. With the development of DNA analysis, it is now known that following bone marrow transplantation in leukemia

patients, normal host lymphocytes can be detected in most transplanted patients even many months after the transplant. It is believed that in such long-term, stable chimeras, host and donor lymphocytes are not reactive towards each other.

Likewise it has been suggested recently that resident 'passenger' leukocytes of hemato-lymphoid origin that migrate from whole organ grafts subsequently establish systematic microchimerism that is essential for graft acceptance and for the induction of donor specific nonreactivity. This hypothesis has been based primarily on observations in organ transplant recipients in whom transplantation was under postoperative immunosuppression with FK506 and prednisone. More recently, attempts to enhance hematopoietic chimerism in recipients of organ transplants by using bone marrow transplantation from the organ donor under postoperative immunosuppression led to some improvement of solid organ acceptance. Nevertheless, enhancement of chimerism was still very limited and not sufficient to afford acceptance of organs in about 50% of the patients, even though they were treated with immunosuppressive drugs. Clearly, if post-transplant immunosuppression had been omitted in these patients, the results are likely to have been even less successful.

Though the mechanisms underlying the development of mutual tolerance have been studied for more than a decade, they are not yet completely understood, although great progress has recently been made in the molecular characterization of the T cell receptor structure, and the selection of its repertoire during thymic differentiation. It is interesting to note that once stable and substantial mixed lymphoid chimerism is established, transplantation of any other organ from the same donor will be accepted without further need for immunosuppression.

Since the early 1970s, many studies have involved attempts to achieve mixed lymphoid chimeras under conditions less severe than those employed in bone marrow transplantation of leukemia patients. Attempts to induce classical transplantation tolerance in sublethally conditioned animals have generally made use of donor bone marrow cells. Traditionally, in these studies more attention was dedicated to achieving lymphoid chimerism than a complete donor-type hematopoiesis. Pierce and colleagues have demonstrated that infusion of unseparated bone marrow cells, comprising GVHD-producing T cells, into sublethally irradiated allogeneic recipients results in mixed lymphoid chimera which were tolerant to donor-type skin grafts. This approach, shown to be mediated by Thy-1⁺ cells, is not only associated with a potential risk of GVHD, but is also limited to the lymphoid lineage due to the

insufficient myeloablation associated with the conditioning (3×2.5 Gy) regimen used. Further progress has been made by Cobbold and colleagues, who were able to induce, with T cell-depleted bone marrow, lymphoid chimerism in sublethally irradiated recipients conditioned with a nonmyeloablative regimen, including 6 Gy total body irradiation (TBI) and *in vivo* treatment with anti-CD4 plus anti-CD8. Likewise, Sharabi and Sachs demonstrated that transplantation tolerance can be induced after a very low dose of TBI (3 Gy) if the conditioning is supplemented with booster irradiation of the thymus with 7 Gy and with anti-T cell therapy, similar to that employed by Cobbold and coworkers. Again, mixed chimerism was only documented in the peripheral blood and was based on monitoring of donor-type cells within the Thy-1 positive and negative cells, whereas analysis of chimerism in the myeloid compartment in bone marrow or spleen is missing.

Very recently, Bachar and colleagues attempted to broaden the 'megadose' approach and to test whether large inocula of T cell-depleted mismatched bone marrow transplants can induce stable donor-type chimerism in sublethally irradiated mice. We addressed this goal, which represents a difficult challenge due to the marked number of host-type lymphocytes surviving the sublethal irradiation, by investigating whether escalation of the bone marrow cell dose can effectively promote the establishment of complete donor-type hematopoietic chimerism across full MHC barriers. Our results demonstrated that full donor-type hematopoietic chimera, which are stable for over 6 months in both the myeloid and the lymphoid lineages, can be produced by this approach in the bone marrow and in the spleen without any post-transplant immune suppression and in the absence of GVHD. Furthermore, anti-host antibody therapy, which was found to be necessary in previous studies, is not required and the marked numbers of residual host-type lymphocytes which survive 6.5 Gy TBI, are probably overwhelmed and paralyzed by the megadose transplant.

Future directions

Despite the importance of allogeneic bone marrow transplantation in humans, its use presently remains restricted to the treatment of fatal disorders such as cancer, SCID and aplastic anemia. A wider use of this curative approach is limited by the occurrence of GVHD if T cell purging is not used, and by the graft rejection associated with T cell depletion.

However, two major approaches to overcome these barriers, namely the use of B7-CD28 blockade or the use of megadose T cell-depleted transplants,

which have been proven successful in sublethally irradiated mice, lend the hope that the use of allogeneic hematopoietic stem cells will become safer in the near future, thereby expanding its use to the treatment of genetic disorders other than SCID, which are less acute but which can, nevertheless, be lethal over the course of years.

In addition, it is hoped that by the same approach, using nonlethal preparative regimens, induction of specific immunological tolerance towards donor-type antigens, as a prelude for transplantation of solid organs from the same donor, will also become possible.

Meanwhile, an alternative approach has captured the interest and imagination of both scientists and the public at large. Experiments in mice have shown that it is possible to insert a particular gene into the hematopoietic stem cell pool by means of retroviral vectors. It is hypothesized that a diseased bone marrow could be corrected *in vitro* by such 'gene therapy', and then reinfused successfully into the patient. By employing such an approach, the problems of GVHD or graft rejection could be entirely avoided. However, due to the low frequency of pluripotent stem cells in the marrow preparations, and the difficulty in stimulating self renewal of such cells *in vitro*, this new approach is still in the embryonic stages of development. If successful, we may have to define a new chimerism status in which transplanted cells differ from the host cells by a very minute piece of DNA.

An alternative approach to achieving hematologic chimerism involves replacing the bone marrow or peripheral blood stem cells with cord blood hematopoietic stem cells. In 1990, Gluckman and colleagues demonstrated for the first time that cord blood can correct Fanconi anemia in a matched sibling. Both the HLA and the disease-free status of the embryo were established by prenatal testing, and transplantation could be planned for before birth. Thus, the potential number of matched sibling donors in the family could be increased. Moreover, if large numbers of cord blood preparations could be collected and cryopreserved, it could potentially increase the availability of matched unrelated donors. While this possibility is gaining increasing enthusiasm worldwide, it is still hampered by a marked incidence of engraftment failure, associated with the limited number of hematopoietic stem cells which can be collected in cord blood. Clearly, the potential of this approach could be greatly enhanced if it were possible to expand the pluripotential stem cells *ex vivo*. Results in animal models regarding the self renewal capacity of expanded stem cell populations are encouraging but still controversial.

Finally, studies in animals have clearly demonstrated that hematopoietic chimerism can be achieved before birth by *in utero* transplantation of allogeneic stem cells. The success of this approach is based on the relative ease by which tolerance can be induced in the embryonic state, compared with in adults. In the future, it is hoped genetic diseases may be corrected *in utero*, thus eliminating the need for intensive conditioning protocols required for transplantation in adult recipients.

See also: Alloantigens; Bone marrow and hematopoiesis; Hematopoietic stem cell transplantation; Cell separation techniques; Graft-versus-host reaction; Immunodeficiency, primary; Radiation, effects on immune system; Tissue typing; Transplantation.

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CHLAMYDIA, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

The genus *Chlamydia* of the order Chlamydiales con-

sists of four species: *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and *C. pecorum*. *C. trachomatis* is comprised of three biovars, two of which are natural parasites of humans and the remaining biovar a pathogen solely for mice. The two human biovars

are subdivided into 12 oculogenital serovars which are responsible for trachoma (serovars A, B, Ba and C) and genital infections (serovars D, E, F, G, H, I, J and K). Genital infections result in urethritis in males and cervicitis, endometritis and salpingitis, potentially leading to tubal obstruction, in females. The remaining three serovars (LGV 1–3) are responsible for lymphogranuloma venereum, a more aggressive and systemic genital infection. *C. pneumoniae* has recently been found to be a major cause of community-acquired pneumonia. Of even greater interest, *C. pneumoniae* has been found in association with atherosclerotic plaques in coronary artery disease. Whether this organism actually causes coronary artery disease has not been confirmed. *C. psittaci* and *C. pecorum* are diseases of veterinary importance although *C. psittaci* may cause a serious and potentially lethal zoonotic infection in humans. Phenotypic and genotypic differences are recognized among the *C. psittaci* but the taxonomic differentiation has not been clearly defined. A wide variety of animals, including but not limited to birds, ruminants, cats, and guinea pigs are infected with different biovars. *C. pecorum* is primarily a pathogen of swine although koalas have also been found to be infected.

Chlamydiae are obligate intracellular parasites and as such are incapable of replicating outside of a host cell, apparently depending upon the host pools for growth. They are able to infect a variety of host cells both *in vivo* and *in vitro* and survive intracellularly by preventing phagosome–lysosome fusion. Chlamydiae are unique among pathogenic bacteria in that they undergo a developmental growth cycle which consists of two stages. The elementary body (0.3 μm) is metabolically inert and is the infectious form of the organism. It attaches to the host cell via a mechanism(s) which is not yet clearly defined. Potential adhesins including the major outer membrane protein (MOMP) (≈ 40 kDa), a cysteine-rich 60 kDa protein (Omp2), and the 70 kDa protein have been suggested although binding via heparan sulfate and surface glycoproteins has also been proposed. After internalization within an inclusion vacuole, the elementary body differentiates into a reticulate body (1 μm) which is metabolically active and divides via binary fission. By 25–30 h after infection, reticulate bodies begin to differentiate back to elementary bodies. Ultimately, the host cell dies with the release of the elementary bodies which go on to infect other cells, the complete cycle taking approximately 40 h.

The major structural component of the chlamydial outer membrane is the MOMP, comprising about 60% of the outer membrane proteins. Rigidity is provided by extensive intramolecular disulfide bonds and the protein is folded so that four segments,

termed variable domains, are surface-exposed. These domains vary among different serovars of *C. trachomatis*, while the remaining regions are conserved among the serovars. MOMP contains genus-, species- and serovar-specific epitopes. Chlamydiae produce a lipopolysaccharide (LPS) which is also associated with the outer membrane and contains a genus-specific antigen. The LPS is immunologically and chemically related to the ‘deep rough’ (Re) mutant LPS of enteric bacteria. Also highly immunogenic are the Omp2 protein and the 57 kDa heat shock protein.

Immune responses of the host

Because chlamydiae infect a wide array of animal species as well as different anatomical species, it is difficult to define a single pattern of host response. There are undoubtedly similarities in the effector mechanisms involved in eliminating the organism, but the critical effector mechanisms appear to vary with the site of the infection, the host species, the particular strain of a host species, and even the infecting strain or serovar of the chlamydiae. The majority of our information regarding immune responses to chlamydial infections has been derived from *in vitro* studies and from genital, respiratory and ocular animal models of infection which can basically be divided into protective host responses and pathologic host responses.

In virtually all chlamydial infections, the initial host response is an acute inflammatory reaction consisting of polymorphonuclear leukocytes (PMNs) which can be chlamydicidal but also may be responsible for tissue pathology. The mechanism by which PMNs are attracted to the local site is not clear, but recent data have shown that by infecting epithelial cells, chlamydiae effect the release of interleukin 1 (IL-1) and IL-8, the latter being a powerful chemo-attractant for PMNs. Infection of macrophages by chlamydiae results in the lipopolysaccharide triggering the production of tumor necrosis factor α (TNF α), IL-1 and IL-6. Chlamydiae have also been shown to activate the alternative complement pathway. Thus, IL-8, TNF α , and complement can potentially mediate the chemotaxis of PMNs to the local site either directly or by upregulating the expression of ICAM-1. In studies in the murine model of genital tract infection, ICAM-1, VCAM-1 and MAdCAM-1, have been found to be expressed early in the infection and could be addressins which are responsible for the extravasation of PMNs and other inflammatory cells at the site of infection.

In both genital tract and respiratory murine models, natural killer (NK) cells have been found to

appear at the local site very early in infection, as early as 24 h after infection in the genital tract. The NK cells are the source of interferon γ (IFN γ) which has also been detected early in the infection and could play a role in the control of the infection until the acquired immune response develops. IFN γ has been shown to be chlamydiostatic *in vitro* and to drive the organisms into a nonreplicating state. TNF α has also been observed *in vitro* to inhibit the growth of chlamydiae. Some data suggest that IFN γ produced by NK cells may be important in the ultimate induction of a T_H1 response.

Both humoral and cell-mediated immune responses are elicited by chlamydial infection. In some models, it appears that antibody is important for the resolution of the infection. It has been well-documented that antibody can neutralize chlamydial elementary bodies *in vitro*. Passive immunization with antibody has met with varied success but at least in the *Chlamydia psittaci* strain GPIC:guinea pig model, serum immunoglobulin G (IgG) was actually able to reduce the level of genital infection. Specific IgA is commonly found at local mucosal sites in association with the development of infection and could play a role in the protective response, especially in chlamydial ocular infections. Cell-mediated immunity (CMI) has been found to be important in both murine and guinea pig models. In the mouse, CMI is solely responsible for the resolution of the infection, since B cell-deficient mice are able to recover normally from the infection, while nude mice and CD4 knockout mice do not resolve their infections. The majority of the evidence indicates that CD4 cells are the primary subset responsible for protective cell-mediated immunity. Adoptive transfer of CD4 cells, CD4-enriched lines and a CD4 T_H1 clone are effective in resolving chlamydial genital infections. Nevertheless, adoptive transfer of CD8 cells, CD8 cell lines and a CD8 clone can also resolve infections, albeit not as effectively as CD4 cells. In genital infections, the dominant CD4 subtype is the T_H1 cell, resulting in high levels of IFN γ and minimal levels of IL-4. IFN γ produced by CD4 and CD8 cells most likely plays a major protective role in the acquired immune response. While cytotoxic T lymphocyte (CTL) activity of CD8 cells has been described, their *in vivo* function has not been confirmed. Interestingly, in the primate models of eye and genital infection, equivalent numbers of CD4 and CD8 cells have been reported at the local site, while in the murine genital tract, the dominant cell type is CD4.

Immunity to chlamydial infections is short-lived in humans as well as animal models. In trachoma, the number of organisms isolated is reduced upon sub-

sequent infections, but pathology persists. There is evidence in the murine and guinea pig genital tract models that susceptibility to reinfection is associated with the loss of T cells in the genital tract. However, it also appears from studies in the guinea pig that reduction in the level of a challenge infection may be associated with the antibody response.

While animal models indicate that CMI is important in the resolution of and resistance to infection, it may also be a critical factor in the development of immunopathology. Trachoma has been clearly defined as a disease caused by repeated antigenic challenge and production of a delayed-type hypersensitivity response, even when few organisms can be isolated. Several animal models have also implicated a similar mechanism in fallopian tube damage resulting from multiple chlamydial genital infections. Of importance has been the observations by several groups that the course of infection may vary in different strains of mice and that different serovars of *C. trachomatis* may produce infections of differing severity. The reasons for the differing response among strains has not been clearly defined.

Evasive strategies by the organism

A key feature of chlamydiae is their unique replication cycle and their ability to replicate inside host cells. Obviously, growth intracellularly protects the organism to some extent from antibody although the elementary body is susceptible to neutralization. In order to survive in the host cell, the organisms have developed a mechanism for preventing phagosome-lysosome fusion. Nevertheless, the prevention of phagosome-lysosome fusion is overcome when chlamydial elementary bodies are opsonized by antibody. A potentially significant mechanism of host immune response invasion may be associated with the apparent ability of chlamydiae to remain in a persistent or latent infection state. Evidence for such a form has been provided by the detection of chlamydial DNA, RNA and antigen in the absence of organisms which could be isolated by standard technology. A variety of other data suggest that a latent form may be present. While the mechanism for latency or persistence has not been defined, it is interesting to note that IFN γ is bacteriostatic *in vitro* for chlamydiae, in that it prevents the organism from dividing. However, upon removal of the IFN γ , the organism again begins to replicate and to complete its growth cycle. It is tempting to speculate that the presence of IFN γ from NK or T cells drives the organism into a non-replicating form until the cellular source of IFN γ leaves the local site and thus brings about a decrease in IFN γ and its control of the infection.

Vaccines

Currently, there is no effective vaccine for chlamydial infections in humans, but a whole-organism vaccine has been available to the veterinary industry for years against the *C. psittaci* feline conjunctivitis organism and ovine abortion agent. An inactivated whole-organism vaccine against trachoma was tested in humans in the 1960s and was to some extent effective. However, the trials were discontinued when some individuals developed more severe disease. These data were supported by primate studies.

Recently, the thrust of vaccine design for chlamydiae has been aimed at determining the protective antigen. The major target for immunization because of its surface exposure and potential involvement in adherence has been the MOMP. The MOMP has been shown to have epitopes which elicit neutralizing antibodies, although to date, subunit vaccines have not been able to elicit protective immunity in animal models. It is important to note that purified MOMP has been found to be protective in animal models, but only if the protein retains its native conformation. Denatured or recombinant MOMP has not been found to be protective. Thus, it appears that with regard to the humoral response, protective antibodies are directed against conformational epitopes on MOMP. While MOMP appears to be the best target to date, there are other chlamydial components which may also be useful targets for a vaccine. Because *in vivo* data indicate an important role for

cell-mediated immunity, it will be important to develop a vaccine which also elicits a protective T cell response. Nevertheless, a major consideration in the development of a vaccine needs to be the possibility that the vaccine may actually elicit more severe pathology or that the vaccine may expedite the development of persistence. Thus, a successful vaccine for chlamydial infections may be one that prevents pathology, although it would be ideal to have a vaccine which prevents infection altogether.

See also: Bacteria, Immunity to; Bacterial cell walls; Cytokines; T lymphocytes.

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CHROMOSOME TRANSLOCATIONS OF IMMUNE GENES

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The uniqueness of the immune system lies in the immense diversity of the reactive proteins that the immune cells can synthesize. The immune genes, as we know now, are one exception to the notion that DNA is immutable. Indeed, the cutting and repasting of DNA segments in lymphocytes is necessary to provide the immune system the varied repertoire to discriminate between the vast array of antigenic moieties that an organism encounters. The reconfiguration of the 'germline' immune genes is achieved by the action of a normal recombination machinery that allows this process to be restricted to developing lymphocytes. This recombination program relies

upon nucleotide sequences in the recombining substrates to provide regional specificity to the cutting and repasting. The process of recombination is influenced by several other factors, including the regional organization of chromatin, the coordinated expression of the recombinase enzymes and the components in the nuclear matrix.

Although the recombinational program that provides for the DNA breakage and rejoining is monitored to prevent errors, some baseline of error-prone recombination may ensue. The probability that this instability will occur at a higher frequency is greater in cell lineages that are prone to such recombination.

It is easy to see how this error-prone recombination could juxtapose regions that are not meant to be ordinarily recombined. The consequences of such illegitimate recombination could range from none to severe depending upon the kind of substrates that recombine. For example, the accidental fusion of sequences that enhance transcription to genes that control proliferation, could result in uncontrolled growth. Thus, the occurrence of error-prone recombination, mediated either because of errors in selecting the sequences that need to be cut and ligated or because of errors in the configuration of the chromatin, is of significant consideration in both leukemogenesis and in lymphomagenesis.

Immune genes as substrates for recombination

The unrearranged immune receptor genes (immunoglobulin and T cell receptor) share the same basic organization – the genes are discontinuous. The chains of the immunoglobulin and T cell receptor (TCR) molecules are encoded as small segments (V, D, J and C) that must associate with each other to provide the complete coding sequence. The genes for the immunoglobulin heavy chain and for the light chains κ and λ are located on chromosomal bands 14q32, 2p12 and 22q11, respectively. The TCR genes are encoded on 7p11 (TCR γ), 7q34 (TCR β) and 14q11 (TCR α and TCR δ).

Recombinational pathway in the rearrangement of immune genes

During the process of generating a functional heavy chain, the intervening genomic DNA between the randomly selected D (diversity) and J (joining) segments is excised, bringing together the D_H and the J_H segments. This process is thought to occur simultaneously on both alleles. Following this rearrangement, a V (variable) region on one of the alleles is approximated to the DJ region to form the VDJ exon. The coupling of the VDJ regions is an imprecise mechanism which includes the insertion of a few extra nucleotides (N) and may result in an 'out-of-frame' rearranged gene. If so, the V region on the second allele is allowed to recombine to give the B cell a second chance to generate a productive allele, before undergoing apoptosis. The light chain and the TCR genes go through a similar VJ/VDJ rearrangement.

Normally, the mechanism of recombination necessary for the production of an immunoglobulin or a T cell receptor molecule allows intralocus recombination to assemble the VDJ regions of the immune genes. In B cells an additional recombination path-

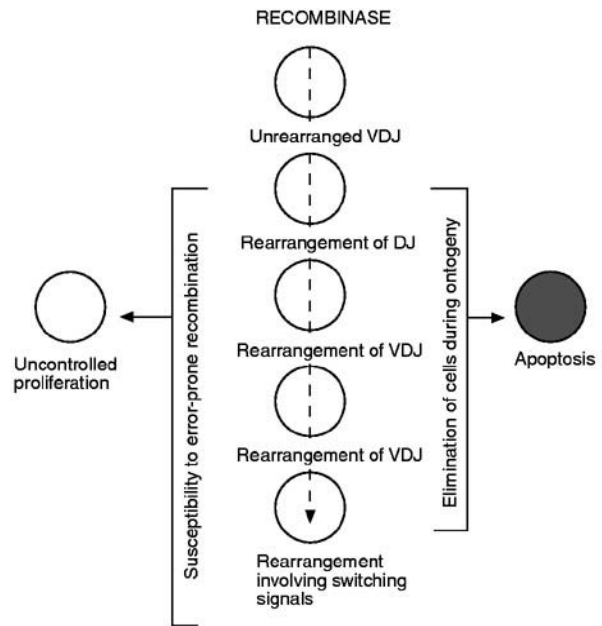


Figure 1 The generation of a productive immune receptor gene is linked to the activity of the recombinase complex. The multi-step rearrangements of the immune locus that takes place during maturation of lymphocytes provides the 'window' during which error-prone recombination may result in a translocation that will allow the clonal proliferation of the cell that has undergone such a translocation. It is possible that normal monitors of the differentiation process that determine the suitability of the lymphocyte at each step also need to be neutralized in the cell with an immune gene translocation in order to resist apoptosis.

way, which again is intralocus, carries out the juxtaposition of the various constant regions to the rearranged VDJ region, allowing the Ig molecule to switch from IgM to any other class. A third pathway that provides additional diversity to the immune molecules is the hypermutation pathway.

Several factors subscribe to the catalysis of these recombination mechanisms. The recombinase machinery interacts with signal sequences on the two segments that need to be cut and pasted, which consist of a heptamer, defined by the consensus sequence CACAGTG, and a nonamer (GGTTTGT) present on each site. It seems reasonable that the substrates for the recombination should be accessible to the recombinase enzymes, thus it is likely that structural features, e.g. the chromatin structure, will mechanistically influence the recombination pathway.

Error-prone recombination: cause of chromosomal aberrations

Given the high frequency of all recombinational events in the lifetime of an organism it is likely that in some instances errors in the recombinase machinery will lead to interlocus rather than intralocus

recombination. The time window of such errors would, of necessity, mirror the time window in the cell lineages where recombinase machinery is active (Figure 1). The recombinational events that are more likely to result in such mistakes include the juxtaposition of the D to the J regions, the recombination of the V and the rearranged DJ region and the switch recombinations.

The occurrence of interlocus recombination within the same chromosome but involving relatively distant sites has been documented for the inversions of chromosome 14, between the immunoglobulin heavy chain and the TCR α genes, and of chromosome 7, involving the TCR β and γ genes. Interlocus recombination that involves two chromosomes has also been documented for the translocations that involve chromosomes 7 and 14 where there is a VDJ recombination that juxtaposes V γ to DJ δ or VD δ to J γ . It has been suggested that this interchromosomal event may occur at a frequency as high as 1×10^{-3} .

The possibility that the presence of a heptamer sequence alone may in some circumstances catalyze the locus to act as a substrate of recombinase albeit at a low frequency, increases the likelihood of recombination involving immune genes and nonimmune partners.

Chromosomal translocations in hematological malignancies

In several neoplastic entities, the development of a malignant phenotype has been intimately related to the gain of abnormal chromosomal patterns. Chronic myelogenous leukemia is the first disease in which an association with a consistent chromosomal abnormality (chromosome Philadelphia) was established. This results from a reciprocal translocation involving chromosomes 9 and 22. Subsequently, this translocation was also shown to occur in acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML) at low frequencies. The consequence of this t(9;22) is the expression of a chimeric protein that contains sequences from both the partner genes c-ABL (9) and BCR (22).

As can be seen in Table 1, the list of nonrandom chromosomal abnormalities in hematological malignancies has grown significantly. Several points can be made from cytogenetic correlations. In most cases there is a high specificity between the morphological classification and the presence of a given abnormality, e.g. the t(14;18) is specifically observed in follicular lymphomas. A more impressive correlation is the restricted involvement of chromosomal loci that harbor immune genes: 2p12, 7q34, 14q11, 14q32 and 22q11 in tumors of lymphoid lineage rather than

Table 1 The most frequent nonrandom chromosomal translocations in hematological neoplasms

Translocation	Genes involved	Neoplasms
t(1;7)(p32;q34)	SCL; TCR β	T-ALL
t(1;7)(p34;q34)	LCK; TCR β	T-ALL
t(1;11)(p32;q23)	AF-1P; MLL	Pre-B ALL, AML
t(1;14)(p32;q11)	SCL; TCR δ	T-ALL
t(1;19)(q23;p13)	PBX-1; E2A	Pre-B ALL
t(2;3)(p12;q27)	Ig- κ ; Bcl-6	B-NHL
t(2;5)(p23;q35)	ALK; NPM	NHL – anaplastic large cell
t(2;8)(p12;q24)	Ig- κ ; MYC	B-ALL, BL
t(2;18)(p12;q21)	Ig- κ ; Bcl-2	CLL; B-NHL
t(3;3)(q21;q26)	EVI-1; ?	AML-M6
t(3;5)(q25;q34)	MLF1; NPM	AML
t(3;14)(q27;q32)	Bcl-6; IgH	B-NHL
t(3;21)(q26;q22)	EVI-1; AML-1	CML-bc, t-AML
t(3;22)(q27;q11)	Bcl-6; Ig- λ	B-NHL diffuse
t(4;11)(q21;q23)	AF-4/FEL; MLL	Early B precursor ALL; AML
t(5;12)(q33;p13)	PDGFR β ; TEL	CMML
t(5;14)(q31;q32)	IL-3; IgH	B-ALL
t(5;17)(q32;q21)	NPM; RAR α	AML-M3
t(6;9)(p23;q34)	DEK; CAN	AML-M2,4
t(6;11)(q27;q23)	AF-6; MLL	AML-M5; pre-B ALL
t(7;9)(q34;q32)	TCR β ; TAL-2	T-ALL
t(7;9)(q34;q34)	TCR β ; TAN-1	T-ALL; T-NHL
t(7;10)(q34;q24)	TCR β ; HOX-11	T-ALL
t(7;11)(q34;p13)	TCR β ; RBTN-2	T-ALL
t(7;19)(q34;p13)	TCR β ; LYL-1	T-ALL
t(8;14)(q24;q11)	MYC; TCR α	T-ALL, T-CLL
t(8;14)(q24;q32)	MYC; IgH	B-ALL, BL
t(8;21)(q22;q22)	ETO; AML-1	AML-M2
t(8;22)(q24;q11)	MYC; Ig- λ	B-ALL, BL
t(9;11)(p21;q23)	AF-9; MLL	AML-M4,5, ALL
t(9;22)(q34;q11)	ABL; BCR	CML, AML-M1,2,4, B-ALL
t(10;11)(p12;q23)	AF-10; MLL	AML-M4,5
t(10;14)(q24;q11)	HOX-11; TCR δ	T-ALL
t(10;14)(q24;q32)	LYT-10; IgH	B-NHL
t(11;14)(p13;q11)	RBTN-2; TCR δ	T-ALL
t(11;14)(p15;q11)	RBTN-1; TCR δ	T-ALL
t(11;14)(q13;q32)	BCL-1; IgH	B-NHL – mantle cell, B-CLL, MM
t(11;14)(q23;q32)	MLL; IgH	B-NHL
t(11;17)(q23;q21)	MLL; AF-17	AML
t(11;17)(q23;q21)	PLZF; RAR α	AML-M3
t(11;19)(q23;p13)	MLL; ENL	AML-M4,5, pre-B ALL
t(11;22)(q13;q11)	Bcl-1; Ig- λ	B-NHL
t(14;14)(q11;q32)	TCR α ; TCL-1	T-ALL, T-NHL
t(14;18)(q32;q21)	IgH; Bcl-2	B-NHL – follicular
t(14;19)(q32;q13)	IgH; Bcl-3	B-CLL, PLL
t(15;17)(q22;q11)	PML; RAR α	AML-M3
t(16;16)(p13;q22)	MYH11; CBFB	AML-M4
t(16;21)(p11;q22)	TLS; ERG	AML-M4; CML
t(17;19)(q22;p13)	HLF; E2A	Pre-B ALL
t(18;22)(q21;q11)	Bcl-2; Ig- λ	B-NHL
t(X;11)(q13;q23)	AFX1; MLL	T-ALL

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; NHL, non-Hodgkin lymphoma; BL, Burkitt's lymphoma; CLL, chronic lymphocytic leukemia; CML, chronic myelocytic leukemia; CMML, chronic myelomonocytic leukemia; PLL, plasmacytic leukemia; MM, multiple myeloma; CML-bc, CML in blast crisis; t-AML, AML secondary to therapy.

Table 2 Translocations involving immune loci in T cell neoplasms

<i>Neoplasia</i>	<i>Immune locus</i>	<i>Partner locus</i>	<i>Gene involved</i>	<i>Deregulated oncogenic function/domain</i>
ALL	7q34	1p34	LCK	Signal transduction/Tyrosine kinase
ALL	7q34	1p32	SCL	Transcriptional/bHLH
ALL	7q34	9q32	TAL-2	Transcriptional/bHLH
ALL, NHL	7q34	9q34	TAN-1	Signal transduction/'Notch'-like protein
ALL	7q34	19p13	LYL-1	Transcriptional/bHLH
ALL	7q34	10q24	HOX-11	Transcriptional/Homeobox
ALL	7q34	11p13	RBTN-2	Transcriptional/LIM
ALL	14q11	10q24	HOX-11	Transcriptional/Homeobox
ALL	14q11	1p32	SCL	Transcriptional/bHLH
ALL, NHL	14q11	8q24	MYC	Transcriptional/bHLH-LZ
ALL	14q11	10q24	HOX-11	Transcriptional/Homeodomain
ALL	14q11	11p15	RBTN-1	Transcriptional/LIM
ALL	14q11	11p13	RBTN-2 (LMO-2)	Transcriptional/LIM
ALL, NHL	14q11	14q32	TCL-1	?
ALL, NHL	14q11	Xq28	MTCP-1	?

in tumors of myeloid origin. Furthermore, there is a lineage dependency even among lymphoid malignancies for the immune loci involved. Thus, translocations involving 7q34 and 14q11 are restricted to T cell malignancies (Table 2) whereas translocations that involve 2p12, 14q32 and 22q11 are specifically associated with B cell neoplasms (Table 3). Since loci 7q34 and 14q11 harbor the TCR genes and in T cells these genomic loci undergo rearrangements, errors in recombination that allow interchromosomal translocations are targeted to these regions in T cells. A similar conclusion can be derived about translocations associated with the immunoglobulin genes in B cells. It is also significant that these immune

loci contain powerful transcriptional regulatory sequences. The major function of a mature immune cell is to synthesize immune molecules – immunoglobulin or T cell receptors – thus, recombinase-mediated rearrangements that will erroneously juxtapose genes that are normally silent in these cells will result in the forced expression of such genes. Depending upon the nature of the gene translocated, this deregulated expression of the rearranged gene could result in various phenotypic changes. If the phenotype conferred by the deregulated expression is survival advantage, it will cause a clonal expansion of the lymphoid cells with this particular rearrangement and provide a pool of cells that are prone to

Table 3 Translocations involving immune loci in B cell neoplasms

<i>Neoplasia</i>	<i>Immune locus</i>	<i>Partner locus</i>	<i>Gene involved</i>	<i>Deregulated oncogenic function/domain</i>
CLL, MM, NHL – mantle cell	14q32	11q13	Bcl-1	Cyclin
NHL – follicular	14q32	18q21	Bcl-2	Inhibitor of apoptosis
CLL, PLL	14q32	19q13	Bcl-3	Transcriptional/REL/NFκB
NHL	14q32	3q27	Bcl-6	Transcriptional/Zinc finger
BL, ALL-L3	14q32	8q24	c-myc	Transcriptional/bHLH-LZ
pre-B ALL	14q32	5q31	IL-3	Cytokine
NHL	14q32	10q24	LYT-10	Transcriptional/REL/NFκB
NHL	14q32	11q23	RCK	Helicase
NHL, CLL	2p12	18q21	Bcl-2	Inhibitor of apoptosis
NHL	2p12	3q27	Bcl-6	Transcriptional/Zinc finger
BL, ALL-L3	2p12	8q24	c-myc	Transcriptional/bHLH-LZ
NHL	22q11	11q13	Bcl-1	Cyclin
NHL, CLL	22q11	18q21	Bcl-2	Inhibitor of apoptosis
NHL	22q11	3q27	Bcl-6	Transcriptional/Zinc finger
BL, ALL-L3	22q11	8q24	c-myc	Transcriptional/bHLH-LZ

MM, multiple myeloma

additional genetic insults, eventually leading to transformation.

There are two types of interlocus abnormalities that can be mediated by the VDJ recombinase: one that involves an immune loci as a partner and one that allows recombination between two nonimmune loci. Examples of the latter include translocations involving the MLL locus on chromosome 11q23 and an interstitial deletion on chromosome 1 which involves two independent transcription units, SIL and SCL, and allows the expression of SCL to be promoted by SIL regulatory sequences. In both these recombinations, it has been proposed that the regions of breakage and rejoining contain cryptic recombinase signal sequences and extra nucleotides, supporting the idea that these translocations are mediated by the recombinase machinery.

Chromosomal translocations that do not involve immune loci frequently result in the formation of novel chimeric fusion products and are not usually a result of recombinase errors. Examples of such translocations include the BCR-ABL and the ALK-NPM fusion products resulting from the t(9;22) and the t(2;5) respectively. The hybrid proteins may have unique biochemical properties that are not derived from their respective normal partner gene products, thus the chimeric genes result in a gain of function lesion that promotes leukemogenesis/lymphomagenesis. Thus, recombinational errors resulting in translocations could either promote malignancy via gene activation or via structural alteration, as

depicted in Figure 2. When immune loci are involved, the mechanism of tumorigenesis is likely to be of a quantitative nature and is limited to gene activation.

Pathogenetic mechanisms: contribution of translocations involving immune genes

A common theme in the molecular pathology of neoplasia is that tumor-associated genetic abnormalities provide the neoplastic cell with the ability to overcome the normal controls of homeostasis. There are three basic pathogenetic mechanisms: the abnormal expression of genes that result in cell proliferation, the silencing of growth inhibitory genes, or the loss of the ability of cells to self-destruct through programmed cell death (apoptosis). Frankly neoplastic cells accumulate more than one abnormality since multiple lesions allow the tumor cell to avoid or pass through the checkpoints present in normal cells that regulate such intricately balanced events as cell cycle, cell differentiation, cell migration and apoptosis. Cells which are lacking some essential attributes are diverted at these checkpoints for repair or self-destruction.

Translocations involving immune genes result in the abnormal expression of proteins either with respect to the cell lineage or the particular stage during differentiation or even with respect to the amount of protein synthesized. This deregulated expression may result from a positive influence – because of the juxtaposition of strong transcriptional regulators like

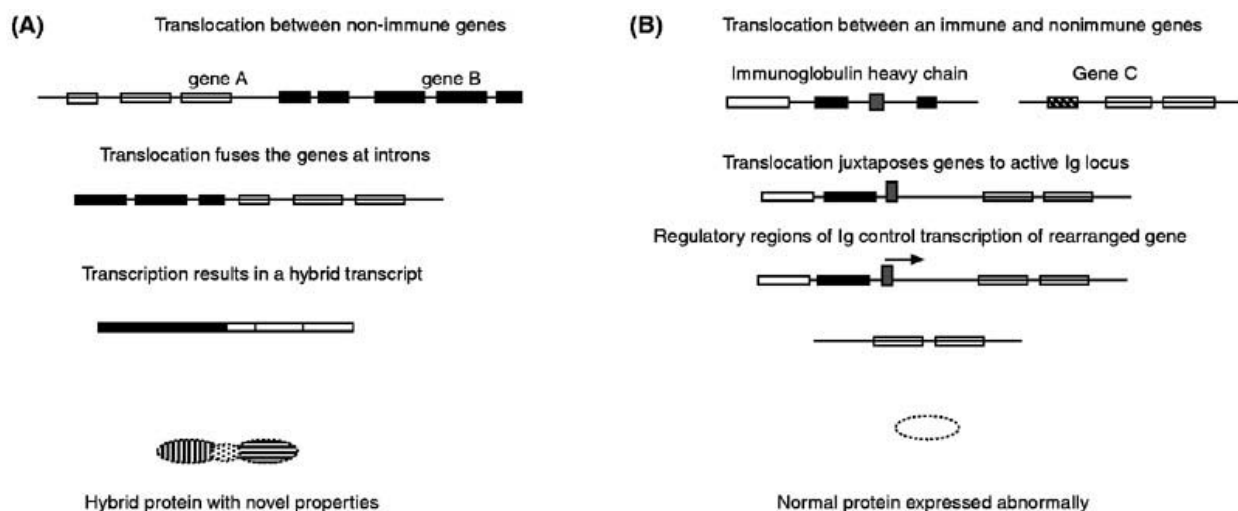


Figure 2 The recombinase machinery, which normally allows only intralocus recombination, is error prone and may cut and paste either two nonimmune genes (A), or juxtapose an immune locus with a nonimmune locus (B), by utilizing sequences present within these loci that serve as signal. If the recombinant product provides a survival advantage, the translocation will often be detected as a marker of clonal proliferation. The mechanisms by which the juxtaposition of an immune locus with a nonimmune locus or of two nonimmune loci provide a survival advantage are discrete. The former translocation often results in the formation of a fusion protein which carries some properties of both the partner proteins so that it acts as a novel protein. The latter translocation very often does not alter the expressed protein but nonetheless results in the inappropriate expression of this protein.

the enhancer elements present in the immune receptor genes, such as that which occurs in juxtaposition of *c-MYC* to *IgH* – or may result from loss of negative elements, e.g. the rhombotin 2 on 11p13 loses an upstream regulatory region during translocation with the TCR locus on 14q11.

The pathogenetic contribution of many, if not all, the translocations is the provision of a dominant genetic lesion. None of the translocations examined so far have resulted in the silencing of a growth-inhibitory or a tumor-suppressor gene product. The ultimate mechanism of lymphomagenesis or leukemogenesis is dependent upon the specific partner gene(s) involved in the translocation. The contribution of the immune genes appears mainly to be that of a switch to turn on the expression of the ‘oncogenic’ gene partners. As seen in Tables 2 and 3 four major classes of partner genes have been recognized: 1) genes that are master regulators of the expression of other genes (transcription factors); 2) genes that affect signal transduction mechanisms; 3) genes that

directly influence cell cycling and proliferation; 4) genes that affect cell survival.

Translocations that deregulate transcription factors
Among the various lesions that are a hallmark of oncogenic events, deregulation of those genes that themselves coordinate the expression of other genes are by far the most common of all lesions.

Transcription factors are versatile proteins that are essentially DNA-binding proteins. A common feature of these proteins is their modular structure which allows two unique domains to be independently defined, the DNA-binding domain and the transcriptional activation domain (Figure 3). The DNA-binding domain allows these proteins to specifically bind regulatory elements, through which they modulate gene transcription. Several classes of transcription factors have been identified as partners in translocations involving immune genes, these include the basic helix-loop-helix (bHLH) proteins, exemplified by *SCL*; proteins that contain zinc fingers (e.g. *BCL-6*), proteins with a cysteine-rich LIM domain like the rhombotin proteins (which also include a zinc finger-like structure), and proteins with specific leucine repeats termed as a leucine zipper, a domain that is found in the *c-MYC* protein; the *c-MYC* protein also contains a bHLH motif.

In translocations that involve immune genes and a transcription factor, the expression of these master regulators is deregulated. Since these genes themselves may control the expression of a set of genes that determine cell differentiation and proliferation, it is not surprising that such genetic lesions are oncogenic (Figure 3). In those translocations where the transcription factors are not juxtaposed to immune receptor genes, the deregulation of the transcription factor is often achieved by the formation of novel hybrid proteins where there is an exchange, for example, of the DNA binding domain conferring upon the hybrid protein an altered specificity of transcriptional regulation.

There appears to be a remarkable correlation between the specific type of transcription factor involved in the translocation and the progenitor cell lineage in which it occurs, suggesting strongly that these lesions disrupt regulatory cascades that control cellular differentiation programs. Thus, translocations involving *TCR β* and *LYL-1* are confined to pro-T ALL while those involving *HOX-11* are often seen in pre-T ALL.

In several cases these rearrangements have been directly correlated to mistakes in VDJ recombination. The ‘smoking gun’ for such mistakes include the presence of nucleotides inserted at the breakpoint junction and the identification of target heptamer-

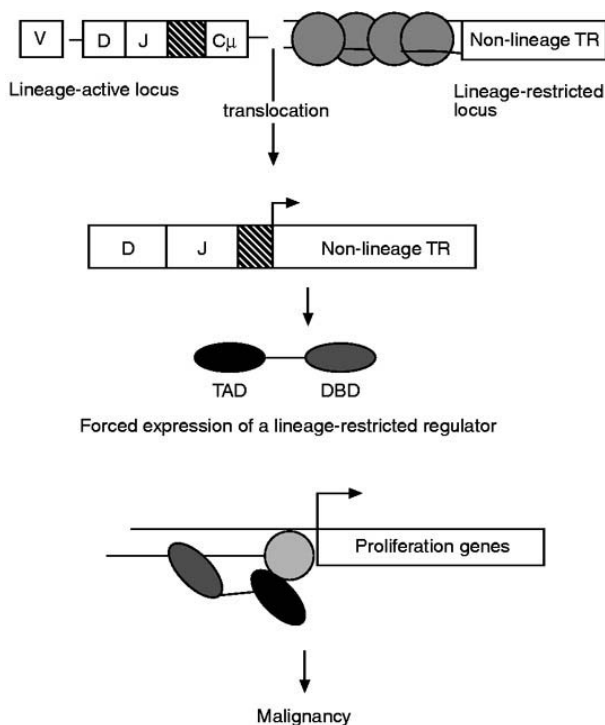


Figure 3 Schema of a possible translocation in a pro-B cell. The translocation juxtaposes a powerful transcriptional regulator (TR), not otherwise expressed in this cell lineage, and an immune gene that is normally active in this cell lineage. As a result of the translocation the TR gains a powerful enhancer from the immune locus and is no longer subjected to lineage restriction. The out-of-lineage expression of the transcriptional regulator provides the switch to turn on other target genes initiating a cascade of events that leads to immortalization of this cell and eventually to neoplasia.

like sequences in the breakpoint region. Thus, recombinase errors have been demonstrated in translocations involving HOX-11 and SCL. One of the most common rearrangement involving SCL (1p interstitial deletions) occurs in as much as a quarter of all pediatric T-ALL and although it does not involve the immune genes, it is nonetheless presumed to be mediated by recombinases. This presumption is again based upon sequencing data that demonstrate the presence of extra nucleotides at the breakpoint junctions. The regions of chromosome 1 that are 'stitched' together also harbor heptamer-like recognition sequences, which catalyze erroneous recombination processes.

Translocations that induce an aberrant signal transduction pathway When the extracellular environment is conducive to proliferation, the cell orchestrates various sets of events that result in growth. This communication from the cell surface, which senses the environment through specific receptors, to the nucleus is transduced by pathways specific for a given signal(s). If the molecular switches within these pathways are locked in the 'on' position, the cell will play out all the downstream events and proliferate even in the absence of a signal.

Translocations that involve the immune genes and thereby dysregulate the signal transduction mechanisms include the t(7;9) (q34;q34) present in about 5% of T-ALL and resulting in a rearrangement of the TCR β locus with TAN-1, and the less frequent t(1;7) (p34;q34) which juxtaposes the LCK gene to the TCR β locus. The LCK gene product, which is a receptor tyrosine kinase homologous to the SRC protein, is predominantly expressed in lymphoid cells. TAN-1 is homologous to the *Drosophila* 'notch' protein which is thought to affect development and cellular differentiation. Unlike LCK, however, the expression of TAN-1 is not preferentially lymphoid. In addition to relocating TAN-1 under the transcriptional control of TCR β , this translocation results in decapitation of the gene, such that the 5' portion of TAN-1 is lost, permitting the redistribution of the protein from its normal location in the plasma membrane to the nucleus.

Translocations that cause a constitutively 'on' cell cycle switch The commitment to enter the cell cycle is an active process initiated by signals received from the extracellular environment. The cell can develop lesions within the cell cycle commitment program, such that it is induced to proliferate even in the absence of upstream signals. A critical phase of the cell cycle commitment occurs in G₁. Following commitment to cycle, several proteins are expressed early

in the G₁ phase. These include c-MYC, c-MYB, c-FOS, c-JUN and PCNA. The critical role these proteins play is highlighted by the observations that when expressed inappropriately, a large number of them promote cell transformation. Deregulation of the proto-oncogene *c-myc* by juxtaposition to an immune gene is seen in both B and T cell lymphomas. In B cell lymphomas, typically in Burkitt's lymphoma, this deregulation in most cases results from transposition of one allele of *c-myc* – normally present on chromosome 8 – into the Ig heavy-chain locus on chromosome 14. Breakpoint locations vary with respect to geography, thus endemic Burkitt's lymphomas very frequently have breakpoints far upstream of the *c-myc* gene, whereas sporadic Burkitt's tumors often carry breakpoints within the transcriptional unit of *c-myc*. It is possible that these breakpoints may be influenced by other factors like the presence of the Epstein-Barr virus, which in certain circumstances may actually influence error-prone recombinations involving the immune genes. Less frequently, the light-chain genes on chromosomes 2 and 22 are involved in deregulation of the expression of *c-myc* in B cell lymphomagenesis. In these cases the light chain genes are juxtaposed to a site downstream of the *c-myc* gene. In T cell lymphomas the deregulation of *c-myc* is achieved by juxtaposition with TCR α .

Among the several proteins that act in concert in allowing the normal progression of the cell cycle, the cyclins play a critical role. Cyclins are expressed transiently during specific phases in the cell cycle and then degraded. Key cyclins mediating G₁ transition are cyclins D1, D2, D3 and E. One error during DJ rearrangement may occur in precursor B cells, leading to a t(11;14) that gives rise to a low-grade B cell lymphoma classified as mantle cell lymphoma. The partner locus on chromosome 11, known as Bcl-1 or PRAD1, has homology to cyclin D1 which associates with CDK-4 and CDK-6. Following activation of the cyclin D/CDK complex, RB is phosphorylated leading to the release of E2F which is now available to transactivate genes like *c-myc*, *c-myb*, thymidylate kinase and thymidine synthetase that are essential for the G₁-S transition.

Death-defying translocations A reciprocal translocation between chromosomes 14 and 18 was found to occur frequently in follicular lymphomas. Molecular analysis indicated that the heavy-chain J segments were juxtaposed to a novel locus designated Bcl-2. The majority of breakpoints on chromosome 18 cluster in a short span of about 300 nucleotides termed as 'major breakpoint region' (MBR). Subsequently, another less frequent cluster of breakpoints was

identified 30 kb from the MBR and was designated as 'minor cluster region' (mcr). The translocation did not compromise the integrity of the open reading frame of the major transcript from the Bcl-2 locus. Detailed sequence analysis of the recombinational sites revealed the presence of extra nucleotides at the junctions of the derivative chromosomes suggesting an error mediated by recombinases. However, the actual recombination in this translocation may be catalyzed by chi-like sequence elements. Thus, the t(14;18)(q32;q21) may involve homologous recombination and errors in the action of the recombinases.

Functional analyses to elucidate the pathogenic contribution of the Bcl-2 gene provided insights into a fundamentally novel pathway in tumorigenesis. Aggressive uncontrolled proliferation of cells is one aspect of tumorigenesis, and accumulation resulting from a loss of susceptibility to dying is another pathway to neoplasia. Normal architecture is usually maintained within organs from early in embryogenesis, because all normal cells are subject to homeostasis. An important mechanism that contributes to this regulation is an inherent ability to selectively deplete cells. This ability is derived from the capacity of cells to activate pathways that result in suicide. These pathways are referred to as apoptosis. It is now apparent how the aberrant loss of this program in a cell in multicellular organisms can lead to neoplasia. The involvement of Bcl-2 in overcoming apoptosis suggested that the deregulation of this death-defying gene interfered with normal B cell ontogeny and prevented the otherwise normal loss of B cells due to programmed cell death. It now appears that resistance to death in several normal lymphoid cells relates to overexpression of Bcl-2. Recently, several other proteins with homology to Bcl-2 have been described. Although none of these members have been associated with chromosomal translocations, it is clear that several members of the Bcl-2 family interact with each other in orchestrating the cell death program.

Immune gene translocations associated with DNA repair deficiency

The cutting and repasting of DNA segments during the process of legitimate immune gene rearrangements must surely involve some components of the normal DNA repair machinery. Increased frequency of chromosomal aberrations are indeed observed in normal cells following severe DNA damage and in lymphocytes of individuals with a DNA repair deficiency. Several DNA repair deficiencies are also characterized by an increased risk of malignancies. Patients with ataxia telangiectasia (AT), for example,

are significantly prone to accumulating chromosomal aberrations and 10% of these patients demonstrate an abnormal karyotype in their peripheral blood lymphocytes. In a large proportion of these lymphocytes, the chromosomal aberrations are nonrandom and are often limited to breakpoints on 14q11, 14q32 and 7q34, loci that harbor immune receptor genes.

Molecular analyses have implicated TCR α , TCR β and IgH to be intimately involved in the recombinational processes that generate these aberrations. Translocations and inversions affecting chromosome 14 are the most frequent clonal rearrangements in AT lymphocytes. Some cases of AT clonal T cell expansion have been reported to progress to T cell leukemias. Interestingly, AT inversions that give rise to chimeric TCR are not associated with leukemogenesis, whereas the t(14;14)(q11;q32) or the inv(14)(q11;q32) clonal proliferations, both of which involve the TCL-1 locus, can progress to malignancy. In addition, some cases of T cell leukemias in AT patients carry a t(X;14)(q28;q11) which involves a locus termed as MTCP-1 and possibly the J region of TCR α .

Translocations involving immune genes in 'normal' cells

Several of the interlocus recombinations that involve immune receptor genes have also been observed in a small fraction of lymphocytes of healthy individuals. These recombinations include those that form hybrid immune receptor complexes by stitching together VDJ regions between different immune receptors genes. Our ability to detect these rearrangements suggests that they may be positively selected for. The finding that the hybrid receptors frequently recombine to be in-frame is in support of this argument. The analyses of these rearrangements (translocations or inversions) also suggest that they are mediated by the VDJ recombinases at a frequency of about 10^{-4} and predominantly involve chromosomes 7 and 14. It has been demonstrated that the frequency increases following exposure to pesticides. This increase is intermittent and reversible. Exposure to pesticides may also increase the risk of developing lymphoid malignancy. These observations, coupled with the increase of such rearrangements in AT patients, indicate that rearrangements involving immune receptor loci are subject to both exogenous and endogenous influences on DNA stability and that some of these rearrangements, for example the frequency of hybrid receptors, may serve as monitors of error-prone recombinase activity.

The functional consequences of these interlocus

rearrangements are not clear. At least one of the translocations described in lymphomas, the t(14;18) which juxtaposes the Bcl-2 locus within the transcriptional influence of the immunoglobulin heavy chain gene, has also been shown to occur in lymphocytes of healthy individuals, and also bears the 'signature' of recombinase activity. The frequency of this translocation increases with age and parallels the age-dependent incidence of follicular lymphomas. Whether this particular rearrangement is affected by exogenous influences such as exposure to pesticides is not known. However, its frequency is not increased in lymphocytes of AT patients. It is entirely possible that an error in recombinase will promote illegitimate recombination between interlocus immune genes more readily than between an immune and a nonimmune locus.

Summary

The principal function of T and B lymphocytes is to support the immune defense mechanisms of the organism. To do this effectively, these cells have acquired the capability of recognizing and neutralizing a diverse array of 'nonself' antigenic epitopes. This diversity is inherent in the germline structure of the immune genes, which contain several different regions. These discrete regions of the immune genes undergo several recombinational steps and reassortment of multiple segments to express the primary immune protein products.

The reassortment of the immune regions is achieved via a cutting and pasting mechanism that draws its specificity from the presence of specific nucleotide signal sequences, modulation of the accessibility of the genomic regions necessary for recombination, developmentally regulated expression of enzymes that will catalyze the recombination and possibly monitoring mechanisms that will prevent nonlegitimate substrates from recombining. In order to ensure the fidelity of the reaction, all productive recombinational processes are limited to be intralocus. Nonetheless there is a baseline error rate in the recombination process and some interlocus recombinations are also catalyzed. When these recombinations are catalyzed between immune and nonimmune partners, the expression of the nonimmune gene is deregulated. If the nonimmune partner is a growth-stimulatory protein, the recombinational

event can lead to a clonal expansion and eventually transformation of a lymphoid cell. As an extreme, these errors may also involve two nonimmune genes that contain nucleotide sequences that are similar to the recombinase signal sequences. Mistaken recombination of such partner genes may also be transforming if the two genes form a novel product with growth-stimulatory properties or if the expression of one of the growth-supporting partner genes is rendered lymphoid specific by the rearrangement. It is possible that the error-prone mechanisms by which these lymphoid-specific transformation events occur may also overlap with errors in other mechanisms that cause genetic instability and promote transformation of nonlymphoid tumors.

See also: Apoptosis; B lymphocyte repertoire; B lymphocyte differentiation; B lymphocytes; Bone marrow and hematopoiesis; Diversity, generation of; Genetic analysis at the molecular level; Immunoglobulin class switching; Immunoglobulin genes; Immunoglobulin structure; Leukemia; Lymphocytes; Lymphoma; T lymphocytes.

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CHRONIC ACTIVE AND AUTOIMMUNE HEPATITIS

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Early descriptions

Autoimmune hepatitis (AH) is now the preferred name for the disease originally described as chronic active hepatitis (CAH) in the 1940s in reference to a protracted viral hepatitis among military personnel. However, CAH, as later understood, emerged from descriptions in the 1950s of a relapsing or progressive and usually fatal liver disease of young women of no ascertainable cause, but perhaps the result of subclinical viral hepatitis. Emphasis was given to endocrine abnormality, amenorrhea, striae and acne due to the diseased liver failing to metabolize steroid hormones, and to hyperglobulinemia with reversal of the albumin–globulin ratio in serum, a then popular liver functional index. Autoimmunity was later implicated because of findings of lupus erythematosus (LE) cells in the blood, and a positive serum complement fixation test with human tissue homogenates as antigen. Clinical and serological resemblances between CAH and systemic lupus erythematosus (SLE) led to the name lupoid hepatitis, but the full expression of SLE was seldom seen, and the LE cell test was usually weaker and more transiently positive. Immunofluorescence testing in the 1960s for antinuclear antibody (ANA) replaced the LE cell test. Later, a serological reaction with smooth muscle was recognized as another identifying marker.

Heterogeneity of CAH

It was realized in the early 1970s that ‘chronic active hepatitis’ was a generic term for a liver disease with a variable expression and etiology. The hepatitis B surface antigen (HBsAg) had been identified as a marker of infection with hepatitis B virus (HBV), so that some cases of CAH could be characterized as HBV-associated CAH, in addition to autoimmunity and other causes.

Several categories of CAH can now be listed, as follows: 1) The archetypic CAH described in the 1950s as lupoid hepatitis, and now identifiable by autoimmune serological markers; 2) CAH due to persistence of hepatitis B virus (HBV) in the liver, marked (usually) by the presence of HBsAg in serum; 3) CAH due to persistence in the liver of hepatitis C virus (HCV); 4) CAH due to persisting immune-

mediated hepatic reactions to a drug; 5) histological CAH in accompaniment with liver diseases of known toxic, genetic or metabolic cause; 6) a category with neither autoimmune nor viral markers, nor any identifiable cause, referred to as ‘cryptogenic’. The relative frequency among populations of these categories of CAH varies. Chronic active hepatitis due to virus infection (HBV, HCV) is now numerically more frequent overall than the autoimmune type and, in some localities, virus-related CAH is seen almost exclusively.

General features of CAH

Chronic hepatitis ranges from an asymptomatic disease recognized only by biochemical abnormalities to one that is severe and progressively cirrhotogenic. Some cases of autoimmune hepatitis can have a long asymptomatic preclinical course, as pertains for other autoimmune disorders. Likewise, an insidious onset and clinical latency is usual for CAH associated with infection with HCV and, in many instances, with HBV. Cases of chronic active hepatitis will have some common general features related to liver dysfunction or cirrhosis, whatever the cause, and other features related to the individual etiological agent or process.

The general features include symptoms of hepatitis, including nausea, anorexia, jaundice, hepatosplenomegaly, and biochemical evidence of liver parenchymal damage, in particular high levels of transaminase enzymes in serum. The biopsy of the liver may show either of two histological lesions, named as chronic *persistent* hepatitis which is indolent and nondestructive, or chronic active hepatitis in which the main morphological feature is the disruption of the peripheral limiting plate of the liver lobule, with a periportal ‘spillover’ of the inflammatory exudate into the liver parenchyma: ‘piecemeal necrosis’ (Figure 1). This lesion generates scarring and eventually macronodular cirrhosis (Figure 2). Hepatocellular carcinoma is a frequent late sequel in CAH due to chronic virus infection but is seldom seen in autoimmune hepatitis. There are features unique to each of the individual categories, as indicated below.

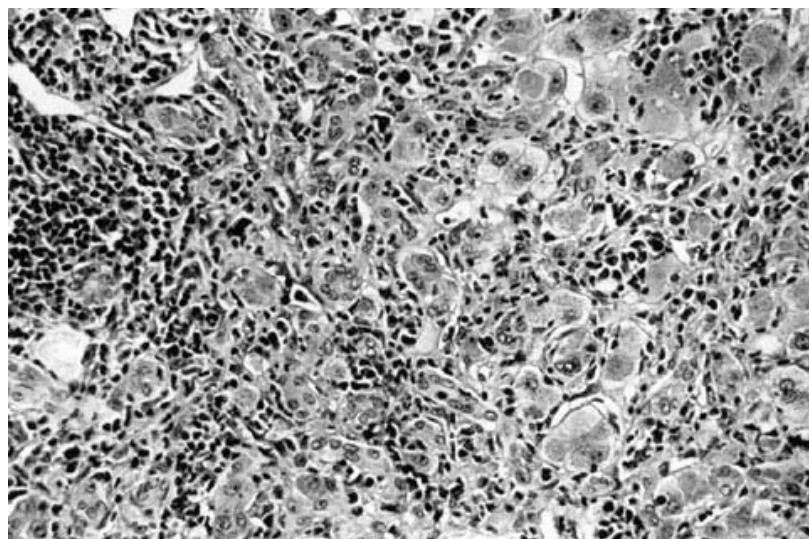


Figure 1 Histological appearance of liver biopsy specimen from a female aged 65 years with recent-onset autoimmune hepatitis, showing periportal and lobular hepatitis and extensive hepatocellular necrosis (HE $\times 200$).

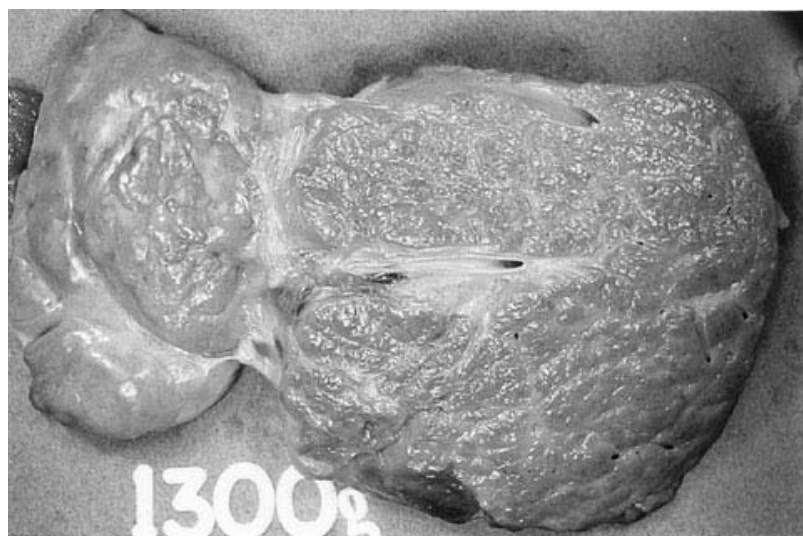


Figure 2 Liver from a female aged 45 years with autoimmune hepatitis, showing scarring and a coarse macronodular cirrhosis.

Features of specific types of CAH

Autoimmune hepatitis

Diagnostic criteria are currently being developed for autoimmune hepatitis. Characteristics that provide a 'disease profile' are: seronegativity for viral markers; female sex; northern Caucasian ethnic origin; multi-system disease expression (arthritis, rashes, thyroiditis, hemolytic anemia, ulcerative colitis); histologically large areas of periportal necrosis and prominence of plasma cells; pronounced hypergammaglobulinemia, $>30 \text{ g l}^{-1}$; serum autoantibodies; HLA-B8, -DR3 and -DR4; rapid response to, and dependency on, corticosteroid drugs; and 'resistance' to hepatocellular carcinoma compared with other types of chronic hepatitis or cirrhosis. Most weight attaches to the autoantibody markers, which are predominantly to antigens of nuclei and smooth muscle

(Figure 3). The multiple nuclear antigenic reactants include DNA (rarely), histones and lamins, and the smooth muscle reactant is the cytoskeletal filament, F-actin. Autoantibody to neutrophil cytoplasmic antigen (ANCA) is also seen. In a variant now called autoimmune hepatitis type 2, the marker antigen is enriched in liver and kidney microsomes (LKM). The LKM antigen has been identified as a particular cytochrome P450 isoform, CYP450 2D6. There is suspected to be a liver-specific autoantigen, perhaps membrane-associated, and one candidate is the asialoglycoprotein receptor (ASGP-R); however this to a degree lacks fine disease specificity for association with autoimmune hepatitis. Long-term treatment with prednisolone, combined with azathioprine, is highly effective.

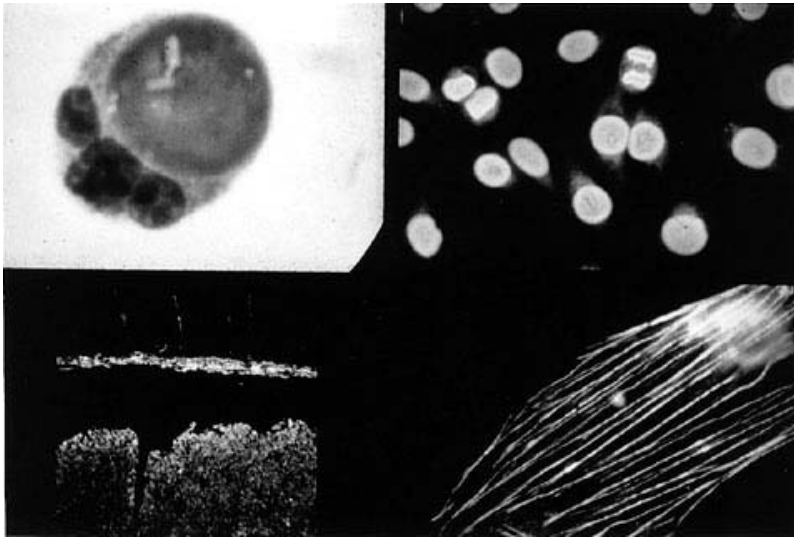


Figure 3 Serological reactions in type 1 autoimmune hepatitis. Top left, lupus erythematosus (LE) cell as the former marker of antinuclear antibody (ANA); top right, homogeneously reactive ANA by immunofluorescence on Hep2 cells; bottom left, smooth muscle antibody (SMA) by immunofluorescence on mouse stomach; bottom right, F-actin specificity of SMA on cultured fibroblast showing staining of cytoplasmic microfilaments.

HBV-associated CAH (CAH-B)

HBV is a DNA virus that is parenterally transmitted. The frequency of chronic infection is higher in males and in localities in which HBV infection is endemic. Infection with HBV is acquired neonatally from infected mothers in high endemicity regions, and sporadically as a parenteral infection in low endemicity regions. The diagnosis of HBV-associated CAH (CAH-B) depends on detecting antigens of HBV in serum, the surface 's' antigen (HBsAg), and in early cases the 'e' antigen (HBeAg). In liver cells, HBV as the core antigen (HBcAg) is demonstrable immunohistochemically or by nucleic acid hybridization. CAH-B has a variable clinical expression and, although often indolent, undergoes progression to cirrhosis, and there is a clear predisposition to supervening hepatocellular carcinoma. Treatment with type 1 interferons may eliminate the virus and arrest the disease.

HCV-associated CAH (CAH-C)

HCV-associated CAH, which accounts for most examples of previously called non-A, non-B viral hepatitis, is due to an RNA virus which is transmitted parenterally. Formerly transmission was mainly by blood transfusion or treatment of hemophilia by blood products; currently the main routes are contaminated equipment of intravenous drug abusers, or close intrafamilial contact. Persisting infection with HCV is frequent after an acute infection. HCV-associated CAH (CAH-C) usually progresses silently to cirrhosis, and primary hepatocellular carcinoma is a risk in the later stages. Cirrhosis and hepatocellular carcinoma appear to be potentiated by coexisting alcoholic liver disease. Many cases formerly specified as cryptogenic CAH

(see below) will probably be attributable to infection with HCV. The diagnosis depends on serologic demonstration of antibody to antigens of HCV, or amplification of viral sequences in blood or liver tissue by the polymerase chain reaction. Treatment with type 1 interferons arrests infection in a proportion of cases. Some instances of CAH-C are associated with low-titer anti-LKM reactions, but this reactivity is not regarded as signifying that autoimmunity plays any role in their pathogenesis.

Drug-associated CAH

Various drugs, of which well-known examples include halothane and chlorpromazine, induce (infrequently) acute idiosyncratic hypersensitivity reactions affecting the liver, with hepatic or cholestatic expressions. As an accompaniment of these reactions, there may be demonstrable serum autoantibodies of the type usually seen in autoimmune liver diseases, directed to antigens of nuclei (ANA), smooth muscle (SMA), mitochondria (AMA) or liver microsomes (anti-CYP450 2D6). In cases with a protracted course, the histopathology of the liver resembles that of autoimmune CAH. However, characteristically, the disease resolves when the offending drug is withdrawn, and cirrhosis seldom eventuates. The immunopathogenesis of drug-induced CAH is uncertain.

'Toxic-metabolic' CAH

Histologically evident CAH may be seen in cases of chronic liver disease with a defined metabolic cause including alcohol abuse, or effects of inborn genetic errors including α_1 -antitrypsin deficiency, Wilson's disease or hemochromatosis. This category is relatively unimportant in terms of immunology, and features of the associated disease are dominant.

Cryptogenic CAH

This term is used for cases of CAH that are negative for markers of autoimmunity or infection with HBV or HCV and for which no other cause is ascertainable. Some cases may be attributable to infection with hepatitis viruses of which markers are absent from serum, to infection with as yet unidentified hepatitis viruses, or to autoimmunity wherein autoantibodies are no longer demonstrable.

Experimental models of CAH

Animal models of CAH of varying fidelity are recognized. HBV-associated CAH has the best models, represented by natural infections of animals in the wild, e.g. wood-chucks or ducks, with a 'hepadna' virus similar to human HBV. Models of autoimmune hepatitis have been induced by neonatal thymectomy and/or immunization with liver preparations, or by other techniques, but none so far has entirely simulated the naturally occurring human disease.

Liver-specific autoantigen

A liver-specific autoantigen as the target for immune-mediated damage in autoimmune hepatitis has not yet been identified. Crude preparations of liver cells and liver cell membrane preparations have been examined in various ways for reactivity with serum or peripheral blood T cells from patients with autoimmune hepatitis with promising but not unequivocal results. No liver-specific autoantigen has yet been identified by techniques applied successfully in other autoimmune disease, namely immunoblotting on electrophoretically separated liver cell membrane preparations, or probing gene expression libraries, using serum from cases of autoimmune hepatitis. Claims for ASGP-R as a liver-specific autoantigen relevant to autoimmune hepatitis need more substantiation.

Mechanisms of hepatocellular damage

The mechanisms of hepatocellular damage in chronic active hepatitis will vary according to the particular cause. In CAH-B there are two likely processes, a specific immune deficiency to critical antigens of the virus, probably the surface antigen, that allows a state of tolerated infection to occur, and a noneliminative attack by cytotoxic T cells on epitopes of the core antigen (HBcAg) expressed with class I major histocompatibility complex (MHC) molecules on the liver cell membrane. In CAH-C, the cytotoxic T cell attack would be on epitopes of HCV expressed with

class I MHC molecules on the liver cell membrane. In autoimmune hepatitis the suspected but as yet unknown processes include a T cell-dependent cytotoxicity due to cytokines released from CD4 T cells or cytotoxic effects of CD8 T cells, and an immunoregulatory failure permitting responses to tolerated hepatocellular autoantigens. Whether any pathogenetic connotations can be ascribed to the serologic reactivities, ANA, SMA, anti-LKM, that serve as disease markers is uncertain. It is intriguing that LKM autoantibodies in type 2 autoimmune hepatitis are highly focused towards one particular isotype (2D6) of CYP450, and that there have been identified linear autoepitopes on this enzyme, with the main site (WDPAQPPRD) at residues 262–270; also, a conformational epitope is suggested by the capacity of anti-LKM to inhibit CYP450 enzyme activity. Pathogenicity of the autoantibodies could depend on their cellular penetration, or the cell surface expression of the autoantigens, which remain under debate.

A final common immunocytotoxic pathway in CAH

The occurrence of autoantibody to various liver cell constituents, together with histological appearances of CAH in diseases that are not primarily attributable to an autoimmune process, has led to the idea that an immune-mediated attack on liver cells can supervene as a 'final common immunocytotoxic pathway' in liver diseases with different initiating causes. Whilst this may indeed occur, the features of this postulated 'secondary' autoimmune hepatitis do not really simulate those of the primary disease. Accordingly it is recommended that autoimmune hepatitis be regarded as an entity *sui generis* for which the nature should be better understood if and when a disease-specific and tissue (liver)-specific autoantigen is identifiable.

See also: Autoantibodies, tests for; Autoimmune diseases; Autoimmunity; Systemic lupus erythematosus (SLE), human; Primary biliary cirrhosis.

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CHRONIC GRANULOMATOUS DISEASE

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Chronic granulomatous disease (CGD) is a syndrome, the uniting features of which are a predisposition to infection, often with otherwise relatively nonpathogenic organisms, a granulomatous tissue reaction and impaired activity of the 'respiratory burst' of phagocytic cells. Although relatively rare, this syndrome has been the focus of intense investigation because it provides an excellent model for the examination of the mechanisms by which phagocytic cells kill and digest microbes, and has been central to our understanding of the structure and function of the NADPH oxidase electron transport chain, components of which are defective in the different subtypes of the disease.

NADPH oxidase of phagocytic cells

The uniting feature of CGD is an absence of the NADPH oxidase activity of professional phagocytic cells. When these cells engulf particles or are activated by a variety of stimuli, including the tetrapeptide formyl leucyl methionyl phenylalanine or the protein kinase C activator, phorbol myristate acetate (PMA), an intense burst of oxygen consumption is observed. This respiration is not mitochondrial, and is not required for the engulfment of microbes, but is important for the efficient killing of a variety of bacteria and fungi, both those classified as pathogenic, and normal commensals. In classical CGD this respiratory burst is completely defective, whereas in a few very rare cases, termed 'variant CGD' a small amount of oxidase activity can be detected.

Composition of the NADPH oxidase

The oxidase system is an electron transport chain that shuttles electrons from NADPH, generated by the hexose monophosphate shunt from glucose, to oxygen in the phagocytic vacuole. The only functionally defined component of the system is a very unusual flavocytochrome *b*, flavocytochrome *b*558. The flavin part of the molecule binds the NADPH and has strong homology with the FNR family of reductases. There are two hemes, which are unusual by virtue of their very low redox potential ($E_m = -225$ mV and -265 mV). It is composed of a 22 kDa subunit (α subunit, or p22^{phox}) and a 76–92 kDa heavily glycosylated subunit (β subunit, or gp91^{phox}). gp91^{phox} is the location of the FAD and NADPH binding sites and most probably both of the hemes. This cytochrome is located in the plasma membrane and membrane of the specific granules, and moves into the wall of the phagocytic vacuole as this forms.

A number of components of the oxidase have been identified; 40 kDa (p40^{phox}), 47 kDa (p47^{phox}) and a 67 kDa (p67^{phox}) proteins are located in the cytosol of the cell and move into association with the cytochrome in the membrane upon activation of the system. A small GTP-binding protein, p21^{rac} seems to be important for activation. The function of these molecules remains to be determined.

Molecular pathology

Chronic granulomatous disease is a syndrome in which the uniting feature is the complete absence of NADPH oxidase activity. The molecular pathology varies, but most cases fall into two main groups.

Approximately 70% have an X-linked pattern of inheritance and 30% are autosomal recessive.

X-linked

In almost all these patients flavocytochrome *b558* is missing from their cells and the lesion is in the gene coding for gp91^{phox}.

Autosomal recessive

The cytochrome is normal in these patients but they are unable to transport electrons on to it from NADPH. Their lesion has been shown generally to involve the p47^{phox}, which is undetectable in their phagocytes. p22^{phox} and p67^{phox} are missing in a minority of patients with this pattern of inheritance. No lesions involving p40^{phox} have been described.

Variant CGD

Unusual subjects have been described in whom a variable but small (less than 20% of normal) amount of oxidase activity can be detected. They are largely X-linked and have reduced expression of the large subunit of the cytochrome.

Clinical features

The respiratory burst of phagocytes is required for the optimal killing of a wide variety of bacteria and fungi. In its absence the microbes are engulfed but killing is impaired. This manifests in the patients in an increased frequency of infection, predominantly in the reticulo endothelial system. The most common sites include cervical lymph nodes, lungs, bone marrow (leading to osteomyelitis), liver and skin. *Staphylococcus aureus* is by far the most common infecting pathogen, although a wide variety of otherwise relatively avirulent organisms, for example *Serratia marcescens* can cause serious infections in these subjects. CGD must be excluded in children presenting with cervical or inguinal adenitis, liver abscesses, or aspergillus or nocardia pneumonia.

The defective function of phagocytes from these patients is not limited to their failure to kill microbes. Digestion by these cells is also grossly defective. This results in the diffuse development in the tissues of the granulomas that have lent their name to this syndrome. Granulomas in biopsies from these patients often result in an incorrect diagnosis of tuberculosis being made. Abnormal digestion and the granulomatous response result in poor healing, with the breakdown of wounds and chronic sinus formation. Exuberant attempts at healing can result in narrowing and obstruction of hollow muscular organs like the duodenum and urinary system. Chronic

inflammation of the gut may mimic inflammatory bowel disease, particularly Crohn's disease, where bowel disease is associated with the presence of granulomas in histological sections.

Diagnosis

The simplest and most rapid test for CGD is the NBT (nitroblue tetrazolium) test. Neutrophils from a small sample of peripheral blood are activated to produce superoxide which is detected by the NBT, which is converted from a yellow water-soluble compound to a dark-blue insoluble formazan that can be clearly detected microscopically. This slide test can be useful for detecting the X-linked type of disease in which the carrier mothers have a mosaic of normal and abnormal cells in their circulation. The respiratory burst can be accurately quantitated by measuring oxygen consumption with an oxygen electrode or superoxide generation by the reduction of cytochrome *c* in the presence and absence of superoxide dismutase. Immunological and molecular biological reagents are becoming available for the characterization of the nature of the defect in terms of the proteins and genes that are defective in each case.

Treatment

The prophylactic use of antimicrobial agents, in particular co-trimoxazole (a compound of trimethoprim and sulphamethoxazole) has transformed the natural history of this condition from one of frequent, life-threatening infections to that resulting in an almost normal life.

Infections that do occur must be treated aggressively with antimicrobial agents. Infusions of neutrophils should be used in addition in severe or systemic infection.

See also: Granuloma; Macrophage activation; Microbicidal mechanisms, oxygen-dependent; Neutrophils; Phagocytosis; *Staphylococcus*, infection and immunity.

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CIRCULATORY SYSTEM INFECTIONS

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The most important infection involving the circulatory system with immunologic sequelae is infective endocarditis. The annual incidence in the USA and the UK is approximately 6 per 100 000 inhabitants, with an overall mortality of 15–30%. In acute endocarditis, most commonly due to *Staphylococcus aureus*, there is a high fever and, if untreated, a fatal rapid destruction of the heart valves. In subacute endocarditis there is a more indolent course and it is here that the immunologic complications occur. These may in part be due to emboli breaking off from the infected heart valve. Clinically these include splinter hemorrhages, Osler's nodes, Janeway's macules, glomerulonephritis, conjunctival hemorrhages (Roth spots) and clubbing of the fingers.

The major causative group of microorganisms is the streptococci. These can be subdivided into the relatively antibiotic-sensitive viridans streptococci and the more antibiotic-resistant enterococci. The viridans streptococci originate in the mouth and are defined by producing partial hemolysis on blood agar plates. They include *Streptococcus sanguis*, *S. mutans* and *S. salivarius*. The enterococci originate in the bowel and include *Enterococcus faecalis*, *E. faecium* and *S. bovis*. The last is associated with underlying bowel pathology.

Immunologic studies

The definitive diagnosis of endocarditis is dependent on the demonstration of vegetations on a heart valve at surgery or necropsy, or a peripheral embolus. Recently this has been augmented by a secondary set of clinical criteria. This consists of major criteria (positive echocardiogram and typical blood culture) and minor criteria (predisposition, fever, vascular phenomena, hematological phenomena, suggestive echocardiogram and suggestive microbiology). In

addition to this there is increasing literature showing that antibody levels, as measured by immunoblotting, are very high in enterococcal or streptococcal endocarditis and that these are species specific. For example, there is a strong immunoglobulin M (IgM) and IgG response in patients with *E. faecalis* endocarditis to an 88–90 kDa band identified as a homolog of heat shock protein 90. In *S. mutans* endocarditis there is an endocarditis-specific pattern of IgM, including antibody against a 190 kDa homolog of the Pac protein of *S. mutans* and the Spa protein of *S. sobrinus*. *S. mutans* antigen I/II (Pac homolog) has been implicated in the bacteria's adherence to teeth surfaces. Furthermore, monkeys immunized with the protein were protected against dental caries. The viridans streptococci have recently been demonstrated as being not only a cause of endocarditis but also a cause of septicemia in cancer patients. There have been changes in the taxonomy which have led to the emergence and description of *S. oralis* both as an important pathogen in neutropenic cancer patients and in infective endocarditis. Studies of the antibody in these diseases have defined two antigens as immunodominant. One of these has been cloned and demonstrated to be a homolog of the Pac protein of *S. mutans* and the Spa protein precursor of *S. sobrinus*. The protein has been further epitope mapped, demonstrating areas to which antibody was seen only in patients with endocarditis due to *S. oralis*. Human recombinant antibodies have been raised against these epitopes and shown to have some therapeutic activity in an animal model of infection. The second immunodominant endocarditis-specific antigen identified was an hsp90 homolog with an apparent molecular weight of 85 kDa. Similar specific antibody patterns have been shown in *Candida albicans* endocarditis, *Corynebacterium jeikeium* endocarditis and endocarditis due to *S. lactis*. In contrast, in

patients with staphylococcal endocarditis the level and pattern of antibody, as measured by immunoblotting, could not distinguish between cases and controls.

In all of the above the antibody patterns were specific to patients with endocarditis and the level of IgM correlated with disease. The resolution of infection by appropriate antibiotic therapy saw a rapid reduction in IgM levels when samples were measured weekly. The description of these patterns has led to a further study in which they were used as a way of diagnosing culture-negative endocarditis. This is a syndrome in which the patient has proven endocarditis but consistently negative blood cultures. Negative serology was found in 28 patients where the diagnosis of endocarditis was rejected or, if proven, staphylococcal, yeast, gram-negative, systemic lupus erythematosus, due to Q fever or *Chlamydia psittaci* or nonbacterial thrombotic. Positive serology was found in 27 of the 34 patients where the response to antibiotics suggested streptococcal or enterococcal infections. In 22 of these there was objective evidence of endocarditis. Positive serology was also found in three out of four further patients with vegetations at necropsy.

In summary, the diagnosis of endocarditis, both positive and negative, has been helped by the study of antibody levels. Individual antigens are now being identified and their role in the pathogenesis of infection characterized.

See also: Molecular mimicry; Oral immunity; Respiratory and cardiac infections; Specificity; *Staphylococcus*, infection and immunity; *Streptococcus*, infection and immunity; Western blotting.

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CLONAL DELETION

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Clonal deletion refers to the process of eliminating unwanted clones of cells by death of the progenitors. At different points in development, the process eliminates lymphocyte (B and T cell) progenitors either through the stimulation or failure to stimulate their clonally unique, membrane-bound antigen receptors. Clonal deletion is an important, general mechanism to limit the development and expansion of auto-reactive lymphocyte populations that would cause autoimmune disease (Figure 1). Clonal deletion may also play an important role in limiting the organism's response to tumors.

Our early understanding of the interaction between pathogens and lymphocytes relied on the 'selective' expansion of appropriate lymphocyte clones in response to antigenic challenge. This allowed the maintenance of a pool of precursor lymphocytes to a broad range of pathogens that could be expanded through proliferation of the most appropriate clones for a specific pathogenic challenge. The lymphocyte clones with the highest affinity for pathogenic antigens would be selected for expansion because their receptor stimulation was the most effective. The paradoxical notions that antigen receptor stimulation could lead to nonresponsiveness through death (clonal deletion) or anergy as well as expansion of the progenitor has changed our fundamental understanding of lymphocyte biology. The antigen receptor can no longer be modeled as a digital on/off switch. Rather, the context in which the antigen is presented as well as the differentiation/activation state of the responding lymphocyte determines the lymphocyte's functional response.

Clonal deletion in thymus-derived lymphocytes (T cells)

An important distinction between T cells and B cells is that T cells can only recognize cell-bound (antigen-presenting cell, APC) antigenic peptides in the context of major histocompatibility complex (MHC) proteins. T cells are involved in surveillance of the peptides bound to MHC molecules in their environment from the earliest period of their development.

Thymus

The primary site for development of T cells is in the thymus. The process begins *in utero* as T cell progenitors migrate from their mesenchymal origin in the yolk sac and fetal liver and continues to a lesser extent after birth with progenitors from the bone marrow. These cells migrate to the thymus and begin to rearrange the genes encoding the heterodimeric T cell receptor (TCR). From this point forward the cells can be selected for complete maturation and export to the peripheral lymphoid organs (lymph nodes and spleen) or they are clonally deleted either because they fail to produce a functional TCR or because their TCR would be self-reactive in a functional, mature lymphocyte and cause autoimmune disease.

One reason T cells may fail to reach full maturity is because they are unable to complete the random rearrangement of the TCR genes. A second reason is that they successfully rearrange their genes and produce a functional TCR on the cell surface, but that protein with its CD4 or CD8 coreceptors does not recognize environmental MHC-peptide complexes sufficiently for completion of maturation. In a sense this failure of clones to be selected for further maturation is because they do not produce TCRs of sufficient affinity to be useful (Figure 2). The molecular basis of this arrested differentiation and subsequent cell death is not well understood, but is often referred to as deletion by 'neglect'. It is clear that the expression of anti-apoptotic proteins such as Bcl-2 in these cells can prolong their life, but does not ultimately rescue them for further differentiation.

Production of rearranged TCR gene products that alone, or in association with the CD4 and CD8 coreceptors, react strongly with MHC-peptide complexes results in the rapid death of the T cell progenitor (Figure 3). The death (active deletion) of these cells takes place at the double positive (CD4⁺CD8⁺) stage of T cell development, shortly after expression of a functional TCR. There appear to be no special APC requirements to induce deletion, but rather deletion is a function of peptide density on the APC and the affinity of the TCR for the MHC-peptide complex. Deletion can take place on any appropriate MHC⁺ cell in the thymus (MHC class I to delete CD8 cells and MHC class II to delete CD4 cells) but is generally thought to occur at the corticomedullary junction.

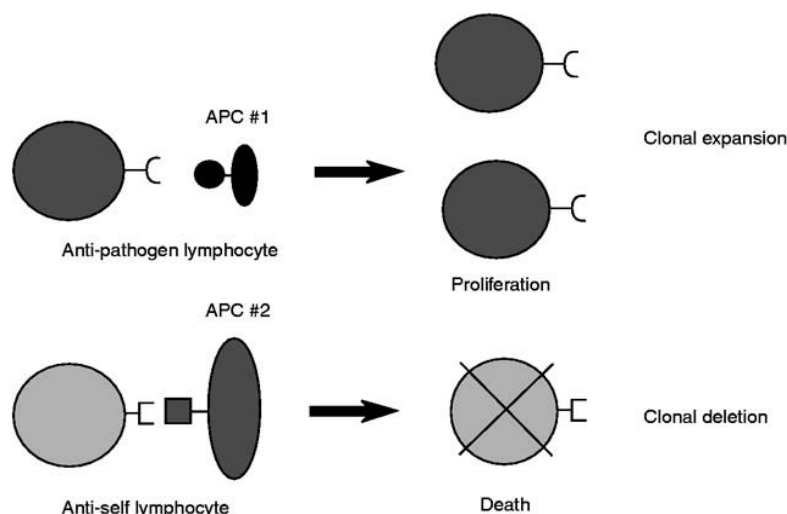


Figure 1 Depending on the activation state of the lymphocyte and the APC, antigen receptor engagement can result in either the selection and expansion of the clone or the death of the lymphocyte.

The molecular details of active deletion are incompletely understood. Evidence suggests that the time the TCR remains bound (k_{off}) to the MHC–peptide complex is the most important kinetic parameter determining the functional outcome. There is evidence from T cell hybridomas and from transgenic mice that *nur77*, an orphan member of the nuclear hormone receptor superfamily, may be involved in clonal deletion. However, animals with a null mutation in the gene for *nur77* demonstrate normal T cell development and function. Thus, *nur77*'s signaling function may be redundant. Some other member of the superfamily may be primarily involved and *nur77*'s physiological involvement is secondary to other pathways leading to T cell progenitor death.

Mature T cells

Antigen-stimulated death of mature T cells was not

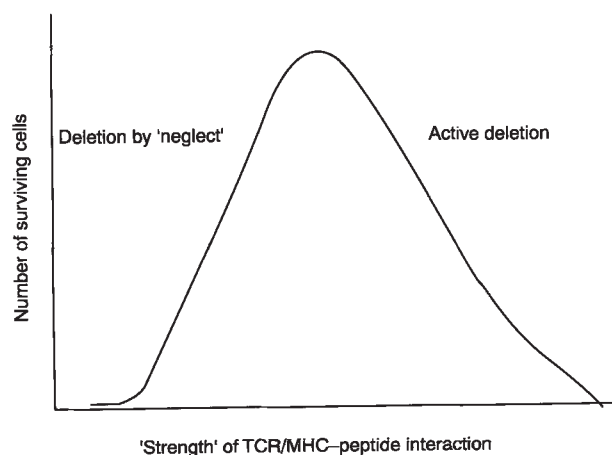


Figure 2 The 'strength' of the interaction, determined by MHC–peptide density and TCR affinity, determines whether the developing thymocyte is clonally deleted or allowed to mature.

recognized until the early 1990s. Shortly thereafter, it was recognized that the process was defective in two mouse mutant strains associated with lymphoproliferative disease and autoimmunity (*lpr* and *gld*). At the same time, Nagata and colleagues determined that the molecular bases for the mutations are in a receptor–ligand pair. The *lpr* mutation functionally eliminates the expression of Fas (CD95/Apo-1) and the *gld* mutation is an inactivating, point mutation in Fas ligand (FasL) (Figure 4). The Fas (Apo-1) protein had previously been identified as a member of the tumor necrosis factor receptor (TNFR) family of proteins and produced apoptosis in sensitive cells when stimulated with agonist antibodies. The recognition of the importance of Fas in the animal models has led to the discovery that defective Fas expression is associated with lymphoproliferative disease and autoimmunity in some human patients.

Fas ligand is a member of the TNF family of proteins that act as membrane-bound or soluble homotrimers to aggregate their receptors into functional signaling units. Yeast two-hybrid analyses have revealed that both Fas and TNFR signal through a series of protein–protein interaction ('death') domains that result in the activation of a unique series of proteases cascading into 'programmed' cell death or apoptosis.

T cells from animals carrying the *lpr* mutation are defective both in culture and *in vivo* in dying in response to chronic exposure to high-dose antigen. It appears that Fas does not regulate most acute responses, but rather is important in limiting the expansion of lymphocyte populations to high doses and/or chronic exposure to antigen. This can be important both in limiting autoimmunity and in limiting the long-term response to immunogenic tumors. There is recent data that the related TNF system also

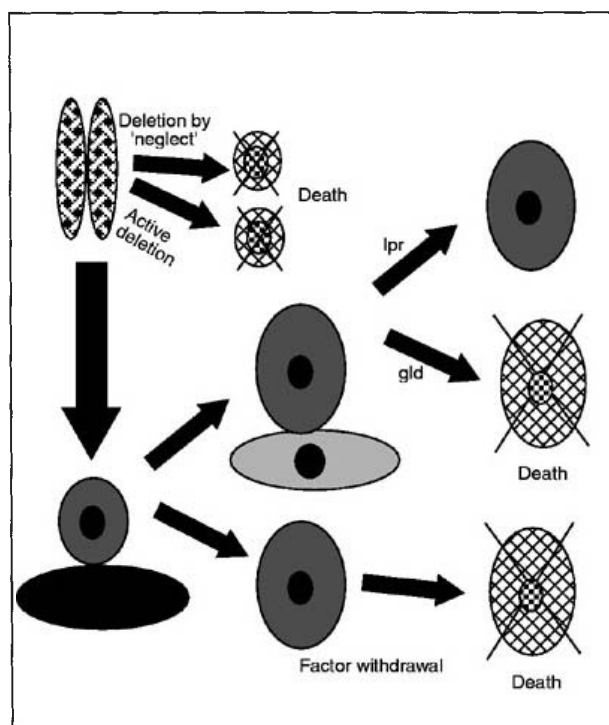


Figure 3 At various stages in T cell development, TCR stimulation or lack of stimulation results in death of those progenitors. Deletion can take place in the thymus by 'neglect' or by active deletion and in the periphery deletion can occur depending on the strength of the TCR/MHC-peptide interaction and on signals from costimulators.

plays an important role in the process of culture and *in vivo*. However, unlike animals with defective Fas or Fas ligand expression, the TNF role in lymphoproliferative disease or autoimmunity is not apparent when there are defects in TNF or its receptors alone, but rather appears to exacerbate the disease in animals with defective Fas or Fas ligand. It is likely that other members of this family will be identified that also contribute to lymphocyte hemostasis.

Fas is expressed at low levels on naive T cells and both its expression and sensitivity to signaling are increased after activation. In contrast, Fas ligand is transiently expressed upon TCR stimulation. The expressed ligand can act as a membrane-bound molecule to kill other cells (murder) or be released from the surface by metalloproteases as functional trimer. This soluble form of Fas ligand is important for the autocrine death (suicide) of the stimulated cell. There appear to be significant species differences in the stability of the soluble trimer. In addition, there appear to be soluble forms of Fas released from cells that neutralize the soluble Fas ligand. Thus under physiological conditions, soluble Fas ligand has a short functional range. Fas and Fas ligand do not

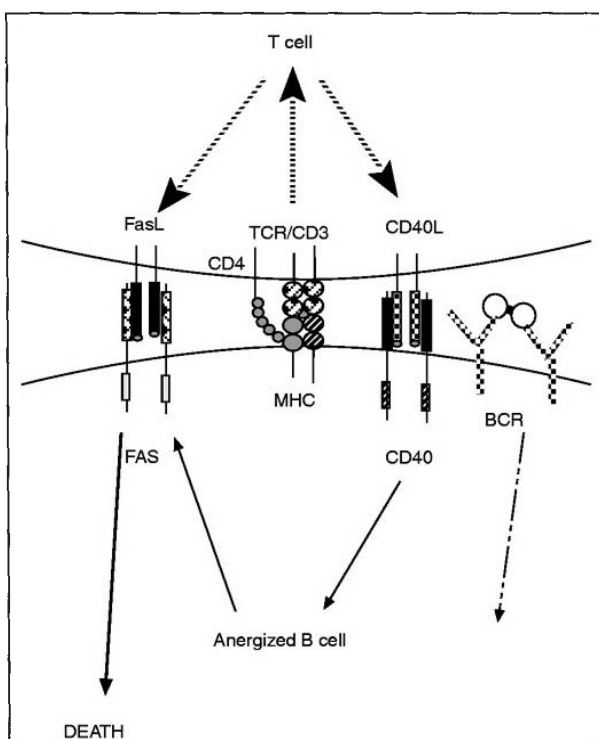


Figure 4 Chronic stimulation of the BCR creates an anergized B cell that increases Fas expression when stimulated by T cell CD40 ligand. It then dies in response to Fas stimulation by T cell Fas ligand. (Adapted from Rathmell *et al.*, 1996.)

appear to play a significant role in clonal deletion of thymic T cells.

Clonal deletion in antibody-producing B cells

Deletion of self-reactive clones of B cells is also an important part of antibody repertoire development. Like T cells, each clone of B cells produces a unique, antigen-specific membrane receptor. For B cells this is a membrane-bound form (BCR, B cell receptor) of the antibody produced and secreted by that cell. The BCR arises by alternative splicing of the antibody mRNA to include a membrane anchoring domain. Like the TCR, the BCR is associated with other proteins involved in transmitting signals into the cell upon stimulation. Unlike the TCR, the BCR binds protein, nucleic acids, carbohydrate and lipid sequences (antigenic determinants) independently of their association with MHC proteins. Thus B cells can be stimulated by both cell-bound and soluble antigens.

Progenitors for B cells develop from mesenchymal tissue in the yolk sac and fetal liver like T cell progenitors. However, there is a nearly continuous influx of new B cell progenitors for most of the life-

time of the animal, in contrast to T cells progenitor production that is largely restricted to embryonic and neonatal life. In avian species the B cell progenitors develop in the bursa of Fabricius (they were first discovered here and thus the term B cell), an appendix-like evagination of the avian gut. The bone marrow is the major source of B cell progenitors in other vertebrate adults. During B cell maturation there are several checkpoints where the developing clone can die either as a result of a negative signal from the BCR or from the lack of a positive signal from either the BCR or its environment (in this case a helper T cell).

Deletion of B cell clones prior to germinal center formation

Much of our understanding of the checkpoints in B cell development resulting in deletion has come from the analysis of BCR transgenic mice (mice carrying genes for a specific, rearranged BCR inserted into their genome and therefore expressed in virtually all developing B cells) by the laboratories of Goodnow, Weigart and Nemanzee. BCR transgenic animals were crossed to animals carrying their antigen as a transgene in various compartments and levels of expression. Antigenic determinants present as highly expressed membrane components cause the apoptotic death of the earliest progenitor with a functional BCR. This process is analogous to clonal deletion of autoreactive T cells in the thymus but the molecular basis is currently unknown.

Clones of developing B cells that fail to successfully rearrange their BCR or whose BCR is not stimulated within 1–3 days after export to the primary lymphoid organs (lymph node and spleen) also die. Death of the newly developed B cell also occurs if it is stimulated by antigen, but does not promptly receive a helper signal (helper T cell cytokines or surface molecules such as CD40 ligand). Cells that are chronically stimulated by soluble antigen (e.g. B cells that react to serum proteins) from their earliest stages of development become anergic. That is restimulation by antigen and helper cells fails to produce normal proliferation and maturation into antibody-secreting cells. These anergized but potentially autoreactive cells are exported to the primary lymphoid organs, but are eliminated in two ways: 1) they remain in the periarteriolar zones and do not compete with normal B cells for space in follicles and germinal centers where continued expansion and maturation occurs; or 2) they are killed by activated T cells.

The lysis of the anergic B cells by T cells is dependent on the Fas/Fas ligand pathway and in *lpr* animals lacking Fas, autoreactive B cells accumulate in

the T cell-rich, periarteriolar sheath. Adoptive transfer studies have indicated that elimination of these anergic B cells requires functional Fas on the B cell and functional Fas ligand on the T cell. In addition, it requires cooperation with other members of the TNF family. In this case CD40 on the B cell and CD40 ligand on the T cell. Stimulation of CD40 on the B cell induces the expression of Fas on that cell and stimulation of B cell Fas by Fas ligand on the T cell causes the death of that anergic B cell (Figure 4). Interestingly, the same sequence of events with normal B cells after acute antigen stimulation is required for B cell proliferation and maturation. Thus the activation state of the B cell (chronic versus acute) determines the functional outcome (death versus expansion and maturation) of the T/B cell interaction.

Deletion of B cells after primary activation

Thus far we have discussed elimination of B cells during the maturation of progenitors. However, mature B cells, unlike mature T cells, can undergo somatic mutation of their BCR after antigenic stimulation. This property allows affinity maturation of the response in germinal centers to produce higher affinity antibodies for a given challenge, but also creates a new risk of autoantibody development. One mechanism for solving the problem is analogous to the costimulator strategy in T cells. In this case the 'costimulator' CD19 dramatically lowers the number of BCRs needed to be occupied for maximal proliferation by allowing synergistic signaling between the complement receptor CD21 and the BCR. Cells stimulated by antigen in the context of complement (e.g. on the surface of a bacterium) or complement on the surface of a specialized antigen-presenting cell (follicular dendritic cell, FDS) do not undergo apoptosis. The FDC can also provide a similar function through CD23 on its surface. Stimulation of the germinal center B cell (centrocyte) by antigen in the absence of complement or CD23 stimulation results in deletion of those clones. This type of clonal deletion of mature B cells is independent of the Fas system. Rather, complement or FDC stimulation of the B cell regulates the expression of antiapoptotic molecules such as Bcl-2 and deletion of the cells not receiving signals from both the BCR and the costimulator are deleted by a pathway more analogous to the 'neglect' pathway of thymocytes.

Summary

Regulated survival of clonal progenitors is a primary mechanism of determining the lymphoid repertoire. Clones that are unwanted either because they do not express useful antigen receptors or express receptors

that would cause autoimmune disease are deleted through a death process. There appear to be two general strategies to eliminate these cells. In some instances (e.g. through Fas and Fas ligand) a clear receptor–ligand interaction actively initiates a death response. In other circumstances such as in the thymus or in germinal center B cells it appears that death is a ‘default’ step in the differentiation pathway that occurs unless there is active intervention by signals from other cell surface molecules that rescue them from death by regulating the expression of anti-apoptotic molecules like Bcl-2.

See also: Antigen-presenting cells; MHC peptide-binding specificity; Apoptosis; Anergy, T cell; Clonal selection; Thymic epithelium: potential role in regulatory T cell tolerance; MHC restriction.

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CLONAL SELECTION

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By the mid-1950s numerous theories had been proposed to account for the remarkable capacity of the immune system to respond specifically to the myriad of foreign antigenic determinants. One group of theories, championed by Alexander, Mudd, Haurowitz, Pauling and Karush, relied on the entry of antigen into antibody-forming cells to serve as a template for the synthesis of complementary antibodies, and, as such, were considered ‘instructional’. A novel ‘natural selection theory’, put forward by Jerne in 1955, suggested that all potential antibodies pre-existed as ‘natural antibodies’ and

appropriate cells, internalizing antigen complexed to a given antibody, would duplicate the internalized antibody. In 1957, Talmage suggested that cells might express their potential antibody as a cell surface receptor, and in 1958 Macfarlane Burnet fully elaborated the ‘clonal selection theory’. This theory, like Jerne’s theory, proposed that appropriate antibodies pre-existed in the absence of antigen ‘instruction’ but that the diverse array of antibodies was the product of multiple cell clones, each capable of expressing a single antibody product. The major tenets of the hypothesis were as follows (all

quotations excerpted from Burnet (1959), see Further reading):

1. 'in the animal there exist clones of mesenchymal cells each carrying immunologically reactive sites corresponding in appropriate complementary fashion to one (or possibly a small number of) potential antigenic determinants'.
2. These clones provide 'a population of cells which, when an appropriate stage of development has been reached, are capable of producing the population of globulin molecules which collectively provide the normal antibodies'.
3. In order that antigen-antibody complexes localize to 'cells, which are genetically determined to produce the corresponding type of antibody molecule - it would simplify matters a great deal if the antigen were in a position to react with natural antibody or a pattern equivalent thereto on the surface of the cell which produced it'.
4. 'When an antigen is introduced it will make contact with a cell of the corresponding clone, presumably a lymphocyte, and by so doing stimulate it to produce in one way or another more globulin molecules of the cell's characteristic type.'
5. That this 'stimulation initiates proliferation'.
6. 'that active sites on cell surface or globulin molecule can be modified to a wider reactivity by somatic mutation, provides the chief agent to allow change in antibody character as immunization proceeds.'
7. 'Self-not-self recognition means simply that all those clones which would recognize (that is, produce antibody against) a self-component have been eliminated in embryonic life. All the rest are retained.'
8. The secondary response is accounted for thus: 'on the simplest form of the hypothesis - the primary stimulus finds only a few examples of the appropriate clones; by the time of the secondary stimulus, many more individuals of the selected clones are accessible'.

Over the past 30 years each of these postulates has to a considerable extent been validated by experimental observation. Although other elements of Burnet's thesis are not as prescient as the above, it should be noted that prior to 1960 neither the molecular basis for antibody formation nor the T cell-B cell dichotomy and cell-cell collaboration were understood.

Unipotentiality of lymphoid cells

The most fundamental tenet of the clonal selection hypothesis, and that which distinguished it from all others, was 'the *existence of multiple clones of globulin-producing cells*, each responsible for one genetically determined type of antibody globulin'. Indeed, in discussing the implication of the theory, Burnet stated 'the clonal selection hypothesis would be completely validated if it could be shown that single cells from a non-immune animal gave rise to clones, each cell of which under proper physiological conditions contained, or could liberate, antibody-type globulin of a single pattern or at most of a uniform small range of patterns'. Early efforts to test this postulate were hardly supportive. Attardi, Horabita, Lennox and Cohn concluded, from single-cell studies of phage-specific antibodies, that single cells were multipotential. Studies from a variety of laboratories, including those of Cunningham, Liacoupoulos, Hiramoto and Sercarz, appeared to demonstrate multiple antibodies per single cell. Additionally, Burnet's own experiments, using a tissue reactivity test devised by Simonsen, were incompatible with a highly diverse repertoire of unipotential cells. However, in retrospect, each of these anomalous findings has been attributable to experimental artifact, the capacity of certain antibodies to bind numerous antigens (multispecificity), or, as in the case of the Simonsen assay, an inordinately high frequency of T cell responses to any given major histocompatibility complex (MHC) alloantigen.

In addition, the numerous examples of the homogeneity of immunoglobulins produced by multiple myeloma cells and the existence of restricted antibody responses to certain antigens, such as Krause's demonstration of restricted antibodies in rabbits responding to A-carbohydrate, implied that the product of single antibody-forming cell clones was homogeneous. A similar conclusion was drawn from findings of numerous laboratories, including those of Nossal, Mäkelä and Biozzi, that single cells bound only one antigen. Using the splenic focus assay, where single antigen-specific precursor cells were isolated in cultures of splenic fragments, Klinman demonstrated, in 1969, the homogeneity of the antibody product of the clonal progeny of a single stimulated precursor cell (Figure 1). These studies demonstrated, by the homogeneity of antigen binding as well as heavy (H) and light (L) chain recombination analyses, that the product of antibody-forming cell clones was homogeneous and displayed binding characteristics that, in composite, could account for the affinity and heterogeneity characteristics of

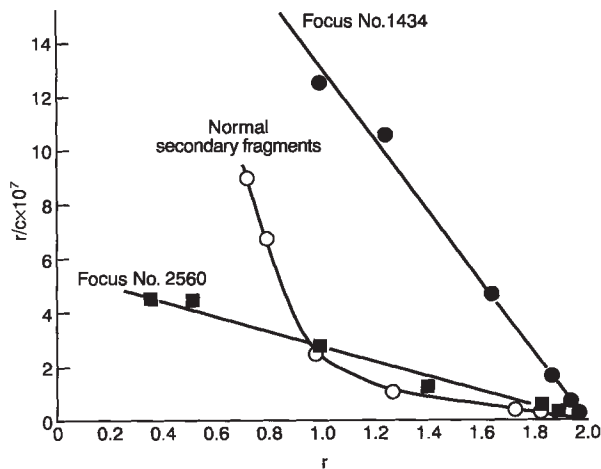


Figure 1 Hapten binding curves constructed from equilibrium dialysis at 7°C with a) antibody from two antibody forming cell clones (foci) generated in splenic fragments, focus No. 1434 (●) and focus No. 2560 (■); and b) pooled antibody from the clonal progeny of numerous secondary B cells (○). r represents the moles of α , N-(³H)-acetyl- δ -DNP lysine bound per mole of antibody at the equilibrium free hapten concentration, c . Antibody concentrations were determined at each point by radioimmunoassay and points represent duplicate samples which agreed to within 5%.

serum antibodies. Although these particular studies used B cells from previously immunized mice, subsequent studies extended these findings to B cells from nonimmune mice, thus fulfilling Burnet's requirements. The only exception to the homogeneity of the antibody produced by single clones was the production of multiple isotypes by most clones; however, Gearhart and Klinman demonstrated that antibodies of different isotypes generated from the same clone shared variable regions. In recent years, the molecular mechanisms that account for the sorting of unique specificities to individual lymphocytes have become well documented and are discussed elsewhere in this volume.

Immunoglobulin receptor on B cells

Essential to 'clonal selection' is the availability of a mechanism to focus the antigen to the appropriate B cells and, in so doing, enable the stimulation of those B cells. In 1967 Mitchison and his coworkers indirectly demonstrated that the fine specificity of antibody produced by B cells could be influenced by selectively stimulating these cells with a cross-reactive hapten. This indirect demonstration of a correlation between the B cell's surface receptor and secreted antibody product was later extended by Klinman, who demonstrated that clonal precursors whose potential antibody product was of high affinity could be selectively stimulated with low antigen

concentrations. That B cells bore surface immunoglobulin was demonstrated by numerous investigators including Naor, Sulitzeanu, Wigzell, Vitetta, Uhr and Marchalonis, and the mobility of these receptors on the cell surface was shown by Raff and Taylor. However, the identity between the B cell's potential antibody product and its surface immunoglobulin receptor awaited the demonstration by Köhler and Cosenza that they shared idiotype.

Tolerance and memory

In part, the clonal selection hypothesis was necessitated by the need to account for two fundamental hallmarks of the immune response: the capacity to respond to nonself or foreign antigens while not responding to self antigens; and the long-term retention of the capacity to mount accelerated and vigorous responses to previously encountered antigens. As anticipated by the theory, numerous studies have demonstrated the inactivation of developing B and T cells by antigens present in their environment, thus validating negative clonal selection as the basis for self tolerance. Although most B cells in an unimmunized animal have the capacity only to generate antibody-forming cell clones and not memory B cells, Linton, Decker and Klinman have identified a subpopulation of precursor cells that do give rise to memory B cells and that fulfill, in detail, Burnet's predictions: 1) 'that a first contact with antigen sets some sort of tooling-up process in train, while secondary contact is needed to evoke actual antibody liberation into the circulation'; and 2) that 'the combination of frequent minor mutation and a highly effective selective process would rapidly improve the accuracy of the complementary relationship to new antigenic determinations'. The process of somatic mutation and antigen selection of these mutations has been described by numerous investigators including Tonegawa, Weigert, Geffer, Rajewsky, Berek and Milstein.

Conclusion

Since its first elaboration, the clonal selection hypothesis has served as the take-off point for experiments, not only to test the theory itself, but also to extend our knowledge of the mechanism of immune responsiveness. While many of the speculations with which Burnet decorated this theory have not stood the test of time, the fundamental aspects of the theory remain and constitute the foundation of our current understanding of the immune response.

See also: B lymphocyte differentiation; B lymphocyte

repertoire; Clonal deletion; Immunoglobulin, cell surface; Memory, immunological; Natural antibodies; Proliferation, lymphocyte; Somatic mutation; Specificity; T lymphocyte clonal expansion; Tolerance, peripheral.

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CLOSTRIDIUM, INFECTION AND IMMUNITY

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Characteristics of the organisms and their antigens

The clostridia are spore-forming rod-shaped gram-positive bacteria that usually grow under anaerobic conditions. An important characteristic of these microorganisms is their ability to survive in high numbers for long periods of time in feces and soil, an ability related to the formation of spores which can be very resistant to temperature and pH extremes. Clostridia are potent pathogens in animals and humans because of their ability to release various exotoxins. Furthermore, many clostridia species release more than one toxin and each toxin is immunologically distinct. For example, eight types of *Clostridium botulinum* and five types of *Clostridium perfringens* have been identified and each type elaborates different toxins. Many similarities exist among clostridial toxins. Amino acid homology and immunologic cross-reactivity exist between *C. difficile* toxins and *C. sordellii* lethal toxins and between tetanus toxins and botulinum B toxin, and these toxins have similar mechanisms of action. In addition, many clostridia produce a variety of enzymes, including collagenases, proteinases, deoxyribonucleases, and neuraminidases, which compromise the host and increase the pathogenicity of these microorganisms.

Although most of the clostridia toxins are large protein exotoxins with distinct biochemical proper-

ties and immunological characteristics, classification of these toxins based on their pharmacologic properties and cellular targets is far from complete. For the purpose of this review clostridia toxins will be categorized according to the diseases they produce into four groups: 1) neurotoxins, represented primarily by *C. tetani* and *C. botulinum* which are associated with tetanus and botulism in humans; 2) enterotoxins, represented by *C. perfringens* enterotoxin produced by some strains of *C. perfringens* type A which mediate acute food poisoning, *C. perfringens* ϵ and ι toxins causing enterotoxemia in sheep, calves, lambs and guinea pigs, and *C. sordellii* hemorrhagic toxin which is associated with diarrhea in cattle and sheep; 3) histotoxins or cytolytic toxins, represented by some of the *C. perfringens* toxins that are associated with gas gangrene and anaerobic cellulitis, and *C. sordellii* lethal toxin (LT), one of the causes of gas gangrene; and 4) cytoskeleton-altering toxins, represented by *C. difficile* toxins A and B, that mediate antibiotic-associated colitis and diarrhea following antibiotic intake in animals and humans, and *C. botulinum* C2 toxin. All cytoskeleton-altering toxins disrupt filamentous (F) actin in target cells. It should be emphasized, however, that many of these toxins have several other biological activities other than the ones listed above. For example, toxin A of *C. difficile* is also a potent enterotoxin that induces fluid secretion when injected into rabbit and rat intestinal loops.

An interesting topic of pathogenicity associated with clostridial toxins is the molecular mechanism by which these toxins modify properties of cellular proteins and, by altering their function, cause human disease (Table 1). Toxins are enzymes which can biochemically modify specific molecules on the plasma membrane or cytosol of target cells. Some of the *C. perfringens* toxins are very good examples of toxins which act on plasma membranes of cells. *C. perfringens* α toxin, a phospholipase C or lecithinase, cleaves lecithin in cell membranes into diglyceride and phosphorylcholine as well as hydrolyzes cephalin and sphingomyelin. *C. perfringens* κ toxin is a collagenase, whereas the μ toxin is a hyaluronidase.

A major breakthrough in our understanding of the pathogenesis of tetanus and botulism has been the recent identification of the molecular mechanism by which *C. tetanus* and *C. botulinum* B toxins cause paralysis. Botulinum neurotoxins act on peripheral motor nerves to block acetylcholine release at neuromuscular junctions, thereby causing muscle paralysis (flaccid paralysis). On the other hand tetanus neurotoxin acts on the central nervous system by blocking neurotransmitter release from inhibitory neurons causing muscle spasms (spastic paralysis). Although these two diseases have different clinical symptoms it is now known that botulinum B and tetanus neurotoxins have identical mechanism in intoxicating

nerve cells. Both toxins are zinc endopeptidases that require a zinc atom for their activity and both have a similar protein structure with a receptor binding B domain and a catalytic A domain. The mechanism of action of these toxins initially involves binding to cell surface receptors and internalization into the cytosol. Once into the cell the toxins are then cleaved by proteases into active fragments which possess catalytic action. The cellular target of botulinum B and tetanus toxin is synaptobrevin, a membrane protein which is important in neurotransmitter release and associated with small synaptic vesicles. The active forms of botulinum and tetanus toxin inactivate synaptobrevin by cleaving it at a specific peptide site.

Another important recent discovery in the field of clostridial toxins is the cellular mode of action of large clostridial toxins including *C. difficile* toxins A and B, and *C. sordellii* and *C. novii* toxins. The common characteristic of these toxins is their large molecular weight (>250 kDa), amino acid similarity, absence of subunits, and their similar cytopathogenic (rounding) effect on cultured cells. These toxins exert their cytopathogenic effect by modifying specific small GTP-binding proteins of the Ras superfamily that regulate cellular actin. Studies with *C. difficile* toxins showed that these toxins bind to specific cell receptors and are then internalized into the cytosol

Table 1 Mechanisms of some clostridial toxins

Organism	Disease	Major lethal toxins	Mechanism of toxicity
<i>C. perfringens</i> type A	Gas gangrene	α toxin	Cytotoxic, calcium-dependent phospholipase C
	Food poisoning	Enterotoxin	Changes permeability of cells.
<i>C. perfringens</i> type C	Gas gangrene	α toxin	Calcium-dependent phospholipase
	Enteritis necroticans	β toxin	Cytotoxin, pore-forming?
<i>C. septicum</i>	Gas gangrene	α toxin	Hemolysin, cytolytic, pore formation
<i>C. novyi</i> type A	Gas gangrene	α toxin	Cytotoxin, actin cytoskeleton breakdown, glucosylation of Rho, Rac, Cdc-42.
<i>C. sordellii</i>	Gas gangrene	Lethal toxin (LT)	Cytotoxin, disruption of actin microfilaments, glucosylation of Ras, Rap and Rac
	Hemorrhagic diarrhea	Hemorrhagic toxin (HT)	Cytotoxin, enterotoxin, glucosyltransferase
<i>C. tetani</i>	Tetanus	Tetanospasmin	Neurotoxin, cleavage of synaptobrevin
<i>C. botulinum</i> types A, B, C1, D, E, F, G	Botulism	Botulinum neurotoxin	Neurotoxin, cleavage of synaptobrevin
<i>C. botulinum</i> type C2	Unknown	C2 or binary toxin	Cytotoxin, inhibition of actin polymerization, ADP-ribosylation of actin
<i>C. difficile</i>	Antibiotic-associated diarrhea and pseudomembranous colitis	Toxin A Toxin B	Cytotoxins, disruption of actin microfilaments, glucosylation of Rho, Rac, Cdc-42

via coated pits. Once into the cytosol these toxins monoglucosylate the small GTP-binding proteins Rho, Rac and Cdc-42 at position Thr37. As a result, these small GTP-binding proteins are unable to regulate actin polymerization thus leading to actin filament breakdown and cell rounding. *C. sordellii* lethal toxin, which also rounds up cells and causes disruption of actin fibers, also glucosylates the small GTP-binding proteins Ras, Rap and Rac, that regulate actin filament formation. These recent discoveries on the molecular mechanism of clostridial toxins not only increase our understanding of their cellular actions, but may also help to design rational prevention or treatment of diseases associated with these toxins.

Evasive strategies by the organism

Clostridia have devised many ways to enter the host and cause disease. In the case of gas gangrene and wound infections *C. perfringens* enters the host through traumatic or surgical wound often taking advantage of poor blood supply in the wound area. Once clostridia grow they release their toxins and cause disease. In *C. difficile*-associated colitis the bacteria multiply when the normal bowel flora is disrupted by antibiotics. *C. difficile* then releases its toxins which target intestinal epithelial cells and cause diarrhea and colonic inflammation. In the case of botulism, however, the organism does not grow in the host but the disease is caused by ingestion of *C. botulinum* toxins present in contaminated food.

Immune responses of the host and vaccines

For most clostridial-mediated diseases, there is no immunity following the experience of the first episode. The reasons for absence of prophylaxis following exposure of patients to clostridia and their toxins is not clear but could involve the small

amount of toxins required to produce disease, which may be inadequate to produce an immunogenic response. Despite the absence of systemic immunity following disease, toxoid immunization against tetanus provides immunity for up to 5 years. Passive immunization with tetanus immunoglobulin is also of great value in suspected cases. An antitoxin antibody has also been available for laboratory workers, and intravenous treatment with gamma globulin preparations containing a high-titer antibody to *C. difficile* toxin was effective in treating patients with relapsing *C. difficile* diarrhea.

See also: Bacteria, immunity to; Toxins; Vaccines.

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CLOTTING SYSTEM

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The clotting system consists of a mixture of components including cells, fragments thereof, zymogens, their activation products and naturally occurring inhibitors, adhesive and structural proteins, phospholipids, lipids, cyclic and noncyclic nucleotides,

hormones and inorganic cations, all of which are normally maintained in a state conducive to blood flow. Upon damage to the vascular wall with disruption of the monocellular layer of endothelial cells, exposing the subendothelial layer, and also permit-

ting bleeding, a series of events is initiated designed to produce clot formation. These homeostatic reactions lead to the generation of the primary platelet plug, followed by a clot principally containing cross-linked fibrin (secondary hemostasis). Subsequently, the blood vessel is repaired and the clot is dissolved by a mechanism which includes fibrinolysis.

Local vasoconstriction

A number of physiologically derived compounds appear to induce vasoconstriction of a transient nature at the site of vascular injury. These include participation of endothelin, a very potent vasoconstrictor which is synthesized by endothelial cells, thromboxane A_2 (TXA $_2$), serotonin and the α -adrenergic system. Thrombin, interleukin 1 (IL-1), transforming growth factor β (TGF β), angiotensin II and epinephrine promote the expression of endothelin mRNA, whereas shear stress, atrial natriuretic peptides (ANP) and brain natriuretic peptides negatively modulate its expression. Endothelial-derived relaxing factor (EDRF), an inhibitor of platelet function, inhibits the release of endothelin. The lung clears endothelin from the circulation. Angiotensin II, also a vasoconstrictor, is synthesized locally, as renin and angiotensinogen are generated within the endothelial cell (EC), and angiotensin-converting enzyme (ACE) is found on the surface of the EC membrane. As a counterbalance to constriction, ECs secrete such vasodilators as adenosine, prostacyclin and EDRF. Nitric oxide (NO) exhibits similar effects as EDRF. However, S-nitrosocysteine appears to behave more akin to EDRF. The final identity of EDRF, is, as yet, unresolved. The ECs also can degrade serotonin.

Platelet components and function

Disk-shaped platelets circulate in the blood, being quite unreactive with the intact vascular wall. Upon injury to the vascular wall, however, components from the platelets, ECs and damaged subendothelial cells (SECs) (SEC matrix), other vascular tissue constituents and circulating plasma components contribute to the formation of a primary platelet plug. The sequence of reactions in response to injury is as follows: initial activation and adhesion of platelets to the damaged SECs, contributing to activation of circulating platelets, and aggregation of platelets to one another at the site of injury, followed by stabilization of the platelet plug by extensive cross-linking of polymeric fibrin molecules. Platelet membrane proteins include numerous receptors for adhesion proteins such as collagen, fibrinogen, fibronectin, von Willebrand factor (vWF), thrombin receptor, and

others. Dense granule constituents in platelets include ATP, ADP, Ca $^{2+}$ and serotonin, while α granule components of platelets include platelet factor 4 (PF4), β -thromboglobulins, fibrinogen, vWF, fibronectin, thrombospondin, coagulation factors V, XI and protein S (PS), α_2 -antiplasmin (α_2 -AP) and plasminogen activator inhibitor 1 (PAI-1). Cytoplasmic proteins which are secreted by platelets are represented by protease nexin I, protease nexin II, tissue factor pathway inhibitor (TFPI), factor XIII, α_1 -antitrypsin (α_1 -AT), C1-inhibitor, α_2 -macroglobulin (α_2 -MG), high molecular kininogen (HMWK) and II-I β). Endothelial cells release prostacyclin (PGI $_2$), an inhibitor of platelet aggregation, as well as platelet activating factor (PAF) and vWF, which contribute to platelet adhesion, in addition to fibrinolytic components, tissue plasminogen activator (t-PA) and PAI-1. The mechanisms for release of these compounds is not yet understood. Thrombomodulin (TM), tissue factor (TF), ACE and binding sites for factors Va, Xa, and XIa are observed on EC membrane surfaces. However, TF is expressed only upon injury to tissue. The subendothelial tissue damage exposes such adhesive proteins as collagen (types IV, V and VI), vWF, fibrinogen and vitronectin, while the damaged media exposes collagen (types I and III), and damaged adventitia exposes collagen and TF.

Flow rate of blood affects adhesion of platelets to the damaged SECs. In areas of low blood flow, collagen and fibronectin play a greater role as adhesive proteins linking the platelets to the SEC. With higher flow areas, however, vWF appears to play a more significant role as an adhesive protein. Upon vascular injury, multimers of circulating vWF bind to platelet receptor glycoprotein GPIb/IX. Subsequently, GPIIb/IIIa receptors are activated. The complex then binds to exposed collagen and fibronectin on the SEC matrix. Additionally, newly exposed SEC matrix-bound vWF binds to circulating platelets. GPIa/IIa receptors on the platelet surface also permit direct binding of platelets to the exposed collagen from the SEC matrix. Additionally, collagen binds fibrinogen, fibronectin and vWF, thereby stimulating further binding with GPIIb/IIIa, GPIc/II and GPIb/IX receptors on the platelet surface.

The activation of platelets is stimulated by strong and weak agonists. Thrombin, which may be locally generated at the site of injury, and collagen have been identified as strong agonists while ADP, epinephrine, TXA $_2$, serotonin, PAF and vasopressin (Vp) have been relegated as weaker agonists. The consequences of platelet activation are: alteration of platelet shape from discoid to globular, with pseudopods; increased cytosolic Ca $^{2+}$; alteration of the GPIIb/IIIa receptor to a high-affinity receptor in

order to effectively bind vWF and/or fibrinogen for adhesion/aggregation; biosynthesis of TXA₂, a vasoconstrictor, and other eicosanoids from arachidonic acid (AA); phosphorylation of platelet proteins; induction of platelet coagulation activity; release of dense granule constituents (ADP, ATP, Ca²⁺, serotonin, pyrophosphate); release of α granule constituents (platelet-specific proteins such as PF4, which interacts with heparin and lowers its anticoagulant activity; β -thromboglobulin, and adhesive glycoproteins such as fibrinogen, vWF, and fibronectin); release of lysosomal constituents, expression to the cell surface of dense and α granule membrane proteins, factors V and XI and PS; inhibitors of fibrinolysis (α_2 -AP, PAI-1); platelet-derived growth factor (PDGF).

Platelet activation involves the mobilization of two enzyme systems in response to agonists. Phospholipase A₂ (PLA₂) and phospholipase C (PLC) act upon platelet membrane phospholipid (PL) substrates. PLA₂ cleaves AA from phospholipids. (Linoleic acid may also serve as a metabolic precursor to AA in mammals.) Upon subsequent successive action on AA by a cyclo-oxygenase and a peroxidase, prostaglandin H₂ (PGH₂) is formed. PGH₂ serves as the precursor to TXA₂ (mediated by TXA₂ synthase) and PGI₂ (mediated by PGI₂ synthase). The EC is a major source of TXA₂. TXA₂ then alters platelet shape and stimulates platelet aggregation. It also promotes the platelet secretion and expression of the fibrinogen receptor. (PGI₂ inhibits platelet aggregation.) The alternative activation pathway involves hydrolysis of membrane PL by PLC to produce diacylglycerol (DAG), inositol triphosphate (IP₃) and some AA. The AA is metabolized by the cyclo-oxygenase and peroxidase in the above described manner. In addition, IP₃ promotes an increase in cytosolic Ca²⁺, which stimulates an alteration in platelet shape, platelet secretion and fibrinogen expression. DAG affects identical changes in platelet shape and function.

A number of factors act counter to the activation of platelets. These include PGI₂, EDRF, NO and ecto-ADPases, derived from ECs which degrade the platelet activator ADP into adenosine, a platelet function inhibitor. Furthermore, substances which negatively modulate the activity of thrombin, the strong agonist for platelet activation, contribute to antiplatelet activation controls. These include antithrombin III (AT III), α_2 -MG, heparin cofactor II (HC II), and activated protein C inhibitor I (APCI). Additionally, 13-hydroxyoctadecadienoic acid (13-HODE), produced in the ECs, inhibits adhesion and aggregation of platelets and TXA₂ synthesis, and promotes the synthesis of PGI₂.

Subsequent to platelet adhesion and activation, additional circulating platelets aggregate to the platelet monolayer. Aggregation involves the participation of fibrinogen which binds to the GPIIb/IIIa receptor site of the platelet membranes and forms a bridge to another platelet, thereby expanding the platelet plug. There are two binding sites for the GPIIb/IIIa receptor on each fibrinogen molecule.

Secretion of the platelet granule components as well as cytosolic proteins (protease nexin I and II, TFPI, factor XIII, α_1 -AT, C1-inhibitor, HMWK, α_2 -MG, vascular permeability factor and IL-1 β) adds components for positively or negatively affecting coagulation in the blood vessel at the site of vascular injury.

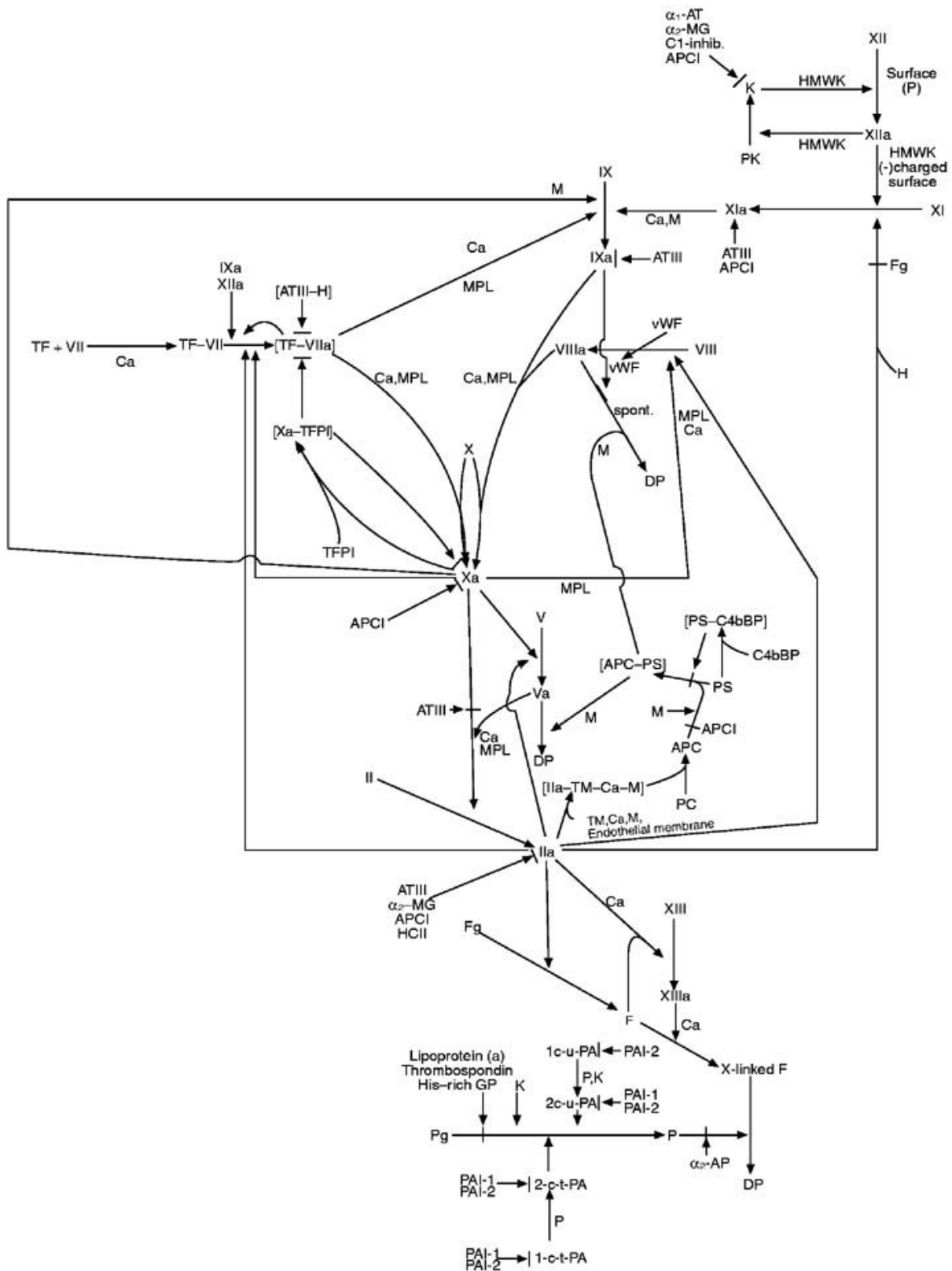
As part of the activation process, a rearrangement of phospholipids occurs whereby phosphatidylserine and phosphatidylinositol position themselves from the inner surface of the platelet membrane to the outer surface as a means of presenting a negative charge to coagulation factors for binding purposes.

Contraction of the clot and secretion of granule proteins are believed to incorporate the use of the contractile protein, actinomyosin.

Coagulation and its modulation

A veritable cascade of limited proteolytic reactions are encompassed in the coagulation process. A series of inactive proteins, namely, zymogens, are sequentially activated by limited proteolysis, culminating

Figure 1 Pathways of coagulation and fibrinolysis. II, Prothrombin, factor II; IIa, thrombin, factor IIa; V, factor V; Va, factor Va; VII, factor VII; VIIa, factor VIIa; VIII, factor VIII; VIIIa, factor VIIIa; IX, factor IX; IXa, factor IXa; X, factor X; Xa, factor Xa; XI, factor XI; XIa, factor XIa; XII, factor XII; XIIa, factor XIIa; XIII, factor XIII; XIIIa, factor XIIIa; α_1 -AT, α_1 -antitrypsin; α_2 -MG, α_2 -macroglobulin; C1-Inhib., C1-inhibitor; APCI, activated protein C inhibitor; HMWK, high molecular weight kininogen; P, plasmin; K, kallikrein; PK, prekallikrein; M, membrane; ATIII, antithrombin III; Fg, fibrinogen; H, heparin; MPL, membrane phospholipid; TF, tissue factor, thromboplastin; TF-VII, tissue factor-factor VII complex; TF-VIIa, tissue factor-factor VIIa complex; TFPI, tissue factor pathway inhibitor; Xa-TFPI, factor Xa-tissue factor pathway inhibitor complex; ATIII-H, antithrombin III-heparin complex; vWF, vonWillebrand factor; spont., spontaneously; DP, degradation products; PC, protein C; APC, activated protein C; PS, protein S; APC-PS, activated protein C-protein S complex; C4bBP, C4b binding protein; PS-C4bBP, protein S-C4bBP complex; HCII, heparin cofactor II; F, fibrin; TM, thrombomodulin; IIa-TM-Ca-M, thrombin-thrombomodulin-calcium-membrane complex; X-linked F, cross-linked fibrin; Pg, plasminogen; α_2 -AP, α_2 -antiplasmin; His-rich GP, histidine-rich glycoprotein; 1c-u-PA, one-chain urinary plasminogen activator; 2c-u-PA, two-chain urinary plasminogen activator; 1c-t-PA, one-chain tissue plasminogen activator; 2c-t-PA, two-chain tissue plasminogen activator; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2.



ultimately in the formation of the stable, insoluble fibrin clot. Many of the activated zymogens are serine proteases, descended from the same evolutionary tree as the digestive enzymes, trypsin, chymotrypsin and elastase. They contain the catalytic triad consisting of histidine, aspartic acid and serine. These include kallikrein, thrombin, factors VIIa, IXa, Xa, XIa and XIIa from the coagulation cascade and plasmin, t-PA, and urinary-plasminogen activator (u-PA) from the fibrinolytic enzyme system. This hemostatic process was historically conceived to meld an *intrinsic* pathway with an *extrinsic* pathway at a juncture resulting in the activation of factor X to factor Xa, whereupon a common pathway concluded with the formation of the fibrin clot. The clotting cascade including the *extrinsic* and the 'historic' *intrinsic* pathways, together with the fibrinolytic system for dissolution of the clot, is depicted in Figure 1. Activation of the historic *intrinsic* pathway could be accomplished upon contact of factor XII with a negatively charged surface to yield the activated factor XIIa. It was observed that factor XIIa could activate two zymogens, prekallikrein (PK) and factor XI, each in the presence of HMWK. Activation of PK yields the protease kallikrein (K). K, in turn, activates factor XII in the presence of HMWK to produce additional factor XIIa, a positive feedback step as the *intrinsic* cascade is initiated. (K activity is inhibited by α_1 -AT, α_2 -MG, C1-inhibitor and APCI.) The activation of factor XI by factor XIIa yields factor XIa. Factor XI is also activated by factors XIa and IIa (thrombin), the latter activation requiring heparin as a cofactor. The activation of factor XIa by thrombin is inhibited by fibrinogen. (Antithrombin III (AT III) and APCI inhibit the subsequent proteolytic activity of factor XIa).

All subsequent steps involving the *intrinsic* system factors occur on membrane surfaces such as the platelet membrane. Endothelial cell membranes also bind coagulation factors. PL is required, serving as the negatively charged surface. The localization of PL in the membrane permits the formation of ternary and quaternary complexes as necessary, for the accomplishment of the clotting steps. Factor XIa activates factor IX in the presence of Ca^{2+} . (AT III inhibits the proteolytic activity of factor IXa.) Factor IXa, in the presence of factor VIIIa and Ca^{2+} , activates factor X to factor Xa. The activation of factor VIIIa will be dealt with below. Factor Xa then activates prothrombin (factor II) to yield thrombin (factor IIa). This occurs with the mediation of factor Va in the presence of Ca^{2+} . Thrombin subsequently acts upon circulating or locally entrapped fibrinogen to generate the initial fibrin gel which is then cross-linked with the assistance of a transglutaminase, fac-

tor XIIIa, in the presence of Ca^{2+} . Fibrinogen is a protein containing six polypeptide chains, two $\text{A}\alpha$, two $\text{B}\beta$ and two γ chains, appropriately linked via disulfide bridges. Initial activation of fibrinogen by thrombin causes the release of fibrinopeptide A from the α chain in a relatively rapid reaction, permitting polymerization of the remaining protein. A rate-limiting cleavage of fibrinopeptide B from the $\text{B}\beta$ chain subsequently occurs. The resultant fibrin molecules may then be cross-linked longitudinally and transversely by factor XIIIa. Factor XIIIa is activated by thrombin from its inactive precursor, factor XIII, in the presence of Ca^{2+} . Factor XIIIa also functions in the presence of Ca^{2+} . The transglutaminase excises the amide groups of select glutamines in the carboxyl terminal region of γ chains and links the resultant glutamic acids with select ϵ -amino groups of lysines in the carboxyl terminal region of the γ chains. It also acts similarly with the α chains. Longitudinal cross-links are established with γ - γ as well as α - α chains, whereas transverse links are formed only with α - α chains. The resultant polymeric product is increasingly insoluble and more resistant to proteolysis. Factor XIIIa cross-links other localized proteins in addition to fibrin, such as fibronectin, thrombospondin, collagen, actin, vWF and α_2 -AP, in forming a tight mesh. Hence, components related to coagulation and fibrinolytic mechanisms may be effectively entrapped at the coagulation site. As will be discussed below in the fibrinolytic section, plasminogen is incorporated in the fibrin clot. Furthermore, t-PA binds to fibrin.

Factor Xa and thrombin have multiple roles in the clotting system. Thrombin activates the inactive factor XIII to the active factor XIIIa in the presence of Ca^{2+} . The initial fibrin gel appears to promote this step. In addition to activating factors XI and XIII, thrombin activates factor V to factor Va, factor VIII to factor VIIIa in the presence of Ca^{2+} , and the tissue factor-VII (TF-VII) complex to its active form, TF-VIIa.

TF is considered as part of the *extrinsic* blood clotting pathway (to be described shortly, after further discussions of factors Va, VIIIa, IIa and Xa). Factor Xa also catalyzes the activation of factor V, as does thrombin. The activation of factor VIII by thrombin is more complex. Factor VIII circulates in blood as a complex with vWF. In the process of activation of factor VIII by thrombin, vWF dissociates from the complex, thereby availing itself of the opportunity to complex with more circulating factor VIII. vWF stabilizes factor VIII, thereby prolonging its circulating half-life. Factor VIIIa can apparently be degraded to degradation products spontaneously. This degradation occurs at a reduced rate in the presence

of high concentrations of factor IXa. Factor Xa also activates factor VIII to factor VIIIa. The activation of factors Va and VIIIa by thrombin and factor Xa serve as additional examples of positive feedback.

Factors Va and VIIIa, which participate in the coagulation system as cofactors of factor Xa and IXa, respectively, are also subject to enzymic degradation. Both factors Va and VIIIa are degraded by a complex of activated protein C (APC) with protein S (PS), APC-PS. The formation of the APC-PS complex occurs on the membrane surface, as do all the reactions in the clotting cascade from the functioning of factor XIa to the activation of prothrombin. The series of reactions begins with the initial formation of a complex comprising thrombin, thrombomodulin (an EC membrane protein) and Ca^{2+} on the membrane. This complex catalyzes the transformation of protein C (PC) to APC. The APC interacts with PS to yield the APC-PS complex in which the APC contains the catalytic degradative potential to fragment factors Va and VIIIa. Under such circumstances, the catalytic functions of factors IXa and Xa are depressed, thereby reducing the generation of thrombin from prothrombin. Hence, thrombin can act not only as a positive modulator (positive feedback) in activating factors Va and VIIIa, but also as a negative modulator by initiating the formation of APC-PS which degrades factors Va and VIIIa, limiting thrombin production. An inhibitor of APC (APCI) has been identified which inhibits the formation of the APC-PS complex from its APC and PS components. It further inhibits the functioning of factor XIa. It therefore functions overall to promote clotting at the Va and VIIIa steps by 'lowering' their 'inactivation' rate. Its effect on factor XIa may be of questionable impact, as discussed below. Another protein, a plasma component, C4b-binding protein (C4b-BP), also negatively modulates the degradation of factors Va and VIIIa by forming a complex with PS, the PS-C4b-BP complex which competitively blocks the formation of the APC-PS complex.

Thrombin activity is inhibited by four naturally occurring proteins: antithrombin III (ATIII), α_2 -MG, HCII, and APCI. The APCI also inhibits factor Xa. ATIII, HCII and APCI are *serpins* (serine protease inhibitors). ATIII is a glycoprotein which is capable of forming a stable tetrahedral complex with thrombin thereby inactivating it. ATIII also inhibits factors IXa, Xa, XIa and XIIa. ATIII contains two domains for binding heparin. Both domains are rich in basic amino acids. Hence they readily bind to the negatively charged sulfate esters in heparin. They also bind to heparan sulfate, a proteoglycan which is synthesized by ECs and expressed to their surface. Hep-

arin is synthesized by mast cells and is detected in granules of these cells beneath endothelial tissue. When ATIII binds to heparin to form an ATIII-heparin complex, the conformation of the ATIII is presumably altered, significantly enhancing reaction with thrombin. Following formation of the ATIII-thrombin complex, heparin is released, thereby permitting it to react with free ATIII molecules. The heparan sulfate-ATIII complex is also a powerful inhibitor of thrombin. HCII inactivates thrombin. Its inactivation of thrombin can be accelerated 1000-fold by complexing with dermatan sulfate, a mucopolysaccharide component of the vascular wall.

As may be seen in Figure 1, factor Xa exerts multiple roles in coagulation, as does thrombin. It activates factors II, V and VIII, thereby promoting the clotting process. It also activates factors IX on a membrane surface, a positive feedback step, thereby stimulating its own activation from factor X, and is further involved in the activation of factor IX by activating the TF-VII complex of the *extrinsic* clotting system. Lastly, it is a component of a negative feedback system whereby it complexes with tissue factor pathway inhibitor (TFPI) to form the Xa-TFPI complex which inhibits factor Xa function as well as TF-VIIa function (Factor Xa is also inhibited by ATIII and by APCI.)

The significance of the contact system for the initial activation of blood clotting in the intrinsic clotting pathway has been questioned as a functional pathway *in vivo* as a deficiency in factor XII does not lead to a clotting abnormality. In addition, a deficiency in factor XI leads to a mild clotting abnormality, if at all. Hence, current views accept the proposal that the exposure of damaged vascular tissue initiates the clotting cascade in what historically was considered as the *extrinsic* clotting system. Damaged vascular tissue causes an immediate response with the induction of mRNA for TF. TF is a glycoprotein which is associated with the membrane of medial smooth muscle cells and adventitia. It has not been observed in the EC. However, it is inducible in the EC upon vascular injury, and exposure to thrombin. Other factors inducing enhancement of TF are platelets, granulocytes, macrophages, IL-1, and tumor necrosis factor α . Exposed TF binds to factor VII to form the TF-VII complex, a mostly inactive complex. It is activated by thrombin, which may be present in small amounts at the initial site of damage, as well as factor Xa. The TF-VII complex can also be activated by factors IXa and XIIa, and by its product, TF-VIIa, the latter being a positive feedback step. The TF-VIIa complex reacts with factor X on the membrane surface to transform factor X to the active factor Xa. Subsequently factor Xa activates

Table 1 Inhibitors of thrombotic and fibrinolytic enzymes

Coagulation/ fibrinolytic enzyme	ATIII	ATIII-H	α_2 -MG	HCII	TFPI	TFPI-Xa	α_1 -AT	Fg	α_2 -AP	PAI-1	PAI-2	C1 inhibitor	Heparan sulfate-ATIII	Protein C inhibitor
TF														
TF-VIIa					X									
IIa	X	X	X	X			X	X	X	X			X	X
VIIa														
IXa	X	X												
Xa	X	X	X		X	X	X	X	X			X	X	X
XIa		X					X					X		X
XIIa														
XIIIa														
Kallikrein			X				X					X		X
Plasmin	X		X				X		X	X		X		
APC			X				X		X	X				X
One-chain t-PA										X				
Two-chain t-PA										X				
One-chain u-PA											X			
Two-chain u-PA										X				

TF, tissue factor; APC, activated protein C; t-PA, tissue plasminogen activator; u-PA, urinary plasminogen activator; ATIII, antithrombin III; H, heparin; α_2 -MG, α_2 -macroglobulin; HCII, heparin cofactor II; TFPI, tissue factor pathway inhibitor; α_1 -AT, α_1 -antitrypsin; Fg, fibrinogen; α_2 -AP, α_2 -antiplasmin; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2; C1 inhibitor, complement protein 1 inhibitor.

prothrombin into thrombin with the mediation of factor Va. (The TF-VIIa complex is inhibited by ATIII-heparin and by the Xa-TFPI complex.) The TF-VIIa complex has the capacity to activate factor IX to factor IXa.

Since TF-VIIa is capable of activating factor X, the necessity for the utilization of factor IX in the clotting system has been raised and addressed. However, individuals with deficiencies of factors VIII and IX do exhibit abnormal clotting times and clinical bleeding. Therefore, current beliefs suggest that the TF-VIIa complex activates both factors IX and X, and that factor IXa, in conjunction with factor VIIIa, activates factor X as the need arises in order for normal coagulation times to be experienced.

Fibrinolysis and its modulation

The fibrinolytic system is a process which modulates the degradation of the clot as the organism repairs and replaces damaged vascular tissue. This process encompasses the degradation of the fibrin clot and its cellular components to soluble degradation products. Many of the constituent zymogens, activators and enzyme inhibitors are either intimately located within the clot or within the adjacent endothelial tissue associated with the clot. t-PA is a secretory product of the EC whose secretion is promoted by thrombin. Its inhibitor, PAI-1 is also observed in ECs, as well as in platelets, plasma and the EC matrix. (PAI-2 has only been seen in extracts of placental tissue in the third trimester of pregnancy.) Plasminogen, the inactive zymogen for the fibrinolytic plasmin, is readily bound by thrombospondin, a glycoprotein produced by platelets and expressed to the membrane surface upon activation. Thrombospondin acts to inhibit interaction of plasminogen with fibrin, as well as the activation of the plasminogen-fibrin complex. Plasminogen is entrapped in the clot and acts locally upon appropriate activation. α_2 -AP is a component of the α granules of platelets. Accordingly, all the components necessary for fibrinolysis and its control are strategically placed at the site of the clot. Circulating α_2 -AP, for example, is not believed to penetrate the clot and affect fibrinolysis, whereas α_2 -AP in the entrapped platelet (granules) can be released upon activation of the platelets. Likewise, fibrinolysis occurs only within the clot, and not systemically. Hence, the postulated pathway for fibrinolysis and its modulation envisions the degradation of fibrin into soluble degradation products by plasmin. (Plasmin activity may be inhibited by α_2 -AP.) Plasmin is activated from the inactive plasminogen by two-chain t-PA. It may also be activated by two-chain u-PA and by kal-

likrein. The two-chain t-PA and two-chain u-PA are both generated from their one-chain precursors by plasmin. Kallikrein transforms the one-chain u-PA into its two-chain metabolite. PAI-1 and PAI-2 inhibit the catalytic activity of one-chain t-PA, two-chain t-PA and u-PA. Only PAI-2 inhibits one-chain u-PA. The activation of plasminogen to plasmin is also inhibited by lipoprotein(a), thrombospondin and histidine-rich glycoprotein (His-rich-GP).

A summary of the active proteases associated with coagulation and fibrinolysis, together with their naturally occurring inhibitors, is given in Table 1.

See also: Erythrocytes; Fibronectin; Macrophage activation; Plasma; Platelet-activating factor (PAF); Platelets; Prostaglandins.

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COBRA VENOM FACTOR

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Cobra venom factor (CVF) is a constituent of cobra venom that specifically interacts with the serum complement system, causing its continuous activation. Whereas numerous studies on the effect of cobra venom on blood and blood components, including complement, were performed during the early part of this century, CVF was not purified until the late 1960s. The molecular interaction of CVF with the complement system became understood in subsequent years, simultaneously revealing the reaction sequence of the alternative complement pathway, and CVF has been extensively biochemically characterized. The molecule is a structural and functional analog of complement component C3. CVF is also often used to deplete laboratory animals in order to investigate biological functions of complement. The depleting activity of CVF has recently been exploited in animal models of xenotransplantation where it has been shown that complement is a major contributor to the hyperacute rejection of the transplanted organ. Another application of CVF is its use in antibody conjugates to introduce cytotoxic activity to monoclonal antibodies, e.g. as a concept for experimental immunotherapy.

Structure of CVF

Cobra venom factor has been isolated from the Asian cobra (*Naja naja*, including several subspecies: *N.n. naja*, *N.n. kaouthia*, *N.n. atra*) and the Egyptian cobra (*Naja haje*). The isolated proteins have been biochemically characterized and found to exhibit only minor structural differences. CVF is a three-chain glycoprotein with a molecular weight of

~136 kDa, as determined by equilibrium sedimentation. SDS polyacrylamide gel electrophoresis yields a molecular weight of ~149 kDa as the sum of the α chain (~68.5 kDa), β chain (~48.5 kDa), and γ chain (~32 kDa). The γ chain of CVF shows size heterogeneity, with usually 3–5 bands being detectable. The oligosaccharide portion of the molecule represents ~7.4% (w/w). The major oligosaccharide was found to be a fucosylated symmetric N-linked chain of the complex type with an unusual α -galactosyl residue at the nonreducing end. Circular dichroism spectroscopy revealed a secondary structure of CVF of 11% α helix and 47% β sheet. The molecule is an irregular and somewhat elongated ellipsoid structure with dimensions of 137×82 Å, as revealed by transmission electron microscopy.

More recently, the molecular cloning of CVF was accomplished using a cDNA library from cobra venom glands. The CVF mRNA contains a single open reading frame of 4926 nucleotides, coding for a single-chain preproprotein of 1642 amino acid residues. The mature three-chain protein is derived from the single-chain prepro-CVF by removal of a 22 residue signal peptide, an 83 amino acid peptide consisting of four arginine residues and a 79 amino acid peptide resembling a C3a anaphylatoxin generating the C-terminus of the CVF α chain and the N-terminus of the CVF γ chain, and of an approximately 279 residue peptide resembling the C3d domain of C3, yielding the C-terminus of the CVF γ chain and the N-terminus of the CVF β chain (Figure 1).

Several methods have been reported to purify CVF. Phospholipase A₂ has been identified as a common contaminant of CVF preparations. Certain uses of CVF require the removal of the contaminating

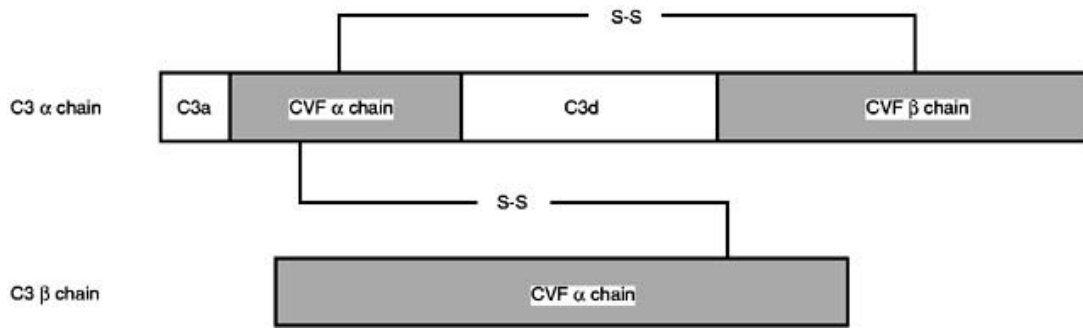


Figure 1 The two-chain structure of human C3 and the three-chain structure of CVF, indicating regions of homology (see text for further details).

phospholipase A₂; and several methods have been reported for that purpose.

The molecule is immunogenic, and antisera can easily be raised in several species of laboratory animals. These antisera can be used in antibody-based immunoassays for the detection and quantification of CVF. A simple hemolytic assay has been devised to determine the activity of CVF.

Molecular interaction of CVF with complement

When CVF is added to human or mammalian serum it activates complement and leads to complement consumption. It binds to factor B of the alternative pathway. When factor B is in complex with CVF, factor B is cleaved by factor D into Ba, the activation peptide, which is released, and Bb, which remains bound to CVF. The bimolecular complex CVF,Bb is a C3 convertase which cleaves C3 into C3a and C3b (Figure 2). CVF,Bb generated with CVF from *N. naja*, but not from *N. haje*, is also a C5 convertase, cleaving C5 into C5a and C5b. The generated C5b will consume the terminal complement components in plasma by formation of the membrane attack complex. In conclusion, through the formation of the CVF,Bb enzyme CVF will consume C3, C5 and the

terminal complement components, leading to the depletion of complement activity in serum.

Functional and structural similarity of CVF and C3

The formation and function of the CVF-dependent C3 convertase is analogous to the formation and function of the C3b-dependent C3 convertase which is formed during activation of the alternative pathway of complement (Figure 2). Both enzymes, CVF,Bb and C3b,Bb are C3/C5 convertases, consisting of a structural subunit (CVF or C3b) and the identical active site-bearing subunit Bb. Despite this similarity, the two enzymes exhibit several functional differences. The C3b,Bb enzyme is very short lived with a half-life of decay into the two inactive subunits of 1.5 min, while the CVF-Bb enzyme is rather stable, with a decay half-life of 7 h. In addition, the C3b,Bb enzyme is subject to rapid and efficient regulation by the complement components factor H and I. Factor H dissociates C3b,Bb and serves as cofactor for the proteolytic inactivation of C3b by factor I. In contrast, the CVF,Bb enzyme and CVF are completely resistant to the regulatory actions of factors H and I. Due to these two properties, the CVF,Bb enzyme is stable and present in plasma for prolonged periods of time. This is the basis for its ability to activate efficiently, and thereby consume, complement in serum.

Extensive structural homology has been found between CVF and mammalian C3. The structural homology includes immunologic cross-reactivity, similar amino acid compositions, similar secondary structures, and a similar shape and dimension under the electron microscope. The N-termini of the three chains of CVF show sequence homology with human C3, leading to the conclusion that the CVF α chain and the C3 β chain are homologous structures, and

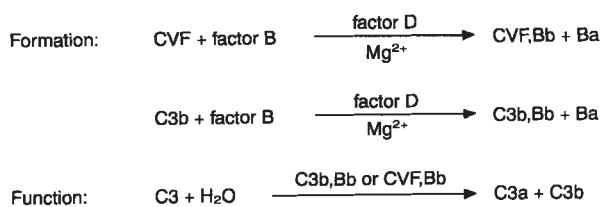


Figure 2 Formation and enzymatic activity of alternative pathway C3 for convertases, CVF,Bb and C3b,Bb.

that the CVF β and γ chains are derived from a C3 α chain (Figure 1). Molecular cloning has recently confirmed the chain relationships and structural homology of CVF and C3.

Both C3 and CVF code for single-chain precursor molecules which are then processed to yield the mature two-chain C3 or three-chain CVF, respectively. The sequence identity between pre-pro-CVF and mammalian pre-pro-C3 molecules on the protein level is ~51% and increases to ~70% if one allows for conservative amino acid replacements. In the case of pre-pro-C3 from cobra the similarity at the protein level is 91.6%, corresponding to a sequence identity on the DNA level of 93.3%. These results strongly suggest that the gene for CVF was generated by gene duplication from a C3 gene.

Use of CVF as an experimental tool to study the biological functions of complement

Cobra venom factor can be safely administered to laboratory animals. With the exception of an acute and transient reaction due to massive complement activation at high dosages, no toxic effects of CVF are known. Injection of CVF causes consumption of complement components *in vivo* and depletes complement activity in the plasma of treated animals. The complement activity will remain low for 1 to several days, and resynthesis of consumed complement components will restore normal complement activity within 5–10 days. Accordingly, temporary suppression of complement activity by CVF has become a widely used experimental tool for studying the biological functions of complement by comparing normal with complement-depleted animals. Questions addressed include the role of complement in the generation of an immune response, in host defense against infections and in the pathophysiology of disease.

Antibody conjugates with CVF

The property of the CVF, Bb enzyme to activate complement exhaustively has been exploited for the selective killing of tumor cells by coupling of CVF to monoclonal antibodies with specificity for surface antigens of tumor cells. Antibody conjugates with CVF will target CVF to the cell surface where the CVF, Bb enzyme forms from complement factors B and D of the host complement system. The antibody-bound and therefore cell surface-bound CVF, Bb enzyme will continuously activate C3 and C5 and

elicit complement-dependent target cell killing through the formation of membrane attack complexes. Antibody conjugates with CVF have been shown to kill human melanoma cells, human lymphocytes and leukemia cells, and human neuroblastoma cells. The concept of coupling CVF to monoclonal antibodies seems particularly promising in light of the fact that many monoclonal antibodies against tumors bind with good specificity but do not activate biological effector mechanisms such as complement. The coupling of CVF to monoclonal antibodies therefore introduces a biological effector function to the antibody.

Outlook

As CVF is a structural analog of C3, it may be possible to engineer a human C3 derivative that has the property of CVF to form a stable C3/C5 convertase. Such a 'human CVF' can be expected to be significantly less immunogenic. A 'human CVF' might be a useful therapeutic agent for modulating the complement activity in certain diseases where complement is known to be involved in the pathogenesis. One particular application of a 'human CVF' might be the depletion of complement in patients prior to xenotransplantation. 'Human CVF' could also be used in antibody conjugates as an immunotherapeutic agent in cancer patients.

See also: Complement, alternative pathway; Complement, classical pathway; Complement deficiencies; Complement fixation test; Complement, membrane attack pathway; Immunotherapy of tumors; Venoms; Xenotransplantation.

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COCCIDIOIDES, INFECTION AND IMMUNITY

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Coccidioidomycosis is a fungal disease of humans and animals caused by the dimorphic fungus *Coccidioides immitis*. It is acquired by inhalation of arthroconidia in the southwestern USA and Central and South America. After exposure, most infected persons (60%) are asymptomatic and some 40% will develop a primary illness, usually pneumonic. Most of these resolve without sequelae. Up to 8% of infected individuals will have prolonged or later emergence of disease with, usually, pulmonary nodules or cavities (5%), but occasionally (1%) meningitis, osteomyelitis, cutaneous, lymph node, urogenital and other disease sites affected. Men, pregnant women, immunocompromised hosts, Blacks and persons of Filipino ancestry (dissemination 175 times more common than Whites) tend to have a higher frequency of dissemination and more severe disease. Disseminated and chronic forms of the disease require therapy. The mortality of coccidioidal meningitis is reduced by treatment from 100 to 30%. Pathologically, the spherule (the tissue phase of the fungus), which may contain many endospores, is surrounded by varying degrees of granulomatous reaction with or without caseation.

Antigens

At least 15 antigens can be found on immunoblots of SDS-PAGE gels from both soluble mycelial and spherular extracellular preparations. These include the tube precipitating antigen (detected by immunoglobulin M (IgM) antibody) which is a mycelial phase glycoprotein containing about 20% protein and the sugars 3-O-methylmannose (unique to *C. immitis* among pathogenic fungi), mannose and glucose, and the antigen recognized by complement fixing IgG antibody, a 110 kDa protein which runs at 48 kDa in a denaturing gel. IgM and IgG antibodies can also react in immunodiffusion with these two

specific antigens. These two antigens are present in both fungal phases. Recently, a 33 kDa protein antigen has been purified, its gene cloned and expressed in bacteria. It is distinct from the antigens used in conventional complement fixation, tube precipitin and immunodiffusion procedures. It has been localized to the inner conidial wall of arthroconidia, within the cell wall and septa of spherules and on the wall surface and interconnecting glycocalyx of endospores.

Cellular and cytokine responses

T lymphocyte responses to *C. immitis* are critical for host defense. Thymectomy in rodents leads to severe disease. Immunity can be transferred to naive mice by infusions of T cells from immune mice. Patients infected with human immunodeficiency virus (HIV) who develop coccidioidomycosis have low CD4 T cell counts, median <150 cells per cubic millimeter. Macrophage killing of arthroconidia by enhanced phagosome-lysosome fusion only proceeds (in mice) in the presence of antigen-stimulated T lymphocytes or soluble mediators produced by these T cells. Natural killer cells are able to kill endospores and spherules *in vitro*.

DBA/2 mice are relatively resistant to *C. immitis*; BALB/c mice are highly susceptible. The lungs of infected DBA/2 mice have relatively increased interleukin 6 (IL-6) and interferon γ (IFN γ) and their splenocytes produce greater amounts of tumor necrosis factor α (TNF α), IL-6 and IL-1 α in response to stimulation with formalin-killed spherules *in vitro*. Treatment of the DBA/2 mice with an IFN γ -specific monoclonal antibody (mAb) increases their susceptibility. Infected BALB/c mice have increased levels of IL-4 in the lungs. Treatment of BALB/c mice with an IL-4-specific mAb provides significant protection. Treatment of susceptible BALB/c mice with recombinant IFN γ provides significant protection. *In vitro*,

treatment of murine peritoneal or alveolar macrophages with rIFN γ augments anti-*Coccidioides* activity.

Independent of the immune status of the donor, human peripheral blood monocytes phagocytose arthroconidia and inhibit growth of the fungus and kill it. Monocytes may therefore play a role in limiting infection before a specific immune response develops. Peripheral blood mononuclear cells from skin test-positive individuals display increased lymphocyte transformation, IL-2 production and IFN γ production in response to spherule antigen. Spherules and arthroconidia elicit production of TNF α from human monocytes *in vitro*. TNF α alone or in combination with IFN γ enhances killing of spherules by human monocytes *in vitro*. The cytokine responses of a small number of patients with overwhelming infection with *C. immitis* have been studied. IL-1 and IL-2 were undetectable but levels of TNF α and IL-6 were elevated. T Lymphocyte coccidioidal-specific responses are suppressed in some patients and various lines of evidence suggest that antigen overload, specific suppressor T lymphocyte activity, circulating humoral suppressive substances and spherule-produced suppressive substances may all be involved.

A positive skin test is a marker of previous infection, and remains positive for life in the absence of severe immunosuppression such as the acquired immune deficiency syndrome (AIDS). Spherule-phase antigens may be more sensitive in eliciting cell-mediated responses. Cell-mediated responses *in vitro* are mirrored by intradermal skin testing which correlates reasonably well with clinical outcome – persons with disseminated disease are often anergic and those who are anergic are more likely to have a poor outcome.

Arthroconidia, endospores and particularly spherules are ordinarily quite resistant to killing by neutrophils or products of oxidative metabolism. A residual hyphal wall layer on arthroconidia and an extracellular matrix (acidic glycoproteins which are both carboxylated and sulfated) surrounding spherules and endospores appear to protect the fungus from phagocytosis and killing. Neutrophils comprise part of the initial response to arthroconidia in the lower airways and inhibit the growth of arthroconidia. Phagocytosis of arthroconidia and endospores is augmented by immune serum. Neutrophils fail to kill any phase of *C. immitis* unless immunologically activated, such as by IFN γ , in which instance they are capable of killing endospores but not arthroconidia. There appears to be some strain-to-strain variation in intrinsic susceptibility to neutrophil activity.

Humoral responses

Both asymptomatic infection and disease are marked in over 90% of all infected persons by an antibody response to *C. immitis*. Mycelial phase antigens are most useful in serodiagnosis. IgM is detected earliest by precipitation, latex agglutination or immunodiffusion in 75% of infected individuals. Detectable IgM usually disappears within 6 months of infection. In identifying progression of disease, IgG is useful: elevated titers indicate dissemination, and titers will fall with successful therapy in serum and in cerebrospinal fluid (CSF) in patients with meningitis. Antibody to the 33 kDa antigen can be detected in the CSF of patients with *Coccidioides* meningitis, including about half of those without detectable coccidioidal complement-fixing (CF) antibody. The antibody against the antigen appears to persist longer than detectable CF antibody. For patients who were failing therapy, a significant increase in antibody to the 33 kDa antigen was demonstrated over time; for patients responding to therapy, a significant decrease. Circulating immune complexes are found in up to 70% of patients with active, disseminated disease. Circulating free antigen has also been detected in patients with active disease. Total serum hemolytic complement activity is depressed early in primary infection in some patients but later recovers. Both classic and alternate pathways are probably activated.

Hypersensitivity

Erythema nodosum may occur during the primary infection and is a marker of hypersensitivity to coccidioidal antigens. Serum IgE is elevated in patients with active disease and tissue eosinophilia (or eosinophilic pleocytosis in CSF) may accompany severe disseminated disease.

Immunosuppression

Solid organ transplantation, corticosteroid therapy and AIDS may lead to severe, often fatal, disease, due to either recent infection or reactivation. The serologic response in compromised hosts is usually, but not always, at least qualitatively intact.

Vaccines

Formalin-killed spherule vaccine is highly effective in a murine model. Unfortunately, a recent trial of formalin-killed spherules in humans failed to demonstrate a significant protective effect. Work is proceeding to identify protein immunogens as potential vaccines.

See also: Avian immune system; Fungi, immunity to.

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COCCIDIOSIS

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Coccidiosis is the generic term given to the disease caused by infection of domestic animals with *Eimeria* species. These are apicomplexan protozoa that parasitize mainly epithelial cells, principally those of the intestinal tract. They exhibit a high degree of host and site specificity and it is usual for a given species of animal to be host to several different *Eimeria* spp., each with its distinct location in the gut. Because of the self-limiting nature of the life cycle and the potential of the host to develop resistance to reinfection, coccidiosis is rarely a problem in nature but may be important in highly intensive systems of animal husbandry. In modern poultry production, where it is estimated to cause annual losses of \$US1.5 billion, most flocks are prophylactically medicated. In humans, closely related organisms (*Isospora* spp.) cause a similar, but rarely significant, condition.

Characteristics of the organism and its antigens

The complex life cycle is monoxenous and sporozoan. Within the host, a period of asexual replication (schizogony) is followed by a sexual phase (gametogony). The number of cycles of schizogony, usually fixed, varies with the species but is commonly three or four, the last being followed by the formation of gametes (Figure 1). After fertilization, the zygote, protected within a resistant shell (oocyst), is voided in the feces. A period of maturation outside the host (sporogony), including a meiotic nuclear division, is necessary for the oocyst to become infective. Transmission is fecal–oral.

Infection causes enteritis of varying severity, depending upon the number and the biology of the

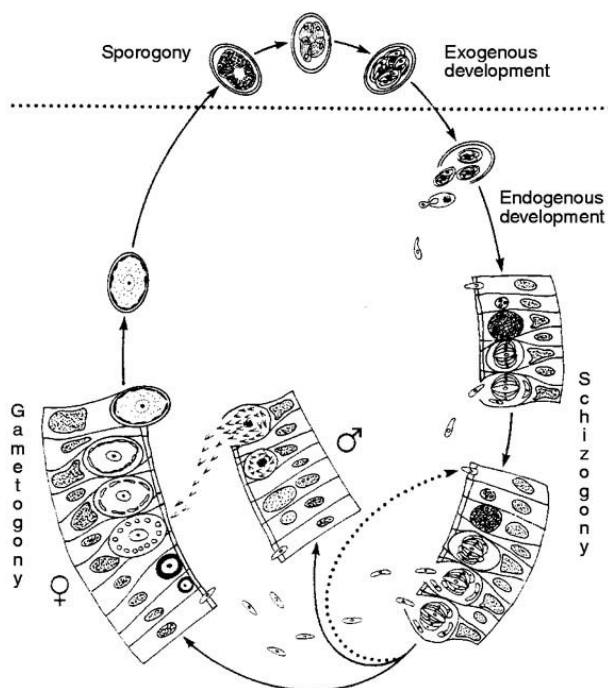


Figure 1 Life cycle of *Eimeria* species. (Reproduced with permission of Springer-Verlag GmbH & Co. KG from Rose ME (1985) *The Eimeria*. In: Parkhouse RME (ed) *Parasite antigens in protection, diagnosis and escape*. *Current Topics in Microbiology and Immunology* 120: 7–17.

Eimeria spp. ingested. Those that develop in crypt epithelium, resulting in hemorrhage, are highly pathogenic and may cause serious mortality. Anorexia and stunted growth are common consequences of infection.

The various developmental stages of the parasite are morphologically distinct and possess both stage-specific and common antigens. Some of these have been purified and characterized, mostly those from species parasitizing the domestic fowl, such as *E. tenella* and *E. maxima*. The emphasis has been on the initial infective stage, the easily accessible sporozoite; this is a major target for immune inhibition and, once development has been initiated, is capable of inducing immunity.

Sporozoite antigens of current interest are those associated with some of the organelles, i.e. the refractile globule and those forming the apical complex (rhoptries, dense granules and micronemes) and presumably involved in invasion of the host cell. Treatment with a refractile body antigen of 26–28 kDa has been reported to protect chickens against challenge infections with four different *Eimeria* spp.; similar antigens are present in sporozoites of *E. bovis* (infecting cattle), becoming distributed within the cytoplasm of the invaded host cell. Rhoptry polypeptides migrate in the 45–65 kDa range and differ anti-

genically between species, paralleling the species-specificity of immunity induced by infection. A merozoite protein from *E. maxima* of 230 kDa has been reported to induce maternally transmissible immunity (to homologous and heterologous challenge), as have gametocyte-derived 56, 82 and 230 kDa antigens of the same species.

Immune responses of the host

Species of *Eimeria* differ widely in the degree of resistance to challenge induced by infection, even within the same species of host. Immunity induced in this way is species- and, in some cases, strain-specific but other methods of immunization, e.g. injection of purified antigens (above), may produce heterologous protection. The specificity, or otherwise, of immunity to the different developmental stages is not known.

Immunity is T cell mediated, apparently with little involvement of natural killer cell activity or reactive oxygen/nitrogen intermediates. Both humoral and cell-mediated immune responses participate in resistance but the latter is the more important. Antibodies are probably most effective against extracellular parasites, thereby reducing invasion. Experimentation in mice has shown that CD4⁺ T lymphocytes, which play the major role in controlling primary infections, are able to do so without the participation of inflammatory cells, and that CD8⁺ lymphocytes appear to be more important in the later stages of infection. The cytokine-release profile of responding lymphocytes does not indicate any correlation between T_H1 and T_H2 type responses and resistance/susceptibility. Although IFN γ , acting via the host cell, is very effective in halting the development of the parasite, it does not seem to mediate the prompt suppression seen in immunized animals. Work in chickens indicates that this may be due to the action of CD8⁺ lymphocytes in preventing sporozoites from reaching their developmental site in crypt enterocytes.

Evasive strategies by the organism

Eimerian coccidia are not known to use mechanisms for avoiding the immune response of the host. The brevity and finite nature of the life cycles, coupled with antigenic differences between the developmental stages, probably accounts for the ability of the parasite to complete its cycle, relatively unhindered, in the naive host; however, in a few (unusual) species additional asexual cycling occurs in immunocompromised hosts.

Some *Eimeria* spp. isolated from chickens in geo-

graphically distinct areas may show intraspecific variation, demonstrable by incomplete cross-protection, but their antigens have not been studied. Variation of this type has not been induced experimentally, nor has it emerged over time in any one site.

Vaccines

Vaccination, as a means of controlling coccidiosis, is an attractive alternative to prophylactic medication which is, inevitably, accompanied by the emergence of drug-resistant strains of the parasite. Until recently the only type of 'vaccine' commercially available (for chickens) consisted of wild-type, fully virulent organisms. Now there are two vaccines whose constituents have been attenuated by selection for abbreviated developmental cycles, or by adaptation to growth in developing chicken embryos. These are being successfully used in various types of flocks but, in view of their cost, are likely to be most useful for breeders and layers. The possibility of deriving live, attenuated strains suitable for vaccinating rabbits or sheep is being pursued.

Although live vaccines are effective, for reasons of cost, convenience (e.g. shelf-life) and safety, the ultimate goal of immunoprophylaxis for coccidiosis must be the availability of subunit vaccines, produced by recombinant DNA technology. Crude parasite extracts (primarily of sporozoites), given parenterally or orally, are capable of inducing protection against subsequent homologous challenge infection,

but the results are influenced by the resistance phenotype of the host. Various purified candidate peptide antigens have afforded partial protection, either when given directly or via the hen (above).

See also: **Avian immune system; Parasites, immunity to.**

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COLD AGGLUTININS

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Cold agglutinins are autoantibodies of low affinity which agglutinate erythrocytes at temperatures below 37°C, and optimally at 4°C. Sera of the majority of healthy adults contain harmless cold autoagglutinins at low titers (1 : 2 to 1 : 32). Sera of newborns usually do not contain cold agglutinins; they become detectable during the first year of life. The majority of these naturally occurring autoantibodies are directed at the I or i antigens. Raised levels of cold agglutinins occur a) transiently following certain infections, for example with *Mycoplasma pneumoniae* and Epstein–Barr virus (anti-i), and b) persistently in a chronic relatively benign lymphoproliferative disorder termed chronic cold agglutinin disease. Raised levels of cold agglutinins may go

unnoticed, and are frequently detected incidentally during blood cross-matching or other hematological investigations. The antibodies of certain individuals however, cause clinical symptoms: cyanosis (blueness) with impaired circulation at the extremities upon exposure to the cold resulting from erythrocyte autoagglutination, or an autoimmune hemolytic disorder associated with a positive Coombs' (antiglobulin) test resulting from coating of erythrocytes with the complement components C3 and C4. The severity of the symptoms does not necessarily correlate with the cold agglutinin titers measured at 4°C, rather it is thought to be a reflection of the relatively high binding affinities of the antibodies in certain patients.

Restricted clonality and cross-reactive idiotypes

Cold agglutinins have been the subject of considerable interest since the late 1950s when it was realized that they are usually monoclonal or oligoclonal antibodies. Those occurring in chronic cold agglutinin disease are almost always monoclonal, but even the transiently occurring, postinfective cold agglutinins, when isolated with care to avoid contamination with serum immunoglobulins, have been found to be monoclonal or oligoclonal antibodies. Cold agglutinins with anti-I and anti-i specificities are almost always of the IgM class; the majority have κ light chains, and only a minority, λ . Cold agglutinins with other specificities, designated anti-Pr, anti-Gd, anti-Fl etc. occur less commonly. The majority of these are also of the IgM class but several monoclonal IgA and IgG have been described.

There is a predominance of κ light chains among monoclonal anti-I, whereas λ chains are not uncommon among anti-i and other types of cold agglutinin. Cold agglutinins were the first antibodies among which cross-reactive idiotypes were demonstrated. Two distinct idiotypic systems have been described: one found among anti-I and anti-i, and a second among anti-Pr antibodies; the latter included proteins of the IgM and IgA classes. The first demonstration of idiotypic antigens on blood lymphocytes corresponding to those serum antibodies in the same individuals was made in patients with cold agglutinin disease. The idiotypic antigens among anti-I and anti-i are located predominantly on the variable regions of their heavy chains. Restricted V_H and V_L subgroups are represented among these antibodies, and the cross-reactive idiotypes are almost certainly associated with the framework rather than the complementarity determining regions, since an occasional myeloma protein lacking erythrocyte cold agglutinin activity also has been found to express the same idiotypic determinants.

Combining specificities towards carbohydrates

The I and i antigens are expressed in a developmentally regulated manner on human erythrocytes. In neonates i predominates, and in adults I. The level of i decreases on erythrocytes in the course of the first year of life while I increases. These antigens were the first among developmentally regulated antigens to have been characterized at the molecular level. They are carbohydrate structures: i is expressed on a linear hexasaccharide sequence (structure 1, **Figure 1**) consisting of alternating galactose and N-acetyl-

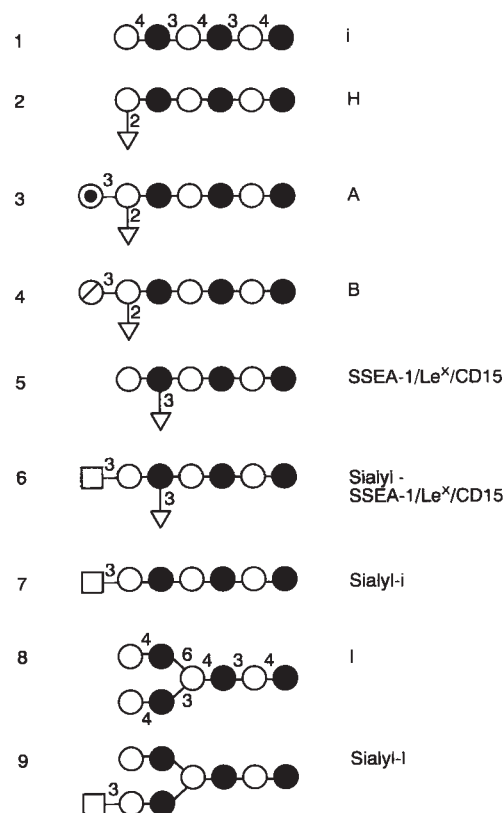


Figure 1 Schematic representation of oligosaccharide sequences which express the i and I antigens and are the most common targets of cold agglutinins. These sequences occur as the backbone domains of oligosaccharides that are O- or N-linked to proteins, or linked to lipids. Also shown are examples of several related oligosaccharides which are based on the i and I backbones and express the major blood group antigen A, B and H, or carbohydrate differentiation antigens SSEA-1 (also designated Le^x or CD15) and sialyl-SSEA-1. The latter two structures are among ligands for leukocyte to endothelium adhesion molecules, the selectins. *Mycoplasma pneumoniae* binds to oligosaccharide structures 7 and 9, but with a stronger affinity for the latter. Symbols for monosaccharides: ○, galactose-β; ●, galactose-α; ◐, N-acetylglucosamine-β; ▽, fucose-α; ⊙, N-acetylgalactosamine-α; □, N-acetylneuraminic acid-α. The numbers over the glycosidic bonds indicate the positions of linkage of the monosaccharides.

glucosamine residues, and I on the related branched octasaccharide (structure 8). Each monoclonal antibody recognizes a particular domain on these oligosaccharide sequences. These sequences occur not only on glycoproteins but also on glycolipids. They are not confined to erythrocytes, but have differing patterns of expression in various tissues of the body. They occur not only as accessible structures but also as internal structures (backbone structures) of the major blood group antigens A, B and H. As illustrated for the i sequence, fucosylation at galactose masks the i antigen activity but gives rise to the blood group H antigen (structure 2). In blood group A

persons there occurs addition of an N-acetylgalactosamine residue to the H antigen structure to form blood group A (structure 3), and in B individuals there is a galactose residue instead of N-acetylgalactosamine (structure 4). Less detailed information is available on the target antigens of cold agglutinins anti-Pr, Gd, etc. The majority of these are directed at various sialo-oligosaccharide sequences on glycoproteins or glycolipids. For example anti-Gd binds to structure 7 on glycolipids.

Clues to pathogenesis

What triggers the production of cold agglutinins? Why are they monoclonal? The precise details are not yet known. The transient cold agglutinin syndrome that follows *M. pneumoniae* infection offers a unique opportunity to study aspects of the mechanism of stimulation of anti-I production. Negligible amounts of I antigen can be detected on cultured *M. pneumoniae*, and there is evidence that this represents passively adsorbed material, probably in association with other lipids and glycolipids, from the serum. As autoantibodies to other glycolipids in the serum, e.g. those with blood group A, B, H, and Lewis activities, do not prevail in this infection, it is unlikely that the anti-I are directed against this passively adsorbed form of the I antigen. On the other hand, the receptors on host cells to which the mycoplasma adheres are sialo-oligosaccharide sequences such as structures 7 and 9, that are based on backbones of Ii type, the highest affinity binding being to the branched I type, structure 9. It seems likely that the selective production of high titer anti-I (i.e. 'antireceptor' antibodies) following *M. pneumoniae* infection is somehow related to these interactions. Possibly the lipid rich mycoplasma serves as an adjuvant overcoming tolerance to self-antigen I. It should be noted that the sialo oligosaccharide receptor sequences occur not only on erythrocytes but also on B and T lymphocytes as well as monocytes, for example. Thus there remains considerable scope for dissection of the macromolecular interactions that culminate in the production of auto-anti-I.

Clues to the biological importance of the target antigens of cold agglutinins and related carbohydrate structures

More recent work with hybridoma-derived monoclonal antibodies that were raised to differentiation antigens of human leukocytes and other cell types, and to various tumor-associated antigens of epithelial tissues has shown that the I and i sequences constitute backbone structures of an array of differ-

entiation antigens and tumor-associated antigens in addition to the blood group antigens discussed above. For example, fucosylation of the penultimate N-acetylglucosamine as in structure 5 forms the antigen that has been variously designated SSEA-1, Le^x or CD15. This antigen is a stage-specific embryonic antigen (SSEA-1) in the mouse; it is a distinctive marker of granulocytes (CD15) in the human, while the corresponding sialylated structure which occurs on monocytes as well as granulocytes is also a tumor-associated antigen in certain epithelial malignancies.

Much progress is being made in the cloning of the glycosyltransferases that give rise to this family of oligosaccharides. There is much current interest in the fucosylated and sialylated oligosaccharides in this series (e.g. structures 5 and 6) as ligands for the endothelium to leukocyte adhesion molecules, the selectins which have important roles in leukocyte recruitment to sites of inflammation.

Conclusions

Cold agglutinin syndrome on the one hand presents excellent opportunities for studying mechanisms that operate in the generation of an autoimmune response in humans. On the other hand, the carbohydrate sequence-specific monoclonal autoantibodies in this disorder have served as remarkable reagents, and work with these antibodies opened the way to a whole new field of carbohydrate onco-developmental antigens, a field that expanded rapidly with the advent of the hybridoma technology and advanced further with the demonstration of roles for oligosaccharides as ligands in biological recognition.

See also: Antiglobulin (Coombs') test; Autoimmune diseases; Autoimmunity; Carbohydrate antigens; Lewis^x/sialyl-Lewis^x (CD15/CD15s); Idiotype: *Mycoplasma*, infection and immunity.

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COLONY-STIMULATING FACTORS

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Colony-stimulating factor (CSF) is the operational term for a class of molecules that stimulate hematopoietic progenitor cells to divide and generate colonies of differentiated progeny such as neutrophils or macrophages. Colony-stimulating factors were originally detected and assayed by culturing a source of hematopoietic progenitor cells, such as a cell suspension derived from the bone marrow, in medium that has been jellified by the addition of a substance such as agar or methyl cellulose. Provided a CSF or a source of a CSF is present, hematopoietic progenitors survive and grow, and over a period of a week or so, give rise to colonies of 100–10 000 differentiated cells. The number of colonies and the extent to which a preparation can be diluted and still retain activity, can be used to quantitate the CSFs in that preparation. Moreover the morphology of the differentiated cells in the colonies can be determined by picking off and staining the colonies or by staining the whole culture.

Colony assays were developed in the 1960s by Pluznik and Sacks and Bradley and Metcalf, and have in large part provided the data that have generated our current picture of the molecular regulation of hematopoiesis and of the cellular steps in the differentiation of hematopoietic cells. Colony assays also have allowed the identification of different types of committed progenitor cells and determination of their physical and antigenic characteristics.

All CSFs share three basic properties:

- 1 they support the survival of hematopoietic progenitor cells and mature cells such as neutrophils by suppressing apoptosis;
- 2 they stimulate growth of stem/progenitor cells and in some cases of mature cells, e.g. mast cells or macrophages;
- 3 they regulate the function of cells, for example activating mature granulocytes.

Nomenclature

Originally, CSFs were given names that reflected in some measure their function. Thus a preparation that stimulated progenitor cells and generated colonies containing both neutrophils and macrophages was said to contain granulocyte-macrophage CSF (GM-CSF); one that stimulated the growth of colonies containing only neutrophils was said to contain granulocyte CSF (G-CSF). The various CSFs are now recognized as members of a large family of cytokines and hormones united by a common evolutionary origin and similarities in their three-dimensional structure (discussed below). Both GM-CSF and G-CSF have gained wide acceptance as names of two, well-characterized molecules that have been produced in large quantities by recombinant DNA techniques and are now in clinical use. It should be noted, however, that activities of these molecules can be broader than implied by their name or by their action in one particular species. Thus whereas in the mouse, GM-CSF generates colonies containing neutrophils and macrophages, in the human it also generates colonies of eosinophils. Likewise, not only does G-CSF stimulate the growth of progenitors of neutrophils, but it also affects more primitive cells. Interleukin 3 (IL-3) acts as a CSF with a broad target range and the capacity to stimulate progenitors of megakaryocytes, mast cells, eosinophils, macrophages, neutrophils, basophils and erythroid cells, as well as those multipotent progenitors generating colonies containing various mixtures of these cell types. Because of these properties IL-3 has also been termed 'multi-CSF'. A fourth CSF, widely called CSF-1, stimulates colonies of macrophages and has also been termed macrophage CSF (M-CSF). As noted below CSF-1 differs slightly from the GM-CSF, G-CSF and IL-3 in its overall molecular structure and greatly in the type of cell surface receptors that it stimulates. It also differs from the other CSFs in that it occurs in the blood-

stream in normal animals and in that it has targets outside the lymphohematopoietic system, in the female reproductive tract.

It should be noted that operationally a number of different CSFs can stimulate one type of hematopoietic cell (Table 1). For example, a colony of macrophages can develop from a macrophage progenitor that has been stimulated by GM-CSF or by IL-3, or by CSF-1. Thus, in the earlier literature, prior to the molecular characterization of CSFs and the subsequent development of specific neutralizing antibodies, an activity that was referred to operationally as 'GM-CSF' may in fact have reflected the presence, not of the molecule now defined as GM-CSF, but instead of another molecule such as IL-3 that could also stimulate the development of colonies containing neutrophils and macrophages. Likewise, in earlier studies, the reader will encounter terms such as 'eosinophil CSF' or 'megakaryocyte CSF'. No distinct molecular species that specifically stimulate the development of large colonies of eosinophils or megakaryocytes have yet been characterized. Many of the earlier studies of eosinophil CSF or megakaryocyte CSF were assaying the ability of IL-3 to stimulate colonies of these cells.

Other molecules are able to stimulate progenitor cells and act operationally as CSFs. In the human, but not the mouse, IL-5 can stimulate the growth of small colonies of eosinophils although its major action appears to be to enhance differentiation of eosinophils. Under some circumstances, IL-6 can give rise to small colonies. Steel locus factor (SLF) or stem cell factor, which is the ligand of the c-kit protein, can stimulate the growth of small colonies of mast cells and of neutrophils.

An important principle is that mixtures of factors often exert synergistic effects on colony formation.

Interleukin 3 and CSF-1, for example, in the human strongly synergize in the production of macrophage colonies. Other peptide regulatory factors such as SLF, IL-1, IL-6 and IL-4 all have been reported to have synergistic activity in colony formation in various situations. IL-11 and thrombopoietin synergize with other cytokines in generating colonies of various hematopoietic lineages, but have a particular propensity to stimulate the generation of megakaryocytes and platelets.

Sources

The colony-stimulating factors are made in many cell types, including epithelial cells, endothelial cells, fibroblasts, smooth muscle cells and cells of the hematopoietic system, in particular T lymphocytes. In general, CSFs are only produced in response to stress, for example infection, although there are exceptions such as SLF or CSF-1, which are produced in the body under normal conditions. Microbial products such as endotoxin or nucleic acids are potent stimuli for CSF production. Other peptide regulatory factors such as IL-1 can induce the production of colony-stimulating factors, for example GM-CSF, from cells such as endothelial cells or fibroblasts. The antigen-activated T lymphocyte is a source of two CSFs: GM-CSF and IL-3. Macrophages can produce GM-CSF, G-CSF and CSF-1. When stimulated by LPS B lymphocytes produce small amounts of GM-CSF. Mast cells and eosinophils produce small quantities of IL-3, IL-5 and GM-CSF when activated by cross-linking the Fc receptor for immunoglobulins.

Table 1 Stimulation of proliferation and differentiation of hematopoietic cells by colony-stimulating factors

Target cells acted on in bone marrow	Multi-CSF (IL-3)	GM-CSF	G-CSF	M-CSF (CSF-1)
Pluripotent stem cell	+	(+)	(-)	(-)
Granulocyte-monocyte progenitor	+	+	+	(+)
Monocyte progenitor	+	+	-	+
Neutrophil progenitor	+	+	+	-
Eosinophil progenitor	+	+	-	-
Basophil progenitor	+	+	-	-
Mast cell	+	-	-	-
Megakaryocyte	+	-	-	-
Erythroid progenitor	+	+/-	-	-
Lymphoid				
B progenitor	+	-	-	-
T progenitor (thymus)	-	-	-	-

Physiological role of CSF

Physiologically, the CSFs can be regarded as part of a larger family of peptide regulatory factors or cytokines that play a major role in regulating the body's response to infection or injury. A general characteristic of the CSFs is that they stimulate not only the growth of progenitor cells leading to production of various differentiated progeny, but also the effector functions of the mature differentiated cells. Thus GM-CSF can prime for a respiratory burst in neutrophils, and GM-CSF and IL-3 can enhance phagocytosis by macrophages.

Some of the CSFs have been shown to enhance immune responses. Both GM-CSF and IL-3 have these effects which probably are mediated via actions on accessory cells. For example, IL-3 induces the release of substances such as IL-1 from macrophages and upregulates the expression of class II major histocompatibility complex antigens. GM-CSF, with IL-4, promotes the differentiation of monocytes to dendritic cells.

A minority of CSFs have a role in the normal development of the hematopoietic system as opposed to its activation during stress. Mice with mutations blocking production of CSF-1 exhibit defects in the development of macrophages and osteoclasts. Genetic lesions in SLF or its receptor leads to defects in the development of erythrocytes and mast cells as well as in nonhematopoietic tissues. In the absence of GM-CSF, a lung disease called alveolar proteinosis occurs.

Structure of the CSFs

The CSFs are small polypeptides of M_r around 15 000 which are heavily and variably modified by glycosylation, although the functional significance of this carbohydrate has not been established. IL-3, IL-5, GM-CSF, G-CSF erythropoietin, thrombopoietin and IL-11 are monomers, whereas CSF-1 and SLF are homodimers, linked by disulfide bonds. As discussed below, CSF-1 and SLF also differ from the other CSFs in binding to a different structural class of cell surface receptors. Although there are few similarities in the amino acid sequences of different CSFs, analysis of their three-dimensional structures has revealed that they all belong to one structural family of proteins, characterized by four α helices, bundled in a particular way. This family also includes most interleukins, as well as hormones such as growth hormone, thrombopoietin, erythropoietin and leptin.

IL-3, GM-CSF and G-CSF bind to members of a structural family of receptors termed the cytokine receptor superfamily, members of which also bind

IL-2, erythropoietin, IL-4, IL-5, IL-6 and IL-7, as well as other cytokines such as LIF, CNTF and leptin.

GM-CSF and IL-3 each bind with low affinity to specific receptor α chains belonging to this cytokine receptor family. High-affinity binding and signal transduction, however, depends upon interaction with a second β receptor chain, which is also a member of the cytokine receptor family and is a common component of the receptors for IL-3, GM-CSF and IL-5. Ligand-binding induces or stabilizes dimerization of α and β chains leading to activation of cytoplasmic tyrosine kinases that associate with the intracytoplasmic portions of these chains. Activation of these tyrosine kinases, which include members of the JAK and Src families of kinases, triggers a cascade of intracellular events that regulate cellular growth, survival and differentiation.

The receptors for CSF-1 and SLF do not belong to the cytokine receptor family, but instead to a distinct family of receptors that are characterized by immunoglobulin-like domains in the extracellular portion of the molecule, and a tyrosine kinase domain in the intracytoplasmic part of the molecule. These receptors resemble those for platelet-derived growth factor and, more distantly, insulin. Ligand-induced dimerization of the CSF-1 and SLF receptors leads to activation of the intrinsic kinase activity of the receptors themselves. Despite these differences in the two major families of receptors used by CSFs, ligand binding in both cases triggers activation of the same intracellular paths that regulate cellular growth and apoptosis. Much more is known about the similarities of the intracellular signals activated by CSFs than their differences.

Clinical use

G-CSF and GM-CSF are already at a relatively advanced stage of their clinical evaluation. They have proven their value in accelerating the recovery of white blood cell counts following myelotoxic procedures such as bone marrow transplantation or chemotherapy. Further indications, for example in the treatment of infections, are being explored. Given the role of colony-stimulating factors in inflammatory processes, it is possible in the future that small molecules that antagonize their action will find use as modulators in diseases in which inflammation plays a role. Thrombopoietin (TPO) – also known as megakaryocyte growth and differentiation factor or megapoeitin – is a new member of the four-helix bundle cytokine family that is able to stimulate increases in levels of platelets in normal animals. In myelosuppressed animals TPO also stimulated recovery of neutrophils, probably reflecting the synergistic

effects of combinations of TPO and other hematopoietins on the *in vitro* proliferation of hematopoietic progenitors of multiple lineages. Administration of IL-11 also accelerates recovery of platelet levels in myelosuppressed animals and, like TPO, also accelerates the regeneration of neutrophils.

See also: Bone marrow and hematopoiesis; Cytokine assays; Granulocyte colony stimulating factor (G-CSF); Granulocyte-macrophage colony stimulating factor (GM-CSF); Interleukin 3; Macrophage colony stimulating factor (CSF-1); Organ culture of lymphoid cells.

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COMMON VARIABLE IMMUNODEFICIENCY

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Common variable immunodeficiency (CVI) is a heterogeneous syndrome characterized by hypogammaglobulinemia and recurrent bacterial infections. This rare syndrome has an estimated prevalence ranging from 1:50 000 to 1:200 000. Although classified as a primary antibody deficiency syndrome, CVI represents a generalized state of immune dysregulation characterized by functional abnormalities of both T and B cells. Clinically, this is reflected in the susceptibility of these patients to a variety of conditions not easily explained on the basis of an isolated humoral immune defect.

Clinical features (Table 1)

Patients with CVI present with recurrent bacterial infections of the respiratory tract, such as sinusitis, otitis media, bronchitis and pneumonia. The most common etiologic agents are encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. The onset of these abnormally frequent infections may occur at any age but most patients do not come to medical attention until the second or third decade. This is in contrast to X-linked agammaglobulinemia, where recurrent infections develop in the first 2 years of life. The recurrent bacterial infec-

tions in CVI are a direct result of the deficiency in antibody production (specifically immunoglobulin G, IgG) that is the hallmark of this syndrome. If CVI goes undiagnosed (and hence untreated), recurrent pulmonary infections can lead to irreversible chronic lung disease with bronchiectasis. Septicemia and recurrent infections of the skin, urinary tract, joints or central nervous system also occur in patients with CVI, but are less frequent. In rare instances, patients with CVI can become infected with a variety of opportunistic fungi, mycobacteria and *Pneumocystis carinii*. An unusual syndrome of severe enteroviral meningoencephalitis is seen in patients with primary antibody-deficiency syndromes. This type of infection is most commonly associated with X-linked agammaglobulinemia, but several well-documented cases have occurred in patients with CVI. Other viral infections that can occur with increased frequency in CVI include recurrent attacks of herpes simplex and herpes zoster.

Patients with CVI suffer from a variety of infectious and noninfectious gastrointestinal disorders. The protozoan *Giardia lamblia* is a common cause of infectious diarrhea in these patients. Malabsorption, of unknown etiology, leading to severe weight loss and diarrhea, is another major gastrointestinal

Table 1 Clinical features of common variable immunodeficiency

Relative frequency	Recurrent infections		Autoimmune disease	Other
	Manifestation	Pathogens		
Most common	Sinusitis, otitis media, pneumonia	<i>Streptococcus pneumoniae</i> <i>Haemophilus influenzae</i>	Hemolytic anemia Neutropenia, thrombocytopenia	Lymphadenopathy Splenomegaly Nodular lymphoid hyperplasia
	Infectious diarrhea	<i>Giardia lamblia</i> <i>Salmonella</i> spp. <i>Campylobacter</i> spp. <i>Cryptosporidium</i> Dysgonic fermentor-3	Pernicious anemia Autoimmune thyroid disease Rheumatoid arthritis Juvenile rheumatoid arthritis	Bronchiectasis
Less common	Septic arthritis	<i>Staphylococcus aureus</i> <i>Mycoplasma</i> spp. <i>Haemophilus influenzae</i>	Systemic lupus erythematosus Sjögren's syndrome Ulcerative colitis Crohn disease	Malignancy Cholelithiasis Idiopathic malabsorption Sarcoid-like granulomatous disease
	Meningitis	<i>Streptococcus pneumoniae</i> <i>Haemophilus influenzae</i> <i>Neisseria meningitidis</i>		
	Encephalitis	Enteroviruses		
	Pneumonia	<i>Pneumocystis carinii</i>		

manifestation of CVI. Small bowel biopsy reveals flattening of the villi with a lymphocytic infiltration in the lamina propria. Neoplasms of the gastrointestinal tract (specifically, adenocarcinoma of the stomach and intestinal lymphomas) appear to occur with increased frequency in CVI.

Approximately 25% of patients with CVI will develop one or more autoimmune diseases, indicating that CVI is a disease of abnormal immune regulation as well as immunodeficiency. Autoimmune (Coombs'-positive) hemolytic anemia and idiopathic thrombocytopenic purpura (ITP) are the two most common autoimmune diseases seen. Neutropenia is also seen in a significant number of patients with CVI, and in some cases antigranulocyte antibodies have been demonstrated. A variety of other autoimmune diseases can occur in association with CVI (Table 1).

Patients with CVI frequently develop lymphoproliferative disorders, which can take several forms. Malignant lymphoma occurs with increased frequency in these patients, although the exact magnitude of this increase is unclear. This high incidence of lymphomas (and cancers in general) is an indication that some patients with CVI may have clinically important defects in cell-mediated as well as humoral immunity. More common than malignant lymphoma is the occurrence of benign lymphoid hyperplasia manifested by one or more of the follow-

ing: splenomegaly; diffuse lymphadenopathy; or nodular lymphoid hyperplasia of the intestinal tract.

Immunogenetics

Evidence for a genetic influence was noted very early in the study of CVI, when family studies disclosed that a significant number of patients had first-degree relatives with other immunologic diseases such as isolated IgA deficiency and various autoimmune diseases. Recent studies have found an association of CVI and IgA deficiency with certain major histocompatibility complex (MHC) haplotypes. These results suggest that CVI and IgA deficiency are related disorders, susceptibility to which is determined by a gene or genes in or near the MHC region.

Immunologic features

The hallmark of CVI is a marked decrease in serum IgG, which is usually associated with significant depression of serum IgM and IgA levels. Despite this panhypogammaglobulinemia, the majority of patients with CVI have normal numbers of B cells in the circulation and peripheral lymphoid tissues. However, the numbers of surface IgG⁺ and IgA⁺ B cells are often reduced, suggesting an impaired ability of CVI B cells to undergo immunoglobulin class switching.

Although the exact nature of the immunologic defect(s) that give rise to CVI are unknown, a number of *in vitro* immunologic abnormalities have been identified in these patients. In early studies, peripheral blood lymphocytes from patients with CVI were stimulated *in vitro* with the lectin pokeweed mitogen, and culture supernatants were assayed for immunoglobulin production. A consistent finding in this type of study was the inability of peripheral blood lymphocytes from patients with CVI to secrete normal amounts of immunoglobulin. Since pokeweed mitogen-induced immunoglobulin secretion is strictly dependent on the presence of functional helper T cells, it was unclear whether this decreased immunoglobulin production by patient B cells was due to an abnormality intrinsic to the B cell or secondary to a T cell abnormality. In subsequent studies partially purified T cells and B cells of patients were separately analyzed in pokeweed mitogen-driven cultures containing allogeneic normal T cells and B cells. This approach showed that most patients with CVI appeared to have a B cell defect, in that coculture of their B cells with normal allogeneic T cells did not correct the defect in immunoglobulin production, and T cells from most patients were able to support immunoglobulin production by normal B cells. Occasional patients whose T cells were capable of suppressing normal B cell immunoglobulin production were identified, but in most patients shown to have excessive suppressor T cell activity, coculture of their B cells with normal T cells failed to rescue B cell function, thereby indicating that these patients also had a B cell defect.

More recent *in vitro* studies indicate that B cells from patients with CVI can be induced to proliferate and secrete immunoglobulin if appropriately stimulated. Studies using combinations of B cell mitogens or monoclonal antibodies (anti-CD40, anti-IgM) and various cytokines have shown that the severity of the defect in B cell immunoglobulin secretion varies among patients. In most cases B cells from patients with CVI can be induced to secrete at least some immunoglobulin; however, only a minority of patients produce normal amounts of all immunoglobulin isotypes. The *in vitro* defect in immunoglobulin secretion seen in CVI tends to be hierarchical, with IgM secretion affected least, IgG secretion affected to an intermediate degree, and IgA secretion reduced to the greatest extent. These results indicate that the B cell defect in CVI is not absolute and suggest that B cells from a subset of patients with CVI may only require the appropriate stimuli (which are presumably lacking *in vivo*) to mature into immunoglobulin-secreting plasma cells.

In addition to abnormalities of B cell function,

many patients with CVI also exhibit *in vitro* abnormalities of T cell function. In about half of CVI patients, a selective abnormality of T cell activation can be demonstrated; it appears to arise from a signaling defect and is manifest as reduced expression of genes directing the synthesis of interleukin 2 (IL-2), IL-4 and IL-5. In addition, T cells from a subset of CVI patients fail to express normal levels of CD40 ligand following activation. Since both CD40 ligand and lymphokines are essential ways by which T cells provide help to B cells for antibody secretion, the fundamental immunologic abnormality in some patients with CVI may be a defect in T cell help.

While most patients with CVI have a normal CD4:CD8 T cell ratio, approximately one-third of patients exhibit a reduced CD4:CD8 T cell ratio due to elevation in the absolute number of CD8 cells, most of which also express CD57 and HLA-DR. The expanded population of CD8 T cells in these patients have phenotypic and functional properties that are characteristic of activated cytotoxic T lymphocytes. The origin and functional significance of the expanded population of CD8 T cells in this subgroup of CVI patients is unclear.

Treatment

Standard therapy of CVI currently consists of intravenously administered immunoglobulin, which is less painful and more effective at preventing infections than intramuscular immunoglobulin. Vigorous treatment of infections and readiness to promptly evaluate and treat any unusual complications are also important aspects of managing these patients. Treatment of noninfectious complications is the same as in nonimmunodeficient patients.

See also: Gammaglobulin; Immunodeficiency, primary.

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COMPLEMENT, ALTERNATIVE PATHWAY

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The term 'alternative pathway of complement activation' is used to describe the activation pathway that is alternative to the so-called 'classical' pathway of activation which was the model of complement activity used to characterize the system. The classical pathway is the reaction sequence by which antibody-sensitized sheep erythrocytes are lysed by human or guinea pig complement. It was already inherent in the original description of the third component of complement, by Coca in 1914, that there must be a separate pathway of complement activation used by yeast cell walls which depleted complement activity without depleting either C1 or C2. It was not until the 1950s, however, that Pillemer and his colleagues recognized that there was a novel pathway of complement activation which they described as the 'properdin pathway'. This pathway did not require antibody for its initiation and they postulated it to be a major mediating pathway for nonspecific immunity. They described a new protein – 'properdin' – which they ascribed the central role in this pathway and major importance in resistance to infection and tumors. Towards the end of the 1950s this group also described the existence of other new factors that were needed for the inactivation of C3 by zymosan. Although much of this work later turned out to be essentially correct, it was much contested at the time, largely by RA Nelson (in 1958), and the existence of

the alternative pathway was not fully accepted until the discovery of sera genetically deficient in C2 and C4 in the 1960s. It was also at this time that the complement components were first isolated as proteins and as antigens so that the biochemistry of their interactions could be worked out. It was not until the 1970s, however, that the biochemistry and reaction pathway of the alternative pathway was clarified after a period of considerable confusion.

At the heart of the alternative pathway is the C3 feedback cycle (**Figure 1**). The primary large cleavage product of C3 is C3b, which combines in the presence of magnesium ions with factor B – the C2-like protein of the alternative pathway – to form a bimolecular complex, C3b,B. This acts as a substrate for factor D – the C1 analog of the alternative pathway – which cleaves a bond in factor B splitting off a fragment, Ba, and leaving the complex C3b,Bb. This is the 'C3'convertase' of the alternative pathway and is the homologous enzyme to C4b,2b; the C3-converterase of the classical pathway. Generating a C3 cleaving enzyme from C3b provides a positive feedback amplification loop, generating more C3b and consuming more factor B until one or other of these components is exhausted. Factor D is a single domain serum protease occurring in plasma in active form. It was a great surprise when it was recently discovered, following the cloning of the molecule,

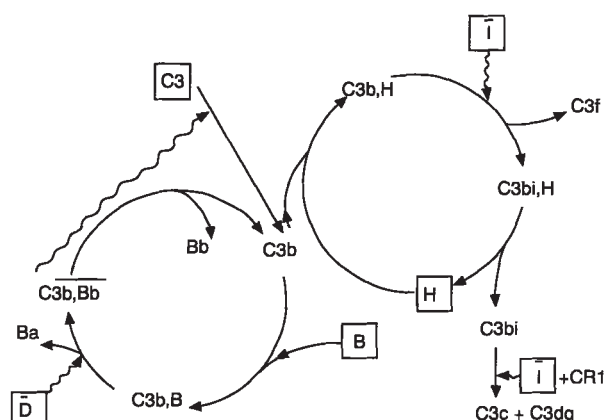


Figure 1 C3b feedback and breakdown. (From Lachmann PJ and Hughes-Jones NC (1984) *Seminars in Immunopathology* 7: 143–162.)

that factor D is identical to a serine protease known as 'adipsin' which occurs in fat tissue and in nerves. The function of adipsin is unknown, but its levels are strikingly reduced in certain genetic forms of obesity and of diabetes.

The C3b feedback cycle is subject to homeostatic control by an analogous reaction cycle where C3b combines with another normal serum protein – factor H – to give a complex C3b,H which is cleaved by another serine protease present in active form in plasma – factor I. Factor I cleaves two bonds in C3b, cleaving out a small fragment – C3f – and leaving the large fragment iC3b which still has important functions as an inflammatory mediator but is no longer capable of combining with factor B or giving rise to a C3 convertase. The rate of C3 activation by the alternative pathway is controlled by the relative velocities of the C3b feedback cycle on the one hand and the C3b breakdown cycle on the other.

The alternative pathway is therefore fired by any mechanism which either increases the rate of C3b production or reduces the rate of C3b breakdown. Mechanisms that increase C3b production include:

1. *The generation of extrinsic enzymes that cleave C3.* The principal of these is the C3-convertase of the classical complement pathway, C4b,2b. Kallikrein and plasmin also split C3 as does elastase and a number of proteases from phagocytic cells or from bacteria. At inflammatory sites a number of these enzymatic systems may be active.
2. *Mechanisms that stabilize the alternative pathway C3 convertase.* Physiologically this enzyme is stabilized by properdin which binds to it. This is the part that properdin plays in the alternative pathway – not at all what the Pillemer group had envisaged. Although properdin levels have not

been found of great diagnostic value, the total absence of properdin leads to (usually severe) immunodeficiency. A more complete stabilization of C3b,Bb is produced by autoantibodies that react with it. Such autoantibodies are called 'nephritic factors' since they were first described in mesangiocapillary glomerulonephritis. However, the renal disease follows the formation of the nephritic factors rather than causing it and the mechanisms underlying the formation of the nephritic factor remain unknown.

An artefactual way of producing a stable C3-convertase is the use of cobra venom factor. This is a reptilian analog of a C3 breakdown product that functionally resembles C3b and has the curious property that it combines with mammalian factor B but is not dissociated by mammalian factor H and therefore produces a stable C3-convertase not susceptible to homeostatic control which causes the C3b feedback cycle to run to exhaustion.

The principal mechanism that decreases the rate of C3b breakdown is the 'protected surface' phenomenon; C3b bound on certain surfaces is relatively poorly reactive with factor H and therefore resistant to breakdown whilst supporting the C3b feedback cycle by reaction with factor B. Such protected surfaces include particulate polysaccharides (for example zymosan and particulate inulin); the surfaces of most parasites and of many virus-infected and some tumor cells; and certain immunoglobulin aggregates particularly those of IgA. More dramatic failure of C3b breakdown occurs in the genetic deficiencies of factor I or of factor H. A further mechanism of reducing C3b breakdown is provided by local removal of factor H. This was first described for 'sulfated sephadex' and has been seen also with an intestinal glycoprotein and a myeloma light chain dimer. The physiological or pathophysiological relevance of this mechanism is still unclear.

The view that the alternative pathway is essentially governed by the velocity of competing reactions requires that there is a minimal level of C3b (or of a C3b-like protein) always available. This is the essential feature of the 'tickover' model of the alternative pathway. It was originally proposed that the tickover was maintained by minimal levels of proteolysis of C3 by any of the enzymes that can cleave it and indeed this is likely to be the case. However, another mechanism has been suggested: namely that the hydrolysis by water of the internal thioester bond generates a C3b-like molecule and that this spontaneous hydrolysis of C3 may be sufficient to keep the tickover working. The levels of C3b or C3b-like proteins that are required for the maintenance of the

tickover are so low that it has not so far been possible to define critically the relative roles of proteolysis and thioester hydrolysis in maintaining the tickover *in vivo*.

See also: Immunoconglutinins; Complement, alternative pathway; Clotting system; Cobra venom factor; Complement, classical pathway; Complement deficiencies; Complement, genetics; Complement receptors.

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COMPLEMENT, CLASSICAL PATHWAY

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The complement system consists in total of about 20 soluble proteins in plasma and other body fluids, and a similar number of cell surface receptors and control proteins, present on blood cells and other tissues. Complement has a major role in controlling the uptake and removal, by phagocytosis, of materials such as immune complexes, microorganisms or host cell breakdown products, which are recognized by, and activate the system. Complement proteins, and their fragments generated during activation, also have roles in regulating cellular immune responses. Individual complement proteins serve the functions of recognizing and binding to target material. Recognition is followed by activation of serine proteases of the complement system (Figure 1), which assemble on the surface of the target. One such protease is termed C3 convertase and is capable of cleaving the most abundant soluble complement protein, C3. C3 is then cleaved and activated, and a major fragment of C3, called C3b, binds covalently to the target. Covalent binding occurs by direct nucleophilic attack of surface hydroxyl or amine groups of the target on an activated thioester within C3b. Surface-bound molecules of C3b, or of its proteolytic breakdown product iC3b, serve as ligands for C3 receptors on phagocytic cells, and so act as labels for phagocytosis

of the target. Surface-bound C3b also forms part of the binding site for C5, which is cleaved by the same protease which cleaved C3. The proteolytic fragment C5b binds to C6, C7, C8 and C9 to form the membrane attack complex, which, if the target material contains a lipid bilayer, associates with the membrane and may cause cell lysis.

The activation of the complement system occurs by either or both of two distinct routes (Figure 1). The first route for which the sequence of reactions was elucidated was termed the *classical pathway*, which consists of the proteins (or 'components') C1, C2, C4 and C3 (named in order of their sequence of action, which by historical accident, differs from numerical order). C1 is a large complex made up of three distinct proteins (or 'subcomponents') called C1q, C1r and C1s. A more recently elucidated route is called the *alternative pathway*, and consists of the proteins factor B, factor D and C3, together with the regulator proteins factor H, factor I and properdin.

Both pathways contain a target recognition mechanism, and both pathways lead to assembly of proteases which cleave C3 and C5. Cleavage of C5 by either pathway results in activation of the lytic pathway (components C5–C9). In the last few years, it has been shown that another protein similar in shape

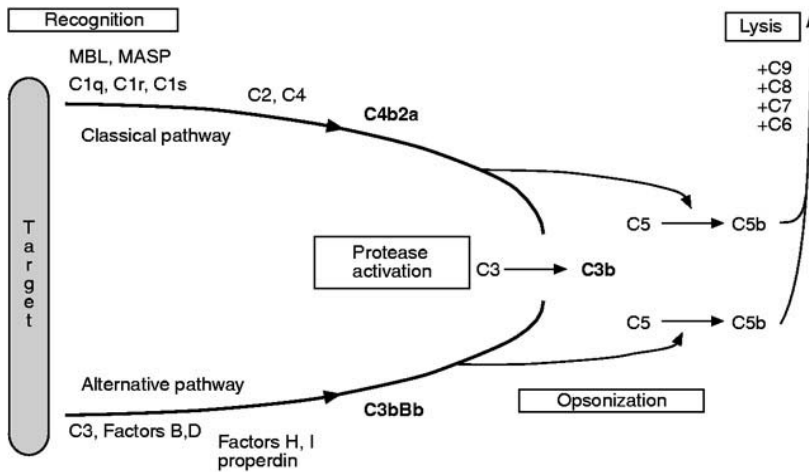


Figure 1 Pathways of the complement system. The major stages of target recognition, protease activation, opsonization by deposition of C3b, and cell lysis are shown. Interactions of the classical pathway components are discussed in the text. The 'C3 convertase enzymes', C4b2a and C3bBb are shown prominently.

to C1q can also participate in classical pathway activation. This is mannose-binding lectin (MBL), a member of the collectin protein family. MBL appears to be associated with two proteases, named MASP-1 and MASP-2, which are homologous to C1r and C1s, and appear to have similar function. In some publications, activation of complement via MBL is regarded as a third, separate pathway (the lectin pathway), but it is discussed here as a 'branch' of the classical pathway. Discussion of the classical pathway thus is limited to the interactions of C1, C2, C4 and C3, MBL and the MASPs, together with 1) the soluble regulatory proteins C1-inhibitor, C4b-binding protein (C4bp) and factor I, which control activation of this part of the complement system, and 2) the cell surface receptors or regulatory proteins which interact with classical pathway components. These include complement receptor type 1 (CR1), membrane cofactor protein (MCP), decay-accelerating factor (DAF) and C1q receptor (C1q-R).

Distinction between classical and alternative pathways

In experimental work, the two pathways of activation can be readily distinguished, in that both pathways require Mg^{2+} , but only the classical pathway requires Ca^{2+} ions: if these are chelated by EGTA (ethyleneglycol-bis-(β -amino-ethylether) tetra-acetic acid) and Mg^{2+} ions are added in excess, only the alternative pathway will function. Further, the activation of the alternative pathway is prevented by dilution of human serum beyond about ten-fold, and is generally considerably impaired after 2–3 days storage of human serum at 4°C: the classical pathway, via C1q, operates at lower concentrations of serum and is stabler on storage. The stability of

activation occurring via MBL has not been extensively examined.

The proteins of the classical pathway and their mechanisms of action

C1

C1 is a multimeric protein consisting of two molecules each of the serine protease proenzymes C1r and C1s bound to a single molecule of C1q. The associations of C1q, C1r and C1s are the Ca^{2+} ion-dependent stages of the pathway. C1q itself consists of 18 polypeptide chains, of three different types, A, B and C, which are encoded by separate, closely linked genes on the short arm of human chromosome 1. The A, B and C chains are each about 225 amino acids long, and are homologous to each other. Each consists of a short N-terminal region containing a cysteine residue, followed by a stretch of 81 amino acids of collagenous sequence (i.e. repeating triplets of sequence Gly-X-Y, where Y is often a hydroxylated proline or lysine residue). The rest of the sequence is noncollagenous. The collagenous regions of one A, one B and one C chain align to form a short stretch of collagen triple helix structure, and the rest of the three chains fold to form a globular head, which consists of three lobes, one derived from each of the A, B and C chains (Figure 2). The collagenous stalk is bent in the middle, because of irregularities in the collagenous sequences. Fully assembled C1q (Figure 2) has a single cylindrical core, made up of the N-terminal ends of the six collagen triple helices: at the bend in the middle of the helices, they splay apart, forming six stalks, which join the six globular heads to the core. The overall appearance of C1q is likened to a bunch of six tulips. The globular head regions of C1q are responsible for recognition and binding to most classical pathway activators,

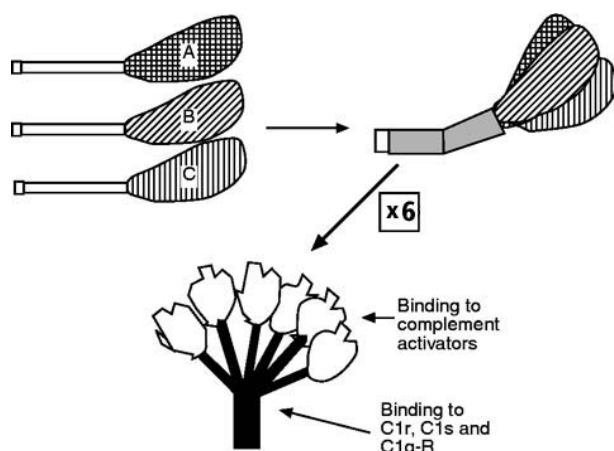


Figure 2 The assembly of C1q from three types of polypeptide chains. The assembly is discussed in the text. MBL has similar structure and assembly, but only one type of polypeptide chain.

while the collagenous regions interact with the next complement proteins in the pathway, C1r and C1s.

C1r and C1s are serine protease proenzymes, encoded by closely linked genes on human chromosome 12p13. Each monomer consists of a single polypeptide chain of about 700 amino acids: the C-terminal approximately 250 amino acids in each is a serine protease domain, homologous to trypsin or chymotrypsin. Like most complement proteins, they are mosaic proteins, i.e. segments of the primary sequence can be recognized as being homologous to segments of many other proteins, and are likely to represent independently folding domains or modules. The approximately 450 N-terminal amino acids of C1r and C1s are homologous to each other, and are made up of five domains (see Figure 3). Each con-

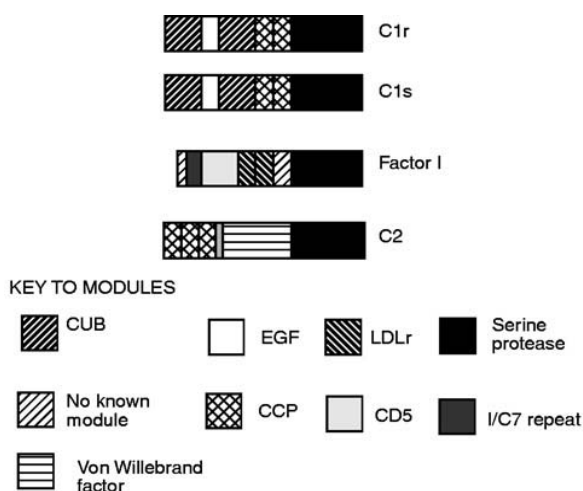


Figure 3 The modular structure of C1r, C1s, C2 and factor I. MASP-1 and MASP-2 have the same modules as C1r and C1s. (This diagram is adapted from the work of Dr A. J. Day.)

tains an EGF (epidermal growth factor) module, which is the Ca^{2+} -binding site. Each also contains two internal repeats of about 100 amino acids, known as CUB (C1r/C1s, Uefg, Bmp1) modules, and two CCP (complement control protein) modules, each about 60 amino acids long. CCP molecules are found in many complement proteins, and are a particular feature of the proteins which interact with C3 and C4 turnover, such as factor H, C4bp, CR1, MCP and DAF. The CUB domains are likely to be involved in binding to C1q.

The overall structure of each C1r or C1s monomer is a dumb-bell shape, with two globular regions of unequal size connected by a short strand. Isolated C1r exists as an elongated dimer, with the dumb-bells joined end-to-end. Two molecules of C1s bind, one to each end of the C1r dimer, in the presence of Ca^{2+} ions. This forms the very elongated C1r₂C1s₂ tetramer, which binds to the collagenous regions of C1q. The structure of the large C1 complex is not clearly established, but several models which indicate how C1r₂C1s₂ might interact with C1q have been proposed (see Colomb *et al.*, 1989 for review).

Activation of C1 occurs when two or more globular heads of C1q bind to a complement activator: movement of the heads is transmitted by the collagenous stalks to the core region and thence to C1r. A conformational change is induced in C1r, which causes the C1r molecules to cleave and activate each other: these then cleave and activate the C1s molecules. During this process, each C1r and C1s monomer is cleaved at the N-terminal end of the serine protease domain, forming a structure with two disulfide-linked polypeptide chains of approximately 56 and 28 kDa. The activated forms have very restricted proteolytic activity: activated C1r will cleave only proenzymic C1s, and activated C1s will cleave only C2 and C4. Both proteases have arginyl specificity. Both also have a low level of esterase activity towards esters of basic (C1r) or basic and hydrophobic (C1s) amino acids. In older literature, activated C1s is often described as C1 esterase, and the nomenclature C1r, C1s, and occasionally C1r₂, C1s₂ denoting the activated forms, is also frequently encountered.

C4

Once C1s has been activated, it will cleave and activate C4. C4 is a 205 kDa protein, synthesized as a single polypeptide chain, but processed on secretion into a form with three disulfide-linked polypeptides, named α , β and γ (97, 75 and 33 kDa). C4 is homologous to C3 and C5, and also to the family of protease inhibitors related to α_2 -macroglobulin. This group of proteins do not have an obvious mosaic

structure, and little can be predicted of their tertiary structure. Low-resolution analysis, by S.J. Perkins and colleagues, of the structure of C4 or C3 in solution, show that they are flat, ellipsoid molecules, about 18 nm long and 10 nm wide. C4, like C3, contains an internal thiolester in the α chain, formed between the Cys and Gln residues of a sequence Gly-Cys-Gly-Glu-Gln.

Activated C1s cleaves a 9 kDa fragment, C4a, from the N-terminus of the α chain of C4. C4a has weak anaphylatoxin activity. The rest of the molecule, C4b, contains the thiolester, which becomes exposed on proteolytic cleavage. The thiolester is very reactive, and will be attacked by any nucleophilic (electron-donating) species in the vicinity. Since C4 is normally activated by C1s which is within C1 bound to a complement activator, the reactive C4b (often called nascent C4b) is generated close to the surface of the activator, and can react with surface OH, NH₂ or SH groups. These form ester or amide linkages to the carbonyl group of the thiolester, and the carbon-sulfur bond in the thiolester is broken, forming a free SH group on the C4b (Figure 4). In this way, C4b becomes covalently bound to the surface of the complement activator. Much of the nascent C4b generated, however, simply reacts with the most abundant nucleophile present, water, and does not become surface-bound. This species, in which the thiolester is hydrolysed, plays no further part in complement activation.

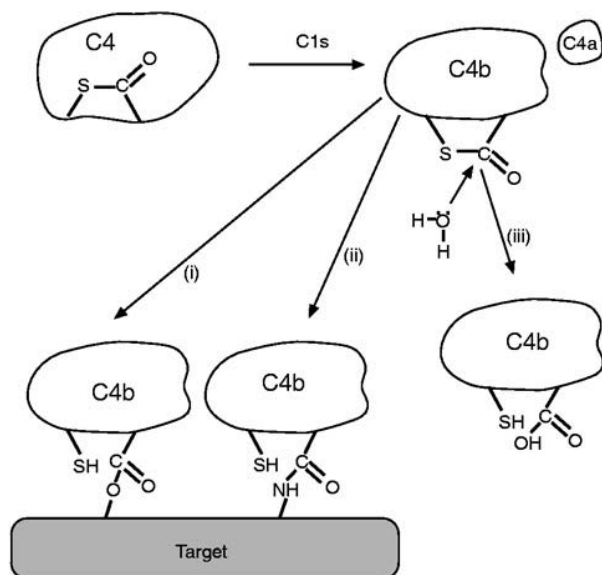


Figure 4 The activation of C4, and covalent binding of C4b. C4 contains an internal thiolester which becomes exposed on cleavage of C4 by activated C1s. The exposed thiolester may react with OH or NH₂ groups on the surface of the complement activator (i) and (ii) or may simply react with water (iii). The activation and binding of C3 occurs in the same way.

The major histocompatibility complex (MHC) class III region of chromosome 6 contains two functional genes for C4: these encode the C4A and C4B isotypes of C4. The sequences of C4A and C4B differ by less than 1%, and both function as C4 in the complement system. They have differences, however, in their covalent binding reaction: activated C4B binds with similar efficiency to hydroxyl and amino groups, while C4A reacts much more readily with amino groups. In a standard assay of complement activity, in which erythrocytes are lysed by complement, C4B is more active than C4A, probably because C4B binds more readily to the carbohydrate-rich red cell surface than does C4A. In contrast, C4A binds better than does C4B to immunoglobulin G (IgG) immune complexes containing nonglycosylated protein antigen.

The selectivity of covalent binding of C4A and C4B appears to be controlled mainly by an Asp (C4A) to His (C4B) amino acid interchange at position 1106 in the sequence. These residues, although not adjacent to the thiolester in the primary sequence, are close in the tertiary structure, and affect polarization of nucleophilic groups approaching the thiolester.

The C4A (acidic) and C4B (basic) nomenclature is based on electrophoretic mobility and corresponds to the older terms 'fast' and 'slow' (C4F, C4S). Each isotype of C4 has many allotypes, distinguishable by agarose electrophoresis, which are designated C4A1, C4A2, C4B1, C4B2, etc. Null alleles (termed C4AQO or C4BQO) at either C4 locus are relatively common. The A and B nomenclature must be carefully distinguished from the a and b nomenclature used to designate proteolytic fragments: terms such as C4Aa, C4Ab, used to denote the proteolytic fragments of the A isotype, are also encountered. C4 fragments bound to erythrocytes carry the Chido/Rodgers blood group. In general C4A allotypes carry the Rodgers blood group, while C4B carries Chido determinants.

C2

C2 is a serine protease proenzyme, which is highly homologous to factor B of the alternative pathway. Both proteins are encoded by genes in the MHC class III region of human chromosome 6, closely linked to the C4A and C4B genes. C2 and factor B are, like C1r and C1s, mosaic proteins (see Figure 3). The N-terminal portion of C2 is made up of three CCP modules, followed by a segment of about 200 amino acids which is homologous to structures in von Willebrand Factor (and also in complement receptor type 3, CR3), and is referred to as a vWF-A domain. This is followed by a serine protease domain. C2 is

cleaved by activated C1s at an arginyl bond situated between the third CCP module and the vWF domain. This creates two nondisulfide-linked fragments, C2b (34 kDa, from the N-terminus) and C2a (74 kDa, C-terminal). There is some confusion in C2 fragment nomenclature, and the term C2a was sometimes used for the smaller fragment. Although C2 can be cleaved by isolated, activated C1s in solution, the C2 fragments produced have no proteolytic activity. To be active, C2 must form a complex with C4b before it is activated. During normal complement activation, C1s, within C1, bound to an activator, will cleave several molecules of C4, some of which will bind covalently to the activator. These can then bind C2, but in only a very few of the C4b–C2 complexes will the C2 be in an appropriate position to be activated by the surface-bound C1s. This may occur, for example, when C1 is fixed to the IgG Fc in an immune complex, and the C4b is fixed to the Fab arms. C2 associates first with C4b via the CCP-domain region at the N-terminus: this is Mg^{2+} -dependent. Cleavage of C2 by C1s then occurs, and the C2a portion remains bound to C4b by a secondary non- Mg^{2+} -dependent interaction, probably through the vWF-A domain. The complex C4b–C2a (generally written C4b2a) is proteolytically active, and is the C3 convertase enzyme of the classical pathway. The proteolytic active site is the serine protease domain of C2a. Proteolytic specificity is restricted to cleavage of single arginyl bonds in C3 and C5. Activated C2, whether free in solution or bound to C4b, has a very low level of esterolytic and amidolytic activity.

C3

C3, as noted above, is homologous to C4: it is a major component of the alternative, as well as the classical pathway. C3 is synthesized as a single polypeptide chain, and processed on secretion into two disulfide-linked chains, α (110 kDa) and β (75 kDa). C3 is cleaved and activated by C4b2a (or by the alternative pathway C3 convertase, C3bBb). Cleavage releases the anaphylatoxic fragment C3a (77 amino acids) from the N-terminus of the α chain. The rest of the molecule, C3b, has like C4b (Figure 4), an exposed reactive thiolester, and will react with surface hydroxyl or amine groups on the complement activator, or with water. Covalent binding to an activator is called the 'acceptor binding reaction' of C3b to distinguish it from noncovalent binding to specific receptors. Again only about 10% of the 'nascent' C3b generated binds to the activator: the rest reacts with water and diffuses away. The half-life of the reactive thiolester is $<100 \mu s$, and so nascent C3b can diffuse only a short distance before

it reacts with a nucleophile. The surface-bound C3b is therefore clustered around the site of proteolytic activation: such clusters are likely to be important in promoting adhesion to C3 receptors. Among the C3b molecules which become activated, one may become bound to the C4b portion of the activating enzyme. This forms a C4b2a3b complex. The covalently associated C4b and C3b molecules form a binding site for C5, which orients C5 for cleavage by the protease domain of C2a, thus initiating the lytic phase of the complement system. C4b2a3b is referred to as the C5 convertase of the classical pathway.

The lectin pathway

Recent work has shown that a protein similar in structure to C1q, mannan- or mannose-binding lectin (MBL), can substitute for C1q in the activation of complement. MBL was formerly called mannose- or mannan-binding protein (MBP) or Ra-reactive factor (RaRF). MBL is made up of 18 identical polypeptide chains, which like those of C1q, contain an N-terminal collagenous segment, and a C-terminal noncollagenous region. The chains associate in groups of three to form collagen triple helices, and six of these groups associate to form the whole molecule (as shown for C1q in Figure 2). MBL, like C1q, has a collagenous core and stalks, and three-lobed globular heads. The major difference from C1q is that the MBL globular heads are lectins, and bind to carbohydrate in the presence of calcium ions. MBL binds mainly to mannose-rich structures, such as yeast mannan. MBL is thought to occur in forms with three, four, or five globular heads instead of six, but this may be an artefact of purification. *In vitro*, MBL bound to a target such as mannan binds and activates $C1r_2C1s_2$, and so activates the complement classical pathway. Other evidence strongly suggests however that *in vivo* MBL is associated with other proteases. One of these, MASP (MBL-associated serine protease) or MASP-1 is known to form a complex with MBL, and is highly homologous to C1r and C1s. Another highly homologous protease MASP-2 has been characterized by cDNA sequencing, and appears also to be involved in complex formation with MBL. The activities of MASP-1, and particularly MASP-2 are not yet known in detail. However MASPs can cleave and activate C2 and C4, and are thought also to cleave C3 at a low rate. MBL therefore probably mediates activation of the classical pathway via the MASPs. In density-gradient centrifugation of plasma, MBL sediments as a 19S complex, of the same size as the C1 ($C1qr_2s_2$) complex. C1q or MBL which are not associated with proteases migrate as 11S proteins. This indicates that MBL

forms a complex like C1 in plasma. This is probably an MBL–MASP complex, although formation of MBL–C1_{r2s2} complexes might also be possible. This MBL complex has previously been characterized under another name, Ra-reactive factor (RaRF) which has been identified in sera from several vertebrates, particularly rodents. RaRF was detected by its binding to the Ra polysaccharide found on enterobacteria, e.g. *Salmonella*, and was shown to consist of MBL and a serine protease (presumably corresponding to MASP-1, or possibly MASP-1 and -2) which activates C2 and C4.

Activators of the classical pathway

The classical pathway is activated, via C1q or MBL, by a wide range of materials. Both C1q and MBL mediate antibody-dependent and nonantibody-dependent activation. These are summarized in Table 1. For C1q, activators include immune complexes containing IgG or IgM, or *in vitro*, aggregated IgG. Human subclasses IgG3 and IgG1 are more efficient activators than IgG2 and IgG4. Interaction with IgG is via the C_γ2 domain, and protein engineering studies by Winter and Duncan indicate that charged residues Glu318, Lys320 and Lys322 in IgG are of major importance in C1q binding. In IgM, the C_μ3 domain is implicated in C1q binding. MBL binds to glycosylation variants of IgG (IgG

lacking terminal galactose residues on the Fc oligosaccharides), and activates complement. MBL also interacts with high-mannose oligosaccharides on IgM, although it is not known whether this interaction does lead to complement activation.

A wide range of gram-negative bacteria have been reported to bind C1q directly, and activate C1, without need for antibody. Mycoplasma species may also activate C1, as do some viruses, including Epstein–Barr virus and Rauscher leukemia virus, and some parasites, such as *Entamoeba*. Many bacteria, viruses and single-cell or multicellular parasites activate complement without need for antibody: alternative pathway activation is the commoner mechanism, but in many cases the contribution of the classical pathway has not been fully studied. Yeasts activate the classical pathway via MBL, as well as activating the alternative pathway. MBL also binds to saccharides on a wide range of bacteria, viruses and fungi, but complement activation via MBL has not generally been examined in these systems.

Tissue-damage products, such as mitochondrial and other subcellular membranes, cytoplasmic filaments, polynucleotides and components of myelin have also been reported to activate C1. The acute phase protein CRP (C-reactive protein), when bound to phosphorylcholine-containing phospholipids, binds and activates C1. Since C1q recognizes a very wide range of targets, mostly via its globular heads,

Table 1 Classical pathway activators

The classical pathway is mainly involved in recognizing charge groupings. This is the case for C1q and CRP, but not for MBL which recognizes neutral sugars. Charges may be on proteins, like IgG, or on complex charged carbohydrates found on surfaces of microorganisms, or on nucleic acid–protein complexes, or on lipids and glycolipids. Sialic acid on cell surfaces tends to promote activation of the classical pathway, but to inhibit activation of the alternative pathway.

Classical pathway activation occurs via:

C1q:	C1q binds to: - Fc portions of antigen-fixed IgG and IgM - Nucleic acid and chromatin - Cytoplasmic intermediate filaments (vimentin-type) - Mitochondrial membranes possibly via cardiolipin or via mitochondrial proteins - Some viruses, e.g. MuLV - Gram-positive bacteria, e.g. some pneumococci, streptococci, via capsular polysaccharide (neuraminidase treatment reduces activating capacity) - Gram-negative bacteria via the lipid A component of the lipopolysaccharide of the cell wall
CRP + C1q:	CRP (C-reactive protein) recognizes phosphate groups in choline phosphate of pneumococcal C-type polysaccharide, and also binds to phosphocholine (PC)-containing and non-PC-containing microbial polysaccharides and lipids, polyanion/polycation complexes, and chromatin. It is also reported to bind to galactans. Bound CRP interacts with C1q, and activates the classical pathway
Mannose-binding lectin (MBL):	MBL binds to high mannose and other polysaccharides and oligosaccharides on yeasts, bacteria and viruses, and activates the classical pathway. It also binds to carbohydrate on glycosylation variants of IgG (IgG-Go). MBL probably acts, <i>in vivo</i>, via the proteases known as MASPs, but <i>in vitro</i> it can act via C1r and C1s. RaRF, formerly reported as being a specific factor mediating classical pathway activation by <i>Salmonella</i> Ra polysaccharide, is the same as MBL.

it is likely that the three homologous 'lobes' of each head have different binding specificities, but this has not yet been proved.

The quantitative significance of complement activation via MBL is not yet clear. MBL and the MASPs are at very low concentration in plasma relative to C1q, C1r and C1s (see Table 2).

Control mechanisms

The activation of the classical pathway has multiple control mechanisms. The proteolytic activity of activated C1 is controlled by C1-inh, which irreversibly activates C1r and C1s. It is also a physiological inhibitor of kallikrein and activated factors XII and XI. C1-inh is a 100–110 kDa single polypeptide chain protein, containing about 50% carbohydrate. It is homologous to other serpins, such as antithrombin III. It acts as a pseudosubstrate for the proteases which it inhibits: these cleave an Arg–Thr bond 34 residues from the C-terminus of C1-inh, but C1-inh remains covalently bound to the protease, probably by an ester bond formed by the arginine carbonyl and the active-site serine of the protease. C1-inh attacks activated C1 by first reacting with C1s, then with C1r: the C1r₂C1s₂ complex dissociates to form two C1inh–C1r–C1s–C1inh complexes, which no longer bind to C1q. C1q, still bound to the complement activator, can now bind to C1q receptor, or can participate in a further slow cycle of binding, activation and inactivation of C1r₂C1s₂. MASP-1 is

also inhibited by C1-inh, but this has not been investigated for MASP-2.

The C3 and C5 convertase enzymes are also subject to control: one aspect of control is the inefficiency of binding of nascent C4b and C3b to appropriate sites to form the convertases. Once formed, the convertases are unstable: C2a dissociates from C4b or C4b3b with a half-life of 2–5 min. Once C2a has dissociated, more C2 can bind to C4b, but the adjacent C1 molecule required to cleave the C2 may already have been inactivated. The stability of the convertases is further decreased by a group of control proteins which includes the plasma proteins factor H and C4bp, and the membrane proteins CR1, MCP and DAF. These proteins, together with CR2, are composed mainly, or only of CCP domains, and are all encoded by genes in the RCA (regulation of complement activation) cluster on human chromosome 1q31–32.

CR1, DAF and C4bp bind to C4b2a or C4b2a3b and cause C2 to dissociate: they also compete with C2 for the initial binding to C4b. CR1 and C4bp, and also MCP, once bound to C4b, render C4b susceptible to attack by the control serine protease, factor I, which cleaves at two sites in the α chain of C4b, forming C4c (145 kDa) and C4d (45 kDa), which no longer participate in convertase formation. CR1 and MCP also act as cofactors for the factor I-mediated cleavage of C3b to form iC3b, as does factor H. In the alternative pathway, CR1, DAF and factor H accelerate the decay of the C3 convertase,

Table 2 Properties of classical pathway proteins

Protein	Mol. wt (kDa)	Chromosomal localization of gene	Plasma concentration ($\mu\text{g ml}^{-1}$)
<i>Components</i>			
C1q	462	1	80
C1r	83	12	50
C1s	83	12	50
C4	205	6	600
C2	102	6	20
C3	185	19	1300
MBL	500	10	<1
MASPs	80–90	?	<1
<i>Regulatory proteins</i>			
C1-inh	110	11	200
C4bp	540	1	250
Factor I	88	4	35
<i>Membrane proteins</i>			
CR1	160–250	1	<i>Distribution on blood cells</i> Erythrocytes, polymorphs, monocytes, B lymphocytes
MCP	45–70	1	B and T lymphocytes, monocytes, polymorphs
DAF	70	1	Erythrocytes, leukocytes and many other tissues
C1q-R	60	?	B and T lymphocytes, monocytes, polymorphs

C3bBb, by displacing Bb, and by competing with factor B for binding to C3b.

Receptors and classical pathway components

C3b and its breakdown products, iC3b, C3dg and C3d, bind to several receptors, including CR1, CR2 and CR3: these are discussed in more detail elsewhere. C4b binds to CR1, and immune adherence to erythrocytes can be mediated by a CR1–C4b interaction, rather than by CR1–C3b. During normal complement activation, much more C3b is fixed than C4b, and it is unlikely that C4b has an important role in receptor-mediated adhesion or ingestion mechanisms. C1q also binds, via its collagenous region, to a receptor, a widely distributed 60 kDa species (C1q-R). C1q-R also binds other ligands (collectins) which are structurally similar to C1q: these include MBL, lung surfactant protein A and conglutinin. All of these ligands are reported to function as opsonins, and a major role of C1q-R may be a phagocytic uptake.

Pathological alterations of the classical pathway

The functioning of the classical pathway is disrupted in many circumstances. These include genetic deficiencies of single components: complete deficiencies of C1q, C1r, C1s, C4, C2, C3, factors H and I have been reported. These are associated with susceptibility to infection and/or inappropriate deposition of immune complexes. Low concentrations of MBL are associated with poor opsonization of yeast and some bacteria, and with recurrent infection in infants. Diminished synthesis of C1-inh, or synthesis of inactive C1-inh mutants is the cause of hereditary angioedema, and causes secondary consumption of C2 and C4. Autoantibodies to C1-inh have been reported, which destabilize the C1-inh-protease complex releasing free active protease: these give rise to autoimmune angioedema. Autoantibodies to C3 convertases, termed nephritic factors, occur in membranoproliferative glomerulonephritis. These generally recognize C3bBb, but a few reports indicate reactivity against C4b2a. They stabilize convertases, leading to excessive consumption of C3. Autoantibodies against bound C1q occur in systemic lupus erythematosus (SLE), but it is not clear to what extent they affect the function of C1q.

Further effects on the classical pathway arise from the presence of reactive thioesters in C3 and C4: the thioesters can be cleaved by low molecular weight nucleophiles without the need for proteolytic ac-

tivation of C3 or C4: this occurs at a low rate with water, causing hydrolysis of the thioester and generating species named C4(H₂O), C4u or C4b-like C4 (and similarly for C3). Ammonia production in ischemic tissue, or during kidney malfunction, may cause extensive formation of these species. C3b-like C3 can bind factor B, and cause alternative pathway activation; formation of C4b-like C4 does not cause further complement activation.

Activated (nascent) C4b and C3b, as well as reacting with water or binding to complement activator surfaces, will react with any strong nucleophile present; any such species will inhibit the binding of C3b and C4b to its real target, the complement activator. A number of drugs which are nucleophilic or have nucleophilic metabolites induce SLE as a toxic side-effect, and these may act by inhibiting C4b and C3b binding, diminishing the opsonization of immune complexes.

See also: Acute phase proteins; Anaphylatoxins; Bacteria, immunity to; Immunoconglutinins; Complement, alternative pathway; Clotting system; Complement deficiencies; Complement, genetics; Complement, membrane attack pathway; Complement receptors; Decay-accelerating factor (CD55); HLA class III region; Immune complexes; Immunglobulin, functions; *Neisseria*, infection and immunity; Phagocytosis; Systemic lupus erythematosus (SLE), human.

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COMPLEMENT DEFICIENCIES

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Inherited deficiencies of all known complement (C) components, of most regulator proteins and of the respective receptors have been found in humans. Table 1 is a compilation of the defects. Although acquired deficiencies – be they by consumption or otherwise – may also cause clinical conditions comparable to those listed in Table 1, they will not be specifically addressed in this entry.

Inherited deficiencies are rare. Since complement is not routinely measured in healthy persons, the incidence is difficult to determine and so is the disease incidence in a given component deficiency. In a Japanese study of healthy blood donors, C5, C6, C7 and C8 have each been found missing in about 3 per 100 000. C9 was absent in 3 per 1000. The prevalence of deficiencies of the late-acting components together was calculated at approximately 12 per 100 000. Some combined deficiencies – total or sub-total – of two or more components were reported when the terminal complex was afflicted. Deficiency in C7 in some cases was combined with the additional absence of C4 (C4a and/or C4b).

Considering the many biological activities associated with complement activation (Figure 1), it initially came as a surprise that clinical symptoms were absent in many cases of missing components. However, experimentation with defective animals clarified the situation by showing that redundant pathways are operative to assure complement-dependent life-preserving functions. Thus, a missing link in one pathway may cause defect symptoms only if other pathways are also affected, or if control of a given condition requires mobilization of all reserves.

By and large, the syndromes expected in complement-deficient states may be subdivided into three groups.

1. Recurrent and/or persistent infections

Bacteria may cause life-threatening conditions in the absence of C3 or factor I or any part of the alternative pathway. Since absence of factor I results in total consumption of C3, the outcome in functional terms is identical with a primary lack of C3. Opsonization and phagocytosis are drastically diminished in these conditions. A contributory factor in combined deficiencies may be a defective immune response, as shown in C4-, C2- and C3-deficient states.

Deficiencies of the later reacting components C5, C6, C7 or C8 are often associated with infections, almost exclusively with meningococci or gonococci, suggesting that the normal defense against these strains rests mainly with the bactericidal (membrane attack) function of complement. In this light, the apparent health of the C9-deficient individuals may be explained with the intact membrane attack function of C5b678 in the absence of C9, although proceeding somewhat more slowly.

2. Connective tissue or immune complex diseases

Most striking is the association of unclear nature of systemic lupus or other autoimmune conditions with C1qrs, C4 or C2 defects alone or in combination with other factor defects. It is surmised that the pathophysiology in these disease states is a defect in

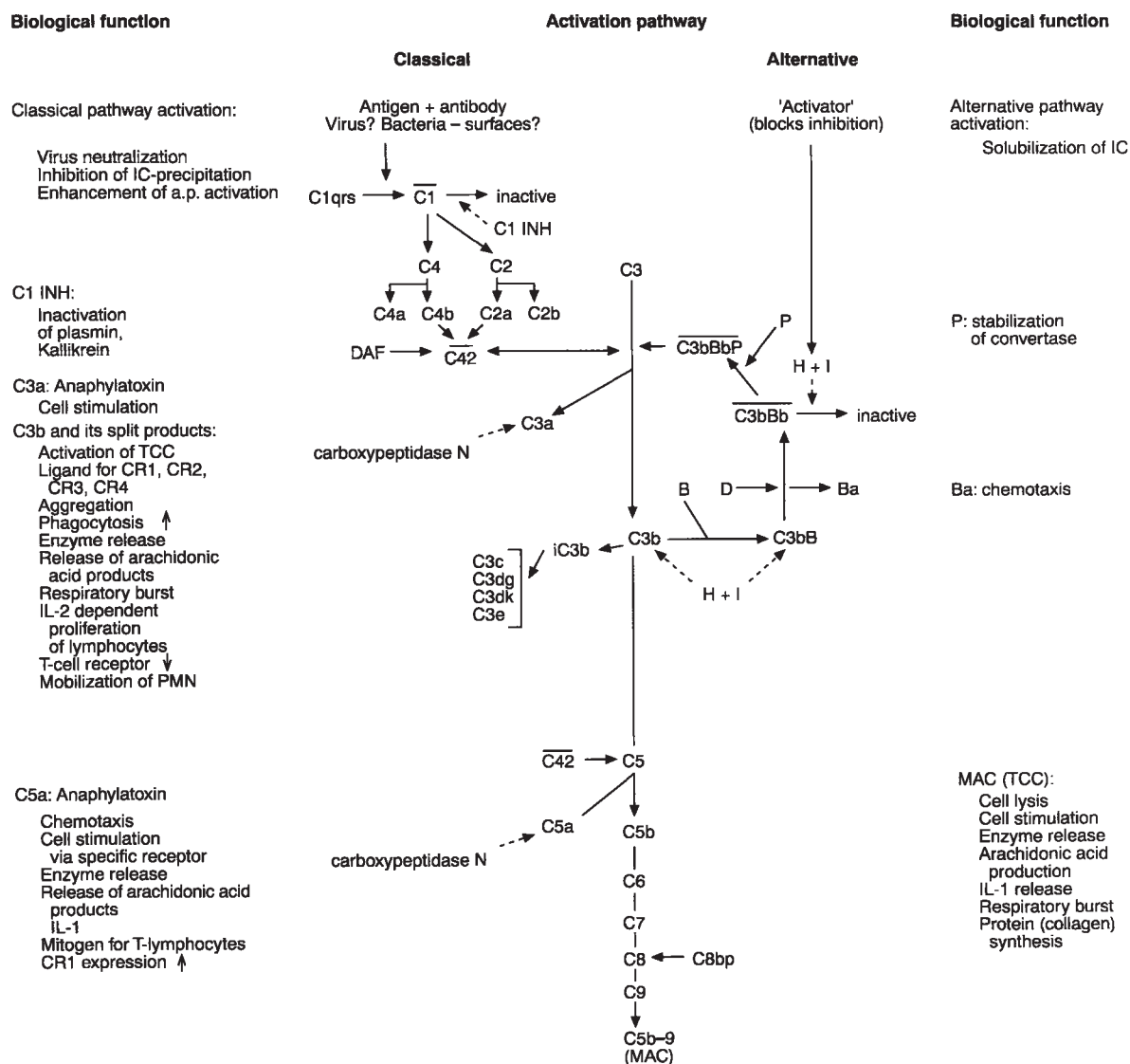


Figure 1 Reaction pathways of complement and biological activities. ----▶, Inhibition; a.p., alternative pathway; C1 INH, C1 inhibitor; IC, immune complexes; B, factor B; D, factor D; H, factor H; I, factor I; DAF, decay-accelerating factor; MAC, membrane attack complex; P, properdin; TCC, terminal complement complex (see article entitled Complement, Classical Pathway for further clarification).

complement-dependent clearance or solubilization of immune complexes due to a failure to enhance the feedback loop in alternative pathway activation.

One of the most common abnormalities involves the C1 inactivator which regulates the classical pathway as well as the kinin and clotting systems. It is this threefold intervention that has so far prevented a clear-cut opinion on whether the symptoms of hereditary angioedema (HANE) are causally related to the complement or to the other two systems. The defect is, so far, the only one in which the infusion of the missing factor ameliorates the clinical condition.

Little is known of receptor deficiencies, except for a decreased expression of receptor molecules such as

CR1 – whether inherited or acquired – in certain immune complex diseases. It seems reasonable to predict a pathogenic role for the lack of the receptors, as CR1 not only serves as a cofactor for factor I (Figure 1) but is also the main receptor molecule on the surface of erythrocytes used to transport immune complexes. A deficiency of CR3 has been described in a boy with severe infections.

Another cell surface constituent involved in complement regulation is the C8 binding protein (C8bp). It controls lysis of homologous cells. Along with other similar factors, C8bp (Table 1) is missing in paroxysmal nocturnal hemoglobinuria (PNH) cells, which are therefore abnormally sensitive to lysis by

Table 1 Inherited complement deficiencies in humans

Component	Inheritance	Remarks	Observed clinical condition	Laboratory findings
C1q	Autos. codom.	Three types: 1) partial, 2) complete, 3) inactive protein: combined with immunoglobulin deficiency	Infections, SLE	CH50 = 0 Bactericidal activity = 0 C1 $\uparrow$ C1s $\uparrow$
C1r, C1s	Autos. codom. not MHC linked	Mostly combined C1r + C1s	Infections (meningitis); Collagen diseases	CH50 = 0 C1 NH $\uparrow$
C4	Autos. codom. (4 genes) MHC linked	Degree dependent on number of 'null' alleles	Collagen diseases; some combined with IgA disorders	CH50 = 0; Bactericidal activity = 0; Chemotaxis $\downarrow$ Opsonization $\downarrow$: no IgM $\rightarrow$ IgG switch; MLC $\downarrow$
C2	Autos. codom. MHC (A25, B18, DR2) linked	30–70% activity in heterozygotes	Often none; Infections when combined with low factor B; SLE; juvenile rheumatoid arthritis	CH50 = 0 Bactericidal activity = 0 Phagocytosis $\downarrow$
C3	Autos. codom. not MHC linked		Severe infections; collagen diseases; nephritis	C3 < 0.1% CH50 = 0; bactericidal activity = 0; immune adherence = 0; opsonization $\downarrow$ Mobilization of PMN $\downarrow$ Factor B cleavage = 0 Chemotaxis $\downarrow$
C5	Autos. codom. not MHC linked (regulatory gene?)	13–65% activity in heterozygotes	Collagen diseases; Infections with meningococci, gonococci	CH50 = 0; bactericidal activity = 0; chemotaxis = 0 Platelet aggregation = 0 Opsonization normal (staphylococci)
C6	Autos. codom. not MHC linked; linked to C7 and C9 on chromosome 5		Gonococcal and meningococcal infections; streptococcal meningitis; chronic meningococcemia	CH50 = 0 Bactericidal activity = 0 Chemotaxis normal Opsonization and intracellular killing = normal Subtotal deficiency: truncated proteins hemolytically functional CH50 = 0; Reactive lysis positive; sera activatable up to C5,6
C7	Autos. codom. not MHC linked		Some healthy: 50% <i>Neisseria</i> infections: 12% connective tissue disorders	
C8	Autos. codom. not MHC linked C8 $\alpha\gamma$ and C8 β controlled by two independent loci	C8 $\alpha\gamma$ or C8 β missing; can be reconstituted with missing part; dysfunctional C8 β chain?	Meningococcal infections (often uncommon serogroups)	CH50 = 0
C9	Autos. codom. not MHC linked		None	Normal lysis and bactericidal activity at reduced rate (erythrocytes and <i>Escherichia coli</i>)

C1 NH	Autos. codom. structural gene	Three types: I) synthesis deficiency II) inactive protein III) C1 NH complexed to albumin (acquired deficiency sometimes by autoantibodies)	Angioedema following trauma or stress. Therapy: danazol, C1 NH infusion	C1 NH function↓ C1 NH protein↓/normal C4↓C2↓ C1↓ in acquired form
Factor I	?		Pyogenic infections, meningitis. Therapy: plasma infusion	CH50↓ C3↓ C3b↓ Factor B↓ properdin↓ Factor H↓ Bactericidal activity↓ Chemotaxis↓; phagocytosis↓ CH50↓ C3↓ factor B↓ C3d present on erythrocytes C5–C9 normal CH50 = normal Properdin = protein/0 apH50↓ Opsonization↓ CH50 normal during attacks: Histamine↑; C3a↑ C4↓; C3↓; Factor B cleavage
Factor H	Autos. recessive? codom.?	Low levels present	Hemolytic uremic syndrome; dense deposit disease; lupus nephritis	
Properdin	X-linked	1) Complete, or 2) partial Low titers present	Meningococcal meningitis; septicemia None (?) Chronic idiopathic urticaria/angioedema	
Anaphylatoxin inactivator (carboxypeptidase N)	Autos. recessive			
CR3 (CD18 deficiency)	Autos. codom.	Three proteins missing; relative contribution unclear	Recurrent infections, LAD	Proteins missing on leukocytes; zymosan response of monocytes↓
CR1	? Acquired?		Immune complex disease	Diminished CR1 on erythrocytes. Defective immune clearance
C8bp ('HRF')	Acquired	Reduced in PNH	Intravascular lysis	Acid lysis
C59 ('p18', 'MIRL' or 'HRF20')	Acquired	Reduced in PNH	Intravascular lysis	Acid lysis
DAF	Acquired	Reduced in PNH	Intravascular lysis	Acid lysis

apH50, Hemolytic test for alternative pathway activity; Autos. codom., autosomal codominant; CH50, hemolytic test for classical pathway activity; DAF, decay-accelerating factor; HRF, homologous restriction factor; Ig, immunoglobulin; LAD, leukocyte adhesion deficiency syndrome; MHC, major histocompatibility complex; MIRL, membrane inhibitor of reactive lysis; MLC, mixed lymphocyte culture; PNH, paroxysmal nocturnal hemoglobinuria.

homologous complement. Intravascular lysis is the clinical syndrome.

One of the enzymes regulating split-product activity of the effector phase is the anaphylatoxin (C5a) inactivator (carboxypeptidase N). It cleaves the terminal arginine off the C5a peptide, resulting in the C5a_{desarg} form. *In vitro* C5a_{desarg} has lost its spasmogenic and basophil-stimulating activities, as well as a large part of its chemotactic activities. *In vivo*, however, the chemotactic activity is not diminished by the loss of the arginine. The clinical role of the regulator defect is doubtful. One family with anaphylatoxin inactivator deficiency and HANE symptoms has been described.

With a few exceptions (see above) the therapy in complement-deficient conditions is symptomatic. A more direct approach such as gene therapy is untried.

3. Immune response

Antibody production following administration of limited amounts of antigen to mice was drastically reduced in the cobra venom factor-induced absence of C3 and this was true for both T cell dependent and T cell independent antigens. Both primary and secondary immune responses were affected by the treatment. Similarly, guinea pigs genetically deficient in C3 were poor responders in the primary phase and later failed in the switch to IgG. Analogous observations were made in C2-deficient guinea pigs or C4-defective mice. In mice with an inherited absence of C4, a fully functional immune response could be elicited following administration of C4. A boy deficient in C4 was analyzed for antibody production with similar results. Since C5-deficient mice were normal immune responders the impaired functions must be dependent on C3. This was further supported by studies on the role of the CR1 or CR2 receptors for C3 split products. Experimental blockade *in vivo* by monoclonal antibodies resulted in markedly reduced primary and secondary antibody responses to low concentrations of antigen. Memory formation was also diminished but T helper cells were not affected. As far as tested in these studies, larger amounts of antigen elicited undiminished responses. It is thus postulated that as yet unidentified enhancing functions are associated with C3 split products in the antigen-presenting pathways and that large-dose immunization does not need the enhancement.

Animal experimentation was the key tool in analyzing the biological functions of complement (Figure 1). While decomplementation (inactivation) of components by various means was helpful, the advent of strains with hereditary deficiencies provided more clearly defined conditions. Presently available are strains with a deficiency in C2 (guinea pigs), C4 (guinea pigs, including a coisogenic +/- colony), C3 (guinea pigs, dogs), C3a receptor (guinea pigs), C5 (mice, including a coisogenic +/- colony) or C6 (rats, pigs, rabbits). Genetic engineering to delete production of defined components is in progress. So far gene knockout mice with no C3 or factor B have been constructed. Further advances are expected from the production of recombinant species-compatible fluid phase inhibitors such as, for example, the CR1 receptor for C3. The trans-species genetic transfection of cell surface control factors may help in circumventing the otherwise effective complement attack against xenografts.

See also: Anaphylatoxins; Complement, alternative pathway; Clotting system; Complement, classical pathway; Complement fixation test; Complement, genetics; Complement, membrane attack pathway; Complement receptors; Decay-accelerating factor (CD55); Kallikrein-Kinin system; Systemic lupus erythematosus (SLE), human.

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COMPLEMENT FIXATION TEST

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The complement fixation (CF) test has traditionally been used for the determination of antimicrobial (viral, bacterial, parasitic and fungal) antibodies in patient sera. In a broader sense, CF refers to any technique that exploits the binding and activation of complement components as a reporter system for antigen-antibody reactions. Potential applications thus include: 1) direct measurement of complement activity using antibody-sensitized erythrocytes; 2) indirect complement consumption assay to detect antibody or antigen; and 3) determination of complement-activating ('fixing') autoantibodies (such as islet cell antibodies in diabetes). Although the CF test could be considered as a piece of history of immunology transferred to the modern world, it still has a significant role in diagnostics and research in human and veterinary medicine. Despite the fact that the method represents a multicomponent biological assay with many easily disturbed components, it works well in professional hands. The method is thoroughly known, reliable, inexpensive in material costs and especially well suited for screening purposes. On the negative side are the long time it takes to obtain results and the labor intensity of the assay.

Principle of the test

The CF test is based on the ability of patient serum antibodies to form complement-activating immune complexes upon binding to their antigens. In immune complexes the complement component C1q binds to neighbouring Fc regions of antibody molecules with at least two of its six globular heads. In subsequent reactions some of the early complement components (C1s, C4, C2 and C3) are consumed in sequential proteolytic cleavages. The antibody assay (Figure 1) is performed in two stages: first, a dilution series of antibody (the serum to be studied) is mixed with a standard amount of antigen and complement (guinea pig serum is usually used as a source of complement components); second, a detection system for unconsumed complement (rabbit antibody-coated sheep erythrocytes, EA) is added. Lack of hemolysis in the detection system indicates the presence of antibodies. The test is quantitative and the result is usually given as the highest titer of patient serum that still prevents

lysis of the indicator cells. Critical components in the assay are the complement (fresh frozen or lyophilized guinea pig serum) and the sheep red blood cells (E). The latter requires the use of cells from a single or a couple of selected animals. The amount of antibody on E needs to be optimized to a saturating, but subagglutinating, level so that the assay is sensitive to changes in complement activity. The amount of complement is titrated to a level that typically corresponds to twice the amount of the 50% hemolytic dose (CH_{50}) under the test conditions. The amount of complement should be just enough to lyse the indicator cells. Controls for the viability of the detection system (EA + complement), background lysis (no complement) and 100% lysis (lysis by water) need to be included. Reproducibility of the CF assay requires that the ionic strength, pH, Ca^{2+} and Mg^{2+} content remain constant. Chelators (EDTA, EGTA) should not be used in the test samples because they directly block complement activity. As the test sera must be void of their own complement, they are heat-inactivated before the assay. For most applications the method utilizes 96-well microtiter plates; it can be automated. A detailed description of the standardization and procedure can be found from the further reading listed below.

Application and interpretation of the assay

The CF test is often used for the screening of antibodies against a variety of possible pathogenic microbes (especially viruses); for this a pool of 15–20 different antigens can be used. Most antigens are commercially available and reasonably priced. Relatively crude antigens (infected cell lysates) are mostly sufficient for the test. Test sera need to be screened in at least two dilutions because in high titer sera an excess of antibody may fail to generate complement-activating immune complexes (the "prozone" effect).

The test is relatively simple to perform and easy to interpret. The CF test broadly measures the humoral immune response (immunoglobulin M (IgM) and IgG class antibodies) against specific microbial antigens. A diagnosis of an acute infection can be made by demonstrating a significant increase in the anti-

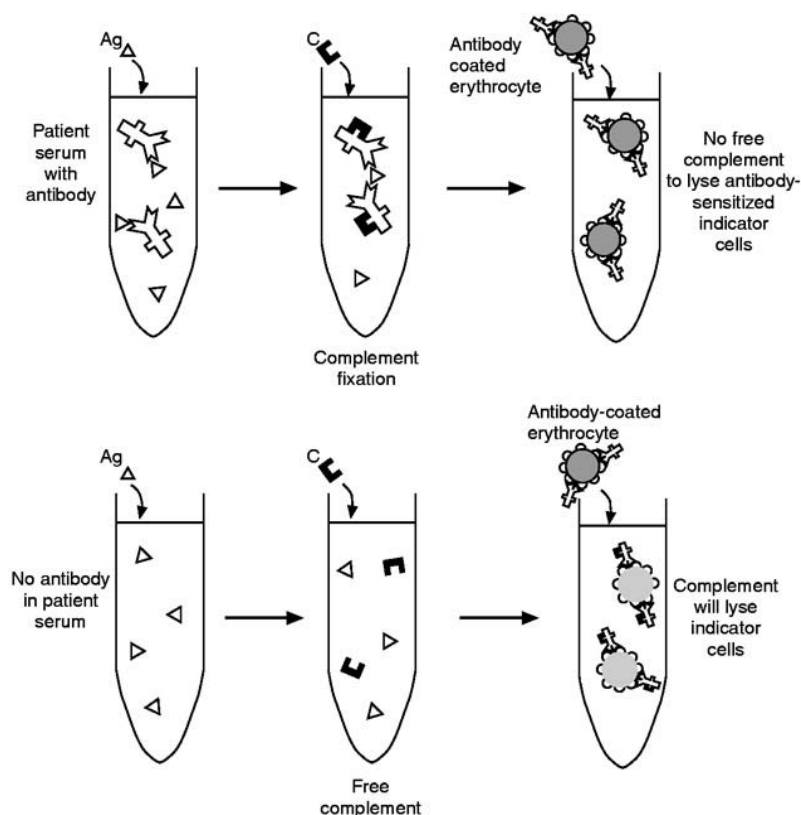


Figure 1 Principle of the complement fixation test.

body titer against a given microbe in paired patient serum samples (generally taken 7–10 days apart from one another). A minimum of a fourfold increase (or sometimes decrease) in antibody level is diagnostic. In screening, a practical way of testing is to analyze the second serum sample at low dilutions (e.g. 1:8 and 1:16) with all the antigens in use and subsequently to test both sera at higher dilutions for the antigens which are positive. A high titer in a single serum sample can be indicative of the state of immunity against the microbe. A declining titer may be the sign of a somewhat older infection. Because of its relatively low sensitivity the complement fixation test may not be applicable for the demonstration of protective immunity acquired a long time ago. Other tests, such as ELISA, are more suitable for this purpose.

The use of the CF test is hindered if the patient serum sample turns out to be 'anticomplementary', i.e. it consumes complement from the test mixture without addition of antigen. The anticomplementarity is usually due to the presence of pre-existing complement-activating factors in the patient sera. These include immune complexes and other immune aggregates, cryoglobulins, contaminating bacteria or bacterial products such as endotoxin. For example, acute sera from patients with a parvovirus B19 infection often contain immune complexes and are anti-

complementary. Polyclonal B cell activation in patients with an Epstein-Barr virus infection may generate, for example, antibodies against sheep erythrocytes that also can give a false (positive) result in the assay. An additional control to exclude direct anticomplementarity of the test serum (test mixture in the absence of antigen) is thus essential.

See also: Antibodies, detection of; Antigens; Complement, classical pathway; Humoral immunity; IgG; IgM; Immune complexes; Immunoassays; Viruses, immunity to.

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COMPLEMENT, GENETICS

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In 1919, Moore discovered a strain of guinea pigs that was complement deficient; for example, serum of these animals did not mediate antibody-dependent cytolysis. Since that time substitution of a component either artificially depleted from or genetically deficient in sera of individuals of a given species has been a major tool of complement research. In humans, a number of autoimmune diseases have been associated with complement deficiencies of the classical pathway. Additionally, fulminant or recurrent bacterial infections are directly correlated with genetic deficiencies of early and late complement components, respectively. The first polymorphism of a complement component was found by Alper and Propp for the third component in humans in 1968. Subsequently, knowledge about deficiencies and polymorphisms in different species at the phenotypic and genotypic level has continuously accumulated.

Definition of genetics

Polymorphism was defined by Ford as 'the occurrence together in the same habitat of two or more discontinuous forms, or "phases", of a species in such portions that the rarest of them cannot be maintained by recurrent mutations'. Polymorphism of complement components can be studied at four different levels:

1. by allotyping of native complement components in serum (the phenotypic level);
2. by defining their subunit composition (the subphenotypic level);
3. by establishing the population and formal genetics (frequency of allotypes, and frequency and segregation of the respective genes/alleles);
4. by gene mapping and DNA sequencing to reveal the gene structure and define sequence variants (the genotypic level).

For this purpose, a complex methodology including electrophoretic and isofocusing techniques with functional or immunologic detection of individual

components, serology, quantitation, Southern blot and restriction fragment length polymorphism (RFLP) analysis, polymerase chain reaction (PCR) amplification and sequencing of DNA is applied (Table 1). By these means, insights into the evolution of polymorphisms and deficiencies and structure–function relationships, as well as into genetically determined disease susceptibilities, are gained. The analysis of complement polymorphisms at the DNA level has provided an even more complex picture by revealing further variants, which subdivide alleles defined at the protein level. DNA sequence analysis of protein allotypes of a number of complement components has demonstrated that most sequence variations are not confined to short stretches of genomic DNA, but rather are scattered throughout the entire gene; therefore it appears to be technically difficult completely to replace protein typing methods by direct DNA typing using the PCR methodology. A combination of typing methods, both at the protein and the DNA level, should be applied to obtain complete analysis for complement component polymorphisms, as well as for the detection of complement deficiencies.

Gene clusters of complement components and their function

In humans, most genes coding for complement components and related proteins have been cloned, partially or completely sequenced and assigned to chromosomal locations, providing further insight into the evolutionary origin of the large variety of complement components (Table 2). This is also reflected by their genomic location and gene structure, as a number of structurally and/or functionally closely related components are organized in major gene clusters in the human genome. These findings support assumptions, based on homology studies at the protein level, that the majority of complement components have evolved by duplication from only a small number of precursor genes.

Table 1 Summary of polymorphisms and deficiency of complement components in humans and animals

Complement component	Typing technique	Total no. of known alleles	Deficiency	Polymorphism in other species	Disease associations
C1q	Imm/Funct	—	Yes	—	Yes
C1r	IEF+WB	>10	Yes	—	Yes
C1s	IEF+WB	2	Yes	—	Yes
C2	IEF+WB RFLP/PCR	<10	Yes	Ch,Rh,Gp,Hm	Yes
C3	HVAGE+IFX RFLP/PCR	>30	Yes	Ch,Rh,Ba,Mc,HI, Ms,Rb,D	Yes
C4	HVAGE+IFX/WB HVAGE+HOV MAB SDS-PAGE (α/β chains) HAI (Rodgers/Chido) RFLP/PCR	>30	Yes	Ch,Mc,D,Ms,Gp,XI	Yes
C5	IEF+WB	2	Yes	—	Yes
C6	IEF+WB	>20	Yes	Ch,Rh,Rb,Ms	Yes
C7	IEF+WB/HOV MAB	>10	Yes	—	Yes
C8	IEF+WB/HOV SDS-PAGE RFLP/PCR	<10	Yes	Ch	Yes
Factor B	HVAGE+IFX/HOV IEF RFLP/PCR	>20	Partial	Ch,Rh,HI,Ba, Gp,Ms	No
Factor I	IEF+WB RFLP/PCR	<5	Yes	—	Yes
Factor H	IEF+WB	<5	Yes	—	Yes
P	Imm/Funct	—	Yes	—	Yes
C4BP	IEF+WB/IFX	<5	No	—	No
CR1	SDS-PAGE EXP RFLP/PCR	<5	Yes	—	Yes
CR2	SDS-PAGE RFLP/PCR	<2	No	—	No
CR3	SDS-PAGE	—	Yes	—	Yes
DAF	Imm (Cromer blood group antigen) RFLP/PCR	<2	Yes	—	Yes
MCP	EXP	<2	No	—	No
MBL	RFLP/PCR	3	No	—	Yes

Typing techniques: EXP, expression polymorphism (number of membrane-associated molecules per cell); Funct, Functional assay; HAI, Hemagglutination inhibition with human alloantisera; HOV, Complement component-dependent hemolytic overlay; HVAGE, High-voltage agarose gel electrophoresis; IEF, Isoelectric focusing; IFX, Immunofixation; Imm, Immunologic detection with specific antisera; MAB, Monoclonal antibodies; RFLP/PCR, DNA restriction fragment length polymorphism detected by Southern blot or polymerase chain reaction analysis; SDS-PAGE, SDS polyacrylamide gel electrophoresis; WB, western blot.

Animal species: Ba, Baboon; Ch, Chimpanzee; D, dog; Gp, guinea pig; Hm, hamster; HI, Hanuman langur monkey; Mc, Macaque; Ms, mouse; Rb, rabbit; Rh, Rhesus monkey; XI, *Xenopus laevis*.

Regulators of complement activation (RCA) gene cluster on human chromosome 1

This cluster includes genes for complement receptors CR1 and CR2, the decay-accelerating factor (DAF), the membrane cofactor protein (MCP), factor H (HF), and the C4-binding protein (C4BP). All six proteins share common sequence motifs called short consensus repeats (SCR) of about 60 amino acids in

length. In addition, multiple RNA transcripts due to alternative splicing have been described for most members of this cluster. Since this tightly-linked gene family controls the activity of the complement system, it is hard to escape the conclusion that genetic linkage is the evolutionary concept for the maintenance of closely related functions.

Table 2 Chromosomal assignments of complement components and related proteins

<i>Component (or subunit)</i>	<i>Gene symbol</i>	<i>Chromosomal location</i>
C1q: α chain	C1QA	1p34.1–p36.3
C1q: β chain	C1QB	1p34.1–p36.3
C1q: γ chain	C1QG	1p34.1–p36.3
C8: α chain	C8A	1p32
C8: β chain	C8B	1p32
C4-binding protein: α chain	C4BPA	1q32 ^a
C4-binding protein: β chain	C4BPB	1q32 ^a
Complement receptor 1 (CD 35)	CR1	1q32 ^a
Complement receptor 2 (CD 21)	CR2	1q32 ^a
Decay-accelerating factor (CD 55)	DAF	1q32 ^a
Membrane cofactor protein (CD 46)	MCP	1q32 ^a
Factor H	HF	1q32 ^a
Factor I	IF	4q25
C6	C6	5p13 ^b
C7	C7	5p13 ^b
C9	C9	5p13 ^b
C2	C2	6p21.3 ^c
Factor B	BF	6p21.3 ^c
C4A (isotype)	C4A	6p21.3 ^c
C4B (isotype)	C4B	6p21.3 ^c
C8: γ chain	C8G	9q22.3–q32
C5	C5	9q33
Perforin	PRF1	10q22
Mannose-binding protein (lectin)	MBL	10q11.2–q21
Surfactant protein A (SP-A)	SFTP1	10q22–q23 ^d
Surfactant protein D (SP-D)	SFTP4	10q22–q23 ^d
Membrane inhibitor of reactive lysis (MIRL, CD59)	CD59	11p13
C1 Inhibitor	C1NH	11q12–q13.1
C1r	C1R	12p13
C1s	C1S	12p13
Complement receptor 3: α chain	CR3A	16p11.2 ^e
Vitronectin (S-protein)	VTN	17q11
C3	C3	19p13.3–p13.2
C5a receptor 1	C5R1	19q13.3–q13.4
Leukocyte adhesion molecule: β chain (CD18)	ITGB2	21q22.3 ^f
Properdin	PFC	Xp11.4–p11.2

^aRegulators of complement activation (RCA) gene cluster.

^bMembrane attack complex (MAC) gene cluster.

^cMHC class III gene region.

^dSurfactant protein (SP) gene cluster.

^eLeukocyte adhesion α (LAA) gene cluster.

^fCommon β chain for the cell adhesion molecules CR3, LFA-1, and gp150,95.

No map assignment: C1q receptor (C1QR, collectin receptor), factor D (DF), factor J (JF), C8-binding protein (C8BP, HRF).

Membrane attack complex (MAC) components C6, C7 and C9 on human chromosome 5

Another gene cluster of complement components was assigned recently to chromosome 5. It has been

known for some time that C6 and C7 of the terminal complex are genetically linked, evidence of which was mainly derived from a case of combined C6/C7 deficiency. Whereas the C6 and C7 genes are closely linked (only about 160 kb apart), the C9 gene is

located at a distance of >2.5 mb. These three genes share a number of common structural features with those of the C8 α and β chains (C8A and C8B) on chromosome 1: a central cysteine-poor region flanked by several thrombospondin, epidermal growth factor (EGF) precursor and low-density lipoprotein (LDL) receptor repeats. The genes of the MAC may have evolved by duplication and translocation from an ancestral precursor functionally related to perforin, a pore-forming protein of cytotoxic T cells.

C2, factor B and C4 as class III components of the major histocompatibility complex (MHC) on human chromosome 6

These central components of the classical and alternative pathway are encoded in a 120 kb stretch of DNA flanked by the genes of class I and II MHC antigens, HLA-A, -B, -C and -DR, -DQ and -DP, respectively. Although the C2 and BF genes differ significantly in size, they share extensive homology in the coding sequence (both are precursors of functional serine proteases) and have arisen by gene duplication. No homozygous factor B deficiency has yet been reported. Homozygous C4 deficiency is a rare condition and regularly associated with autoimmune or immune complex disease (e.g. systemic lupus erythematosus (SLE) or SLE-like disease). In contrast, about two-thirds of homozygous C2-deficient individuals appear to be healthy, although cases with SLE-like disease or bacterial infections have been described. Whereas there is only a limited polymorphism of C2 and BF, the fourth component of humans is encoded by two closely linked loci – C4A and C4B – exhibiting an extensive polymorphism with a total of more than 30 alleles. Two duplicated and functionally active C4 loci are found only in higher primates and humans. In all mammals (except for the rabbit), as well as in amphibians, the class III complement genes are located in the MHC.

Complexity of the fourth component of human complement with regard to molecular basis, functions and disease associations

For a number of reasons, human C4 is unique among all complement components with regard to its genetic basis, the subtle functional differences of isotypes and allotypes as well as its potential role in the pathogenesis of disease:

1. Human C4 has the most complex molecular basis: the C4A and C4B genes encode proteins with 99% sequence homology but clearly differ-

ent functions. The two isotypes differ only in a single serologic determinant defined by amino acid residues of the α -chain. A single amino acid substitution from Lys to Pro at position 1101 is responsible for an apparent molecular weight change of 2 kDa on SDS-PAGE; the change from Asp to His at position 1106 alters the hemolytic activity by three- to fourfold. Further, both loci contain so-called 'null' alleles C4Q0 (= quantitative zero), which express no detectable gene product, at remarkable high frequencies (10–20%). These 'silent' alleles are due to: deletions of an entire C4 gene including one of the adjacent genes of steroid 21-hydroxylase, CYP21 (such as C4AQ0 in the most common Caucasoid HLA haplotype A1-Cw7-B8-C2C-BFS-C4AQ0B1-DR3), gene conversion from one C4 isotype to the other (e.g. in the HLA haplotype B44-C2C-BFS-C4A3BQ0-DR4), or non-expression of structural genes due to individual point mutations (e.g. in the HLA haplotype B60-C2C-BFS-C4AQ0B2-DR6). In addition, a number of haplotypes with structurally duplicated C4 genes have been found.

2. Different functions have been ascribed to the thiolester bond of nascent C4b: C4A preferentially transacylates on to amino group nucleophiles, whereas C4B prefers hydroxyl groups for ester formation. Furthermore, C4A is 1.7-fold more efficient at inhibiting immune precipitation than C4B. Complete C4-deficient serum has almost no neutralizing activity in a mumps virus neutralization assay; purified C4A enhances the reaction about tenfold compared with C4B. Immunization experiments with complement-deficient guinea pigs have provided evidence that both C4 isotypes play distinct roles in the induction and amplification of the T cell-dependent immune response.
3. There is a steadily growing list of associations (of variable confidence) of C4 deficiency with a wide variety of diseases such as SLE, rheumatoid arthritis, systemic sclerosis, subacute sclerosing panencephalitis (SSPE), primary biliary cirrhosis, chronic polyarthritis, multiple sclerosis, visceral leishmaniasis, chronic glomerulonephritis, leprosy, Brazilian paracoccidioidomycosis, common variable immunodeficiency, immunoglobulin A deficiency, insulin-dependent diabetes mellitus, Chagas disease and the acquired immune deficiency syndrome (AIDS). A number of autoimmune diseases are associated with the HLA haplotype A1-Cw7-B8-C4AQ0B1-DR3.

Possible mechanisms of complement and disease associations

Since only some, but not all, complement-deficient individuals show disease manifestations, it appears legitimate to conclude that the deficiency is the common genetic basis, not the primary cause in the pathogenesis of a given disease. Also, there is no direct evidence for a role of single genetic variants of a given component in the pathogenesis of autoimmune or infectious diseases. However, there are several lines of evidence suggesting an important physiologic role of complement in immune defense:

1. It has been shown that the intact classical pathway is needed to prevent formation of insoluble immune complexes (ICs); the components of the classical pathway play an important role in this function. In cases of deficiency, ICs may not be cleared by the reticuloendothelial system and therefore recirculate until being deposited at capillary or basal membranes, leading eventually to tissue damage. This mechanism could be envisaged in some forms of vasculitis and glomerulonephritis, as well as autoimmune disease in the presence of nuclear antibodies.
2. In the case of viral antigens, small ICs may escape from CR1-mediated clearance by erythrocytes if the individual is C4-deficient. Circulating ICs may even, after years, be deposited in the brain, having passed the blood-cerebrospinal fluid barrier, contributing to cytotoxic effects in the central nervous system, as seen in diseases such as SSPE and AIDS.
3. Deficiency of MAC components may render these individuals more susceptible to recurrent infections with bacteria such as *Neisseria*. On the other hand, the lack of complement-dependent bacterial lysis protects the patient from massive levels of bacterial endotoxin, leading to a milder course of disease. Depending upon the structural components of the bacterial membranes, some strains appear to be more resistant to complement-mediated lysis than others.

Concluding remarks

A deep insight into the complex genetic structure of most complement components and their evolution has been gained in the last decade. A number of genetic abnormalities of complement components have been observed in association with disease. It is, however, still premature to bridge the variation or loss of *in vitro* functions to a given *in vivo* situation. It will be an important task to replace the statistical relationship of a disease association by the eluci-

dation of causal factors in the pathogenesis of complement-related diseases, as well as to establish evidence for a functional relevance of polymorphic complement allotypes in the natural selection process.

See also: Complement, alternative pathway; Complement, classical pathway; Complement deficiencies; Complement receptors; Decay-accelerating factor (CD55); H2 class III; HLA class III region; Systemic lupus erythematosus (SLE), human.

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COMPLEMENT, MEMBRANE ATTACK PATHWAY

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The membrane attack pathway represents a common effector mechanism of the classical and alternative pathways of complement activation. Activation of the membrane attack pathway constitutes the last stage of complement activation and results in assembly of a multiprotein complex known as the membrane attack complex (MAC). The MAC is comprised of the complement components C5b, C6, C7, C8 and C9 (Table 1). Assembly of the MAC on the surface of a cell may have two major consequences. First, the MAC may activate a series of cellular processes that result in MAC elimination as well as cellular responses that are mostly pro-inflammatory. Second, if sufficient MAC attaches to a cell membrane, cell death may ensue. The name MAC derives from this latter property as the complex can damage a cell membrane independently of the binding of earlier complement components to that cell.

Assembly, structure and control of the MAC

The first step in assembly of the MAC is the generation of C5b from C5. This is accomplished through cleavage of C5 into C5a and C5b by the C5 convertases of the classical pathway (C4b2a3b) or the alternative pathway (C3bBbC3b) (Figure 1). C5 binds to C3b which serves both as an assembly site and a cofactor for the enzyme site in the C5 convertases that cleave the α chain of C5, generating C5a and C5b. Then C5b interacts with the terminal complement proteins in a nonenzymatic sequential association, resulting in assembly of the MAC (Table 1 and Figure 1). As each protein incorporates into the complex a conformational rearrangement produces a binding site for the next component. After generation C5b exposes a binding site for C6 with a half-life of 2.3 min at 37°C. Binding of C6 to the

Table 1 Properties of the proteins, complexes and inhibitors of the MAC

	Molecular weight (kDa)	Binding sites for
<i>Individual proteins^a</i>		
C5	α Chain: 115 β Chain: 75	C3b, C6, C7, C8
C5b	α' Chain: 104 β Chain: 75	C6, C7
C6	Single chain: 120	C5, C5b
C7	Single chain: 110	C5, C5b
C8	α Chain: 64 β Chain: 64 γ Chain: 22	C5, C9 C5b-7
C9	Single chain: 71	C5b-8, C5b-9
<i>Protein complexes</i>		
C5b6	328	C7
C5b-7	430	Lipid bilayer, C8, vitronectin, clusterin
C5b-8	589	C9, control proteins
C5b-9	660-1850	C9, control proteins
<i>Control proteins</i>		
Vitronectin ^a	Single chain: 83	C5b-7, C5b-8, C5b-9
Clusterin ^a	α Chain: 40 β Chain: 40	C5b-7, C5b-8, C5b-9
CD59	Single chain: 20	C5b-8, C5b-9
HRF	Single chain: 65	C5b-8, C5b-9

^aSerum concentrations are 50-70 $\mu\text{g ml}^{-1}$ for each of the MAC precursor proteins, 500 $\mu\text{g ml}^{-1}$ for vitronectin and 50 $\mu\text{g ml}^{-1}$ for clusterin.

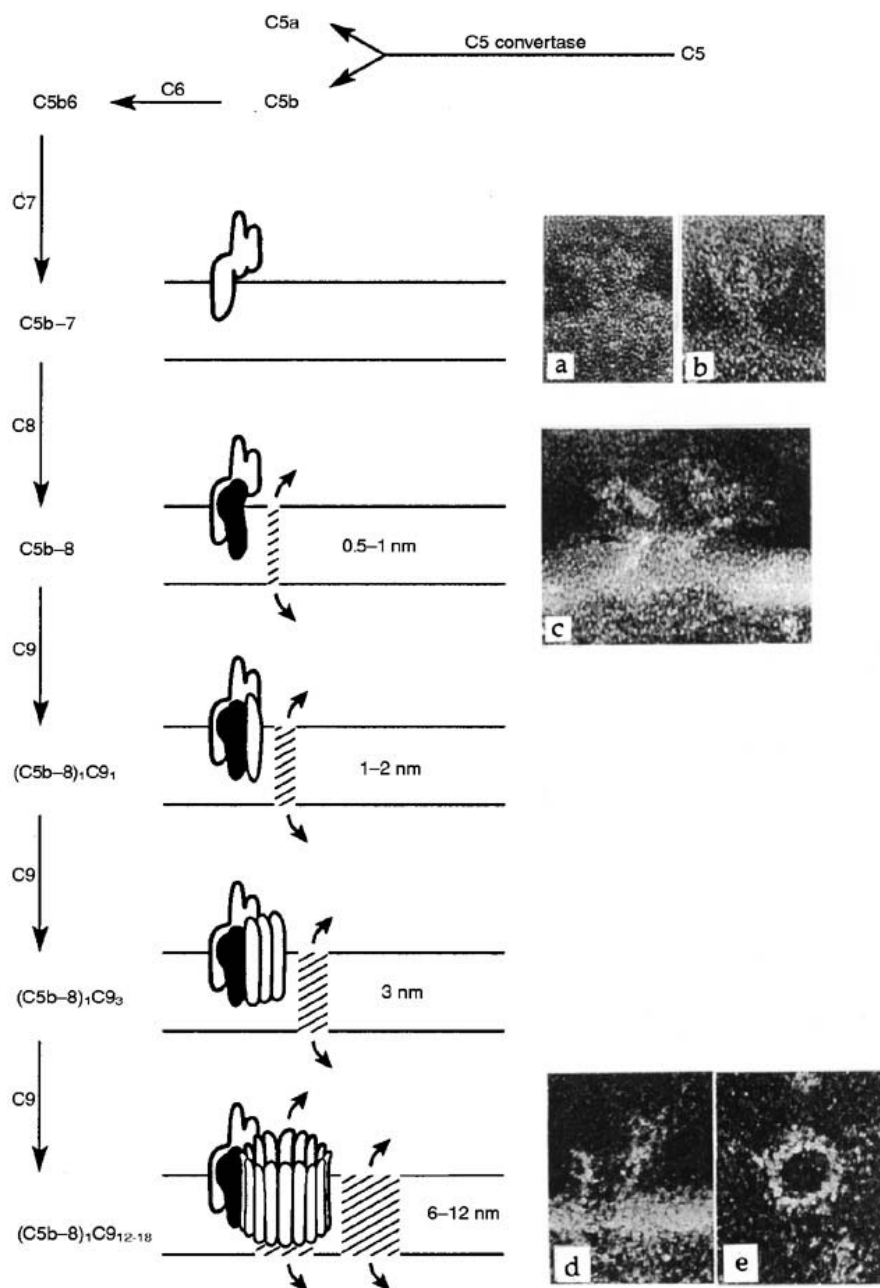


Figure 1 Diagrammatic illustration of the complement membrane attack pathway and electron microscopy of MAC protein complexes. Reaction sequence from activation of C5 through assembly of the complete MAC is shown. Interaction of complexes with a cell membrane is illustrated, with C8 depicted in black. Stippled areas indicate membrane pores of progressively larger size that develop in parallel with progression of MAC assembly. Arrows indicate loss of permeability barrier. Also shown are electron micrographs of membranes bearing C5b-7 (panels a and b), C5b-8 (panel c) and the fully assembled MAC (panel d, lateral view, and panel e, top view). (Electron micrographs are reproduced from RR Dourmashkin (1978), The structural events associated with the attachment of complement components to cell membranes in reactive lysis, *Immunology* **38**: 205-212, with permission from the publisher.)

transient site is facilitated by the weak association of C5 and C6 in solution. The resultant C5b6 is stable and has a binding site for C7. C5b6 can remain loosely associated with C3b, which assists in directing C5b-7 to the adjacent membrane, or detach to the fluid phase. Once C7 incorporates into the

complex a metastable binding site with a high affinity for phospholipids is produced. This site is very short-lived, less than 10 ms, and allows the C5b-7 complex, after detachment from C3b, to anchor in the external surface of the lipid bilayer, primarily through C7. The electron microscopic appearance of

membrane-bound C5b-7 is that of a foliaceous particle (Figure 1). Assembly of the complex can also continue in the fluid phase; however, competition for the lipophilic binding site precludes attachment to a cell membrane. Vitronectin, clusterin, lipoproteins and C8 can all bind to fluid phase C5b-7 and prevent further association with membrane phospholipids.

Membrane-associated C5b-7 binds one molecule of C8, forming the C5b-8 complex, which then may bind multiple molecules of C9. The C5b-7 complex in the membrane bilayer binds a C8 molecule by interaction of the C8 β chain with C5b; possibly a second binding site on C7 promotes a conformational rearrangement in C8 resulting in the C8 α chain penetrating the lipid bilayer. Bound C5b-8 appears as a particle with a variable number of arms (Figure 1), with C5b and C8 β most distal from the membrane. The resultant C5b-8 complex has a greater affinity for phospholipids than C5b-7, penetrates more deeply into the membrane and disrupts membrane integrity to produce small transient ion-permeable channels. C9 binds to the C8 α chain in the C5b-8 complex and undergoes a conformational change that allows C9 to insert through the bilayer and expose additional binding sites for C9 polymerization. The C5b-9 complex has a greater affinity for phospholipids than C5b-8 and the membrane channel becomes more stable. The channels are formed by the disruption of the bilayer structure as membrane phospholipids attach to the complex. Additional molecules of C9 incorporate into the complex in a circular configuration and the size of the channel continues to increase (Figure 1). Incorporation of 12-18 molecules of C9 results in formation of the closed ring, tubular polyC9 structure, which may function as a protein-walled channel. The ultrastructure of this complex on top view corresponds to a ring with an internal diameter of 10 nm and an external diameter of 21 nm. On a lateral view there is a pedicle attached to polyC9 that represents the majority of the C5b-8 complex and extends 16 nm beyond the ring which has a height of 15 nm. Penetration of a membrane by the C5b-9 complex results in loss of membrane phospholipids and a reduction in membrane fluidity. Phospholipids and cholesterol in red cell membranes that have been exposed to the MAC are resistant to dissociation by caotropic agents.

MAC assembly is controlled by several regulatory mechanisms that protect tissues from damage by physiologic activation of autologous complement. Inhibition of early events of complement activation reduces formation of C5 convertases and thus prevents generation of C5b and MAC. Even after C5b has been produced there are mechanisms that control

MAC assembly. Intrinsic control is provided by the lability of the exposed binding sites in C5b and C5b-7. There are also membrane-bound and plasma inhibitors that prevent further assembly of complement proteins into the complex. The membrane-associated inhibitors are homologous restriction factor (HRF) and CD59 (also called protectin, MAC inhibitory factor or HRF20) and they are more effective to protect a cell from lysis by complement of the same species than by complement from other species (homologous restriction). CD59 and HRF inhibit formation of the MAC through interaction with membrane-bound C5b-8 to impair the binding of C9 and with C5b-9 to block additional binding and polymerization of C9. These inhibitors are expressed on most cells that are in contact with blood or other biological fluids containing complement. These proteins as well as the inhibitor of the C3 and C5 convertases decay-accelerating factor (DAF) are anchored to the membrane through a glycosyl phosphatidylinositol moiety, although in certain cells they may also exist as transmembrane proteins. There are also plasma proteins that control MAC formation. Vitronectin (S protein) and clusterin (SP-40,40) are multifunctional proteins that interact with late-acting complement components as complexes assemble in plasma, and inhibit binding of the protein complex to cell membranes. In addition, apolipoproteins A-I and A-II may inhibit MAC formation by interfering with polymerization of C9.

Physiologic and pathologic significance of the MAC

The MAC is endowed with the potential to inflict damage on cell membranes that may result in cell death and to induce cellular responses that are generally proinflammatory. These activities may participate significantly in varied physiologic and pathologic processes. Induction of cell damage is a consequence of the ability of the MAC to generate ion-permeable channels in the cell membrane. Membrane-bound C5b-8 can lyse red cells at a low rate but this process is enhanced by C9. Because of the strong affinity of C5b-8 for phospholipids, the membrane bilayer is disrupted, causing increased permeability, which is further enhanced by larger channels formed by addition of C9. Addition of one C9 molecule per C5b-8 is sufficient for rapid lysis of red cells. The permeability changes may cause osmotic swelling and lysis of red cells. Red cells are able to offer some resistance to osmotic swelling by means of a Ca^{2+} -activated K^{+} channel and may repair lesions through a Ca^{2+} -dependent vesiculation process. Killing of nucleated cells by the MAC is

independent of permeability changes and is associated with an increase in cellular Ca^{2+} and intracellular lipid metabolism, mitochondrial swelling, and hydrolysis of membrane lipids. Nucleated cells are capable of defending themselves from the cytotoxic effect of the MAC and, consequently, lysis is achieved only when large numbers of MAC with multiple C9 are deposited on the plasma membrane. Sublytic amounts of MAC induce activation of the target cell, which, among other effects, results in energy-dependent elimination of membrane-bound MAC by endocytosis or exocytosis. This process requires extracellular Ca^{2+} , with participation of protein kinase C (PKC) and cAMP. Cells with increased levels of cAMP are resistant to MAC-mediated killing. Conversely, inhibition of protein synthesis or lipid metabolism increases susceptibility to MAC-mediated killing.

The MAC plays a role in host defense processes through its ability to kill certain microorganisms and to promote inflammation. The function of the MAC in protection against infections is best demonstrated in individuals with homozygous deficiency of a MAC protein. These individuals have increased susceptibility to develop disseminated disease when infected with the gram-negative organism *Neisseria gonorrhoeae* or *N. meningitidis*. Thus killing by the MAC must be essential to maintain localization and prevent dissemination of neisserial infections. Other gram-negative bacteria, but not gram-positive bacteria, may be killed by the MAC; however, susceptibility is variable. In general smooth strains, but not rough strains, are resistant to killing because they are protected from the MAC by long-chain lipopolysaccharides. For bactericidal activity the deposits of MAC on the bacterial surface must contain at least three molecules of C9 per C5b-8. Enveloped viruses and parasites may also be susceptible to the cytotoxic effect of the MAC. The poor susceptibility to MAC-mediated killing of many microorganisms is due to their defense mechanisms against complement, including certain surface proteins that are homologous to human complement inhibitors.

In vitro studies have shown that formation of sublytic amounts of MAC on the surface of nucleated cells may trigger certain transduction pathways that may result in generation of mediators of inflammation. Even the C5b-7 complex, which binds to membranes but does not form a channel, is able to induce in certain cells an increment in cAMP, diacylglycerol (DAG) and ceramide. C5b-8 and C5b-9 in sublytic amounts can activate several transduction pathways mainly through the participation of Ca^{2+} which first enters the cell through the MAC pore and then triggers the release of Ca^{2+} from intracellular

organelles. Transduction pathways that are Ca^{2+} -independent may also be activated. The MAC can induce activation of PKC and stimulate phosphoinositide metabolism, with increased production of IP_1 , IP_2 and IP_3 , and increase the intracellular concentration of *sn*-1,2-diacylglycerol. The MAC can also interact with pertussis toxin-sensitive guanine nucleotide-binding proteins (G proteins). C5b-9 causes arachidonic acid release, which in part requires external Ca^{2+} , resulting in production of DAG, which may activate PKC and thus stimulate phospholipase A_2 . The MAC may induce cell cycle in oligodendrocytes, which involves *c-jun* activation, and can also cause hydrolysis of myelin basic protein.

Through these and possibly other cell activation mechanisms, the MAC may induce the synthesis of soluble and membrane-associated inflammatory mediators. Thus, it may stimulate the conversion of arachidonic acid into various metabolic products, including prostaglandins, thromboxanes and leukotrienes, and facilitate the production of interleukin 1 (IL-1), tumor necrosis factor α (TNF α) and oxygen-derived free radicals. In endothelial cells, the MAC stimulates the secretion of IL-8 and monocyte chemoattractant protein 1, and inhibits endothelial cell-dependent relaxation, and C5b-7 causes endothelial cell retraction. The MAC may also modify the expression of adhesion molecules in endothelial cells, resulting in upregulation of P-selectin and secretion of von Willebrand factor. In endothelial cells that have been pretreated with TNF α , the MAC enhances the expression of E-selectin and ICAM-1. The MAC is able to directly stimulate mitogenesis in target cells and may also induce the release of mitogenic substances from endothelial cells. In addition to causing cell activation and production of several inflammatory substances, molecules such as cAMP may exit the cell through the C5b-9 channel and modulate the function of neighboring cells.

The MAC plays an important pathophysiologic role in several autoimmune and inflammatory diseases. In some cases this may be due to its cytotoxic effect, as in several immune hematologic diseases. In many instances, however, the pathophysiologic participation of the MAC is more likely related to its ability to elicit cell activation when present in sublytic amounts. The MAC has been implicated in renal diseases such as acute poststreptococcal glomerulonephritis, membranous and membranoproliferative type III nephropathy and lupus nephritis, in neurological diseases such as multiple sclerosis, Guillain-Barré syndrome and myasthenia gravis, in rheumatoid arthritis and immune-complex vasculitis, and in the extension of myocardial infarction lesions and

ischemia-reperfusion injury. The MAC also plays a major role in hyperacute rejection of allografts and xenografts. In xenografts this rejection is in part due to the fact that the complement inhibitors are inhibitory to homologous complement but have little inhibitory capacity upon complement from a different species. For this reason transgenic donor pigs that express human complement inhibitors such as CD59 and DAF have been prepared and are being tested in nonhuman primates. The complement-mediated xenogeneic tissue injury is less severe in pig organs that express the human inhibitors than in organs from control pigs.

The biological importance of the inhibitors CD59 and DAF in protection of host cells against physiologic activation of autologous complement is highlighted by the disease paroxysmal nocturnal hemoglobinuria. This condition is caused by an acquired mutation in a gene for one of the enzymes that participates in the synthesis of the glycan phosphatidylinositol moiety shared by the complement inhibitors and certain other membrane proteins. As a consequence the peripheral blood cells derived from the mutant stem cell have low or absent expression of CD59 and DAF and are highly susceptible to complement mediated lysis. A role for the membrane-associated complement inhibitors, including CD59, in resistance of cancer cells to complement-mediated control has been proposed. However, human malignancies that express little or no complement inhibitors do not appear to have a better course than malignancies that express large amounts of inhibitors.

See also: CD59; Complement, alternative pathway; Complement, classical pathway; Complement deficiencies; Complement, genetics; Cytotoxicity, mechanisms of; Immunopathology; Xenotransplantation.

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COMPLEMENT RECEPTORS

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Complement (C) receptors were first reported by Levaditi and Inmann, in 1905, who found that phagocytic cells exhibited enhanced ability to ingest bacteria after treatment with fresh antisera that served as a source of complement as well as antibacterial antibody. This process, termed opsonization, was subsequently shown to involve the coating of bacteria with C3 molecules that facilitated attachment of the bacteria to membrane C3 receptors expressed on the phagocytes (i.e. neutrophils or monocyte/macrophages). Later, in 1930, Duke and Wallace observed that complement-opsonized trypanosomes bound to primate red blood cells, producing the phenomenon that was named immune adherence by Robert Nelson in 1953. Nelson was the first to demonstrate that immune adherence with red blood cells or neutrophils required only the activation of C3, and not the terminal lytic complement components. The immune adherence receptor of erythrocytes and neutrophils was subsequently named complement receptor type 1 (CR1) when the existence of other types of C3 receptors was appreciated (Table 1). Four additional types of C3 receptors have been named according to their order of discovery. CR2 (1973), CR3 (1979), CR4 (1984) and CR5 (1984). Four receptors for other complement components and for C3a have been named according to their ligand specificity: C1q-R (C1q receptor, 1975), C5a-R (C5a receptor, 1978), C3a-R (C3a receptor, 1979), fH-R (factor H receptor, 1980). In addition, several of the complement receptors have been recognized as leukocyte CD antigens by the International Leukocyte Antigen Workshop: CR1 is CD35, CR2 is CD21, CR3 is CD11b, CR4 is CD11c, and C5a-R is CD88. Complement receptors have a wide range of functions that include, in addition to phagocytosis, enhancement of B cell antigen recognition, removal of immune complexes from the blood, release of histamine from mast cells, and chemotaxis of phagocytic cells to sites of inflammation.

Structure, specificity and cellular distribution of complement receptors

Complement receptor type 1 (CR1)

CR1 (CD35) binds to C3b, and with lower affinity to C4b and iC3b. Proteolysis of iC3b into C3dg

destroys CR1 activity. Several cell types express CR1, including erythrocytes, phagocytic cells, lymphocytes (B cells and some T cells), kidney podocytes and peripheral nerves. CR1 along with CR3, CR4 and immunoglobulin G (IgG) Fc receptors are the major opsonin receptors on phagocytic cells. The CR1 of erythrocytes has two important functions in the clearance of circulating immune complexes. First, immune complexes and particles that activate complement are bound rapidly to erythrocytes via CR1. Erythrocytes then serve as a vehicle that transports the complexes to the liver and splenic macrophages. Second, erythrocyte CR1 function as the obligate cofactor for factor I cleavage of complex-bound iC3b into bound C3dg and fluid-phase C3c. B cell CR1 forms a membrane complex with CR2 that functions to promote the uptake of protein antigens that are coated with C3b or iC3b via natural antibodies and the classical pathway of complement activation. Antigen complexes that are bound to the CR1 portion of this B cell membrane complex via C3b are transferred to CR2 following proteolysis of the C3b into iC3b and C3dg. T cell CR1 may have a similar antigen-trapping function, but only small subsets of CD4⁺ and CD8⁺ T cells express CR1, and the amount of CR1 expressed per cell is ≤10% of that expressed by B cells.

CR1 is a member of a family of proteins known as the 'regulators of C activation' (RCA) that are encoded by chromosome 1 in humans. Other RCA members include CR2, factor H, decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), and C4-binding protein or C4-BP. CR1 exists in four allotypic forms that vary in size from 160 kDa to 250 kDa. Analysis of CR1 cDNA has demonstrated that CR1 consists of 3–5 linked protein segments that exhibit a high degree of internal sequence homology and are termed 'long homologous repeats' (LHRs). Allotypic variation in the number of LHRs per CR1 explains the large size variation in the CR1 from different individuals. Each LHR consists of seven short (60–70 amino acid) repeating sequence motifs ('short consensus repeats', SCRs). These SCR motifs are the common feature of the RCA proteins that apparently explain their C3b- and C4b-binding activity. Variations in SCR sequence determine binding activity, and distinct SCR sequences in CR1 confer either C3b- or C4b-binding activity.

Table 1 Structure, specificity and cellular distribution of complement receptors

Receptor type	Specificity	Structure	Cell type distribution
CR1 (CD35)	C3b > C4b > iC3b	Four allotypes that vary in size from 160 kDa to 250 kDa due to different numbers of repeating sequence motif units	Monocytes, neutrophils: high expression Tissue macrophages: low expression B cells, eosinophils: high expression Kidney podocytes: high expression T cells (~20%): low expression Peripheral nerves: low expression
CR2 (CD21)	iC3b = C3dg > C3d > C3b, Epstein-Barr virus, CD23	Made up of repeating sequence motif units similar to CR1; 140 kDa	B lymphocytes: high expression Lymph node follicular dendritic cells: very high expression Thymocytes, pharyngeal epithelial cells: low expression
CR3 (CD11b; Mac-1; $\alpha_M\beta_2$ -integrin)	iC3b, C3dg, C3d, β -glucan, ICAM-1, fibrinogen, factor X, collagen, heparan sulfate, CD14, CD16, CD87	Two noncovalently linked glycoprotein chains: 165 kDa α_M chain (CD11b), 95 kDa β_2 chain (CD18)	Neutrophils, monocytes: high expression Tissue macrophages: low expression Activated cytotoxic T cells, NK cells, eosinophils: high expression
CR4 (CD11c; p150,95; $\alpha_X\beta_2$ -integrin)	iC3b, C3dg, fibrinogen	Two noncovalently linked glycoprotein chains: 150 kDa α_X chain (CD11c), 95 kDa β_2 chain (CD18)	Neutrophils, monocytes: low expression Tissue macrophages: high expression Activated B cells: high expression Activated cytotoxic T cells, NK cells, eosinophils: low expression
CR5	C3d portion of fluid-phase iC3b, C3dg, C3d	Unknown; 95 kDa candidate molecule expressed by platelets	Neutrophils: low expression Platelets: low expression
gC1q-R	C1q globular 'head', as well as vitronectin and high molecular weight kininogen	33 kDa glycoprotein	All leukocyte types: variable expression Platelets: high expression Endothelial cells: high expression
cC1q-R	C1q collagen 'tail'	60 kDa glycoprotein; homologous to calreticulin	All leukocyte types: variable expression
C1q-R _p	C1q collagen-like fragment, mannose-binding protein, lung surfactant protein A	126 kDa glycoprotein	Neutrophils: high expression Monocytes: high expression
C3a-R	C3a, C4a	Guinea pig platelet: 95–105 kDa Human mast cell: 57 kDa and 97 kDa Human leukocyte: 482 amino acids, ~54 kDa, seven membrane-spanning domains	Mast cells: high expression Eosinophils, basophils: high expression Neutrophils, monocytes: low expression Guinea pig platelets: high expression
C5a-R (CD88)	C5a, C5a _{des Arg}	47 kDa glycoprotein binding unit with seven membrane-spanning domains expressed as an oligomer of 150–200 kDa	Mast cells, neutrophils: high expression Monocytes and tissue macrophages: high expression Eosinophils: high expression Hepatocytes, astrocytes, endothelial cells: high expression

In the mouse there is a closer relationship between CR1 and CR2, and the two membrane glycoproteins are derived from a single gene by alternative splicing of mRNA. As a result, the larger 190 kDa murine CR1 molecule contains the entire 140 kDa CR2

sequence as part of its structure. Monoclonal antibodies to CR2 are therefore equally reactive with CR1 and unable to distinguish the two types of C3 receptors. Murine CR1 appears to be restricted to B cells, and is not expressed on neutrophils or macro-

phages for phagocytosis, nor is it expressed on erythrocytes or platelets where it could function in immune complex clearance. Murine neutrophils and platelets express instead distinct C3b/C4b-binding molecules that appear structurally unrelated to CR1 or CR2.

Complement receptor type 2 (CR2)

CR2 (CD21) is expressed by B lymphocytes, lymph node follicular dendritic cells, pharyngeal epithelial cells and thymocytes. Only minor subsets of T lymphocytes express CR2, and the amount per cell is only 10% of the amount expressed by B cells. CR2 is not only a receptor for C3 fragments but also functions as the cellular attachment site for Epstein-Barr virus (EBV) and the counterligand for CD23. CR2 is specific for the C3d portion of iC3b, C3dg or C3d, and has only a low affinity for C3b. B cell CR2 has been shown to play a major role in primary and secondary antibody responses to protein antigens. In a primary response, natural antibodies form immune complexes with protein antigens, resulting in activation of the classical complement pathway and covalent fixation on to the antigen of C3b that is rapidly degraded by factor I into iC3b and C3dg. This bound C3 promotes efficient antigen uptake by B cells via CR2, and the resulting CR2 stimulation of the B cell lowers the antigen concentration threshold required for antigen recognition by specific surface Ig on the B cell. CR2 signaling results from a B cell membrane complex of glycoproteins that includes CR2, CD19 and TAPA-1 in which transmembrane signaling occurs via the CD19 portion of the complex. This pathway for use of complement and CR2 for antigen recognition was initially demonstrated with blocking antibodies to C3 or to CR2, and then later with gene knockout mice deficient in either C3, C4 or B cell CR2. Although an immune response could be generated in these mice with greatly increased antigen doses as compared with those required in normal mice, antibody levels were abnormally low, isotype switching did not occur, and there was no immunologic memory. This pathway for humoral immunity to protein antigens represents an important link between the innate and specific immune systems.

B cell isotype switching to IgE production also involves CR2 and its ability to form membrane complexes with the IgE receptor CD23. However, in a subversion of its normal function, CR2 is utilized by EBV to gain entry into B cells, permitting virus infection (infectious mononucleosis) or a malignant transformation of B cells or epithelial cells (Burkitt's lymphoma or nasopharyngeal carcinoma, respectively).

CR2 consists of a single polypeptide chain of

140 kDa with the same type of SCR structure as CR1. The CR2 gene is closely linked to the CR1 gene, and, in the mouse, a single gene transcribes both receptor proteins via alternative RNA splicing.

Complement receptor type 3 (CR3)

CR3 (also known as CD11b/CD18, Mac-1, or $\alpha_M\beta_2$ -integrin) is a multifunctional membrane protein that serves as both a membrane receptor to trigger phagocyte and natural killer (NK) cell cytotoxic events and as an adhesion molecule used by leukocytes for diapedesis across the endothelium (via attachment to ICAM-1) or the extracellular matrix (via attachment to heparan sulfate or fibrin). The I domain of CD11b binds with high avidity to opsonizing iC3b attached to microorganisms, and with lower avidity to opsonizing C3dg or C3d. However, its receptor function is complex, because CR3 not only recognizes exogenous target cell-associated ligands directly but also extends its effective ligand specificity by coupling itself to certain other endogenous leukocyte membrane receptors that utilize CR3 for their transmembrane signaling function. Critical to both forms of receptor activity is a lectin site located C-terminal to the I domain of CD11b and involved in recognition of either microbial cell wall polysaccharides or the leukocyte membrane glycoproteins that couple to CR3 for transmembrane signaling. Bacteria or yeast that bear opsonizing iC3b trigger CR3 activation because of simultaneous recognition of microbial cell wall polysaccharides via the CR3 lectin site and bound iC3b via the I domain. Potential target particles that bear fixed iC3b but lack specific polysaccharides recognized by this lectin site of CR3 do not stimulate cellular activation, despite avid adhesion of the particles to CR3 via the fixed iC3b. Ligation of polysaccharides to the lectin site of CD11b induces a tyrosine kinase- and magnesium divalent cation-dependent conformational change in CD11b that is associated with generation of a primed state of CR3 capable of stimulating cellular cytotoxic activation events (e.g. phagocytosis, degranulation) when CR3 subsequently attaches to target cell-bound iC3b via its I domain-localized binding site. Three neutrophil membrane receptors that are attached to membranes via surface phosphatidylinositol glycolipid (PIG) bridges acquire transmembrane signaling function by forming lectin site-dependent complexes with the transmembrane CR3 molecule: 1) the IgG Fc receptor Fc γ RIIIA (CD16), 2) the urokinase plasminogen activator receptor (uPAR or CD87), and 3) the lipopolysaccharide (LPS) receptor (CD14).

Unlike C-type (calcium-dependent) lectins, the polysaccharide-binding site of CR3 does not require divalent cations and has a relatively broad reactivity

with certain polysaccharides containing mannose or N-acetyl-glucosamine, as well as glucose (β 1,3-glucans).

Unstimulated phagocytes express small amounts of membrane CR3 and retain large stores of CR3 in cytoplasmic granules. Following stimulation with C5a or a variety of cytokines, much of the initial membrane surface CR3 becomes linked to the actin cytoskeleton in a way that renders it capable of mediating phagocytosis or extracellular adhesion to ICAM-1. Simultaneously, specific granules come to the cell surface, where their membranes fuse with the outer cell membrane, greatly increasing the expression of membrane surface CR3. However, this granule-derived CR3 is not linked to the cytoskeleton and is thus incapable of mediating firm cellular adhesions or phagocytosis. This more abundant but loosely associated CR3 is very mobile within the membrane and functions to promote phagocyte attachment to iC3b-coated target cells, allowing their subsequent phagocytic recognition by the less mobile but cytoskeleton-bound CR3 (or CR4).

CR3 consists of two noncovalently associated glycoprotein chains known as α_M or CD11b (165 kDa) and β_2 or CD18 (95 kDa). CR3 is one of four members of the leukocyte β_2 -integrins that share the common β_2 -integrin structure linked to a distinct α -chain type. The other three leukocyte β_2 -integrins are LFA-1 (CD11a/CD18), CR4 (CD11c/CD18 or p150,95) and CD11d/CD18.

Complement receptor type 4 (CR4)

CR4 (CD11c/CD18 or p150,95), the third member of the leukocyte β_2 -integrins, is closely related to CR3. The CR4 α chain (CD11c, 150 kDa) is ~87% homologous in sequence to CD11b, and recognizes similar ligands including iC3b, fibrinogen and, according to some reports, even ICAM-1. CR4 is expressed preferentially on tissue macrophages that bear only small amounts of CR1 and CR3. Phagocyte CR3 and CR4 differ in cytoplasmic domain structure and attachment to the actin cytoskeleton, and this is thought to provide a mechanism for differential regulation of cellular adhesion or phagocytosis. The relative importance of CR4 as a receptor for iC3b is unclear, as most of the iC3b receptor function of leukocytes, including tissue macrophages, is blocked by use of antibodies to CR3 alone.

Complement receptor type 5 (CR5)

CR5 appears to be specific for the C3d portion of iC3b, C3dg and C3d, but reacts only with these fragments in the fluid phase and not when they are fixed via their normal covalent site. CR5 activity has been defined with fluid-phase dimers of C3dg labeled with

^{125}I . Neutrophil uptake of ^{125}I -C3dg dimers was blocked by competing unlabeled iC3b, C3dg and C3d, but not by C3b. At one time it was believed that the C3dg dimer receptor might be the same as the receptor for erythrocyte C3dg rosette formation, and so both receptor activities were designated CR4. Later, however, the receptor activity responsible for rosette formation was shown to have several properties that distinguished it from the C3dg dimer receptor, and so the C3dg dimer receptor was named CR5. The binding of CR4 to fixed iC3b was prevented by ethylene diamine tetraacetic acid (EDTA), whereas EDTA had no effect on the uptake of ^{125}I -C3dg dimers. Although CR5 activity has been identified on neutrophils and platelets, it is unknown whether the same molecule is responsible for this activity on both cell types. A platelet membrane glycoprotein of 95 kDa identified by affinity labeling techniques has been shown to function as a C3dg receptor. In the absence of sequence data established from a molecular clone, the existence of CR5 as a distinct entity is less certain than the other C3 receptors.

C1q receptors (C1q-R)

Three distinct types of C1q-R have been described: one that binds to the globular 'head' portion and two that react with the collagen 'tail'. All three of the receptors have been shown to react with other molecules in addition to C1q, such as vitronectin, kininogen and mannose-binding protein. A 33 kDa glycoprotein with affinity for the C1q globular head, 'gC1q-R', and a 60 kDa glycoprotein that reacts with the collagen tail, 'cC1q-R', were first identified on Raji B lymphoblastoid cells. Antibodies to these two molecules have now shown a wider cell type distribution, and gC1q-R is particularly expressed on platelets and endothelial cells, as well as eosinophils. A second type of receptor for the collagen portion of C1q is a 126 kDa monocyte and neutrophil membrane glycoprotein that has been termed C1q-R_p because of its ability to enhance phagocytosis mediated via CR1. The cDNAs for all three of these molecules have been cloned, and gC1q-R has been expressed as a recombinant molecule that retains C1q-binding activity. Although the two have novel sequences, the 60 kDa cC1q-R has considerable homology with calreticulin and may represent a membrane surface variant of this cytoplasmic protein. The 33 kDa gC1q-R is the product of a single gene localized to chromosome 17 and has a specificity similar to the Fc region of Ig. By contrast, cC1q-R and C1q-R_p do not compete with Ig Fc, and thus can promote cellular uptake of immune complexes bearing C1q linked to Ig. These latter types

of C1q-R bind immune complex-associated C1q preferentially, because single C1q molecules have a low affinity for cC1q-R or C1q-R_p, and the C1q binding site in native C1 is masked by C1r and C1s. The collagen binding site in C1q is exposed by C1 inhibitor that complexes with activated C1r and C1s, separating them away from C1q.

Factor H receptor (fH-R)

A fH-R activity has been detected on B lymphocytes, monocytes and neutrophils, but no structural comparison of the fH-R on these cell types has been carried out and the molecular entity representing fH-R has not been identified. Since plasma did not block cellular uptake of radiolabeled factor H, it was proposed that fH-R were unreactive with 'native' plasma factor H, and that either purification procedures or immune complex attachment might alter factor H in a way that exposed a binding site for fH-R. Subsequently, purified factor H preparations were shown to contain two species of factor H, designated ϕ_1 and ϕ_2 , that could be separated by phenyl-Sepharose hydrophobic affinity chromatography. Only the ϕ_2 form of factor H bound specifically to lymphoid cells, but it is unknown whether this ϕ_2 form of factor H exists in plasma or if it is generated by H interaction with immune complexes. The first attempt at isolation of the fH-R from lymphoblastoid cells used either antifactor H idiotype antibody or factor H-Sepharose and identified a protein complex of ≥ 150 M_r with 100 kDa and 50 kDa components. A second study using factor H agarose affinity chromatography detected a single protein species of 170 kDa on the surface of B lymphoblastoid cells and tonsil B cells. This 170 kDa protein bound specifically to factor H agarose, and in soluble form, prevented rosettes with factor H-coated erythrocytes. B cells have been shown to release endogenous stores of factor I in response to fluid-phase or immune complex-associated factor H, and purified H has been reported to serve as a growth factor for the maintenance of B cell lines.

C5a receptor (C5a-R)

The inflammatory functions of C5a are mediated via specific C5a-R (CD88) expressed on a variety of cell types including mast cells, phagocytes, bronchial and alveolar epithelial cells, hepatocytes, astrocytes and vascular endothelial cells. The action of C5a is regulated both by serum carboxypeptidase N (SCPN), which removes the C-terminal arginine (forming C5a_{des Arg}), and by neutrophil catabolism of C5a following specific uptake by C5a-R. Although C5a_{des Arg} has reduced binding affinity as compared with C5a, it is a major ligand for C5a-R *in vivo* because of the

serum protein ('cochemotaxin') that combines with the C5a_{des Arg} and enhances its action with C5a-R. Cochemotaxin is identical to GC globulin or vitamin D-binding protein. The ~47 kDa C5a-R glycoprotein is a member of the family of related G protein-linked chemokine receptors that have seven membrane-spanning domains. A two-site model has been suggested in which part of the N-terminal extracellular domain of the receptor recognizes the N-terminal and disulfide-linked core of C5a. This is then thought to be followed by interaction of the C-terminus of C5a with a second region of the receptor that is linked to G protein and responsible for the activating signal. Both the contractile responses of smooth muscle cells and the increased vascular permeability produced by C5a are thought to result from histamine release by mast cells or basophils that express the same C5a-R structure. C5a-R mediate a wide range of leukocyte responses, including chemotaxis, homotypic aggregation (clumping), activation of CR3 to expose its high-affinity binding site for ICAM-1 (thus promoting leukocyte adhesion to endothelium), and upregulation of the number of CR1, CR3 and CR4 exposed on external membrane surfaces. Larger doses of C5a cause degranulation and a respiratory burst. Because of the importance of C5a in mediating inflammatory responses, C5a-R antagonist drugs have been widely sought as therapeutic agents for autoimmune and inflammatory disease syndromes.

Receptor for C3a and C4a (C3a-R)

A single type of receptor is believed to be responsible for responses to C3a or C4a. This receptor activity was first characterized on mast cells, and later a similar C3a-R was demonstrated on basophils, eosinophils, neutrophils and guinea pig platelets. The first successful molecular cloning of the C3a-R was reported with cDNA libraries generated from differentiated HL-60 cells or U-937 cells. As with C5a, smooth muscle spasmogenic responses occur with nanomolar concentrations of C3a or C4a that stimulate mast cell secretion of histamine. Although disputed for many years, the chemotactic activity of C3a has now been shown with eosinophils and is likely to occur also with neutrophils or monocytes that express C3a-R. As with C5a, SCPN removes a C-terminal arginine from both C3a and C4a, greatly reducing, if not totally eliminating, the ability of these anaphylatoxins to stimulate cellular responses. Not only are all spasmogenic and leukocyte-associated activities of C3a and C4a absent in C3a_{des Arg} and C4a_{des Arg}, but also no cochemotaxin-like serum factor has been identified that restores C3a/C4a_{des Arg} activity in the same way as has been shown for C5a_{des Arg}. Attempts to identify a C3a-R with

¹²⁵I-C3a coupled to heterobifunctional cross-linking reagents identified a putative C3a-R only on guinea pig platelets and failed to identify a C3a-R on human platelets and neutrophils; however, similar methods were successful with a human mast cell line, HMC-1. These studies identified two putative guinea pig platelet C3a-R candidate molecules of 95 kDa and 105 kDa, and two human mast cell C3a-R species of 57 kDa and 97 kDa. Sequence homology with both the C5a-R (37% at the nucleotide level) and the fMLP-receptor allowed molecular cloning of the C3a-R. Cells expressing recombinant C3a-R exhibited the same reactivity with C3a as the native receptor, including the failure to bind C5a. Northern blotting with the 4.3 kb cDNA for C3a-R demonstrated transcripts of both 2.3 kb and 3.9 kb, perhaps explaining the two different sized receptors noted in cross-linking experiments. Based on the 2.3 kb transcript, a polypeptide of 482 residues was predicted with the typical seven membrane-spanning domains of the G protein-linked receptor family.

See also: Adhesion molecules; Anaphylatoxins; Complement, alternative pathway; Chemokines; Complement, classical pathway; Complement, genetics; Epstein-Barr virus, infection and immunity; Immune adherence; Integrins; Opsonization; Phagocytosis; Viruses, infection of immune cells by.

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CONGENIC MICE

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Inbreeding is the mating of individuals more closely related than mates chosen at random from a population. An inbred strain is a population that is almost genetically identical. Two animals (lines) that are genetically identical save for a difference at one locus

are called coisogenic (L. *co*, together; Gr. *iso*, identical).

When George D. Snell, working at the Jackson Laboratory, realized that rejection of a graft between two mice was affected by multiple genes, he

developed the concept of 'congenic mice', by transferring resistance genes from one mouse to another from a different genetic background. He produced lines by genetic backcrossing, and calculated that 12 generations were needed to obtain a line that differed from the original at one chromosomal region carrying an identified marker. While working on this breeding, Snell noticed that not only was there a locus intimately related to the rejection of tumor grafts, but another gene seemed to be retained from the donor strain, the *Fu* locus, a marker for a deformity of the tail. He named it the histocompatibility locus. During the development of the first congenic lines, Snell observed that some of the mice retained not only the tumor resistance but also marker genes of the donor strain. For example, the congenic line ACA, was produced from the inbred strain A and an outbred stock CA carrying the mutation fused tail (*Fu*). This not only carried the resistance to A strain tumors but also had the shortened tails caused by the *Fu* gene. This was the first demonstration of genetic linkage in mammals and the first genetic marker for the major histocompatibility complex later to be found by serology as H2.

At the same time, Peter Gorer in England discovered a hemagglutinating antibody associated with the rejection of transplantation of tumors. It appeared that a cytotoxic antibody responsible for this rejection was associated with blood group antigens. This was not considered a general rule for rejection, but the gene encoding this antigen was indeed located at the named 'H' locus. Gorer named the antigen 'antigen II'. In 1948, Gorer and Snell, working together, discovered that the H locus was linked to the tail marker (*Fu*) and was associated with rejection. Using congenic strains they found that skin grafts were rejected from the mice at different periods after the transplant. Experiments with different congenic strains produced support to the concept that some genes were associated with different degrees of influence in the rejection of allografts.

The H2 genes were identified as major genes and later were located in a single region at chromosome 17 named the major histocompatibility complex (MHC).

Congenic mice are useful tools for the analysis of the genetics of tissue and bone marrow transplantation, the genetics of the immune response including cellular interactions and the role of genes of the MHC in autoimmunity and longevity.

Production of congenic strains

Coisogenic strains are produced when an inbred mouse becomes genetically identical to another,

except with a difference at a single locus produced by mutation. This can be achieved by crossing mice in order to bear or introduce a small dominant region of one donor into a common genetic background. Therefore, congenic mice are two strains that are identical except for a short chromosomal segment. Coisogenic strains differ in one locus and congenic differ in a small variable region of a chromosome including several loci.

Backcrossing

The backcrossing procedure is applicable to the introduction of an autosomal dominant or codominant allele. The original pair, one inbred strain which donates the genetic background (first parent or inbred partner) and one donor strain which donates the chromosomal segment (second parent), are crossed. The F1 progeny are backcrossed to the inbred partner. For the next generation, a donor that poses the desired allele is selected. After 12 generations heterozygote siblings are mated, originating the desired genotype. At this point of the backcrossing, the offspring will be the progenitors of the desired congenic strain, which will be perpetuated by standard sibling mating (intercross) or by further backcrossing to the inbred parent.

The production of congenic strains of mice is based on the introduction of a short chromosomal segment into a second strain. The resulting strain is congenic. Molecular techniques are required to identify the marker gene of the genetic region that will be introduced into a common genetic background. Most of the existing congenic strains were named congenic resistant to indicate the introduction of genes that induced resistance to an incompatible tumor originating from a parent of the donor strain involved in the backcrossing. They differed from their inbred part at the histocompatibility locus. However, they can be produced for any genetic marker. The production of the strain B10.D2 is given as an example (Figure 1). In this example the genetic background is given by the B10 (C57BL/10SN) strain which is H2-b and the donor of H2-d of the strain DBQ/2J. The diagram shows that by a number of backcrosses, usually 10–15, it produces strains that differ from their inbred partner by a chromosomal segment 12–20 centimorgans (cM) long. In the case of the H2 (2 cM long) it would require at least 30 backcrosses to produce strains differing at less than 10 cM.

Essentially the backcrosses are tested for H2 markers (usually antibodies specific for MHC gene products). In the example of the figure, the homozygotic H2-b are discarded. The backcrosses are always between a b/d and the B10 inbred. It is important

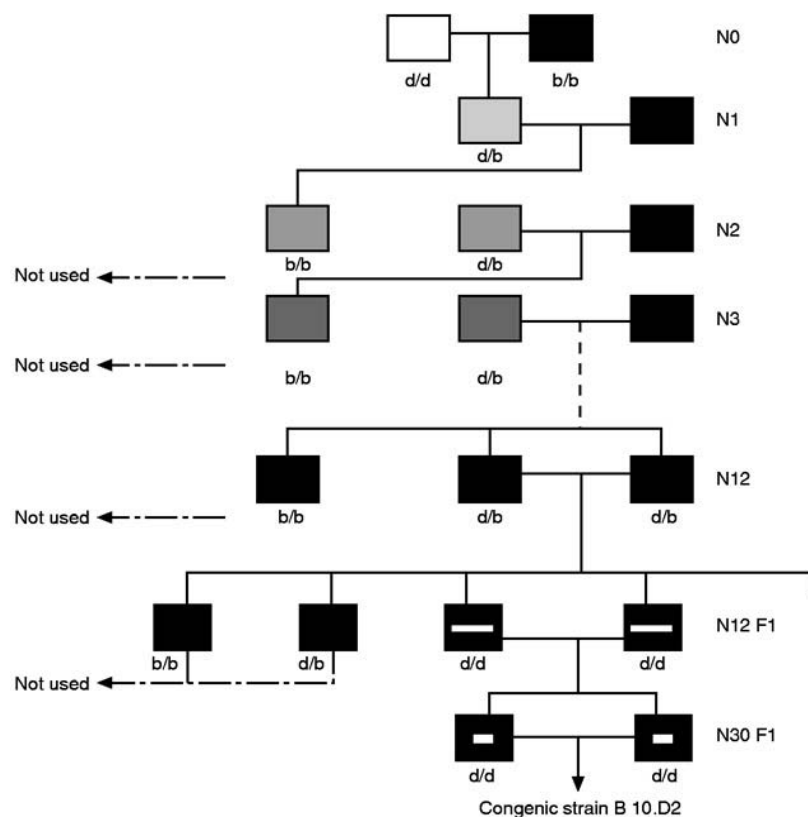


Figure 1 Breeding system for producing congenic mice.

that the coisogenic strains differ only by a point mutation. When they are produced the level of probability is defined by the equation:

$$P_n = 1 - (1 - c)^{n-1}$$

where p = probability, n = number of generations and c = recombination frequency.

The higher the number of backcrosses the smaller the length of the differential chromosomal segment.

Genetics of the donor strains in congenic mice

The majority of the congenic mice were produced in the C57BL6 or C57BL10 mice (Table 1 gives examples of congenic and A mice background).

Symbols

The H2 complex occupies a segment of the chromosome 17 at a distance of some 15 cM from the centromere. The segment is between 0.1 and 1.5 cM long and contains a minimum of 10–15 loci. (The length of the segment and the number of loci in it depend on which loci one counts as belonging to the H2 complex.) To understand the designation of the congenic lines, Table 1 illustrates the strains (background partners) from which they originated

with the locations. These are some of the congenic strains that are available today at the Jackson Laboratory in Bar Harbor, Maine.

Congenic strains can be named by the symbols of the parents' strains used in the original mating. They are separated by a period, i.e. B10.D2; this means that B10 originated from C57BL/10SN and D2 from DBA/2J. They can also be designated by using the name of the inbred partner followed by a hyphen and then the name of the differential allele contributed by the donor strain as described.

Numbers and letters in parenthesis are used when several different strains are derived from one initial cross.

There are also symbols that originated for laboratory purposes only but subsequently were used in the literature, i.e. (5M), (HW80), (HW19).

For designation of substrains, the addition of an abbreviation of the laboratory where they originated from is used, e.g.: Sn for Snell and By for Bailey.

When congenic strains differ at nonhistocompatibility loci, designations are used to distinguish the differential loci; e.g. Ea for erythrocyte antigen, Ly for lymphocyte, Thy for thymocyte and Tla for thymus-leukemia antigen.

See also: Allelic exclusion; Genetic analysis at the phenotypic level; H2 class I; Inbred strains; Linkage

Table 1 Examples of congenic strains

Strain	Genotype*	Donor strain
<i>Congenic strains with differential locus in the H2 complex</i>		
A.BY/Sn	H2 ^b Tla [?]	Non-inbred
A.CA/Sn	H2 ^f Tla ^a	Non-inbred
A.SW/Sn	H2 ^a Tla ^b	Non-inbred
B6.AKR-H-2 ^b /FlaEg	H2 ^k	AKR/JBy
B6.C-H-2 ^d /aBy (HW19)	H2 ^d	BALB/cBy
B10.A/SgSn	H2 ^a Tla ^a	A/WySn
B10.AKM/Sn	H2 ^m Tla ^k	AKR.M/oSn
B10.Br/SgSn	H2 ^k Tla ^a	C57BR/cdJ
B10.D2/nSn	H2 ^d Tla ^c Hc ^l	DBA/2
B10.HTG/2Cy	H2 ^g	HTG/GoSfSn
C3H.HTG/Sn	H2 ^g Tla ^{b?}	HTG/Sn
C3H.NB/Sn	H2 ^p Tla ^{c?}	Non-inbred
C3H.SW/Sn	H2 ^b Tla ^{b?}	Non-inbred
<i>Congenic strains with differential locus at H loci other than H2</i>		
B6.H-1 ^b /By (HW80)	H-1 ^b , c, Hbb ^d	BALB/cBy
B6.C-H-7 ^b /By (HW23)	H-7 ^b , Mod-1 ^a	BALB/cBy
B6.C-H-8 ^c /By (HW96)	H-8 ^c	BALB/cBy
B6.C-H-15 ^c /By (HW13J)	H-15 ^c	BALB/cBy
<i>Congenic strains with differential locus that determines nonhistocompatibility cell membrane alloantigens</i>		
B6.RIII(76NS)/Sn	Ea-2 ^a	RIII
B6.PL-Ly-2 ^a Ly-3 ^a /Cy	Ly-2 ^a Ly-3 ^a	PL/J
B6.PL-Thy-1 ^a /Cy	Thy-1 ^a	PL/J
BALB/c-Igh ^b /Smn (C.B-17) Igh ^b		C57BL/Ka
BALB/c-Igh ^d /Smn (C.B-20) Igh ^d		AL/N
C57BL/6-Igh ^a (B6.C20) Igh ^a		BALB/cAn

*The genotypes listed are from The International Committee on Standardized Genetic Nomenclature for Mice. Genetic mutations of each gene are identified by superscript numbers or letters. For example, H2^b is a specific mutation of the histocompatibility locus 2 (H2) gene.

disequilibrium; Mouse inbred strains; Mouse inbred strains, origins of; Transgenic animals.

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CONTACT HYPERSENSITIVITY

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Contact hypersensitivity is a form of delayed-type or cell-mediated hypersensitivity expressed in the skin. It is initiated by agents/antigens that penetrate through the outer epidermal barrier (stratum corneum) and reach the viable layers of the epidermis. Once the immune system has been activated via the epicutaneous route, topical re-exposure to the relevant antigen elicits an inflammatory response, characterized by edema formation, which reaches a

maximum 48–72 h later. In humans, immunological memory to contact antigens is long-lived, whereas in mice it is usually reported as being transient, lasting only a few weeks.

Functional significance

Contact hypersensitivity is probably important for protective immunity against tumors and microbes,

such as viruses, fungi and parasites (ticks and mites), that invade the skin; however, there is also a response to environmental substances that enter the skin, causing the clinical condition of allergic contact eczema/dermatitis.

Immunobiology

Activation of the immune system via the epicutaneous contact route is dependent upon several factors.

Antigen potency

Some antigens, such as 2,4-dinitrochlorobenzene (DNCB), pentadecylcatechol (urushiol of poison ivy) and diphenylcyclopropenone (diphenylpyrone) are potent and can sensitize all subjects. Many organic compounds and inorganic salts of metals such as nickel, cobalt and chromium are weak antigens and appear only to sensitize some subjects.

Genetic factors

In experimental animals there are clearly genetic controls of immune responsiveness. Thus, inbred guinea pigs of strain XIII can be sensitized with mercury but not chromate or beryllium, whereas animals of strain II can be sensitized with chromate and beryllium but not with mercury. Evidence in humans is less clear-cut. With regard to a strong antigen (DNCB), it seems that everyone can become sensitive but there is a normal distribution of the degree of sensitivity. Family studies have indicated that 'high responder' characteristics are transmitted to children. Studies of HLA linkage in subjects with the propensity to form numerous allergic sensitivities to environmental substances have not found positive evidence of genetic control.

Intact lymphatic drainage

Guinea pigs cannot be sensitized if antigen is applied to islands of skin lacking lymphatic drainage. This is because the early stages of the induction process involve circulation of antigen-bearing cells via the lymphatics to the draining lymph nodes.

Antigen-presenting cells

The epidermis is populated with Langerhans cells. These are leukocytes whose function is presentation of antigens to the appropriate CD4⁺ T lymphocytes. They have long dendritic processes ramifying between the epidermal cells, forming a network likely to be encountered by any organism or antigen which penetrates through the stratum corneum barrier. They are characterized by a formalin-resistant membrane ATPase, a specific cytoplasmic organelle,

the Birbeck granule, membrane antigens including CD1a (T6), class II major histocompatibility complex (MHC) antigens and costimulatory molecules B7-1 and B7-2. Langerhans cells also express various adhesion molecules including E-cadherin, probably necessary for maintaining position in the epidermis, and ICAM-1 and LFA-1, necessary for their migration to the regional lymph node and interaction with T cells.

Induction of contact sensitivity

When epicutaneously applied antigen reaches the Langerhans cells they exhibit signs of activation, as shown by formation of coated pits, appearance of Birbeck granules and loss of surface ATPase activity. During the next 1–4 h antigen-bearing Langerhans cells emigrate from the epidermis and are found in the superficial dermis. They are observed in the lymphatics leaving the site and by 24 h they have reached the draining lymph node. This is the site of interaction with the appropriate antigen-specific CD4⁺ T lymphocytes, which are activated to proliferate. Langerhans cell migration is dependent upon expression of ICAM-1 and LFA-1; mice treated with antibodies against these adhesion molecules fail to develop contact sensitivity and antigen-bearing Langerhans cells fail to reach the regional lymph nodes. Also, evidence suggests that tumor necrosis factor α (TNF α) is an important mediator in stimulation of Langerhans cell migration. The interaction between Langerhans and T cells is via the T cell receptor and antigen–MHC complex and also includes LFA-1/ICAM-1, LFA-3/CD2 and B7-1/CD28 recognition. Additional cytokine signals including interleukin 1 β (IL-1 β) and possibly IL-12 result in differentiation of T cells of the T_H1 type. It is not known to what extent Langerhans cells have to 'process' the antigen. It seems likely that small molecules (haptens), such as DNCB or nickel, may not be processed as they could bind directly to the MHC class II epitopes important for recognition by the T cell receptor. However, evidence is emerging that even small molecules may require metabolic conversion from the prohaptens to the immunogenic haptens.

The sequence of events in which Langerhans cells become activated and leave the epidermis *en route* for the regional lymph nodes appears to be determined at least in part by the antigen. Thus, in contrast to DNCB, a potent antigen, 2,4-dichloronitrobenzene (DCNB), a nonsensitizer, does not induce emigration of Langerhans cells, although it is absorbed through the epidermis equally, and has equal protein-binding properties. It would be inter-

esting to know if DNCB can bind directly to the MHC class II determinants.

Dose-response relationships

As for other physiological systems, the immune system obeys a variety of dose-response relationships. These have been studied in mice and humans using experimental contact sensitivity. Firstly, increasing the concentration of antigen per unit area leads to an increase in the proportion of reactive subjects. Thus, using a 3 cm diameter circle on the forearm, application of 62.5 µg DNCB sensitized 8%, 116 µg sensitized 50% and 500 µg sensitized 100% of experimental volunteers. Not only are more subjects sensitized by increased concentrations of antigen, but also their degree of sensitivity increases linearly with the log of sensitizing dose. By contrast, when concentration is kept constant then, over a wide range, changes of total dose and area of application have relatively little effect. These observations imply that, although the number of Langerhans cells reaching the lymph node is important, it is actually the number of antigen molecules per Langerhans cell that is the major determinant in the activation of specific T cells and their subsequent clonal expansion. Of course, at small areas (less than 1 cm diameter) this ceases to be true and the number of Langerhans cells also becomes critical.

Expression of contact hypersensitivity

When a sensitized animal or person re-encounters an antigen, a series of events occurs which results in the clinically recognizable response of contact hypersensitivity. The response is characterized by vasodilation (erythema), edema of both dermis and epidermis, fibrin deposition and the accumulation of an inflammatory infiltrate of monocyte/macrophages and lymphocytes. Detectable sensitivity reflects the presence of specifically committed CD4⁺ lymphocytes circulating and trafficking through the tissues. Their numbers presumably determine the magnitude of the reaction to antigenic challenge; thus, in fact, subclinical sensitization could be present if the clone of T cells had not expanded sufficiently to leave the lymph node, although 'memory' would have been established. There is clear evidence that such subclinical sensitization can occur. The extent of the clonal proliferation appears to be proportional to the 'drive' from antigen-bearing Langerhans cells, reflected in the increasing degree of reactivity induced by increasing sensitizing doses of antigen.

One of the unanswered questions is whether Langerhans cells have to participate in the secondary pres-

entation of antigen to passing T cells to initiate the response. It seems probable that they are required.

The normal traffic of T cells through the tissues is light and hence the likelihood of the appropriately committed T cell passing by and encountering a few antigen-bearing Langerhans cells in the superficial dermis must be so low that the efficiency and reliability of the system as one of protection would be quite hopeless; therefore there must be initial processes, independent of the physical presence of T cells, which can increase cellular traffic. In rodents, mast cells have been implicated as providing such a mechanism. In humans, the evidence is against the involvement of mast cells. However, the skin 'senses' physical or chemical perturbation, whether by irritant or antigen, by activation of a range of mechanisms aimed at increasing the speed and efficiency of T cell traffic and hence immune surveillance. The mechanisms activated include production of cytokines, upregulation of adhesion molecules on endothelial cells and translocation of Langerhans cells from the epidermis to the superficial dermis.

Cytokines

Within 15 min of application of a contact sensitizer to mouse skin, IL-1β gene transcription is activated in Langerhans cells. Other cytokines including IL-1α, TNFα and chemokines IP-10 and MIP-2 are also upregulated within a few hours. In mice, macrophage migration inhibition factor is also expressed. Some evidence suggests that keratinocytes rather than Langerhans cells are the source of these, and that they are induced by contact sensitizers but not by irritants.

Adhesion molecule expression

Dermal microvascular endothelial cells show upregulation of expression of E-selectin, first observable, 2 h after application of provoking chemicals. ICAM-1 and VCAM-1 increase from 4–6 h onwards and reach a maximum by 24 h. The upregulation of adhesion molecules is seen with both irritants and antigens, and whether individuals are sensitive or 'naive'. These events precede any cellular infiltration. Lymphocyte infiltration is first detectable by 8 h. In individuals sensitive to the provoking substance, a few activated T cells expressing CD25 (IL-2 receptor α chain) are seen at 8 h. After this the reaction builds into a full 'positive response' with heavy infiltration of mononuclear cells. In nonsensitive subjects the various features all subside over the next 12–24 h. Thus, the tissue elements in skin respond to perturbation by activating mechanisms designed to increase the traffic of T cells through the tissues. If an appropriate T cell encounters its target antigen on a Lang-

erhans cell, it is activated to release interferon γ (IFN γ), and a general and nonspecific recruitment of other lymphocytes occurs. This continued accumulation of cells appears to be mediated in large part by IFN γ , as inoculation of rats with a monoclonal antibody to IFN γ reduced by 50–90% the migration of lymphocytes into reaction sites.

Dose–response relationships

As for the induction component of the immune response, so the expression component also exhibits predictable dose–response relationships. Application of a graded series of challenge doses to sensitized subjects elicits a graded series of inflammatory responses. There are several methods for quantifying responses, including measurement of thickness of the edema with calipers or ultrasound, measurement of the redness with reflectance instruments, measurement of the blood flow with a laser Doppler flowmeter, and in experimental animals accumulation of radiolabeled cells can be determined. None of the methods are perfect and various caveats apply to them all. The main one is that at large responses, when edema formation is great enough to cause blistering, all the methods, with the possible exception of radio-labeled cell accumulation, break down.

The mechanisms by which the inflammatory reaction of contact hypersensitivity resolves are not clear. Postulated mechanisms include induction of immunosuppressive cytokines, such as IL-10, or apoptosis of the effector T cell.

See also: Antigen, entry into the body; Antigen-presenting cells; Antigen presentation via MHC class II molecules; Mast cells; Cell-mediated immunity; Delayed-type hypersensitivity; Eczema; Hapten;

Macrophage migration inhibitory factor (MIF); Skin, contribution to immunity.

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CONTRACEPTION, IMMUNOLOGICAL

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A major technological advance in the twentieth century was the development of a number of methods to prevent an unwanted pregnancy. Amongst newer methods currently under development are those which employ immunological approaches. Vaccines inducing appropriate immune response have been made, with demonstrated ability to control fertility in experimental animals, including subhuman primates. Extensive safety and toxicology studies have shown the safety and reversibility of some of these

vaccines, and these, with due approval of the drugs regulatory authorities and institutional ethics committees, have undergone clinical trials in humans. Six vaccines have completed phase I clinical trials documenting their safety and reversibility – three vaccines in women and three in men. One vaccine has also successfully completed the phase II trials in women, providing evidence for its efficacy in preventing pregnancy. These trials have established the titers of antibodies and other characteristics (avidity, immuno-

dominant epitopes, bionutralization capacity) to achieve efficacy. Of interest is the application of some of these vaccines in hormone-dependent cancers (carcinoma of the prostate) and lung cancers secreting human chorionic gonadotropin (hCG).

Principle

The rationale behind birth control vaccines is the generation of antibodies and/or cell-mediated immune responses (CMI) against either a hormone or gamete antigen(s) important for the success of reproduction. The production of the gametes in both males and females is critically regulated by hormones. A cascade of hormones is involved, starting from the gonadotropin-releasing hormone (GnRH or LHRH) secreted by the hypothalamus. This decapeptide hormone, common to both males and females, stimulates the production and secretion of the two gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary. These act in concert on the gonads to generate eggs or sperm from the ovaries or the testes, respectively (Figure 1).

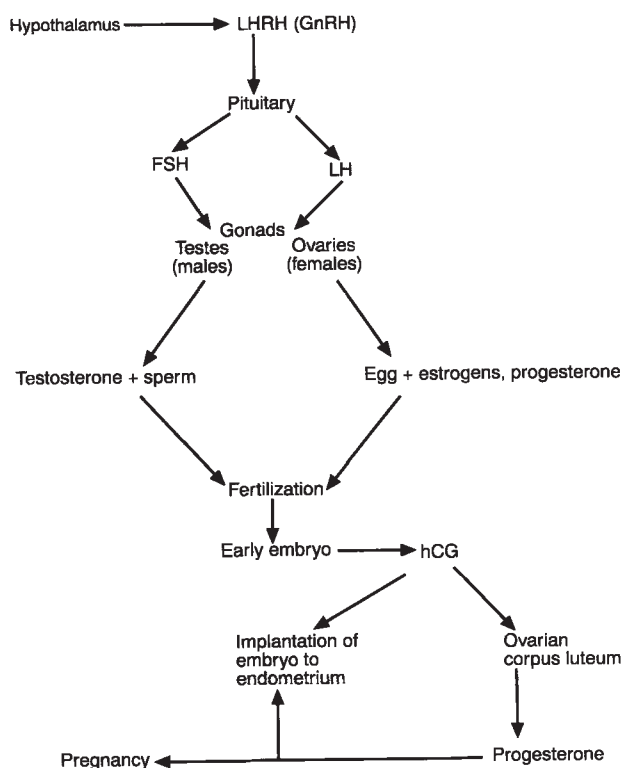


Figure 1 Hormones involved in the production of gametes and sex steroids. Antibodies inactivating one or more of these hormones would block fertility. hCG secretion from the embryo starts at the preimplantation stage. It has a role in nidation (initiation of pregnancy) and in its sustenance for 7 weeks by production of progesterone from ovaries.

The fertilized egg also makes the hormone hCG, secretion of which by the preimplantation human embryo is detectable in culture following *in vitro* fertilization prior to transfer to the uterus. Evidence points to a crucial role of this hormone in implantation, and antibodies inactivating hCG intercept implantation. Following implantation, the amount of hCG secreted increases sharply until 7–9 weeks of pregnancy, after which it declines to a lower level and stays so throughout pregnancy in humans. A second important biological role of hCG is in sustenance of the ovarian corpus luteum and in continued production of progesterone for the first 7–9 weeks, after which the placenta takes over the production of progesterone. hCG thus has a dual role, in establishment of pregnancy (implantation of the embryo into the endometrium) and in its sustenance for at least 7–9 weeks. Antibodies against hCG can prevent the establishment of the pregnancy if these are pre-existing in the circulation by virtue of active immunization. Antibodies given passively at the postimplantation stage but during the first 7 weeks of pregnancy have been shown, in baboons, to terminate pregnancy and thus act as an abortifacient.

Vaccines under development

Antibodies inactivating any one or more of the hormones depicted in Figure 1 would theoretically result in impairment of fertility. However, intervention in the action of LHRH would also block the production of sex steroids. This may be desirable for the control of fertility of animals (dogs, cats, animals raised for meat) but not acceptable for contraception in humans. It should be noted, however, that LHRH secretion remains suppressed during lactational amenorrhea. Family planning advocates recommended spacing of children by breast-feeding, which in ancient societies was a 'natural' method of stopping ovulation. But to keep LHRH, and thereby ovulation, suppressed, the breast suction must take place frequently during the day and the night, a *modus vivendi* not easily practicable for modern women. An alternative would be to inactivate LHRH by circulating antibodies by immunization with the LHRH vaccine to prolong lactational amenorrhea and interchild interval. LHRH vaccine is thus conceivable as a postpartum vaccine and can have a special niche in the contraceptive array. Fraser and colleagues have reported the block of ovulation in breast-feeding women for 9–11 months by using an LHRH agonist.

Blocking ovulation has been the principal strategy for contraception in oral pills and contraceptive steroids delivered as injections. Most of them inhibit the

production of the normal sex steroids by the woman, and their deficiency is made good by the synthetic hormonal analogs administered. It will be counter-productive to have a vaccine which would also demand synthetic hormones as implants for retention of libido and other metabolic functions. Hence an LHRH vaccine will not in general be suitable for contraception, other than in the period following delivery of the child and during lactation where physiologically LHRH secretion remains inhibited.

In contrast, if immunological interception is targeted at hCG, the normal hormonal cascade would stay intact. The woman would ovulate and produce normal levels of sex steroids. The intervention occurs only following fertilization at the preimplantation stage. These were amongst the considerations that led to hCG being the preferred target for the first type of birth control vaccines devised.

Tables 1 and 2 summarize the many vaccines being made. It will be noted that the most advanced vaccines are those directed against hormones. Four

vaccines are directed against hCG, two against LHRH and one against FSH.

A large number of vaccines are being made against sperm and egg antigens; the nature and status of these are summarized in Table 2. Additionally, one vaccine aims to block the uptake of an essential vitamin for embryo development.

Antibody response to 'self' hormones – need for carriers

β hCG or LHRH used as vaccines in humans would not by themselves induce appreciable antibody responses, especially when employing permissible but relatively weak adjuvants such as alum. Their immunogenicity is substantially enhanced, however, by linkage with an immunogenic carrier. Tetanus toxoid (TT), diphtheria toxoid (DT) and cholera toxin chain B (CTB) have been used for this purpose with successful results. They increase the immune response against the hormone and at the same time they generate antibodies against the carrier, which

Table 1 Birth control vaccines reaching clinical trial

Target	Vaccine	Destined use	Status	Principal investigator(s)
hCG	HSD-TT/DT	Women for reversible fertility control	Phase I safety and Phase II efficacy trials completed; prevents pregnancy at $>50 \text{ ng ml}^{-1}$ titers; no significant side-effects; ovulation maintained with regularity of menstrual cycles	G.P. Talwar and colleagues
hCG	β hCG-TT	Women for reversible fertility control	Phase I trials completed in India, Sweden, Finland, Brazil and Chile	G.P. Talwar and ICCR of Population Council
	β hCG-TT	Women for reversible fertility control	Phase I trials determining hormone profiles which remained normal	Horatio Croxatto Vivian Brache Rose Marie Thau
hCG	CTP-DT	Women for reversible fertility control	Phase I clinical trials conducted in Australia	V. Stevens Warren Jones
hCG	Vaccinia- β hCG anchored	Men and women, lung cancer-secreting hCG	Experimental efficacy <i>in vitro</i> and <i>in vivo</i> in nude mice on Chago cells; preliminary clinical trials in three lung cancer patients in Mexico	G.P. Talwar Debajit Biswas Carlos Gual
LHRH	LHRH-6 DLys-DT	Men, carcinoma of prostate	Phase I/phase II trials conducted in India and Austria on advanced stage patients with carcinoma of the prostate	G.P. Talwar S.N. Wadhwa S. Sharma J. Frick
LHRH	LHRH-1-TT	Men for fertility control with androgen supplement, and for carcinoma of the prostate	Experimental studies completed; phase I trials initiated in USA	Rose Marie Thau, Y. Tsong and colleagues
FSH	FSH	Men for fertility regulation without loss of libido and decline in testosterone	Phase I trials initiated in India	N.R. Moudgal

Table 2 Birth control vaccines under development at the experimental stage

<i>Target</i>	<i>Vaccine</i>	<i>Destined use</i>	<i>Status</i>	<i>Principal investigator(s)</i>
Riboflavin carrier protein (RCP)	Chicken RCP, CTP and RCP-DT	Pregnancy interception in females; renders males subfertile	Tested in rodents and primates. Immunodominant epitopes determined	P.R. Adiga
Sperm	LDH-C ₄	Female fertility control	Immunodominant epitopes identified; protection studies in rabbits and baboons with partial success	E. Goldberg
Sperm	RSA	Male fertility control	Inhibition of sperm-egg interaction by monoclonal antibodies	M.G. O'Rand
Sperm	PH-20	Males and females	Fully effective in guinea pigs	P. Primakoff
Sperm	SP-10	Females	Intra-acrosomal protein. Vaccine trials in baboons caused reduction of fertility	John C. Herr
Sperm	HSA-63	Females	Antibodies inhibit human sperm penetration. Cross-reactive with SP-10	M.S. Liu
Sperm	FA-1	Females	Conserved across species. Antibodies prevent human sperm penetration. Present in infertile patients; functional epitope identified	R.K. Naz
Sperm	FA-2	Females	Antibodies block human sperm penetration	R.K. Naz
Sperm	CS-1	Females	Antibodies inhibit first cleavage of pronuclear stage zygotes; antibodies present in infertile patients	R.K. Naz
Oocyte	Rabbit recombinant 55 and 75 kDa	Females	Immunization of cynomolgus monkey with 55 kDa protein leads to infertility without effecting ovarian function; 75 kDa protein causes ovarian dysgenesis	B.S. Dunbar
Oocyte	Human/cynomolgus macaque ZP3 peptide	Females	Immunization of cynomolgus macaques leads to infertility with normal ovarian functions	C.A. Mahi-Brown
Oocyte	Recombinant human ZP3/porcine ZP3	Females	Immunization of marmoset monkey leads to infertility associated with ovarian dysfunction characterized by suppression of folliculogenesis and depletion of the primordial follicle pool	R.J. Aitken
Oocyte	Pig zona pellucida	Females	Demonstrated effect of immunization for control of wild-life population	J.F. Kirkpatrick
Oocyte	Synthetic peptide corresponding to mouse ZP3	Females	Efficacy to block fertility in female mice without concomitant autoimmune oophoritis	K.S.K. Tung
Oocyte	Recombinant pig ZP1 and synthetic peptides	Females	Mapped the epitope for a monoclonal antibody inhibiting binding of pig and human sperm-egg interaction	K. Koyama

Table 2 Continued

Target	Vaccine	Destined use	Status	Principal investigator(s)
Oocyte	Porcine ZP3	Females	Immunization of bonnet monkey with porcine ZP3 along with permissible adjuvants leads to reversible block of fertility	S.K. Gupta
Oocyte	Bonnet monkey ZP1/ZP2/ZP3; synthetic peptides	Females	Cloning, sequencing and expression of bonnet monkey ZP1, ZP2 and ZP3. Active immunization with ZP3 and its synthetic peptides corresponding to monoclonal antibody identified epitopes	S.K. Gupta
Oocyte	Mouse ZP3 sythetic peptide KLH conjugate	Females	Immunization of Swiss mice leads to reversible block of fertility	J. Dean
Oocyte	Dog, cat, cow, pig, cynomolgus monkey and human ZPA/ZPB/ZPC	Females	Cloning, sequencing and expression of the various zona proteins. Testing efficacy to block fertility	Zonagen Inc.
Oocyte	Porcine ZP3 cynomolgus deglycosylated form	Females	Demonstrated contraceptive efficacy in squirrel monkeys and rabbits	A.G. Sacco

brings in an immunoprophylactic benefit to nonimmunized individuals. TT and DT are good carriers. However, repeated immunization with conjugates containing these carriers can cause hyper-responsiveness to the carrier, shutting off the immune response to the attached hormone. In humans, about 11–15% of subjects developed carrier-induced immunosuppression upon repeated use of TT-containing conjugates. The suppression could, however, be overcome by presenting the ligand on an alternative carrier, such as CTB. As has been shown by Eli Sercarz and colleagues, a protein such as lysozyme has both stimulating and inhibitory determinants. Proteins have epitopes stimulating T and B cells. A number of T helper determinants have been mapped in viral, bacterial and parasite coat proteins. Their judicious choice can enable communication with the entire major histocompatibility complex (MHC) spectrum, so that vaccines constructed using these peptides as carriers can elicit immune responses in individuals of different genetic background. Experimentally it has been found that the heterospecies dimer (HSD) hCG vaccine linked to peptides containing three different T cell epitopes induces as good, if not higher, antibody responses than TT or DT conjugates in mice of different genetic strains.

Vaccines in clinical trial and their current status

The hCG vaccines

Three vaccines have been developed against hCG for use as antifertility agents. One of them employs the carboxy-terminal 37 amino acid peptide (CTP) of the β subunit of hCG, another the entire β hCG, and the third hCG vaccine is an HSD composed of the β -hCG associated noncovalently with the α subunit of ovine LH. The ability of the subunits to combine is conserved across species. In fact, the HSD has a higher steroidogenic potency than the homospecies dimer hCG. The α peptide of another species would be expected to enhance immunogenicity of the vaccine in humans. Data in Table 3 summarize the highest antibody titers attained in humans using the three types of hCG vaccine which have undergone phase I clinical trials. HSD is indeed the most immunogenic of the three constructs. Another feature of this vaccine is the higher bionutralization capacity of the antibodies that it generates. Different epitopes in a macromolecular hormone antigen induce antibodies with variable capacities for inactivating the hormone. High titers of antibodies (as determined by radioimmunoassay) obtained against the CTPs of

Table 3 Characteristics of the antibodies generated by the three hCG vaccines during phase I clinical trials

	CTP37-DT ^b (Squalene + MDP + Arlacel A) ^a	β hCG-TT ^c (Alum) ^a	HSD-TT/DT ^d (Alum + SPLPS) ^a
Peak titers (ng ml ⁻¹)	36–127	120–1800	222–6000
Avidity (M ⁻¹) ^e	10 ⁸	10 ¹⁰	10 ¹⁰
B/I index ^f	?	52	61
Cross-reaction with:			
hTSH and hFSH	–	–	–
hLH	–	+	+
Pancreatic cells	+ ^g	–	–

^aAdjuvant used.^bJones *et al* (1988).^cThau *et al* (1989).^dOm Singh *et al* (1989).^eAvidity data from collaborative studies.^fB/I bionutralization index data from Talwar *et al* (1988).^gData from Rose *et al* (1988).

β TSH or β hCG using strong adjuvants such as Freund's complete adjuvant do not always inactivate the bioactivity of the parent hormone, possibly due to the poor avidity of these antibodies. The affinity of hCG for its ovarian receptors is of the order of 10⁹. Antibodies ten times less avid may not be able to prevent hCG binding to its receptors.

The lead work on the β hCG CTP vaccine was carried out by Vernon Stevens in Ohio. Phase I clinical trials of this vaccine were conducted by Warren Jones in Australia with the support of the WHO. The vaccine was employed at different doses in 20 women who had elective tubal ligation, another ten women served as vehicle controls. No significant side-effects ascribable to immunization were reported by the investigators. However, this vaccine could not proceed to phase II efficacy trials because it produced unacceptable side-effects in the first seven subjects enrolled in Sweden. Its efficacy in humans thus remains untested. Dirnhofer and colleagues have cast serious doubts on the efficacy of this vaccine.

The β hCG-TT vaccine was initially conceived and developed by Talwar and coworkers in New Delhi. Its early phase I trials were conducted in India. The same vaccine was taken up by the International Committee on Contraception Research of the Population Council and two series of phase I clinical trials were carried out, the first in Finland (Tapani Luukkainen), Sweden (Elof Johansson), Brazil (Elsimar Coutinho) and Chile (Horacio B. Croxatto), and the second in the Dominican Republic (Vivian Brache), Finland (Pekka Latteenmaki) and Chile (Horacio Croxatto). Both the trials in India and in other countries demonstrated the safety of the vaccine and lack of side-effects. Sequential bleeds provided data on the levels of FSH, LH, estradiol and

progesterone, which remained normal with ovulatory cycles. In all centers the antibodies declined to near zero levels in the course of time in the absence of boosters, thus indicating the reversibility of the vaccine.

The HSD vaccine was made by Talwar and colleagues with the aim of further improving the immunogenicity of the β hCG-TT vaccine (Table 3). After confirming its safety, lack of side-effects and noninterference in ovulation and regularity of menstrual cycles, phase II efficacy trials were conducted with this vaccine in 148 women of proven fertility (at least 2 children) who were sexually active; 119 of them (80%) made antibodies above 50 ng ml⁻¹ bionutralization capacity, a threshold fixed for determining the effective titers. Women had the option of leaving the trial or taking booster injections as and when antibody titers declined towards or below 50 ng ml⁻¹. Only one pregnancy occurred in 1224 cycles, showing the high efficacy of the vaccine in preventing pregnancy as long as the titers remained above 50 ng ml⁻¹. The ability of antibodies to deter pregnancy was also confirmed by postcoital tests. The block of fertility was reversible. On decline of antibody titers below 35 ng ml⁻¹ conceptions readily occurred (Figure 2). Those desirous of having another child, after a gap with the use of the vaccine, have carried pregnancy to term and have given birth to normal babies.

Optimization of the HSD The HSD-hCG vaccine has provided the scientific foundations of a birth control vaccine which prevents pregnancy without impairing ovulation or deranging menstrual regularity. Its characteristics render it usable at all stages of reproductive life. It is, however, not yet ready as a product. Further optimization of the vaccine is

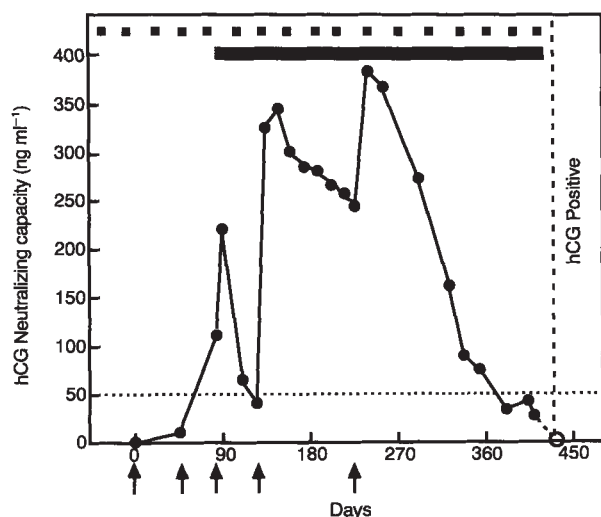


Figure 2 A representative subject showing the kinetics of anti-hCG titers (●—●), menstrual events (top squares) and the period of protection from pregnancy (solid line). This lady of 30 years of age with two live children and one elective termination of pregnancy was among the 119 subjects on which the efficacy of the HSD vaccine was determined in phase II trials. The graph also shows the reversibility of the vaccine with return of fertility in the cycle when her antibody titers declined to less than 5 ng ml⁻¹ bionutralization capacity. (Reproduced from Talwar *et al* (1994).)

necessary to enhance the percentage of high responders making antibodies above 50 ng ml⁻¹. The present vaccine is adsorbed on Alhydrogel. As more potent adjuvants get regulatory approval, their use may facilitate the enhancement of antibody titers. Another strategy will be the substitution of the DT/TT by T cell determinants communicating with the entire MHC spectrum.

We have determined the joint ability of three T cell epitope peptides derived from circumsporozoite protein of *Plasmodium falciparum*, respiratory syncytial virus and purified protein derivative (*Mycobacterium tuberculosis*) to generate higher titers of antibodies in seven genetic strains of mice than those obtained with DT as a carrier for the HSD. Encapsulation of the vaccine in biodegradable microspheres can also add to the immunogenicity and at the same time enable the delivery of multiple doses of the vaccine at a single contact point. To make the vaccine at low cost and in a reproducible manner on a large scale, it will be necessary to use recombinant DNA technology. β hCG has been expressed with and without fusion proteins in vaccinia as a naturally glycosylated peptide, in baculovirus with higher yield but somewhat different glycosylation, and in *Escherichia coli* and *Vibrio cholerae* with still higher yield unglycosylated. Live recombinant vaccines employing attenuated pox viruses,

adenoviruses, salmonella or BCG as vectors may offer very low cost vaccines generating a protective immune response over long periods. Ivan Roitt and colleagues have employed recombinant technology to make a mutant of β hCG lacking in reactivity with a panel of anti-hLH antibodies but preserving the immunoreactivity with monoclonal antibodies recognizing hCG.

Applications of the hCG vaccine in hCG-synthesizing lung cancers Although hCG is known mainly as a trophoblast product and its increased levels in serum or urine are employed as a diagnostic test for pregnancy, its synthesis and secretion has also been reported in about 30 different types of cancers. The function, if any, of hCG or its subunits made by tumor cells is unknown. Debajit Biswas and coworkers at Harvard Medical School observed that Chago cells derived from a human lung cancer produced hCG α chain, which promoted the growth of these tumor cells. Antibodies reacting with the hCG subunit, or antisense RNA, caused inhibition of cell proliferation. Cell clones derived from lung cancer make either isolated α or β chains or whole hCG. Antihormonal subunit antibodies given passively to nude mice bearing tumors formed from Chago cell implants have been shown to cause necrosis of the tumor. When tumor cells were exposed to antibodies from the time of implantation, growth was inhibited in a dose-dependent manner, with total absence of tumor growth at high doses of antibody.

Given that no effective chemotherapy is at present available for hCG-synthesizing lung cancers, regulatory permission was given in Mexico to carry out clinical trials with the hCG vaccine in three patients characterized on the basis of clinical manifestations backed by radiography, histopathology and serum β hCG levels. All three patients tolerated immunization with the vaccine well. The serum β hCG declined to near zero levels after immunization. No further metastasis occurred during 2.5 years of follow up and the Karnofsky index improved for one of the patients. These studies clearly need to be extended to a larger number of individuals in order to draw any conclusions regarding the efficacy of the hCG vaccine in such patients.

LHRH vaccines

Two vaccines against LHRH are in clinical trials in carcinoma prostate patients. The vaccine made by Rose Marie Thau, Y. Tsong and colleagues at the Population Council in New York has TT tagged at the N-terminus of the decapeptide, whereas the vaccine developed by Talwar and coworkers in New Delhi employs a modified LHRH with the glycine at

position 6 replaced by D-lysine. This is linked through a spacer to DT.

The rationale employed in the New Delhi vaccine is to preserve the natural conformation of the LHRH, where N- and C-terminal amino acids are proximal to each other with a bend in the middle of the molecule. Substitution of glycine at position 6 by D-lysine stabilizes this conformation and also slows the catabolic degradation of LHRH by the enzyme cleaving at glycine 6.

Both vaccines are immunogenic in rodents, have passed through toxicology studies, and are in clinical trials. The Population Council vaccine (LHRH-1-TT) was tried in four orchietomized men to determine the immunogenicity of the vaccine, its reversibility and immediate and delayed side-effects. These studies have shown that the vaccine was well tolerated and that although there was a large variability in antibody titers between the four subjects, they all made antibodies to LHRH. In order potentially to use this vaccine for male fertility control, these investigators have also carried out studies in rodents to assess the dose of androgens required to restore libido without reversing the inhibition of spermatogenesis.

The New Delhi LHRH vaccine (LHRH-6D-Lys-DT) has undergone phase I/phase II clinical trials in advanced stage patients with metastatic carcinoma of the prostate. In one study 24 patients received either 200 or 400 µg LHRH equivalent per injection. No ill-effects of immunization were reported in any of the studies despite the advanced age of the patients and their low health status. All the subjects made antibodies against LHRH and many, but not all, produced sufficiently high titers to cause depression of testosterone to castration levels. Prostatic-specific antigen serum levels declined significantly. Ultrasonography showed shrinkage of prostatic mass. These studies indicate the potential of this vaccine in prostatic hypertrophy as an inexpensive alternative to the LHRH synthetic analogs. Immunotherapeutic use could extend to endometriosis and precocious puberty, however clinical trials remain to be undertaken in these conditions.

The LHRH vaccine, with stronger adjuvants, also has applications for reversible fertility control of domestic pets. Given that this decapeptide hormone is identical in males and females, the same vaccine could be used in male and female animals.

FSH vaccine

This vaccine was conceived by Moudgal and colleagues for male fertility control on the basis of the requirement of FSH for spermatogenesis in primates. Passively administered antibodies to FSH caused a

significant reduction of bonnet monkey sperm count without affecting testosterone. Ovine FSH was chosen as the antigen, which induced in monkeys both monkey and human FSH cross-reactive antibodies of high affinity. Immunization caused a reduction in sperm count and the fertilizing capacity of the remaining sperm was significantly reduced. A study conducted in three laboratories demonstrated that 25 out of 27 monkeys immunized with ovine FSH became infertile. Fertility was regained on decline of antibodies.

After toxicology studies and regulatory ethical approval, a phase I study with this vaccine was initiated in 19 adult human males in three centers in India. Antibodies were made by all of the subjects but the titers of antibodies reactive with human FSH were low. The mean avidity of the antibodies was also an order of magnitude lower than in the previous studies in primates. Vaccination was, however, well tolerated. There were variations in the levels of pituitary hormone such as prolactin in some subjects. No significant effect of immunization on sperm count was noted, presumably owing to the low titers of antibodies or an insufficient duration of the study. Repeat phase I trials are planned with the vaccine employed at different doses.

See also: Autoimmunity; Infertility, immunological causes of; Neutralization of biological reactions by antibodies; Sex hormones and immunity; Vaccines.

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CONTRASUPPRESSION

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Contrasuppression refers to any immunoregulatory activity that facilitates immune responses by interfering with suppressive influences, without otherwise helping or augmenting the response. While this is the original definition given by RK Gershon who coined the term, it came to refer more specifically to a regulatory T cell activity, i.e. the function of T contrasuppressor cells. As is true for other regulatory T cells (e.g. suppressor T cells), the existence of contrasuppressor T cells as a discrete functional population is controversial.

In this brief overview, the concept of contrasuppression is reviewed from two perspectives. The first is an uncritical view of the original literature on the phenomenon; the second is from the standpoint of current thinking on the functions of T cells. From the outset, however, the reader should be aware that while most of the observations on contrasuppression have eluded biochemical and molecular characterization, they were described from a number of laboratories and continue to be reproduced. Although this state of affairs demands that all of the conclusions regarding contrasuppression be treated with caution, this regulatory phenomenon exists. Questions concerning whether or not contrasuppression is *important* are discussed in more detail below.

Background

The study of immunoregulatory T cells began in 1970, with the experiments of Gershon and Kondo, showing that T cells can adoptively transfer some forms of immunological tolerance. Work from a large number of laboratories focused on such suppressor T cells and their soluble mediators ('suppressor factors'). During the 1970s, complex regulatory pathways were described in which different T cell subpopulations induce, transduce and effect antigen specific suppression of immune responses. Intensive study of the regulatory cells and factors implicated their involvement in a range of immunologic phenomena.

Based on the idea that tolerance is due to active suppression of immunity and on the observation that cross-reactive antigens can sometimes break tolerance, in 1974 Gershon predicted that there is an immunoregulatory activity that functions to interfere with suppression. In 1981 his laboratory published a series of papers describing such an activity, and since then T cells with contrasuppressive activity have been identified in a number of different systems. In all cases, contrasuppression allows immune responses to proceed in the presence of demonstrable, active suppression.

Research on suppressor and contrasuppressor T

cells suddenly slowed in the mid- to late-1980s for several reasons. Most significantly, efforts to fully characterize antigen specific regulatory T cell factors uniformly failed, despite encouraging results early on. A second confounding problem was I-J, a marker that was associated with many regulatory T cells (including T contrasuppressor cells). Molecular analysis of the region of the major histocompatibility complex (MHC) to which I-J was mapped revealed an absence of any structural MHC gene encoding the molecule. These and other problems cast the study of regulatory T cells in a poor light. Nevertheless, several groups continue to successfully study these regulatory phenomena with interesting results, and therefore an open-minded outlook is warranted.

Characteristics of cells with contrasuppressive activity

The single discriminating characteristic of contrasuppressor T cells is their function: the ability to interfere with immune suppression *without* providing active help. Depending upon the system studied, this functional activity may appear to be antigen specific or nonspecific. Contrasuppressor cells act prior to suppressor cells, i.e. contrasuppressor cells do not restore responsiveness to previously suppressed cells, but rather interfere with inhibition. In some systems, contrasuppression has been shown to render target cells (helper T cells or B cells) resistant to subsequent suppressive influences. However, this does not rule out the possibility that some contrasuppressive effects are mediated by direct inhibition of suppressor functions in some systems.

In general most murine cells with contrasuppressive activity are Thy-1⁺ and where examined, human and rodent contrasuppressor cells have also been found to be CD3ε⁺. The cells are therefore likely to bear T cell receptors. In addition, most contrasuppressor cells are bound by the *Vicia villosa* lectin, but while this trait distinguishes them from other functional T cells (such as helper T cells) it does not provide a unique marker. It is important to note therefore, that binding of this *N*-acetyl-D-galactosamine-specific lectin does not *define* contrasuppressor cells, but rather provides a convenient means of enriching cells with functional contrasuppressive activity.

Contrasuppressive T cells are phenotypically heterogeneous with respect to CD4 and CD8. Functional contrasuppressor cells may be CD4⁺, CD8⁺, or CD4⁻8⁻, depending upon the system studied and the cellular interactions involved. Where examined, CD8⁺ T contrasuppressor cells require the presence of a population of CD4⁻, CD5^{hi}, CD8⁺ T cells in order to mediate contrasuppression. Therefore, such

CD8⁺ cells have been designated as inducer cells, or Tcsi. In contrast, where examined, CD4⁺ T contrasuppressor cells have been found to act without a requirement for an intermediary (or 'transducer') population, and have therefore been designated as effector cells, or Tcse. The relationships between Tcsi and Tcse have not been rigorously explored.

Little is known about the T cell receptors of contrasuppressor cells. In one series of studies, a population of CD3⁺, CD8 intraepithelial cells from murine intestine were found to have contrasuppressive activity *in vitro* and *in vivo*. Immunoprecipitation from the cell surface suggests that this population of T cells express the γδ T cell receptor (TCR). Other contrasuppressor cells are likely to express the αβ TCR, as has been observed in a T cell hybridoma with contrasuppressive activity.

One marker that has been associated with contrasuppressor T cells is 'I-J'. As mentioned above, the nature of I-J is controversial. Nevertheless, antisera and monoclonal antibodies with anti-I-J activity have been shown to remove cells with contrasuppressive activity in many different systems.

It is important to note that since contrasuppression is defined by function and not by cell phenotype, there is little evidence to suggest that contrasuppressor T cells represent a discrete T cell population. Although this remains a possibility, it is equally likely that contrasuppression represents a functional property of many T cells that may have other functions as well.

Experimental studies of contrasuppression

In order to study contrasuppressive effects, it is necessary (by definition) to employ a system involving active suppression of a measurable immune response. This necessarily complex situation is one source of the problems associated with this phenomenon (see below). Nevertheless, contrasuppression has been demonstrated in a number of different systems, both *in vitro* and *in vivo*.

In vivo studies of contrasuppression often take advantage of protocols for the induction of antigen-specific tolerance. For example, antigen-coupled spleen or peritoneal exudate cells, injected intravenously, induce an active state of tolerance that is blocked by the administration of T cells with contrasuppressive function. Such contrasuppressor cells may act either antigen specifically or nonspecifically, depending upon the source of the cells. Tolerance induced by oral administration of xenogeneic red blood cells is similarly blocked by contrasuppressor T cells derived from gut-associated lymphoid tissue.

In this system the contrasuppressive effect favors immunoglobulin A (IgA) responses, and the contrasuppressor cells appear to bear $\gamma\delta$ T cell receptors (as determined by immunoprecipitation of the receptor on the enriched functional population).

Studies of the regulation of the adoptive transfer of contact hypersensitivity have also demonstrated the effects of contrasuppression *in vivo*. T Cells from tolerized animals inhibit the ability of T cells from immune animals to adoptively transfer immunity into naive recipients, and contrasuppressor T cells block this inhibitory effect. These hapten-specific contrasuppressor T cells have been generated by injection of haptenated Langerhans cells, haptenated murine IgG, or haptenated peritoneal exudate cells treated with antihapten antibody of the IgM, IgG1, or IgG3 subclasses. In each case *V. villosa* lectin-adherent T cells from the treated animals were found to be incapable of adoptively transferring immunity, but capable of blocking suppression of immune T cells. Further, these contrasuppressor T cells were found to release a cell-free factor that renders immune T cells resistant to suppression, either by inhibitory T cells or by the environment of a tolerized recipient.

A murine model of autoimmune nephritis has implicated contrasuppression in the development of autoimmunity. In the nephritis-prone kdkd mutant, T cells capable of contrasuppressing delayed-type hypersensitivity to renal tubule antigen appear with onset of autoimmunity. When these *V. villosa* lectin-adherent T cells were mixed with wild-type spleen cells and injected into kidney capsules, destruction of tubules and mononuclear infiltration followed. This was not observed with either population alone, nor with spleen cells plus lectin-nonadherent (and noncontrasuppressive) T cells. One interpretation of these results is that antitubule immunity is normally suppressed by an active mechanism, and that contrasuppressor cells from the autoimmune mice are capable of interfering with this suppression.

Another series of studies suggest that contrasuppressor T cells may be important in some forms of tumor immunity. Two fibrosarcoma cell lines, one regressive and one progressive, have strikingly different effects when haptenated and injected into animals. The haptenated progressor line was observed to induce T cells capable of suppressor hapten-specific responses *in vivo*, while the haptenated regressor line instead induced hapten-specific contrasuppressor cells. These effects were studied using the contact hypersensitivity systems mentioned above. The contrasuppressor T cells induced by the regressor line displayed markers common to such cells, and released a cell-free, hapten-specific, contrasuppress-

ive factor. The cells were used to generate a T cell hybridoma that constitutively produces this factor. Injection of hapten-immune mice with either the *in vivo* generated contrasuppressor cells or with the hybridoma-derived factor rendered these animals immune to haptenated progressor tumor cells (that otherwise grow in and eventually kill naive or hapten-immune animals).

A number of studies have characterized contrasuppression *in vitro*, including human contrasuppressor cells. As in the murine system, human contrasuppressor T cells were found to be adherent to the *V. villosa* lectin and to interfere with active, T cell-mediated inhibition of immune responses. The induction of these cells was found to be dependent upon antigen dose (the optimum dose depending upon HLA-DR type) and they have been shown to block suppression of both antibody responses and T cell proliferation *in vitro*.

Problems with contrasuppression

As mentioned above, the phenomenon of contrasuppression is controversial for a number of reasons. One of the most important reasons lies in the fact that contrasuppression is defined by its effects on suppression. However, the mechanisms of suppression (let alone contrasuppression) are unknown.

Regulatory cells are by their nature a property of the immune system whose activity can only be measured as a function of the immune activity of other cells. This complexity carries special problems of completely characterizing the activity of even a single component of these interactions. On the other hand, the demonstration of this functional activity *in vivo*, and the common features of the cells responsible for contrasuppressive function, suggest that this may be an important, if poorly understood phenomenon.

No known lymphokine or other well-defined molecule, either alone or in combination with other molecules, can fully account for the phenomenon of contrasuppression. The suggestion that at least some contrasuppressor cells release antigen-specific contrasuppressor factors presents additional problems, since no such factor (suppressor or contrasuppressor) has been well characterized. The alternative, that some contrasuppressor T cells act via the release of one or more lymphokines has not been fully explored. Thus the molecular basis of contrasuppression in any system is unknown.

It is unlikely that the problems associated with the study of contrasuppression will be solved in the near future. It is likely, however, that the phenomenon will return in a different guise as we learn more

about the workings of the immune system as a whole.

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See also: **CD3; CD4; CD5; CD8; I-J; Lectins; Suppressor T lymphocytes; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; Thy-1.**

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COOPERATION, MECHANISMS OF CELLULAR

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Various pathogens, ranging from viruses to helminths, can invade the host by a number of pathways, and usually the pathogen is cleared through an orchestrated immune response. Moreover, protection from a repeat infection is provided by a memory response, which, relative to the primary response, is rapid and highly specific. This coordination of multiple effector cells relies on communication between cells of the immune system and cells in their environment. Many of the components involved in cellular cooperation are molecules that are produced by the cells which bind to receptors on other cells. We can roughly divide these components into two major categories: membrane-bound and secreted. This discussion will focus on certain membrane-bound molecules and their influence on the immune response.

The immune response has many examples of cellular cooperation. Tissues secreting chemokines alert the immune response to areas of damage and potential pathogen invasion. Adhesion molecules, such as selectins and integrins, present on cells of the tissues and leukocytes mediate cellular homing, margination and migration. Once these cells are in the site of damage, membrane-bound receptors in conjunction

with secreted cytokines activate immune cells and enable them to develop a potent effector reaction. As a prototypical example of cellular cooperation in the immune response, we will focus on germinal center (GC) development in the response to T cell dependent antigens. Consider the formation and maintenance of GCs developed during primary immune responses. This entire process relies upon communication between multiple cells, including T cells, B cells, interdigitating dendritic cells (IDCs), germinal center dendritic cells (GCDCs) and follicular dendritic cells (FDCs). Resting T cells interact with activated IDCs presenting antigen initially captured from local sites of damage, such as mucosal or epidermal tissues. B cells, also activated through antigen encounter, are further helped along their maturation pathway by interaction with the T cells. The activated B cells undergo affinity maturation by interaction with antigen bound on FDCs. Early in this process, somatic hypermutation of B cell antigen receptors (BCRs) generates novel specificities, some of which have greater affinity for antigen. Antigen uptake mediated by the BCRs, followed by processing and presentation in class II major histocom-

patibility complex (MHC) molecules to the cognate T cells results in T cell help that propagates the B cell. B cells bearing novel specificities which have lost affinity to antigen, or have acquired affinity to another antigen, must be eliminated. Through types of receptor-mediated 'reciprocal dialogues' in the GC, lymphocytes can be induced to proliferate and differentiate, or, equally as important, can be instructed to undergo programmed cell death.

Membrane-bound molecules are critical for lymphocyte activation

Lymphocyte activation by a foreign antigen is the initial step in the humoral response against T-dependent antigens. We generally regard activation of a resting B or T cell as a multistage process involving multiple signals transduced through membrane receptors. This model is based on the historic two-signal model initially proposed by Bretscher and Cohn. For example, a T cell requires costimulation in addition to the signal it receives from its antigen receptor binding to its cognate MHC/foreign antigen complex. If the T cell fails to receive costimulatory signals, the model proposes that the cells cannot be activated. Similarly, multiple signals are required to activate B cells. Some studies have suggested that resting lymphocytes which receive one signal are rendered unresponsive, or anergic. Characterization of single molecules which synergize with antigen receptor signals to activate lymphocytes has remained elusive. This fact emphasizes the dialogue of multiple receptor signals between cells during lymphocyte activation. The following discussion will focus primarily on the recent body of evidence dealing with specific membrane-bound coreceptors that appear to be necessary for activating or downregulating T cells or B cells. Two receptor/counter-receptor families will be discussed: members of the immunoglobulin superfamily (IgSF), and members of the tumor necrosis factor receptor (TNF-R) and tumor necrosis factor (TNF) family. The IgSF molecules, CD80/CD86 (B7-1/B7-2) interact with two distinct counter-receptors, CD28 and CD152 (CTLA-4), which either activate or downregulate T cells, respectively. Currently, novel members of the TNF-R/TNF family are rapidly being characterized. Functionally, these molecules appear to have an important role in determining cell fate, as exemplified by CD40 and CD95 (Fas).

CD80/CD86: CD28/CD152 interactions have dual roles in regulating lymphocytes

CD80 and CD86 are members of the IgSF. They are approximately 60 kDa in size. Both bear similar

overall structure, with two extracellular Ig-like domains, a transmembrane domain and cytoplasmic tail; however, at the amino acid level they have only 25% homology to each other. Interestingly, the cytoplasmic domains of CD80 and CD86 barely resemble each other, which suggests different signaling functions.

Consistent with their role as critical costimulatory molecules in the initial steps of T cell activation, CD80/86 are expressed on antigen-presenting cells (APCs), such as IDCs, GDCs, Langerhans cells and activated monocytes. The expression of these molecules can be induced upon activation of other APCs. In particular, CD80/86 are upregulated on B cells by cytokines, such as interleukin 4 (IL-4), or by receptor cross-linking of the BCR, MHC class II or CD40. There are differences in expression between CD80 and CD86, although the functional relevance of this is unclear. For example, during activation of B cells, CD86 is expressed more rapidly than CD80.

While CD80 and CD86 have both been demonstrated to bind their counter-receptors CD28 and CD152, there is suggestive evidence that CD80 and CD86 may have different biologic functions. This variance may, in part, be due to differences in ligation of receptor – such as variation in binding kinetics and avidity – and in induction and expression of CD80 and CD86. For example, in the GC, expression of CD80 and CD86 differs between B cell centroblasts and centrocytes. Ectopic overexpression of CD86 results in deletion of B cells by CD28⁺ T cells which can be prevented by introduction of hen egg lysozyme (HEL)-specific B cells, T cells and the HEL antigen. This suggests that CD86 can play a role in elimination of nonspecifically activated B cells. Comparing immune responses of mice which are CD80^{-/-}CD86^{+/+}, CD80^{+/+}CD86^{-/-} or CD80^{-/-}CD86^{-/-} suggests that either molecule is required to mount a humoral response, depending on the route of antigen administration. Either CD80 or CD86 is sufficient for immune responses to soluble antigen inoculated intradermally. However, CD86, but not CD80, is required for antigens inoculated intravenously.

CD28 costimulates T cells

CD28, one of the two known ligands for CD80/86, is also a member of the IgSF. It is expressed on the surface as a 90 kDa, disulfide-linked homodimer. CD28 is mainly expressed on T lineage cells, including thymocytes, but may also be found on plasma-blasts. The level of expression varies, depending on the lineage and the activation state. Upon activation of a mature T cell, either by mitogen or by cross-linking of the TCR, the expression of CD28

increases. However, following this upregulation, ligation of CD28 by its counter-receptors apparently induces a transient negative feedback to downregulate expression and render the cells unresponsive to CD28 signaling.

CD28 usually transduces a positive signal that promotes proliferation of TCR-stimulated T cells. Studies demonstrating its importance in the initial steps of T cell activation include *in vivo* blockade of its counter-receptors, CD80 and CD86, and genetic deletion of CD28 expression. In addition to its costimulatory role, CD28 ligation can enhance the production of various cytokines, such as IL-2, IL-4 and IL-10. CD28^{-/-} mice have reduced IgG responses to T-dependent antigens and reduced GC formation. Taken together, these observations suggest that CD28 is critical for T cell-dependent antibody responses. Responses generally attributed to T_H1 cells remain. In some experimental systems, CD28 ligation rescues T cells from anergy. CD28 may also play a role in prolonging an activated T cell's fate and initiating its differentiation into the memory phenotype. In the absence of CD28 signaling, activated T cells do not survive as long. CD28 costimulation can upregulate the expression of Bcl-xL, a protein that prevents CD95-induced apoptosis.

CD152 downregulates T cells

In addition to their role of providing costimulation to T cells, CD80 and CD86 may also play a role in downregulating the immune response. A second counter-receptor, CD152, binds to both CD80 and CD86 with approximately 20-fold higher affinity than CD28. This molecule has significant sequence identity to CD28. The *cd152* and *cd28* loci are located on the same chromosome and share similar intron/exon structure. They probably arose from a gene duplication. CD152 is also a member of the IgSF of molecules.

CD152 is expressed on most activated T cells. Its cell surface expression pattern is unusual in that it appears to be regulated post-translationally. At the mRNA level, CD152 transcription is detectable hours after T cell stimulation; however, the protein is retained intracellularly. Truncation or replacement of the cytoplasmic region of CD152 increases its cell surface expression. Temporally, the expression of CD152 is upregulated during a period of transient downregulation of the CD28 molecule expression.

CD152 can function as a negative regulator of the immune response. Antibody blockade of this receptor enhances T lymphocyte proliferation and delays apoptosis, suggesting a negative regulatory function for CD152. The generation of CD152-deficient mice has confirmed this. These mice have massive lympho-

proliferation defects and multiorgan destruction. This lymphoproliferation in CD152^{-/-} mice appears to be antigen driven. An accumulation of lymphocytes in secondary lymphoid organs is noticeable about 1 week after birth. By 4 weeks, the number of lymphocytes in these organs is up to five times the number found in wild-type controls. Also by this time, most organs are infiltrated by lymphocytes. These observations, taken together, suggest that CD152 plays a critical role in the regulation of autoreactive T cells, as well as in the downregulation of activated T cells.

TNF/TNF-R receptor/counterreceptors influence cell fate

CD154:CD40 ligation costimulates B cells

CD154 (CD40L, gp39 or T-BAM) is a member of the TNF gene family, which includes TNF, CD30L, FasL and TRAIL. Similar to other members of this family, it is a type II membrane protein of 30 kDa in size. CD154 can be detected on activated T cells, monocytes, natural killer (NK) cells, human mast cells, basophils, some DCs and some CD8 T cells. CD154 is not detectable on resting T cells. Upon ligation of the TCR, CD154 becomes transiently expressed. The expression of CD154 on T cells is consistent with its importance of providing help to B cells during a humoral response to T-dependent (TD) antigens. CD154 expression on DCs can also promote B cell maturation. The function of CD154 on other cell types is currently unknown.

Trimeric CD154 binds to and cross-links CD40. CD40 is expressed on all APCs, such as DCs, Langerhans cells, macrophages and B cells. It is also found on basal epithelial cells, FDCs, endothelium and thymic epithelium. CD40 is approximately 45–50 kDa in size and is a member of the TNF-R family, which includes CD95, TNF-RI, TNF-RII, CD30, CD27, TRAIL-R, NGF and 41-BB. These proteins are characterized by a cysteine-rich extracellular domain and a unique cytoplasmic domain. Currently, it is generally believed that regions in the cytoplasmic domain mediate the receptor's association with other proteins that transduce signals from the receptor. One molecule with which CD40 has been shown to associate is termed TRAF3. This protein has a number of putative DNA-binding motifs; however, its function is currently unknown. It may mediate activation of MAP kinase family members, because stimulation of CD40 causes activation of NF- κ B, SAPK (JNK) and p38 MAP kinase pathways.

The importance of the CD154/CD40 interaction in the generation of a humoral response is highlighted by a naturally occurring congenital defect of

CD154 in humans, and is paralleled by the CD154-deficient mouse. The human disease, X-linked hyper-IgM syndrome, is signified by lack of IgG, IgA and IgE isotypes, GC formation and memory B cells in patients. Consequently, these patients have severe immune system defects and are susceptible to numerous immune system disorders and to opportunistic infections.

The transduced signal through CD40 has different effects, depending on the cell type studied. Generally, ligation of CD40 by T cells expressing the counter-receptor results in activation of the cell. In terms of APCs, this leads to the expression of cytokines that stimulate cells in a paracrine or autocrine manner. CD40 also upregulates cell surface proteins, such as the costimulatory molecules, CD80 and CD86 and other adhesion molecules. Notably, this interplay between costimulatory molecules CD154:CD40 and CD80/86:CD28 forms the basis of reciprocal dialogue between T cells, B cells and other APCs (Figure 1).

For the humoral response to T-dependent antigens, the CD40 signaling pathway apparently complements signals from the BCR that are necessary to promote proliferation. Wheeler and colleagues demonstrated that the signal generated through CD40 effectively lowers the threshold for activation through the BCR. Mice that are genetically deficient for CD40 clearly have abnormal B cell responses to TD antigens, and part of this defect lies in the inability of T cells to stimulate CD40^{-/-} B cells.

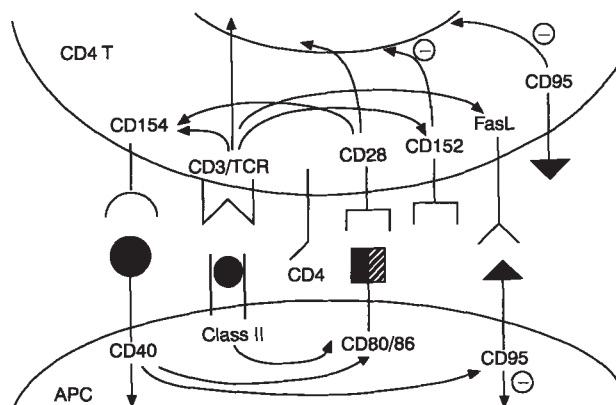


Figure 1 Receptor-mediated reciprocal dialog between a T cell and an antigen-presenting cell. Activation of T cells and APCs cause upregulation of costimulatory molecules and their counter-receptors. These molecules enhance the activation process and can lead to proliferation of lymphocytes. Downregulatory molecules such as CD95 and CD152 are important for eliminating lymphocytes after they have served their function. The APC in this figure represents a class of cells, including dendritic cells, macrophages and activated B cells. Not shown in this figure are other surface molecules and secreted compounds which also play an important role in the communication process.

In addition to its costimulatory role, these synergistic signals between CD40 and the BCR may play an integral role in the positive selection of highly antigen-specific B cells during the GC response. As the B cells undergo hypermutation of their antigen receptor, novel specificities are generated. Positive selection of B cells bearing high-affinity receptors is mediated through ligation of the BCR, presumably by interacting with FDC-bound antigen, and ligation of CD40, provided by helper T cells bearing CD154. In studies where *ex vivo* GC B cells are isolated, in the absence of T cell help, these cells eventually undergo programmed cell death. These cells can be rescued via engagement of CD40 with or without BCR ligation. Consistent with this observation is the fact that signaling through CD40 can increase the expression of Bcl-xL, a negative regulator of apoptosis.

CD40 has also been implicated in B cell differentiation. GC B cells which have been positively selected for their high-affinity antigen receptors differentiate by switching of Ig isotype and maturing into long-lasting memory B cells or terminally differentiating into Ig-secreting plasma cells. Cytokines influence the differentiation pathways, especially in the process of isotype switching. There is evidence from *in vitro* systems that demonstrate specific cytokine profiles that result in the development of certain Ig isotypes. The role that CD40 plays in any of the differentiation steps is currently unknown. CD40 activates expression of IL-12 in DCs, which can promote a T_H1 response, yet CD40 can also promote IgE class switch, typical of a T_H2 response.

In addition to the severe defects in humoral immunity, X-linked hyper-IgM syndrome patients appear to have defects in the T cell compartment. They are susceptible to diseases that are typically associated with T cell immunodeficiency. Whether this defect is due to a direct effect from the lack of CD154 signaling on the T cell, or an indirect effect from the lack of CD40-induced activation of the APC, is not clear. There is evidence that CD154 can transduce a signal to T cells. Alternatively, CD154-mediated stimulation of APCs has been shown to be critical in their ability to counterstimulate the T cell.

FasL/CD95 interactions downregulate the immune response

CD95 (Fas, APO-1) is also a member of the TNF-R family. As previously mentioned, TNF-R family members are distinguished by a cysteine-rich extracellular domain. In the cytoplasmic region, some family members contain a 'death domain' which is important for initiating programmed cell death. The death domain mediates binding of the receptor to

intracellular proteins that also contain death domains. These cytoplasmic proteins operating in conjunction with members of a specialized protease family, the caspases, are important in transducing the apoptotic signal.

The expression of CD95 is broadly distributed. It can be detected on the surface of myeloid, stromal and lymphoid cells. High expression of CD95 is observed upon activation of B cells and T cells. Also, experiments with cell lines suggest that expression of CD95 can be upregulated if cells are treated with interferon γ (IFN γ) alone, or in combination with TNF α .

The ligand for CD95, FasL, is a member of the TNF family, and, like the other characterized family members, is a type II membrane protein. FasL can also be secreted, similar to other members such as TNF and LT. The expression of FasL appears to be limited, in contrast to the broader expression pattern of CD95. FasL can be detected on the surface of DCs, activated, mature T lymphocytes and on NK cells. This is consistent with its suspected role in immunoregulatory or killer function. Interestingly, the other tissues which FasL has been detected are small intestine, eye, lung and mouse or rat (but not human) testes. The function of FasL in these tissues is not known but it might protect some tissues, such as eye and testes, from infiltration by activated T cells. Another proposed function for FasL is in T cell deletion by DCs following an immune response. One of the possible sites of activated T cell deletion is believed to be the gut. One particular paradox with the expression of FasL concerns the thymus: FasL expression is not detectable there, despite the fact that thymocytes express high levels of CD95. There is considerable controversy about whether CD95 plays a role in thymocyte selection.

In numerous experimental systems, antibody or soluble FasL ligation of CD95 induces a cell death program. CD95 plays a role in CD8⁺ T cell and NK cell-mediated cytotoxic function. In this context, cytotoxic cells expressing the counter-receptor to CD95 ligate CD95 present on the target cells.

Another interesting role of CD95 is in the regulation of T and B cell maturation. As previously mentioned, activation of mature T or B lymphocytes increases the expression of CD95. This renders the activated cell susceptible to CD95-mediated cell death. Thus, CD95 offers an attractive explanation for how activated lymphocytes are eliminated after they have served their function. For example, during the GC response, CD95 expression is increased on activated B cells which are undergoing the process of somatic hypermutation. CD95 renders them susceptible to FasL-mediated death, unless the B cell is

stimulated effectively through the BCR and CD40. According to this model, anergic B cells may be deleted, as their BCR signaling pathway is inoperative, thereby preventing BCR-mediated rescue from death.

Activated T cells also need to be downregulated, especially following an effective immune response. A possible mechanism by which the expression of CD95 and its counter-receptor on activated, responding T cells could cause apoptosis is by an autocrine or paracrine mechanism. One possibility is that after an effective immune response and the number of target cells becomes limiting, FasL on the surface of the activated killer cell will begin to ligate CD95 on itself or on neighbouring cells. This mechanism is a form of self-limiting effector function.

The importance of CD95-mediated downregulation of the immune response is underscored by the phenotype of mice with naturally occurring *lpr* and *gld* mutations which have genetic disruption of either CD95 or FasL, respectively. The phenotypes are generally similar in either mutant strain: they display an accumulation of lymphocytes in the peripheral lymphoid organs that resembles a lymphoproliferative disorder. While the immune response to antigen is apparently normal, the elimination of the responding cells is impaired in these mice. In the B cell compartment, a defect in the elimination of activated and maturing B cells is reflected by a high production of IgG and IgM antibodies. In *lpr* or *gld* mice that have been bred on to the MRL background (MRL/*lpr* and MRL/*gld* mice), the inability to control autoreactive cells in the periphery is apparent. These mice develop an autoimmune disease resembling that of systemic lupus erythematosus (SLE) in humans. This is marked by the production of autoreactive antibodies such as anti-DNA and rheumatoid factor.

Conclusions

By describing the function of certain IgSF and TNF/TNF-R family of molecules as 'stimulatory' or 'downregulatory', we have presented them in a conventional manner. However, as these molecules become further defined, their function in the process of cellular cooperation must be considered in the context of a cell's activation state and its location. In considering their relevance to the biology of the entire immune response, we have metaphorically compared them to words used by cells to communicate. Depending on the context, these words can effect a different meaning to a message. An example of this is recent evidence suggesting that CD95 may actually have a costimulatory function. Much

remains to be learned, not only about the range of definitions that receptor 'words' may have, but also of the syntax and semantics of this intercellular language.

To conceptualize the cellular communication process, we have described only a small fraction of molecules that are involved. Our intent is to highlight key molecules that function during the discrete steps of the immune response: activation, differentiation and elimination. However, at each step, there are clearly other molecules that have a role in modifying the signals. For example, the CD19/CD21 complex, CD22 and CD45 have been shown to regulate signals through the BCR. At the time of this writing, there are over 160 molecules with a cluster of differentiation (CD) designation. Many of these play important roles in intercellular communication during immune responses. In addition, secreted compounds also play a role in enhancing or modifying cellular fate. Cytokines secreted by APCs and T cells are crucial in determining the type of immune response to a particular antigen. Again, the number of characterized secreted compounds is growing. The challenge that immunologists face is to decipher the multiple signals these molecules provide to a cell during a given maturation step, with the hope of eventually being able to comprehend the intricate language by which cells of the immune system communicate.

See also: Adhesion molecules; Antigen-presenting cells; Apoptosis; B lymphocyte activation; B lymphocyte, antigen processing and presentation; B lymphocyte differentiation; B7 (CD80 & CD86); CD2; CD22; CD28; CD40 and its ligand; CD45 (the leukocyte common antigen); Chemokines; Chemotaxis; Cytokines; Dendritic cells; Fas (CD95) and fas ligand; Germinal center; Hyper-IgM syndrome; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Langerhans cells; Lymphocyte function-associated antigen 1 (LFA-1); Lymphocyte function-associated antigen 3 (LFA-3); Microenvironment; Second signals for lymphocyte activation; Selectins (CD62-E/L/P); T lymphocyte activation; TNF receptors.

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COPPER AND THE IMMUNE SYSTEM

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Human studies

Copper is a trace metal necessary for the development and maintenance of the immune response. The human requirement for copper is approximately 1 mg day^{-1} . Copper is an essential component of many important enzymes, including ceruloplasmin, tyrosinase, lysyl oxidase, cytochrome *c* oxidase, dopamine- β -mono-oxygenase, Cu-Zn superoxide dismutase, and peptidylglycine α -amidating mono-oxygenase.

Copper insufficiency in humans leads to known pathologic states. Menkes' syndrome, an X-linked trait characterized by *pili torti* and cerebral degeneration, is due to a defect in copper metabolism which leads to decreased copper levels in blood, liver and brain. Infants with this defect are extremely susceptible to infection and usually succumb by 2 years of age. Neonatal copper deficiency, though rare, is characterized by a marked neutropenia and is often associated with infections. Children suffering from severe malnutrition do not regain full immunocompetence unless copper supplementation is also included. Recent studies have shown that a low-copper diet in normal adult males causes a significant decrease in the proliferative response of cultured blood leukocytes to mitogen and an increase in the percentage of circulatory B cells.

Animal studies

Impairment of immunity as a consequence of nutritional copper insufficiency is now firmly established in both domestic and laboratory animals. Domestic animals with insufficient copper intake show decreased bactericidal activity, impairment of neutrophil function and a higher susceptibility to a variety of bacterial and fungal infections.

Laboratory models of copper deficiency have been useful in elucidating the potential defects in the immune system. In mice, the effects of copper deficiency are most readily observed in 6-week-old mice that are on a copper-deficient diet from birth. While body weight of animals on deficient diets is equal to copper-supplemented control animals, marked anemia is observed, thymus weights are routinely lower and spleen weights are usually increased.

Electron microscopy of these lymphoid organs shows abnormalities of the nucleus and mitochondria. Ceruloplasmin activity is significantly diminished within a matter of days on the deficient diet and there is a concomitant loss in cytochrome *c* oxidase and Cu-Zn superoxide dismutase activity.

Copper-deficient mice and rats exhibit an alteration in lymphocyte subpopulations, marked by a substantial overall increase in the number of surface immunoglobulin (Ig)-bearing B cells and a relative decrease in the helper T cell subset. Copper deficiency leads to an impairment of the antibody response to sheep erythrocytes and keyhole limpet hemocyanin. Lymphocytes from copper-deficient animals show a decreased response to both B and T cell mitogens. The degree of impairment in antibody production and mitogen responsiveness is proportional to the level of copper deficiency. Copper-deficient mice have a decreased cell-mediated immune response to tumor challenge and impaired natural killer cell activity.

Studies of one-way mixed lymphocyte reactions showed that responder cells from copper-deficient mice had decreased activity to inactivated control cells. Of particular interest was the observation that inactivated copper-deficient cells were poor stimulators for normal responder cells. Since it is now generally accepted that the stimulating antigens in mixed lymphocyte reaction are predominantly the class II histocompatibility antigens, copper deficiency may cause a change in the cell surface density or functional properties of these antigens. The purified lymphocyte plasma membrane from copper-deficient mice is known to have alterations in both protein and lipid content.

Analysis of serum immunoglobulin isotype levels in copper-deficient mice and rats indicate IgG levels to be statistically below control animals and IgM levels to be equivalent.

Cytokine production is altered in copper-deficient mice and rats. Production of interleukin 1 (IL-1) is increased (approximately twofold) relative to copper-supplemented controls, while IL-2 production is substantially lowered (one-third of control values). IL-2 receptor levels and interferon γ production in these copper-deficient animals is equivalent to controls.

Table 1 Cuproenzymes and immunomodulation

<i>Copper enzyme</i>	<i>Function</i>	<i>Immunomodulatory role</i>
Ceruloplasmin	Iron oxidation	Membrane integrity
Cu-Zn Superoxide dismutase	Removes superoxide	Membrane integrity
Cytochrome <i>c</i> oxidase	ATP synthesis	Energy metabolism
Dopamine- β -mono-oxygenase	Norepinephrine synthesis	Affector molecule
Peptidylglycine- α -amidating mono-oxygenase	Vasoactive intestinal peptide synthesis	Affector molecule

Possible mechanisms

Several possible mechanisms may be operative that could explain the pathogenesis of immunologic dysfunction which accompanies copper deficiency (Table 1). These mechanisms may be direct, i.e. the result of flux-limiting changes in specific cuproenzymes, or indirect. The levels of the antioxidant enzymes, Cu-Zn superoxide dismutase and ceruloplasmin, are decreased in copper deficiency and may leave the immunoreactive cells vulnerable to the increased oxygen metabolism known to accompany an activated immune response. It is also conceivable that decreased activity of cytochrome *c* oxidase may limit ATP flux and thus impair lymphocyte and macrophage energy metabolism. The complex interaction between the immune, nervous and reproductive systems may depend on copper. For example, norepinephrine synthesis depends on dopamine- β -mono-oxygenase. Norepinephrine metabolism, known to be altered by copper deficiency, is essential for immune function. Another cuproenzyme, peptidylglycine α -amidating mono-oxygenase, may also be involved; it is responsible for post-translationally modifying a large number of neuroactive peptides, including vasoactive intestinal peptide. Barnea and colleagues have shown that the release of luteinizing hormone-releasing hormone from the median eminence area is greatly amplified in the presence of copper and prostaglandin E_2 . Release of luteinizing hormone from the pituitary has a major effect on

gonadal hormone release, which in turn affects the thymus. The most significant impact of altered copper status may well be the level of production of IL-2.

See also: Antioxidant micronutrients and the immune system; Mixed lymphocyte reaction (MLR).

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CORONAVIRUS, INFECTION AND IMMUNITY

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The family of Coronaviridae was defined around 1968, primarily by morphologic criteria. The virions are pleomorphic to spherical-shaped particles (120–160 nm diameter), which are surrounded by a fringe of club- or pear-shaped surface projections (12–

24 nm long; **Figure 1**). The virus has a lipid envelope and contains a single-stranded RNA genome of positive polarity. Coronaviruses infect humans, mammals and birds. Based on molecular criteria, the toroviruses represent a second genus within this family.

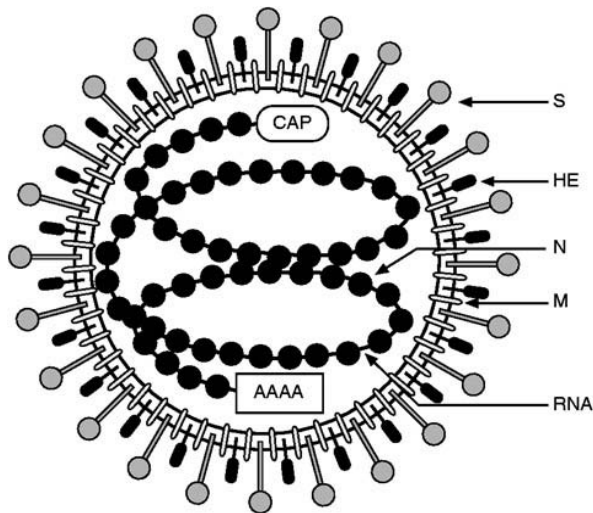


Figure 1 Coronavirus particle. The HE protein is expressed in a subset of strains only. A small membrane protein, sM, is present in the envelope of TGEV, IBV and MHV (not shown). For explanation, see text.

Furthermore, evolutionary relationships to the Arteriviridae are discussed within the concept of a 'coronavirus-like' superfamily.

Structure and replication of coronaviruses

Coronaviruses replicate in the cytoplasm and mature by budding through the Golgi apparatus and the rough endoplasmic reticulum. Their genome – a large, single-stranded molecule (size 27 000–31 500 nucleotides) – has the properties of a mRNA, namely a 5' cap structure and a polyadenylated 3' end. The replicase or polymerase gene 'Pol' is located at the 5' end and contains two large open reading frames (ORF 1a and 1b, about 20 000 nucleotides) which are expressed as a fusion protein involving ribosomal frame shifting. The polymerase products generate RNA templates for replication of genomic and subgenomic mRNAs. These mRNAs contain a common 5' leader sequence (65–100 nucleotides) that is derived from the 3' end of the negative-sense template and is essential for a discontinuous transcription mechanism. The viral proteins are translated from a nested set of 5–7 overlapping subgenomic mRNAs with common 3' ends. In general, only the 5' end of each mRNA is translated into a single viral polypeptide. Certain mRNAs for nonstructural viral proteins can employ up to three ORFs via internal initiation.

All coronaviruses contain a large surface protein (S, spike, about 200 kDa), a matrix protein M (20–30 kDa) and a nucleocapsid protein N (50–60 kDa).

Bovine coronavirus (BCV), human coronavirus (HCV)-OC43, hemagglutinating encephalomyelitis virus (HEV), turkey coronavirus (TCV) and some mouse hepatitis virus (MHV) strains express a hemagglutinin esterase glycoprotein termed HE (65 kDa). A small membrane protein sM (9.1–12.4 kDa) was demonstrated for transmissible gastroenteritis virus (TGEV), infectious bronchitis virus (IBV) and MHV. The typical gene order for coronaviruses is Pol-S-M-N. The position and expression of genes for HE, sM and nonstructural proteins varies between strains in a complex pattern. The helical nucleocapsid contains the genome and is surrounded by a lipid envelope (Figure 1). The M glycoprotein (20–30 kDa) spans this envelope three times. Peplomers (large bulbous projections) are formed by the glycoprotein S, which is cleaved in many coronaviruses to subunits of about 90 kDa (S1 and S2). The S protein induces cell fusion and binds to the host-cell receptor. A smaller fringe of spikes is formed by the HE protein, which promotes binding by interaction with 9-O-acetylated neuraminic acid.

Antigenic relationships

The majority of coronaviruses fall into three serologically distinct clusters (Table 1). Within each cluster, different antigenic relationships exist. The evolutionary relationships indicated by sequence data correlate by and large with this classification. Human and porcine coronaviruses are distributed in two clusters. The large number of serotypes for some coronaviruses (e.g. HCV, IBV and MHV) may result from recombination and high mutation frequencies. The S protein in particular displays a strong polymorphism. Enteric coronaviruses comprise only one serotype per group.

Diseases caused by coronaviruses

A variety of diseases of clinical and economic importance are caused by coronaviruses (Table 1). The majority of diseases are associated with infections of the respiratory and gastrointestinal tract. Most infections lead to acute and self-limiting diseases. Human coronaviruses are responsible for up to 30% of upper respiratory diseases, which display some periodicity in winter and spring. These viruses are difficult to isolate in tissue or organ cultures. The association of coronavirus-like particles with non-bacterial gastroenteritis is not clear.

Of veterinary importance are avian IBV, BCV, several porcine strains (e.g. TGEV, HEV and porcine respiratory coronavirus (PRCV)) and feline infectious peritonitis viruses (FIPV). Enteric coronaviruses

Table 1 Coronavirus-induced diseases

<i>Antigenic cluster</i>	<i>Virus family</i>	<i>Designation</i>	<i>Natural host</i>	<i>Typical diseases</i>
I	Canine coronavirus	CCV	Dog	Enteritis
	Feline enteric coronavirus	FECV	Cat	Enteritis
	Feline infectious peritonitis virus	FIPV	Cat	Peritonitis, granulomas
	Human coronavirus (229E)	HCV	Human	Respiratory disease
	Porcine epidemic diarrhea virus	PEDV	Pig	Respiratory disease
	Porcine respiratory coronavirus	PRCV	Pig	Respiratory disease
	Rabbit coronavirus	RbCV	Rabbit	Cardiomyopathy, enteritis
	Transmissible gastroenteritis virus	TGEV	Pig	Enteritis
II	Bovine coronavirus	BCV	Cow	Enteritis
	Human coronavirus (OC43)	HCV	Human	Respiratory disease
	Hemagglutinating encephalomyelitis virus	HEV	Pig	Vomiting and wasting, encephalomyelitis
	Mouse hepatitis virus	MHV	Mouse	Hepatitis, encephalomyelitis, enteritis, vasculitis
	Rat coronavirus	RCV	Rat	Respiratory disease
	Turkey coronavirus	TCV	Turkey	Enteritis
	Infectious bronchitis virus	IBV	Chicken	Respiratory disease, nephritis, gonaditis

are only of limited pathogenicity for older animals; respiratory viruses affect adults as well. Some strains are neurotropic, others can involve many different organs. Several coronaviruses readily establish chronic infections, which can remain inapparent or modulate immune responses. In this context, MHV infections of small rodents are interesting models for investigating pathogenetic mechanisms such as demyelination or immunosuppression. Furthermore, FIPV infections in particular induce a variety of antibody-mediated immunopathologic mechanisms.

Immune responses to coronaviruses

Antibodies are generated against all structural proteins. The peplomer protein S is the major antigen for induction of neutralizing and fusion-inhibiting antibodies. Furthermore, antibodies specific for HE, N, M and sM have also been implicated in neutralization, especially in association with complement factors. Important antigenic sites of major coronavirus families are in general well defined. The bulbous S1 subunit of IBV displays high antigenic variability and induces serotype-specific neutralizing antibodies which bind to discontinuous epitopes. More conserved sites forming linear epitopes are present on the S2 subunit (stalk) and an immunodominant region was identified which is recognized by antisera against different strains. The most immunogenic sites of BCV and MHV are concentrated on the S1 subunit of the molecule. The S2 subunit of MHV also induces neutralizing and fusion-inhibiting antibodies. The neutralization of TGEV involves four major

antigenic sites in the S protein, which help to differentiate members of this cluster.

BCV-specific antibodies against the HE protein are involved in neutralization. Furthermore, the M protein of MHV and TGEV induces complement-dependent neutralizing antibodies. The sM protein of TGEV appears also to be involved in neutralization. T cell responses against defined sites in the S and N protein contribute to virus elimination and can confer protection against encephalomyelitis in mice and rats. An immunodominant T cell antigenic site in the N protein of MHV helps to elicit neutralizing S protein-specific antibodies. Such T cell epitopes within the N protein have also been defined for TGEV and IBV.

Epidemiology and prevention

Coronaviruses are distributed throughout the world. Transmission does not involve vectors other than the host and occurs mostly by the oral-fecal route. The frequency of reinfection by human coronaviruses is high and vaccination may be indicated only in special cases. Administration of recombinant interferon α to volunteers prevented disease when treatment was started before infection. Mucosal immunity plays a pivotal role for the defense against respiratory and enteric coronavirus infections. The serum antibody profile does not correlate well with protection.

Licensed vaccines with attenuated live viruses exist for BCV, FIPV, IBV and TGEV. Protection against IBV is most efficient if the virus is administered to the trachea, but the continuous emergence of antigenic

variants prevents effective vaccination. Recombinant vaccinia viruses which express the S protein of IBV or FIPV can induce specific immunity; however, protection experiments with FIPV failed because antibodies elicited by defined antigenic sites of S protein accelerate the disease (antibody-mediated enhancement). Attempts to vaccinate cats with related coronaviruses were also without success.

In the mouse model, protection against encephalitis can be achieved by vaccination with a peptide which represents a surface domain of the S protein. Furthermore, immune responses against other viral proteins contribute to protection. For example, BCoV can be neutralized *in vivo* by monoclonal antibodies against HE protein, and adenovirus recombinants expressing HE are capable of inducing both systemic and mucosal HE-specific immunity in cotton rats.

Enteric coronavirus infections of piglets under the age of 10 days cause high mortality that cannot be prevented by vaccination of sows. Suckling animals are naturally protected by maternal antibodies contained in milk and colostrum. This protection is mediated mainly by neutralizing S-specific IgA antibodies. Protection against TGEV can also be achieved by administration of specific serum or monoclonal antibodies. Immunization of pregnant animals with attenuated live virus is a feasible approach to control the spread of virus.

Newer experimental vaccines are mainly based on three approaches: noninfectious antigens which can be targeted to the mucosa; live vectors with enteric tropism; or internal image anti-idiotypic antibodies

which mimic protective epitopes on the virus. Examples for these strategies are antigenic constructs based on peptides which represent viral immunodominant sites combined with T cell factors or bacterial components which selectively induce mucosal immunity. Furthermore, vaccines employing live bacterial vectors, adenoviruses, porcine poxviruses or genetically engineered coronaviruses are under investigation.

See also: Influenza virus (orthomyxovirus), infection and immunity; Viruses, immunity to.

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CORYNEFORM BACTERIA, INFECTION AND IMMUNITY

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Coryneform bacteria from mammalian sources can be assigned to five genera: *Brevibacterium*, *Corynebacterium*, *Dermabacter*, *Propionibacterium* and *Rhodococcus*. All are gram-positive, nonspore-forming bacilli, distinguished chiefly on their cell wall composition; speciation within the genera is incomplete.

Brevibacterium spp. are aerobic, nonlipid-requiring organisms which are sometimes referred to as 'large colony coryneforms'. Their pathogenic role is uncertain except in forms of tinea pedis associated

with malodor caused by proteolytic destruction of the skin. Bloodstream infections are reported on rare occasions.

Corynebacterium spp. include many of the best speciated organisms; they frequently need lipid for adequate growth *in vitro* and are often microaerophils; they may be called the 'small colony coryneforms'. The localized skin disease erythrasma is caused by an overgrowth of the normal skin inhabitant *C. minutissimum*, whilst a variety of normal axillary inhabitants are associated with pronounced

axillary odor which results from microbial modifications of apocrine steroid secretions.

Corynebacterium diphtheriae, the best studied and most serious pathogen, gives rise to toxinogenic pharyngeal diphtheria and also to less severe skin infections. Toxin production occurs following lysogenization of *C. diphtheriae* by tox⁺ phages, several of which have been described though most have similar DNA structures. Tox⁺ phages are also able to lysogenize *C. pseudotuberculosis* (*C. ovis*) and *C. ulcerans*, causing these organisms to produce diphtheria toxin. The toxin is a 535 amino acid polypeptide of 58.3 kDa. When nicked by trypsin at specific sites, it forms two dissimilar fragments which remain joined to form an enzymically active molecule catalyzing ADP ribosylation of elongation factor 2 (EF2) with nicotinamide adenine dinucleotide as substrate. EF2 is an enzyme present in the cytoplasm of eukaryotic cells and is essential for peptide synthesis. Ribosylation of this enzyme inhibits protein synthesis and results in the necrotic lesions of diphtheria. A single molecule of the A fraction of toxin, which contains the ribosylating activity, is lethal to a cell within hours if introduced into the cytoplasm but neither A nor B fragment is toxic extracellularly. Since the toxins produced by all strains of *C. diphtheriae* are immunologically identical, immunity is achieved by immunizing with a single toxoid; there is no type-specific immunity. The immunized individual is not protected against infection but against the systemic and local necrotic effects of the toxin; the degree of immunity conferred is high but not complete.

Caseous lymphadenitis in sheep and goats and ulcerative lymphangitis in horses results from infection with *C. pseudotuberculosis*. The disease is pyogenic despite the fact that *C. pseudotuberculosis* may produce its own toxin ('*C. ovis* toxin') as well as diphtheria toxin. The toxins appear to play little part in animal disease. *C. ulcerans* is also able to produce both these toxins and has given rise to classical diphtheria in humans. The *C. renale*, *C. pilosum*, *C. cystitidis* group cause bovine cystitis and pyelonephritis associated with extensive necrosis.

Corynebacterium jeikeium is most frequently characterized by resistance to most common antibiotics. Although found on normal human skin it causes low-grade bacteremias, generally in the immunosuppressed and particularly in males with leukemia. The mechanism of pathogenesis is unknown. *C. urealyticum* is similarly a skin resident and resistant to many antibiotics, it is a rare cause of kidney stones.

Dermabacter hominis is a recently described large colony coryneform from human skin which is re-

cognized as a rare cause of bacteremia and deep infection.

Propionibacterium spp. require lipid and anaerobic conditions for successful cultivation *in vitro*. *P. acnes* and *P. granulosum* are isolated from highly sebaceous areas of human skin in all those past puberty; *P. avidum* is similarly found in the axilla. The first two species are associated with acne and are occasionally recovered from deep infections in humans. There is no consensus on the role of propionibacteria in acne. Not all acne lesions contain microorganisms but a potential role for propionibacteria may be as follows: they reside in the ducts of sebaceous glands which become blocked as a result of changes in keratinization; sebum continues to be produced so that the blocked duct forms a closed comedo. *In vitro*, *P. acnes* has sharp pH and pO₂ optima for the production of hyaluronidase and protease; production of these enzymes *in vivo*, if it occurs, would render the comedo wall leaky to host-defense mechanisms; triggering of the alternative complement pathway by the cell wall of *P. acnes* or by protease would account for erythema and the recruitment of white cells to form a pustule. The serological picture is complicated by the almost universal existence of antibody to *P. acnes*. There is switch from predominantly immunoglobulin M (IgM) in children to IgG in adults. Antibodies to hyaluronidase are found in only about 10% of adults and this may indicate that exocellular enzymes have little role in acne. Cellular immunity in acne is a late event which probably does not contribute to lesion formation. The immunostimulator '*Corynebacterium parvum*' is a mixture of propionibacteria similar to that found on the skin.

Propionibacterium propionicus (*Arachnia propionica*) is a normal inhabitant of the human oral cavity which causes lachrymal duct infections.

Rhodococcus spp. may be difficult to distinguish from *Corynebacterium* spp. unless red pigmented colonies are seen or details of mycolic acid structure obtained, since the two grow under similar conditions *in vitro*. *R. equi*, a facultative intracellular organism, produces a potentially fatal suppurative bronchopneumonia in foals with intense inflammation of the lymph nodes. *Rhodococcus* spp. are rare pathogens of humans, occurring most frequently in the severely immunosuppressed.

See also: Bacterial cell walls; Immunopotentiality; Toxins.

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C-REACTIVE PROTEIN

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C-reactive protein (CRP) is a member of the pentraxin family of plasma proteins, which are unrelated to other known proteins but which are themselves stably conserved in vertebrate evolution. There is even a homologous CRP-like protein which is a major constituent of the blood of the so-called horseshoe crab, the arachnid *Limulus polyphemus*. The other pentraxin in humans is serum amyloid P component (SAP), which shares 55% identity of amino acid sequence with CRP. CRP is composed of five identical nonglycosylated 206 amino acid polypeptide subunits, each of M_r 23027 Da, which are noncovalently associated in a disc with cyclic pentameric symmetry. The crystal structure of CRP is very similar to that of SAP and shares with it the characteristic tertiary fold that identifies these proteins as members of the 'lectin fold' superfamily, which includes concanavalin A, pea lectin, and a variety of human, animal, plant and bacterial carbohydrate-binding proteins. There is a single functional copy of the human gene for CRP, located on chromosome 1, and no polymorphism of either the coding sequence or of the protein itself has yet been described, nor has any individual lacking CRP been identified. CRP is secreted only by hepatocytes and its production is regulated by cytokines, particularly interleukin 6 (IL-6). In regions flanking the CRP gene IL6-responsive promoter elements, and hepatocyte-specific nuclear proteins which interact with them, have been identified in studies of human CRP gene expression in transgenic mice and in human hepatoma cells.

In normal healthy subjects CRP is a trace plasma protein, median level 0.8 mg L^{-1} , with 90% of individuals having levels less than 3 mg L^{-1} and 99% less than 10 mg L^{-1} . In response to bacterial infection, trauma, necrosis of tissue, and most forms of inflammation and malignant neoplasia, CRP production is rapidly and dramatically increased and

serum levels can exceed 300 mg L^{-1} by 48 hours after an acute event. In chronic infections and inflammatory disorders CRP levels may be persistently high, but with resolution or remission of pathology, either spontaneously or in response to treatment, the CRP concentration falls rapidly to normal. Although there is a range of variation in CRP levels for a given apparent state of disease activity in groups of patients with any particular condition, the CRP values in individual patients closely reflect the extent and activity of their disease.

Severe viral infections are usually associated with a significant acute phase response but localized viral infections are generally a much less potent stimulus for CRP production than bacterial infections. In addition there are some important disorders in which CRP levels are usually normal or only modestly increased, even in the presence of severe, active, tissue-damaging pathology. These include systemic lupus erythematosus (SLE), ulcerative colitis, dermatomyositis, scleroderma, Sjögren's syndrome and leukemia. However, in all of them, intercurrent microbial, and especially bacterial, infection always stimulates a major acute phase response. The mechanisms underlying these phenomena are not known. Studies of SLE-like autoimmune disease in inbred mouse strains suggest that the acute phase response to this type of autologous tissue damage may be genetically determined, but the locus of gene action, whether at the level of mediators, receptors, acute phase protein synthesis or catabolism has not been established.

Despite ignorance of the underlying pathophysiological processes, the enormous accumulated experience of clinical CRP measurement underlies its very widespread and rapidly increasing use in routine medical practice. It is the single most sensitive non-specific screening test for organic disease, it is

exceptionally valuable as an objective marker of disease activity, and it is a unique and sensitive test for intercurrent microbial infection, especially in immunocompromised and other at-risk patients. The recent discovery that low grade CRP responses, demonstrable only with high sensitivity immunoassays, are of prognostic significance in coronary heart disease has opened a whole new field of research and practical application.

For clinical purposes it is particularly important that the rapid turnover of CRP (plasma half-life 19 hours) is constant and unaffected by intercurrent pathology. The only significant determinant of the plasma level is the synthesis rate, which generally reflects closely the extent and activity of the disorder stimulating the acute phase response. Furthermore, except in severe hepatocellular insufficiency, CRP production *per se* is impaired neither by disease nor by any treatment, unless that treatment affects the underlying disease activity. Thus CRP measurements are greatly superior to the use of erythrocyte sedimentation rate (ESR), which when used as a marker of the acute phase response is imprecise, variable, insensitive and slow to respond to changes in disease activity or effects of treatment.

CRP was discovered and named because of its interaction with the somatic C-polysaccharide of pneumococci, a ribitol teichoic acid containing phosphocholine. In the presence of calcium, CRP binds specifically and with greatest affinity to phosphocholine. It also reacts with phosphoethanolamine, and less avidly with some galactans, zwitterions and polyanions. In the absence of calcium, that is under nonphysiological conditions, it can also bind to polycations such as histones and protamine. Avid binding of CRP to chromatin has been observed at low ionic strength but does not occur under physiological conditions, in contrast to SAP which is the physiological chromatin and DNA-binding protein of the plasma. CRP does, however, bind specifically and selectively to small nuclear ribonucleoprotein particles (snRNPs).

Although CRP binds to phosphocholine with much greater affinity than to all its other known ligands, and such binding is very likely to be of functional significance, CRP does not interact indiscriminately with any phosphocholine-containing molecule. There is no interaction, for example, with the plasma membrane phospholipids of normal healthy cells or with plasma high density lipoprotein particles. On the other hand CRP does bind strongly to platelet-activating factor (PAF), the potent inflammatory mediator, and aggregated CRP selectively binds low density and very low-density lipoprotein from whole plasma.

Having bound to its ligands CRP produces a number of secondary effects which resemble those of antibodies and include neutralization of biological activity (in the case of PAF), precipitation of soluble ligands, agglutination of particulate ligands and activation of the classical complement pathway. CRP in some circumstances can act as an opsonin, particularly as a result of complement activation, although the existence and importance of receptors on phagocytic cells for bound CRP is not entirely clear. Human CRP passively administered to mice confers dramatic protection against pneumococcal infection, and CRP may be a factor in host resistance to this or other infections in autologous systems. There is evidence that in particular circumstances CRP may have proinflammatory activity, but there is little to suggest that this is a general phenomenon. On the contrary, CRP can inhibit chemotaxis and accumulation of neutrophils in *in vivo* models of lung inflammation. CRP stimulates production of tissue factor by mononuclear phagocytes and may therefore have effects on coagulation.

A very large number of other biological properties have been ascribed to CRP in a variety of different experimental systems *in vitro* and *in vivo*, involving platelets, phagocytic cells and lymphocytes, inflammation, infection and neoplasia. Many of these reports have not been reproducible and others are unlikely to be of physiological relevance. As a result, the function(s) of CRP in health and disease are still unknown. They may well be multiple, and include modulation of inflammation, participation in handling of damaged or dead cells and involvement in lipid metabolism.

See also: Acute inflammatory reaction; Acute phase proteins; Amyloid; Cytokines; Platelet-activating factor (PAF).

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CRYOGLOBULIN

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The first case of cryoglobulin was described in 1933 by Wintrobe and Buell in a patient with multiple myeloma complicated by hepatosplenomegaly, Raynaud phenomenon, purpura and retinal thrombosis, whose serum formed a precipitate when cooled. It was, however, Lerner and Watson in 1947 who, having characterized the precipitate in a patient suffering from 'allergic purpura' as immunoglobulin, coined the term cryoglobulin.

Cryoglobulins are now defined as immunoglobulins which precipitate or form a gel below 37°C (Figure 1). This is usually reversible on rewarming. At low concentrations they precipitate only around 4°C but, in general, the higher the concentration the higher the temperature of precipitation. Small amounts of cryoglobulin of about 30 mg l⁻¹ occur normally in human serum, while symptoms may be associated with concentrations above 100 mg l⁻¹. Concentrations of several grams per liter may, however, occur in the absence of symptoms.

Classification

Immunochemical analysis of purified cryoglobulins allows their classification into three groups. Different studies show rather discrepant figures for the incidence of the various types; those from a recent large study are used here (Monti *et al*, 1995).

Type I cryoglobulins, made of isolated monoclonal immunoglobulins, are found almost exclusively (90% of cases) in patients with malignant B cell tumors. They account for 5–10% of cryoglobulinemias. Most often the monoclonal immunoglobulin is

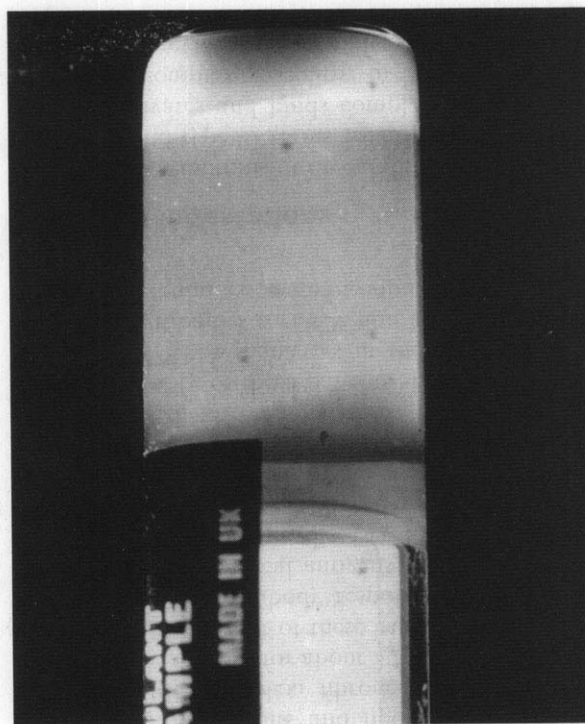


Figure 1 Serum from a patient with type I cryoglobulinemia as seen after standing at 4°C (courtesy of Professor JR Hobbs). (Reproduced with permission from Whicher JT (1994) *Plasma Proteins*. In: Williams DL and Marks V (eds) *Scientific Foundations of Biochemistry in Clinical Practice*, 2nd edn. Oxford: Butterworth-Heinemann.)

IgM, less often IgG (usually IgG2 or IgG3), occasionally IgA and very rarely Bence Jones protein.

Type II cryoglobulins are mixed cryoglobulins with a monoclonal component possessing antibody activity towards polyclonal IgG. They account for about 50–65% of cryoglobulinemias. The monoclonal component is most commonly IgM κ and possesses 'rheumatoid factor' activity and frequently anti-idiotypic activity. Occasionally it might be IgG or IgA in type. Such cryoglobulins are most often idiopathic and (along with some type III) are referred to as 'essential mixed cryoglobulinemias' though some may be associated with immune responses to infectious agents or with chronic inflammatory disorders. About 40% of type II cryoglobulins are secondary to B cell tumors and such a pathology must be rigorously excluded before labeling a case as 'essential'.

Type III cryoglobulins are mixed cryoglobulins composed of one or more classes of polyclonal immunoglobulins, most commonly IgM, in the form of immunoglobulin–anti-immunoglobulin complexes. Nonimmunoglobulin molecules such as complement components and lipoproteins may be included in the complex: fibronectin is invariably present. They account for about 30–40% of cryoglobulinemias. About half of these cases are associated with either active antibody responses to infectious agents (e.g. streptococcal antigens) or cirrhosis and certain connective tissue diseases (notably Sjögren syndrome, systemic lupus erythematosus and progressive systemic sclerosis).

Essential mixed cryoglobulinemia is frequently associated with chronic hepatitis. Studies suggest that the majority of these patients show hepatitis C virus (HCV) RNA sequences in the serum. About 50% have antibodies to HCV and also suffer from more cryoglobulin-associated symptoms.

Clinical manifestations

The classical symptoms of cryoglobulinemia arise in the type II and type III forms when the mixed cryoglobulin activates and binds complement, resulting in immune complex vasculitis. In both types 90% of cases show evidence of complement consumption (decreased CH50 C3 or C4 in the serum) and the vasculitic lesions invariably contain bound complement. The commonest features are shown in Table 1. Purpura is intermittent, nonitchy and associated with cooling of exposed parts of the lower extremities. Chronic leg ulcers may be associated with purpura. Arthralgia is intermittent, symmetrical and nonmigrating. Renal damage may be progressive with proteinuria, hypertension and renal failure.

Table 1 Clinical features of mixed cryoglobulinemia

Signs and symptoms	Approximate %
Purpura	90
Hepatomegaly	70
Splenomegaly	50
Arthralgia	60
Weakness	60
Raynaud phenomenon	40
Leg ulcers	30
Proteinuria	50
Renal failure	40
Abnormal liver function tests	30

Renal biopsies show proliferative or membranoproliferative glomerulonephritis.

In type I cryoglobulinemia symptoms are less marked, with purpura, arthralgia and renal involvement often being absent. When they do occur they result primarily from increasing viscosity in the peripheral capillaries causing Raynaud's phenomenon, extensive gangrene and recurrent distal necrosis (Figure 2). Occasional arterial thrombosis may occur. *In vitro* cryoprecipitation can be demonstrated quite clearly in many sera containing monoclonal immunoglobulins, particularly after storage for several days at 4°C, with no obvious clinical sequelae.

Laboratory investigations

The laboratory investigation of cryoglobulinemia is aimed at detecting cryoglobulins in patients with suggestive symptoms and characterizing them according to type. It is important to appreciate that



Figure 2 Digital gangrene in a patient with cryoglobulinemia (Kindly supplied by Dr P Copeman.) (Reproduced with permission from Whicher JT (1994) *Plasma Proteins*. In: Williams DL and Marks V (eds) *Scientific Foundations of Biochemistry in Clinical Practice*, 2nd edn. Oxford: Butterworth-Heinemann.)

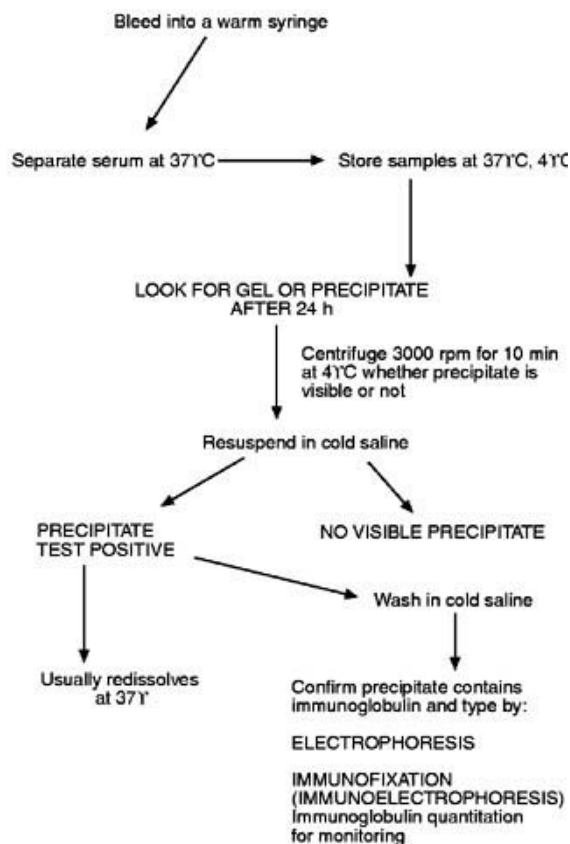


Figure 3 The laboratory investigation of cryoglobulin.

in the absence of obvious underlying disease, the diagnosis of 'essential cryoglobulinemia' must be kept under continuous review as it is very likely to represent 1) the preclinical stage of a lymphoproliferative disease; 2) the only sign of an underlying infection or chronic inflammatory disease; 3) the preclinical stage of cirrhosis or chronic active hepatitis.

To detect cryoglobulins, blood samples should be sent to the laboratory warm and separated at 37°C. The investigative procedure is summarized in **Figure 3**. The precipitate does not always redissolve easily, probably due to intermolecular disulfide bond formation. Electrophoresis of the warmed serum and the redissolved precipitate reveals a monoclonal component in about 60% of cases (type I or type II). In type II cases this is most often IgM κ but confusion may arise as the monoclonal band seen on electrophoresis may be found to contain IgM, IgG, κ and λ on immunofixation or immunoelectrophoresis owing to the presence of bound polyclonal IgG.

The analysis of cryoprecipitates in trace amounts

using two-dimensional polyacrylamide gel electrophoresis, has shown that polyclonal IgG are associated with a mixture of polyclonal and monoclonal IgM. These cryoglobulins have been tentatively named type II–III cryoglobulins. They are theoretically relevant since they may represent the transition from type III (mixed-polyclonal) to type II (mixed-monoclonal) cryoglobulins. This transformation could be induced by the chronic antigen stimulation caused by persistent HCV infection documented in most patients with 'essential' mixed cryoglobulinemia.

Patients with cryoglobulins containing a monoclonal component should be investigated for an underlying lymphoproliferative disease and the presence of hypogammaglobulinemia and/or Bence Jones proteins are useful indicators.

Mixed cryoglobulins frequently have anticomplementary activity and low serum complement levels, especially of C4, may be seen.

Immunohistological studies of skin lesions in cryoglobulinemia show IgM, IgG and/or C4 in the blood vessel walls.

See also: Bence Jones proteins; Idiotype; Immune complexes; Immunoelectrophoresis; Lymphoma; Plasmapheresis; Rheumatological disorders; Sjögrens syndrome; Systemic lupus erythematosus (SLE), human.

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CRYOPRESERVATION OF IMMUNE CELLS

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Primitive experiments in cryopreservation date to the mid-eighteenth century but it was not until 1949 that Polge first used glycerol to successfully cryopreserve both animal and human spermatozoa at -79°C . After the demonstration of glycerol's effect on the low-temperature storage of spermatozoa, early cryopreservation studies focused on the use of glycerol and then dimethylsulfoxide (DMSO) to cryopreserve murine bone marrow for syngeneic bone marrow transplantation. The goal was to circumvent the lethal effects of radiation on the bone marrow, which were first seen as a result of the atomic bombs that had been dropped on Japan a few years earlier. These initial studies demonstrated the efficacy of these agents in the cryopreservation of marrow cells frozen at a constant rate of $1^{\circ}\text{C min}^{-1}$ to -79°C , with storage for periods up to 6 months. Viability was determined by the demonstration of viable cells post-thaw by dye exclusion studies and the successful engraftment of the bone marrow when injected into syngeneic animals after lethal irradiation. This technology now forms the basis of numerous successful autologous bone marrow transplant programs worldwide, with clinical successes not only in the leukemias and lymphomas but also in solid tumors.

The use of cryopreserved lymphocytes for *in vitro* studies is also now commonplace. The initial studies were reported in 1964 by Ashwood-Smith, who cryopreserved murine lymphocytes using 15% DMSO. Cells were held in liquid nitrogen (-196°C) for 3 months and then assayed for viability, both by using phase microscopy and by the demonstration of splenomegaly after they were injected into irradiated hybrid mice. With the need for standardization of cytochemical staining, surface marker analysis and *in vitro* stimulation studies of lymphocytes, cryopreservation subsequently became the easy way to store cells for comparative studies. It is now easy to follow and compare changes over time or after therapy in individual patients using cryopreserved cells, with all assays done at the same time. While cryopreservation is used mainly for *in vitro* testing of lymphocytes, with the advent of recombinant cytokine therapy, cryopreservation may permit the storage of large numbers of either resting or 'stimulated' lymphocytes treated *ex vivo*, either after or before cryopreservation, in an attempt to augment the *in vivo* immune response to tumor cells. When infused later at a time of minimal residual disease, they might decrease

relapses when used as consolidation therapy following conventional or transplant therapies.

Cryoprotectants and the freezing process

The initial use of glycerol for the freezing of spermatozoa was a 'chance observation' in 1949; it and other cryoprotectants work by stabilizing cell membranes and intracellular contents during the freezing and thawing processes. Glycerol and related compounds and DMSO are intracellular cryoprotectants in that they diffuse readily into the cell when added prior to cryopreservation, while hydroxyethyl starch (HES), dextran and polyvinylpyrrolidone (PVP) act as extracellular cryoprotectants by stabilizing the cell membrane during the freezing and thawing process. Lethal damage to cells can occur at various points in the freezing-thawing process, with the majority of damage occurring during the phase change from liquid to solid, and during the thawing and manipulation of the previously frozen cells. When cells in suspension are cooled, extracellular ice crystals form, raising the intracellular solute concentration. This osmotic change, if it occurs in the absence of a cryoprotectant, can lead to their death with cell membrane lysis. While rapid cooling decreases water shifts, it causes cell death due to intracellular ice formation. By diffusing into the cell, intracellular cryoprotectants decrease the solute shifts that occur during the freeze-thaw cycle, thereby decreasing the potential damage caused by high intracellular solute concentrations. This permits the use of slow cooling rates ($1-3^{\circ}\text{C min}^{-1}$), which in turn decreases the risk of damage caused by intracellular ice formation when faster rates are used.

The phase change from liquid to solid, with the liberation of the heat of fusion that occurs during the freezing process, can also be damaging to the cells. Without the use of a controlled-rate freezing apparatus, the temperature can actually rise several degrees (heat of fusion), and does not begin to fall again until the entire solution has been frozen. A rapid transit during this transition at $1^{\circ}\text{C min}^{-1}$ appears to be optimal for the preservation of marrow stem cells, but seems to be less critical for other cells, including lymphocytes and platelets, especially when small aliquots with a greater surface area available for the dispersion of this heat are available.

While little damage is felt to occur during storage

at low temperatures, provided that there are no significant temperature fluctuations, significant damage can occur during the thawing of the cryopreserved cells. During the thawing process, cells swell because of the reversal of the water shifts that occur during freezing. Intracellular cryoprotectants that diffuse out of the cells rapidly, extracellular cryoprotectants and proteins, including albumin, appear to decrease the damage to cell membranes that occurs during this swelling. While some controversy exists as to the best way to thaw frozen cells, in general most cells thaw rapidly and, once thawed, should be diluted slowly, 1:1 over 3–10 min, and then more rapidly to minimize further swelling. When used for transplantation purposes the thawed cells are infused rapidly, unfiltered and undiluted.

While effective at low temperatures, intracellular cryoprotectants such as DMSO are toxic to cells at room temperatures or higher. For optimal use, DMSO should be added at 4°C just prior to freezing. In addition, for *in vitro* work it should be diluted or washed to a concentration of <1% as soon as possible post thaw. A recent analysis of the mechanism of action of DMSO by Arakawa suggests a reason for the disparity of the effects of DMSO at different temperatures. At low temperatures, DMSO is excluded from the hydration cover of proteins which leads to their stabilization during the freezing process; however, at room temperatures DMSO interacts in a hydrophobic way with these same proteins, leading to denaturation or destabilization. Exposure at concentrations >1% for as little as 30–60 min is sufficient to cause irreversible cell membrane damage.

Cryopreservation techniques

Hematopoietic stem cells

There are currently two methods of hematopoietic stem cell cryopreservation in use worldwide. The most commonly employed method is based on the animal studies of the 1950s. After bone marrow is harvested in the operating room, it is brought to the cryopreservation laboratory where, using a cell sorter or density gradient separation, the majority of the red cells are removed. After adjusting the cell count to 60–70 million ml⁻¹, using tissue culture media admixed with 20% autologous plasma, an equal volume of media/plasma solution containing 20% DMSO is slowly admixed with the cells at 4°C; 100 ml aliquots are then placed into polyolefin freezing bags, sealed, placed into aluminum freezing frames and then frozen using a programmable rate-controlled freezer, at a constant freezing rate of

1°C min⁻¹ to –80–120°C, at which time the bags are placed into the liquid or vapor phase of liquid nitrogen. At the time of reinfusion, the cells are thawed at the bedside and infused rapidly over 5–15 min. Rapid reinfusion is required because this method does not successfully cryopreserve granulocytes, which lyse rapidly after thawing and release nucleoproteins which cause clumping and a gel formation if left at room temperature for even 30 min.

A newer method, which has also been proven to be effective for both marrow and blood stem cells, uses a combination of both DMSO, an intracellular cryoprotectant, and low molecular weight HES, an extracellular cryoprotectant. This combination successfully cryopreserves both stem cells and terminally differentiated granulocytes, permitting the cryopreservation of large volumes of bone marrow in a single bag. In addition, this method does not require the use of a rate-controlled freezing process, nor does it require storage at liquid nitrogen temperatures (although recent data suggest that cells preserved in DMSO alone can also be frozen without a rate-controlled freezer or stored for short periods at –80°C). After harvesting, the majority of the red cells are removed as described above. The cells are brought to a final volume of 300 ml and are admixed with an equal volume of a cryoprotectant solution containing 10% DMSO, 12% HES and 8% human albumin in lieu of autologous plasma; 300 ml aliquots are placed into freezing bags, which are then placed into aluminum freezing frames. The marrow is frozen and stored simply by placement in a –80°C freezer, where it is kept until it is autotransfused. Reinfusion is done by infusing the 300 ml aliquots over 30 min each. Successful engraftment has been seen for cells stored at –80°C for periods of 2 years.

The newest source of stem cells being utilized for transplantation is cord blood, obtained at birth and used primarily for unrelated allogeneic transplantation. Cord blood banks are being developed in many countries, using standard cryopreservation techniques; successfully thawed, these cells are used as a transplant source primarily for children who have neither a related sibling donor nor an unrelated donor available through national and international computer registries. While standard cryopreservation techniques are used, many programs concentrate the stem cells (HES sedimentation) prior to cryopreservation to maximize freezer space. This type of transplant will likely increase as there appears to be a lower incidence of graft versus host disease. Furthermore, techniques have been developed to expand hematopoietic stem cells *ex vivo*, making these grafts available to adults.

Lymphocytes

While post-thaw recovery of cryopreserved lymphocytes appears to be optimal when the cells are frozen in 7.5–10% DMSO using a rate-controlled freezing process at $1^{\circ}\text{C min}^{-1}$, many routinely freeze lymphocytes by simple placement into a -80°C freezer, or even by simple placement in insulated containers into the vapor phase of liquid nitrogen. Alternatively, lymphocytes can be held at room temperature for several days if nutrient media are properly buffered at pH 7.0 with HEPES buffer, and care is taken to avoid bacterial contamination. The cryopreservation process using rate-controlled freezing is similar to that for bone marrow; however, after freezing to -30°C at $1^{\circ}\text{C min}^{-1}$ the rate is increased to $5^{\circ}\text{C min}^{-1}$ to -80°C , at which time the cells are transferred to the vapor phase of liquid nitrogen. Alternatively, good results can be seen if the cells are put in a styrofoam box and placed in either a -80°C freezer for a minimum of 2 h before transfer to the vapor phase of liquid nitrogen or left in the mechanical freezer, or directly in the vapor phase of liquid nitrogen for 30 min and then transferred to the liquid phase of liquid nitrogen. Thawing and processing is done in a similar manner to marrow cells, with dilutions performed slowly over 5–10 min using a buffered, protein-containing solution. To wash the cells free of the residual DMSO, they are centrifuged at 150g for 10 min and then gently resuspended with the same media. Cells can also be frozen in tissue culture wells for later use, e.g. lymphocytotoxicity assays.

Evaluation of cryopreserved cells

Depending on the cryoprotectant used to preserve hematopoietic stem cells, post-thaw recovery of nucleated cells varies from 50 to 90%. Unfractionated marrow cells cryopreserved in DMSO alone are associated with a lower recovery because terminally differentiated granulocytes are not successfully preserved, lyse and release nucleoproteins, causing clumping which entraps additional cells. This problem is eliminated if this cell population is removed prior to cryopreservation, or the DMSO/HES cryoprotectant is used, which successfully preserves these cells. Post-thaw recovery of committed stem cells such as CFU-GM and BFU-E also appears to be higher using the DMSO/HES method, ranging from 80 to 90% of prefreeze assays. However, post-transplant engraftment times appear similar for the two methods, with time post-transplant for a granulocyte recovery $>500 \text{ cells } \mu\text{l}^{-1}$ and platelets $>20\,000 \text{ cells } \mu\text{l}^{-1}$ of 17–22 days. Engraftment times appear to be shorter (5–7 days) for blood stem cell transplants,

largely due to the increased number of cells that are mobilized, collected and cryopreserved.

Cryopreserved lymphocytes have many uses, including histocompatibility studies, functional assays to compare differences over time or therapy given *in vivo* for determination of cell surface markers. Having a reproducible source of cells, and the ability to improve quality control by assaying all target cells at the same time under the same conditions, is of significant value. Total recovery of viable lymphocytes, cryopreserved as described above, is in the range of 70–80%, and as early as 1962 Pegg demonstrated that thawed lymphocytes could be used in stimulation assays, with little difference compared with fresh controls.

A variety of lymphocyte assays have been performed on cryopreserved lymphocytes. The relative number of B and T cells, the number of E (sheep erythrocyte) and EAC (erythrocyte–antibody–complement) rosettes, and the response to the mitogens PHA, PWM and Con A are not affected by cryopreservation but their recovery appears to be optimal when programmed freezing methods are used. The handling of the cells prior to cryopreservation appears to be important: specifically, the use of ammonium chloride is toxic to T cells when used prior to cryopreservation; it can, however, be safely used post thaw for separation studies.

Cryopreservation with DMSO appears to decrease the number and intensity of CD4^{+} , CD7^{+} , CD13^{+} and CD33^{+} cells assayed by flow cytometry, but appears to have no effect on B-lineage cells or on CD8^{+} cells. Multiple studies have demonstrated a decrease in natural killer (NK) cells, i.e. cytotoxicity against K562, as well as antibody-dependent cellular cytotoxicity (ADCC) and mixed lymphocyte responses for cryopreserved lymphocytes. NK cell recovery is 50–70% compared with that of fresh cells; however, this value significantly improves when the thawed cells are incubated for 18 hours prior to assay. Lymphokine-activated killer (LAK) cell activity may also be reduced following cryopreservation. Activity levels vary widely, ranging from 33 to 87%, depending on the effector:target ratios and the target cell line used for the assay. Cytotoxicity, however, improves upon incubation with recombinant interleukin 2 (rIL-2) for several days after thawing, and, if stimulated both before and after cryopreservation, activity levels may exceed that for fresh mononuclear cell populations due to loss of macrophage function during cryopreservation. The cause for the lowered NK and LAK activity appears to be a lowering of the binding to target cells early after thawing which recovers upon incubation for 5–24 h. Thymocyte recovery is also poor following standard

cryopreservation using DMSO but preliminary studies suggest a benefit to thymocyte recovery if both DMSO and HES are used together as cryoprotectant agents.

With the advent of many recombinant cytokines, the ability to cryopreserve both bone marrow and lymphocytes will attain even more significance. Clinical trials in which cryopreserved marrow is incubated for 24 h prior to the reinfusion into patients as part of an autologous transplant are under way.

See also: Antibody-dependent cellular cytotoxicity; Hematopoietic stem cell transplantation; Cell separation techniques; Flow cytometry; Hybridomas, B cell; Hybridomas, T cell; Leukocyte culture; Lymphokine-activated killer (LAK) cells; Rosetting techniques; Tissue typing; Viability, methods of assessing leukocyte.

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CRYPTOCOCCOSIS, INFECTION AND IMMUNITY

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Cryptococcosis is a chronic, subacute or rarely acute infection caused by the round encapsulated yeast, *Cryptococcus neoformans*, measuring 15–30 µm. Cryptococcal meningitis, with or without disseminated infection of other organs, is the best known form of this mycosis. Pneumonia developing after inhalation of infectious particles is more common but frequently takes a subclinical course. During fungemia the organism can lodge in the central nervous system and in any other organ system, for example skin, bone, adrenals, eyes or prostate. The central nervous system provides an immunologic sanctuary for the infection from which the fungus cannot be eradicated by host defenses. The microbiology, ecology, epidemiology and immunology of cryptococcosis have interesting aspects illustrating many important general principles of infectious diseases.

Cryptococcus neoformans is a saprophyte isolated from environments contaminated with bird (particularly pigeon) droppings and from the tree *Eucalyptus camaldulensis*. Among the four serotypes A–D serotypes A and AD from *C. neoformans* var. *neoformans* are associated with bird droppings. Ser-

otype B from *C. neoformans* var. *gattii* is associated with *E. calmadulensis* and its geographic distribution is associated with this tree, which is abundant in Australia. The ecology of serotype C, common among clinical isolates in southern California, is unknown. Serotypes A and AD are the common serotypes in clinical material from Asia, Europe and the east of the USA. Up to 5×10^7 yeast cells can be recovered from 1 g of pigeon feces. *C. neoformans* has been isolated from the environmental air in the vicinity of bird colonies but its prevalence is not comparable to the ubiquitous abundance of spores from pathogenic molds such as *Aspergillus* spp. On the other hand *C. neoformans* is more abundant in the environment than the dimorphic fungi causing endemic mycoses such as *Coccidioides immitis*, *Blastomyces dermatitidis* or *Histoplasma capsulatum*.

Before the sexual form, the perfect state *Filobasidiella neoformans* was discovered, inhalation of yeast cells, shrunk during their sojourn in hyperosmolar alkaline bird feces, was regarded as the sole mode of infection. The present view is that this assumption holds true for serotypes associated with bird drop-

pings, while the infectious particle for serotype B could well be the basidiospore of *F. neoformans*. Both the shrunken yeast, with a diameter of 3–7 μm , as well as the spore, with a diameter of 2–3 μm , are of a size that permits inhalation and deposition in the alveolar space distal to the mucociliary apparatus of the airways.

The ecology in addition to the virulence of *F. neoformans* govern the occurrence of cryptococcosis relative to other mycoses in normal and compromised hosts (Table 1). Accordingly also a positive skin test to cryptococcal in contrast to candidal antigens is commonly only found in people with exposure to bird droppings, such as pigeon breeders. *C. neoformans* takes an intermediate position between the primary pathogenic dimorphic agents of the endemic mycoses and the opportunistic molds and yeasts with regard to its virulence (Table 1). In the past, about 50% of patients with cryptococcal meningitis did not have any recognizable underlying condition explaining their vulnerability to this fungus. With the advent of the human immunodeficiency virus (HIV) epidemic, the fraction of patients without underlying disease has dwindled and the acquired immune deficiency syndrome (AIDS) is the most frequent predisposing condition, documenting the opportunistic propensities of *Cryptococcus*, particularly for patients with defective cell-mediated immunity. Cryptococcosis is the most important deep mycosis in AIDS patients and can be one of the presenting manifestations of HIV infection. It is of note that AIDS patients are not, however, particularly prone to deep-seated, invasive infections with *Candida*, *Aspergillus* or *Mucoraceae*, which, in contrast to *Cryptococcus* or the primary pathogenic agents of the endemic mycoses (Table 1),

can be controlled by phagocytic defenses alone. Clinical experience has not linked defective humoral immunity, complement deficiencies or isolated phagocytic defects to cryptococcosis.

An essential property enabling *C. neoformans* to cause human disease is its ability to grow at temperatures of 37–39°C. The elevated body temperature of mammals precludes *in vivo* propagation of nonvirulent species of *Cryptococcus*. Similarly, it appears plausible that a body temperature of 42°C explains the absence of deep-seated cryptococcosis in pigeons.

A second property essential to the virulence of *C. neoformans* is its polysaccharide capsule (Figure 1). Acapsular mutants are avirulent and by restoring encapsulation regain their virulence for mice. In analogy to encapsulated bacteria, the capsule of *C. neoformans* has antiphagocytic properties. The capsule masks binding sites recognized by phagocytic receptors for β -glucan and mannan that could mediate induction of phagocytosis and secretion of tumor necrosis factor α (TNF α). The capsule can also mask cell wall bound immunoglobulin G (IgG). Yet the capsule is also the site of complement activation via the alternate pathway, resulting in the presentation of iC3b fragments that might contribute to opsonization. Accordingly, small capsule variants can be sufficiently opsonized for phagocytosis or extracellular killing by phagocytes by the complement system alone. *In vitro* several types of leukocytes display anticryptococcal activity, including neutrophils, monocytes and natural killer (NK) cells. *In vivo* higher levels of interleukin 2 (IL-2) and interferon γ (IFN γ) are generated in response to nonencapsulated strains of *C. neoformans*. In addition it has been proposed that the polysaccharide capsule of *C. neoformans* might contribute to virulence by inducing sup-

Table 1 Comparison of *C. neoformans* with other common pathogenic fungi

Fungal pathogen	Distribution	Virulence	Infection
<i>Cryptococcus neoformans</i>	Worldwide, restricted natural habitat	Intermediate	Rare in normal host, increased incidence in AIDS patients and other compromised hosts
Monomorphic molds <i>Aspergillus fumigatus</i>	Worldwide, ubiquitous, abundant	Low	Common in host with phagocytic dysfunction
Opportunistic yeasts <i>Candida albicans</i>	Worldwide, normal endogenous flora, ecologically linked to humans	Low in regard to invasive infection	Common in compromised hosts
Dimorphic primary pathogenic fungi <i>Histoplasma capsulatum</i> <i>Coccidioides immitis</i> <i>Blastomyces dermatitidis</i> <i>Paracoccidioides brasiliensis</i>	Geographically restricted, restricted habitat	High	Common in exposed normal individuals, opportunistic propensities

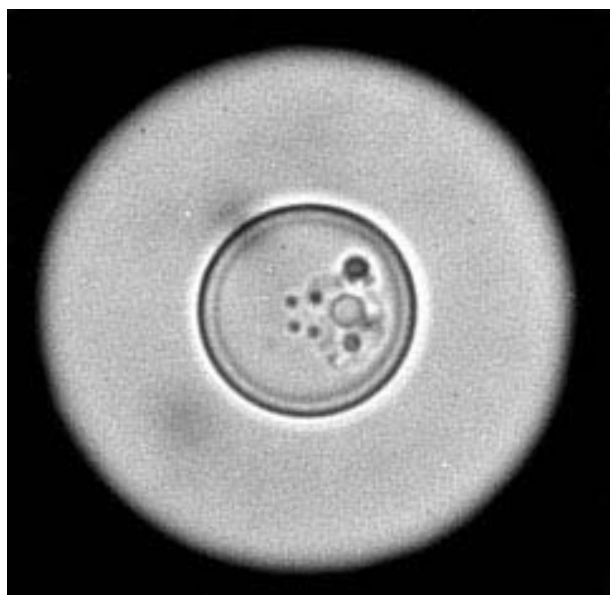


Figure 1 The outstanding virulence factor of *C. neoformans* is its capsule protecting the yeast cell from recognition by phagocytes. India-ink preparation of native yeast cell in cerebrospinal fluid from a patient with cryptococcal meningitis.

pressor T cells. Antigen-specific suppressor CD8⁺ T cells produce a factor that inhibits binding of *C. neoformans* by macrophages. But while immunosuppressive mechanisms have been found to attenuate host defense mechanisms, true immunosuppression affecting the course of cryptococcosis has not been documented.

Recognition of encapsulated *C. neoformans* by antigen-specific mechanisms is the decisive element of immunity to this fungus. Natural antibody and complement have some opsonic activity, permitting phagocytosis or inhibition by alveolar macrophages of lightly or nonencapsulated variants in natural resistance, but a specific immune response is essential for an efficient control of encapsulated *C. neoformans*. Antibody-dependent inhibition or killing by phagocytes involves reactive oxygen intermediates and, in some species such as the mouse, nitric oxide. Melanin-like pigments of *C. neoformans* appear to contribute to its virulence by reducing the susceptibility of the yeast to hydrogen peroxide and nitric oxide. The anticryptococcal mechanisms responsible for the direct antifungal effect of NK and T cells against *C. neoformans* appear to be independent of oxygen or nitrogen radicals, but their nature has not been elucidated. Observations in immunodeficient

hosts make it clear that T cell-mediated immunity is essential for acquired immunity to *C. neoformans*. Athymic nude mice are unable to control an infection with this yeast and develop chronic progressive cryptococcosis; up to 8–9% of patients with AIDS go on to develop disseminated cryptococcosis. The observation that beige mice that are deficient in NK cell activity are more susceptible to cryptococcosis, but not to other fungal infections that are controlled by phagocytes alone, such as aspergillosis or invasive candidiasis, indicates that NK cells also play an important role. Extracellular inhibition of *C. neoformans* by secretory products of macrophages other than nitric oxide may also occur.

See also: Acquired immune deficiency syndrome (AIDS); Fungi, immunity to; Natural killer (NK) cells; Opportunistic infections; Opsonization; Suppressor T lymphocytes.

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CRYPTOSPORIDIOSIS

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Natural history

Cryptosporium, a protozoon found in the gastrointestinal tract of most vertebrates, was first described by Tyzzer in 1907 in the gastric mucosa of the domestic mouse. The name signifies that the parasite belongs to the class Sporozoasida in which spores are absent or concealed within the oocyst. *Cryptosporidium* is a genus which now consists of several species, of which the mammalian species *C. parvum* is the most important. *Cryptosporidium* was considered as a harmless opportunistic parasite for mammals and birds until the late 1970s, after which time evidence began to emerge implicating it as a possible cause of diarrhea in calves. The history of cryptosporidiosis in humans is more recent, the first two cases being described in 1976 by Nime *et al* and Meisel *et al*. By 1980 further cases had been described in individuals with clinical evidence of acquired or congenital immunodeficiency. The first case of cryptosporidiosis in an immunocompetent adult, who suffered severe, watery but self-limiting diarrhea and vomiting, was described in 1980 by Tzipori *et al*. This patient was the first in whom the diagnosis was made by detection of oocysts in stools. The transmission of this human isolate to mice and lambs not only showed that it was biologically indistinguishable from *Cryptosporidium* of other mammals, but also indicated indirectly the zoonotic potential of this parasite, as was subsequently confirmed by Reese and colleagues in 1982. The emergence of acquired immune deficiency syndrome (AIDS) and AIDS-related infections promoted human cryptosporidiosis to the forefront of interest in 1982. By 1985 *Cryptosporidium* had been observed in the intestines of most mammals, birds, several species of fish and reptiles. The prevalence of the infection in the general human population also became apparent with the publications of the first epidemiological studies in 1983 by Jokipii and coworkers, and Tzipori and colleagues. The early epidemiologic studies were followed by numerous reports from around the globe which established the parasite as an important cause of diarrhea in healthy and in immunodeficient individuals.

Characteristics of the organism

Cryptosporidium is a genus in the family Cryptosporidiidae, suborder Eimeriorina, order Eucoccidiorida, subclass Coccidiasina, class Sporozoasida, phylum Apicomplexa. Other important members of the suborder Eimeriorina include *Eimeria*, *Isospora*, *Toxoplasma* and *Sarcocystis*. Until 1980 *Cryptosporium* was considered to be host specific and was named after the animal species from which it was isolated. At present two mammalian species *C. muris* and *C. parvum* are recognized, the latter being the most commonly isolated from humans and domestic animals. *C. baileyi* is the only other confirmed species, infecting chickens and turkeys; others undoubtedly will emerge in the future.

The life cycle of *C. parvum* follows closely the pattern characteristic of other coccidia: asexual followed by sexual endogenous stages resulting in production and discharge of oocysts in the feces. The oocysts, however, are minute (approximately 4 µm), surrounded by a thick and extremely resistant capsule, and are shed sporulated and therefore infectious. Immediate sporulation and the fact that a portion of oocysts are produced with a thin capsule which breaks up within the host, releasing naked infectious sporozoites, contribute to the parasite's unique potential for autoinfection; a crucial phenomenon in the pathogenesis of persistent infection in immunologically compromised hosts.

Unlike other enteric coccidia, *C. parvum* does not become internalized within the epithelial cell cytoplasm but remains physically located on the cell surface. After excystation in the stomach or the duodenum, sporozoites and later merozoites become attached to the epithelial cell surface, displacing the microvillous border, and become enveloped by a membrane (parasitophore) which is derived from the host cell (Figure 1), a phenomenon termed by Gocbel and Braendler in 1982 as 'intracellular-extracytoplasmic'. By becoming internalized the parasite not only now derives all its nutrient and energy requirements from the host cell, but also evades the host immune response.

Infection and disease

C. parvum infection is extremely common in nature among domesticated and wild mammalian species.

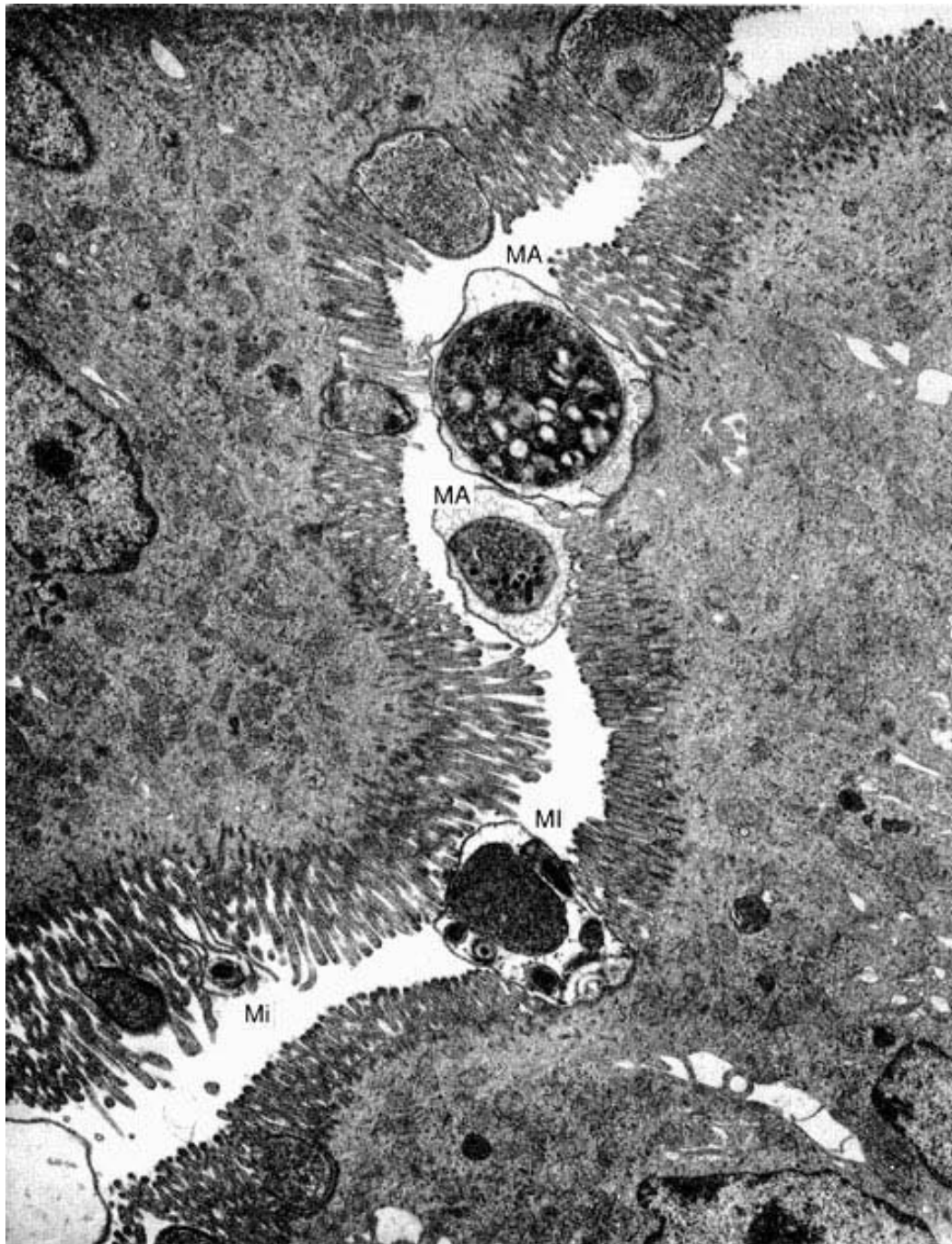


Figure 1 An ultrathin section of ileum from a lamb experimentally infected with *Cryptosporidium*. Note endogenous forms firmly attached to the luminal surface of enterocytes displacing the microvillus border. MA represents two macrogamonts—female forms—each containing one macrogamete; MI represents a microgamont—the male form—which contains five visible, of 16, microgametes. Mi is a single microgamete, while all the others are trophozoites (the asexual form) at various stages of development ($\times 12750$).

The infection can cause disease in many species of animals, particularly in the very young and in the immunocompromised host; however, it is most commonly associated with diarrhea in humans and newborn ruminants. *C. baileyi*, the avian species, often causes respiratory symptoms in turkeys and chick-

ens. *C. parvum* infection in newborn rodents and other laboratory animals is largely asymptomatic.

Cross-transmission experiments among animal species, and reports of several cases of human infection contracted from animals, indicate that the majority of isolates perpetuating among humans,

their domestic animals and their immediate environment are interchangeable and probably belong to the genus *C. parvum*. Avian isolates (*C. baileyi*) were shown to differ on the basis of cross-transmission studies, sporozoite chromosomal DNA and some antigenic variation in the oocyst wall.

C. parvum has been observed in part or in all of the gastrointestinal tract, including the biliary system and bile duct, gallbladder, colon and rectum. The mechanism by which it induces diarrhea is not entirely clear. The endogenous stages, which heavily colonize the surface epithelium, destroy the microvillous border, leading, in the proximal small intestine, to loss of membrane-bound digestive enzymes. There is also a variable degree of villous contraction and increased cellularity in the lamina propria, both of which contribute to reduced absorption from the lumen. In humans at least, there is clinical and physiologic evidence to implicate the action of an enterotoxin in the pathogenesis of what essentially is a watery diarrhea.

While the prevalence of the infection in the general population is high, the incidence of diarrhea associated with cryptosporidiosis is 4–7% in children under 5 years of age. In infants it can be as high as 25% in outbreaks in daycare centers, and at certain times of the year in children in some African countries. The incidence is highest during the summer dry months. Oral-fecal is the most common mode of transmission – from other persons, from domestic pets and wild animals, and from contaminated water. Recently several major waterborne outbreaks of diarrhea due to *C. parvum* have highlighted water contamination as a major vehicle for exposure. The diarrheal outbreak in 1993 in Milwaukee, Wisconsin, USA, involving some 403 000 people, was a major turning point with regard to *C. parvum* in municipal water supply. Aerosol transmission may also be a factor because infection of the upper respiratory tract in humans has also been described.

The immune status of the host appears to be a major determinant of whether the infection is self-limiting or becomes persistent. It is clear that both branches of the immune system are required for complete recovery because CD4 blood lymphocyte counts below 200 per mm³, as seen in AIDS or hypogammaglobulinemia, can both lead to persistent infection. Because of the continuing increase in the incidence of AIDS, the occurrence of persistent *C. parvum* infection in this subpopulation has substantially increased.

Studies on serum antibodies against sporozoites identified the presence of antibodies to a common 20 kDa antigen – a major sporozoite surface determinant – in humans, horses and calves for up to 5

months after infection; however, levels remained high in individuals which had frequent exposure. Studies in mice have shown that passive antibodies from previously infected mothers did not prevent experimental infection; nor did treatment with monoclonal antibody against sporozoites prevent infection in mice, although there was a reduction of parasite load compared with control animals. However, bovine hyperimmune colostrum containing specific antibodies did modify the clinical outcome of infection in humans and when tested in mice. These studies indicate that high levels of antibody may be effective in reducing the infectivity of sporozoites, presumably by blocking surface receptors, but not sufficient to prevent or eliminate an existing infection. As the majority of endogenous forms are masked by a host-derived parasitophore, presumably only the free forms (sporozoites and merozoites) are exposed to host defenses.

Diagnosis

Cryptosporidiosis causing self-limiting illness is clinically indistinguishable from other forms of diarrhea and therefore diagnostic procedures are required to demonstrate an infection. *C. parvum* infection should be considered in all cases of persistent diarrhea, with or without evidence of immunodeficiency, particularly in malnourished children. Demonstration of oocysts in stools stained with modified acid-fast, remains the simplest, cheapest and the most rapid procedure. The much smaller size of the *C. parvum* oocyst, compared with other enteric coccidia, often makes the diagnosis difficult to an untrained person. The production of monoclonal antibody against various components of the oocyst wall has facilitated the development of immunofluorescence assays. These assays, now available commercially, are useful for screening morphologically damaged, excysted or nonviable oocysts. Recently several laboratories have described polymerase chain reaction (PCR) techniques which make diagnosis rapid, sensitive and specific – and safe, as they can be applied to formalin-fixed stools.

Laboratory cultivation

C. parvum has been propagated successfully in most laboratory animals and to a lesser extent in chick embryos. In cell culture the growth is restricted to a limited number of cycles and decreased yield. Persistent, asymptomatic infection can be induced in immunodeficient mice such as the nude or the severe combined immunodeficient (SCID) mouse, or after prolonged treatment with immunosuppressive drugs.

Such animals have been used to test the efficacy of therapeutic drugs. Drugs can also be tested in diarrhea models such as gnotobiotic piglets and calves, which are more expensive models than rodents.

Treatment

An effective treatment against cryptosporidiosis will undoubtedly be beneficial for humans and calves. It would greatly benefit individuals with AIDS, in whom *C. parvum* infection can be life threatening. So far, however, attempted chemotherapy in animals and humans has been disappointing. Treatment of the infection in AIDS with either hyperimmune bovine colostrum or paromomycin has produced mixed results.

Immunology

While the incidence of the disease in the general human population does not warrant the development of a vaccine, prevention of the disease in AIDS with repeated mucosal immunizations may be beneficial. Prevention of *C. parvum* infection via effective passive transfer of maternal antibody from mother to calf will be economically enormously beneficial to the cattle industry. However, the development of effective vaccines or immunotherapy require knowledge of the immune mechanisms responsible for clearance of infection and prevention of reinfection, including identification of the key parasite antigens responsible for stimulating protective immunity. Our knowledge on the host immune response to *C. parvum* infection is still very limited. The increased severity and duration of illness observed in immunosuppressed animals or immunodeficient individuals indicate that resistance to, and resolution of, *C. parvum* infection requires activation of a parasite-specific mucosal immune response in the host. Studies with adoptive transfer of *C. parvum*-immune histocompatible cells results in complete elimination of infection. Administration of cytolytic anti-CD4⁺ and/or interferon γ (IFN γ)-specific monoclonal antibodies provides evidence that IFN γ may act to limit the level of infection, while CD4⁺ T cells may limit the duration of infection. The significant role of CD4⁺ T cells is further supported by the enhanced susceptibility to *C. parvum* of major histocompatibility complex (MHC) class II-deficient mice which fail to develop CD4⁺ T cells. The role of CD8⁺ T cells, which also produce IFN γ , remains unresolved. While IFN γ and CD4⁺ are both important in establishing resistance to, and resolution of, *C. parvum*

infection, precisely how this occurs is unknown. Recently it was shown that administration of interleukin 12 (IL-12), which activates natural killer cells and cytotoxic T lymphocytes and stimulates IFN γ production, prevented *C. parvum* infection in mice. It provided the first evidence that treatment with exogenous IL-12 can prevent *C. parvum* infection by an IFN γ -dependent, specific immune system-independent mechanism, and that endogenous IL-12 production has a role in limiting *C. parvum* infection.

In summary, much more information is now available on the epidemiology and transmission of the infection, and on the nature of the disease in humans and in other mammals, *C. parvum*, however, remains enigmatic from the point of view of its biological characteristics, and, for a life-threatening disease in individuals with AIDS, the infection remains untreatable. This is despite concerted efforts by scientists to develop an effective immunotherapeutic and/or chemotherapeutic treatment. Important issues that remain unresolved include understanding the underlying mechanisms of attachment of the parasite to cell receptors and of diarrhea, and defining the immune mechanisms required to either resist, or resolve, *C. parvum* infection.

See also: Acquired immune deficiency syndrome (AIDS); CD4; Gastrointestinal tract infections; Immunodeficiency, animal models; Immunosuppression; Interferon γ ; Interleukin 12 and its receptor; Opportunistic infections; Parasites, immunity to; Severe combined immunodeficiency.

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CUTANEOUS ANAPHYLAXIS

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In 1902, Portier and Richet injected foreign protein into animals without incident, but found that when injected a second time a fatal systemic reaction was induced. The phenomenon was called anaphylaxis and may be defined as a specific immunoglobulin E (IgE)-mediated antigen-induced reaction by mast cells and basophils, the type 1 immediate hypersensitivity reaction of Gell and Coombs. Clinically, the term anaphylaxis is used to describe a syndrome resulting from the release of a variety of preformed and newly generated, mast cell-derived chemical mediators (**Table 1**). The trigger may be mediated by specific antibodies such as IgE, or by nonimmunologic mechanisms. The hallmarks of a cutaneous anaphylactic reaction are urticaria and angioedema and such a reaction may remain localized to the skin, or become generalized, causing pruritus, diffuse erythema, urticaria, angioedema, laryngeal edema, bronchoconstriction, hypotension and cardiac arrhythmias. In addition, the skin may either be the source of an anaphylactic reaction, or be a target organ in a systemic reaction. Causes of systemic anaphylaxis in which the skin is the origin of the reaction are listed in **Table 2**.

Table 1 Mediators released from activated mast cells

Preformed mediators
Histamine, heparin, tryptase, kininogenase, carboxypeptidase A
Proteoglycans, eosinophil and neutrophil chemotactic factors
Newly generated mediators
Prostaglandin D ₂ , thromboxane A ₂
Leukotriene B ₄ , C ₄ , D ₄ and E ₄
Platelet-activating factor
Cytokines
Interleukin 3,4,5,6
Granulocyte-macrophage colony-stimulating factor

Table 2 Causes of cutaneous anaphylaxis

Immediate wheal and flare prick test responses
Insect stings or bites
Immunologic contact urticaria
Physical urticaria: cold, aquagenic, solar, cholinergic, delayed pressure

Urticaria

Data on the prevalence of urticaria are few, but it is thought to affect 15–23% of the general population. It is an eruption caused by exudation of fluid into the dermis which presents as itchy, erythematous or edematous swellings known as wheals, surrounded by a bright red flare (**Figure 1**). Clearing of the central area may result in an annular pattern and lesions vary in size from 2–5 mm up to several centimeters in diameter (**Figure 2**). They may occur anywhere on the skin and usually last between 12 and 24 h. In angioedema the edema extends subcutaneously or submucosally and swellings are larger, usually not itchy and may last up to 72 h. It most commonly affects the eyelids, lips and tongue, but any part of the body may be affected. Urticaria and angioedema occur together in nearly 50% of patients and both fade, leaving completely normal skin. There are many causes of urticaria (**Table 3**) but the end-result is a release of mediators that cause increased permeability of capillaries and small venules.

Urticaria is traditionally divided into acute and chronic forms (**Table 4**). Acute urticaria may be idiopathic, IgE mediated, or result from nonimmunologically mediated reactions. The latter may be anaphylactoid reactions, or be due to direct mast cell activation or to altered arachidonic acid or kinin metabolism.

Chronic urticaria is arbitrarily defined as the occurrence of widespread wheals daily, or almost daily, for at least 6 weeks. It is idiopathic in approxi-



Figure 1 Acute urticaria with marked erythema and whealing. (See also color **Plate 7A**.)



Figure 2 Urticaria showing central clearing and an annular pattern. (See also color **Plate 7B**.)

mately 75% of patients, and caused by physical stimuli in a further 15% of patients. In the past, it was suggested that up to 30% of patients with chronic urticaria reacted to food additives such as benzoic acid compounds, azo dyes and penicillins; more recently, in a placebo-controlled oral challenge study, Kobza Black and colleagues found an incidence of no more than 10%. Such reactions may be more common in children and, if the history is suggestive, appropriate challenge testing is justified. Other exacerbating factors include aspirin (in 30%), psychological stress, intercurrent febrile illness and, more rarely, parasitic infestations. Urticarial vasculitis is an important differential diagnosis and, if suspected, a skin biopsy should be performed. A prolonged history of repeated cutaneous or mucosal angioedema should prompt screening for hereditary or acquired C1 esterase deficiency by measuring plasma complement C4 levels.

Nonsedating H_1 antihistamines are the mainstay of treatment in urticaria. A number of drugs are available, namely terfenadine, cetirizine, astemizole, acrivastine and loratidine. Terfenadine and astemizole are extensively metabolized by cytochrome P450 and can produce elongation of the Q-T interval and arrhythmias in susceptible patients. In view of this, recommended doses should not be exceeded and they should never be prescribed together or in conjunction

Table 3 Classification of urticarias according to underlying mechanism

Immunologic
IgE mediated
Foods: fish, shellfish, milk, eggs, nuts, fruit, vegetables, spices
Drugs: penicillin, porcine insulin, dextran
Insect stings: bees, wasps, hornets, yellow jackets
Inhalants: pollens, animal dander
Contactants: latex
Physical: dermatographism, cold, solar, cholinergic
IgG autoantibodies to high-affinity IgE receptor or IgE or both
Immune complex/anaphylatoxin-mediated
Urticarial vasculitis
SLE, Sjögrens syndrome, hepatitis B or C, serum sickness, drugs, IgM gammopathy
Blood products
Radiocontrast media
Haptens
penicillins, cephalosporins, sulphonamides, tetracyclines, macrolides
Non-immunologic
Direct mast cell activators
Opiates, tubocurarine, radiocontrast media, dextran, polymyxin
Arachidonic acid metabolism
Aspirin, non steroidal anti-inflammatory drugs (NSAIDs)
Kinin metabolism
Angiotensin converting enzyme (ACE) inhibitors
Idiopathic
Miscellaneous
Physical: heat, vibratory, delayed pressure, aquagenic
Thyroid autoimmunity

Table 4 Classification of urticarias

Acute
Idiopathic
IgE mediated:
Immediate wheal and flare prick test response
Insect stings, penicillin,
Transfusion of blood or blood products
Fish, shellfish, milk, nuts, vegetables, fruit, spices
Nonimmunologically mediated:
Aspirin, NSAIDs, paracetamol, radiocontrast media, dextran, food dyes, preservatives, morphine, codeine, tubocurarine
Chronic
Idiopathic
IgG autoantibodies to high-affinity IgE receptor or IgE or both
Contact urticaria
Immunologic
Nonimmunologic
Physical urticaria
Dermographism, delayed pressure urticaria, cholinergic urticaria, cold urticaria, aquagenic urticaria, solar urticaria
Urticarial vasculitis

with drugs such as cyclosporine, erythromycin or ketoconazole. With astemizole adequate contraception is essential during treatment and for up to 4 weeks afterwards. Older, more sedating H₁ antihistamines, such as chlorpheniramine or hydroxyzine, can be used concurrently and may be useful to alleviate pruritus at night. If one particular compound is not effective, others should be tried sequentially as there is a wide variation in response between patients. There is also a variability of response to different antihistamines over time, largely as a result of tachyphylaxis, and breaks from or changes in treatment may be beneficial. The role of H₂ antihistamines is controversial. There is some evidence that in combination with H₁ antihistamines they are of benefit in some patients with symptomatic dermatographism and chronic idiopathic urticaria. Ranitidine is the drug of choice as it does not inhibit cytochrome P450 or seem to have the pronounced antiandrogenic effects of cimetidine with long-term use.

Immediate wheal and flare prick test responses

Prick testing involves the application of commercially prepared diluted antigens as single drops on marked areas of skin on the forearm. The skin is gently pricked through the drop and the drop is then removed with a tissue wipe. The wheal starts within 10 min, reaches a peak within 20–30 min and resolves in 1–2 h. It depends upon the reaction and cross-linking of antigens with specific IgE antibody bound through its Fc to mast cells and basophils. Rarely, prick tests may precipitate a systemic anaphylactic reaction.

Insect bites and stings

Allergic reactions to insect stings may occur as extensive localized swelling (>10 cm in diameter) lasting at least 24 h, immediate IgE-mediated systemic reactions (urticaria, angioedema, bronchospasm and anaphylactic shock) or more rarely as delayed systemic reactions (vasculitis, polyradiculitis, glomerulonephritis). The prevalence of such reactions varies from 3.1–17% for extensive localized to 0.15–3.3% for systemic reactions. The diagnosis is suggested by the history and the Hymenoptera most often implicated include Apidae (the honeybee and bumblebee), Vespidae (wasps, hornets and yellow jackets) and Myrmicidae (stinging ants). The major allergens in venom are different forms of phospholipase A. In addition, bee venom contains enzymes (acid phosphatase and hyaluronidase), mediators (dopamine and norepinephrine) and peptide 401, which causes mast cell degranulation. Similar toxins are present in wasp venoms. The most sensitive techniques for determining the presence of

venom-specific IgE antibody (i.e. sensitization) are the radioallergosorbent test (RAST) and skin testing with venoms. Such tests are positive in 15–25% of the general population, presumably reflecting normal IgE responses to insect stings in nonallergic subjects. A positive RAST does not discriminate between those patients who develop a local rather than a systemic anaphylactic reaction, and up to 2% of patients with anaphylactic reactions may have negative tests. A majority of patients with large local reactions will continue to have localized reactions regardless of their IgE status, although 5–10% of adults and 2% of children may have systemic reactions following a subsequent sting. The risk of systemic reactions on re-sting in patients with a previous systemic reaction is 29% and is usually of similar severity to the previous systemic reaction in adults and similar or less severe in children. Systemic reactions are more likely to be severe following bee stings (which contain a larger dose of venom than wasp stings), or if re-sting occurs >2–3 weeks and <10 years after a previous sting. Immunization with venom is a safe, extremely effective treatment for preventing future systemic reactions and the aim of such immunization is to increase venom-specific IgG levels and to produce negative skin tests and RAST. Such treatment is mandatory in patients with a history of severe systemic reactions and can be undertaken for milder systemic reactions in adults and children if appropriate. It is not recommended for large local reactions which respond to treatment with nonsedating H₁ antihistamines. In the event of a severe systemic reaction, treatment should be with epinephrine and antihistamines, as for systemic anaphylaxis (see below).

Contact urticaria

This is a localized wheal and flare response, usually occurring within minutes and provoked by the application of a wide range of rapidly absorbable agents to the skin, lips and buccal mucosa. Contact urticaria may occur through immunologic or nonimmunologic mechanisms.

Immunologic contact urticaria Immunologic contact urticaria is distinguishable by its ability to involve other organ systems and its increased prevalence in atopic individuals. It is due to a type I IgE-mediated hypersensitivity and so patients have positive RAST and skin prick tests to the contactant allergen, most of which are proteins or protein complexes. As systemic reactions can occur with skin prick testing in such patients, other tests are often undertaken: casual handling of the suspected provoking agent in exactly the same way as when the symptoms appear and open or occlusive patch testing

(in which the substance is applied to uninvolved skin for 15 minutes either with or without occlusion).

Foods Foodstuffs are common causes, and this type of reaction is common in food handlers who may present with a true contact urticaria or recalcitrant hand eczema (with immediate exacerbations when affected skin is exposed to the responsible food proteins). Common allergens include wheat flour, chicken, turkey, lamb, fish, shellfish, garlic, onions, chives, cucumber, horseradish, leek, parsley, tomato, lettuce, endive, apple and potato.

Some patients experience itching, tingling and/or edema of the lips and tongue and hoarseness, occasionally progressing to systemic symptoms following contact of fruit and vegetables, particularly apple, carrot, parsnip and potato, with the orolaryngeal area. This is particularly common in atopics with hypersensitivity to birch pollen and, in such cases, is thought to be due to cross-reactivity of polypeptide fruit and vegetable allergens with birch allergens.

Latex Although the predominant immunologic response (in 82% of cases) to natural rubber latex is a type IV delayed hypersensitivity to rubber additives which presents as contact dermatitis, IgE-mediated immunologic contact urticaria and anaphylactic reactions are well recognized. IgE antibody to residual rubber tree proteins in latex medical devices are present in 7–10% of nonatopic health care workers. Sensitization usually occurs as a result of regular latex exposure from wearing gloves or inhaling aerosolized latex in the workplace. In children with spina bifida the prevalence ranges from 28 to 67%, presumably as a result of mucosal absorption of latex allergens during multiple surgical procedures early in life. Exposure to latex gloves during surgery, gynecologic examination or dental procedures, and in men use of latex condoms, have also been implicated. Re-exposure of such individuals may lead to contact urticaria and/or systemic reactions such as allergic rhinoconjunctivitis, asthma or anaphylactic shock. Diagnosis is made on positive RAST or skin prick testing, however the latter appears to have an increased risk of anaphylactic reactions and should be performed with very dilute (1:1 million) doses of commercially available extracts. Treatment is by avoidance of latex-containing materials and, in particular, the complete removal of powdered latex gloves from the environment; this dramatically reduces the amount of aerosolized latex particles.

Many other agents are capable of producing immunologically mediated contact urticaria, including drugs; (particularly antibiotics, e.g. penicillin,

cephalosporins, neomycin, chloramphenicol), metals (e.g. nickel), animal hair and dander, textiles and cosmetics.

Nonimmunologic contact urticaria This type of contact urticaria is much more common than the immunologic variety. Its mechanisms are less well understood, although direct effects on vascular endothelium or non-antibody-mediated release of prostaglandins, leukotrienes and other inflammatory mediators have been suggested. Prior sensitization is not required. Common precipitants include salicylates, parabens, sulfites, benzoic acid, sorbic acid, cinnamic acid, cinnamic aldehyde and nicotinic acid esters present in foods, such as ice cream and chewing gum, shampoos, perfumes, mouthwashes, creams and ointments. Plants such as nettles, primula and seaweed, as well as moths, caterpillars, sea anemones, corals and jellyfish may also be responsible. Symptoms remain localized and include burning, stinging, itching, edema and erythema. Skin prick testing results often do not correlate with clinical findings and should be considered in conjunction with the history. Oral challenge testing is not performed routinely, but can be done on a placebo-controlled basis if the history is suggestive. It should not be undertaken in patients with a history of severe angioedema, asthma or systemic anaphylaxis. Sixteen different food additives, alternating with placebo, are administered on a daily basis. The patient keeps a daily diary of wheal counts and continues the usual dose of antihistamine if this has already been prescribed. If an exacerbation of urticaria occurs within 24 h of the ingestion of a numbered capsule, the potential culprit is administered once more to confirm the findings.

Chronic idiopathic urticaria

Up to 75% of patients seen with urticaria in dermatology clinics have chronic idiopathic urticaria, lasting more than 6 weeks. Clinically it resembles acute urticaria, usually occurs in adults and is twice as common in women. It frequently follows a relapsing-remitting course and up to 40% of patients who have chronic urticaria for over 6 months still have lesions 10 years later. It frequently occurs in association with physical urticarias, particularly symptomatic dermographism and delayed pressure urticaria. Appropriate challenge testing should be performed in such patients to assess the predominant cause of their symptoms. Autoimmune thyroid disease occurs in 14% of patients and recent studies have suggested an autoimmune basis for chronic idiopathic urticaria. In up to 60% of patients an endogenous histamine-releasing factor has been identified. IgG auto-

antibodies causing mast cell degranulation have been found in nearly half of these patients: anti-IgE, anti-FcεRIα (directed against the α subunit of the high-affinity IgE receptor expressed by basophils and mast cells) and a further antibody thought to interact with FcεRI but not FcεRIα. These findings have therapeutic implications and in patients with autoantibodies and severe disease unresponsive to conventional therapy, plasmapheresis, intravenous immunoglobulin and cyclosporine have induced remissions. Nonsedating H₁ antihistamines remain the first-line treatment. Sedating antihistamines may be added if symptoms are troublesome at night. Doxepin (10 mg three times daily or 25 mg at night), which, in addition to its tricyclic antidepressant activity, is a potent H₁ antagonist, a weak H₂ antagonist and a weak muscarinic agonist, is useful in patients with associated anxiety or depression. Doxepin should not be given with monoamine oxidase inhibitors or terfenadine. The addition of H₂ antihistamines to H₁ antihistamines can produce additional benefit in some patients. Patients should avoid aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs) and angiotensin-converting enzyme inhibitors, which may all aggravate symptoms. A salicylate-free diet may be of benefit and a 4 week trial of a strict exclusion diet can be performed to assess this.

Urticarial vasculitis

This disorder presents clinically as urticaria but histopathologically the features are those of a cutaneous leukocytoclastic vasculitis of variable degree. It may be impossible, clinically, to differentiate ordinary urticaria from urticarial vasculitis but important pointers include a longer duration of lesions (up to 3–7 days), a burning or painful sensation at the site of lesions and the fact that lesions may resolve with residual purpura, bruising or scaling. Frankly vasculitic or bullous lesions may rarely occur. In addition, there may be evidence of a systemic vasculitis with fever, arthralgia and more rarely arthritis, glomerulonephritis, obstructive pulmonary disease or gastrointestinal, ophthalmic or central nervous system involvement. Many patients are hypocomplementemic and in some an IgG autoantibody to C1q has been demonstrated. In others, the pathogenesis is that of an immune-complex vasculitis resembling a type III hypersensitivity. The antigen involved is unknown in most cases, although patients with serum sickness, the collagen-vascular diseases systemic lupus erythematosus and Sjögrens syndrome, and infections such as hepatitis B and infectious mononucleosis may present with this clinical picture. Treatment with H₁ antihistamines alone or in combination with H₂ antihistamines is only

rarely of benefit. Additional therapy with dapsone, hydroxychloroquine, sulfasalazine, NSAIDs, systemic corticosteroids or azathioprine may be required.

Physical urticaria

In the physical urticarias, specific physical stimuli trigger mast cell degranulation, with the release of mediators. In some cases there is evidence of a passively transferable reactivity which is thought to involve IgE, or more rarely, IgM or IgA, recognizing a molecule released or conformationally altered by the physical stimulus. Often more than one type coexists in the same patient, suggesting abnormal tolerance of self antigens.

Dermographism Simple dermographism is a transient erythematous and whealing response which develops in 5% of the population after application of only a moderate pressure to the skin. It appears to represent a combined effect of pressure and shearing forces on the skin and is therefore an exaggerated triple response of Lewis. In symptomatic dermographism, the threshold to induce dermographism is lowered and there is marked pruritus (**Figure 3**). Lesions may be precipitated by the pressure caused by the elastic in underwear or from shower jets. Symptomatic dermographism is more common in young adults (mean age of onset 26 years) and is not associated with atopy. The diagnosis can be confirmed using a calibrated dermographometer. It may last for months or indefinitely. Treatment with the nonsedative H₁ antihistamines usually provides good symptomatic relief, cetirizine and astemizole being the most effective. Addition of an H₂ antihistamine may be of benefit in this group.

Delayed pressure urticaria This type of urticaria often occurs in association with chronic idiopathic



Figure 3 Symptomatic dermographism, marked linear whealing has occurred at sites of scratching. (See also color Plate 7C.)

urticaria. There is erythema, dermal and subcutaneous edema developing 4–6 hours after application of sustained pressure perpendicular to the skin. Lesions occur after activities such as climbing ladders, walking or sitting on a hard surface, carrying heavy items, wearing tight-fitting clothes, using a screwdriver or hammer or after kissing or sexual intercourse. Maximum swelling occurs after 10 h and the lesions may last up to 48 h. Patients often complain of itching and a painful or burning sensation in the lesions; generalized symptoms of malaise, fever, headache or generalized arthralgia occur in about 70% of patients. The diagnosis is confirmed using a calibrated dermatographometer. Cetirizine (10 mg daily) is now the treatment of choice. It is the only H_1 antihistamine of any proven value, presumably because it also inhibits eosinophil and neutrophil chemotaxis. Stanazolol (2.5 mg daily) and dapsone (50–100 mg daily) are of benefit in some patients, although their mechanisms of action are unclear.

Cholinergic urticaria This urticarial eruption occurs in situations where there is activation of cholinergic sympathetic autonomic nerve fibers supplying eccrine sweat glands, after exercising or hot baths, or during periods of mental stress or anxiety. The itchy lesions are 2–5 mm diameter monomorphic wheals surrounded by a bright red flare which lasts no more than 20 min to 1 h. In some cases angioedema, flushing, faintness, bronchospasm or hypotension may occur. Cholinergic urticaria may be induced by intradermal injections of acetylcholine and can be abolished by local anesthetics and topical applications of atropine. The exact pathogenesis is unknown. The diagnosis is confirmed by reproduction of the eruption by exercising sufficiently to cause sweating or by immersion in bath water at 42°C for 10–15 min. Treatment with nonsedating H_1 antihistamines, either continuously or before an activity known to precipitate urticaria, is recommended. Danazol has also been reported to be effective. It raises protease inhibitor levels and clinical improvement can persist for several months after cessation of treatment. The condition may last for months or years and then spontaneously resolve.

Cold urticaria This urticaria develops after exposure of the skin to cold and may be idiopathic or secondary to serologic abnormalities. The majority of patients with the idiopathic form (over 90%) have the primary acquired form of cold urticaria.

A. Idiopathic cold urticaria

Primary acquired cold urticaria This occurs 5–30 min after exposure to cold and may be precipitated by wind, cold bathing, handling cold objects, immersion of the hands in cold water, and drinking cold foods or drinks. The urticaria may develop during the period of exposure but more often develops during rewarming. It lasts for about 30 min and resolves, to leave normal appearing skin. In up to 50% of patients systemic symptoms may occur, characterized by syncope, headache, palpitations and wheezing. Loss of consciousness and drowning may occur during swimming. The diagnosis can be confirmed by the induction of lesions by the application of a polythene-wrapped ice cube to the skin for 5–10 min with a 5 min period allowed for rewarming.

Rarer forms of primary acquired cold urticaria:

1) delayed cold urticaria – lesions occur several hours after exposure; 2) localized cold urticaria – lesions occur at sites of injections and bites; 3) reflex cold urticaria – widespread whealing occurs in response to a fall in core temperature.

Familial cold urticaria This is rare and is inherited as an autosomal dominant condition. It starts at an early age and is a lifelong problem. Urticaria develops 30 min after patients are exposed to generalized cooling rather than local cold application, and lesions may persist for up to 48 h.

B. Secondary cold urticaria This type accounts for about 5% of all patients with cold urticaria and may occur in association with cryoglobulinemia, cryofibrinogenemia and cold hemolysins. Other triggering factors such as viral infection, syphilis, infestations or allergies to drugs, such as penicillin, oral contraceptives and griseofulvin, can sometimes be identified.

The pathophysiology of cold urticaria is unclear but between 10 and 50% of patients with primary acquired cold urticaria demonstrate the phenomenon of passive transfer by a serum factor which may be either IgE or IgM. The mainstay of treatment is the avoidance of precipitating factors, but nonsedating H_1 antihistamines may be required. Ketotifen (2–4 mg daily), which is both an H_1 antihistamine and a mast cell stabilizer, and doxepin (10–25 mg three times daily) are both effective. Finally, induction of cold tolerance by repeated cold exposure may be attempted but this usually involves taking one or two cold showers or baths a day and requires that patients be strongly motivated.

Aquagenic urticaria Contact with water, irrespective of its temperature, may induce an urticarial eruption which looks very similar to cholinergic urticaria. The lesions are frequently confined to the neck, arms and upper trunk, develop within 5–30 min of exposure to water and resolve over the next hour. The condition is partly responsive to antihistamine therapy with terfenadine, cetirizine or loratidine (10 mg daily) and barrier preparations may be used for aquatic sports.

Solar urticaria This is an uncommon condition in which urticaria develops within 2–3 min of sun exposure and then fades within an hour or so. It occurs particularly in areas of exposed skin which are usually kept covered. All parts of the ultraviolet light spectrum may induce solar urticaria. Passive and reverse passive transfer tests have been demonstrated in such patients. A precursor is converted to an antigenic photoproduct which triggers an IgE-mediated response and the diagnosis is confirmed by monochromator or solar stimulator testing. Treatment is often disappointing as only in the mildest cases are sunscreens beneficial, and, with the exception of astemizole (a highly specific H_1 antagonist) antihistamine therapy is usually ineffective. Tolerance may be induced by the appropriate ultraviolet source and, as the condition is due to a circulating photoallergen, plasmapheresis can be used in severely affected patients during the desensitization phase. In combination, PUVA (psoralens and ultraviolet B) and plasmapheresis produce a more prolonged improvement than if used alone.

Hereditary angioedema

This is a rare disorder, inherited as an autosomal dominant trait in which there is a deficiency in the activity of C1 esterase inhibitor, resulting in continued complement activation. There is usually a family history, although not invariably, and it usually presents in early childhood but may be delayed until late adult life. Patients present with nonitchy and sometimes painful swellings on the limbs or face, attacks of abdominal pain, vomiting and diarrhoea (which may be blood stained). The most serious manifestations are edema of the larynx, pharynx, trachea and bronchi, leading to respiratory obstruction and asphyxia in up to 30% of patients. Hypotension and urticaria are usually absent, helping to distinguish such attacks from acute, generalized anaphylaxis. Attacks may be precipitated by trauma, stress, infections or cold and usually last between 48 and 72 h, although lesions may not resolve for days or even weeks.

The diagnosis can be confirmed by the finding of low C4 and C2 levels in all patients. In type I hereditary angioedema, which accounts for 85% of patients, low functional and antigenic levels (5–31% of normal) of the C1 esterase inhibitor occur, due to decreased synthesis and increased metabolism. In the remaining 15% of patients, as a result of point mutations in the reactive center, the functional level is low but the antigenic level is within normal limits.

The treatment of choice in an acute attack is with intravenous purified C1 esterase inhibitor (24 000–36 000 units). If this is unavailable, fresh frozen plasma may be used, although, because this contains more C4 and C2, patients may initially deteriorate before they improve. In the presence of laryngeal edema, epinephrine, oxygen, H_1 antagonists and corticosteroids are also recommended (see treatment of anaphylaxis below). For prevention of recurrent attacks danazol (100–400 mg daily for 1 month, then 5 days on, 5 days off) or stanazolol (2.5–10 mg daily to control attacks then 2.5 mg three times a week), which increase hepatic synthesis of C1 esterase inhibitor, tranexamic acid (1–3 g daily), which carries a risk of thromboembolism, or epsilon aminocaproic acid may be prescribed. Patients should avoid taking angiotensin-converting enzyme inhibitors which alter kinin metabolism.

Acquired form of hereditary angioedema

In the nonhereditary form of angioedema, deficiency of the C1 esterase inhibitor occurs either as an autoimmune process in systemic lupus erythematosus or in association with hematological malignancies such as lymphoma or myeloma. In the autoimmune variety, anti-C1 esterase inhibitor antibodies predispose it to cleavage. In lymphoproliferative disease there is a 2–3-fold increase in C1 esterase inhibitor metabolism, possibly as a result of continuous activation of C1 by anti-idiotypic antibodies directed against surface immunoglobulin on the malignant B cells, leading to C1 esterase inhibitor-complex formation and removal. Characteristically, in the acquired disease C1 levels are very low, whereas in the hereditary form C1 levels are normal. Treatment is aimed at the underlying cause.

Treatment of anaphylaxis

The first-line treatment of systemic reactions, which may include bronchospasm, hypotension and angioedema, is an intramuscular injection of epinephrine in a dose of $10 \mu\text{g kg}^{-1}$ up to 1 mg (1 ml of 1:1000 solution), which may need to be repeated every 10 min until improvement occurs. Epinephrine acts as a positive inotrope, blocks the end-organ effects of

histamine, and inhibits release of histamine and leukotrienes from mast cells. Administration of high-dose oxygen is also of primary importance. Additional second-line treatment consists of H_1 antagonists, initially intravenously (chlorpheniramine 10–20 mg) and then orally (e.g. cetirizine 10 mg once daily or terfenadine 60 mg twice daily) for 24–48 h to prevent relapse, and corticosteroids (intravenous hydrocortisone 100–300 mg or 5 mg kg^{-1}), which may help suppress the late-phase and persistent responses. Continuing deterioration may necessitate treatment with bronchodilators, either nebulized or intravenous β_2 agonists (nebulized salbutamol 2.5–5 mg four times daily, intravenous salbutamol 3–20 $\mu g \text{ min}^{-1}$) or intravenous aminophylline infused slowly over 10–15 min (up to a total dose of 500 mg or 4 mg kg^{-1}). Occasionally, slow intravenous infusion of low dose adrenaline (5 ml of 1:10 000 adrenaline at a rate of 1 ml = 100 $\mu g \text{ min}^{-1}$ until improvement occurs) is required. Fluid replacement with colloids such as hemacel, monitoring of central venous pressure (CVP) and/or pulmonary capillary wedge pressure (PCWP) and in the presence of a raised CVP or PCWP, inotropic support with dopamine (5 $\mu g \text{ kg}^{-1} \text{ min}^{-1}$) and occasionally norepinephrine may be necessary. The use of H_2 antagonists is controversial, but it has been suggested that in combination with H_1 antagonists these agents suppress late-phase cutaneous responses.

For patients with previous or recurrent anaphylactic reactions, for example to bee stings, emergency self-treatment kits are available (e.g. Epipen which contains 300 μg epinephrine in a fully assembled syringe) and patients should be taught to give their own injections. Epinephrine inhalations (e.g. Medi-haler-Epi which contains 280 μg per metered inhalation – adults should take a minimum of 20 puffs) are much less effective but may be useful in patients without other symptoms who are beginning to develop bronchospasm or edema in the orolaryngeal area.

See also: Allergens; Anaphylatoxins; Arachidonic

acid and the leukotrienes; Atopic allergy; Autoantigens; Autoimmune disease, pathogenesis; Autoimmune diseases; Basophils; Blood transfusion reactions; Complement deficiencies; Contact hypersensitivity; Drugs, allergy to; Eczema; Eosinophil chemotactic factors; Exercise and the immune response; Food allergy; Granulocyte-macrophage colony stimulating factor (GM-CSF); Histamine; Hypersensitivity reactions; IgE; Interleukin 3; Interleukin 4; Interleukin 5 and its receptor; Interleukin 6; Kallikrein-Kinin system; Mast cells; Platelet-activating factor (PAF); Prostaglandins; Serum sickness; Venoms.

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CYCLOSPORINE

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Ciclosporin (WHO)/cyclosporine (US Adopted Name Council)/cyclosporin A (British Approved Name) (CsA) is a cyclic undecapeptide containing a novel amino acid together with several *N*-methylated amino acids, which is produced as a major secondary metabolite of the fungus *Tolypocladium inflatum* Gams. This strain of fungi imperfecti was isolated from a soil sample collected in the Hardanger Vidda (Norway). Extracts of cultures exhibit a narrow spectrum of antifungal activities *in vitro*, but exert only limited effects *in vivo*. The striking selective immunosuppressive activity discovered in 1972, in an additional pharmacological screening, led to an extensive investigation of this fungal metabolite. CsA is the first immunosuppressive drug to selectively and reversibly act only on a limited population of lymphocytes and to be devoid of myelotoxicity. This explains its immediate impact in the field of transplantation. Today, however, its potential in the treatment of autoimmune diseases is steadily growing. CsA is also widely used as an experimental probe in basic research.

Pharmacology of CsA

The structure of CsA (Figure 1) was established by chemical degradation and X-ray analysis of an iodo-

derivative. Having a molecular weight of 1202, $C_{62}H_{111}N_{11}O_{12}$, CsA is a neutral, hydrophobic, cyclic peptide with 11 amino acid residues, all having the *L*-configuration of the natural amino acids, except for the *D*-alanine in position 8 and the achiral sarcosine in position 3. One amino acid had never been seen before. Located in position 1, it is abbreviated as MeBmt, for (4*R*)-4-[(*E*)-2-butenyl]-4, *N*-dimethyl-*L*-threonine.

The molecular mechanism of action of CsA in the antigen-driven T cell activation process is shown in Figure 2. In contrast to previous immunosuppressive drugs (mostly cytostatic drugs), CsA exerts a specific action on lymphocytes, but it does not influence the functions of phagocytes or hematopoietic stem cells. It is neither lymphocytotoxic nor mutagenic, and its action is reversible. Besides being accepted today as the first-line immunosuppressive drug for clinical use, CsA is also widely used as an experimental probe in basic research. Especially for immunologists, it has become an important pharmacologic tool for defining *in vitro* the respective roles of cell interactions and mediators in lymphocyte activation, analyzing the cell regulatory mechanism of the genes, and studying the different steps involved generally in an immune response. Though *in vitro* several pathways can lead to the induction of gene transcription in lymphocytes, only pathways involving mobilization of intracellular calcium are CsA sensitive. There is compelling evidence that CsA acts by preventing the activation of resting lymphocytes at an early stage of the cell cycle (G_0 to G_1 transition), thus inhibiting primarily the production and release of interleukin 2 and several other lymphokines by helper T cells.

CsA has been widely used *in vivo* to evaluate the contribution of various T cells not only in normal but also in pathological conditions. Since the drug can induce and maintain a remission in many models of autoimmunity as well as in clinical autoimmune diseases, it is evident that it also inhibits fully activated T cells, possibly by suppressing their continuous *de novo* restimulation. The *in vivo* mechanism of action is still being debated. However, most observations resulting from many different types of *in vivo* models suggest that CsA acts by suppressing lymphocyte activation within the allograft in transplantation or at the site of (chronic) inflammation in autoimmunity. Although necessary and often helpful, *ex*

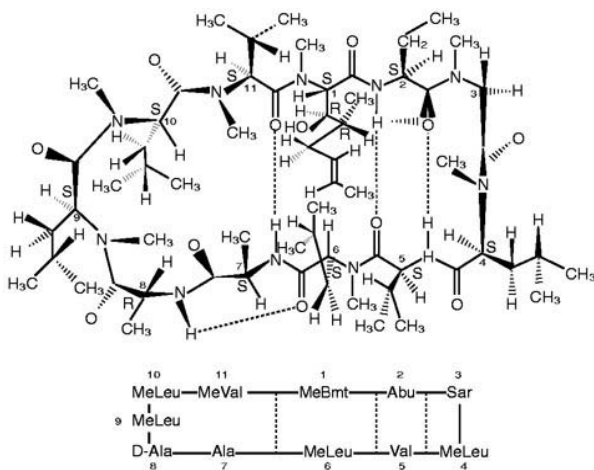


Figure 1 Structure of CsA corresponding to the conformation observed in the crystal. (Reproduced with permission from Borel JF (ed) (1986) *Ciclosporin*. *Progress in Allergy* 38: 48. Published by Karger, Basel.)

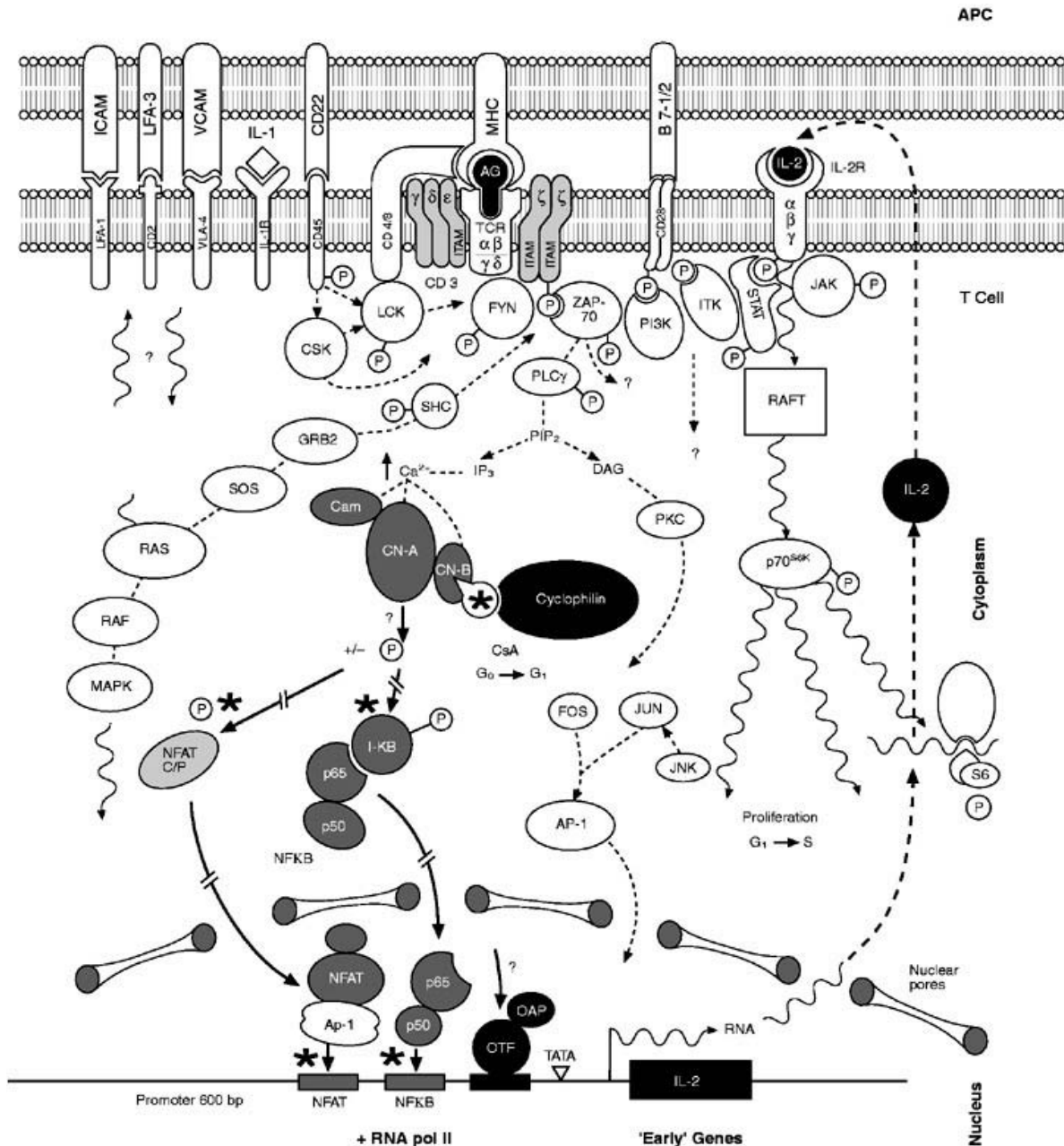


Figure 2 General overview of T cell signaling pathways following ligation of the T cell receptor (TCR) or the IL-2 receptor (IL-2R). Mode of action of CsA. In the presence of costimulatory signals provided by multiple cell surface receptor–ligand interactions (e.g. CD28/B7-1, 2; LFA-1/ICAM; CD2/LFA-3; CD45/CD22; IL-1R/IL-1) antigen (AG) presented by an antigen-presenting cell (APC) in the context of major histocompatibility complex (MHC) proteins induces a T cell differentiation process that results ultimately in lymphokine secretion (G_0 to G_1 transition of cell cycle) and proliferation (G_1 to S) of the antigen-specific T cell. Signal transduction involves a number of biochemical events, including phosphorylation and dephosphorylation (P) of tyrosine, threonine or serine residues of intracellular signaling proteins, leading to gene transcription of 'early' genes like IL-2. CsA exerts its immunosuppressive effects downstream from the very early membrane-associated events, such as activation of phospholipase C_γ (PLC γ) and recruitment of tyrosine kinases (e.g. LCK, FYN, CSK, ZAP-70) and SHC to the 'immunoreceptor tyrosine-based activation motifs' (ITAM) in the ϵ and ζ chains of the T cell receptor (TCR)/CD3 complex. It is hypothesized that cyclophilin-bound CsA inhibits the calcium-dependent (Ca^{2+}) phosphatase activity of calcineurin (CN-A, -B and Cam, calmodulin) as a crucial step in the activation (*) and nuclear translocation of cytoplasmic transcription factor subunits like NFAT $_{c/p}$ and/or NF κ B required for 'early' gene transcription. IL-2R-mediated signaling processes involving activation of JAK kinases, STATs (signal transducers and activators of transcription), RAFTs (rapamycin and FKBP target) and p70 S6 kinase (p70 S6K) are not affected.

vivo results may be misleading, because the experimental reactions are occurring outside the organism in which CsA is present in biologically effective levels.

CsA in transplantation

CsA was first registered in 1983 (trade name Sandimmune®) for use in organ transplantation. Recently, a new microemulsion formulation of CsA (trade name Sandimmun Neoral® or Neoral®) which shows more consistent oral absorption, is bile independent, and has dose linearity, has become available. This has the advantage that its absorption is not affected by food intake and it shows reduced variability between as well as within patients. Neoral® has been shown to be safe and well tolerated.

CsA is used for the prevention of acute rejection of kidney, liver, heart, heart-lung, lung and pancreas allografts. It may also be used to treat rejection episodes in patients who have already received other immunosuppressants. In bone marrow transplantation, CsA is used to prevent rejection of the transplanted bone marrow as well as for prevention and treatment of graft-versus-host disease (GVHD). In transplantation CsA has long become established in all drug regimens and is today the drug of first choice in immunosuppression. However, it is seldom used alone but generally in combination with other immunosuppressive agents. These drug combinations have the advantage of exploiting additive and synergistic drug effects while concomitantly minimizing the adverse reactions.

From the very beginning the steroid-sparing potential of CsA has been a main consideration in all clinical situations. In transplantation a state of true tolerance has not been achieved (with the exception of bone marrow transplants) and, therefore, as with other immunosuppressants, lifelong therapy is necessary. Although acute rejection can now be effectively controlled, there remains, in the long-term, the problem of chronic rejection which is considered as one of the leading causes of late allograft failure.

The kidney is the most frequently transplanted organ. Before the CsA era the overall one-year graft survival rate was about 60%, depending on the center. Since the introduction of CsA it has now risen to 80–90%. In kidney recipients followed for up to 10 years renal function tended to worsen during the first year, but remained stable thereafter with no progressive renal damage. The use of CsA in liver transplantation had the effect of revitalizing the field and

soon this procedure was accepted as a treatment option for end-stage liver disease and no longer considered experimental. The 5-year survival of patients, which was about 20% before the use of CsA, increased to 60%. With CsA the number of heart transplantations increased rapidly and currently a 5-year survival rate of approximately 70% is obtained. Heart-lung and lung transplantation was never successful without CsA. The one-year survival of the former has been about 60–65% with CsA. Transplantation of the pancreas simultaneously with a kidney offers a successful treatment for patients with type I diabetes mellitus and end-stage renal failure. A one-year graft survival of about 80% can be achieved at the best centers. In bone marrow transplantation the use of CsA has resulted in a reduction in the severity of graft-versus-host disease. Its use is also associated with prompt and sustained engraftment, quicker hematological recovery and a shorter hospital stay.

CsA in autoimmunity

Since 1987 CsA has also been registered for the treatment of several autoimmune disorders. The use of CsA in this field was based on a solid pharmacological rationale and was also a logical consequence of its superior efficacy in organ transplantation. By their very nature the autoimmune diseases demand a different evaluation of the risk-benefit ratio inherent in all forms of therapy. Indications subjected to investigation were selected on the basis of the experimental results in animal models of autoimmunity and on the medical need. The efficacy of CsA has been proven in several such diseases with clinical benefit being demonstrated in some and remaining uncertain in others as shown in Table 1.

CsA is efficacious in many autoimmune diseases, both for induction and maintenance of remission. Common features of the clinical effects of CsA are relatively quick onset of effect and also the occurrence of relapses when treatment is stopped. The autoimmune reaction seems temporarily 'frozen', without correction of the intrinsic autoimmune defect. Nevertheless, it is evident that many patients who do not respond satisfactorily to conventional therapy, in terms of efficacy or toxicity, may profit from CsA. However, in therapy-resistant end-stage patients who present with substantial pathology which is irreversible, the full potential cannot be realized. The use of the drug may thus eventually be extended to less advanced disease stages.

Table 1 Effect of CsA on autoimmune diseases in humans*Efficacy proven by controlled studies*

Established benefit in severely affected patients resistant to conventional therapy

Uveitis (autoimmune, Behçet disease)
 Psoriasis
 Idiopathic nephrotic syndrome
 Rheumatoid arthritis

Unclear benefit

Severe aplastic anemia
 Crohn disease
 Atopic dermatitis
 Asthma
 Primary biliary cirrhosis
 Myasthenia gravis
 Insulin-dependent diabetes mellitus

Efficacy suggested by uncontrolled studies

Systemic lupus erythematosus
 Dermatomyositis/polymyositis
 Ulcerative colitis
 Pyoderma gangrenosum
 Membranous nephropathy and other autoimmune glomerulonephritides

Efficacy questionable

Multiple sclerosis
 Endocrine ophthalmopathy
 Sjögren syndrome
 Sarcoidosis
 Pemphigus
 Polychondritis

Adverse events

One of the most important issues is safety on long-term treatment. The adverse events recorded are similar in all indications. Apart from the general risks of immunosuppression (opportunistic infection, malignancy) the most important side-effects of CsA are nephrotoxicity and hypertension. Others include anorexia, nausea, vomiting, paresthesia, hypertrichosis, gingival hyperplasia and tremor.

Although infections do occur with CsA, they appear to be more amenable to treatment. The occurrence of lymphomas or lymphoproliferative disorders is now recognized to be the result of over-immunosuppression. Experience gained so far indicates that the incidence of cancers following the use of CsA is no higher than with other types of immunosuppressive therapy. Today the nephrotoxicity of CsA is considered to be manageable. This is achieved, in transplantation, by dosage adjustments based on the monitoring of CsA blood concentrations. Added to that is the increased understanding of the nephrological findings in kidney biopsies

from patients exposed to the drug. In autoimmune diseases CsA is kept at the lowest effective dose, i.e. exceeding $5 \text{ mg kg}^{-1} \text{ day}^{-1}$, and this is decreased whenever serum creatinine increases by more than 30% over the individual baseline value. In addition, regular monitoring of blood pressure is recommended. Hypertension, if it occurs, is responsive to the standard therapy. One must also be aware of pharmacokinetic interactions which may result in either an increase (e.g. ketoconazole) or decrease (phenytoin) of the CsA blood concentration.

See also: Anti-inflammatory (nonsteroidal) drugs; Antilymphocyte serum; Autoimmune diseases; Cytokines; Effector lymphocytes; Glucocorticoids; Graft rejection; Graft-versus-host reaction; Immunosuppression; Lymphoma; Monoclonal antibodies (mAbs); Opportunistic infections; T lymphocytes; Tolerance, peripheral; Transplantation.

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CYSTICERCOSIS

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Characteristics of the organisms and their antigens

The term 'cysticercosis' is a general one applied to the occurrence of cystic (larval) stages of cestodes (tapeworm parasites) in humans and domestic and laboratory animals. Cysticercosis due to the cysticercus (known by veterinarians as *Cysticercus cellulosae*) of the pig tapeworm, *Taenia solium*, is the most common simple cystic form in humans. The life cycle of *T. solium* is depicted in Figure 1. Pigs act as the intermediate host and the adult worms occur in the human gut. In pigs the cysticercus may lodge anywhere in the body but shows predilection for muscle. When undercooked or raw pork infected with cysticerci (measly pork) is eaten by humans, the scolex (activated by bile) evaginates and becomes attached to the duodenum, the bladder of the cysticercus is digested and the worm differentiates into an adult. The fully developed adult (up to several meters long) sheds single segments or proglottids (proglottis = single segment) each containing 50 000 eggs and these are voided in the feces. On ingestion by a pig, the egg hatches in the gut to release the oncosphere which bores its way through the mucosa and into the general circulation, finally localizing and developing in body muscle.

Neurocysticercosis

The eggs of *T. solium*, when ingested by humans, can also develop into cysticerci. Their presence causes a serious and often fatal disease due to the frequent involvement of the nervous system (neurocysticercosis), resulting in 'cysticercus epilepsy'. *T. solium* occurs in most pork-eating countries where sanitary conditions are primitive and scavenging pigs have access to human fecal material. Neurocysticercosis is now the most prevalent disease of the central nervous system in the USA, although most of the patients appear to have originated from Mexico or Latin America. The disease is also a major health hazard, and has been reported from Africa, China, Japan, Indonesia, Poland, Italy, Spain and Central and South America but has virtually disappeared from most other areas of Europe.

Other cysticercoses

Other important cysticercoses are caused by *T. saginata* in cattle (adult in humans), *T. ovis*, *T. hydatigena* and *T. multiceps* in sheep (adults in dogs). Another human *Taenia* has recently been identified in South-East Asia; new genetic evidence and earlier biological studies suggest this 'Asian *Taenia*' is a distinct entity, but that it is closely related to, and should best be regarded taxonomically as, a subspecies or strain of *T. saginata*. Valuable experimental models for immunologic studies of cysticercosis include *T. taeniaeformis* in rats, *T. crassiceps* in mice and *T. pisiformis* in rabbits. This group of worms collectively belong to the family Taeniidae (the taeniids) but the account given here has especial emphasis on *T. solium* because of its medical importance. Hydatid disease, caused by dog tapeworms of the genus *Echinococcus* is, strictly speaking, also a cysticercosis.

Antigens

Few antigens have been characterized from tapeworms and much work has been aimed at providing reagents suitable for specific and sensitive immunodiagnosis. Antigen B (95–105 kDa) (paramyosin, see below), an immunodominant antigen of *T. solium*, believed previously to be specific, has now been shown to cross-react with antibodies produced to a number of other human helminth infections. Two low molecular weight antigens (26 kDa and 8 kDa) have been identified as specific for *T. solium* and appear to provide requisite diagnostic sensitivity. Most encouragingly, seven major lentil lectin-binding glycoprotein antigens (13–50 kDa) have been identified in *T. solium* and these have been applied very successfully in a highly specific and sensitive western blot assay for diagnosing human cysticercosis, and for immunodetection of infected pigs. ELISA tests have also been developed for detection of *Taenia* antigens in the feces of human carriers. Some protective antigens have been partially characterized and these include molecules from *T. taeniaeformis*, *T. saginata* and *T. ovis* (see below). Very few of these antigens have been localized and further work in this area is clearly warranted.

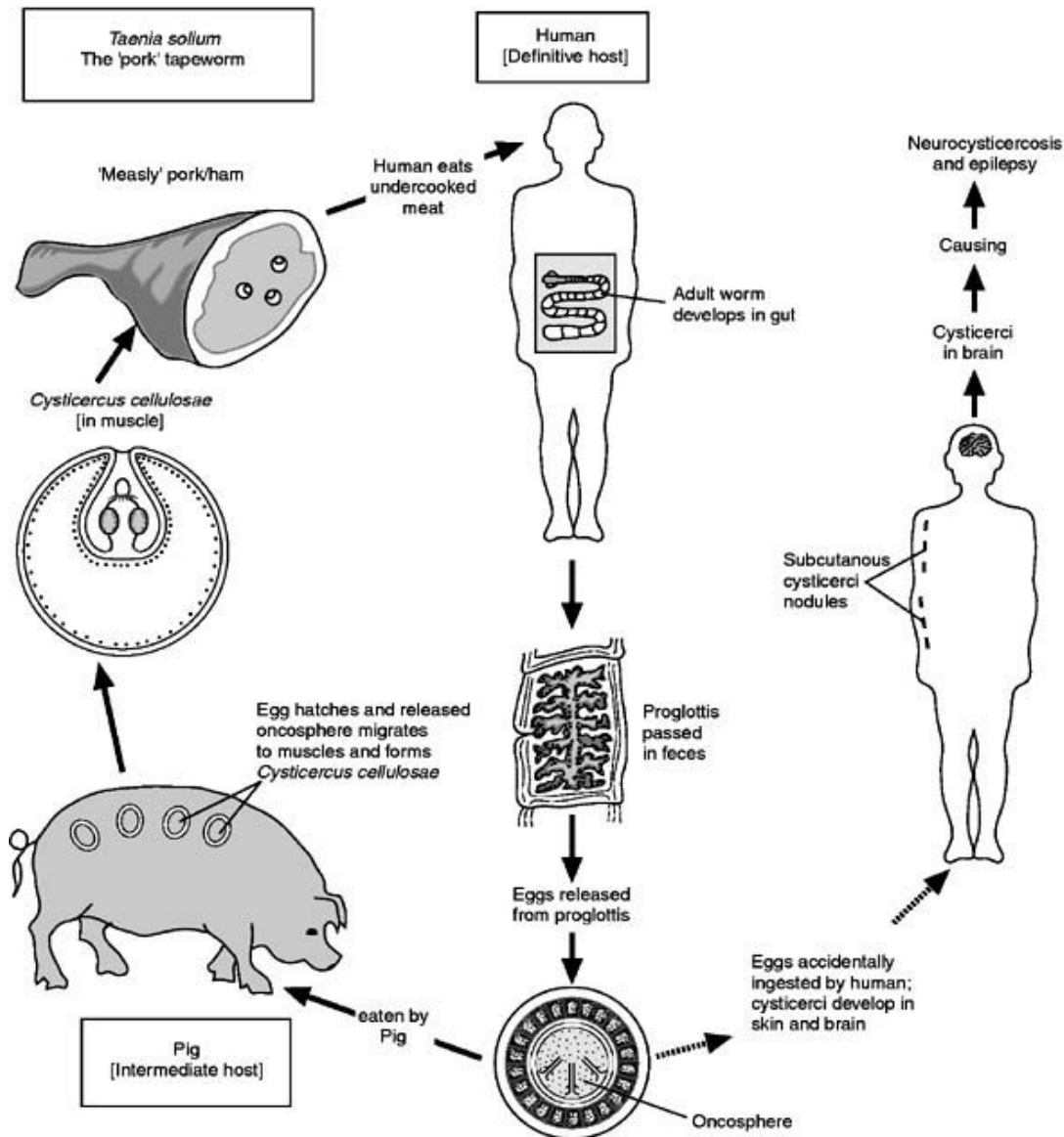


Figure 1 Life cycle of the 'pork' tapeworm, *Taenia solium*. Note that the larval stage (cysticercus) can develop in humans, causing the dangerous and often fatal disease neurocysticercosis. (After Smyth (1994), with permission.)

Immune response of the host

T. solium, similar to other larval cestodes in mammals makes intimate contact with host tissue at two sites (Figure 2): 1) during the early phase of infection, when the recently hatched oncosphere penetrates the intestine; and 2) at the final encystment site, commonly in muscles, viscera and the nervous system. The immune response at the oncosphere penetration site is often referred to as 'early' or 'pre-encystment' immunity, and that at the final establishment site as 'late', 'postoncospherical', or 'postencystment' immunity (Figure 2).

Secretory immunoglobulin A (IgA) in gut secretions and (where relevant) in colostrum prob-

ably plays an important role in attacking invading oncospheres, a situation related to the fact that this antibody may be resistant to intestinal enzymes. Thus infection of mice with *T. taeniaeformis* results in the production of intestinal IgG antibodies protective against challenge infection, and oral immunization results in a protective immune response against this species. An IgA antibody response has also been reported following *T. taeniaeformis* infection in rats and *T. pisiformis* infection in rabbits.

Mast cells accumulate around both invading oncospheres and developing larvae and it is possible that IgE may react with antigen, causing degranulation of these cells with a consequent increase in vascular permeability; this would allow IgG anti-

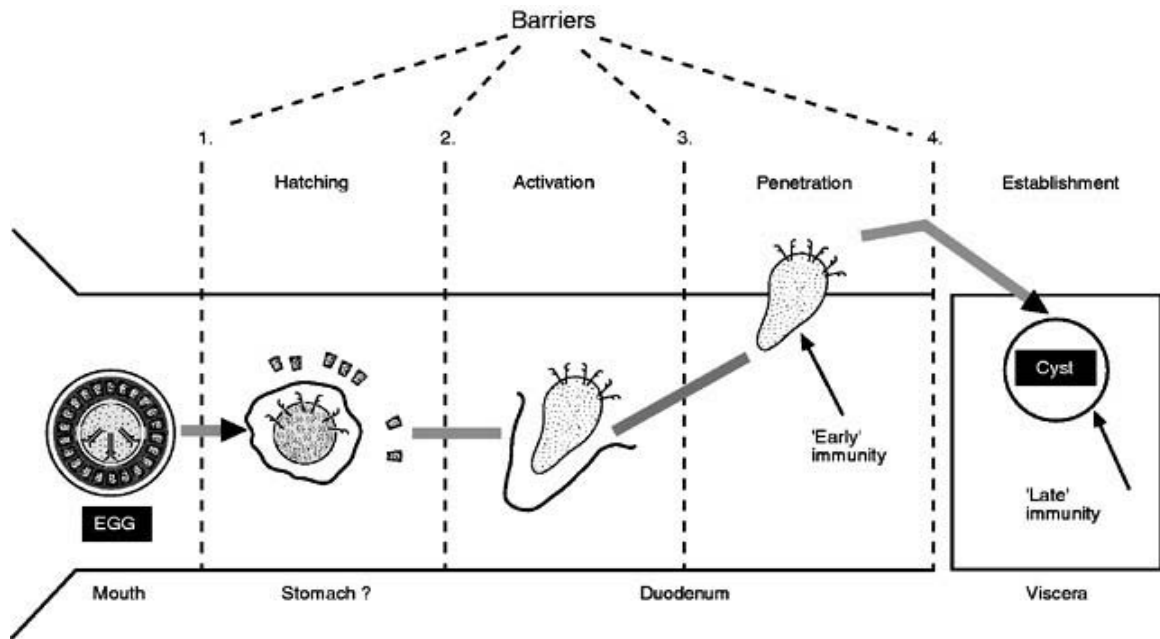


Figure 2 Pattern of immune responses on an oncosphere of a taeniid cestode such as *Taenia solium* during initial penetration of the hatched oncosphere (initiating 'early immunity') and its subsequent migration to, and establishment in, its definitive site, where it is subject to 'late immunity'. The exact site of hatching in humans is unknown but most eggs probably hatch in the upper duodenum. (After Smyth and McManus (1989), with permission.)

bodies to more easily reach the invading site. Eosinophils may accumulate around invading oncospheres, but there is no evidence that these cells actually damage oncospheres or postoncospherical stages. The role of macrophages or neutrophils, which may accumulate at the site of dying oncospheres, remains unknown.

When newly hatched oncospheres reach their site of establishment, they become transformed from a stage of being highly susceptible to attack to one of almost complete insusceptibility. The cellular and humoral response in *T. solium* cysticercosis is heterogeneous in humans and pigs. IgG represents almost 90% of the serum antibody detectable by ELISA but some patients show no antibody or cellular response whatsoever. Cysticerci of *T. solium* elicit a chronic granulomatous reaction in pig muscle. There is extensive eosinophil infiltration and degranulation on to the parasite surface but no apparent damage ensues. As in *T. solium*, eosinophilic response to *T. taeniaeformis* is antibody dependent. Eosinophils have been shown to adhere to and damage *T. taeniaeformis* *in vitro* but it is not clear if this occurs *in vivo*.

Little is known of T cell immunity to larval cestodes, although CD4⁺ T helper cells have been implicated in the early phase of resistance to infection with *T. taeniaeformis* in mice. Such cells may be activated by parasite to produce cytokines involved in

maturation and activation of eosinophils. Alternatively they may mediate interaction between hepatocytes and the parasite, thereby causing an alteration in the lipid metabolism of the former, resulting in the production of an eosinophilic chemotactic factor. Much work remains to be done in this important area.

Evasive strategies

The ability of tissue parasites, such as the larval taeniids, to survive in an immunologically hostile environment represents a phenomenon of special interest and controversy.

Blocking antibodies

In larval cestodes, host proteins have been demonstrated on the surface, including antibodies, these being referred to as 'blocking' antibodies. Thus, on the surface of the cysticerci of *T. solium*, a number of immunoglobulin isotypes have been found. In the case of *T. crassiceps* larvae, the predominating immunoglobulins appear to be IgM and IgG but, with this species, complete protection against immune attack may not be provided.

Molecular mimicry

The theory of molecular mimicry proposes that

parasites disguise themselves as host (i.e. self) tissues by synthesizing host-like antigens on their surface, with the result that they are not recognized as foreign (i.e. nonself) by the host. There has been much controversy as to whether larval cestodes are capable of synthesizing such antigens or whether they have simply been absorbed from the host. Some evidence suggests that parasites can synthesize receptors or binding proteins capable of associating with host molecules. For example, an IgG (Fc γ)-binding protein has been identified and purified from *T. crassiceps*.

There is evidence that the fact that the postoncospherical stages rapidly outgrow their susceptibility to attack may be due to development of stage-specific antigens related to profound alterations in the surface structure of the developing larva. Thus, a strong immune response is generated against *T. taeniaeformis* in the liver, and larvae become insusceptible by 8 days – a time that coincides with the surface replacement of microvilli by microtriches.

Secretory products

Some larvae (e.g. *T. pisiformis*, *T. taeniaeformis*) release substances which inhibit proteolytic enzymes such as trypsin and chymotrypsin. Taeniaestatin, a 19.5 kDa secretory protein from *T. taeniaeformis*, in addition to inhibiting these enzymes, also inhibits neutrophil chemotaxis, the alternative and classical complement pathways, and interleukin 2 stimulation of T-cell proliferation. Antigen B (AgB) of *T. solium*, a secretory protein which has the ability to bind collagen, can inhibit, *in vitro*, the classical pathway of complement through inhibition of C1 function and may play a role as a modulator of the host immune response in cysticercosis. Interestingly, AgB has immunologic and sequence homology with the invertebrate muscle protein paramyosin. Whether secretory products of other species can potentially modify the host cellular responses in a similar fashion remains to be established.

Immunosuppression

There are several reports that some cestode larvae can induce immunosuppression in their hosts. For example, the sera of rabbits heavily infected with *T. pisiformis* have an inhibitory effect on the lectin-mediated blast transformation of lymphocytes. Furthermore, polyclonal B cell activation has been suggested as an immunosuppressive mechanism in human *T. solium* infections, while mice infected with *T. crassiceps* have impaired ability to make antibodies against sheep red blood cells.

Vaccines

The development of successful vaccines against the highly pathogenic larvae of *T. solium* (in humans and pigs) and those of economic importance, such as *T. saginata* (in cattle) and *T. ovis* and *T. multiceps* (in sheep) would clearly revolutionize the control of these important parasites. Studies with *T. saginata*, *T. ovis* and other taeniid species have shown that vaccination with living eggs, the parasite-free supernatant from *in vitro* culture of hatched and activated oncospheres, or detergent solubilized oncospherical antigens can induce complete protection against challenge infection. A major difficulty has been the limited supply of eggs, which has prevented the development of a practical vaccine.

Recently, recombinant DNA techniques have been applied to the problem of large-scale production of taeniid vaccine antigens, and the expression in *Escherichia coli* of cDNAs encoding taeniid antigens has been achieved. In particular, a highly effective recombinant antigen vaccine against *T. ovis* has been developed. Vaccination of sheep with this molecule, expressed as a soluble glutathione *S*-transferase fusion protein (GST-45W), stimulated 94% protection against challenge infection with *T. ovis*. Initial trials with the 45W clone as a β -galactosidase fusion protein proved unsuccessful. The antigen was stabilized subsequently by modification of the original

Table 1 Results of a pilot field trial showing numbers of *Taenia ovis* cysts found in the carcasses of vaccinated lambs naturally (field) or artificially infected with *T. ovis* eggs

Group	Number	Range of cysts	Mean	Protection (%) [*]
Controls – field infection	12	36–348	92	—
Controls – artificial challenge	10	74–254	156	—
Vaccinated – field infection	12	0–11	1.8	98
Vaccinated – artificial challenge	18	0–203	11.7	92

^{*}Expressed as percentage reduction of infection compared with respective controls. Modified from Rickard *et al* (1995).

cDNA; the modified antigen (GST-45B/X) was also highly effective in vaccinating sheep. Field trials with *T. ovis* recombinant antigen GST-45B/X (Table 1), in conjunction with saponin, the most effective adjuvant, again provided encouragingly strong protection against infection and this formulation is currently commercially registered. GST-45B/X does not cross-protect against *T. hydatigena*, *Echinococcus granulosus* or *Taenia saginata* and maternal antibody has been shown to interfere with development of immunity in vaccinated lambs. Other potentially protective, cloned *T. ovis* antigens (GST-16 and GST-18) have been identified, and it is likely that similar recombinant products could be developed against *T. solium* and *T. saginata*. Indeed, some cloned *T. saginata* and *E. granulosus* antigens are already under trial; with the latter, more than 95% protection has already been obtained in two separate trials with sheep.

See also: *Echinococcus*, infection and immunity; Molecular mimicry; Parasites, immunity to.

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CYTOKINE ASSAYS

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Cytokines may be assayed by biological properties, immunological recognition (ELISA or radioimmunoassay), competitive binding to the receptor molecule and by inference from the transcription of the mRNA. The advantages and disadvantages of each technique are summarized in Table 1. In general, bioassays are very sensitive and by implication verify biological activity of the cytokine; they are not however always very reproducible or specific. In contrast immunoassays, although reproducible and specific, are not nearly as sensitive as bioassays, although more recently the sensitivity of immunoassays has improved with the use of amplification techniques.

Immunoassays

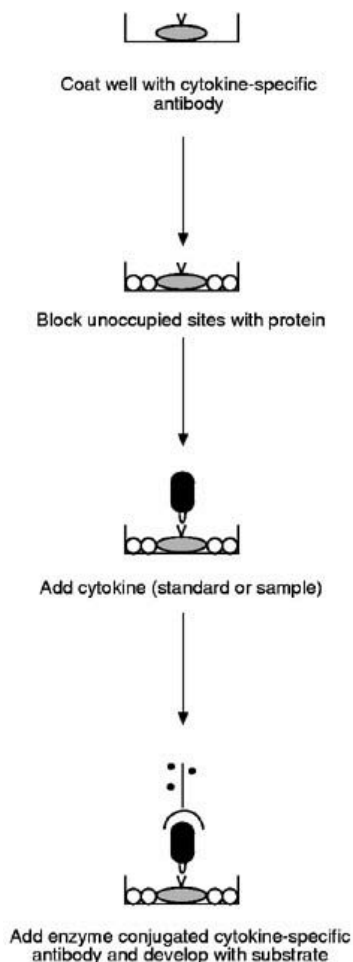
Immunoassays detect cytokines using a combination of polyclonal and monoclonal antibodies directed against the cytokine or two different complementary monoclonals. In either case the antibodies should be raised against purified cytokines, preferably recombinant products or peptide derivatives to ensure specificity. There are essentially two main types of immunoassay; the enzyme-linked immunoabsorbent assay (ELISA) and the immunoradiometric assay (IRMA). In both cases the cytokine is detected by antibody capture.

In the ELISA the first antibody (polyclonal or

Table 1 Comparison of methods of cytokine detection

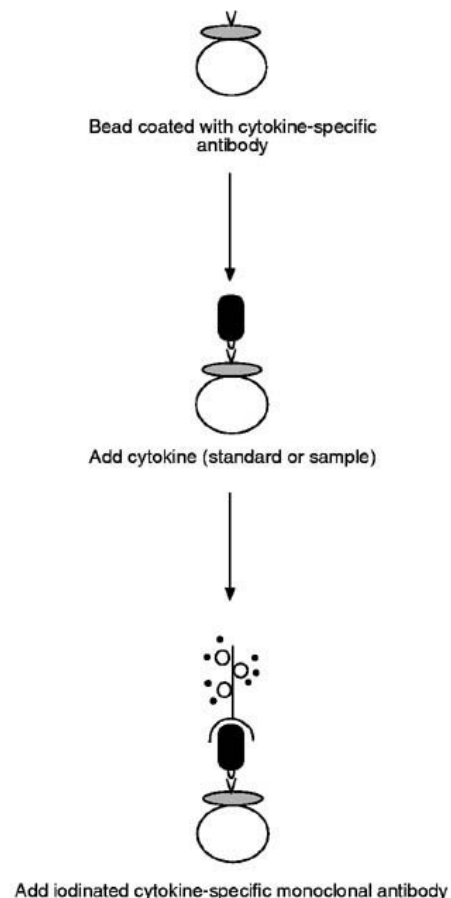
	<i>Evidence of bioactive protein</i>	<i>Sensitivity</i>	<i>Technical skill</i>	<i>Specificity</i>	<i>Effect of inhibitors</i>
Radioreceptor	+	+/++	++	+	+
Immunoassay	—	+	+	++	—
Bioassay	+	++	Variable	±	+
mRNA	—	Variable depending on technique	++	++	

monoclonal) is coated to plastic, followed by incubation with the sample or cytokine standard. The cytokine is then detected using the second antibody (monoclonal) conjugated to an enzyme and visualized using the appropriate substrate (Figure 1). Further layers on this sandwich technique can be applied to increase the sensitivity. For example unconjugated second monoclonal is detected using an anti-mouse immunoglobulin G (IgG) enzyme conjugate followed by the substrate. Alternatively the second monoclonal is conjugated with biotin followed by streptavidin enzyme complex.

**Figure 1** The enzyme-linked immunosorbent assay (ELISA).

The IRMA technique is also based on antibody capture as in the ELISA but the detection system depends on ^{125}I -labeled antibodies. In this case the first antibody is coated to a bead, followed by incubation with the cytokine in the fluid phase which is detected by iodinated cytokine-specific antibody (Figure 2).

In either system the relevant dilution of the different components in the assay require to be determined first and then the detection limits of the immunoassay established. This part is time consuming but ensures that the assay is reproducible and that the assay is as sensitive as possible. The limits of detection for such capture assays are normally around 30–50 pg ml⁻¹ with the upper range at approximately 10 000 pg

**Figure 2** The immunoradiometric assay (IRMA).

ml⁻¹. The detection limit depends upon a number of factors including the affinity of the relevant polyclonal and/or monoclonal antibodies used, in addition to the sample diluent which may contain inhibitory binding proteins. The use of high-affinity monoclonal antibodies and amplification systems has increased the sensitivity of many cytokine ELISAs to 10 pg ml⁻¹.

Advantages and disadvantages

The advantage of the ELISA method over the IRMA is that the reagents have a longer shelf-life as their detection system does not depend on radiolabeled antibodies. However, as the ELISA is a solid-phase assay the detection limit is not as good as the fluid-phase IRMA (presumably due to steric hindrance). In both cases the assays only measure antibody binding to the cytokine and not necessarily biological activity. This is an important fact to consider as inhibitors in the sample or bound to the cytokine itself may not interfere with immunobinding but render it biologically inactive. Furthermore it is important (as in any assay) to use the appropriate standards. Many native cytokines are glycosylated (e.g. interferon γ) which may have different biological and immunobinding properties to nonglycosylated recombinant products. Thus it is important to measure cytokines in immunoassays using a standard (e.g. glycosylated recombinant cytokine) which reflects the cytokine in the sample.

Standard preparations

In both immunoassays and bioassays (discussed in the next section) it is important to use reference or standard preparations of the cytokine both to establish the assay and further to allow interassay comparison. Cytokines are defined both in terms of protein concentration (pg ml⁻¹) and specific activity (units mg⁻¹). A unit is defined as that amount of cytokine which gives 50% maximal activity in the bioassay. As certain cytokines can be measured by more than one bioassay (which can vary up to tenfold in sensitivity) this measurement is purely arbitrary depending on the bioassay used. In conclusion, in both immunoassays and bioassays the standard preparation (recombinant material) should be used to standardize the technique and the results expressed both as protein concentration and units mg⁻¹.

Bioassays

Bioassays (Table 2) are necessary to establish that the system under investigation is producing active protein and the design must take the following into account:

- Cytokines have overlapping bioactivity and there-

fore the assay must be designed to eliminate contributions from other cytokines.

- Specific inhibitors may be produced in the same experimental systems that produce the active cytokines e.g. interleukin 1 inhibitor.
- Nonspecific inhibitors may exist in the system, e.g. α_2 -macroglobulin or enzymes which degrade cytokines, e.g. encephalinase.

Further it is advantageous if the system:

- uses a target system which is easily available;
- has clear endpoints;
- requires only small amounts of test material.

Cytokine bioassays will be discussed with reference to the following:

- choice of target system
- choice of property to be assayed
- ensuring specificity
- inhibitor proteins.

Target cells

The choice usually lies between purified and nonpurified populations of normal cells versus cell lines. The latter have the advantage that if the appropriate cell line exists it can be available in large amounts, with comparable sensitivity between experiments (though drifting of sensitivity of cell lines may take place), and secondary effects from accessory cell populations are eliminated. However, when no cell lines exist or when new activities are being sought in physiological circumstances normal cells may be more useful. It is often necessary to enrich the target cell population to ensure that the assay is sensitive and linear over an adequate range. Enrichment may be done *in vivo*, e.g. the assay for mouse IL-5 (Eo-CSF) activity done on bone marrow cells enriched for eosinophil precursors by infecting the animal with *Mesocostoides corti*. When it is necessary to completely eliminate accessory cell effects target cells must be rigorously separated from contaminating cells, e.g. by FACS sorting. Cytokines have a wide variety of patterns of interspecies cross-reactivity viz:

- cytokines that are species nonspecific, e.g. TGF β s;
- human cytokines acting in human systems only, e.g. interferon γ ;
- human cytokines acting in mouse systems (but not vice versa), e.g. hematopoietic growth factors, IL-1, IL-2, IL-4, IL-6. Thus some human cytokines can be tested against mouse cells.

Properties

The choice of property of the cytokine to be tested will be influenced mainly by the ease and sensitivity

Table 2 Bioassays used to detect cytokine activity

<i>Cytokine</i>	<i>Target cell</i>	<i>Read-out</i>	<i>Important contaminating cytokines</i>
IL-1	D.10 (mouse T cell)	[³ H]thymidine incorporation	IL-2 (human and mouse), IL-4, GM-CSF, IL-7, TNF (mouse only). IL-6
	Mouse thymocyte	[³ H]thymidine incorporation	IL-2 (human and mouse), IL-4, IL-7 (mouse), IL-6. Inhibited by interferon γ (mouse), TGF β 1, TGF β 2, TNF (mouse).
	EL.4	IL-2 induction read in CTLL assay	IL-4 and IL-2 carry over to CTLL assay
IL-2	CTLL	[³ H]thymidine incorporation	IL-4 (mouse) TGF β 1, TGF β 2 inhibit at low IL-2 levels
IL-3	Hematopoietic cell lines e.g. FDCP.1 (mouse) Mo7E (human)	[³ H]thymidine incorporation	GM-CSF
	Bone marrow	20 α steroid dehydrogenase	
	Hematopoietic colony assays in semi-solid agar	Colony numbers	
IL-4	B cells (mouse only)	CD23 induction HLA class II	Inhibited by interferon γ
	Co-mitogenesis with PHA-stimulated mouse T cells	[³ H]thymidine incorporation	
	IL-4-dependent T cell lines	[³ H]thymidine incorporation	
IL-5	Induction of eosinophil maturation from bone marrow in semi-solid or liquid culture (human and mouse)	Microscopy	IL-3
	Normal mouse B cells treated with anti-immunoglobulin	IgM secretion	
	BCL-1	[³ H]thymidine incorporation	
IL-6	B9 mouse hybridoma (sensitive to human, and mouse IL-6)	[³ H]thymidine incorporation	IL-4 (mouse), IL-11
	Hepatocytes (Hep G 2)	Fibrinogen production	
	CESS cells (EBV transformed human B cells)	IgG production	
	SKW cells	IgM production	
IL-7	Bone marrow pre-B cells from Whitlock–Witte cultures	[³ H]thymidine incorporation	
IL-8	Neutrophils	Chemotaxis	Other neutrophil chemotactic factors including G-CSF, M-CSF
IL-10	MC9 mouse mast cell line Sensitive to mouse and human IL-10	[³ H]thymidine incorporation	IL-3
IL-11	B9 mouse hybridoma or B9/11 (subclone with higher sensitivity)	[³ H] thymidine incorporation	IL-6
IL-12	PHA-activated lymphoblasts	[³ H] thymidine incorporation	IL-2
IL-13	Human B cells costimulated with CD40 antibody	[³ H] thymidine incorporation	IL-4
IL-15	Activated T cells	[³ H] thymidine incorporation	IL-2, IL-12
TGF β ₂	NRK cells	Anchorage-independent growth	TGF α
Activin/inhibin	Cultured pituitary cells	FSH release $\uparrow/\downarrow$	
GM-CSF	Normal bone marrow progenitor cells	Granulocyte macrophage colonies	IL-3, inhibited by TGF β , TNF
	GM-CSF-dependent cell lines (see IL-3 above)	[³ H]thymidine incorporation	
G-CSF	Normal bone marrow progenitors Neutrophils	Granulocyte colonies Activation, e.g. superoxide release	GM-CSF GM-CSF, IL-6
TNF/LT	WEH1 164 clone 13 L929	Cell viability – by MTT cleavage by mitochondria and OD reading	

Table 2 Continued

<i>Cytokine</i>	<i>Target cell</i>	<i>Read-out</i>	<i>Important contaminating cytokines</i>
PDGF	Fibroblasts, e.g. cell lines SS, WHG, WI26 or 28	Proliferation	FGF (1, 2, 5), EGF, TGF β , IL-1, TNF, amphiregulin, oncostatin, Kaposi growth factor
IFN γ	Many, e.g. human diploid fibroblasts	Resistance to viral cytopathic effect, e.g. VSV	IFN α and β
IFN α /IFN β	DAUDI human lymphoblastoid B cell line	Inhibition of cell growth [3 H]thymidine incorporation	TGF β
MIF	Macrophages	Inhibition of migration in agar	IL-4/IFN γ
VEGF	Endothelial cells	[3 H]thymidine incorporation	
<i>C-C chemokines</i>			
I-309	Monocytes	Chemotaxis	Other monocyte chemoattractants
MCP-1/2/3	Monocytes or basophils	Chemotaxis	Other monocyte chemoattractants
MIP-1 α	Eosinophils or inhibition of CFU-A	Chemotaxis [3 H]thymidine incorporation	
MIP-1 β	Memory T cells	Chemotaxis	RANTES
RANTES	Memory T cells eosinophils	Chemotaxis	MIP-1 β and others
<i>C-X-C chemokines</i>			
γ IP10	Monocytes	Chemotaxis	Other monocyte chemoattractants
MIP-2	Neutrophils	Chemotaxis	Other PMN chemoattractants
CTAP-III	Neutrophils, fibroblasts	Chemotaxis	Other chemoattractants

of the read-out assay, the extent to which other cytokine activities overlap and the ease with which their contribution can be eliminated. In general the multiple functions of individual cytokines are associated with the same epitope and therefore properties of individual molecules do not vary with respect to each other, e.g. the collagenase activity of IL-1 is parallel to its T cell activity. The properties which are usable in assays may be divided into two groups: (stimulation and inhibition of) proliferation and function. The former may be assayed by growth stimulation (usually [3 H]thymidine incorporation) into sensitive cells or by assaying mitochondrial function as an indicator of cell viability, e.g. by cleaving 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) to form a dark blue formazan product. Such assays have the advantage of similarity of technique for a variety of cytokines and therefore many can be assayed with semiautomated equipment.

Functional assays are much more heterogeneous. They are necessary when the cytokine to be tested has no growth related properties (e.g. IL-8 must be assayed in neutrophil migration assays), or where the functional properties are more specific than the growth-related properties. Some cytokines can only be specifically bioassayed in organ culture (e.g. Mullerian inhibitory substance by assaying regression of 14-day fetal rat embryo urinogenital tracts) or *in vivo* (e.g. EGF-induced eyelid opening in neonatal

mice or MIP1- and 2-induced neutrophil chemotaxis in mouse cerebrospinal fluid).

Specificity

It has already been stated that many biological effects are mediated by more than one cytokine, e.g. bone resorption is induced both by IL-1 and TNF α and many cells respond to more than one cytokine as a growth factor, e.g. mouse thymocytes respond to IL-1, IL-4, IL-6 and IL-7 to varying degrees, in addition the test preparations may contain both stimulatory and inhibitory cytokines, e.g. TGF β is inhibitory in many of the IL-1 assays and assays where early hematopoietic cells are the target, and interferon γ inhibits many IL-4 assays.

Specificity may be increased by a variety of techniques:

1. Selection of assay systems where two cytokines with overlapping properties are likely to coexist, nonoverlapping properties may be selected for assay, e.g. human GM-CSF and G-CSF may be distinguished as the former has growth stimulatory properties on the megakaryoblastic leukemia cell line Mo7E.
2. The cells may not have linear responses to the different cytokines at the same dilutions of the test material, therefore the assay conditions can be arranged to favor the cytokine under test.
3. Use of mouse target cells may reduce the range

of human cytokines influencing the assay (see above).

4. When known cytokines are likely to influence the assay addition of excess neutralizing antibodies (plus appropriate control antibodies for monoclonal antibodies or serum for polyclonal antibodies) may be used to enhance specificity.
5. To further ensure that the activity assayed is attributed to the correct cytokine, neutralizing antibodies to the cytokine must completely abrogate all bioactivity.

Inhibitor proteins

Specific physiological inhibitors of, for example, IL-1 and TNF of the relevant cytokine influence bioassays.

Radio-receptor binding assays

Cytokines may also be assayed by competitive binding for the receptor between the test material and radiolabeled (usually ^{125}I) ligand. This assay requires availability of cells expressing high levels of the receptor and that optimal conditions of ligand and receptor binding are understood. In addition, non-specific interference in receptor ligand binding must be excluded by including controls for the media and buffers in the materials to be tested.

The assay involves incubation of iodinated ligand with varying dilutions of test or standard material under optimal conditions which must be predetermined for each receptor ligand pair, possibly at 4° to reduce internalization. This is followed by wash-

ing to remove excess ligand, solubilization of the ligand receptor complexes and counting in a γ -counter.

Problems intrinsic to this assay are:

- cytokines and their receptors may exist in several forms, e.g. PDGF and PDGF receptor with different binding coefficients;
- cytokines can cross-compete for the same receptor, e.g. IGF1 and IGF2, and MGSF (melanocyte growth-stimulating factor) and IL-8;
- cytokine inhibitors may bind to the cytokine receptor with equal avidity to the cytokine.

See also: Cytokine inhibitors; Cytokine receptors, soluble; Cytokines; Cytokine receptors; Enzyme-linked immunosorbent assay (ELISA); Granulocyte colony stimulating factor (G-CSF); Granulocyte-macrophage colony stimulating factor (GM-CSF); Immunoassays; Interferon γ ; Interleukin 1 and its receptors; Interleukin 2; Interleukin 3; Interleukin 4; Interleukin 5 and its receptor; Interleukin 6; Interleukin 7 and its receptor; Interleukin 8 and its receptor; Transforming growth factor β (TGF β); Tumor necrosis factor α ; TNF receptors.

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CYTOKINE GENES, REGULATION OF

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Cytokines play a central role in the immune response by promoting the activation of antigen-specific and nonspecific effector mechanisms and tissue repair. Selective cytokine production in particular can determine the outcome of a response by stimulating protective or exacerbative immune mechanisms. On the other hand, cytokines can be pathogenic when produced in excess, by causing overproduction of inflammatory cells, fever, vascular leakage, tissue damage and lethal shock. Multiple levels of cytokine gene regulation therefore exist to ensure that, in most circumstances, cytokine synthesis is limited in time and space.

Cell lineage-specific cytokine synthesis

Most, if not all, nucleated cell types can synthesize cytokines but different cell lineages vary in the combinations they produce. T lymphocytes collectively produce many of the known cytokines of immunologic interest, including most of the interleukins, interferon α (IFN α), β and γ , granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF α) and β , lymphotoxin α , lymphotoxin β and transforming growth factor β (TGF β); they apparently do not produce IL-7, IL-11,

IL-12, IL-14, IL-15, G-CSF or M-CSF. Different but overlapping sets of cytokines are produced by other cell lineages, including B lymphocytes, dendritic cells, monocytes and macrophages, natural killer (NK) cells, mast cells, neutrophils, eosinophils, megakaryocytes, fibroblasts, endothelial and epithelial cells. Whereas expression of some cytokines (notably IL-1, IL-6, GM-CSF and TNF α) is widely distributed among lineages and tissue types, others are highly restricted. For example, IL-2 and IL-4 production appears limited to T cells and mast cells and IL-12 production has only been reported in B cells, dendritic cells and macrophages. Further heterogeneity is seen within cell lineages. While individual T cells can simultaneously express multiple cytokine genes, different CD4⁺ and CD8⁺ T cells can synthesize many different cytokine combinations. Two extreme T cell cytokine profiles are the type 1 and type 2 patterns, distinguished by their mutually exclusive expression of IL-2, IFN γ and TNF β in the former case and IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 in the latter case.

Inducibility of cytokine synthesis

Production of most cytokines does not occur constitutively but depends on appropriate stimulation of the producer cell. For example, naive peripheral T cells do not synthesize detectable levels of any known cytokines but acquire this function as a result of combined stimulation through the T cell receptor for antigen (TCR) and receptors for costimulatory signals, such as CD80 or CD86 acting through CD28 on the T cell. Once activated, subsequent bursts of cytokine synthesis can be induced through the TCR or certain other receptors alone. Induction of cytokine synthesis is also antigen dependent in some other cell types, such as NK cells, mast cells and eosinophils, which can be triggered by binding of antigen-antibody complexes to Fc receptors. Other physiologically important ligands that can stimulate cytokine synthesis include bacterial lipopolysaccharide, extracellular matrix and cytokines themselves; the last can in some cases stimulate their own synthesis or initiate a cascade of synthesis of other cytokines.

Both coordinate and differential cytokine production can be observed in a single cell type. In previously activated T cells, for example, TCR cross-linking induces a transient wave of mRNA and protein synthesis for multiple cytokines with comparable kinetics. Costimulatory signals and cytokines can modulate this TCR-dependent cytokine synthesis

and, in some cases, can also differentially stimulate the TCR-independent expression of certain cytokines. Conversely, some physiologic regulators (such as glucocorticoid hormones) and therapeutic agents (such as cyclosporine) can inhibit cytokine synthesis in a coordinate or selective manner. Patterns of cytokine synthesis therefore depend not only on the lineage and activation status of the producer cell, but also on the combination of signals it receives.

Signal transduction

The signal transduction pathways that couple surface receptor stimulation to inducible cytokine gene expression have been most extensively studied in T cells. The common theme that has emerged is that a variety of distinct pathways converge at the level of transcription factor regulation to control cytokine gene transcription. Pathways that might regulate post-transcriptional events are much less well defined.

Three major signaling pathways activated by TCR ligation regulate cytokine production in T cells. These pathways and the transcription factors through which they act are as follows: 1) a calcium-dependent pathway regulating NFAT family members; 2) a protein kinase C (PKC)-dependent pathway that acts through AP-1 and NF- κ B; 3) pathways activated by p21^{ras} that work primarily through AP-1. The calcium-dependent and PKC pathways share a common activator, phospholipase-C- γ -1. Tyrosine phosphorylation activates phospholipase C- γ -1 resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate and diacylglycerol. The released inositol triphosphates stimulate the release of calcium ions from intracellular stores, activating the calcium-calmodulin dependent serine/threonine phosphatase calcineurin, which dephosphorylates NFAT proteins, resulting in their translocation to the nucleus. The importance of this pathway is evidenced by the presence of NFAT consensus binding sites in the regulatory regions of all cytokine genes studied, and the devastating effect of the calcineurin inhibitor cyclosporin A upon cytokine production. Diacylglycerol activates PKC- α and - ϵ isoforms, which activate cytokine gene expression via the NF- κ B and AP-1 transcription factors. NF- κ B activation is achieved by phosphorylation of the inhibitory subunit I κ B, which promotes dissociation of I κ B from the inactive cytosolic complex and allows the nuclear translocation of NF- κ B. AP-1 regulation is achieved through activation of the MAP kinases, which are downstream effectors of both PKC and p21^{ras}.

Stimuli that affect T cell commitment to the production of particular cytokines, such as IL-12 for the promotion of type 1 responses and IL-4 for the promotion of type 2 responses, utilize a set of signaling molecules distinct from those activated by the TCR. These include members of the JAK family of tyrosine kinases and their substrates, the STAT transcription factors. There is evidence that the establishment of stable cytokine production phenotypes is associated with modulation of these signaling pathways.

Transcriptional activation

Expression of most of the known cytokines is regulated primarily at the levels of *de novo* transcription and mRNA turnover. Multiple DNA-binding proteins have been identified as important regulators of cytokine transcription. Cytokine enhancer and promoter elements usually contain several transcription factor binding sites, sometimes with multiple sites for each factor, and the interplay between individual transcription factors, and complexes containing these factors, is an important feature of cytokine gene regulation. Many of these transcription factors regulate multiple cytokine genes in a variety of cell types, and specificity is thought to be achieved through the formation of specific transcription factor combinations acting through specific cytokine gene regulatory elements.

The activity and composition of these complexes can be regulated by five distinct means. The first is transcription factor accessibility. DNAase I hypersensitive sites reflect chromatin accessibility, are associated with active transcription and can vary between cell types in accordance with lineage-restricted cytokine production. Transcription factor access may also require demethylation of genomic DNA bearing 5-methylcytosine at CpG dinucleotide sequences. The initiation of enhancer- and promoter-driven transcription in a cytokine gene may be preceded by release from the influence of specific silencer elements. Second, transcription factor activity is affected by the DNA sequences with which the complexes interact, an example being the preference of the composite NFAT/AP-1 binding site in the IL-4 promoter for AP-1 components other than *c-fos* and *c-jun*. Polymorphisms in regulatory sequence elements of the human IL-1 β and IL-4 genes have been shown to be associated with variations in transcriptional activity between individuals. The third level of control is the availability of transcription factors in the nucleus, for example NFAT and NF- κ B family members, whose translocation to the nucleus requires dephosphorylation and phosphorylation/proteolysis-dependent mechanisms respect-

ively. The fourth level of control is achieved by phosphorylation of transcription factor components, which enhances the ability of complexes containing these factors to activate transcription. The differential composition and activities of transcription factor complexes provide one mechanism whereby differential cytokine production patterns can be achieved. Finally, transcription factors that act both positively and negatively have been demonstrated, as have both cooperative and competitive interactions. This can therefore be a point of integration of synergistic or counteractive signals in the induction of cytokine expression.

RNA processing and transport

Regulation of cytokine expression can potentially occur at the level of splicing, capping, polyadenylation and/or transport of mRNA from the nucleus to the cytoplasm. Stimulus-dependent effects on pre-mRNA splicing have been shown for IL-1 β and IL-2. Alternative splicing of some cytokines occurs and, for IL-4, can yield an inhibitor of IL-4-mediated stimuli (see below). Transport of IL-2 mRNA can be affected by CD28-mediated costimulation in T cells.

mRNA degradation

Post-transcriptional mRNA decay is a major level of regulation of expression for many inducible cytokines. Rapid mRNA deadenylation and degradation are mediated by sequence motifs in the 3' untranslated region of the transcript. The key motif has recently been redefined from AUUUA to UUAUUUA(U/A)(U/A). Multiple constitutively expressed or inducible proteins capable of binding these motifs have been partially characterized but their precise function(s) is unknown. There is some evidence that the degradation of certain cytokine mRNAs may also be achieved by endonucleolytic cleavage involving RNases with incompletely understood sequence preferences.

Translation and secretion

Translational control of cytokine expression has been less frequently described but is a major level of regulation for certain cytokines, including IL-1, TNF α and β and TGF β . Sequence elements in both the 5' and 3' untranslated regions of cytokine mRNAs can positively or negatively influence translation rates. Cytokines can also be modified post-translationally by glycosylation and/or phosphorylation and, although glycosylation does not appear to be essential for biological activity, differences in the

activities of different cytokine glycoforms have been described. Both IL-1 α and - β are synthesized as pro-hormones, which, in the case of IL-1 β , must be cleaved to be biologically active. TGF- β is similarly secreted as an inactive latent complex which requires specific extracellular activation. Secretion of a splice variant of IL-4 (see above) or a homodimer of the IL-12 p40 chain can antagonize the activity of the normal forms of these cytokines. Inducible cytokine secretion can occur in some cells, notably mast cells and platelets, through release of preformed molecules stored in granules.

Several mechanisms serve to focus or sequester cytokines at the site of production. For example, some cytokines (including IL-1 α , TNF α , lymphotoxin α/β trimers and M-CSF) can be expressed as membrane-bound forms, and many secreted cytokines are bound nonspecifically by extracellular matrix. Directional secretion of IL-4 by T cells towards the region of contact with antigen-presenting cells has also been reported and may be an important general mechanism for maximizing the effects of transiently-produced cytokines in tissue microenvironments.

See also: Colony stimulating factors; Cytokines; Cytokine receptors; T lymphocyte activation; T lymphocytes; Transforming growth factor β (TGF β); Tumor necrosis factor α .

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CYTOKINE INHIBITORS

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Cytokines deliver information to target cells when the appropriate receptors are expressed at the cell surface. Upon cytokine binding and cytokine-induced receptor aggregation, the signal is transduced resulting in gene activation and/or phenotypic or functional changes of the target cell. Thus, cytokine activity ultimately depends on its concentration in the microenvironment (i.e. in the extracellular fluid or in membrane-bound form) and on the expression level of specific receptors at the surface of

responding cells. Cytokine inhibitors may act in several ways and at multiple levels, inhibiting cytokine synthesis and release, modulating the amount of biologically active cytokine in the extracellular milieu, and inhibiting the capacity of the target cell to respond to cytokines (**Figure 1**). For a better understanding of the field of cytokine inhibitors, it is important to realize that they were discovered as natural products in biological fluids, blocking interleukin-1 or tumor necrosis factor.

Levels of inhibitory mechanisms

Cytokine production (see Figure 1) can be regulated at the gene level (1a), where promoters or inhibitors of transcription act, and at the mRNA level (1b), where the stability and translation of the message can be modulated by hormones, cytokines or drugs. Members of the MAP kinase family and serine-threonine kinase are among the factors involved in the signal transduction pathway for cytokine production and are therefore the target of drug intervention. In addition, the intracellular protein level can be affected by proteases (1c) needed to process propeptides into mature, functionally active polypeptides or to release cytokines from the cytoplasmic membrane. Once released, the biologically active amounts of a cytokine can be modulated by soluble ligands (2), in the form of nonreceptor cytokine-binding proteins (2a), autoantibodies (2b), soluble specific receptors (2c), and by proteolytic enzymes (2d). Soluble receptors can be derived from proteolytic cleavage of membrane-bound receptors (i.e. tumor necrosis factor α (TNF α) and interleukin 1 (IL-1) receptors), by alternative splicing of the mRNA coding for membrane receptors (i.e. sIL-4R), and by post-translational modification (i.e. glycosyl phosphatidylinosi-

tol (GPI)-anchored receptors by removal of the signal peptide). All the soluble ligands with cytokine-binding capacity potentially participate in the regulation of cytokine delivery and homeostasis. Whether their net effect is inhibition or enhancement of the cytokine action may depend on specific characteristics of the binding protein and on the kinetics of binding. As long as the cytokine is prevented from reaching membrane receptors, inhibition is maintained. However, soluble receptors and other soluble ligands can actually increase the concentration of cytokines in body fluids by increasing their stability and decreasing their proteolytic degradation. Upon dissociation from soluble ligands, biologically active cytokines can again reach and activate specific membrane receptors. Thus, soluble ligands can function as carriers of cytokines. Binding affinity therefore plays an important role in determining the relevance of soluble ligands in cytokine homeostasis. For instance, very high concentrations of soluble receptors are needed to effectively compete with membrane receptors when these receptors are associated with transducing subunits which increase their binding affinity (i.e. sIL-2R α). In contrast, the soluble forms of single chain receptors (sIL-1R; sTNF-R) are strong competitors and lesser amounts of them are required.

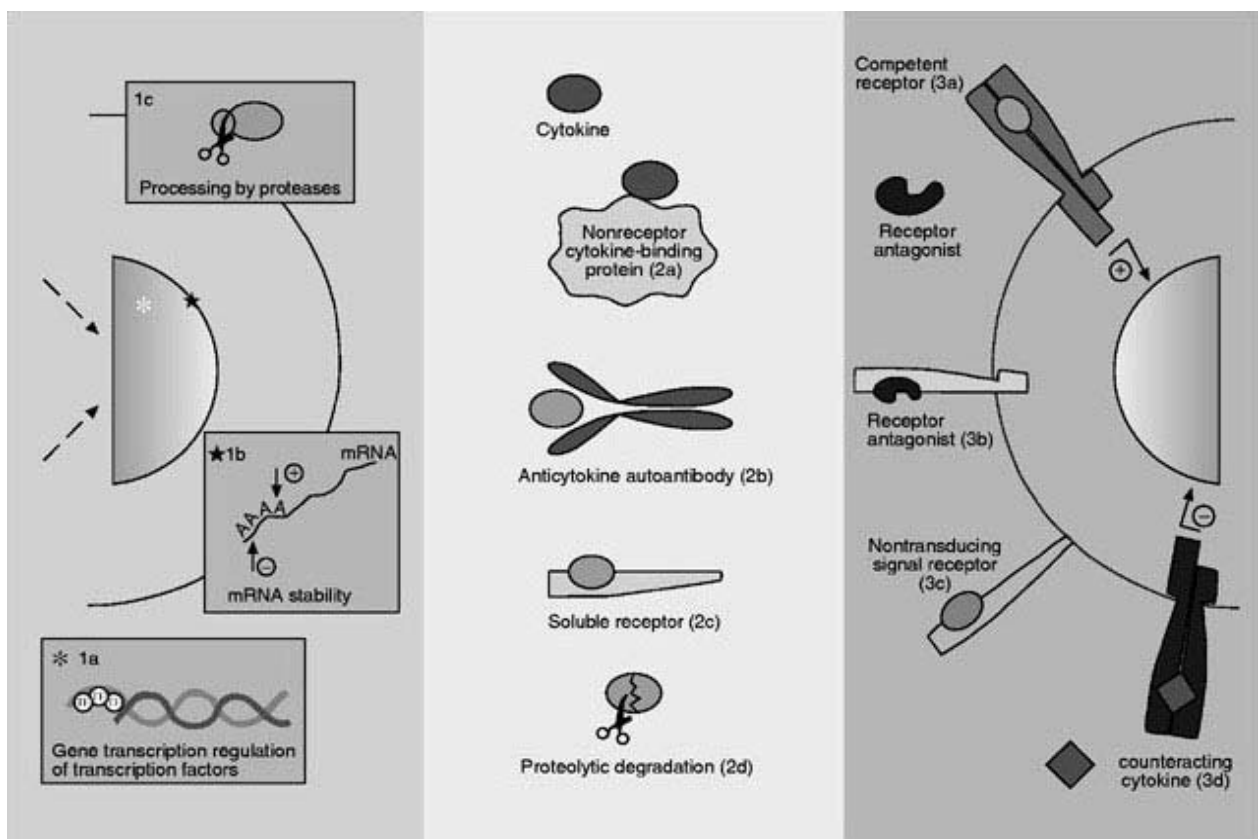


Figure 1 Diagrammatic representation of the various levels at which cytokine inhibitors can act (see text for details).

An additional mechanism of inhibition is generated by molecules that specifically bind to membrane receptors (i.e. IL-1Ra) thus competing with biologically active cytokines for binding. Such receptor antagonists (3b) are unable to generate a signal upon binding resulting in the inhibition of the cytokine activity. The receptor expression level at the surface of the target cell (3a) can be affected and regulated by several stimuli, including the stage of activation of the target cell, hormones, cytokines, drugs. Furthermore, the expression of surface receptors that bind with high affinity but do not signal has been documented (3c). Lastly, the signal transduced upon receptor occupancy (3d) can be regulated by concomitant stimuli delivered by other cytokines or drugs. Finally, cytokine bioactivity can be controlled by transcription-factor inhibitors (e.g. NF- κ B). A peculiar characteristic of cytokines is that they generate and act within a network through synergistic and antagonistic interactions. The balance or homeostasis between agonists and antagonists (or inhibitors) is probably more important than their absolute amounts. All the above levels of cytokine regulation have to be considered in order to understand the cytokine network and the mechanisms of cytokine inhibition within. The following sections contain typical examples illustrating known mechanisms involved in the inhibition of cytokine activities (Table 1). However, the regulation of cytokine production by xenobiotics will not be discussed.

Inhibition of interleukin-1

IL-1 is a potent mediator of inflammation affecting the function of many different cell types. At least five different levels of regulation of IL-1 activities have been documented.

IL-1 receptor antagonist (IL-1Ra), a glycoprotein of 22 000 molecular weight, is a member of the IL-1

gene family and binds IL-1R type I, a receptor capable of transducing signals, with similar affinity to that of IL-1 α and IL-1 β . However, unique in cytokine biology, no signal is transduced upon IL-1Ra binding to IL-1R. It is speculated that no signal is transduced because IL-1Ra fails to bind the IL-1 receptor accessory protein (IL-1R-AcP), thus inhibiting the formation of the IL-1 receptor heterodimer complex required for signal transduction. IL-1Ra is produced by the same cells that secrete IL-1; however, the expression of IL-1Ra is regulated by different types of stimuli including complexed IgG and anti-inflammatory cytokines. In addition, IL-1 and IL-1Ra exist in various forms (i.e. secreted and intracellular). The actual extent of the inhibitory activity of IL-1Ra *in vivo* is unclear. Due to the fact that occupancy of only a few membrane IL-1 type I receptors is sufficient to generate a biological response, the concentration of IL-1Ra (compared to IL-1) needed to interfere with IL-1 activity has to be at least 100 times higher.

IL-1R type II binds with high affinity both IL-1 β and IL-1Ra. However, it lacks a cytoplasmic domain and does not transduce signals. Therefore, this type of receptor functions as a decoy, inhibiting IL-1 β activities.

Both type I and type II IL-1 receptors exist in soluble form after proteolytic cleavage of the full length membrane forms. The rank of binding to type II IL-1 soluble receptor is IL-1 β > IL-1 α > IL-1Ra and for type I IL-1 soluble receptor it is IL-1Ra > IL-1 α > IL-1 β . IL-1 soluble receptors with bound IL-1 species do not have signaling capacity; instead, they act by decreasing the levels of biologically active IL-1.

Naturally occurring autoantibodies that bind IL-1 α (but not IL-1 β) have been described in about 25%

Table 1 Mechanisms involved in cytokine inhibition

Level	Process	Mechanism
Cytokine synthesis and secretion	Transcriptional regulation	Transcription factors
	mRNA stability	3'-AU-rich motifs
	Transcription/translation	Hormones (e.g. corticosteroids) Cytokines
	Processing and release	Proteases (e.g. metalloproteases)
Expression of membrane cytokine R	Competition	Nonsignaling receptors (e.g. IL-1R type II)
Interaction of cytokines with membrane R	Interference	Soluble cytokine R
		Receptor antagonists (e.g. IL-1Ra)
		Nonreceptor cytokine-binding protein (e.g. uromodulin)
		Anticytokine autoantibodies

of normal individuals, and some of them have proved to neutralize IL-1 α *in vitro*. The prevalence of such autoantibodies is increased in some inflammatory autoimmune diseases (e.g. rheumatoid arthritis). Their function *in vivo* remains unclear but they could contribute to regulating the levels of bioactive IL-1 α so that disease activity may inversely correlate with the amounts of blocking autoantibodies.

The production of IL-1Ra can be enhanced by anti-inflammatory cytokines such as IL-4, IL-13, IL-10, and transforming growth factor β (TGF β). These cytokines increase IL-1Ra production while decreasing lipopolysaccharide (LPS)-induced IL-1 production *in vitro*. The levels of type II IL-1 soluble receptor are also increased in response to IL-4. Thus, the coordinate expression and upregulation of the receptor antagonist and of type II soluble receptor may regulate and decrease the responses to IL-1. *In vivo*, infusion of IL-4 results in the suppression of gene expression and synthesis of inflammatory cytokines while inducing high levels of IL-1Ra and type II soluble receptor.

Large amounts of IL-1 α may exist at the intracellular level, both in the cytoplasm and in the nucleus, in the form of pro-IL-1 α which is processed by the interleukin-1 converting enzyme (ICE), in turn controlled by inhibitors. This is well documented in keratinocytes where pro-IL-1 α is thought to drive terminal cell differentiation. An intracellular form of IL-1Ra (icIL-1Ra) has also been documented in keratinocytes and monocytes, that is supposed to regulate pro-IL-1 α -driven cell proliferation and differentiation. IL-1Ra, easily detectable in human serum, is also produced by hepatocytes, behaves as an acute-phase protein and probably is an important factor in host defense. Its monitoring could be useful in assessing inflammatory diseases.

Interestingly, several vaccinia strains of the poxviruses have encoded in their genome the capacity to secrete a soluble protein which binds to IL-1 β selectively and with high affinity. Although structurally different, this viral protein shares 33% homology with human IL-1 receptors at the amino acid level. The virus strains capable of releasing this protein are less virulent, and it is speculated that evolutionary selective pressure has favored the development of these strains because they are substantially less pathogenic, thus ensuring the survival of both host and virus. These data emphasize the importance of IL-1 in both protection and pathology. They stress the relevance of fine tuning the levels of bioactive agonistic IL-1 and explain the redundancy of the molecules implicated in its control.

Inhibition of tumor necrosis factor and lymphotoxin

At the ligand level, an important control mechanism for the production of TNF α is the release of the membrane-bound (26 kDa) precursor controlled by the metalloproteinase TNF α -converting enzyme (TACE). TNF α and lymphotoxin (LT α) (previously called TNF β) act after binding to membrane-bound p55 (or p60, or type I) and p75 (or p80 or type II) receptors expressed by numerous types of cells. Both exist in soluble forms, detected *in vitro* and *in vivo*, and are released from the cell surface after proteolytic cleavage, possibly mediated by an unidentified metalloproteinase. Of the two receptors p75 is shed more frequently. Among the stimuli that enhance TNF receptors shedding is TNF α itself. Since soluble receptors have a longer half-life than TNF α , it is easier to detect in body fluids the levels of soluble TNF receptors which reflect previous or ongoing TNF responses. The affinity of soluble receptors for TNF α and LT α nearly equals that of membrane receptors. Thus, they may act as buffers, although they may also act as carriers and prolong TNF α bioavailability. Soluble TNF receptors circulate in nanogram amounts in human blood, but are increased in various inflammatory diseases and control the bioactivity of TNF α . Chimeric protein molecules consisting of an Fc portion of the human IgG1 coupled to p55 or p75 TNF soluble receptors are at present being evaluated in clinical trials to treat chronic inflammatory autoimmune disorders such as rheumatoid arthritis and severe acute inflammatory or infectious diseases.

Inhibition of interleukin-4

IL-4 signals through an heterodimeric receptor composed of the IL-4Ra and the IL-2 R γ_c chain. IL-4Ra exists in soluble form and protects IL-4 from proteolytic degradation. Because IL-4 soluble receptor has a lower affinity for IL-4 than the cell-bound receptors it seems likely that the soluble form serves as a carrier of IL-4, facilitating its presentation to cell-bound receptors. In addition, an alternative splice variant of human IL-4, called IL-4 δ -2, inhibits *in vitro* IL-4-stimulated T cell proliferation. This splice variant seems to be preferentially expressed in the thymus and bronchoalveolar lavage cells. This finding suggests that the generation of cytokine variants by alternative RNA splicing due to exon-deletion mechanism might give rise to receptor antagonists and may be an important mechanism for the regulation of cytokine activity.

Inhibition of interleukin-12

The biologically active IL-12 is a heterodimer composed of two disulfide-linked subunits, p35 and p40. The naturally occurring p40 homodimer which retains the capacity to bind the IL-12 receptor prevents signaling, thus acting as an antagonist of the IL-12 heterodimer.

Ambivalence of the two soluble IL-6 receptors: inhibitory activity of gp130 receptor

gp130 is the common receptor β subunit shared by IL-6, ciliary neurotrophic factor (CNTF), oncostatin M, IL-11, cardiotrophin 1, and leukemia inhibitory factor (LIF). Its soluble form has been shown to inhibit the action of IL-6 *in vitro*. This is interesting because the soluble IL-6R α acts in the opposite way. Indeed, upon binding to IL-6 soluble IL-6R α allows signaling through membrane-bound gp130.

Anticytokine autoantibodies

Natural anticytokine autoantibodies occur in 10–25% of normal donors and with increased prevalence and titers in patients with autoimmune or rheumatic disorders. Antibodies specific for IL-1 α , IL-6, TNF α and type I interferon have been principally reported. However, the capacity to act as blocking antibodies is still debated for many of them.

Nonreceptor proteins

Uromodulin, an 85 kDa protein found in the urine of pregnant women binds both IL-1 and TNF α , thus acting as a nonspecific, partial inhibitor whose mechanism of action is still unknown. In contrast, serum α_2 -macroglobulin is mentioned as a transporter rather than an inhibitor. It binds several cytokines (i.e. IL-1, IL-2, IL-6, PDGF, FGF β) and inhibits proteases. The role of nonreceptor proteins *in vivo* is far from clear.

Inhibitory biological activities

As mentioned above, cytokines and their receptors are enmeshed in a network of agonistic, synergistic and antagonistic biological activities. A single cell type can be the target of multiple cytokines with similar or opposite effects. In turn, the target cell responds to received stimuli by releasing in the milieu soluble products that may affect the original producer cell. Thus, positive and negative feedback loops regulate the responses to cytokines.

Concerning the immune response, the concept of T_H1 and T_H2 cells has developed in recent years. These are T lymphocytes characterized by the polarized production of cytokines with opposite functions. Thus, T_H1 cells exclusively produce the T_H1 cytokines interferon γ (IFN γ), lymphotoxin and IL-2, whereas T_H2 cells produce the T_H2 cytokines IL-4, IL-5, IL-10 and IL-13. Broadly speaking, in terms of B cell help, of proinflammatory and anti-inflammatory activities, T_H1 and T_H2 cytokines can be considered reciprocal inhibitors. Although obviously incomplete and not restricted to the T helper lymphocyte subset, this dichotomic and simplified division provides a handle on the complexity of the regulation of the immune system. For example, both IL-4 and IL-10 inhibit the release and function of IL-1, TNF α and other proinflammatory cytokines such as IL-8 and matrix metalloproteinases (MMP). Conversely, they increase the secretion of natural cytokine inhibitors such as IL-1Ra, soluble IL-1 receptors and soluble TNF receptors. Thus IL-4 and IL-10 may serve as feedback control mechanisms of the activities of several proinflammatory cytokines. Beside releasing soluble mediators, TH1 and TH2 cells cross-regulate inflammatory responses also upon direct cell-cell contact with monocytes. TH1 enhance the production of proinflammatory cytokines while TH2 favor the production of anti-inflammatory cytokines.

Therapeutic implications

Based on the aforementioned concept, a more rational approach to interfering with acute and chronic human diseases has been developed that has led to numerous clinical trials which may culminate in clinical use. The general principle is that the absolute level of a cytokine has to be analyzed in terms of balance with regard to that of its natural inhibitor(s). Thus, IL-1Ra, sIL-1R and sTNFR may guard against the proinflammatory cytokines. IL-4 and IL-10 are at present being assessed in clinical trials. An attempt to increase local expression of cytokine inhibitors has also been made through gene therapy. At present, the overexpression of IL-1Ra is being tested in animal models of arthritis.

Recombinant forms of natural inhibitory cytokines such as recombinant IL-1Ra, recombinant soluble TNF-R p55 or recombinant soluble TNF-R p75 with human Fc constructs (rTNFR:Fc p60 or rTNFR-Fc80 fusion protein), short motifs of such peptides, or polyethyleneglycol (PEG-rsTNF-RI) or TNF-binding protein (TNF-bp-PEG) are now available in large amounts and their pharmacokinetics and safety profiles are being tested. Approaches using

gene therapy to reach the local site of the inflammatory destructive lesion are under investigation. A complete assessment of the efficacy of these new agents will comprise their evaluation for long-term treatment, not only in patients with refractory and longstanding disease, but also in those in the early stages of disease. Thus, potentially better therapies are provided along with a better understanding of the pathogenetic mechanisms of immune inflammatory diseases.

See also: Antibodies, specificity; Cytokine genes, regulation of; Cytokine receptors, soluble; Cytokines; Cytokine receptors; Interferon γ ; Interleukin 1 and its receptors; Interleukin 2; Interleukin 2 receptor; Interleukin 3; Interleukin 4 receptor; Interleukin 6; Interleukin 6 receptor; Interleukin 12 and its receptor; Poxvirus, infection and immunity; Tumor necrosis factor α ; Lymphotoxin; TNF receptors.

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CYTOKINE RECEPTORS

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Cytokines exert their biological effects on responsive tissues by binding to high-affinity cell surface receptors. Prior to 1988, little was known of the cytokine receptors, as only the CSF-1 (colony-stimulating factor) receptor (R) and the low-affinity binding chain (α chain) of the interleukin (IL-) 2 R had been cloned and characterized. However, the cloning, purification and large-scale production of cytokines and the rapid development of expression cloning technologies have led to the molecular characterization of their receptors. In general, the cytokine receptors share some common characteristics, a high affinity for ligand (K_d 10^{-11} – 10^{-9} M) and normally very low levels of expression, typically a hundred to a few thousand receptors per cell. The majority of cytokine receptors are glycosylated integral type 1 (N-terminus extracellular, C-terminus cytoplasmic) membrane proteins. Functional cytokine receptors are also typically complex structures requiring the formation of homologous or heterologous associations between receptor chains. The exceptions to this are the receptors of the chemokine family, e.g. those for RANTES and IL-8. These receptors belong to the serpentine superfamily that span the membrane seven times and do not appear to form multimeric complexes.

Apart from these general features the characterization of the cytokine receptors has led to elucidation of a variety of structural features that has permitted the identification of many distinct receptor families (Table 1). In some cases these families can be further subclassified by the nature of the subunit structure used in the active receptor (see below).

Distribution of receptors

As most cytokines were originally isolated from the hematopoietic system, initial studies on receptor expression largely concentrated on this tissue. However, with time and the generation of suitable reagents, cytokine receptors have been detected in many other tissues. The expression of individual cytokine receptors on different cell types is highly variable. For instance, the receptors for IL-2, IL-3, IL-5, IL-7 and IL-9 have so far been mainly restricted to the cells of the hematopoietic system, whereas the receptors for IL-1, IL-4, and IL-6 are more widely expressed on other cells (e.g. fibroblasts), and the receptors for TNF (tumor necrosis factor) and

IFN α/β (interferon) are found on most cell types. However, it is likely that with further studies and improved quantitative technology our perception of the tissue distribution of some cytokine receptors will change considerably.

Receptor structure: five major families

Hematopoietic growth factor receptor family (cytokine receptor class I)

The hematopoietic receptor family (cytokine receptor class I) forms the largest group of cytokine receptors. This family includes the receptors for IL-2/IL-15 (β chain), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), OSM (oncostatin M), LIF (leukemia inhibitory factor), GH (growth hormone), Epo (erythropoietin), prolactin, CNTF (ciliary neurotrophic factor), thrombopoietin (c-mpl) and CT-1 (cardiotropin 1), as well as nonligand binding accessory subunits such as the common gamma (γ c) chain, gp130 and KH97 (see below). These receptors all share a degree of structural homology in their extracellular domains, and in particular have a number of conserved cysteine residues. In addition, the extracellular domains express one or two copies of a common pentameric motif, Trp-Ser-X-Trp-Ser, contained within a fibronectin type III domain. The precise role this motif plays in receptor function is unclear. Between the cytoplasmic domains of these receptors there is generally little homology except for two short amino acid sequences, known as the box 1 and box 2 regions, which are expressed by most receptors. The box 1 region provides the site of interaction for the Janus family of tyrosine kinases. The role of the box 2 region is unclear. None of these receptors have any identifiable enzymatic activity but intracellular tyrosine kinases of both the *src* and/or the Janus family of tyrosine kinases can associate with these receptors (see below).

A general feature of these receptors is oligomerization of receptor subunits, often leading to the conversion of lower affinity binding of the ligand-specific chain to a higher affinity. Another feature of receptor assembly is the promiscuous use of common receptor subunits by various groups of functionally related cytokines. Three such families have so far been defined. The receptors for IL-2, IL-4, IL-7, IL-9 and

Table 1 Cytokine receptor families

<i>Family</i>	<i>Members</i>	<i>Comments</i>
HGFR/class I	IL-2R, IL-4R ^a , IL-7R ^b , IL-9R, IL-15R ^c IL-3R ^d , IL-5R, GM-CSFR IL-6R ^e , IL-11R, LIFR, OSMR, CNTFR, CT-1R IL-12R G-CSFR, EpoR, prolactin R, GHR Thrombopoietin-R (c-mpl) IL-13R	All associated with γ_c chain All associated with common β chain All associated with gp130 Two chains β_1 , β_2 related to gp130 Homodimer formation Association unknown Forms complex with IL-4R
IFNR/class II	IFN α / β R, IFN α R, IFN γ R, IFN γ R accessory IL-10R ^f	Forms heterodimers Forms homo- and heterodimers Homodimer
TNFR family	LT/TNF-R (p55, p75), CD27, CD30, CD40, 4-1BB, Fas, LT β R, OX40, NGFR	Forms homodimers/oligomers; ligands mainly cell surface bound
Ig superfamily (no tyrosine kinase)	p60 IL-1R, p80 IL-1R, IL-1R-associated protein, IL-6R	IL-1R and IL-1R-associated protein Forms heterodimers
Ig superfamily (tyrosine kinase)	PDGFR($\alpha\beta$), FGFR (1,2,3,4), CSF-1R (c-fms), SCFR/c-kit	Forms oligomers on ligand binding
Tyrosine kinase/non-Ig superfamily	EGFR, insulin R, insulin-like growth factor R, neurotrophin receptors (trkA, B, C)	Forms oligomers on ligand binding
Serpentine/chemokine receptor	High-affinity IL-8R, low-affinity IL-8R, Duffy antigen, MIP-1 α R (CC-CKR-5)	Seven transmembrane spanning regions
IL-17R	Unknown	
Serine/threonine kinase	TGF β RI, TGF β RII	Forms heterodimers
Proteoglycan	TGF β RIII	
IL-2R α , IL-15R α		Structurally similar to CD35 ⁺ E-selectin

^aAlso forms complex with IL-13R.

^bBinds another ligand TSLP (murine only).

^cAlso uses IL-2R β chain.

^dAssociates with two forms of β chain in mouse (AIC2A/B).

^eAlso has Ig superfamily domains.

^fAdditional chain thought to exist.

IL-15 all utilize the γ_c chain. Members of a second group, containing the IL-3, IL-5 and GM-CSF receptors, all utilize the common β chain (KH-97/AIC2A/B), and those of a subfamily that contains the receptors for IL-6, IL-11, LIF, OSM, CNTF and CT-1 all share gp130. It is likely that the complexity of these receptor subfamilies may grow, as the IL-4R can associate with the IL-13R instead of the γ_c to form an alternative IL-4/IL-13 receptor.

Interferon receptor family (cytokine receptor class II)

A smaller family of receptors has been termed the interferon or cytokine receptor type II family, and includes the IFN α / β R, the IFN α R, the IFN γ R, the IFN γ R accessory protein and the IL-10R. This family is characterized by the presence of either one (IFN α / β R, IFN γ R, IFN γ R accessory protein, IL-10R) or two (IFN α R) homologous regions of 200 amino acids in the extracellular domain, each of

which contains two fibronectin type III domains. As with the class I family, these receptors have no integral enzymatic activity but can associate with the Janus family of tyrosine kinases. The IFN α / β R and the IFN α R form heterodimers, whereas the IFN γ R and the IL-10R homodimerize. In addition, the IFN γ R requires the IFN γ R accessory protein to form a functional receptor.

TNF receptor family

A third group is represented by the type I (p60/p55) and the type II (p75/p80) TNF/LT (lymphotoxin) receptors. This family also includes the NGFR (nerve growth factor receptor), CD27, CD30, CD40, the LT β receptor, OX40, 4-1BB and the Fas antigen. These proteins are characterized by the presence of three or four cysteine rich repeats of about 40 amino acids in their extracellular domains. With the exception of the two TNFRs and the NGFR, the other members of this family have not previously been

considered to be cytokine receptors, having ligands that themselves are expressed on the cell surface rather than being soluble proteins. In addition, the TNFRs, in particular the type II receptor, can also bind surface-bound TNF. A common feature of this family is that activation involves oligomerization. In the case of the TNFR this involves the homotrimerization of the receptor on binding of the trimeric ligand(s). Another feature of the TNFR is the presence of two distinct receptor forms that are degenerate for the binding of TNF and LT. As with the type I and type II cytokine receptor families, the TNF family has no intrinsic enzymatic activity and does not appear to associate with any of the known families of tyrosine kinases.

Immunoglobulin and receptor tyrosine kinase superfamily

The fourth group of receptors belong to the immunoglobulin (Ig) superfamily of proteins. These receptors have multiple Ig-like disulfide bridge-stabilized subdomains in their extracellular portion and include the p80 IL-1R (type I), the IL-1R-associated protein and the p60 IL-1R (type II), as well as the IL-6R (which also has features of the cytokine receptor class I family). The p80 IL-1R and the IL-1R-associated protein form active heterodimers. These receptors have no integral enzymatic activity.

Another group of receptors that also contain Ig-like structural domains in their extracellular region are a number of growth factor receptors that possess a tyrosine kinase domain in their cytoplasmic region. These include the PDGF (platelet-derived growth factor) receptor (two receptor forms α and β), FGFR-1, -2, -3, -4 (fibroblast growth factor receptor), CSF-1R (colony stimulating factor 1 receptor) and SCFR/c-kit (stem cell factor receptor). On ligand binding, receptor homodimer and heterodimer formation results in the activation of the tyrosine kinase.

Another group of receptors which contain tyrosine kinases includes the EGFR (epidermal growth factor receptor), which also binds TGF α , the insulin R, the insulin-like growth factor R and the neurotrophin R (three forms; trkA, trkB and trkC). These receptors do not have Ig-like domains in their extracellular region. Ligand binding induces receptor oligomerization and activation of the tyrosine kinase.

The chemokine receptors

The last major receptor family are the serpentine receptors. These molecules have seven transmembrane spanning regions and are the receptors for the chemotactic cytokines. Several receptors have been identified, including a high-affinity IL-8R, a low-

affinity IL-8R and a MIP-1 α receptor. There appears to be some degree of degeneracy in ligand binding by these receptors. For example, the low affinity IL-8R also binds the related GRO and NAP-2 chemokines, and the MIP-1 α receptor also binds RANTES. The erythrocyte Duffy antigen also has a serpentine structure and binds IL-8 and related chemokines. The cytoplasmic regions of this receptor family associates with heterotrimeric G proteins which are activated on ligand binding.

Other cytokine receptors

In addition to the cytokine receptor families featured above, there are a few cytokine receptors that fall outside of these categories. These include the α chains of the IL-2 and IL-15 receptors, which share certain structural features with each other and with CD35 and L-selectin. The TGF β (transforming growth factor) receptors I and II are similar to each other in that they have a serine/threonine kinase in their cytoplasmic domains. They are also related to the activin receptors. Lastly, the TGF β R type III has a proteoglycan-like structure. This receptor does not appear to be involved directly in signal transduction. The structural variety of the cytokine receptors is likely to increase further as the latest addition to the cytokine family, IL-17, also has a receptor unrelated to any detailed above.

Cytokine receptor signaling

For many classes of cytokine receptors the first detectable intracellular signaling events are the tyrosine phosphorylation of proteins (Figure 1). This tyrosine kinase activity is either provided by the integral tyrosine kinase domains of the receptors, as for the PDGFR and EGFRs, or by the recruitment to the receptor of intracellular kinases. For example, the src family of tyrosine kinases (lck, fyn, lyn, hck) are recruited by many members of the class I family, while the Janus tyrosine kinase family (Jak1, Jak2, Jak3, Tyk2) are recruited by receptors of both the class I and the class II families. Tyrosine kinase activation appears to occur as a consequence of receptor homo- or hetero-oligomerization and is often accompanied by the phosphorylation of tyrosine residues in the intracellular domain of both the receptors and kinases themselves. The kinases in turn phosphorylate and activate other intracellular enzymes, such as phosphatidylinositol-3' kinase or PLC γ . These enzymes are brought into the proximity of the tyrosine kinases by binding via their SH2 domains to the tyrosine phosphorylated receptors. Another class of proteins that binds to the phosphorylated receptors are the SH2 and PTB

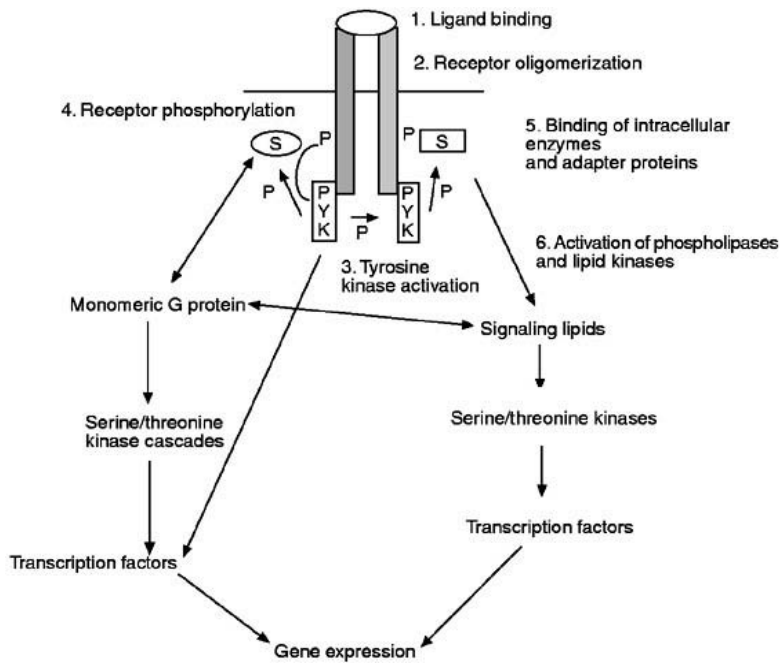


Figure 1 Signaling mechanisms induced by tyrosine kinase, class I and class II cytokine receptors. P, Tyrosine phosphorylation; PTK, protein tyrosine kinase.

containing 'adapter' proteins such as Shc and Grb2, which in turn bind other cellular proteins (which may become kinase substrates) either by SH2 or SH3 interactions (SH3 domains bind proteins with proline-rich sequences). A third class of proteins that can interact with tyrosine phosphorylated receptors are the STAT (1–6) (signal transduction and activation of transcription) proteins. The STATs are substrates for the Janus kinases and homodimerize or heterodimerize on phosphorylation, after which they migrate to the nucleus and bind DNA. The STATs also require serine/threonine phosphorylation for transactivational activity.

The activation mechanism of the TNF receptor family and the IL-1R is less clear: as yet, no tyrosine kinases have been shown to associate with these receptors. The cytoplasmic domain of the TNF receptor has been shown to associate with several proteins termed TRAP (TNF receptor-associated protein), TRAF 1, 2 and 3 (TNF receptor-associated factor), TRADD (TNF receptor-associated death domain) and FAD (Fas-associated death domain). The functions of these proteins is still under investigation, although the TRAFs and TRADD and FAD are involved in NF κ B activation and TNF-induced apoptosis. The chemokine receptors signal via the activation of receptor-associated trimeric G proteins where both the α subunit and the $\beta\gamma$ complex can have functional activity. Distal targets include PLC β and adenylate cyclase, the activation of which leads to increases in intracellular calcium and cAMP.

Distal events in cytokine signaling generally involve the activation of small monomeric G proteins

(e.g. p21 ras), the production of signaling lipids such as diacylglycerol (DAG), phosphatidylinositol-3' phosphates and ceramide (by the action of sphingomyelinase). The effectors of the monomeric G proteins and the various lipids are a number of serine/threonine kinases such as those within the PKC family, c-raf and others. Many of these kinases are organized into cascades, leading to the activation of p42/44, p38 and p54 mitogen-activated protein kinases (MAPKs). The substrates for many of these kinases are transcription factors such as ATF-2, c-Fos, c-Jun and Elk-2 which are activated as a result of phosphorylation. Other key kinases involved in cytokine signaling are the p70S6K and I κ B kinases (essential for the activation of NF κ B). However, the mechanism leading to the activation of these kinases remains largely obscure. It is clear that cytokine receptors are involved in the parallel activation of multiple signaling pathways with different functional end-points. The full elucidation of the relationship between the activation of the various pathways to the different functions of a given cytokine is a future challenge.

Cytokine receptors and disease

High levels of cytokine receptors have been observed on tumor cells. For example, human T cell lymphoma virus 1 (HTLV-1)-infected cells (acute cutaneous lymphoma) express high levels of the IL-2R α chain, whereas plasmacytomas have been found to express high levels of the IL-6R. Another intriguing area where cytokine receptors may play a role is in

viral pathogenicity. A number of homologs of cytokine receptors have been identified encoded in viral genomes, either as membrane-associated or as soluble-receptor forms. It is anticipated that by the inappropriate expression of virally encoded receptors, or the secretion of soluble receptors as potential inhibitors, the cytokine network can be manipulated to the advantage of the virus. A third area in which cytokine receptors are implicated is that of congenital hematopoietic disease. X-linked severe combined immunodeficiency disorder (X-SCID), for example, is the result of genetic defects in the expression and function of the γ c chain which results in a failure of B lymphocyte development. Furthermore, congenital defects in the expression of gp39/CD40 ligand are associated with X-linked hyper-IgM syndrome. Cytokine receptors have also been shown to have a key role in the pathophysiology of human immunodeficiency virus (HIV) infection. It has been shown that the chemokine receptors, CCR-5 and CCR-3, act as cofactors for virus entry into macrophages, whereas the LESTR/SDF-1 receptor is required for the infection of T cells. Moreover a naturally occurring mutation of CCR-5 appears to confer resistance to acquired immune deficiency syndrome (AIDS).

See also: **Cytokine inhibitors; Cytokine receptors, soluble; Cytokines; Enzyme-linked immunosorbent assay (ELISA); Granulocyte colony stimulating factor (G-CSF); Granulocyte-macrophage colony stimulating factor (GM-CSF); Human immunodeficiency viruses; Immunoassays; Interferon γ ; Interleukin 1 and its receptors; Interleukin 2; Interleukin 3; Interleukin 3 (IL-3) receptor; Interleukin 4; Interleukin 5 and its receptor; Interleukin 6; Interleukin 7 and its receptor; Interleukin 8 and its receptor; Interleukin 9**

and its receptor; Interleukin 10 and its receptor; Interleukin 11 and its receptor; Interleukin 12 and its receptor; Interleukin 13 and its receptor; Interleukin 15 and its receptor; Transforming growth factor β (TGF β); Tumor necrosis factor α .

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CYTOKINE RECEPTORS, SOLUBLE

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Cell-bound and soluble forms of ligands and receptors

The term 'soluble receptors' (SRs) is an apparent paradox. Originating from the field of hormone research, the terms 'receptors' and 'ligands' initially stood for features that are peculiar to the endocrine mode of intercellular communication. In this mode, the communicating cells play different roles; cells of

the endocrine gland serve as the source of the message and the cells of the target tissue respond to it. Accordingly, the ligands and the receptors that participate in this communication also have distinct features, the ligand molecules serving solely to transfer the information and thus occurring only in a soluble, secreted form, and the receptors acting solely for receiving this information and thus, by definition, occurring solely in association with the target cell.

With the increasing knowledge of the cytokines and the ways in which they act, it became clear that in most cases transfer of molecular information does not occur, as in endocrine regulation, between cells that serve solely an effector or a target role. In fact, all nucleated cells serve both as producers of cytokines and as targets for their action. Nor is the transfer of molecular information restricted only to interaction between remote cells. Much of the action of cytokines occurs in close proximity to their site of formation, namely, in autocrine, juxtacrine and paracrine ways. The physical states and functional roles of receptor and ligand molecules may therefore be quite different from those that, prior to the era of cytokine research, were considered as definitive. Both receptors and ligands can occur in cell-bound as well as in soluble forms. Moreover, some cell-bound molecules have the ability to function at the same time both as receptors and as ligands. Their binding to receptors expressed on other cells results both in triggering of signaling by the latter receptors and in triggering of signals that they themselves produce (reversed signaling; illustrated in Figure 1(2)).

Another former distinction which we now view

differently concerns the functional significance of the various regions in the receptor molecule. According to the initial concepts of the way in which receptors act, their extracellular domains appeared to serve merely a subsidiary role to that of the signaling intracellular domains. The discovery of the SRs highlighted the fact that recognition of the ligand by the extracellular domain can have some functional consequences that are independent of the function of other regions in the receptor. In a few cases, this independent role of the extracellular domain can be observed even when it is still cell bound. The extracellular domain of the type II interleukin 1 (IL-1) receptor, for example, has been shown to act as a 'decoy receptor'; that is to say, it serves solely as a means of restricting the availability of IL-1 to the other (type I) IL-1 receptor, thereby decreasing signaling by it. Another example, opposite in nature, is an apparent ability of the extracellular domain of CD120b (the p75 tumor necrosis factor receptor, TNF-R) to assist binding of TNF to coexpressed CD120a molecules ('ligand passing'). However, most of our knowledge of such 'extracellular domain-specific' functions concerns SRs.

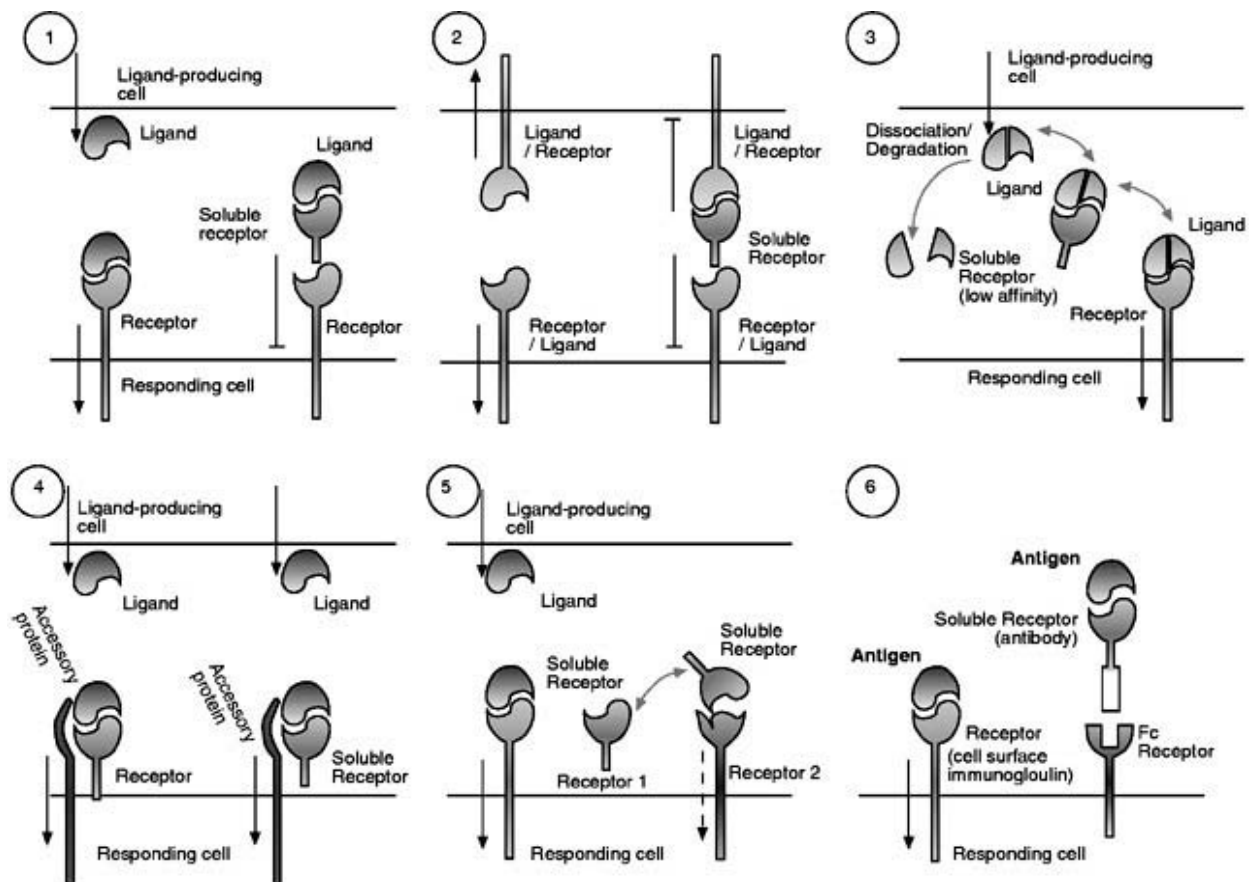


Figure 1 Structural forms and modes of action that can be attained by cell-bound and soluble forms of receptors and ligands.

In a wide sense, the term 'soluble receptors' also relates to the antibodies, whose ligand-binding portion can occur both in a soluble form (immunoglobulin, Ig) and in association with cells (the cell-surface immunoglobulins in B cells). Indeed, it was while attempting to describe the mechanisms by which antibodies are formed that Paul Erlich first suggested, about a hundred years ago, that cells are able to produce ligand-binding molecules both in soluble and in cell-associated forms (Figure 2).

Receptors known to occur in soluble form

A partial list of the receptors that are known to exist in soluble form is presented in Table 1. Some of these receptors were identified in body fluids or in the growth media of cultured cells. Others, for which specific messages exist (see below), were identified by cloning of their cDNAs. A unique example of receptors that exist only in soluble form are the 'viroceptors' – coding regions for soluble forms of cytokine receptors – found in certain pox viruses that have apparently usurped these sequences from host cells. All known SRs are derived either from single-transmembrane domain proteins or from glycosylphosphatidylinositol (GPI)-anchored cell surface proteins. Other than that, there are no evident structural or functional features common to these receptors.

Structure and mechanisms of formation of soluble receptors

The SRs whose cell-bound forms are transmembrane proteins can be formed in one of the following two ways: 1) proteolytic cleavage of the cell surface form; 2) transcription of a distinct mRNA derived by alternative splicing from the gene that encodes the cell surface receptor. Some of these receptors are formed in both ways, while the *c-kit* receptor (as well

as its ligand, the stem cell factor, which also occurs in both cell-bound and soluble forms) provides an example of a composite mechanism. It is derived proteolytically from a particular splice variant of the cell surface form in which one of the exons, which is lacking in the other splice variants, codes for the proteolytic cleavage site. Soluble forms of GPI-anchored receptors are produced by escaping the post-translational modification (removal of the signal peptide) required for anchorage, and perhaps also by phospholipase C-mediated cleavage of the anchor. They can also be formed by proteolytic cleavage of their polypeptide chain.

Traces of the mechanism by which the SRs are formed can sometimes be discerned in their amino acid sequences. Those SRs derived proteolytically from the cell surface receptors have exactly the same amino acid sequences as those found in the extracellular domains of the cell surface receptors (upstream of the site of proteolytic cleavage). On the other hand, SRs encoded by distinct messages can have additional, unique sequences, downstream of the site of alternative splicing, resulting from alteration of the reading frame. This unique sequence is usually short and nonfunctional. The only known exceptions are the antibodies, whose unique C-terminal parts (The Fc regions) are encoded by exons that are different from those found in the message for the cell surface immunoglobulins and serve unique and important functions. The viroceptors also contain regions of unique sequence downstream of the region homologous to that of the cell-bound receptors. In some cases self association of these regions results in formation of dimers of the viroceptors. Such dimers have the ability to bind to the ligand more effectively than the monomeric molecules.

The cleavage process by which SRs are formed was shown to occur following transport of the cell-bound receptor to the cell surface. In some of these cases

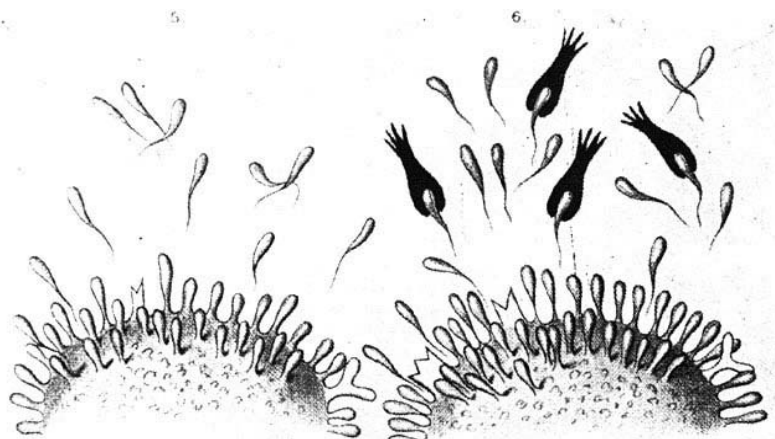


Figure 2 Free side-chains (circulating antitoxins) unite with the toxins and thus protect the cell. (Reproduced from Ehrlich P (1900) On immunity with special reference to cell life. *Proceedings of the Royal Society*.)

the proteases involved are soluble and are found in the extracellular milieu (e.g. elastase, released by activated granulocytes). In others, however, they are cell associated. Inhibitory effects of hydroxamic acid inhibitors on the shedding of several receptors pointed to the involvement of metalloproteases. The enzyme responsible for the shedding of TNF α (TACE) was recently cloned, and indeed found to be a metalloproteinase-disintegrin of the adamalysin family. To date, however, no other 'sheddase' has been identified, nor is there any knowledge of the specificity of action of these enzymes, except that their function seems to be restricted by some conformational features of the membrane-proximal region of the extracellular domain at which the cleavage occurs.

Both the proteolytic production of SRs and their formation as distinct splice variants can occur in an inducible manner. Often the ligand to which the soluble receptors bind serves to induce their formation, allowing the SRs to act as a feedback control mechanism.

Functions of soluble receptors

Current ideas of the function of SRs are largely speculative, being based primarily on *in vitro* effects of these molecules and only to a small extent on direct information about their *in vivo* effects.

In vitro effects

In vitro studies of SRs have revealed several different potential roles of these molecules.

1. SRs that may act as inhibitors of signaling (Figure 1(1)) All SRs bind their ligands with lower affinity than that of their cell surface forms. The binding affinity of some, for example the soluble IL-2R α , is rather low, raising doubts as to their ability to affect significantly the function of the cell surface receptors. Other SRs, however (e.g. the receptors for IL-1, IL-4 or TNF), have binding affinities high enough to allow them to compete effectively with the cell surface receptors for the ligands. Except in cases where the SRs themselves have the ability to act as initiators of signaling (see below), binding of the ligand to such SRs is expected to result in blockage of signaling.

2. SRs that may act as inhibitors of reversed signaling (Figure 1(2)) Although not documented so far, it is possible in theory that the SRs may also act as inhibitors of 'reversed signaling'. As illustrated in Figure 1(2), in the case of cell surface ligand molecules that exhibit reversed signaling (when aggre-

gated as a consequence of their binding to the cell surface receptors), binding of the monomeric SR molecules to the ligand may impede the ligand's interaction with the cell surface receptors and thus block signaling.

3. SRs that may assist the function of their ligands (Figure 1(3)) IL-4 dissociates from its SR much more rapidly than from its cell surface receptors. The soluble IL-4 receptors also provide IL-4 with protection against proteolytic degradation. Therefore, although at high concentrations these receptors can interfere with IL-4 function, it seems likely that at their physiological levels the soluble IL-4 receptors actually serve as carriers of IL-4 in the circulation and facilitate its presentation to the cell-bound receptors. A similar role was ascribed to the soluble form of the growth hormone receptor (the growth hormone-binding protein). This SR is believed to increase the functional duration of its ligand by withholding its clearance from the circulation. The soluble TNF receptors are also found to increase the functional duration of their ligands, though by a different mechanism: they prevent dissociation of the trimeric TNF molecules into inactive monomers and thus, despite their ability to compete with the cell surface TNF receptors for TNF binding, they augment those TNF effects that are slowly induced. The fact that SRs can have such positive effects on ligand function, as well as the consistent occurrence of some SRs in body fluids and the existence of mechanisms that coordinate the extent of SR formation with that of their ligands, suggests that some SRs may play an integral role (as 'buffers' or carriers) in the normal functioning of their ligands.

4. SRs that can substitute for the cell-surface receptors (Figure 1(4)) The α chain of the IL-6 receptor is devoid of signaling activity. After ligand binding, however, it associates with another transmembrane protein, gp130 (which constitutes the β chain of the IL-6 receptor as well as of other receptors of the IL-6 family); and the latter initiates signaling within the cell. In this case, the soluble forms of the two components of the receptors affect ligand function in opposite ways. The soluble form of gp130 blocks the function of IL-6 (as well as of the other cytokines that employ gp130 as part of their receptors). The soluble form of the α chain of the IL-6 receptor, however, can substitute for the cell surface receptor. The situation is similar in the case of the CNTF-R and CD14, a cell-surface lipopolysaccharide (LPS)-binding protein. Application of the soluble forms of the IL-6 receptor α chain, CNTF-R or CD14 to cells that do not express the cell surface forms of the

Table 1 Examples of known soluble forms of receptors, their presumed functions and mechanisms of formation

Receptor	Structural motifs in extracellular domain (receptor family)	Function of cell surface form	Presumed function	Soluble form		
				Proteolytic cleavage	Lack of GPI anchorage/PLC cleavage of GPI	Specific transcripts allowing cleavage
IL-1, type 1-R	Immunoglobulin-like domain	Inflammatory and acute phase reactions. Thymocyte and T cell activation	Blocks ligand binding	+		
IL-1, type II-R	Immunoglobulin-like domain	'Decoy' receptor (blocks ligand binding to coexpressed type I receptor)	Blocks ligand binding	+		
IL-2R α	Two CCP domains	T and B cell growth, macrophage activation	Blocks ligand binding	+		
IL-2R β	Cytokine receptor and fibronectin type II domains	T and B cell growth, macrophage and natural killer activation	Blocks ligand binding	+		
IL-4R	Cytokine receptor and fibronectin type II domains	Regulation of allergic reactions, IgG1 and IgE responses, growth and differentiation of hematopoietic cells, suppression of macrophage-mediated immune functions	Blocks ligand binding? 'Transport' molecule?		+	
IL-5R α	Cytokine receptor and fibronectin type II domains	Growth and differentiation of eosinophils and B and T lymphocytes	Blocks ligand binding		+	
IL-6R α	Immunoglobulin-like, cytokine receptor, and fibronectin type II domains	Acute phase protein induction, stimulation of growth of activated mature B lymphocytes, regulation of inflammation	Signals (substitutes for the cell surface receptor)	+		
gp130 (IL-6R β)	Immunoglobulin-like, cytokine receptor domain, cytokine receptor motif and four fibronectin type III domains	Acute phase protein induction, regulation of growth and differentiation of cells through effects of the various IL-6 subfamily receptors	Blocks ligand binding to the various IL-6 subfamily receptors	+		
Ciliary neurotrophic factor (CNTF)-R α	Class I cytokine receptor, GPI anchored	Growth and differentiation of cells of the neuronal system	Blocks ligand binding (substitution for the cell surface receptor)		+	
Growth hormone-R	Class I cytokine receptor	Bone growth, lactogenic, insulin-like, diabetogenic, lipolytic, protein-anabolic and sodium/water-retaining effects	Decreases rate of clearance of the ligand from the circulation	+		+

TNF, p55R (CD120a)	TNF/NGF receptor family	Signaling for cell death, growth, various inflammatory and immune functions	Blocks ligand binding; stabilizes ligand	+	
TNF, p75R (CD120b)	TNF/NGF receptor family	'Ligand passing'? (assists ligand binding to CD120a?) Cell death, growth, various inflammatory and immune functions	Blocks ligand binding (less effectively than the soluble p55-R); stabilizes ligand	+	
Fas/Apo-1 (CD95)	TNF/NGF receptor family	Cell death, growth, various inflammatory and immune functions	Blocks ligand binding	+	
NGF, p75R	TNF/NGF receptor family	Growth, differentiation and death, particularly in cells of the neuronal system	?	+	
IFN α -R (IFNAR-2)	Class II cytokine receptor	Antiviral and other defense mechanisms	Blocks ligand binding	+	
IFN γ -R	Class II cytokine receptor	Antiviral and other defense mechanisms, mainly related to interactions with cells of the immune system and among them	Blocks ligand binding	?	
CD14	Leucine-rich repeats, GPI anchored	Receptor for the complex LPS-LPS binding protein	Signals (substitutes for the cell surface receptor)	+	
CD23	Type II transmembrane, homologous to C-type animal lectins	Low-affinity IgE receptor, mediates IgE-dependent cytotoxicity and phagocytosis by macrophages and eosinophils	Acts like a cytokine with pleiotropic effects: regulates IgE production, affects macrophage activation through cytokine formation and various aspects of hematopoiesis	+	+
c-Kit	Immunoglobulin gene family	Proliferation, differentiation and survival of several hematopoietic lineages, mast cell activation	Blocks ligand binding		
T2	Viroceptor, homologous to CD120b, C-terminal dimerization region		Blocks binding of TNF α and LT α	+	
CrmC	Viroceptor, homologous to CD120b, without C-terminal dimerization region		Blocks binding of TNF α but not of LT α	+	
v-CD30	Viroceptor, homologous to CD30		Blocks binding of the CD30 ligand	+	

receptors can endow them with responsiveness to IL-6, CNTF or LPS.

5. Receptors that gain novel ligand activity upon their shedding (Figure 1(5)) Such a situation seems to exist in the case of the soluble form of CD23. In its cell-bound form, this type II transmembrane protein acts as a low-affinity receptor for IgE and also binds a cell surface protein, CD21. In its soluble form, CD23 was reported to have a variety of cytokine effects on cells. Some seem to be due to triggering of 'reversed signaling' by CD21. Others, however, seem to be mediated by another, as yet undefined, receptor. The cytokine effects of the soluble CD23 molecules are mediated by an epitope distinct from the IgE-binding site.

6. Receptors which, when in soluble form, can serve functions unrelated to signaling (Figure 1(6)) The only known example of SRs that act in this way is provided by the immunoglobulins. In their cell surface form, these molecules initiate signaling activities within the cells that express the receptors (B lymphocytes). In their soluble form, on the other hand, the immunoglobulins modulate the function of the antigens to which they bind by neutralizing or opsonizing them, by facilitating their transport, or – in the case of antigens that are expressed on the surface of cells – by causing their destruction (through effects of complement or of antibody-dependent cellular cytotoxicity). These modulatory functions are exerted through binding activities of C-terminal regions unique to the soluble immunoglobulins (the Fc region). An example of the unique binding ability of the Fc region is seen in its ability to bind to specific Fc receptors.

***In vivo* function**

As mentioned above, there is still hardly any direct information on the *in vivo* significance of the SRs. In the absence of such information, one cannot exclude the possibilities that at least some of the SRs have no functional significance at all, or (in the case of SRs that are derived proteolytically from the cell-bound forms) are merely side-products of the down-regulation of cell surface receptors upon their cleavage. A major obstacle to objective evaluation of the functional significance of SRs is the lack of technical tools for evaluating the impact of specific elimination of these molecules. Use of knockout mice or neutralizing antibodies, which have proved very useful in evaluating the *in vivo* relevance of the cytokines, cannot be applied in studies of the function of their SRs unless a way is found to maintain the function of the cell surface receptors while eliminating or

blocking the soluble forms. There are, however, a few noteworthy items of indirect evidence which suggest that SRs have important functions *in vivo*:

1. Assessment of the effect of human sera on the *in vitro* function of TNF confirmed that the soluble TNF receptors reach serum levels that are high enough to effectively suppress TNF effects (at the TNF concentrations attained in the serum).
2. Deletion of the genes that encode viroreceptors resulted in significant reduction of the pathogenesis of the viruses that harbor these genes, confirming that the viroreceptors act to suppress immunoprotective effects of cytokines.
3. As mentioned above, the soluble IL-6 receptor can substitute for the cell surface IL-6 receptor. The physiological significance of this mode of action of SRs is supported by findings about the structure of IL-12. This cytokine is composed of two subunits that show considerable sequence similarity to IL-6 and its soluble receptor, and probably act together in a way analogous to that of the IL-6–IL-6 SR complex.

Therapeutic implications

While there is only limited evidence for the physiological significance of effects of SRs observed *in vitro*, there is extensive documentation that, by injecting sufficient amounts of SRs, it is possible to reproduce artificially *in vivo* at least one of the effects that SRs can mediate *in vitro*, namely, blocking of signaling. Use of chimeric soluble receptor-Fc regions ('immunoadhesins'), or of SRs coupled to polyethylene glycol, whose clearance rate is lower than that of the natural SRs, allows prolonged maintenance of these molecules in the body and, in the case of the soluble receptors for TNF, more effective binding to the ligand (by virtue of the ability of the dimeric SRs to bind simultaneously to two protomers of the trimeric TNF molecules). Ongoing clinical trials demonstrate effective therapeutic effects of SRs in humans. Blocking of TNF function by its SRs, for example, has been found to be highly beneficial in rheumatoid arthritis and in chronic inflammatory bowel diseases.

Diagnostic implications

Data acquired in multiple studies of the *in vivo* levels of various SRs in health and disease, as well as following injection of cytokines, indicate that, as in cultured cells, formation of the SRs in the body is inducible by various stimulating agents. These data, particularly the evidence indicating that formation of several SRs is most strongly induced by the ligands

to which they bind, are consistent with the notion that formation of the SRs constitutes a physiological mode of regulation of response. This evidence prompted investigators to employ the determination of the levels of various SRs in body fluids as a diagnostic tool for assessing disease activity and for examining the involvement of the ligand of the SR in the particular pathological situation. Information gained in this way can be particularly useful in the case of SRs that have pronounced effects on the function of their ligands. Probably the most informative diagnostic parameter in such cases is the quantitative ratio of the SRs and their ligands. Indeed, there are a few reports demonstrating correlation between the severity of a particular pathological situation to which a given cytokine contributes and the occurrence of an abnormally low quantitative ratio of SR and ligand levels in the serum.

Various cancer cells are known to shed some of their cell surface proteins far more rapidly than do their normal counterparts. This may be due to an increased expression by tumor cells of some proteases capable of cleaving these cell surface receptors. Because of this particular feature of tumor cells, and perhaps also because of the activation of certain immune cells in cancer patients, their sera exhibit increased levels of certain SRs (e.g. the soluble TNF receptors, or the carcinoembryonic antigen, which is a shed form of a GPI-anchored cell surface protein). Determination of the levels of these receptors can thus be of diagnostic value not only in inflammatory and infectious diseases but also in cancer.

See also: Antibodies, synthesis; Cytokine inhibitors; Cytokines; Cytokine receptors; Fas (CD95) and fas ligand; Fc receptors; Immunoglobulin, cell surface; Immunoglobulin structure; Interferon γ receptor; Interleukin 2 receptor; Interleukin 4 receptor; Interleukin 5 and its receptor; Interleukin 6 receptor; TNF receptors.

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CYTOKINES

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Nomenclature

Low molecular weight protein mediators involved in cell growth, inflammation, immunity, differentiation and repair are now generically termed 'cytokines'. There have been many previous attempts at a suitable nomenclature, but 'cytokines' is the current most widely used compromise. Previous attempts include 'lymphokines', 'monokines' and 'interleukins'. 'Lymphokines' dates from the 1970s, for products of lymphocytes, designed to contrast with 'monokines' for products of monocytes. However, it is now known that there is considerable overlap – for example, tumor necrosis factor α (TNF α), interleukin 6 (IL-6) are made by both cell types – so this nomenclature is no longer in wide use.

'Interleukins' describes molecular messengers acting between leukocytes. The term dates from 1979, at a conference in Switzerland where it became apparent that a variety of biological activities termed 'lymphocyte-activating factor', 'endogenous pyrogen', 'catabolin' and so on, were due to molecules with similar biochemical characteristics, and the term 'interleukin 1' was adopted. Currently there are 17 molecules with a wide spectrum of activities, designated interleukins 1–17. However, there are deficiencies in this nomenclature; it is becoming abundantly clear that these molecules are often made by nonblood cells, and act on cells apart from blood cells, e.g. IL-6, IL-1.

Two other related groups of molecules have been

described: 'interferons' and 'growth factors'. 'Interferons' are molecules first described in the 1960s as protein, with nonspecific anti-viral effects. These are now known to have many other effects; thus interferon γ (IFN γ) is one of the most potent immunoregulatory mediators known; all interferons have immunoregulatory and cytostatic as well as antiviral actions. 'Growth factors' are molecules which stimulate the growth of a variety of cells. Some are more specific than others, for example granulocyte-macrophage colony-stimulating factor (GM-CSF) acts on monocytes/macrophages and granulocyte cells to promote their growth, while platelet-derived growth factor (PDGF), which is three related molecules, acts on most connective tissue cells. Hematopoietic growth factors, also termed 'colony-stimulating factors' from their initial bioassays, are, in common with the other molecules in this category, not restricted to mediating growth. They are all also involved in regulating cellular activation. Thus 'growth factors' is not an accurate terminology either.

There is a large family of chemotactic cytokines of low molecular weight (8–10 kDa) which are now termed 'chemokines'. These include IL-8, MCP-1, etc, and are grouped into two major families, depending on the position of cysteine, as C-C or C-X-C chemokines. The latter act chiefly on granulocytes, the former on monocytes and lymphocytes.

Overview of cytokine action

Unlike hormones, which are carried by the bloodstream over the whole body, cytokines are chiefly involved in local effects. Some do have systemic effects, IL-6 being the most notable, but the major physiological role of cytokines is short range, within a few cell diameters. Most cytokines are not constitutively produced, but are produced after activation of the cells. Production after activation is short lived, usually for only a few days. Typically, cytokines are effective in the picogram (pg) to nanogram (ng) per milliliter range. Studies with cytokines produced by recombinant DNA technology have shown that their half-life in the circulation is brief, reinforcing the belief that systemic distribution is not part of their normal physiology. Unlike hormones, most cytokines are not produced by specialized cells, and probably all cells produce some cytokines. T cells and macrophages are particularly rich sources of cytokines.

There is no consensus structure for cytokines. Most are glycosylated, but not all (e.g. IFN α/β , TNF α); many are in the 10–25 kDa mass range. Some cytokines are members of a family, e.g. IL-1 α

and β and the IL-1 receptor antagonist, IFN α (~15 molecules), TNF α and TNF β (also known as lymphotoxin). The family relationship is usually clear-cut if the members of the family bind to the same receptor, e.g. IL-1 α and β . Amino acid sequence homology sufficient to permit binding to a common receptor need not be very high: of the order of 30% suffices for IL-1 α and β . Recently a third member of this family, the IL-1 receptor antagonist protein (IL-1ra), has been described, which binds to the same receptor but has antagonistic effects. Should IL-1ra be considered a cytokine? If it does not activate the IL-1 receptor, most would consider it is not a cytokine but the first member of the cytokine antagonist family.

Cytokines typically induce multiple actions, on different cells. This property is often termed 'pleiotropy'. Different cytokines, e.g. IL-1 and TNF α , have closely related and overlapping actions; this is sometimes termed 'redundancy'. This is an inappropriate term, as gene knockout and antibody neutralization studies have shown that cytokines have distinct roles.

Cytokines mediate their effects after binding to high-affinity receptors on the surface of the target cell. The affinity of these receptors is generally very high, in the 10^{-9} – 10^{-11} molar range, which accounts for their potency. Receptors are usually present in low numbers (100–1000 cell $^{-1}$), but with their high affinity this clearly suffices. Increased knowledge of cytokine receptors has recently emerged from biochemical and molecular cloning studies. The cytokine-binding molecules have been described for most cytokines, nevertheless for the majority of cytokines it is not clear how signal transduction is mediated. Exceptions include the receptor for CSF-1 (M-CSF) and the PDGF receptor, which are tyrosine kinases. For the other receptors, it is becoming evident that they are multichain complexes, which assemble cytoplasmic signaling molecules upon ligand binding.

Cytokine receptors are subject to a variable degree of upregulation upon cell activation. For the IL-2 receptor α chain (p55 or Tac antigen) this is considerable, from ~0 to 10–50 000 per activated T cell in 3 days. This upregulation is usually transient, lasting only a few days in the absence of further stimulation. Upon interaction with their ligand, cytokine receptors are typically internalized, taking the ligand with them. Receptors become re-expressed after a few hours delay. Thus, after cytokine exposure, cytokine receptor expression varies considerably, initially diminishing due to internalization and then increasing due to cytokine-induced receptor upregulation.

For some cytokines, e.g. IL-1 and TNF, there are two distinct binding chains, expressed differentially on different cell types. The two TNF receptors have

Table 1 Properties of cytokines

<i>Cytokine</i>	<i>Biochemistry (kDa)</i>	<i>Properties</i>
IL-1 α	17, nonglycosylated	Activates lymphocytes, acute phase response, strongly proinflammatory, radioprotective
IL-1 β	17, nonglycosylated	
IL-2	15, glycosylated	Activator of T, natural killer (NK), B, macrophages: 'T cell growth factor'
IL-3	15, glycosylated	Multiple actions, hematopoietic cell growth – precursors of mast cells
IL-4	20, glycosylated	B cell activating factor, T cell activator, growth and stimulating factor
IL-5	45, glycosylated	B cell growth and differentiation factor, regulates production of granulocytes and macrophages
IL-6	26, glycosylated	B cell differentiating factor, activation of T and B cells, induces acute phase response
IL-7	25, glycosylated	Pre-B and pre-T cell growth factor, T cell growth factor
IL-8	8–10, family of related proteins	Involved in migration of neutrophils and T cells
IL-9	35, glycosylated	Supports growth of mast cell progenitors
TNF α	17, nonglycosylated, functions as a trimer	Proinflammatory cytokine, induces acute phase response, thrombosis, cachexia
Lymphotoxin (TNF β)	25, glycosylated, functions as a trimer	Proinflammatory cytokine, induces acute phase response, thrombosis, cachexia
IL-10	17, nonglycosylated (in humans), functions as a dimer	Inhibitor of monocyte and T cell function, activator of B cells
IL-11	23, nonglycosylated	Growth factor for plasmacytomas, hematopoietic multipotential and committed megakaryocytic and macrophage progenitor cells
IL-12	75 (heterodimer of 35 and 40 subunits), glycosylated	Induces IFN γ production by T and NK cells, enhances NK and antibody-dependent cell-mediated cytotoxicity, induces T $_H$ 1 subset development
IL-13	17, glycosylated	Inhibits monocyte function, activates B cells
IL-15	14, glycosylated	Activates T and LAK cells and gut epithelium
IL-16	14, functions as a tetramer	Chemoattractant for eosinophils and CD4 $^+$ lymphocytes
IL-17	20, glycosylated	Proinflammatory cytokine
IL-18	18	Enhances NK cytotoxicity, induces IFN γ production
IFN γ	17, glycosylated, functions as a dimer	Induces MHC class I and II, upregulates antigen presentation, macrophage activator, antiviral, antiproliferative, inhibits T $_H$ 2 responses

homologous binding domains, but nonhomologous cytoplasmic domains, suggesting a different mechanism of signal transduction, and probably different functions.

Cytokine receptors appear to have evolved in families. The largest of these is termed the 'hematopoietin' family, and includes the erythropoietin receptor, IL-2R p75 (β chain), and receptors for GM-CSF, IL-3, IL-4 and IL-7, etc. The extracellular domains of these receptors all contain conserved cysteines, as well as a Trp-Ser-X-Trp-Ser motif near to the transmembrane region. The two TNF receptors, nerve growth factor receptors and lymphocyte surface antigens (CD27, CD30, CD40 and OX40 Fas) belong to another family. The extracellular region of these molecules contain a number of repeated cysteine-rich 40 amino acid domains.

How is cytokine action controlled?

Since cytokines have such potent effects, it is clearly important that their action be limited to avoid the pathogenic effects of cytokine overdose. This subject is only beginning to be understood. For IL-1 there is a receptor antagonist which interferes with IL-1 binding to its receptors, but by far the most common inhibitors of cytokines appear to be truncated forms of the extracellular domain of the receptors. Such molecules have been demonstrated for IL-1 (type II), IL-2 (p55), IL-4, IL-7, TNF-R (both chains) and IFN γ , and are not limited to cytokines; similar molecules are also found for hormones, e.g. growth hormone receptor and IGF-1 receptor and adhesion molecules. There is also a soluble extracellular domain of the IL-6 receptor, but it is costimulatory as

opposed to inhibitory. The current concept is that circulating cytokine inhibitors prevent small concentrations of cytokines from exerting prolonged or diffuse effects. It is likely that the balance of cytokine-to-cytokine inhibitor will be of paramount importance in determining whether cytokines exert physiological or pathological effects. There is now clear-cut evidence that excess TNF α signaling is involved in the pathogenesis of rheumatoid arthritis.

Summary

Table 1 summarizes the main properties of the best known cytokines.

See also: **Colony stimulating factors; Cytokine assays; Cytokines; Eosinophil chemotactic factors; Granulocyte colony stimulating factor (G-CSF); Granulocyte-macrophage colony stimulating factor (GM-CSF); Interferon α ; Interferon γ ; Interleukin 1 and its receptors; Interleukin 2; Interleukin 3; Interleukin 4;**

Interleukin 5 and its receptor; Interleukin 6; Interleukin 7 and its receptor; Interleukin 8 and its receptor; Interleukin 10 and its receptor; Interleukin 11 and its receptor; Interleukin 12 and its receptor; Interleukin 13 and its receptor; Interleukin 15 and its receptor; Macrophage colony stimulating factor (CSF-1); Macrophage migration inhibitory factor (MIF); Monocyte chemotactic protein 1 (MCP-1); Platelet-activating factor (PAF); Transforming growth factor β (TGF β); Tumor necrosis factor α ; Lymphotoxin.

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CYTOMEGALOVIRUS, INFECTION AND IMMUNITY

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Cytomegalovirus (CMV) is a common infection which usually only causes damage if the host immune response is immature or compromised. It is thus an important pathogen to the fetus, to allograft recipients and to those with the acquired immune deficiency syndrome (AIDS).

Characteristics of the organism and its antigens

Cytomegalovirus is a typical herpesvirus. It contains double-stranded DNA of 2.4×10^5 base pairs which makes it the largest virus known to infect humans in terms of genome size. The proteins are expressed in an infected cell in an ordered cascade sequence termed α (immediate early), β (early) or γ (late). Typically, they function as regulators of cell metabolism, enzymes and structural proteins of the virus, respectively. Many of the primary translation products are modified by glycosylation, cleavage or phosphorylation. Chee and colleagues have analyzed the complete sequence of one strain of CMV which encodes approximately 202 proteins. Recently, an additional 22 open reading frames have been

described in one or both of two further strains of CMV.

Approximately 25% of the total open reading frames can be assigned functions, with many more having sequence homologies to known proteins. The surface structures which may be important for immunity exist as high molecular weight glycoprotein complexes which have been termed gCI, gCII and gCIII. The gCI complexes have also been termed gB because they have homology with herpes simplex virus (HSV) glycoprotein B, while the gCIII complexes are called gH. Like HSV, the gH of CMV requires gL for its expression.

A characteristic feature of the virus is its ability to establish latency for the life of the individual. There are probably multiple sites the virus can reactivate from at multiple occasions. It often does so when the patient is otherwise debilitated, i.e. CMV acts as an opportunist.

Immune responses of the host

Cell-mediated immunity

Cytotoxic T lymphocytes are predominantly directed

against structural phosphoproteins of the virus which are revealed immediately CMV enters the cell. It can be inferred that cell mediated immunity (CMI) is the major means of controlling CMV replication, as CMV is a major pathogen in groups of patients whose CMI responses are depressed. In the murine model of CMV, natural killer cells have also been shown to be important. Genetic investigations have mapped susceptibility to infection to the class I loci of the major histocompatibility complex (MHC), which probably represents an immunologic phenomenon rather than a receptor interaction as postulated in the past.

Humoral immunity

Some of the humoral responses to viral proteins can be documented by western blot or immunoprecipitation. Two surface glycoproteins, gB and gH, have so far been shown to have neutralizing domains.

Note that the combined humoral and CMI responses of the host are not sufficient to prevent reactivation of latent virus nor to protect against reinfection from exogenous sources. However, there is increasing evidence that humoral immunity may ameliorate disease.

Immunopathology

There is considerable evidence from both mice and humans to suggest that CMV pneumonitis is an immunopathologic condition. In addition, CMV is associated with infection of solid organ grafts and with graft-versus-host disease after bone marrow transplantation. One possibly causal mechanism is that CMV upregulates adhesion molecules.

Evasive strategies by the organism

1. Latency. This probably allows no or very minimal expression of virion proteins which could be presented as peptides in the context of MHC class I molecules. The cell with latent CMV genome would therefore be undetectable by circulating cytotoxic T cells.
2. The virus can spread by cell-to-cell fusion, so avoiding an extracellular phase where it may be susceptible to neutralization by antibody.
3. Murine and human CMVs decrease surface display of mature HLA complexes as a final common pathway for avoiding cytotoxic T lymphocyte (CTL) recognition. The methods are complex and multiple. For human CMV, these include the input UL83 (pp65) protein phosphorylating the major immediate-early protein and so preventing its presentation. At

immediate-early times, the US3 gene product retains Class I molecules in the endoplasmic reticulum (ER). At early times, gene products US2 and US11 re-import Class I molecules from the ER back into the cytosol where they are degraded. At early/late times, gene product US6 blocks peptide transport into the ER. At late times, gene UL18 (which encodes a Class I-like protein) is displayed to decoy NK cells which would otherwise destroy a cell lacking Class I molecules.

4. CMV is generally accepted to cause 'immunosuppression' in clinical practice and is associated with bacterial or fungal infections. While the mechanism(s) remain undefined, this may decrease the host's ability to control CMV infection.

Vaccines

One live attenuated vaccine strain (Towne) was subjected to a well-conducted placebo-controlled trial carried out by Brayman and colleagues. Unfortunately, the vaccine had no effect on the incidence of CMV infection or CMV disease, although the severity of disease was reduced. Due to the theoretical risks of the DNA from this virus containing transforming regions, further studies with this vaccine outside high-risk populations are unlikely.

A recombinant gB vaccine has proceeded to phase I clinical trials and is well tolerated. Seronegative volunteers seroconverted and seropositive volunteers had significant increases in antibody levels. The vaccine may be tested in transplant patients and may be used to immunize normal blood donors to produce specific immunoglobulin.

There is evidence for adoptive transfer of immunity following bone marrow transplantation from immune donors. CD8⁺ CTL clones isolated from the marrow donor can be reinfused safely into bone marrow recipients and may provide protection against CMV disease in the critical early weeks after transplantation.

See also: Acquired immune deficiency syndrome (AIDS); Adenovirus, infection and immunity; Hematopoietic stem cell transplantation; Herpes simplex virus, infection and immunity; Opportunistic infections.

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CYTOTOXIC T LYMPHOCYTES

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Cytotoxic T lymphocytes (CTLs) are a functional subset of the cellular arm of antigen-specific immune responses. They play a major role in protection and recovery from viral infection, mediate allograft rejection, are implicated in certain autoimmune diseases, contribute to protection and recovery from certain bacterial and parasitic infections, and to tumor immunity. Our understanding of CTLs has evolved from their characterization as solely CD8⁺, class I major histocompatibility complex (MHC)-restricted, nonproliferative endstage effectors, to T cells that can employ one or several mechanisms of cytolytic activity (perforin/granzyme, FasL/Fas, tumor necrosis factor α (TNF α)), produce different subsets of lymphokines (CD4⁺ T_H1, CD4⁺ T_H2), and recognize foreign antigen in the context of either class I or class II MHC molecules (CD8⁺CTL, CD4⁺CTL, respectively).

Historical perspective

The effector cells in cell-mediated immunity which demonstrate specific cytotoxic activity as described by Govaerts in 1960 are cytotoxic T cells (also called cytolytic T lymphocytes, killer T cells, and abbreviated as CTLs). Some of the major questions involving cytotoxic T cells in the past included the nature of MHC restriction, the nature of the T cell receptor and diversity of the T cell repertoire, the enigma of alloreactivity and Mls reactivity and learning of self MHC in the thymus. Zinkernagel and Doherty in 1974 (working with CTLs specific for a lymphocytic choriomeningitis virus (LCMV)), and independently, Shearer and colleagues in 1975 (working with MHC-restricted responses specific for hapten (TNP)-modified spleen cells), extended the phenomenon of MHC-restricted recognition by T cells to CTLs. At that time, in the absence of biochemical and structural information on the T cell receptor, the two prevailing hypotheses were that 1) there were two separate receptors, one for the foreign antigen and one for self MHC (the restriction molecule) or 2) a single receptor could recognize an altered self MHC determinant. It is now known that the T cell receptor is comprised of an $\alpha\beta$ heterodimer coexpressed with several nonpolymorphic chains (CD3) which exhibits both the foreign antigen specificity and the MHC-restriction specificity. The 'altered-self' MHC molecule is now known to be an MHC molecule which

binds and presents a fragment of antigen to the T cell.

Also at question at that time was how diverse is the T cell repertoire since there are CTLs with exquisite MHC restricted foreign antigen recognition as well as a high frequency of CTLs which recognize allo-MHC molecules independent of self MHC restriction. It was estimated that ~1% of cytolytic T cells recognize a given allogeneic MHC molecule while as many as 20% could recognize Mls loci determinants. The precursor frequency for a given foreign antigen is merely ~1/10 000. Examination of clonal CTL populations revealed that a given T cell could recognize foreign antigen in an MHC-restricted fashion and additionally recognize allo-MHC. This dual specificity was also demonstrated for Mls reactivity. The current view is that alloreactivity may be directed to allo-MHC molecules which bind and present self peptides.

Nature and characteristics

Classically described cytotoxic T cells are class I MHC-restricted (i.e. foreign antigen is recognized in the context of H2-K, D or L molecules in the murine system and HLA-A, B or C in the human). These effectors also express the cell surface molecules Thy-1, Lyt-1, Lyt-2,3 (CD8), TCR $\alpha\beta$, CD3 and LFA-1. In the 1980s, it was then reported in the human for virus-specific CTLs and subsequently confirmed in the murine model that class II MHC-restricted CTLs are elicited in response to viral antigens. These effectors bear the cell surface molecules Thy-1, Lyt-1, LFA-1, L3T4 (CD4), CD3 and TCR $\alpha\beta$. Concomitant with these reports, the suggestion was independently put forth that Lyt-2 (CD8) and L3T4 (CD4) expression are more clearly correlated with class I and class II MHC restriction, respectively, than with cytotoxic effector and helper functions, respectively. Exceptions to the rule have been found (e.g. Lyt-2⁺ class II MHC-restricted CTLs), but the correlation holds for the bulk of CTLs. The class II MHC-restricted CTLs may have been overlooked in earlier studies because many tumor cell and fibroblast cell lines and macrophages used as target cells did not express class II MHC molecules. Target cells for human CTLs, however, are usually EBV-transformed B lymphoblastoid cell lines (LCL), which generally express class II MHC molecules.

Clonal CTL populations have been mapped for their MHC restriction molecule by comparing lysis of class I MHC-bearing targets with targets which bear both class I and class II MHC molecules (e.g. B lymphoma cells). Transfected murine cell lines where a given class I or class II MHC molecule is expressed in a cell bearing MHC molecules of a different haplotype have proven useful as target cells to map MHC restriction. In the human, homozygous typing lines have been used as targets, but the serological classification of class I and class II MHC molecules does not always help to distinguish restriction molecules recognized by CTLs. Transfected murine fibroblast lines which express human class I or class II MHC molecules have proven useful in these cases. Antibodies directed towards the class I or class II MHC molecules generally inhibit the cytolysis of specific target cells (antibodies directed towards the foreign antigen generally do not inhibit). In addition, antibodies directed to CD8 or CD4 molecules have been shown to inhibit cytolysis by class I MHC-restricted and class II MHC-restricted T cells, respectively. Anti-LFA-1 specific antibodies block cytolysis mediated by either CD8⁺ or CD4⁺ CTLs.

CTL induction and growth

Most MHC-restricted CTL responses directed to viral antigens, tumor antigens, soluble protein and minor histocompatibility antigens require *in vivo* immunization or priming. In the case of strong responses, MHC-restricted, antigen-specific T cells can be obtained by day 7 postimmunization. Upon *in vitro* secondary restimulation with limiting numbers of primed precursors one can obtain clonal CTL populations. In general, class I MHC-restricted CTLs require an exogenous source of T cell growth factor, interleukin 2 (IL-2), in addition to the antigenic stimulus, while class II MHC-restricted CTLs do not. If cloned in the absence of exogenous IL-2 and at a high precursor density per well, rare CD8⁺, class I MHC-restricted CTLs which produce IL-2 can be selected. CTL antigen specificity and MHC restriction can also be determined in proliferation assays. At one time, cytotoxic T cell effectors were considered to be end-stage cells incapable of proliferating (much like antibody-secreting plasma cells). It has been shown, however, that CTLs can continue to proliferate but only upon specific antigenic stimulation. Some general features of CTL induction are depicted in **Figure 1**. The events are the same for both class I MHC-restricted CD8⁺ CTLs and class II MHC-restricted CD4⁺ CTLs. The first signal in the activation of precursor CTL (pCTL) is the antigen/MHC complex recognized by the pCTL T

cell receptor (TCR). The costimulatory (or second) signal once considered to be IL-1 (soluble or membrane-bound) for CD4⁺ pCTL, is now thought to be B7(CD80), the ligand for CD28 expressed on the pCTL. The activated T cells undergo RNA synthesis, protein synthesis and expression of IL-2 receptor α (IL-2R α) resulting in expression of high-affinity IL-2 receptors (IL-2R $\alpha\beta\gamma$). Upon engagement of IL-2 by IL-2R the T cell blasts undergo DNA synthesis and subsequent cell division. Whether an additional cytotoxic differentiation factor (CDF), such as IL-12, is required at this time to allow the T cells to gain cytolytic effector function remains to be demonstrated. Whether mature CTLs and memory CTLs are one and the same also is not known. These mature/memory CTL eventually downregulate IL-2R α expression and stop proliferating until they receive an antigenic stimulus once again.

Also depicted in **Figure 1** the current scheme that there are two predominant pathways of antigen presentation, one for class I MHC-restricted recognition and the other for class II MHC-restricted recognition. Crossover between the two pathways have been described to occur. For the most part, however, antigen synthesized endogenously within the antigen-presenting cell or target cell (or somehow introduced into the cell cytoplasm) can be processed and presented in the context of class I MHC molecules. Antigens which are taken up by an endocytic route by the antigen-presenting cell or target cell are processed and presented in the context of class II MHC molecules. As a result of this distinction, infectious virus can elicit both class I and class II MHC-restricted CTLs *in vivo* while inactivated virions generally elicit just class II MHC-restricted T cells *in vivo*.

Mechanisms of cytolysis

Recent advances in studies of the mechanism of CTL cytolytic effector function have resolved a long-standing controversy. Two opposing hypotheses, the cytolytic granule exocytosis mechanism and the calcium-independent, perforin-independent mechanism are now known to be independent or overlapping but not mutually exclusive mechanisms. The granule exocytosis hypothesis describes the cytolytic event as resulting from a calcium-dependent secretion of cytoplasmic granules into the extracellular space bounded by the cytotoxic T cell membrane/target membrane junction. The perforin inserts into the target cell membrane and forms pores through which granzymes are introduced into the target cell allowing for events that result in target cell apoptosis and nuclear DNA fragmentation. In 1993, the mechanism of Fas ligand/Fas-mediated cytolysis

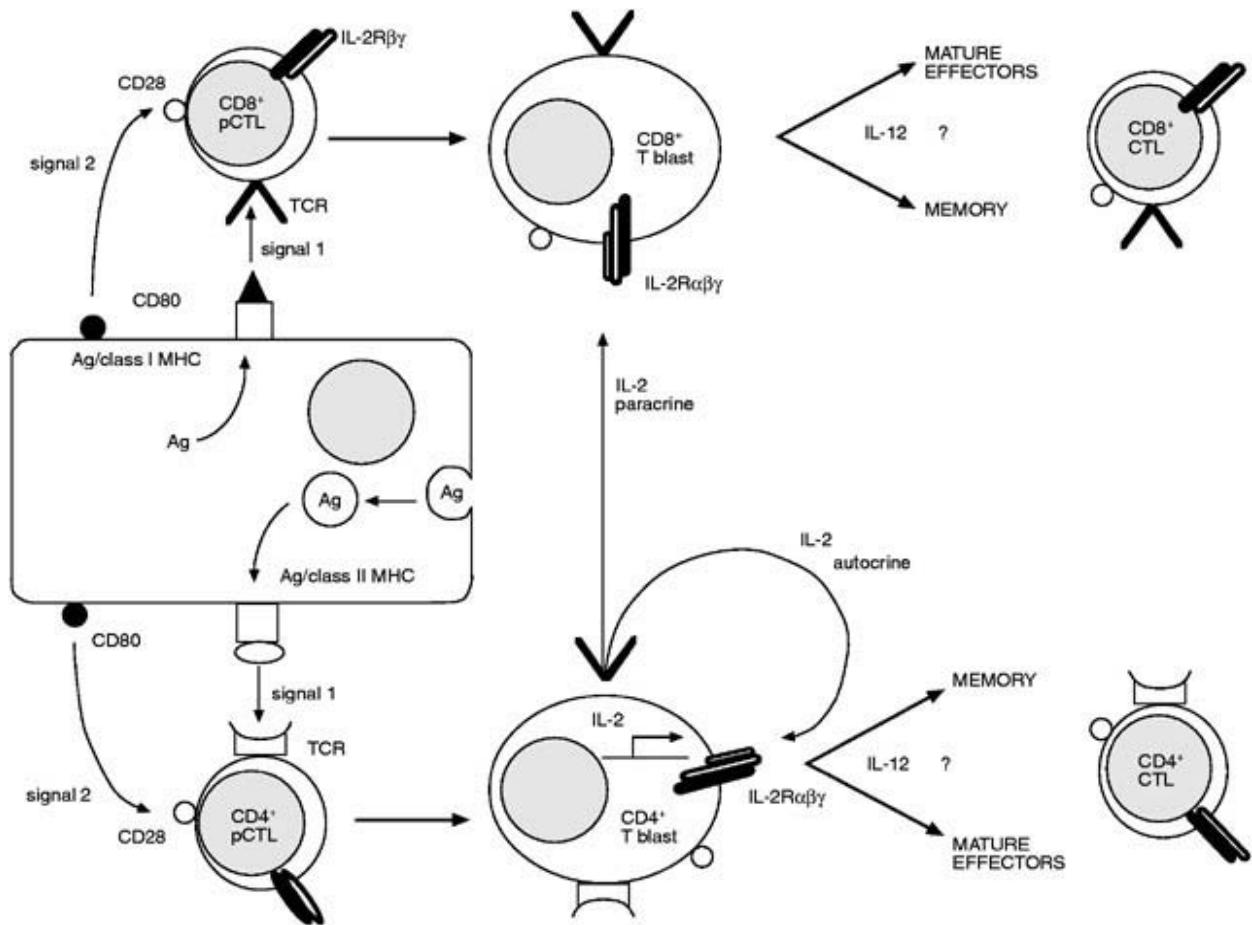


Figure 1 Induction of CD8⁺ and CD4⁺ cytotoxic T lymphocytes.

was described which accounts for the previously described calcium-independent, perforin-independent cytolytic mechanism. Fas (CD95) is a member of the tumor necrosis factor receptor family (TNFR) expressed on the target cell and while FasL (CD95L) is a member of the tumor necrosis factor (TNF) family expressed by CTL upon antigen-specific activation. CTL can mediate cytolysis of target cells by FasL/Fas-mediated cytolysis and/or by exocytosis of cytolytic granules containing perforin/granzymes in short-term *in vitro* assays. Thus, calcium-independent cytolysis can be achieved by CTLs that lack perforin-containing granules, via the FasL/Fas mechanism. This form of cytolytic effector function remains intact in mice that are deficient in perforin. Thus far, there are CD8⁺ CTLs that employ both the perforin/granzyme and FasL/Fas mechanisms of cytolysis (**Figure 2**) and CD8⁺ CTLs that employ the FasL/Fas mechanism only. In a mutant strain of mice, *gld*, the T cells express defective FasL and thus are defective in FasL/Fas lysis. CD4⁺ CTLs employing both mechanisms of cytolysis have been reported for viral-specific T cells (**Figure**

2) and CD4⁺ CTLs that employ solely the FasL/Fas mechanism have been reported for alloreactive CTL. In addition to these CD4⁺ CTLs, which are generally of the T_H1 subset, viral-specific CD4⁺ T_H2 cells have been reported to demonstrate FasL/Fas cytolytic activity. CD4⁺ (**Figure 2**) T_H1 cells produce IL-2 and interferon γ (IFN γ) while CD4⁺ T_H2 cells produce IL-4 and IL-5. Although not as systematically characterized *in vitro*, it is believed that most of these CTLs can mediate cytolysis via membrane-bound or soluble TNF α *in vitro* and *in vivo*. TNF α -mediated lysis of target cells is not detectable in the classical short-term cytotoxicity assays. Another feature of TNF α and FasL/Fas lysis is that the target cells must express TNFR or Fas respectively, whereas perforin/granzyme cytolysis requires only the specific Ag/MHC complex to be presented.

Lysis mediated by CTL is generally quantitated using the classic ⁵¹Cr-release assays developed in 1968 by Brunner and colleagues where lysed cells release ⁵¹Cr into the medium. Target cell DNA fragmentation which occurs before cell lysis can be detected using a modified ¹²⁵IUDR assay (Russell and

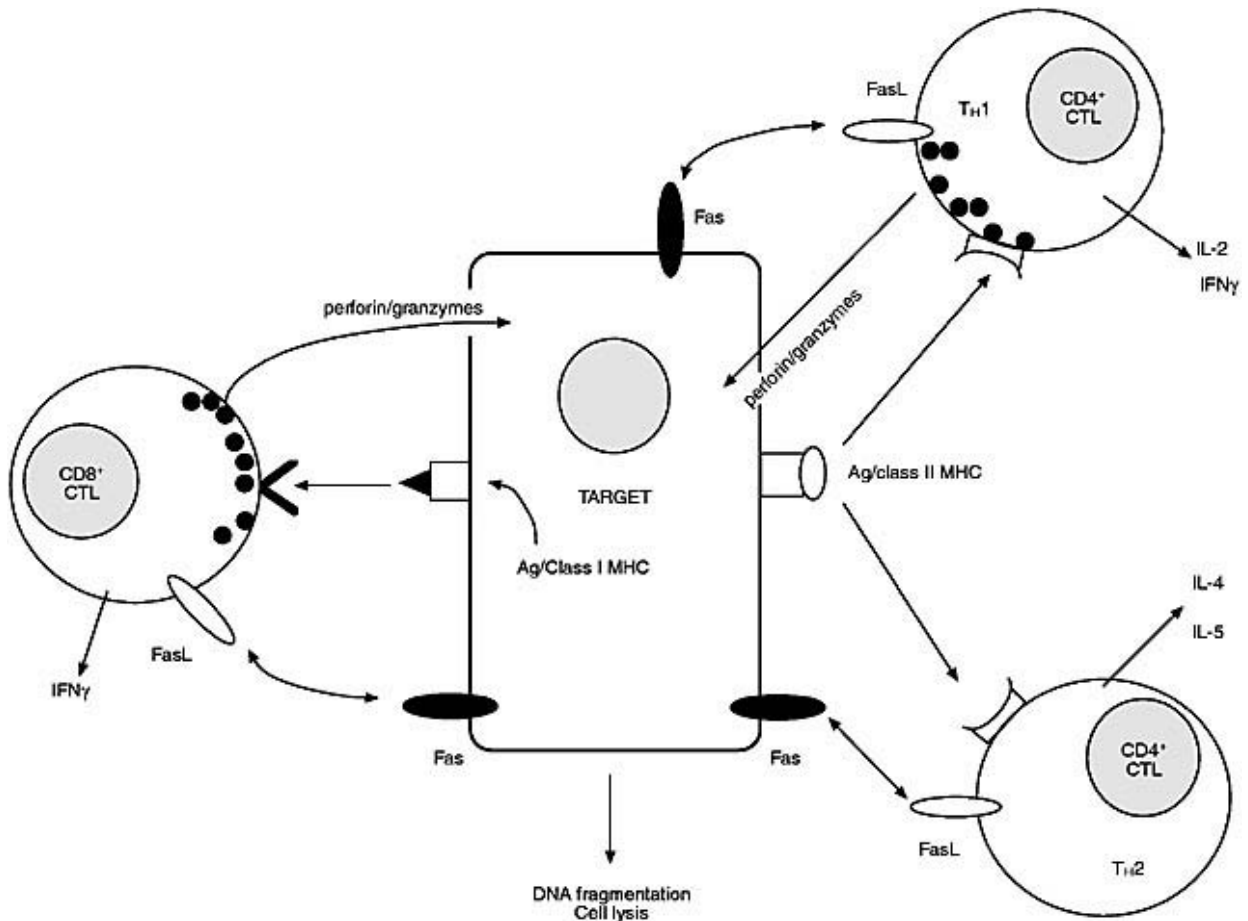


Figure 2 Perforin/granzyme granule exocytosis and FasL/Fas cytotoxicity mediated by CD8⁺ and CD4⁺ CTLs.

Dubos) using nonionic detergent to dissolve the target cell membranes. The lytic activity by particular cytotoxic T cell populations can be compared with that of another population by comparing lytic units. A lytic unit (LU) is the number of effectors required to achieve an arbitrary amount of lysis (e.g. 50%) in a particular amount of time (e.g. 4 h) using data derived from a range of effector-to-target ratios. Events in cytotoxicity generally fall into three stages termed 1) conjugate formation or adherence, 2) lethal hit delivery, and 3) target cell lysis or disintegration. A detailed discussion of these stages can be found elsewhere (see Mechanisms of cytotoxicity above). Both specific and nonspecific conjugates can form between T cells and target cells in the presence of Mg^{2+} via LFA-1 and its coreceptor ICAM-1, CD2 and its coreceptor LFA-3. For perforin/granzyme cytotoxicity, the lethal hit delivery is both temperature and calcium dependent. Target cell disintegration can occur in the absence of the CTL following the lethal hit. CTLs can recycle, i.e. kill more than one target cell. *De novo* protein synthesis is not required for perforin-mediated cytotoxicity as the perforin exists

in preformed granules, but it is required for FasL expression. A hallmark of short-term CTL assays (4–6 h), is that there is no bystander killing of nearby nonspecific target cells. T cell-mediated cytotoxicity is unidirectional killing in that CTLs are spared the lethal hit delivery by some unknown mechanism.

Cytoplasmic granules in CTLs contain perforin (or cytolyisin) and members of a family of serine esterases (granzymes). Secretion of granzyme A activity into the medium following cytotoxic T cell/target cell interaction has been used as an assay of granule exocytosis but not necessarily cytotoxicity. There is clonal variation with regard to granzyme A content and mice genetically manipulated to be granzyme A deficient retain cytotoxic activity while mice rendered granzyme B deficient are deficient in cytotoxic T cells.

Cytokines reported to be released following killer target cell interaction are IFN- γ and lymphotoxin (LT) in addition to the previously mentioned TNF- α . The *in vivo* release and function of these cytokines are under investigation. It is unlikely that they play a direct role in short term *in vitro* cytotoxicity since there is little bystander killing and cytotoxic activity is not

demonstrable in supernatants of killer T cells cocultured with specific target cells.

In vivo function

As mentioned above, cytotoxic T cells have been shown to effect allograft rejection, mediate autoimmune tissue damage, and protect against and promote recovery from viral and bacterial infections. CTLs with viral specificity have been shown to lyse virus-infected target cells before virus budding and sometimes before viral genome replication. This serves to limit the release of virus particles and also limit cell-to-cell spread of virus. Perforin-deficient mice are unable to efficiently clear virus in infected animals yet are able to reject allografts. This points to a differential use of cytolytic effector function. It has been suggested that perforin-mediated cytolysis may be employed to control spread of infectious pathogens while FasL/Fas-mediated cytolysis as well as TNF α -mediated cytolysis may be involved in immunoregulatory events that limit lymphoproliferation and dysregulated autoimmune responses.

Methods to manipulate CTL populations have included vaccine designs to elicit *in vivo* class I MHC restricted as well as class II MHC restricted CTL responses as well as introduction of *in vitro* manipulated CTL populations. When cloned CTLs with influenza virus specificity and class I or class II MHC restriction were adoptively transferred into mice given an intranasal dose of influenza virus, the lung viral titers were 100-fold lower and the mice recovered while the infected group that did not receive the T cell clones died within one week. Other *in vivo* studies indicate that there is little or no bystander killing *in vivo*. Since *in vitro* cultured CTLs can demonstrate *in vivo* function, studies are underway in a number of model systems to elicit CTLs to tumors and viral antigens, propagate these effectors *in vitro* and transfer them back *in vivo*. Progress on this front has been reported for cytomegalovirus-specific CTL transfer into bone marrow transplant patients and experimental protocols to introduce *in vitro* expanded human immunodeficiency virus (HIV)-specific CTLs in patients with the acquired immune deficiency syndrome (AIDS) are currently under development.

See also: Adhesion molecules; Alloantigens; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Apoptosis; Bystander effects; CD2; CD3; CD4; CD8; CD28; B7 (CD80 & CD86); Cell-mediated immunity; Helper T lymphocytes; Cytokines; Cytotoxicity, assays for; Cytotoxicity, mechanisms of; Effector lymphocytes;

Exocytosis; Fas (CD95) and fas ligand; Granzymes; H2 class I; H2 class II; Cell-mediated lysis; HLA class I; HLA class II; Immune response *in vitro*; Interferon γ ; Interleukin 2; Interleukin 2 receptor; Interleukin 12 and its receptor; MHC restriction; Perforin; Proliferation, lymphocyte; Second signals for lymphocyte activation; T lymphocyte activation; T lymphocyte clonal expansion; T lymphocyte differentiation; Tumor necrosis factor α ; TNF receptors; Vaccines; Viruses, immunity to.

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CYTOTOXICITY, ASSAYS FOR

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A variety of immunological effectors attack cells, resulting in cytolysis of the targets. These include specific antibody with complement, cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, lymphokine-activated killer (LAK) cells, the effectors of antibody-dependent cellular cytotoxicity (ADCC), macrophages, neutrophils and certain substances secreted by cells such as tumor necrosis factor (TNF) and perforin. CTLs and NK cells are known to utilize dual pathways of cytotoxic machinery: perforin + granzyme mediated and Fas ligand mediated. Cell death is generally categorized into two different types. One, called programmed cell death (apoptosis), involves active metabolic process of the dying cell resulting in disintegration of nuclear DNA and an increase in the permeability of the cell membrane. The other type, called necrosis, does not involve active metabolic process and results in an increase in membrane permeability without immediate nuclear disintegration. Cell-mediated cell lysis generally induces the former type of cell death, whereas cell lysis mediated by antibody and complement causes

the latter type. Accordingly, there are two different principles for detecting cell death: one for measurement of increased membrane permeability and the other for detecting DNA disintegration.

Two types of methods for monitoring membrane permeability of cells are widely used as cytotoxicity assays. One is the dye exclusion test. This assay involves microscopical counting of dead cells stained with a dye, such as trypan blue, to which viable cell membrane is impermeable. These agents, because of their basic nature, stain cell nuclei when they have entered the cells across the damaged cell membrane. By this method, the percentage of dead cells can be directly determined. The other method is the chromium-release assay, in which target cells are labeled with radioactive ^{51}Cr . When cultured in the presence of a trace amount of $\text{Na}_2^{51}\text{CrO}_4$, viable cells take up Cr^{6+} . Once inside the cell, the Cr^{6+} is reduced to Cr^{3+} . The Cr^{3+} binds to multivalent anions, including proteins present in the cells. This results in the formation of molecular complexes which are not easily released from cells unless the

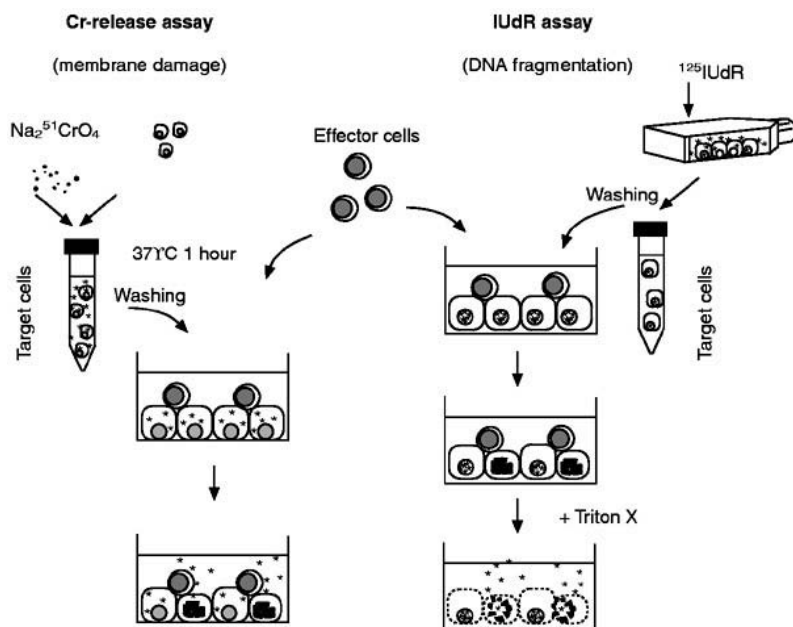


Figure 1 Two types of cytotoxicity assay. In the Cr-release assay, an increase in membrane permeability is measured. In the IUdR-release assay, disintegration of nuclear DNA is measured.

cell membrane is damaged. Thus, cell death can be determined by measuring radioactivity released from dead cells into the supernatant (**Figure 1**). Results are usually expressed as specific Cr release (%) calculated according to the following formula:

$$\frac{(\text{experimental release}) - (\text{spontaneous release})}{(\text{maximum release}) - (\text{spontaneous release})} \times 100,$$

where spontaneous release is the background radioactivity released in the absence of the effector and maximum release is the amount of released radioactivity when the target cells are maximally lysed by acid or detergent. The specific Cr release does not faithfully represent the actual percent of dead cells. This is because the efficiency of Cr uptake and spontaneous release of Cr can significantly vary from cell to cell in a heterogeneous cell population, and the maximum release induced by detergent is, for unknown reasons, usually significantly (approximately 20% on average) higher than Cr release when 100% of the cells are lysed by the effector. In order to quantitate the lytic activity of the effector, titration of the effector activity is necessary. Determination of lytic activity at a single effector: target ratio does not give an accurate estimate of cytotoxicity, as a small difference in the values of specific Cr release (less than 10%) is not reliable. Furthermore, above the plateau (79–80%), differences cannot be detected. Generally, the Cr release assay is used for cell-mediated target cell lysis because the presence of an excessive number of effector (or attacker) cells becomes problematic when using the dye exclusion assay. Furthermore, because of the ease of quantitation, this assay is favored when a large number of samples is involved.

For quantitative measurement of DNA disintegration, labeling nuclear DNA with ^{125}I UdR or ^3H -thymidine is widely used. Since labeling nuclear DNA depends on cellular synthesis of DNA, this method is applicable only for rapidly dividing cells such as tumor cells or blast cells. When a dividing population of cells are cultured in the presence of ^{125}I UdR for 1–2 hours, a portion of cells incorporate the label into newly synthesized DNA strands. The

chromosomal DNA remains in the nuclear matrix even after lysing cells with detergent. When nuclear DNA fragmentation takes place, small DNA fragments become released from the nucleus. In the assay, after incubation with the effectors, the whole cell mixtures are lysed with detergent and radioactivity in the supernatant is measured after pelleting nuclei by centrifugation. DNA fragmentation is usually expressed as percentage of specific IUDR release, which is calculated in a similar manner as the above-mentioned Cr release.

See also: Antibody-dependent cellular cytotoxicity; Apoptosis; Complement, membrane attack pathway; Cytotoxic T lymphocytes; Cytotoxicity, mechanisms of; Fas (CD95) and fas ligand; Granzymes; Lymphokine-activated killer (LAK) cells; Natural killer (NK) cells; Perforin; Tumor necrosis factor α ; Lymphotoxin.

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CYTOTOXICITY, MECHANISMS OF

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In 1960 Govaerts showed that white peripheral blood cells from alloimmune dogs were able to lyse *in vitro* target cells bearing the corresponding allo-antigens. Four decades and several thousands of publications later, we know that many different cell types have this ability to destroy other cells. This entry is concerned, however, essentially with lymphoid cells (thus not touching in detail upon mechanisms of cytotoxicity by activated macrophages or by polymorphs, for instance). Among lymphoid cells, we consider mostly cytotoxic T lymphocytes. We briefly review, first their *in vivo* roles, then the phenomenology of cytotoxicity, and finally molecular studies leading to the identification of two main mechanisms of T cell-mediated cytotoxicity as assessed in short-term *in vitro* assays, namely a mechanism involving granule exocytosis, perforin and granzymes, and a mechanism involving Fas and its ligand.

Why do cytotoxic cells exist at all? Under physiologic circumstances (i.e. not for instance in a situation of allograft rejection), they can meet and destroy only syngeneic cells. Since cells from a given organism usually cooperate rather than destroy each other, the selective advantage overriding this rule must be considerable. It is now believed that a main role of cytotoxic T cells is to lyse syngeneic cells presenting surface determinants originating from viruses or other intracellular microorganisms. These, when within cells, are out of reach of other immune system effectors. Destruction of infected cells destroys the microorganism or exposes it to other effector mechanisms. A teleological justification for the existence of T cell-mediated cytotoxicity, perhaps in particular perforin based, thus seems to be its role in fighting intracellular pathogens. Another *in vivo* counterpart, of in particular Fas-based cytotoxicity, is downregulation of immune responses, in part through recognition and destruction of Fas-bearing activated lymphocytes. Neither the perforin- nor the Fas-based mechanisms in isolation, nor both together, seem to fully account for graft rejection. A possible role in spontaneous clinically significant tumor cell rejection is not clear; the therapeutic use against established tumors and their metastases of cytotoxic lymphokine-activated killer (LAK) cells or intratumor-derived cytolytic cells is still being investigated.

Another intriguing possibility, stemming from the finding that cytotoxic T cells recognize at the surface of target cells peptides which may derive from any

protein (even intracellular), has been raised in particular by Thierry Boon's group in Brussels. A cell in which a given protein has been modified by somatic mutation would present at its surface correspondingly modified peptides. These would be recognized as foreign by the immune system. Activated cytotoxic T cells may consequently destroy the mutation-bearing cells. An important role of cytotoxic T cells might thus be to 'proofread' the genome against somatic modifications.

Phenomenological studies

How do cytolytic cells destroy target cells? This has been studied in a very analytical, very reductionist manner in model systems *in vitro*. A large variety of techniques have been used, while cytotoxicity itself has been most frequently assessed using the ^{51}Cr -release assay introduced in this field by T. Brunner and colleagues in Lausanne. Briefly, target cells are labeled by incubation with radioactive chromate, then washed of unincorporated label. Addition of cytotoxic cells induces lysis of target cells, and therefore release of intracellular chromium-labeled molecules. The percentage of radioactivity released in the supernate reflects the percentage of lysed target cells and thus indirectly the lytic efficiency of the cytotoxic cells under test. Studies on the mechanisms of cytotoxicity include phenomenological descriptions, some of which date back a number of years, and more recent molecular investigations.

Phenomenological studies concurred to provide a minimal description, a basic scenario of the lysis of a target cell by a cytotoxic T cell. The first stage of lysis is recognition, which involves an array of diverse molecules at the surface of the effector cell (such as TCR/CD3/peptide, CD4 or CD8, LFA-1 . . .) and of the target cell (class I or class II MHC, ICAM-1 . . .).

Engagement of these molecules triggers a remarkable succession of events in the effector cell. An early sign, and a further trigger, of lymphocyte activation is the increase of free cytoplasmic calcium, shown by Poenie *et al* in Berkeley in 1987 and by other groups, using real-time video an intracellular probes the fluorescence of which varies as a function of calcium concentration. This increase occurs in a matter of minutes and is immediately followed by reorientation, within the effector cell, of the secretory apparatus towards an area facing the target cell, as

shown in particular by S.J. Singer and his colleagues in California in 1985. This suggested the possible participation of secretory phenomena in cytotoxicity. Many cytotoxic T cells indeed contain peculiar cytoplasmic granules. Their content can be released by degranulation in the extracellular fluid upon target cell recognition. Interestingly, reorientation of the secretory apparatus is calcium dependent, and so usually are degranulation systems.

Among the phenomena then observed in the target cell, a calcium influx resulting in a very high cytoplasmic calcium concentration is prominent. Also very striking is the early disintegration of nuclear DNA, first observed in this system by J. Russell (St. Louis). Disintegration of DNA into fragments of around 180 bp, and multiples thereof, corresponding to the size of nucleosome-shielded DNA, is very likely due to the action of one or several endonucleases activated early in the sequence of events leading to target cell death. Other events in this sequence include cytoplasmic condensation, cytoplasmic and nuclear fragmentation, and only very late secondary membrane disruption.

This schematic description leads to three remarks. First, there are several calcium-dependent events in the process described above. Indeed, T cell-mediated cytotoxicity is mostly calcium dependent at the population level. However, some cytotoxicity does occur in the absence of extracellular calcium within alloimmune *in vivo* or *in vitro* raised cell populations, and some clones could be shown to exert considerable levels of cytotoxicity under these conditions. This led in the late 1980s to the speculation that at least two distinct cytotoxic mechanisms may exist, depending or not on extracellular calcium, in the same cells or in different cells.

The second remark bears on the fact that once a cytotoxic T cell has lysed a target cell, it can proceed to lyse another one. Thus, it is clearly itself not permanently affected by the killing process. For the target cell to be lysed without destroying the effector cell, the killing mechanism should be polarized, unidirectional from the effector cell towards the target cell. Alternatively, the killing mechanism may not be polarized (it may for instance include a soluble extracellular substance), but then the killer cell should be resistant to it, and therefore to other killer cells. Experiments testing whether cytotoxic T cells could be lysed by other cytotoxic T cells were performed at the population level and also more recently at the clonal level. The conclusion seemed to be that although cytotoxic cells can indeed be sensitive to some cytotoxic cells, some are resistant to others and particularly to themselves. However, these experiments did not take into consideration the then

unknown fact that two distinct cytotoxicity mechanisms were at play, and the nature of these mechanisms. Resistance/sensitivity of cytotoxic T cells to cytotoxicity mechanisms should be reinvestigated accordingly.

The third remark is that the phenomenology of cell death is very similar, if not identical, whether death is induced by cytotoxic cells or occurs, say, in developmental circumstances. In both cases, the same program seems to govern the course of events in a cell that dies, including as we now know a similar cascade of molecular events, and the same 'apoptotic' morphologic traits described by John Kerr *et al* in 1972. What may be different is merely the signaling of this death program. The nature of the signals produced by cytotoxic cells has been largely unraveled by studies at the molecular level.

Molecular studies

A first approach to the identification of molecules involved in cytotoxicity has been, naturally enough, to look for molecules themselves endowed with cytotoxic activity, and present either in the supernates of cytotoxic cells, or in subcellular fractions of these. This approach, among others, contributed to the identification, and later the cloning, of tumor necrosis factor (TNF- α) and lymphotoxin (TNF β). Using the corresponding antibodies, TNF α , probably in a membrane-bound form, has been shown to be required for cytolysis mediated by natural cytotoxic (NC) cells, by some activated macrophages, and more recently by activated T cells in long-term cytotoxicity tests *in vitro*.

This approach also led to the detection (initially by P. Henkart *et al* and by E. Podack *et al*, in 1983–1985), the characterization and the cloning of perforin. Perforin is a 60 kDa molecule present in granules in the cytoplasm of many cytotoxic T and natural killer (NK) cells; at high concentrations, it can act on membranes as a calcium-dependent channel-former; most interestingly, it shows significant homology to the terminal components of the complement cascade. A formal demonstration that perforin was involved in a mechanism of cytotoxicity was provided by Kägi *et al* in 1994, followed by several other groups, all showing that cytotoxicity mediated by T cells was greatly impaired in mice made perforin deficient through gene targeting.

Other molecules present in granules of cytotoxic T cells were identified by subtractive hybridization cloning techniques. Prominent among molecules isolated this way were a number of serine esterases, such as CTLA-1 (Brunet *et al* 1986, also isolated by Lobe *et al* under the name CCP1 and by Tschopp *et*

al under the name granzyme B) and CTLA-3 (Brunet *et al* 1986, also isolated by Gerschenfeld and Weissman under the name H Factor and by Tschopp *et al* under the name granzyme A). Heusel *et al* demonstrated in 1994, also using gene-targeted mice, that granzyme B is required in a mechanism of cytotoxicity. It would seem that, upon recognition of the target cell, in the effector cell perforin- and granzyme B-containing granules reorient towards the target cell and are triggered to exocytose. In a still unclear manner, perforin would then facilitate the entry of, in particular, granzyme B into the target cell, where it may trigger the programmed cell death cascade by activating members of the ICE family of cysteine proteases.

Meanwhile, a somatic cell genetic approach led Rouvier *et al* in 1993 to the finding that another mechanism of T cell-mediated cytotoxicity required Fas (otherwise called APO-1 or CD95) at the target cell surface. This in turn led to the cloning by Suda *et al* of the Fas ligand, expressed at the effector cell surface. These molecules belong, respectively, to a family of receptors (including Fas, the TNF-Rs, CD30, CD40 . . .; most or all being very efficient at transducing a signal interpreted as a cell death or cell survival signal) and to a family of ligands (including the Fas ligand, the TNFs, the CD30 and CD40 ligands . . .). Involvement of Fas in cytotoxicity may be a reflection *in vitro* of a main role *in vivo* of downregulation of the immune response.

While triggering of cell death in development involves largely unknown signals, the Fas pathway is one of the very few programmed cell death pathways in higher eukaryotes in which signaling is in part molecularly defined. Signaling in the Fas pathway runs in the effector cell from TCR engagement (by target cell MHC) to expression of the Fas ligand, then in the target cell from engagement of Fas (by effector cell Fas ligand) to death. It therefore is a two-cell twice-*trans* process.

The residual cytotoxicity of *in vitro* and *in vivo* induced cytotoxic T cells from perforin-deficient mice seemed exclusively Fas-based, in several systems, as shown by Kägi *et al* in 1994 and by others. In many experimental situations only perforin- and Fas-based cytotoxicity mechanisms could be detected. These two mechanisms thus seem to account for all of the classical cytotoxic T lymphocyte phenomenology, in short-term (i.e. 4 h) cytotoxicity tests *in vitro*. The Fas pathway is used by both CD4⁺ (mostly T_H1) and CD8⁺ effector cells, while the latter also use the perforin/granzyme pathway. Thus, in CD8⁺ T cells, two signals may stem from the TCR/CD3 complex upon antigen-specific recognition, one of them leading to granule exocytosis

and the other one leading to transcription of the Fas ligand gene. Neither the exact nature of these signals nor whether they can be triggered simultaneously in a given cytotoxic cell is known as yet.

More generally, both mechanisms of T cell-mediated cytotoxicity may act by signaling intermediate steps of a conserved programmed cell death cascade within the target cell. This speculation is based, for the Fas pathway, on weak homologies between the death domains (of Fas and TNF-R1) and the death-signaling molecule reaper in *Drosophila*, and for the perforin pathway on recent findings that granzyme B can activate at least one of the ICE family members. In this sense, the emergence in evolution of T cell-mediated cytotoxicity may have required the invention, not of new mechanisms of killing, but merely of new ways of signaling a pre-existing programmed cell death cascade.

Conclusion

How T cells are able to kill other cells is no more a complete mystery. As assessed by short-term cytotoxicity tests *in vitro*, they seem to use two distinct mechanisms, perforin/granzyme-based and Fas-based, respectively. These seem to be *in vitro* correlates of, in particular, *in vivo* defense mechanisms against intracellular pathogens and downregulatory reactions within the immune system, while they do not seem to account entirely for graft rejection.

See also: Adhesion molecules; CD28; Cell-mediated lysis; Cytotoxic T lymphocytes; Cytotoxicity, assays for; Lymphokine-activated killer (LAK) cells; Natural killer (NK) cells; Perforin.

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DECAY-ACCELERATING FACTOR (CD55)

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Decay-accelerating factor (DAF), also termed CD55, is a glycoprotein of 65–70 kDa expressed on the surface of all blood cells, vascular endothelial cells and various other tissue cells. It is one of the membrane-bound complement inhibitors that protect host cells from the destructive action of autologous complement. An inherent characteristic of complement is that its active fragments or their complexes can bind to host cell surfaces accidentally or physiologically. This potentially harmful characteristic is prevented by widely distributed membrane-bound complement inhibitors, such as DAF, membrane cofactor protein (MCP or CD46; a 45–70 kDa cofactor for the C3b/C4b-cleaving enzyme factor I) and CD59 (an 18 kDa inhibitor of the membrane attack complex; also termed MACIF, HRF20 or MIRL). A primary function of DAF is to inhibit C3 convertases (C4b2a and C3bBb) and C5 convertases (C4b2a3b and C3bBb3b) on the cell surface; DAF interacts with the convertases and destabilizes them by inducing rapid dissociation of a catalytic subunit C2a or Bb. The inhibitory action of DAF is restricted to C3/C5 convertases bound directly to host cells; that is, DAF does not inhibit normal complement activation on targets, such as microorganisms and immune complexes.

The peptide portion of DAF consists of four tandem repeats of about 60 amino acids, called short consensus repeats (SCRs), followed by a Ser/Thr-rich region of about 70 amino acids. The SCRs are present in 14 complement proteins that bind to C3, C4 or C5 and in other noncomplement proteins. The Ser/Thr-rich region contains multiple O-glycosylation sites. One N-glycosylation occurs in SCRs. A glycolipid anchor covalently linked to the C-terminus of the peptide attaches the protein to the membrane. The glycolipid anchor consists of ethanolamine, which connects the anchor to the peptide, and three mannose residues, glucosamine and phosphatidylinositol, which is attached to the membrane. This glycolipid structure, called glycosylphosphatidylinositol, is a membrane anchor in 100 or more mem-

brane proteins, such as alkaline phosphatase, acetylcholinesterase, Thy-1, the variant surface glycoprotein of *Trypanosoma brucei* and CD59.

In a soluble form, DAF is present in urine, plasma, tears and saliva. Soluble DAF may be released from membrane DAF by the action of phospholipase C or D, or a protease. Soluble DAF has about 100-fold less activity than membrane DAF on cell-bound C3 convertases, and its physiologic function is unknown.

The DAF gene is located in chromosome 1 in band q32 within a cluster of genes of complement regulatory proteins that include SCRs; i.e. factor H, C4-binding protein, MCP, complement receptor 1 (CR1) and 2 (CR2). DAF bears Cromer blood group antigens. Rare individuals called Inab or IFC-lacking Cromer-related antigens are deficient in DAF.

Blood cells of patients with an acquired hemolytic anemia, paroxysmal nocturnal hemoglobinuria (PNH), are deficient in DAF. In the clonal disorder, PNH, abnormal totipotent or pluripotent hematopoietic stem cells give rise to abnormal blood cells showing deficient surface expression of glycosylphosphatidylinositol-anchored membrane proteins. Because of this surface deficiency of DAF and CD59, in PNH a fraction of the red cells is susceptible to complement, resulting in intravascular hemolysis and hemoglobinuria.

DAF acts as a receptor for some microorganisms. Echoviruses of various serotypes, including 6, 7, 11, 12, 20 and 21 and coxsackieviruses B1, B3 and B5, use DAF as a receptor for cell attachment. Uropathogenic *Escherichia coli* binds to cell surface DAF with its fimbriae-like adhesin.

Mouse DAF, which inhibits C3 convertases, has been purified and molecularly cloned. Similarly to human DAF, it consists of four SCRs, a Ser/Thr-rich domain and a glycosylphosphatidylinositol anchor. There is another closely related mouse DAF gene whose product has transmembrane and cytoplasmic domains instead of a glycosylphosphatidylinositol anchor. Two genes are tandemly organized within

mouse chromosome 1 near the C4-binding protein gene. Mouse Crry/p65 is a 65 kDa protein bearing both DAF- and MCP-like functions and consisting of five SCRs and transmembrane and cytoplasmic domains. The significance of functional redundancy among these mouse regulatory proteins requires clarification.

See also: CD59; Complement, alternative pathway; Complement, classical pathway; Complement, membrane attack pathway; Thy-1.

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DEGRANULATION

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Many cells, particularly those involved in inflammatory and immunological reactions, exhibit morphologically distinct intracytoplasmic granules. Degranulation is usually taken to describe the process by which these granules or their contents are discharged to the outside of the cell. Accordingly the term is often used synonymously with secretion, discharge or release. Strictly, however, degranulation represents a state in which the granules are no longer visible microscopically, only presumptively a result of discharge, and this discharge might as well be into a phagosome as to the extracellular environment.

In vivo the phenomenon is generally assessed by histological (or ultrastructural) determination of the granularity of the cell. Quantitation here has to involve the techniques of stereology. In isolated cell populations *in vitro* it is more usually measured by secretion of the granule contents to the outside of the cell. This has the advantage of being amenable to more accurate quantitative assessment and is generally much faster. With mixed cell populations it is clearly important to use a marker that is unique to the cell or granule in question. However, the concept of degranulation came originally from the response of professional phagocytes such as neutrophils or eosinophils to the process of phagocytosis, wherein elegant cinematographic studies in the 1950s showed

the dramatic loss of phase-dense cell granules as they were discharged into the phagosome. Subsequent cell fractionation analysis placed this upon a quantitative footing.

Granules are membrane-bounded cytoplasmic storage structures in which the contents (for example, enzymes) are generally thought to exist in a state of latency, requiring changes in pH, release from a matrix, and/or hydration for optimal activity changes that accompany degranulation (into an endosome or to the outside). Cells involved in inflammation and immunological reactions tend to have at least two types of granules, those specific for the given cell type and those that represent lysosomes, although these latter may contain unique components in given cell types, for example myeloperoxidase and elastase in the 'azurophil' granule of the neutrophil. The granules that are unique to these inflammatory cells include the amine- and protease-containing granules of the mast cell and basophil, the characteristic crystal-containing granule of the eosinophil, the dense granule of the platelet and the 'specific' granules of the neutrophil. Characteristic staining properties of these granules were generally used to identify and name the particular cell – hence neutrophil, basophil and eosinophil. Similarly, the mast cell granules exhibit the characteristic meta-

chromasia with stains such as toluidine blue. Loss of this staining is used to indicate degranulation. It has been suggested that these cell-specific granules are true secretory structures, whose contents are directly released by exocytosis to the cell exterior (as well as into phagosomes in phagocytic cells). They would therefore be analogous to the secretory granules of chromaffin, pancreatic and neurosecretory cells.

By contrast, the modified lysosomal granules in this simplified concept are primarily involved in fusion with endosomes (including phagosomes). However, it is clear that in stimulation of macrophages or neutrophils, lysosomal contents are released to the exterior, although the mechanisms remain obscure. Certainly degranulation of the two major granule types of the neutrophil (whether to the outside or into the phagosome) is under separate control. The 'specific' granules are discharged earlier, leading to teleologic speculation that their largely neutral pH-effective contents have time to act before the pH of the phagosome drops and the acid-optimum contents of the azurophil granule take precedence. Calcium ionophores stimulate specific granule release, sodium or potassium ionophores secretion of the azurophil granules.

Direct exocytosis involves the movement of the granule to the plasma membrane followed by fusion of the membranes and discharge of the contents. A modification of this process is seen in mast cells in which there is an explosive degranulation involving fusion of a small section of the plasma membrane with many granules at once, either expelling the granule contents to the outside or exposing them to the extracellular milieu through a network of channels. Granule contents such as histamine are liberated from the proteoglycan matrix by ion exchange mechanisms (Figure 1). 'Piecemeal' degranulation is a process described for basophils in which the granule contents are apparently transported to the plasma membrane in vesicles. These two cell types can also regranulate, a property which may not be shared with neutrophils or eosinophils. In professional phagocytes, including macrophages, the mechanisms of secretion are less clear. Probably some granules fuse directly with the plasma membrane as they have been clearly demonstrated to do with the phagosome, some extracellular discharge occurs as a result of phagosome-granule fusion before the phagosome is closed to the extracellular environment, and some granule contents may be released as a result of vesicle transport and recycling, particularly from the modified lysosomes. In all these cell types, the degranulation appears to occur only at the sites on the plasma membrane that are stimulated. Interaction with stimuli on surfaces too large to be internalized

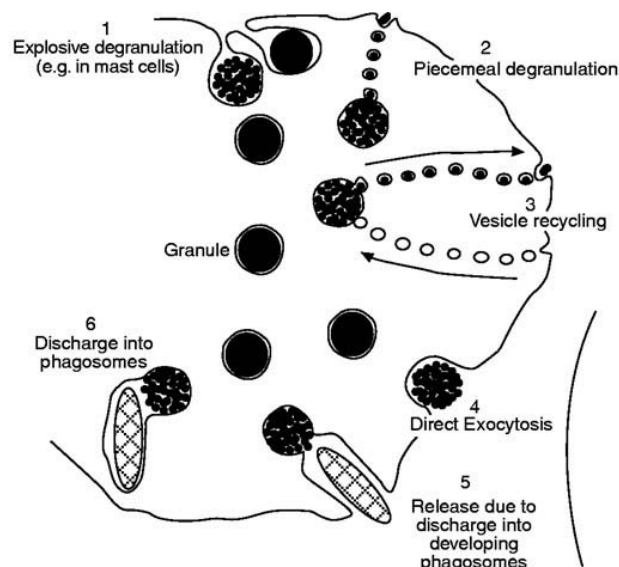


Figure 1 Suggested mechanisms of degranulation in inflammatory cells.

therefore results in release to the outside along the points of contact with that surface, a process termed colloquially, frustrated phagocytosis. These sites represent a protected environment under the cell which may allow secreted constituents to act on the underlying substrata even in the presence of inhibitors in the surrounding tissue fluid.

The signal transduction pathways involved in degranulation are by no means fully identified but do not seem to differ radically from those involved in other responses of these cells and include phospholipid metabolism, calcium mobilization, G proteins and kinase activation. The fungal metabolites cytochalasins B and D significantly enhance secretion in these cells by mechanisms that are not completely understood. However, the effect is employed widely to study secretion of granule contents and caution should be employed in interpreting mechanistic conclusions when these agents are present.

The accumulation of inflammatory cells in a tissue is not by itself enough to provide host defense or induce injury; it is the secretory responses of these cells that are critical, whether into a bacteria-laden phagosome or into the surrounding tissue structures. The early concept that release of contents resulted from cell lysis is now known to be significantly limited; all these cells undergo active, stimulated and highly regulated secretion of their granule contents, as well as of other materials such as newly synthesized proteins and lipids. The granule contents in particular often include highly destructive materials, including nonspecific proteases and phospholipases

as well as potent toxic cations. Their release is commonly associated with tissue destruction and disease.

See also: **Basophils; Eosinophils; Exocytosis; Mast cells; Neutrophils; Phagocytosis; Serotonin.**

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DELAYED-TYPE HYPERSENSITIVITY

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Definitions

‘Delayed-type hypersensitivity’ is generally understood to mean a state of reactivity characterized by inflammatory reactions starting macroscopically 6–12 h after antigen administration in a sensitized individual, reaching their peak after 24–72 h and characterized histologically by lymphocytic and mononuclear cell infiltration. This state of reactivity may not be transferred by serum but by lymphocytes of sensitized animals. Classical examples of delayed-type hypersensitivity are the tuberculin reaction, following intradermal injection of mycobacteria or of their protein extract (tuberculin) in sensitized individuals, or contact dermatitis to eczematogenous substances, such as dinitrochlorobenzene (DNCB). Historically, the tuberculin-type hypersensitivity was described by Koch, who observed that patients with tuberculosis could react with fever and generalized shock, following a subcutaneous injection of tuberculin. This general reaction was accompanied by an area of induration and swelling at the site of tuberculin injection. In the classification of hypersensitivity reactions suggested by Coombs and Gell in 1963, delayed hypersensitivity (also cell-mediated hypersensitivity or type IV) was used as a general heading to describe all hypersensitivity reactions which took more than 12 h to develop. In some more modern textbooks, it can often be read that allergic reactions of the delayed type depend upon a special subset of CD4 T lymphocytes and are essentially due to inflammatory mediators, or lymphokines, produced by these T lymphocytes.

A definition based on the macroscopic kinetics of

inflammatory reactions observed following injection of antigen in a sensitized animal may be misleading. While the macroscopic and morphologic description of classical delayed-type hypersensitivity reaction appears most of the time to follow the kinetics indicated above (onset after 6–12 h, peak after 24–72 h), there are numerous exceptions and the same immunological mechanisms may lead to different time courses. First, the kinetics may vary when the contact between antigen and sensitized lymphocytes occurs in areas other than the skin. Furthermore, some reactions where similar cellular elements are involved may evolve over a period of weeks, or even months (granulomatous reactions). Some authors therefore prefer the general term of ‘delayed-onset hypersensitivity’.

Two modes of delayed-type hypersensitivity are frequently observed:

1. A *permanent* type, persisting for several months or years after completion of the sensitization process, and which corresponds to classical tuberculin-type hypersensitivity. In this case, the inflammatory reaction is still very marked 72–96 h after intradermal injection of antigen.
2. A *transient* type, morphologically very similar, but which disappears 1–2 weeks after induction of sensitization. In this case, the inflammatory lesion peaks at 24 h and has practically disappeared after 48–72 h. This form is also called ‘Jones–Mote hypersensitivity’.

In guinea pigs, reactions with very similar kinetics and also implying cellular infiltrations, but in which basophils may predominate (‘cutaneous basophil

hypersensitivity'), have been described, but they may be sometimes due to antibodies.

The processes leading to delayed-type hypersensitivity reactions may be divided into three phases:

1. A *sensitization phase*, including formation and presentation of antigen to immunocompetent cells.
2. A *development phase*, including the production of specifically sensitized lymphocytes.
3. Upon renewed antigen administration, an *effector phase* in which the interaction of sensitized lymphocytes with antigen leads to the characteristic inflammatory reaction.

Sensitization phase

A large variety of antigens may induce delayed-type hypersensitivity (DTH). Usually, particulate antigens, such as bacteria, viruses or parasites, seem particularly to favor this type of hypersensitivity. Intradermal injection of foreign proteins or application on the skin of reactive chemical substances of low molecular weight, capable of forming conjugates with autologous carrier proteins, also frequently induce DTH. Polysaccharides, on the other hand, are less prone to cause DTH. The mode of administration of antigen also seems to play a role. Intravenous injection is usually not efficacious in inducing DTH. The incorporation of antigen in some adjuvants, in particular in complete Freund's adjuvants containing mycobacteria, is very favorable for establishing DTH. The role of macrophages in antigenic presentation is also important. Failure to provide proper antigenic presentation by macrophages usually leads to lesser induction of DTH, even if antibodies may be formed. The fate of antigen in the organism, for example after application on or injection into the skin, probably also plays an important role in induction of sensitivity. It has been shown that the integrity of afferent lymphatic channels and of regional lymph nodes is needed for establishment of DTH. It is therefore very likely that the first recognition of antigen and presentation to T lymphocytes occurs in the regional lymph nodes. However, to this concept of 'central sensitization' can also be added examples of 'peripheral sensitization'. In the induction of contact eczema or in skin transplantation, it has been shown that local persistence of antigen for a while is required for the induction of the process, even when large amounts of soluble antigens quickly reach the regional lymph nodes. In that case, antigen presentation and recognition may occur at the level of local antigen deposits by T lymphocytes and macrophages freely moving through tissues and returning through afferent lymphatic channels to the

regional lymph nodes. The role of dendritic cells in the epidermis (Langerhans cells), which possess several macrophage-like properties, has been particularly emphasized.

Development phase

Kinetic examination of cellular changes in regional lymph nodes following local administration of antigen has shown that some of the thymus-dependent lymphocytes, which accumulate in the paracortical zone of the lymph nodes, start to proliferate from the 2nd day on and reach, 3–4 days after first contact with antigen, an immunoblastic, proliferative stage. These immunoblasts (large pyroninophilic cells) revert after mitotic division to small lymphocytes, which appear to be specifically sensitized and are the expression of amplification of some predetermined clones. However, the large proportion of immunoblasts in the lymph node cell population suggests that this proliferation is also to some extent polyclonal, probably under the effect of mitogenic lymphokines. The acquisition of specific T lymphocytes, able to produce a DTH reaction, may be evaluated by passive transfer of lymph node cells taken at various times after initial antigen administration. Such passive transfer experiments have shown that specifically sensitized T cells are present in local regional lymph nodes and cause local inflammatory reactions upon renewed contact with antigen. These transfer experiments have also enabled the identification of some T lymphocyte subsets as specifically reactive elements with antigens and responsible for DTH reactions in guinea pigs and in mice. Current evidence suggests that a special subset of CD4⁺ T_H1 lymphocytes is responsible as primary effector cells in DTH reactions.

Recent findings suggest that the nature of antigen presentation by dendritic cells and the lymphokine microenvironment play a decisive role in CD4⁺ T cell development leading to suitable effector T lymphocyte subsets for DTH. Interleukin 12 (IL-12) favors T_H1 development and DTH, while IL-10 antagonizes the development of T_H1 cells and favors T_H2 lymphocytes. However, there is also evidence that several subsets of lymphocytes may be implicated in DTH and that the T_H1/T_H2 dichotomy may be an oversimplification.

Effector phase

Renewed application or injection of antigen into the skin of a sensitized individual induces an inflammatory reaction, which may start within 6–12 h with some erythema, accompanied by progressive tissue

induration reaching its peak after 24–72 h. When the antigen is simply deposited on the skin, such as in contact dermatitis, dermal cellular infiltration is accompanied by epidermic lesions, such as spongiosis and formation of vesicles. Although the morphological aspects of contact dermatitis and tuberculin-type hypersensitivity reactions do differ somewhat, which has led some authors to consider these two types of reaction as entirely separate, the differences appear rather to be due to different local concentrations of antigen at the dermal or epidermal level, but they do not reflect fundamental differences in mechanisms.

Contact hypersensitivity is mainly an epidermal phenomenon in which the main antigen-presenting cell is the Langerhans cell. Langerhans cells are dendritic and express the cortical thymocyte antigen CD1 and major histocompatibility complex (MHC) class II antigens. They can also be found in the afferent lymph and paracortical area of lymph nodes as early as 4 h after epicutaneous application of a contact allergen in mice. The earliest histological change in the challenge reaction of contact hypersensitivity is observed after 3–4 h, when a mononuclear cell infiltrate is noticed around blood vessels. The infiltrate with mononuclear cells consists essentially of CD4⁺ lymphocytes, with a lesser number of CD8⁺ cells. Some increase in the expression of activation antigens (e.g. HLA-DR) by keratinocytes indicates that epidermal cells may also be implicated.

In tuberculin-type reactions, in which antigen is primarily deposited in the dermis, dermal perivascular infiltrates predominate and usually peak around 48 h. In this infiltrate CD4⁺ cells outnumber CD8⁺ cells by about 3:1, CD1⁺ cells (Langerhans-like) are also found. Polymorphs are rather uncommon in humans, but macrophages and monocytes also accumulate around dermal vessels by 12 h. Many of the infiltrating lymphocytes and macrophages express activation antigens, such as HLA-DR.

Functional analysis of such reactions has clearly shown that they occur in two stages. In a first specific stage, antigen comes into contact with sensitized T lymphocytes which are present as a very small proportion of the total T lymphocyte population normally migrating from blood vessels throughout tissues, to return then through lymphatic channels to regional lymph nodes and to the blood circulation. This specific interaction with antigen stimulates lymphocytes to synthesize and release in the tissues various proinflammatory cytokines. One of the first manifestations of inflammation consists of an augmentation of capillar permeability and increased blood flow, which favor cellular influx into tissues as well as liquid extravasation and deposition of certain

blood proteins. Fibrin deposits, not cellular infiltration as usually believed, are responsible for the induration phenomenon characteristic of DTH, as induration may be totally suppressed by anticoagulants. How far the concomitant activity of perivascular mast cells carrying immunoglobulin E (IgE) of the same antigen specificity, as well as vasoactive amines, such as serotonin, or factors produced by T cells enhancing capillary permeability are also involved remain topics for investigation.

During a second nonspecific phase, various cellular types are attracted into the reaction site, essentially monocytes and small lymphocytes, more rarely basophils or eosinophils. Transfer experiments have clearly shown that the great majority of cells participating in the tissue infiltration are nonspecific.

Pathophysiological analysis of DTH reactions has implicated different chemotactic mediators in the accumulation of the cellular infiltrate. There are probably also some factors inhibiting cellular migration, in order to explain accumulation of cells in the tissues, which otherwise would be normally evacuated through lymphatic vessels. Many of the cells involved are in a state of activation, as manifested by the appearance of surface activation antigens (e.g. HLA-DR). The production of various lymphokines such as IL-2 and interferon α has been detected by *in situ* hybridization and is involved in cellular proliferation in DTH reaction sites. There are also manifestations of cellular toxicity at the local level, for example in transplant rejection, which may be due to the local production of lymphotoxins. The role of cytokines in DTH reactions was first supported by indirect arguments, such as: 1) the production of very similar inflammatory lesions upon intradermal injection of lymphokine-containing T cell culture supernatants or of purified lymphokines; 2) similar effects on inhibition of migration of peritoneal macrophages *in vivo* ('macrophage disappearance reaction'); 3) cellular chemotactic effects *in vivo* by extracts from local DTH reactions; 4) the presence of various lymphokines in the serum of animals or humans undergoing a general DTH reaction; 5) the suppression of DTH reactions by some antilymphokine antibodies. Experiments using *in vitro* hybridization techniques now permit us to follow directly the synthesis of various cytokines in DTH reactions. In particular, IL-2 and interferon α seem to play an important role.

The development of DTH lesions in tissues appears increasingly as a complex event in which, beyond antigen-specific T lymphocytes subsets and monocytes, a number of local cells may be implicated. These are: 1) keratinocytes producing proinflammatory cytokines (e.g. interferon γ); 2) mast

cells and platelets; 3) endothelial cells releasing L- and P-selectins and mediating selective local attachment of T lymphocytes. Among mediators released by such cells, histamine, serotonin and leukotrienes all appear to contribute to DTH inflammation.

Delayed-type hypersensitivity reactions may become chronic and protracted, taking the form of granulomatous hypersensitivity, which may be clinically the most important form of DTH. It often results from the presence within macrophages of some persistent agent, usually a microorganism, which the cell is not able to destroy. On other occasions it may be caused by the continuous presence of antigen in particulate or immune complex form. The histological appearance of the granulomatous reaction is quite different from the tuberculin-type reaction and is characterized by the abundance of epithelioid cells, which are believed to derive from activated macrophages. The immunologically induced granulomatous lesions usually possess a core of epithelioid cells and macrophages, sometimes with giant cells; the central area may become necrotic. The macrophage epithelioid core is surrounded by a cuff of lymphocytes; there is often also considerable fibrosis and deposition of collagen fibers.

Clinical manifestations

Delayed-type hypersensitivity reactions are a prominent feature of several chronic diseases in humans, which for the most part are due to infectious agents, such as mycobacteria, protozoa and fungi. Important diseases include tuberculosis, leprosy, listeriosis, leishmaniasis, deep fungal infections (e.g. blastomycosis) and helminthic infections (e.g. schistosomiasis). These diseases are caused by pathogens which represent a persistent, chronic, antigenic stimulus. In such cases, protective immunity and delayed hypersensitivity often occur but are not always coincidental.

Instead of discussing these various DTH-related diseases, it may be more useful to describe under more general terms the various types of DTH reactions which may be encountered.

Local reactions

Most descriptions of DTH are based on local reactions following application or injection of antigen into the skin. These reactions are easiest to follow experimentally and are also frequently used from a diagnostic point of view (cutaneous tests). However, these local reactions, described in detail above, are not the only possible manifestation of clinical DTH.

Focal reactions

In most local reactions, antigen is eliminated after a few hours or a few days, in particular by activated macrophages. When antigen cannot be eliminated, a local chronic inflammation may occur in the form of a granuloma. In that case, any new massive parenteral administration of antigen may provoke a violent focal reaction following interaction of antigen with locally accumulated sensitized lymphocytes: this manifests as marked flare-up of the chronic inflammatory process. A historical example of focal reaction is the sudden aggravation of tuberculous lesions following intravenous injection of tuberculin, first attempted by Koch in the treatment of tuberculosis. Such reactions may still occasionally be observed after intradermal injection of too high a concentration of tuberculin. Similar focal reactions, for example in dental granulomas, sinusitis, etc., are frequently observed clinically. Flare-up reactions after new parenteral administration of antigen at the site of previous local DTH reactions may also be due to similar pathophysiology, but they are sometimes also due to local accumulation of plasma cells and specific antibodies.

Generalized DTH reaction

Generalized DTH reactions following parenteral administration of antigens are also frequent. The most classical example, which is rarely observed nowadays, is the tuberculin shock accompanied by fever. Some of the cutaneous reactions accompanying viral infections (morbilliform exanthema in measles) are probably of the same nature, as well as generalized hypersensitivity reactions to drugs. In that case, lymphokines may be detected in the serum during the acute phase. Such reactions often occur within 4–6 h of antigen administration and are therefore not 'delayed'.

In summary, although the kinetics and characteristics of DTH reactions may vary according to the mode of administration of antigen and to the tissue in which the reaction takes place, a common triggering mechanism in which T lymphocytes and lymphokines play a major role gives a clear-cut identity to this chapter of immunopathology.

See also: Antigen-presenting cells; Cytokines; Effector lymphocytes; Granuloma; Hypersensitivity reactions; *Mycobacteria*, infection and immunity.

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DENDRITIC CELLS

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Dendritic cells (DCs) are a lineage of specialized antigen-presenting cells (APCs) that play a central role in the initiation of adaptive immune responses. Since the first description by Steinman and Cohn in 1973 of the lymphoid DC as a trace cell type isolated from lymphoid tissues of mice, it has become clear that cells of this leukocyte lineage are widely distributed throughout lymphoid and most nonlymphoid tissues of all mammalian species studied. DCs have or can acquire a phenotype distinct from that of other leukocyte lineages (Table 1).

It is convenient to distinguish between different stages of DC according to the cells' specialized functions in different anatomical compartments. 1) DC progenitors originate from the bone marrow (BM) of adult mammals and enter the blood to seed the tissues. 2) Within nonlymphoid tissues the cells develop into the so-called 'immature' or, perhaps better, 'processing' stage, with optimal capabilities for the uptake and processing of foreign antigens, MHC (major histocompatibility complex) production, and the formation of foreign peptide-MHC complexes. 3) The cells begin to mature, and migrate from nonlymphoid tissues into secondary lymphoid tissues. 4) In secondary lymphoid tissues, DCs at the 'mature' or 'costimulatory' stage present foreign peptide-MHC complexes to resting T cells and have acquired the capacity to deliver specialized costimulatory signals for T cell activation, a crucial step in the induction of most adaptive immune responses. 5) DCs within the thymus may present self peptide-MHC complexes to developing thymocytes for tolerance induction.

In contrast to cells of the DC leukocyte lineage, the follicular dendritic cell (FDC) probably represents a distinct cell type that is not BM derived, can express membrane-bound native antigens in immune complexes for prolonged periods, and plays a role in B cell (humoral) responses.

Dendritic cell progenitors

Progenitors *in vivo*

The bone marrow (BM) origin of DCs is well established from studies with radiation-induced BM chimeras, in which replacement of host DCs by donor-derived cells can be visualized. It seems probable that DC progenitors seed most tissues, but the present lack of markers for the cells at this early stage precludes precise estimates of their numbers. Stimuli that recruit DC progenitors into tissues include granulocyte-macrophage colony-stimulating factor (GM-CSF), since, for example, local production of GM-CSF increases the numbers of DCs in human lung and some lung cancers, and increased numbers of DCs are present in the dermis of human skin after intradermal administration of this cytokine. Bacterial lipopolysaccharide (LPS), or cytokines elicited by this agent, recruits DC progenitors to rat lung, and mouse heart and kidney, following aerosol and systemic administration of LPS, respectively.

Following transplantation of rat liver to allogeneic recipients, donor-derived DC progenitors can be detected in host BM, suggesting that DC progenitors from at least some nonlymphoid tissues may seed other sites. After transplantation of human livers,

Table 1 Phenotype of dendritic cells^a

Molecule	Expression on DCs ^c and comments	Status ^b
CD1	CD1a expressed by human DCs cultured from progenitors and by epidermal LCs. CD1c expressed by human lymph node DCs. Structurally related to MHC class I molecules and can act as ligands for some $\alpha\beta$ and $\gamma\delta$ T cells	
CD4	Expressed by a subset of <i>human</i> blood DCs that may be derived directly from the bone marrow (CD4 downregulated on culture), and by human epidermal LCs and DCs in other epithelia. Expressed by <i>rat</i> veiled cells. Binds MHC class II molecules and a receptor for HIV in human	
CD8	Expressed by a subset of <i>mouse</i> DCs in spleen as $\alpha\alpha$ homodimers, and by DCs in thymus, some of which express $\alpha\beta$ heterodimers. Binds MHC class I molecules	
CD10	Expressed by human bone marrow progenitors that apparently give rise to DC, T cells, B cells and NK cells	P
CD11a/CD18: LFA-1	Weakly or not expressed by epidermal LCs, but expressed by many other DCs. Ligands are CD54 (ICAM-1), ICAM-2 (CD102) and ICAM-3 (CD50)	
CD11b/CD18: CR3	Expressed by most DCs but often at low levels. The type 3 complement receptor; ligands include iC3b, fibrinogen and cell wall components of some bacteria and protozoa	
CD11c/CD18: p150,95	In <i>mouse</i> expression is relatively restricted to most or all DCs in lymphoid tissues	+
CD14	Not expressed by most DCs. Transient expression by DCs cultured from progenitors is possible. The receptor for LPS complexed with LPS-binding protein expressed by monocytes/macrophages and FDCs	(0)/F
CD18	See CD11a, CD11b and CD11c. The common β_2 integrin subunit	
CD19	Not expressed by DCs. Expressed by all human B cells, some B cell precursors, and FDCs	0/F
CD21: CR2	Not expressed by DCs. The type 2 complement receptor expressed on mature B cells and FDCs. Receptor for EBV	0/F
CD23: Fc ϵ RII	Human epidermal LCs express the Fc ϵ RIIb form of CD23, the low-affinity receptor for IgE. Note: High affinity IgE receptors (Fc ϵ RI) are also expressed by epidermal LCs	
CD25	Expressed by maturing DCs such as mouse LCs and rat veiled cells, but its function on these cells is unknown. The IL-2 receptor α (p55) chain	
CD32: Fc γ RII	Expressed by human and mouse epidermal LCs but downregulated during maturation. May target antigens to intracellular processing compartments and enhance antigen presentation. The Fc receptor for aggregated and antigen-complexed IgG	
CD34	Expressed by immature hematopoietic cells, including DC progenitors in human and mouse bone marrow and blood	P
CD35: CR1	Not expressed by DC. The type 1 complement receptor expressed for example by B cells and FDCs	0/F
CD40	Expressed by DCs cultured from progenitors and by DCs in lymphoid tissues. A costimulatory molecule for T cell activation. CD40 ligand (gp39) stimulates maturation of presumptive DCs cultured from human blood monocytes	
CD44: Pgp-1	Expressed by mouse epidermal LCs and interstitial DCs (e.g. heart). CD44 is differentially spliced. Different isoforms may be expressed by DCs: for example, CD44-v5 and -v6 may be upregulated during mouse LC maturation, and CD44-v9 is induced during maturation of human DCs grown from progenitors. Ligand is hyaluronate	
CD45: LCA	Expressed by essentially all DCs. CD45 is differentially spliced; isoforms expressed by DCs not yet studied in detail	
CD49	Expressed by epidermal LCs and lymphoid DCs. CD49 is a family of integrin chains of the very late antigens (VLA) associated generally with CD29. Mouse LCs express little VLA-4 (CD49d) but some cells have high levels of VLA-6 (CD49f); the converse is true for DCs isolated from lymph nodes. Ligands include CD106 (VCAM-1) for CD49d/CD29 and laminin for CD49f/CD29	
CD54: ICAM-1	Not detectable on epidermal LCs, expressed on DCs grown from progenitors, and upregulated to high levels during maturation of DCs (e.g. on DCs in lymphoid tissues). A ligand for CD11a/18 (this interaction enhances T cell activation) as well as CD11b/CD18 (CR3). The major rhinovirus receptor in human	
CD58: LFA-3	Expressed by some DCs. Ligand is CD2, e.g. on T cells	

Table 1 Continued

<i>Molecule</i>	<i>Expression on DCs^c and comments</i>	<i>Status^b</i>
CD74: li	Freshly-isolated epidermal LCs exhibit a high rate of CD74 biosynthesis but this is shut off during maturation in culture; may also apply <i>in vivo</i> and to other nonlymphoid DCs. The MHC class II-associated invariant chain	I
CD80: B7-1	Expressed at low levels by mouse and human epidermal LCs but upregulated during maturation, and expressed by lymphoid DCs. A costimulatory molecule. Ligands are CD28 and CTLA-4 on T cells	
CD83	Expressed by <i>human</i> DC cultured from progenitors, human blood DC, LCs and DCs in lymphoid tissues on mature cells	*
CD86: B7-2	No or low-level expression on epidermal LCs but dramatically upregulated during maturation and expressed by interdigitating cells in lymphoid tissues. Reportedly expressed by mouse DCs in other epithelial sites (e.g. lung but not gut) and in some interstitial spaces (e.g. heart but not kidney). A major costimulatory molecule. Ligands are CD28 and CTLA-4 on T cells	
CD88: C5aR	Expression upregulated during maturation of epidermal LCs, and expressed by interdigitating cells in lymphoid tissues. The C5a receptor	
CD95	Expressed by some DCs. Fas receptor	
CDw116	Expressed by DC progenitors (inferred) and by epidermal LCs. The GM-CSF receptor	
CD117: <i>c-kit</i>	Expressed by DC progenitors (inferred). The stem cell factor (SCF) receptor	
CD120	Expressed by DC progenitors and epidermal LCs (inferred). The TNF receptor(s)	
CD2w121	Expressed by epidermal LCs (inferred). The IL-1 receptor(s)	
Chemokine receptors	CCR5 specific for RANTES, MIP-1 α and MIP-1 β , and CXCR4 (or fusin) specific for SDF-1 are expressed by DC; HIV co-receptors in human	
CLA	Expressed by DC progenitors that can give rise to DCs containing Birbeck granules in culture. The cutaneous lymphocyte-associated antigen	P
DEC 205	Expressed by <i>mouse</i> epidermal LCs and by subsets of DCs in lymphoid tissues, and may target antigens to processing compartments. DEC 205 is structurally related to the mannose receptor (see below); ligands under investigation. Identified by the NLDC-145 monoclonal antibody	
HSA, J11d	Expressed by epidermal LCs and perhaps subsets of DCs in lymphoid tissues in <i>mouse</i> . A costimulatory molecule for CD4 ⁺ T cells. The heat-stable antigen	
Mannose receptor	Expressed by presumptive DCs cultured from human blood monocytes, and targets mannosylated antigens to processing compartments. The macrophage mannose receptor. See also DEC 205	
MHC class I	Classical MHC class I molecules are probably expressed by all DCs after the progenitor stage. Expression of nonclassical molecules has not been well studied. See also CD1	
MHC class II	MHC class II molecules may be expressed by some DC progenitors and by all DCs after the progenitor stage, and expression can be upregulated (e.g. during maturation of LCs). In human, lymphoid DCs reportedly express HLA-DP, -DQ and -DR products. See also CD74	
MRC OX62	Expressed by some lymphoid and nonlymphoid DCs in <i>rat</i> . Also expressed by some $\gamma\delta$ T cells. May be an integrin	*
2A1	An intracellular antigen of <i>mouse</i> DCs that is expressed during maturation. Appears localized to a late endosomal compartment. See also M342	*, I, >
33D1	A marker of a subset of DCs in <i>mouse</i> lymphoid tissues	*
F4/80	Expressed by <i>mouse</i> epidermal LCs and downregulated during maturation. A molecule with homology to G protein-linked 7 transmembrane hormone receptor family members	
M342	Expression and distribution similar to 2A1	*, I, >
NLDC-145	See DEC 205	*
S100	An intracellular molecule expressed by <i>human</i> LCs, and often taken as indicative of DCs in other sites	I

^aExamples of molecules expressed by DCs at different stages of development and in different anatomical compartments, focusing on human and mouse DCs but including comments on rat DCs where relevant.

^bStatus of molecule: *, molecule that can be used as a marker of DCs rather than of most other cell types; 0, not expressed by DCs; P, expressed only at progenitor stage; >, expression increased during DC maturation; <, expression decreased during DC maturation; F, molecule expressed by FDCs but not DCs; I, intracellular expression.

^cSpecies specificity is indicated by *italics*. In some cases (e.g. CD4 versus CD8 expression on human and rat versus mouse DCs, respectively; mouse CD11c expression) this is likely to be absolute. In other cases, homologous molecules showing a similar distribution may be identified in other species.

donor-derived cells resembling DCs can also be detected in other recipient tissues such as skin, and it has been suggested that such a state of 'microchimerism' is necessary for transplantation tolerance, although this is not yet proven.

Culture of progenitors

DCs can be generated *in vitro* from BM and blood progenitors cultured in cytokines, but the precise branch point for differentiation of DCs from other leukocyte lineages is not completely determined. 1) DCs can be grown from mouse CD34⁺ MHC class II-negative BM and blood progenitors cultured in the presence of GM-CSF, or from human BM and cord blood progenitors cultured in GM-CSF plus tumor necrosis factor α (TNF α). Production of mixed colonies of mouse DCs, macrophages and neutrophils implies the existence of a common committed stem cell (e.g. the mouse granulocyte-macrophage colony-forming unit or CFU-GM). In human BM, a clonogenic progenitor (termed CFU-DC) has been identified that generates pure colonies of DCs and which can be expanded in the presence of *c-kit* ligand (stem cell factor). Cytokines such as *flt-3* ligand may also induce proliferation of DC progenitors and increase production of DC *in vitro* and *in vivo*. 2) DCs can be generated that either do or do not possess Birbeck granules, an endocytic organelle present in Langerhans cells of several species but not in interstitial DCs (see below), depending on whether CD34⁺ progenitors express CLA (Table 1) or not, respectively. This indicates that the recruitment of DCs to different anatomical sites may be determined, in part, at the progenitor level. 3) Subsets of human CD34⁺ MHC class II-positive BM cells and mouse thymic lymphoid precursor cells may give rise to both DCs and lymphoid (mouse T, or human T, B and natural killer (NK)) cells but not myeloid cells. This suggests either that a distinct DC progenitor(s) seeds the thymus, or that this differentiation pathway is induced by the particular cytokine or hormonal milieu of this tissue. 4) Cells closely resembling DCs can be cultured from human peripheral blood low-density adherent cells (monocytes), with GM-CSF plus interleukin 4 (IL-4) generating an 'immature' or 'processing' stage, and subsequent exposure to TNF α , IL-1, CD40 ligand or LPS promoting further maturation.

Further development of these approaches for production of DCs *in vitro* is likely to lead to new immunotherapeutic strategies using DCs *in vivo*. Such cells grown from BM and pulsed with relevant antigens have been used to induce antitumor immunity in mice, for example, and related clinical trials are now underway.

Nonlymphoid dendritic cells

DCs in nonlymphoid tissues perform a sentinel function in acquiring foreign antigens before maturing and migrating to secondary lymphoid tissues for initiation of many immune responses. Hence, in general, the cells *in situ* represent the immature or processing stage of the lineage.

1) DCs are present within skin and the epithelia of the respiratory, gastrointestinal and urogenital tracts. The DCs of skin, Langerhans cells (LCs), form a ramifying network throughout the epidermis, together, in mouse but not human, with epidermal $\gamma\delta$ T cells whose functions have yet to be ascertained. It is estimated that the half-life of LCs in unperturbed skin epidermis is likely to range from weeks to even a month or two. Precursors to LCs are probably present within the dermis of skin, in which a population of so-called dermal dendrocytes has been identified. Similar cells are distributed throughout the lung and airways, but are juxtaposed to macrophages which appear to regulate DC function by maintaining the transiently resident DCs at an immature stage. In gut, the lamina propria contains large numbers of DCs. In rats, following oral feeding or intraintestinal injection of antigen, and after aerosol administration of antigens to the lung and airways, DCs from these sites can present the antigen to sensitized T cells *in vitro* and can prime naive recipients after adoptive transfer *in vivo*. 2) DCs are present in the interstitial spaces of all solid organs, with the exceptions of the bulk of the central nervous system and some other sites considered to be 'immunologically privileged' such as the testis. Interstitial DCs probably acquire antigens that gain access to these sites. The half-life of interstitial DCs, such as those in heart, may range from a few days to a week or so. 3) The liver may represent a special case, in that DC progenitors may be recruited to the liver sinusoids, develop into the processing stage, and acquire particulate and perhaps other antigens that enter the bloodstream.

Maturation and migration of dendritic cells

Maturation

DC maturation commences in nonlymphoid tissues and may continue within secondary lymphoid tissues. Maturation involves profound phenotypic and functional changes as the cells convert from the processing to the costimulatory stages. These events have been well studied in the case, for example, of LCs. 1) Freshly-isolated epidermal LCs express membrane receptors, some of which can target antigens

into cellular processing compartments (e.g. CD32 and mouse DEC205; Table 1); internalize soluble and certain particulate antigens (pinocytosis and phagocytosis); contain acidic compartments associated with processing of exogenous antigens (endolysosomes and specialized CIIV and MIIC), synthesize MHC class II and invariant chain molecules; assemble peptide-MHC complexes which are transported to the cell surface; and produce some cytokines (e.g. IL-1 β) and chemokines (e.g. MIP-1 α , MIP-1 γ , MIP-2). The cells have little or no costimulatory activity. 2) In the presence of GM-CSF, and in synergy with IL-1 but not TNF α in mouse, cultured LCs downregulate some receptors (e.g. CD32 but not DEC205); lose phagocytic activity and many acidic compartments; shut off MHC class II and invariant chain synthesis; and exhibit a different cytokine profile (e.g. increased IL-1 β and IL-12 but decreased MIP-1 α and MIP-2 production). However, surface expression of peptide-MHC complexes increases and the cells acquire costimulatory activity associated with new or increased expression of molecules such as CD40, CD80 and CD86 (Table 1). Broadly speaking, these events are likely to occur during maturation of LCs *in vivo* and are likely to apply to other nonlymphoid DCs. Mice that lack the *relB* subunit of the NF κ B transcription factor have LCs in skin, but appear to lack lymphoid DCs and have highly disorganized lymphoid tissues. Hence, *relB* expression may be essential for development or maturation of at least some DCs.

Migration

Migration from nonlymphoid tissues (and presumably maturation) is promoted by locally-produced inflammatory cytokines and other agents that may be generated during local infection or trauma. For example, IL-1 β and TNF α (the latter perhaps produced in a paracrine loop induced by IL-1 β *in vivo*) may promote migration of LCs from mouse skin, and LPS or LPS-induced cytokines such as TNF α (or LPS itself) may promote migration of DCs from rat gastrointestinal tract and from mouse heart and kidney and probably other nonlymphoid sites.

As a general rule, DCs from epithelia may migrate exclusively via afferent lymph into regional lymph nodes, whereas DCs from interstitial spaces migrate both via afferent lymph to nodes and via the blood to spleen. 1) The DCs of afferent lymph, so-called 'veiled cells' because of their distinct cytological features (namely the presence of sheet-like lamellipodia), appear heterogeneous in phenotype and function and can be viewed as representing a transitional stage from processing to costimulatory DCs. The flux of DCs in afferent lymph draining normal

skin is considerably enhanced following topical application of agents such as contact sensitizers, probably because of the local inflammatory response induced in the skin. In mouse skin transplants and explants, LCs can be visualized moving from epidermis and into dermal lymphatics before leaving the tissue; this process is accompanied by maturational changes. There is also a considerable traffic of DCs from lamina propria of gut, and these cells can be collected from rat central lymph after mesenteric lymphadenectomy. In general, agents such as LPS or LPS-elicited cytokines appear to recruit DC progenitors into local sites from which the immature cells are leaving, presumably to replace the local DC pool; these events have also been documented for DCs in rat lung following administration of certain bacteria. 2) Peripheral blood contains at least two populations of DC: recently-produced DC progenitors traveling from BM to tissues (these cells may express CD4 in human), and DCs from nonlymphoid tissues traveling to spleen. The latter route is evident from the finding that DCs migrate from mouse cardiac allografts and can be detected in recipient spleens. 3) There may be a specialized route for DC migration from liver sinusoids into celiac nodes for initiation of responses to bloodborne antigens: following celiac lymphadenectomy of rats and intravenous delivery of a phagocytic load, particle-laden DCs can be isolated from central lymph, but these cells are not present after mesenteric lymphadenectomy.

Lymphoid dendritic cells

DCs in secondary lymphoid tissues

DCs isolated from secondary lymphoid tissues such as spleen, lymph node and Peyer's patch are potent stimulators of immune responses *in vitro*. These include: T cell proliferative responses to specific antigens (e.g. in transgenic T cell systems), alloantigens (i.e. allogeneic mixed leukocyte reactions), superantigens, and mitogenic stimuli (e.g. oxidative mitogenesis); T-dependent antibody formation; and cytotoxic T lymphocyte generation through both CD4-dependent and CD4-independent pathways. DCs are probably required for responses of memory, as well as resting T cells, whereas activated T cells can respond to other types of APC as well as DCs. The potency of lymphoid DCs can at least in part be attributed to their capacity to form antigen-independent clusters with resting T cells, expression of relevant peptide-MHC complexes and expression of costimulatory molecules such as CD40, CD80 and CD86. In addition, lymphoid DCs can produce high levels of IL-12, which promotes development of T_H1 responses of CD4⁺ T cells. Bulk populations of

lymphoid DC suspensions are capable of antigen uptake and processing to some extent, indicating that at least some DCs in secondary lymphoid tissues may not be fully mature. For example, some lymphoid DCs are phagocytic *in vitro* (although this activity is lost during culture) and antigen-pulsed lymphoid DCs can sensitize naive recipients after adoptive transfer *in vivo*.

An important and distinctive feature of DCs is their capacity to home to discrete regions of lymphoid tissues *in vivo*. For example, DCs home from afferent lymph into the subcapsular sinus of lymph nodes and thence to the interfollicular and paracortical (T) areas; and from the blood into the marginal zone of spleen and thence to white pulp, probably traversing peripheral white pulp (B areas) before localizing in central white pulp (T areas). Hence at least some DCs in secondary lymphoid tissues originate from peripheral sites, although some cells conceivably develop from progenitors entering directly from the BM.

Lymphoid tissues such as mouse spleen and Peyer's patch contain two phenotypically distinct subsets of DC, and the same is likely to be true of lymph node and to pertain to other species. Within mouse spleen, interdigitating cells (IDCs) are localized in central white pulp (PALS) juxtaposed to the bulk of resting T cells, while in the marginal zone 'nests' of DCs have been described that may ultimately give rise to IDCs. The phenotype of these cells, based on the use of DC-restricted monoclonal antibodies is, respectively, N418⁺ NLDC145⁺ 33D1⁻ 2A1⁻ M342⁺ and N418⁺ NLDC145⁻ 33D1⁺ 2A1⁻ M342⁻ (Table 1). Similar subsets have been described in mouse Peyer's patch, with DCs in deep interfollicular (T cell) regions being clearly distinguishable from DCs in the more superficial subepithelial dome region (and which may acquire antigens transported from the gut across M cells of the patch). A small subset of DCs, distinct from FDCs, has also been identified within germinal centres. It is possible, though not yet proven, that DCs can enter these sites during secondary responses or later in primary responses, for example after antibody has been formed.

DCs do not appear to leave secondary lymphoid tissues, either via efferent lymph from lymph nodes or via blood from the spleen. Instead, it seems likely that the cells may be removed by apoptosis, either as part of a regulated differentiation process or after interaction with activated T cells (e.g. through Fas-Fas ligand interactions). The half-life of DCs in spleen, for example, is short and probably ranges from 2 to 3 days only.

DCs in thymus

DCs isolated from thymus are similar to DCs isolated from secondary lymphoid tissues in their capacity to activate resting T cells. However, within the thymus, DCs may interact with developing thymocytes and induce central tolerance (negative selection). A large body of evidence, from studies with radiation-induced BM chimeras for example, attests to the capacity of BM-derived cells to induce negative rather than positive selection of T cells. Experiments in which organ cultures of mouse embryonic thymus lobes were reconstituted with DCs have shown that these cells can indeed delete potentially autoreactive thymocytes. Similar findings after injection of DCs into mouse thymus *in vivo* have also implicated DCs in tolerance induction, although both clonal deletion and clonal inactivation (anergy) have been reported. Within the thymus, MHC class II-positive DCs are situated at the corticomedullary junction and within the medulla (thymic IDCs), and DCs added to organ cultures of mouse embryonic thymus lobes home to this region. It is possible a distinct DC progenitor within thymus can give rise to both DCs and T cells (see above). Whether or not DCs can also induce peripheral tolerance is currently under investigation.

See also: Antigen-presenting cells; Antigen presentation via MHC class II molecules; Apoptosis; B lymphocytes; Bone marrow and hematopoiesis; Cytokines; Germinal center; Langerhans cells; Lymphatic system; Lymph nodes; Mucosa-associated lymphoid tissue (MALT); Skin, contribution to immunity; Spleen; Thymus; T lymphocytes.

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DEOXYGUANOSINE

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The discovery in 1972 that an inherited disorder of adenine nucleotide metabolism was associated with a specific immunodeficiency disease in humans stimulated a major resurgence of interest in the metabolism and effects of purine nucleosides. The association of disorders of purine nucleoside catabolism with severe immunologic dysfunction provides compelling evidence for the role of these metabolic pathways in the biochemical control of lymphocyte differentiation and function. The link between the biochemical explanations for the immunologic disorders, the remarkable tissue specificity of the diseases, and the pathologic effects of these inborn errors of metabolism remains somewhat uncertain.

Purine nucleoside phosphorylase deficiency

Deficiency of the enzyme purine nucleoside phosphorylase (PNP) usually results in the appearance of signs and symptoms of immunodeficiency between the ages of 3 and 18 months. There is a striking dichotomy between T and B cell immunity in these patients. Humoral immunity, B cells, B memory cells, and plasma cell differentiation are generally intact. Indeed, the presence of autoantibodies and elevated immunoglobulin levels are usually present. In contrast, T cell immunity is markedly affected. Although T cell precursors appear to be present, peripheral T cell lymphopenia in the circulation and lymphoid

tissues is profound, accounting for the rather selective deficiency of cell-mediated immunity. Although the T cells that are present may be immature and proliferate poorly, they are capable of providing helper cell activity for B cell maturation. The most significant metabolic abnormality found in PNP-deficient patients is the elevated deoxyGTP levels in erythrocytes and urine.

Deoxyguanosine metabolism

The structural gene for PNP in humans has been assigned to the q13 band of chromosome 14. The enzyme is ubiquitous in human tissues with the highest specific catalytic activities in peripheral blood lymphocytes, granulocytes, erythrocytes and kidney. In most patients deficiency of enzyme activity is associated with a mutation of the structural gene for PNP. The products of adenosine deaminase – inosine, deoxyinosine, guanosine and 2'-deoxyguanosine (dGuo) – are all naturally occurring substrates for the enzyme. The phosphorylase generates the corresponding purine bases, hypoxanthine or guanine (see Figure 1). Of the substrates that accumulate in excess in the absence of PNP activity, only dGuo can be salvaged directly by phosphorylation to the corresponding nucleotide, 2'-deoxyguanosine monophosphate. Phosphorylation is exclusively by deoxycytidine kinase which results in the accumulation ultimately of deoxyGTP.

Deoxyguanosine metabolism and T lymphocyte ontogeny

An understanding of the toxicity of dGuo in T cells may be derived from knowledge of the distribution of the enzymes important in nucleoside metabolism in different lymphocyte subpopulations and at different stages of maturation. The critical enzymes are adenosine deaminase, PNP and deoxycytidine kinase (Figure 1). The more immature the T cell, the higher the levels of adenosine deaminase and deoxycytidine

kinase and the lower the levels of PNP. Thus immature T cells are at risk for accumulating deoxyGTP.

Deoxyguanosine toxicity

Deoxyguanosine has long been recognized as a potent cytotoxic agent to cultured mammalian cells. Deoxycytidine may reverse or antagonize dGuo toxicity. This toxicity or inhibition of DNA synthesis by dGuo appears to be mediated by deoxyGTP-mediated inhibition of the enzyme ribonucleotide reductase. This results in the intracellular depletion of the DNA substrate deoxyCTP and explains the reversal of the dGuo toxicity by deoxycytidine.

It is now clear that of the four nucleoside phosphorylase substrates, only dGuo can result in inhibition of lymphocyte growth. This was initially shown in phosphorylase-deficient S49 cell mutants. Deoxyguanosine-mediated inhibition of DNA synthesis has also been observed in mitogen-stimulated human and murine T cells. Inhibition of T suppressor cell function has also been observed in human and murine T cells *in vitro*. Following *in vivo* administration of dGuo, inhibition of the development of murine suppressor T cells has been demonstrated. Deoxyguanosine injections have been shown to abrogate T cell-mediated suppression of contact sensitivity. The enhanced capacity of thymocytes to accumulate significant amounts of deoxyGTP has been taken advantage of in other situations. On the basis that dGuo is selectively toxic to proliferating intrathymic lymphocytes, this drug has provided an efficient means of producing lymphocyte depletion in human or murine organ-cultured thymuses. Results show that dGuo treatment leads to selective depletion of the lymphoid cells while allowing survival and function of the stromal and epithelial elements. In recent studies, the toxic effects of dGuo on T cell function in the presence of PNP activity may also be related, to some degree, to the accumulation of GTP. Proliferating lymphocytes exhibit sensitivity to intracellular GTP as well as dGTP.

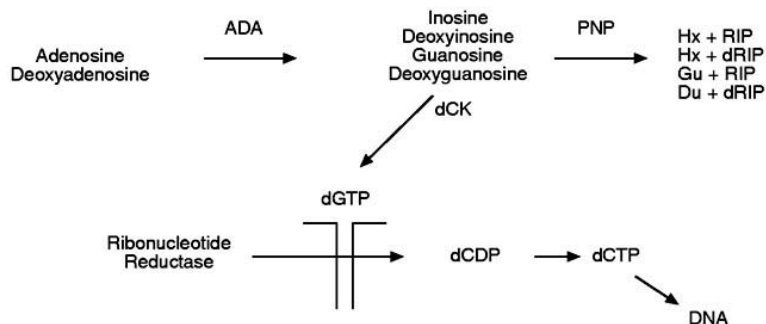


Figure 1 Nucleoside metabolism. ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; dCK, deoxycytidine kinase; Hx, hypoxanthine; Gu, guanine; RIP, ribose 1-phosphate.

See also: Immunodeficiency, primary; Organ culture of lymphoid cells; Suppressor T lymphocytes.

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DERMATOLOGICAL INFECTIONS

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Normal skin provides an effective defense against most infectious agents. Keratinization, skin shedding, basal cell regeneration, lipid composition, low moisture content and the presence of a 'normal' cutaneous flora all contribute to making intact skin an efficient nonspecific barrier to invasion by most infectious organisms. The capacity of skin to mount a targeted immunologic response against individual invading microorganisms also makes skin an efficient specific barrier to most infectious agents. Bone marrow-derived Langerhans dendritic cells migrate throughout the epidermis, comprise 2–8% of all cells in the epithelium, sample antigen, and proceed through the dermis to regional lymph nodes via lymphatics. These cells express major histocompatibility complex (MHC) class II molecules and efficiently process and present antigens to CD4 T cells both locally (in the epidermis and dermis) and distally (in local lymph nodes). Memory/effector (predominantly CD45 RA⁺/RO⁺) T cells, natural killer cells and rare plasma cells are also scattered throughout dermatological structures. Normal skin cells such as keratinocytes, dermal fibroblasts and melanocytes are able to synthesize and secrete transforming growth factors α and β , tumor necrosis factor α , chemotactic and colony-stimulating factors and immunomodulatory cytokines such as interleukin 1 (IL-1), IL-3, IL-6, IL-8 and IL-10. Skin is therefore able to respond to invading microorganisms by directly recruiting and activating T cells and also by modifying dermal

endothelial cells to express molecules such as E-selectin that locally recruit additional cells of the immune system. Such cells themselves in turn affect keratinocytic proliferation, MHC expression and immunomodulatory factor secretion. This complex interaction results in an immune response that is locally directed and carefully tailored to respond to the specific microorganism of interest. It also means that the development of an appropriate dermatological immune response is as dependent upon the immunological capabilities and repertoire of the host, as it is upon the identity and nature of the infecting organism.

In normal skin, extracellular bacteria usually elicit a polymorphonuclear inflammatory response; intracellular bacteria, fungi and protozoa usually elicit a mononuclear immunologic response; and helminthic and ectoparasitic infections usually elicit an eosinophilic and immunoglobulin E (IgE)-mediated immunologic response (Table 1). Still other microorganisms are able to infect human skin while inducing only mild or minimal apparent immunologic changes.

Infections characterized by a minimal or moderate immunologic response

Human papillomaviruses (HPVs) are double-stranded DNA viruses that infect basal epithelial cells. HPVs can be divided into at least 65 genotypes.

Table 1 Skin disorders and their causative organisms

Disorder	Infecting organism
Skin infections characterized by a mild or minimal immunologic response	
Verruca vulgaris	Human papillomavirus
Molluscum contagiosum	Molluscum contagiosum poxvirus
Erythrasma	<i>Corynebacterium minutissimum</i>
Tinea versicolor	<i>Malassezia furfur</i>
Cryptococcosis	<i>Cryptococcus neoformans</i>
Cysticercosis (viable larvae)	<i>Cysticercus cellulosae</i> (larval stage of <i>Taenia solium</i>)
Follicle mites	<i>Demodex folliculorum</i>
Skin infections characterized by a polymorphonuclear inflammatory response	
Impetigo	Group A streptococci, and others
Bullous impetigo	<i>Staphylococcus aureus</i>
Ecthyma	<i>Streptococcus</i> spp., <i>Pseudomonas</i> spp. and others
Erysipelas	<i>Streptococcus</i> spp.
Cellulitis	Many bacterial species (e.g. <i>Staphylococcus</i> spp., <i>Streptococcus</i> spp., <i>Clostridium</i> spp., <i>Vibrio</i> spp., <i>Aeromonas</i> spp., etc.)
Folliculitis (including furuncles and carbuncles)	<i>Staphylococcus aureus</i> and others, including <i>Pseudomonas aeruginosa</i>
Candidiasis	Various <i>Candida</i> spp.
Skin infections characterized by an eosinophil IgE-mediated immunologic response	
Scabies	<i>Sarcoptes scabiei</i>
Onchocerciasis	<i>Onchocerca volvulus</i>
Cutaneous larva migrans	Various nonhuman hookworm species (e.g. <i>Ancylostoma braziliense</i> , <i>A. caninum</i>)
Cercarial dermatitis	Nonhuman schistosomal (e.g. avian) cercariae
Skin infections characterized by the presence of a monocyte/macrophage immunologic response	
<i>Diffuse histiocytic</i>	
Lepromatous leprosy	<i>Mycobacterium leprae</i>
Diffuse cutaneous leishmaniasis	<i>Leishmania</i> spp.
<i>Granulomatous</i>	
Tuberculoid leprosy	<i>M. leprae</i>
Cutaneous tuberculosis	<i>M. tuberculosis</i>
Leishmania recidivans	<i>Leishmania</i> spp. (usually <i>L. tropica</i>)
Tertiary syphilis (gumma)	<i>Treponema pallidum</i>
Dermatomycoses due to primary invasive systemic fungi	Various fungal spp. (e.g. <i>Blastomyces dermatitidis</i> , <i>Histoplasma capsulatum</i> , <i>Sporothrix schenckii</i>)

A nongenital HPV lesion of the skin is usually caused by HPV-1, 2, 3 or 4. In its most common manifestation, HPV infection of the skin presents as verruca vulgaris, the common wart. The immunologic response to HPV infection is poorly understood. A cellular response is undoubtedly important in preventing or limiting infection since individuals deficient in cell-mediated immunity are not only predisposed to HPV infection and dissemination, but also HPV infections in such individuals are often refractory to treatment. Additionally, patients with epidermodysplasia verruciformis, who are congenitally susceptible to disseminated HPV infection of the skin, appear to have a specific defect in cell-mediated immunity. Although affected individuals are not predisposed to infectious processes in general, T cell and natural killer cell responses directed against HPV-encoded antigens have been shown to be defective *in vitro* in such patients. The successful use of the cellular immunity-boosting glycoprotein interferon α in the treatment of HPV-infected individuals also suggests the importance of cell-mediated immunity in controlling HPV infections of the skin.

Even in normal individuals, verruca vulgaris induces minimal inflammatory changes and can persist for months or years. Histopathologic examination of involved skin can disclose an acanthoid dermis with papillomatosis, hyperkeratosis and parakeratosis. Despite the fact that viral HPV DNA and antigens are easily detected in many epithelial cells, a host immune inflammatory response is usually minimal (a mild mononuclear infiltrate may be present) or absent.

Tinea versicolor is another dermatological infection that elicits only minimal apparent immunologic change. The scaly hypo- or hyperpigmented macules of tinea versicolor are caused by the dimorphic, lipophilic fungus *Malassezia furfur* (formerly designated *Pityrosporum orbiculare*). Since the organism is part of the normal skin flora, it is unclear what allows the fungus to cause clinical disease. Defects or alterations in cellular immunity probably play a central role. Histopathological examination of involved skin demonstrates proliferating *M. furfur* organisms in stratum corneum with only minimal, if any, immunological infiltrate.

Infections characterized by a polymorphonuclear immunological response

The majority of 'classic' skin and soft tissue infections are due to extracellular bacteria and are characterized by the presence of a polymorphonuclear inflammatory response. Impetigo, ecthyma, erysip-

elas, cellulitis, folliculitis and furunculosis are examples of such common infections. Recruitment, activation and functionality of polymorphonuclear cells in infected skin is dependent upon the correct production of chemokines and interleukins such as IL-8, the proper display of surface integrin molecules by polymorphonuclear cells, the upregulation of endothelial cell counter-integrin receptor molecules such as intercellular adhesion molecule 1 (ICAM-1), the presence in polymorphonuclear cells of normal phagocytic and enzymatic capabilities, and a preserved ability of polymorphonuclear cells to degranulate.

Impetigo results from a superficial infection of skin, usually by group A streptococci or *Staphylococcus aureus*. Infectious manifestations begin with formation of thin-roofed vesicles that rapidly pustulate and rupture. Dried, purulent discharge results in the formation of a characteristic, adherent, golden-brown crust. The vesico-pustule forms between the stratum corneum and the stratum granulosum, and the inflammatory response is predominately polymorphonuclear in nature. The most serious complication is that of streptococcus-specific antibody-associated glomerulonephritis that can occur approximately 2 weeks after the cutaneous infection. Bullous impetigo also results from a superficial infection of skin but is caused by locally invasive phage-group 2 *S. aureus* that produces a toxin, exfoliatin. The action of this toxin results in the formation of bullae that contain *S. aureus* organisms and many polymorphonuclear cells. Bullous impetigo is not associated with the development of glomerulonephritis.

Erysipelas is a superficial cellulitis of skin and is most frequently caused by group A streptococci. Involved skin is usually red or brawny and is markedly indurated. The classic skin lesion rapidly expands peripherally. Edema is usually marked. Vesicles, bullae and petechiae may follow in involved skin. The patient may be febrile and systemically ill. Histopathologic examination of involved skin discloses marked vascular dilatation, extensive edema, and prominent infiltration by invading streptococci, especially of dermal lymphatic vessels. A polymorphonuclear cell infiltrate predominates.

Cellulitis is an acute, spreading infection of the deeper dermis and subcutaneous tissue and is most frequently caused by staphylococcal or streptococcal species. Infections with many other bacterial and fungal species have, however, been documented. The organisms proliferate in dermal and subcutaneous structures, and infection imparts an edematous, erythematous, inflamed appearance to involved skin. The advancing margin is less distinct than that seen

with erysipelas. Leukocytosis, regional lymphadenopathy, local abscess formation and bacteremia can complicate the infectious process. The dermatological response is predominantly polymorphonuclear in nature.

Folliculitis is an infection of hair structures. Although most cases are due to gram-positive organism such as *S. aureus*, gram-negative bacteria such as *Pseudomonas aeruginosa* and fungal species can also be causative agents. Superficial folliculitis is characterized by pustule formation at the follicular opening. Deep folliculitis can manifest as a furuncle, or boil; or as a carbuncle, with extensive deep pustular involvement of the dermis and deeper structures. A polymorphonuclear cell infiltrate predominates in both superficial and deep folliculitis. Deep folliculitis can be complicated by fever, leukocytosis, bacteremia, local abscess formation, sinus tract formation and local tissue necrosis.

Individuals with defects in polymorphonuclear cell recruitment or functionality often have recurrent and chronic infections of the skin. For example, individuals with chronic granulomatous disease (CGD) are deficient in activity of the multicomponent cellular enzyme NADPH oxidase and are predisposed to skin infections with catalase-positive organisms such as *S. aureus* which are often chronic and recurrent. NADPH oxidase is crucial in generating the oxidative burst required to kill many catalase-positive bacteria, and a deficiency in the activity of NADPH oxidase often necessitates a compensatory host response that is granulomatous in nature.

Infections characterized by an eosinophilic, IgE-mediated immunologic response

Certain skin infections provoke an immunologic response in which eosinophils, mast cells, basophils and IgE antibody-mediated reactions play prominent roles. Such responses are often orchestrated by activated T cells and the production of interleukins such as IL-4 and IL-5. Involvement of the skin by the microfilarial stage of *Onchocerca volvulus* results in such a response. Onchocerciasis is caused by the filarial nematode *O. volvulus*. Infective larval stages of the worm are inoculated into human skin during a blood meal of an infected *Simulium* blackfly. After migration and maturation of larvae, adult worms become localized in palpable subcutaneous nodules, onchocercomata. Female worms release millions of microfilariae that freely infiltrate the skin and subcutaneous tissues. Involvement of ocular structures can cause keratitis and blindness. Microfilarial involvement of the skin results in significant dermatitis and

pruritis. Histopathologic examination of involved skin discloses an eosinophilic infiltrate with extensive extracellular deposition of eosinophil granule proteins. An eosinophilic response in the blood is common. Longstanding infection of skin results in hyper- or hypopigmentary changes, lichenification, skin atrophy and loss of skin elasticity.

An eosinophilic immunologic response is also seen during infection of human skin by the scabies mite, *Sarcoptes scabiei*. During infection, the female mite burrows through the epidermal cornified layer and deposits eggs and feces. These antigens provoke an immunologic response that results in skin lesions that may be papular, vesicopapular, nodular or diffuse (crusted Norwegian scabies). Although the mites and their products do not penetrate below the epidermis, they provoke a prominent dermal perivascular inflammatory response containing lymphocytes, histiocytes and numerous eosinophils. Diffuse dermal infiltration by eosinophils is not uncommon. The increased propensity of patients coinfecting with *S. scabiei* and human immunodeficiency virus 1 (HIV-1) to have diffuse scabetic involvement of the skin, highlights the importance of cellular immunity in orchestrating an appropriate eosinophilic and IgE-mediated antibody response.

Infections characterized by a lymphocytic immunologic response

T cell-mediated immunity is critical in orchestrating and effecting the immune response, and the presence of T cells in skin can be demonstrated in almost all dermatological infections. Although humoral responses may play a pivotal role in the dermatological manifestations of many infectious processes (measles, for example), a dermatological response characterized by the presence of antibody-producing B cells or plasma cells in skin is rare. Dermal involvement during primary syphilis, however, is one such example. A primary syphilitic chancre develops at the site of skin penetration by the spirochete *Treponema pallidum*. The chancre is usually 1–2 cm in size with regular, raised, indurated, sharply defined borders. The lesion is classically painless and resolves within 1–2 weeks with treatment, or 3–6 weeks without therapy. Late manifestations of secondary or tertiary syphilis can occur if the infection is not appropriately treated. Histopathological examination of a primary syphilitic chancre discloses lymphocytic infiltration of the epidermis surrounding the ulcer margin. Cross-sectional examination of the ulcer bed discloses a dense dermal infiltrate that contains many plasma cells and lymphocytes. There is endothelial cell proliferation and swelling, and spirochetes can

be demonstrated throughout the epidermal and dermal areas. Despite this plasma cell response, and despite a rapid increase in serum *T. pallidum*-specific antibodies, cellular immunity undoubtedly plays a critical, and probably pivotal, role in controlling the syphilitic infection.

Infections characterized by a histiocytic immunologic response

There are many skin infections that provoke an immunologic response in which cells of the monocyte/macrophage lineage cells play critical roles. Dermatological immune responses in which macrophages appear to play a central role, however, can be divided into those in which a diffuse histiocytic pattern is present in skin and those in which frank granulomas are present. Some infectious agents are able to elicit a variety of histiocytic responses; a result largely due to differences in incompletely understood host factors. An example of one such infectious agent is *Mycobacterium leprae*, the causative agent of leprosy. Many individuals are resistant to becoming infected with *M. leprae*. Susceptible individuals who do become chronically infected can present with clinical symptoms and signs located anywhere on a continuum of disease whose extremes are referred to as 'tuberculoid' or 'lepromatous' leprosy. Disease in many individuals is manifest somewhere between these two extremes, so-called 'borderline' leprosy. Borderline involvement tends to be clinically 'unstable' and usually progresses toward one immunologic extreme or the other.

Tuberculoid leprosy is characterized by the presence in skin of a single or a few discrete macules or plaques. Such lesions are dry, erythematous or hypopigmented, and hypo- or anesthetic. Histopathologic examination of involved skin discloses granuloma formation with well-differentiated macrophages surrounded by lymphocytes. Many CD4⁺ helper T cells are present and there is prominent production of IL-2. The granuloma is usually surrounded by CD8⁺ cytotoxic and suppressor T cells. Granulomas often involve nerves. Caseation may be present, and bacilli are either absent or extremely rare.

Patients with lepromatous leprosy have diffuse, systemic involvement by *M. leprae*. Examination of skin in early disease discloses symmetric, large, widespread, poorly defined macules. In such an early stage, sensation is usually intact. The skin takes on a diffuse, nodular character in later disease and neural enlargement can then be pronounced. Histopathologic examination of involved skin discloses a diffuse, foamy, histiocytic dermal infiltrate. Granulomas are either absent or disorganized. Non-

differentiated macrophages filled with large numbers of *M. leprae* predominate. Compared to what is found in tuberculous lesions, CD4⁺ helper T cells are less frequent and IL-2 production is much less pronounced. CD8⁺ cytotoxic suppressor T cells are scattered throughout the lesions and large numbers of *M. leprae* organisms are widely disseminated.

In summary, as would be expected from the wide-ranging nature of organisms that can infect dermatological structures, the skin must be able to mount an adequate, efficient and directed immunological response specifically tailored to an invading organism. Careful immunological surveillance of dermatological structures and complex interactions between skin and immune cells insure that most dermatological infections are appropriately handled through the action and intricacies of chemokines, cytokines, growth factors, antibodies and various effector cells.

See also: *Candida*, infection and immunity; **Chronic granulomatous disease**; *Clostridium*, infection and immunity; *Corynebacterium*, infection and immunity; *Cryptococcus*, infection and immunity; *Eosinophils*; *Fungi*, immunity to; *Granuloma*; *Histo-*

plasma, infection and immunity; **Innate immunity**; *Langerhans cells*; *Leishmaniasis*; **Mast cells**; *Mycobacteria*, infection and immunity; *Onchocerciasis*; *Papillomavirus*, infection and immunity; **Parasites, immunity to**; *Pseudomonas aeruginosa*, infection and immunity; **Skin, contribution to immunity**; *Staphylococcus*, infection and immunity; *Streptococcus*, infection and immunity; *Treponema*, infection and immunity.

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DETERMINANT SPREADING

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Definitions

'Determinant spreading' (or epitope spreading) describes an amplification phenomenon observed in inflammatory autoimmune disease in which the original T cell response diversifies through induction of T cells against additional autoantigenic determinants. The response to the initiating determinant is followed by 'intramolecular' spreading, activation of T cells specific for other, often previously cryptic or subdominant self determinants (defined below) within the same antigen during the course of chronic and progressive disease; 'intermolecular' spreading involves determinants on other unrelated self antigens.

A multideterminant protein antigen possesses dominant, subdominant and cryptic T cell determinants. The dominant determinants are those that are most efficiently processed and presented from native antigen, whereas cryptic determinants are inefficiently processed and/or presented, or not at all.

Therefore, a response to a cryptic determinant can only be induced by immunization with the appropriate peptide which usually does not need additional processing. The subdominant determinants lie on the continuum between these two categories.

Examples of determinant spreading

Determinant spreading during the course of experimental autoimmune encephalomyelitis

The term, determinant spreading, was first applied by Lehmann and colleagues to the phenomenon observed in experimental autoimmune encephalomyelitis (EAE), a model of human multiple sclerosis (MS). EAE is an inflammatory demyelinating disease of the central nervous system mediated by CD4⁺ T cells specific for myelin autoantigens. EAE can be induced in susceptible mouse strains by immunization with myelin basic protein (MBP) or a peptide containing its dominant determinant, Ac1-11, or with proteolipid protein (PLP).

EAE in (B10.PL $\times$ SJL/J)F1 mice manifests as an acute disease phase followed by a chronic phase. Lehmann and coworkers induced EAE in (B10.PL $\times$ SJL/J)F1 mice by injection of MBP-dominant determinant peptide, Ac1-11, and observed that in the acute phase of the disease the T cell response was directed against Ac1-11, whereas in the chronic phase the response spread to cryptic determinants of MBP (35–47, 81–100 and 121–140). McCarron and colleagues also observed T cell reactivity to the large 89–169 fragment of MBP in mice immunized with MBP 1–37. T cell reactivity to these two fragments was restricted by different major histocompatibility complex (MHC) class II elements. Perry and coworkers observed that following induction of EAE in (SJL/J $\times$ PL/J)F1 mice by MBP immunization, T cell responses spread to PLP. Raine's and Miller's laboratories have shown that in EAE initiated in the SJL/J mouse by adoptive transfer of T cells reactive with the dominant determinant of MBP, responses spread to the dominant determinant, 139–151, of PLP. Miller's group went on to demonstrate intermolecular spreading between PLP 139–151 and PLP 178–191. Moreover, both groups demonstrated that T cells specific for the new determinants were functionally capable of causing EAE in naive recipient mice by adoptive transfer. Tuohy's laboratory has reported that in EAE in (SWR $\times$ SJL/J)F1 mice, there is a predictable, orderly determinant spreading involving PLP, MBP and myelin oligodendrocyte glycoprotein (MOG).

Intramolecular and intermolecular spreading in a spontaneous disease: insulin-dependent diabetes mellitus (IDDM)

During the very early phase of disease in the non-obese diabetic (NOD) mouse, T cell proliferative responses arise first to certain determinants on glutamic acid decarboxylase (GAD). Subsequently, a spreading of T cell responsiveness occurs to other determinants of GAD as well as to other unrelated antigens present in the β cells of the pancreatic islets (65 kDa heat shock protein, carboxypeptidase H, insulin, etc.). It is still unclear whether GAD is the endogenous initiator molecule, or whether some other proinflammatory event precedes this induction, to ignite a multidimensional conflagration. Interestingly, induction of intravenous or inhalation tolerance to GAD was successful in preventing insulinitis as well as clinical diabetes.

Diversification of T cell response can lead to engaging regulatory T cells: autoimmune arthritis

Adjuvant arthritis (AA) can be induced in the Lewis rat by injection of *Mycobacterium tuberculosis*, and

it serves as an experimental model for human rheumatoid arthritis (RA). T cell responses to the 65 kDa mycobacterial heat shock protein (Bhsp65) have been implicated in the pathogenesis of AA as well as RA. Moudgil and colleagues observed that in the acute phase of AA, T cell responses arise to several dominant and subdominant determinants of Bhsp65. Interestingly, in the late phase of AA, there was evidence for 'diversification' (broadening of responses to new determinants within an antigen after priming with the whole multideterminant antigen) of the T cell responses to certain additional determinants located at the C-terminal end of Bhsp65. Moreover, arthritic rats in the late phase of AA also raised vigorous responses to the corresponding C-terminal determinants within self(rat) hsp65 (Rhsp65). These results suggest that the observed diversification is possibly triggered *in vivo* by induction of Rhsp65-reactive T cells, and that it is critical in regulating acute inflammatory arthritis in the Lewis rat.

Reciprocal T and B cell spreading: systemic lupus erythematosus

In the (NZB $\times$ NZW)F1 mouse model of systemic lupus erythematosus (SLE), Hahn's group has shown that three V-region peptides from a single prototype DNA-specific antibody, under the appropriate conditions, can either accelerate or delay disease significantly. The above effects were explained on the basis of activation of T cells by the peptide-presenting B cells, followed by a T cell induction of B cells displaying a cross-reactive peptide, continuing reciprocally in this manner. Interference with this reciprocal expansion and/or associated regulatory (T_H1/T_H2) interactions could lead to protection. Similarly, both intramolecular and intermolecular immunoglobulin epitope spreading has been reported by Harley's group.

Breaking of self tolerance by raising a B cell response to a foreign homologous protein

Mamula and Janeway have shown that although mice are tolerant at the T cell level to self cytochrome *c* (cyt *c*), coimmunization with human and mouse cyt *c* breaks T cell tolerance to cryptic determinant region 1–80 of self cyt *c*. It was suggested that the foreign cyt *c* induces B cells that are cross-reactive with self cyt *c*; these B cells then bind, process, and present the self molecule to T cells.

Mechanisms of determinant spreading

Several mechanisms of determinant spreading have been proposed. 1) In the case of EAE and diabetes, upregulation of antigen processing and MHC presen-

tation in the local inflammatory milieu at the site of tissue damage may be responsible for the enhanced visibility of previously poorly displayed, cryptic and weak subdominant determinants. Although spreading will usually occur in a T_H1 proinflammatory context, T_H2 cytokines may also activate the B cell spreading mechanism. 2) B cells present antigen differently than conventional antigen-presenting cells (APCs) when they internalize antigen via their antigen-specific immunoglobulin (Ig) receptors. Celada's and Lanzavecchia's groups have shown that, depending upon what portion of the antigenic molecule is bound by the Ig receptor, different T cell determinants on the molecule will be preferentially presented in association with MHC class II molecules by the B cell. Similarly, processing and presentation of certain T cell epitopes could either be enhanced or suppressed when antigen (Ag) is internalized into APCs as Ag-Ig complexes via Fc receptors. 3) Increased internalization of a cell surface molecule as a result of its cross-linking by specific ligands may result in enhanced processing and presentation of previously cryptic determinants within that molecule. 4) In addition, other factors, such as infection of APCs with viruses or intracellular parasites, or the cellular stress response (heat shock response), should be considered as mechanisms that may alter antigen presentation schemes and the hierarchy of determinant display.

Implications of determinant spreading for immunotherapy

Knowledge about the temporal pattern of appearance of new T cell specificities directed against determinants within the same or different antigens is crucial in designing appropriate therapeutic strategies. Therapeutic approaches based primarily on controlling T cell responses to the initial disease-inducing determinant may not succeed if spreading of response has already occurred. Tolerance induction with peptides comprising encephalitogenic determinants appearing later in the disease may prevent subsequent progression of EAE. Recently, new strategies based on blocking the B7.1-CD28 interaction have been successful in blocking determinant spreading and thereby preventing relapses during the course of EAE. In the case of diabetes in the NOD mice, in which responses to several antigens appear in a hierarchical fashion, administration of GAD to young NOD mice has been successful in preventing the development of clinical disease. Some evidence in the EAE system points to the need for continued activity of the inflammatory T cells directed against the initiating determinant for perpetuation of the

spread. Removal or deactivation of the initiating cell, even after determinant spreading has begun, may weaken or eliminate disease progression. Therefore, measures directed to such initiating cells may be therapeutic despite their late administration.

See also: Antigen presentation via MHC class II molecules; Autoantigens; Autoimmune diseases; B lymphocyte, antigen processing and presentation; Epitopes; Immunodominance; Immunotherapy of autoimmune diseases; T lymphocytes, autoreactive.

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DIAPEDESIS

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Diapedesis is defined in immunology as the outward passage of cells through the intact vessel wall. The most prominent cells to traverse the endothelial cell lining of the vessel walls are blood leukocytes, but tumor cells also translocate via the blood circulation through diapedesis from the primary tumor sites to distant sites in the body. Diapedesis of leukocytes is a major event in the migration of leukocytes from the blood circulation to sites of inflammation or tissue injury and in the recirculation of lymphocytes from the blood to the lymphatic compartment. Diapedesis of leukocytes is often confused with the terms 'leukocyte transmigration' or 'leukocyte extravasation'. These terms define the entire process of leukocyte migration from the blood circulation to the extravascular connective tissue, across the vessel wall endothelium. Diapedesis is regarded as the final event in the transmigration, i.e. the actual penetration of the vascular endothelium and the subendothelial matrix.

Diapedesis: the final chapter in a sequence of separate consecutive adhesion events

Elegant intravital microscopy studies revealed that diapedesis of leukocytes is preceded by a coordinated sequence of adhesion events that enables these cells to withstand the hemodynamic forces, known as shear forces, generated by the flow of blood and to make firm contact with the vascular endothelial cells.

At present, three separate consecutive events have been clearly distinguished (Figure 1). Leukocytes first attach to the vessel wall in a rolling interaction (1) and then are arrested, sticking firmly to the surface membrane of a single endothelial cell (2), before diapedesis (3). Specific cell surface glycoproteins on endothelial cells and leukocytes are directly implicated in these events.

1. Leukocyte rolling

The rolling of leukocytes along the endothelial lining of small venules is observed exclusively at inflammatory sites or sites of vascular injury. Leukocyte rolling is a process involving attachment to, and detachment from, the endothelium. Consequently the leukocytes slow down in the main vascular flow. Studies in rodents and *in vitro* experiments using laminar flow chambers showed that leukocyte rolling is initiated by the expression of specific molecules, designated as selectins, on the surface of leukocytes, i.e. L-selectin (CD62L), or activated endothelial cells, i.e. P-selectin (CD62P) and E-selectin (CD62E). The amino-terminal lectin domain of these adhesion molecules recognizes sialyl Lewis^x (CD15s) or related sulfated, fucosylated or sialylated carbohydrate structures on the opposing cell and is thus critically involved in cell–cell adhesion. Selectin-mediated rolling is an obligatory event before leukocytes can move on to the stage of firm adhesion.

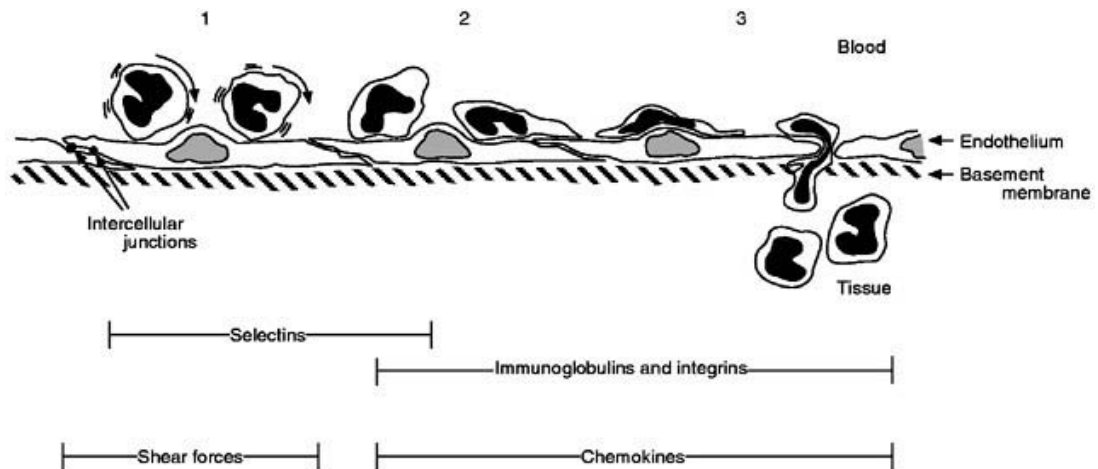


Figure 1 Consecutive events in the binding and transendothelial migration of leukocytes. (1) Rolling of leukocytes along the endothelium of vessel wall adjacent to an extravascular site of inflammation. (2) Arrest of rolling and firm attachment of leukocytes to the endothelial cells. (3) Lateral migration of leukocytes over the surface of the endothelial cells and subsequent diapedesis. Shear forces generated by the flow of blood, chemokines and adhesion molecules belonging to the family of selectins, immunoglobulins and integrins act in sequence, with some overlap.

2. Arrest of rolling and firm adhesion

The arrest of rolling leukocytes at a particular site of the vascular endothelium involves cell surface adhesion molecules that are different from those required for rolling. At least five heterodimeric cell surface proteins, designated as integrins, have been implicated in the firm adhesion of leukocytes to endothelial cells. These proteins are: LFA-1 (lymphocyte-function associated antigen 1; CD11a/CD18); CR3 (complement receptor 3; CD11b/CD18) and p150,95 (CD11c/CD18), which belong to the β_2 subclass of the integrin superfamily of adhesion molecules; the β_1 -integrin VLA-4 (very late antigen-4; CD49d/CD29); and the β_7 -integrin LPAM-1 (lymphocyte Peyer's patch adhesion molecule 1; CD49d/ β_7). These integrins bind their respective counterstructure on the endothelial surface, which include proteins that structurally belong to the immunoglobulin supergene family, i.e. ICAM-1 (intercellular adhesion molecule 1; CD54), ICAM-2 (CD102) and VCAM-1 (vascular cell adhesion molecule 1; CD106). Most of the adhesion promoting molecules are expressed on the surface of endothelial cells at inflammatory sites and are not or are minimally expressed on resting (nonstimulated) endothelial cells.

The integrin-mediated firm leukocyte-endothelium interaction is triggered or amplified by the action of chemotactic cytokines or chemokines, such as IL-8 (interleukin 8) or MCP-1 (monocyte chemotactic protein 1), that are produced by the endothelial cells in response to activation or binding of leukocytes. The present belief is that selectin-mediated rolling of leukocytes will enhance exposure to chemokines that

are bound to, and presented by, proteoglycan-like molecules on the surface of the endothelial cell. This will lead to activation of integrin adhesiveness and firm leukocyte adhesion to endothelium.

Firm adhesion between leukocytes and the endothelium coincides with an increase of the intercellular contact area. These leukocytes change shape, become flattened and move in a lateral direction over the endothelial cell surface. Very little is known about the exact mechanisms and molecules involved in this process, but there are some indications that after initial contact between leukocytes and endothelial cells ICAM-1 diffuses in the plane of the membrane of endothelial cells to the intercellular contact. Whether chemokines provide an additional stimulus is not yet clear.

3. Diapedesis: the actual transendothelial migration

Most endothelia form a continuous and nonfenestrated monolayer of endothelial cells. The peripheral membranes of adjacent endothelial cells overlap each other and at these sites, specialized junctions, e.g. tight and gap or communicating junctions, preserve the integrity of the monolayer. Adherent leukocytes cross the vessel wall by squeezing themselves between tightly apposed endothelial cells in an ameboid fashion. Transendothelial migration of leukocytes through the cytoplasmic compartment of the endothelial cells is not a normal pathway. During diapedesis the barrier function of the endothelium is excellently preserved because of the close interaction between the migrating leukocyte and the

peripheral membranes of the endothelial cells. The maintained integrity of the endothelium during diapedesis of leukocytes *in vitro* can be very well demonstrated by measuring the electrical resistance over the monolayer, which, in fact, does not change during diapedesis. Electron microscopy and time-lapse microcinematography have indicated that the retraction of endothelial cells is restricted to the endothelial cells which are directly involved in the interaction, and that most of the surrounding endothelial cells maintain their integrity and spreading morphology. Although reorganization of the cytoskeletal filaments must be involved, the exact mechanism of endothelial cell retraction, as well as the intracellular signal(s) which coordinate the opening of the interendothelial junctions, are still obscure. It is speculated that the interaction of leukocytes with the endothelial cell surface adhesion molecules is an important initiating signal.

Because leukocytes must adhere to the endothelium before subsequent diapedesis, it is difficult to unravel the contribution of a particular adhesion molecule to firm adhesion of leukocytes to the endothelium versus its role in the actual diapedesis. In experimental settings agents that inhibit firm adhesion will also reduce diapedesis. Clearly, β_2 -integrin molecules are involved in diapedesis. This is concluded from the profound lack of neutrophil and monocyte diapedesis observed in patients with the leukocyte adhesion deficiency (LAD) syndrome, who are genetically deficient in the functional expression of leukocyte β_2 -integrin molecules. Beside β_2 -integrins, other adhesion molecules contribute to the diapedesis. Recent experiments suggest a role for PECAM-1 (platelet-endothelial cell adhesion molecule-1; CD31). This molecule is expressed on leukocytes and it is found in high densities on endothelium at sites of intercellular junctions. An interesting feature is that PECAM-1 can interact with itself. It is suggested that such homophilic binding between PECAM-1 on leukocytes and PECAM-1 on endothelial cells may promote diapedesis, whereby the leukocyte migrates in the direction of the highest density of endothelial PECAM-1, a process called haptotaxis.

Following diapedesis the migrating leukocytes attach to and subsequently traverse the subendothelial matrix, or basement membrane, consisting of fibronectin, laminin, elastin and various types of collagen, and accumulate in the extravascular tissues. The endothelial cells rapidly reconstruct an intact monolayer of tightly connected cells. The time required for leukocytes to attach to and traverse the endothelium varies from several minutes to several hours, depending on the type of invading cell, the

type of endothelium including their basal matrix and the presence of (inflammatory) stimuli.

Diapedesis of leukocytes under noninflammatory conditions

Under normal circumstances the vascular wall, composed of a monolayer of endothelial cells sited on a basement membrane, does not promote the adherence and transendothelial migration of circulating leukocytes. The nonthrombogenic and nonhemostatic surface properties of the endothelium and the narrow spaces (varying from 10 to 200 nm) between adjacent endothelial cells normally prevent cellular adherence and subsequent diapedesis. Diapedesis of leukocytes under noninflammatory conditions is mainly restricted to specific sites, the high-endothelial venules, bearing high-walled endothelium, where recirculating lymphocytes migrate from the blood circulation to the lymphatic compartment.

Diapedesis of leukocytes under inflammatory conditions

At inflammatory sites or at sites of vascular injury, the adhesiveness of endothelial cells for leukocytes is increased and results in enhancement of the number of leukocytes traversing the endothelium and an invasion of the extravascular, inflamed, tissues. Both processes are regulated by the local generation and secretion of inflammatory mediators, which in turn regulate the expression of adhesion molecules on the surface of the cells involved. This can be mimicked by treatment of leukocytes or cultured endothelium, *in vitro*, with cytokines, such as IL-1 α , tumor necrosis factor α or interferon γ , or with chemoattractants such as anaphylatoxin C5a, leukotriene B₄, formylmethionyl peptides (e.g. fMLP) or chemokines (e.g. IL-8 or MCP-1). These studies revealed that expression of the various adhesion molecules on endothelial cells is often transient and depends on the type of stimulus. This is well illustrated in endothelial cells treated with the proinflammatory cytokine IL-1. Within a few minutes of activation these endothelial cells express high levels of P-selectin, which returns to basal levels within an hour; several hours after activation, surface expression of E-selectin is maximal, returning to basal levels within 1 day; about a day after activation, ICAM-1 and VCAM-1 expression reach relatively stable plateau levels. These changes in surface expression of endothelial adhesion molecules most likely correlates with the shift from granulocyte to monocyte and lymphocyte extravasation that is observed *in vivo* about 6–24 hours after the onset of inflammation.

Diapedesis and cell activation or cell differentiation

Close cellular interactions might have consequences for cellular activation and/or cell differentiation. Circulating monocytes and granulocytes traverse the endothelium as a response to a stimulus. In the tissues, monocytes differentiate into different types of macrophages. The exact trigger for the onset of such differentiation is unknown. It has been suggested that the cellular contact occurring during diapedesis provides a signal for differentiation. More likely, however, is that local conditions, which can be anatomical or biochemical, including cytokines or other mediators, and oxygen tension orchestrate monocyte differentiation. This is exemplified by differences between peritoneal macrophages, alveolar macrophages, Kupffer cells, osteoclasts, etc.

Experimental models

Observations on the behaviour of circulating leukocytes are difficult. However, techniques such as time-lapse and intravascular microscopy on rabbit ear, mesenteric tissue of mouse or rat, hamster cheek pouch or bat wing enabled us to monitor intravascular events and diapedesis. Radiolabeled blood cells (^{111}In or ^{92}Tc) are useful tools for studying the kinetics and clearance of cells from the blood circulation and for monitoring differences in cell distribution and extravasation to the distinct tissue compartments.

Unfortunately these methods offer little insight into the molecular mechanism(s) underlying cellular adherence and diapedesis. Progress in identifying the mechanisms involved has been dependent on the development of *in vitro* systems that not only permit the adherence of cells to endothelial cells but also allow these cells to penetrate and traverse the endothelial monolayer, including the underlying basement membrane. One approach involves the interaction of cells with monolayers of endothelial cells grown on nitrocellulose or polycarbonate filters. Biological membranes, such as the mesenteric membrane or amnionic membrane, or layers of cultured smooth

muscle cells also serve as excellent carriers for endothelial cells in this respect. The endothelial cells used for these *in vitro* studies are usually harvested from large vessels, as isolation and cultivation are fairly simple and do not require expensive equipment or advanced techniques. However, diapedesis of leukocytes *in vivo* is more prominent in capillaries, post-capillary venules and small venules than in macrovessels and occurs during constant blood flow. Therefore, during the past few years, there has been enormous interest in the isolation and culture of microvascular endothelial cells from distinct vascular sites and in the development of new techniques to study adhesion and subsequent diapedesis of leukocytes under flow conditions.

See also: Adhesion molecules; Chemokines; Cytokines; Endothelium; Integrins; Lymphocyte trafficking; Recruitment; Selectins (CD62-E/L/P).

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DIGEORGE SYNDROME

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DiGeorge reported in 1965 the occurrence of thymic hypoplasia and hypoparathyroidism in infants with congenital heart disease. Reviews of DiGeorge syndrome in the 1970s described a diverse collection of phenotypic features that, a decade later, came to be understood as a developmental field defect, designated *DiGeorge sequence* (DGS), attributed to failed differentiation and migration of cephalic neural crest cells. Only a few cases were familial so the etiology was generally assumed to involve some environmental damage to the developing embryo. The most significant advance has been the identification of microdeletions on human chromosome 22q in most patients with DGS. Mapping and sequencing of genes on the affected area now promises a better understanding of the mechanisms responsible for the maldevelopment of the third and fourth pharyngeal pouches in DGS.

Genotype

Most affected individuals have deletions in the 22q11-qter region from one of their two copies of chromosome 22. These deletions are large, usually spanning at least 2 Mb. In about 20% of cases the deletion is visible when chromosomes are viewed at resolutions of 600 bands or greater. In most cases the deletions are most readily detected by molecular methods, such as commercially available locus-

specific fluorescence *in situ* hybridization (FISH) probes (Figure 1).

The chromosome 22q11 region is particularly rich in duplicated sequences which facilitate recombination and this may be the mechanism which allows for structural mutations such as deletions and duplications. In about 75% of sporadic cases of DGS, the affected chromosome is inherited from the mother. A recent survey found 5% of neonates with congenital heart disease had deletions at 22q11. These findings support an extrapolated prevalence of 22q11 microdeletions approaching 1:5000. Numerous families have been reported in which deletions passed through several generations causing dominantly inherited abnormal phenotypes including DGS.

The mechanism by which the chromosome 22q11-qter deletions cause the phenotypic features described below is not established. 'Haploinsufficiency' of genes which might be developmentally important in this region is one possibility and DGS has been considered a prototypic 'contiguous gene syndrome' involving an unknown number of genes. Alternatively, the phenotype may result from deletion of a single gene, as is suggested by the recent report of the complete DGS in association with a balanced translocation. A candidate gene disrupted by this translocation has been named HIRA because of homologies with the HIR1 and HIR2 repressors of

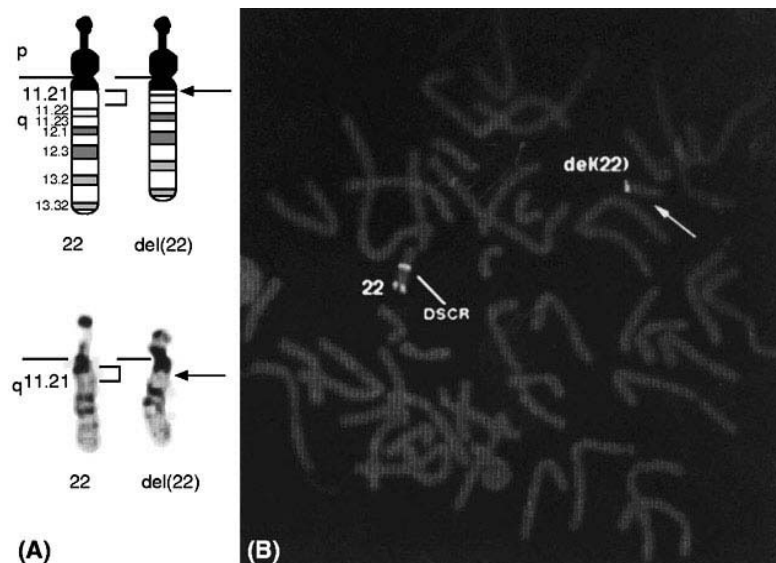


Figure 1 (A) Partial karyotype and ideogram depicting the deletion of bands q11.21 on a number 22 homologue, the microdeletion associated with DiGeorge/Velocardiofacial syndrome. (B) The deletion of the DiGeorge syndrome critical region (DSCR) detected by fluorescence *in situ* hybridization (FISH) using N-25 cosmid (Oncor, Inc.) (Courtesy of the Colorado Genetics Laboratory.)

histone gene transcription. Reports of the DiGeorge syndrome associated with mutations at loci other than 22q11 are accumulating. The most common alternative locus is chromosome 10p13 where, again, deletions producing haploinsufficiency have been documented.

Phenotype

An acronym CATCH 22 (for cardiac defects, abnormal face, thymic hypoplasia, cleft palate and hypocalcemia) has been proposed to describe the commonly affected structures in DGS (Figure 2). Reviews of DGS diagnosed clinically have generally emphasized the variability of each of these features, such that the most immunodeficient patients may have perfectly normal hearts. Even patients diagnosed with chromosome 22q11-qter deletions are phenotypically variable. Concurrence of two or more of the following probably suffices to make a clinical diagnosis of DGS: conotruncal heart defect, hypocalcemia, hypoplastic/absent thymus and typical facial dysmorphism (including small palpebral fissures, a 'beaked' or 'bulbous' nose and small or posteriorly rotated ears).

Features of DGS overlap with other malformations affecting the migration of cephalic neural crest cells. These include the velocardiofacial (VCF; Shprintzen) syndrome, the CHARGE association and various combinations of midline structural defects found in association with hypertelorism (e.g. Optiz-Frias syndrome, Kallmann syndrome). Thymic hypoplasia has also been reported with abnormalities of chromosome 10 and observed in fetuses exposed *in utero* to 13-*cis*-retinoic acid (Accutane®). In the mouse, a DGS phenocopy that includes thymic hypoplasia can be produced by recessive knockout of *hox 1.5* in transgenic embryos. There are no *hox* gene homologs known on human chromosome 22. The DGS pheno-

type is probably best considered a development field defect of heterogeneous etiology, even though it is most commonly encountered in association with chromosome 22q deletions.

The VCF syndrome (with cleft palate, lateral nasal build-up, mild microcephaly, congenital heart disease typically involving atrial or ventricular septal defects, and unusually long and tapering fingers) is also associated with chromosome 22q11 deletions. VCF syndrome patients usually have normal numbers of T cells and are not usually hypoparathyroid. They have a high frequency of otitis media and sinus infections, perhaps related to altered facial development, but the extent to which thymic hypoplasia contributes to this phenotype is unclear.

Individuals affected by chromosome 22q11-qter deletions with either the DGS or VCF phenotypes also share certain neurodevelopmental problems. Expressive aphasia is common and need not be associated with obvious intellectual retardation. When intellectual retardation is apparent, the contribution of expressive aphasia to this problem is incompletely understood. Anxiety and affective disorders also occur commonly and several cases with schizophrenia have been reported. Familial psychoses in some pedigrees have been linked to polymorphisms mapping to the chromosome 22q11-qter region.

Immunologic findings

Patients originally described with DGS had low proliferation of blood lymphocytes in response to the mitogen phytohemagglutinin (PHA). Affected children who died had no thymus in their anterior mediastinum, although some did have ectopic thymus tissue in the neck. Most subjects with DGS, diagnosed either by phenotype or by chromosome 22q11-qter microdeletions, have normal blood lymphocyte counts and normal absolute counts of CD3, CD4 and CD8 cells by 1 year of age. Their serum immunoglobulins are normal. In the first months of life the T cell counts are variable, with some having low absolute numbers of CD3 cells. The subjects who have normal numbers of T cells also appear to make good antibody responses to infections (such as chickenpox) and to immunizations with adjuvanted bacterial toxoids. Reduced responses to polysaccharide antigens in subjects who had low counts of CD4 cells in blood have been reported. A single subject with complete DGS had limited diversity of antibodies, a finding consistent with the known role of T cells in facilitating B cell proliferation and diversification.

A small number of DGS subjects, perhaps 2–5% of the total, have a chronically low number of circul-



Figure 2 DiGeorge phenotype. This affected infant has a dimpled nose and long philtrum of the upper lip.

ating T cells. These subjects seem to be vulnerable to a much more severe disorder characterized by the production of autoantibodies to blood components and/or clotting factors. There is also a clinical impression that these subjects have an increased frequency of upper respiratory tract infections.

Diagnosis

Any patient with two or more features suggesting DGS or VCF should be investigated for chromosome 22q11-qter deletions through high resolution chromosomal studies that include FISH probe analysis at the Shprintzen/DiGeorge locus. This is especially true for infants with conotruncal cardiac malformations. While less than 1% of infants with isolated tetralogy of Fallot are found to have microdeletions on chromosome 22q, more than 40% will be positive for a deletion when additional dysmorphic features are present. If a deletion is found, the parents' chromosomes should also be investigated, even when neither mother nor father appears to be affected. If a deletion is present in a parent the risk of a recurrence in a subsequent pregnancy approaches 50%. In the absence of a parental deletion, recurrence risks for DGS/VCF are low, but not negligible, due to the potential for parental gonadal mosaicism. Cytogenetic prenatal diagnosis should be offered in subsequent pregnancies regardless of parental karyotype. In cases lacking microdeletions, further investigation of parathyroid and thymic hypoplasia can be deferred in the absence of symptoms. In chromosomally normal cases involving congenital heart disease, subsequent pregnancies should be monitored by fetal ultrasonography and echocardiography.

Treatment

Infants with major cardiac defects require corrective or palliative surgery to survive. Blood used to transfuse these children should be irradiated or depleted of T cells (to avoid graft-versus-host disease) until the recipient is known to have functioning T cells. Patients with hypocalcemia may need intravenous calcium to control seizures or tetany, and subsequently most need both oral calcium supplements and vitamin D. By far the majority of affected infants develop normal numbers of T cells in the first 6 months of life and require no immunologic interventions. Those whose T cell numbers remain low and who have either recurrent infections or autoimmune disease are likely to be candidates for some form of immunologic intervention. Perhaps the least controversial is immunoglobulin G (IgG) treatment

for those with recurrent respiratory tract infections, particularly if their antibody responses are known to be impaired. Subjects who have HLA-matched siblings have been treated with blood transfusion and with bone marrow transplantation, with the assumption that sufficient mature T cells would be transferred to colonize the recipient. Although much has been written about fetal thymus transplantation in DiGeorge syndrome, most stems from the late 1960s and the 1970s, before the potential for spontaneous improvement in T cell numbers was appreciated. A few DGS cases with persistently low numbers of blood T cells have been grafted with pieces of child thymus, after these were first cultured *in vitro* to reduce the number of mature donor T lymphocytes. Clinical improvement has been reported in some, whether or not the thymus was matched with the recipient for histocompatibility antigens. The number of DGS patients treated by thymus transplant is still too small for systematic analysis.

See also: Immunodeficiency, primary; Thymus.

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DIVERSITY, GENERATION OF

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Immunoglobulins (Ig) are four-chain glycoproteins composed of two identical heavy (H) and two identical light (L) chains forming a Y shaped molecule (Figure 1). Each chain is divided into a variable (V) domain and either one (light chain) or three or four (heavy chain) constant (C) domains. The tips of the two prongs consist of variable sequences while the remaining portion of the prongs and the stem contain sequences common to antibodies of the same isotype. Antigens are bound by Ig through the V domains, and are eliminated through the effector functions of the C domains. V domains are divided into framework regions (FR) and complementarity determining regions (CDR). Four FRs and three CDRs alternate to give the pattern FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4 (Figure 2). It is predominantly the three CDRs from the H chain, and the three CDRs from the L chain which contact antigen. The FR support the immunoglobulin β barrel structure, and have also been shown to influence antigenic specificity. The folding of a V_H and V_L domain is shown in Figure 3. The residue numbers

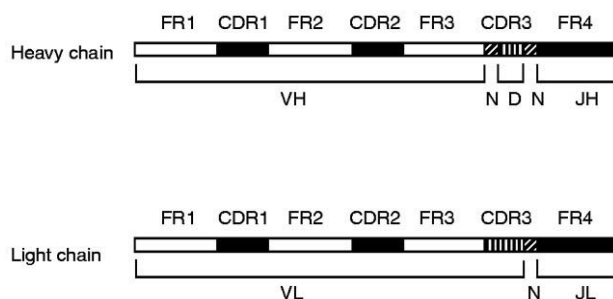


Figure 2 Structure of rearranged V_H and V_L domains. The relationship between the V(D)J gene segments and the framework and complementarity-determining regions is indicated. N segments have been identified at V_LJ_L junctions in about 20% of light chains.

corresponding to their FR and CDR positions are indicated in Table 1.

Immunoglobulins are produced by B lymphocytes either as membrane-bound receptors or secreted Ig. Each B cell produces immunoglobulins of identical specificity. It is necessary for the immune system to

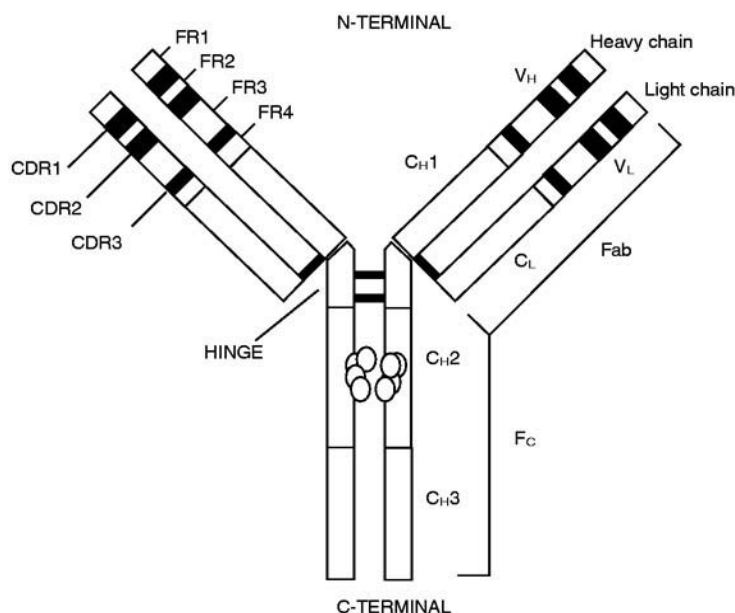


Figure 1 Stick diagram of the polypeptide chain structure of the human IgG antibody. The variable (V) and constant (C) domains are indicated. The alternating pattern of framework (FR) and complementarity-determining regions (CDR) in the heavy (H) and light (L) chains is indicated. The hinge region is located between the C_{H1} and C_{H2} domains of the H chain. The positions of the interchain disulfide bonds (dark bars) and carbohydrate molecules (open circles) are shown. The Fab fragment consisting of an L chain $V_L C_L$ and an H chain $V_L C_{H1}$ joined by disulfide bonds, and the Fc fragment consisting of a constant region dimer lacking the C_{H1} are indicated. (Reproduced with permission from Potter KN, Li Yucheng and Capra JD (1995) In: Wang HY and Imanaka T (eds) *Antibody Expression and Engineering*. Washington, DC: American Chemical Society.)

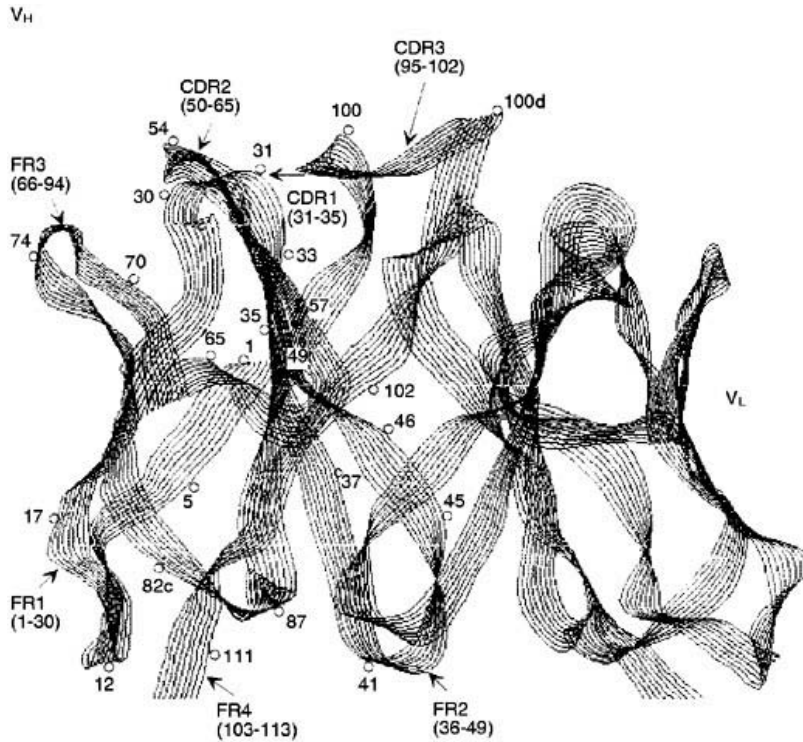


Figure 3 Ribbon drawing of the V_H and V_L domains of the V_H3 -encoded antibody Kol using the HyperChem software (Waterloo, Ontario, Canada) showing the folding and relationship between the framework and complementarity-determining regions.

Table 1 Amino acid residue numbers corresponding to positions in H and L chain variable domains

Region	Chain	Position
FR1	Heavy	1–30
	Light	1–23
CDR1	Heavy	31–35 (31a,b)
	Light	24–34 (27a–f)
FR2	Heavy	36–49
	Light	35–49
CDR2	Heavy	50–65 (52a–c)
	Light	50–65
FR3	Heavy	66–94 (82a–c)
	Light	66–88
CDR3	Heavy	95–102 (100a–k)
	Light	89–97 (95a–f)
FR4	Heavy	103–113
	Light	98–107 (106a)

CDR (complementarity-determining region), FR (framework region). The numbering system serves to optimally align conserved residues. Length variations are labeled with letters.

generate a population of B cells producing antibodies with a diverse array of antigen-combining sites in order to interact specifically with the myriad of antigenic structures which can invade a host. There are several mechanisms used for generating this diversity in the variable domains of human immunoglobulins. They include 1) the innate diversity in the sequences of the V (variable), D (diversity) and J (joining) gene segments, 2) site-specific DNA rearrangement of the

V, D and J gene segments, 3) deletion of nucleotides at joining regions by nucleases, 4) insertion of non-templated and templated nucleotides at joining regions, 5) fusion and inversion of D regions, 6) pairing of light and heavy chains, and 7) somatic hypermutation. The result of these mechanisms is the production of an estimated 10^{14} different antibodies in humans.

Human germline diversity

The production of a human immunoglobulin involves an ordered series of somatic DNA rearrangements involving gene segments (exons) located on three different chromosomes: the heavy (H) chain locus on chromosome 14, the kappa (κ) chain locus on chromosome 2, and the lambda (λ) chain locus on chromosome 22. The human immunoglobulin H chain locus is located on chromosome 14q32.3, and spans approximately 1100 kb. The entire V_H locus has been mapped and the V_{H1} genes sequenced. There are approximately 100 V_H gene segments, of which about only half are functional, approximately 30 D genes, and nine J_H genes, of which six are functional (Figure 4). At the protein level, V_H gene segments encode FR1, CDR1, FR2, CDR2 and FR3 (Figure 2). The 3' end of the V_{H1} , D and 5' end of the J_H gene segment form the CDR_H3, with the remainder of the J segment forming FR4. The V_H gene segments are grouped into seven V_H

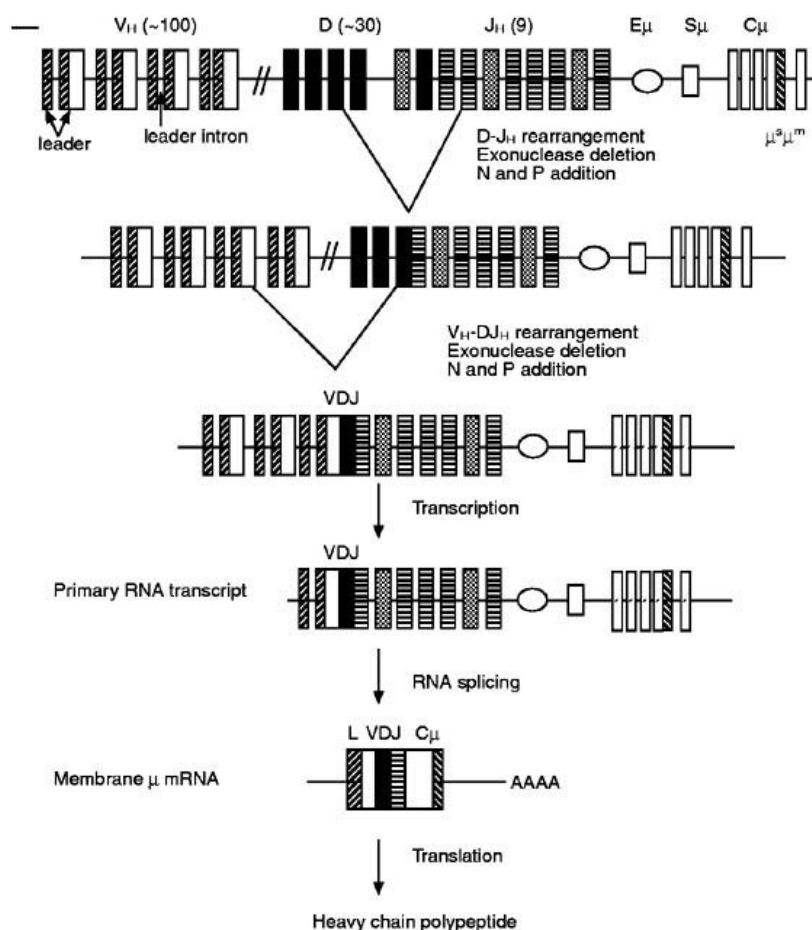


Figure 4 Diversity generated by VDJ recombination. The organization of a portion of the human immunoglobulin heavy chain locus containing the V, D and J gene segments is shown. The numbers refer to total number of gene segments including pseudo-genes. The union of one D gene segment to one J_H gene segment occurs prior to one V gene segment joining to the -DJ_H pair. The leader intron is removed during RNA splicing. Alternative splicing can lead to either membrane bound (m) immunoglobulin, or secreted immunoglobulin (s). The approximate numbers of the gene segments are indicated. E_μ, enhancer; S_μ, μ switch region; μ_m, membrane-bound IgM; μ_s, secreted IgM. The primary repertoire consists of V(D)J recombinations in the absence of antigen, producing predominantly antibodies which are polyclonal but of low affinity. The secondary repertoire consists of antibodies selected from the primary repertoire by antigen. These antibodies are characterized by mutation and high affinity for specific antigens. (Reproduced with permission from Okada A and Alt FW (1995) The variable region gene assembly mechanism. In: Honjo T and Alt FW (eds) *Immunoglobulin Genes*. London: Academic Press.)

families based on greater than 80% homology at the nucleotide level (Table 2). In the human, the V_H family gene segments are interspersed as opposed to the mouse, where the V_H gene segments from particular families are clustered. There are four D_H clusters (D1–D4) each containing seven D_H segments. Two orphan V_H loci are located on chromosomes 15 and 16, some of which are potentially functional, although they are not associated with J_H or C region genes. At least four orphan D_H clusters, each con-

sisting of five D_H segments, are located on chromosome 15. There are approximately 76 (40 of which are functional) V_K gene segments (grouped into seven families), five J_K gene segments, approximately 50 (30 of which are functional) V_λ gene segments (grouped into nine families), and 7–10 J_λ gene segments. L Chain V domains are the product of V_L and J_L gene segment recombination. The junction between the V_L and C_L encodes the CDR_{1,3} (Figure 2).

The mouse V_H locus is located on chromosome 12. As with the human system, the V_H, D and J_H gene segments are arranged in separate clusters. The V_H gene segments are divided into 14 families based on nucleotide homology. A rough estimate of the number of V_H members is 100. On chromosome 12, members of one family tend to remain in groups and are not as interspersed as on the human chromosome. There are three families of murine D_H gene segments, and four J_H segments. There is evidence of J_H proximal usage of V_H gene segments, unlike in the human system where there is no evidence for position playing a role in V_H gene segment usage.

Table 2 Human V_H gene segments

Chromosome	V _H family							Total
	1	2	3	4	5	6	7	
14q32	14	4	48	12	2	1	6	87
15q11	6	0	1	1	0	0	0	8
16p11	4	1	11	0	0	0	0	16
Total	24	5	60	13	2	1	6	111

Approximately 50% of these gene segments are nonfunctional pseudogenes.

From Hongo T and Matsuda F (1995) Immunoglobulin heavy chain loci of mouse and human. In: Honjo T and Alt FW (eds) *Immunoglobulin Genes*, 2nd edn. London: Academic Press.

VDJ combinatorial diversity

Both H and L chain V domains are the product of somatically rearranged V, D (H chain only), and J gene segments, a process specific to lymphocytes. During rearrangement, only one of each type of segment is used. The mixing and matching of gene segments is a main contributor to the generation of a diverse repertoire of antibodies. The result of the recombination event is that three gene segments, which originally were scattered over several kilobases of DNA, are brought together to form a single gene which can then be transcribed and translated. In H chain assembly, V_HDJ_H rearrangement proceeds initially as a $-DJ_H$ rearrangement on both chromosomes in pro-B cells, followed by V_H to $-DJ_H$ on one chromosome in pre-B cells (Figure 4). If that rearrangement is nonproductive (i.e. results in a frameshift), rearrangement occurs on the other chromosome. The production of the μ H chain on the cell surface, in association with surrogate L chains, inhibits further H chain VDJ rearrangements (allelic exclusion), and induces the rearrangement of V_L and J_L gene segments. In general, rearrangement is attempted first at the κ locus, and if rearrangement on the two chromosomes are nonproductive, rearrangement is initiated at the λ locus. Functional rearrangement of an L chain stops further rearrangements of L chain genes of the same isotype (allelic exclusion). Each V and J gene segment has only one correct reading frame. D segments can theoretically be read in all three reading frames in different VDJ recombinations, which contributes to antibody diversity. Since there does not appear to be a mechanism regulating the alignment of V and J gene segments, productive (inframe) joining is accomplished in one out of every three joining events.

The process of V(D)J recombination is site-specific, and involves the enzymatic cutting and ligating of DNA. It is directed by recombination signal sequences (RSSs) adjacent to the DNA segments to be joined. RSSs consist of a conserved heptamer and nonamer separated by a spacer of either 12 or 23 base pairs (Figure 5). The consensus palindromic heptamer sequence is CACAGTG and the consensus nonamer sequence is ACAAAAACC. It is always the heptamer which is directly adjacent to the gene segment. RSSs are only on the 3' side of V_H and V_L gene segments, and have 23 base pair spacers. RSSs are only on the 5' side of J_H , J_κ and J_λ gene segments, and have 23 base pair spacers. D gene segments, which are only in the H chain locus, are flanked by RSSs having 12 base pair spacers. Recombination occurs almost exclusively between two signals of 12

and 23 spacer lengths. This is referred to as the '12/23 rule'.

V_HDJ_H recombination between RSSs is characterized by biases. Forty-six out of 87 mapped V_H segments have been shown to be used. Using D- J_H minilocus substrates, it was determined that the sequence of the signal and coding ends determines the bias. The spacer sequences, and the sequences adjacent to the RSSs are not conserved. The nucleotide composition of the three residues of the heptamer closest to the cleavage site can affect the efficiency of recombination. There is no evidence of a correlation between preferential rearrangement of a V_H gene segment and its position in the V_H locus. It is thought that V(D)J rearrangements are dependent on the accessibility of a locus for the recombination machinery. Transcription may be involved as germline V and J-C transcripts are found prior to the onset of DNA recombination.

During V(D)J rearrangement, double-strand breaks are introduced at heptamer borders which most often religate head-to-head forming a signal joint, and the coding ends next to the heptamers are joined to form a coding joint (Figure 5). If the two RSSs are in the same orientation, the intervening segment is inverted and maintained in the chromosome (inversional rearrangement). If the two RSSs face the opposite orientation, the intervening segment is deleted (deletional rearrangement) (Figure 6). Excised circular DNA is not known to have a function. D- J_H joining predominantly undergoes deletional joining using the J_H proximal RSSs of the D gene segment. V_HDJ_H chain recombinations most often result in the deletion of the intervening DNA, while in many κ chain segments, recombination occurs by inversional joining. Occasionally recombination results in an exchange of RSSs between two gene segments resulting in a hybrid junction. Junctional modifications can involve complex rearrangements including V-D-D-J, V-D-inverted D-J, etc. to generate different CDR_{H3} sequences. Some of these unusual rearrangements break the 12/23 rule.

N-Segment and P-nucleotide additions

During V(D)J recombination, the cleavage of the two heptamer elements generates blunt ends, which are unmodified during ligation to form a heptamer-to-heptamer signal joint. Coding junctions, however, are heterogeneous in sequence. The recombination process creates many different coding junctions from the same RSS pairs. Nucleotides can be either removed or added enzymatically. Exonucleases can delete residues, while nucleotides can be added at junctions by two different mechanisms. These are

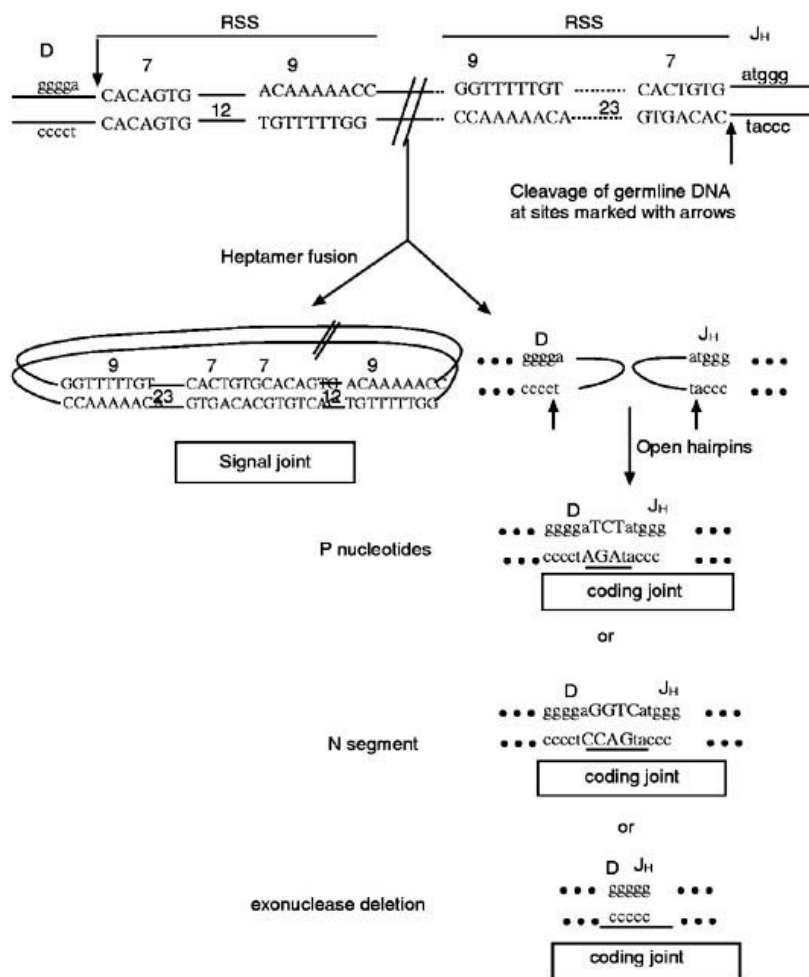


Figure 5 Formation of coding and signal joints. Heptamer fusion leads to an unmodified signal joint, and hairpin formation followed by exonuclease deletion and N and P nucleotide addition leads to a modified coding joint. (Reproduced with permission from Okada A and Alt FW (1995) The variable region gene assembly mechanism. In: Honjo T and Alt FW (eds) *Immunoglobulin Genes*. London: Academic Press.)

referred to as N-segment and palindromic (P)-nucleotide additions. N-Segment diversity results from the addition of untemplated bases by terminal deoxynucleotidyl transferase (TdT). TdT activity is minimal in the fetus and neonate but is upregulated postnatally. As a result, the CDR_H3 is shorter in the fetal compared with the adult repertoire. While TdT appears to act predominantly on H chains, N segments have been identified at L chain VJ junctions in about 20% of light chains. This indicates that TdT, or an analogous enzyme, must be active in a significant percentage of human B lymphocytes during L chain rearrangement. P Nucleotides are templated bases from the noncoding strand in the coding joint as a result of the formation of hairpin structures generated during recombination. These usually consist of single bases or dinucleotides (Figure 5).

Not all of the proteins involved in this highly regulated DNA recombination event have been identified. Two proteins, RAG-1 and RAG-2, the products of the recombination activating genes RAG1 and RAG2, have signal-cutting activity. They are re-

quired for initiating V(D)J recombination. Studies indicate that there is synchronous cleavage at the two RSSs between the signal and the coding border. Cleavage is a two-step reaction: first, a single-strand nick is made at the 5' end of the heptamer, and secondly, a covalently closed hairpin coding end and a blunt 5' phosphorylated signal end are formed (Figure 5). Mutational analysis indicates that the recombination machinery must interact with both RSSs for cleavage to occur at either signal. Ku, a nuclear DNA repair protein, is also required for V(D)J recombination. Ku is a heterodimer composed of a 70 kDa and an 86 kDa subunit. Both subunits are required to form functional Ku. Ku binds DNA ends in a sequence-independent manner. The Ku heterodimer also associates with a DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form a trimeric protein called DNA protein kinase (DNA-PK). DNA-PK has serine/threonine kinase activity and requires DNA for activation. One model suggests that following the cleavages by RAG-1 and RAG-2, Ku is then loaded on to the coding hairpin

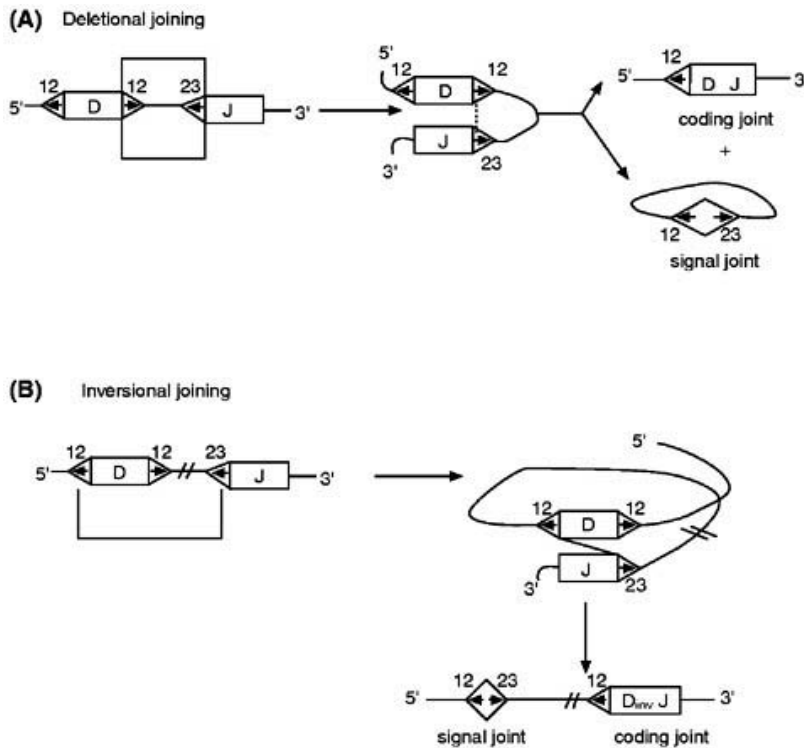


Figure 6 Joining mechanisms. (A) When two RSSs face in opposite directions the intervening sequences are deleted as circular DNA (deletional joining). (B) When two RSSs face the same direction recombination results in the inversion of intervening sequences without deletion of DNA (inversional joining). (Reproduced with permission from Okada A and Alt FW (1995) The variable region gene assembly mechanism. In: Honjo T

ends and acts to stimulate the joining of the DNA breaks. Signal joints require only Ku and not DNA-PKcs.

Somatic mutation

Somatic hypermutation is a key mechanism in generating antibody diversity. The details of the mechanisms and regulation of somatic hypermutation are unknown. In the human, somatic hypermutation occurs in the presence of antigen, unlike in sheep where antigen-independent somatic hypermutation occurs. The germinal centers of peripheral lymphoid tissues are the sites where somatic hypermutation, positive selection and differentiation of B cells with high-affinity receptors occur. Germinal centers are the only places in the body where antigen is retained for months or years in an extracellular location. Antigen is trapped on the surface of follicular dendritic cells which hold it as an antigen-antibody complex in proximity to nearby B lymphocytes. It is in the microenvironment of the germinal center in which the repertoire of the antigen-specific B cell, influenced by signals from T cells, is shaped. Hypermutation is restricted to V regions and their flanking sequences of H and L chains (Figure 7). Since IgM antibodies undergo somatic mutation, H chain class switching is not required for somatic mutation to occur. Most antibodies expressed as germline sequences are of relatively low affinity. After con-

tacting antigen, mechanisms which generate somatic mutations produce antibodies of higher affinity. There is enrichment of B cells with mutations which confer higher affinity for antigen, while B cells which have acquired stop codons or have lowered affinity are removed by apoptosis. Only rearranged, transcriptionally active Ig genes are mutated, at a rate of

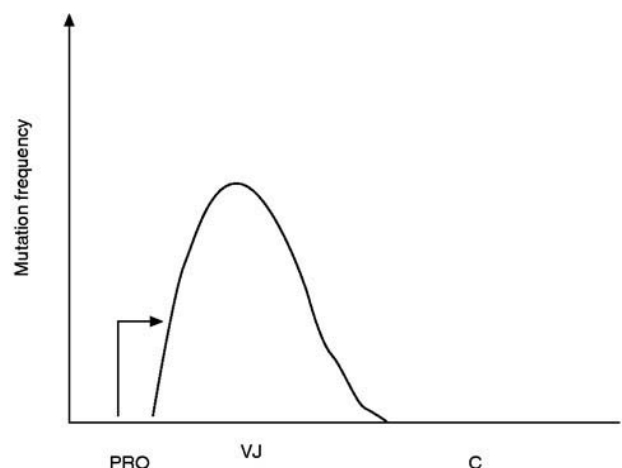


Figure 7 Localization of mutations in the light chain locus. The 5' boundary of mutations is within the leader intron, approximately 150 bp after the transcription promoter (PRO), to approximately 1.5 kb downstream but does not extend into the constant (C) region. (Reproduced with permission from Storb U (1996) The molecular basis of somatic hypermutation of immunoglobulin genes. *Current Opinion in Immunology* 8: 206-214.)

approximately 10^3 per bp per generation. Both productive and nonproductive rearrangements are substrates for mutation. There are several features characteristic of the hypermutation process: 1) mutations are mainly point mutations, with very rare deletions or insertions of mostly single nucleotides, 2) there is one strand of the double helix which is preferentially targeted, 3) foreign DNA in the context of an immunoglobulin transgene is somatically mutated, suggesting that the targeted sequence itself does not initiate the mutation process, 4) hotspots of mutation are observed at different positions in different sequences suggesting that the process is influenced by the primary DNA sequence, 5) the 5' boundary of mutations is within the leader intron, and 6) while there is no defined 3' end-point of the mutation tract, mutation does not extend into the C region. A current model indicates that transcription is necessary for mutation to occur.

Heavy and light chain association

In theory, there is random combination between any H and L chain. The combinatorial pairing of H and L chains does serve to increase the antibody repertoire, but not to the extent contributed by VDJ joining and coding joint manipulation. Antibodies with different specificities are found to have identical V_L domains paired with different V_H domains. Using recombinant antibodies, L chains were demonstrated to modulate or inhibit binding of antigen.

Studies indicate that B cells which produce antibodies having self-reactivity with high-affinity for their antigen can resynthesize a different L chain. This process, referred to as receptor editing, indicates that cells which produce autoantibodies can exchange L chains, and following successive pairing with the H chain, produce a final Ig product which does not have high-affinity recognition for self antigens. It is suggested that this is an antigen-directed mechanism used by immature autoreactive B cells to change the specificity of the receptor in order to escape tolerance. Defects in this editing process may lead to the accumulation of high-affinity, self-reactive antibodies.

See also: Allelic exclusion; Antibodies, bispecific; Antibodies, specificity; Antibodies, synthesis; Anti-

serum; B lymphocyte repertoire; Clonal selection; Immune response; Immunoglobulin genes; Immunoglobulin structure; Somatic mutation; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T lymphocyte clonal expansion.

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DNA VACCINES

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DNA vaccines represent a novel vaccine approach which employs genes encoding proteins of pathogens or tumors, rather than the proteins or pathogens themselves as in more conventional approaches. The vaccines consist of bacterial plasmids containing a strong viral promoter which will function in mammalian cells, a gene encoding the protein antigen, and a polyadenylation sequence and transcriptional terminator, all placed in a vector backbone that permits replication of the plasmid (for purposes of production) in a bacterial host. Thus the plasmid is produced through standard recombinant technology by bacterial fermentation, yet the plasmid does not replicate following injection into a vertebrate. Rather, there, the protein antigen is produced *in situ* and can elicit immune responses. From an immunological standpoint, the advantage of this technology is that both humoral and cellular immune responses are generated, with the production of antibodies, and both helper and cytolytic T cell responses.

Although the initial demonstration of the protective efficacy of such a vaccine in an animal challenge model was published only in 1993, promising pre-clinical results have been observed in a number of disease models representing various classes of disease targets (viral, bacterial and parasitic infections as well as cancer) and various animal species including nonhuman primates. The ultimate value of the technology awaits the results of clinical trials which are at very early stages. Nevertheless, this technology makes it possible to study some fundamental issues of antigen processing and presentation and to evaluate the potential role of various antigens and types of immune responses for protection against certain diseases, as well as offering potential solutions to some of the challenges of vaccine development. The latter include: generation of cytotoxic T lymphocyte responses which can recognize epitopes from conserved proteins on infected cells and thus may provide broader protection against different strains of a virus; presentation to elicit antibodies to antigen that has post-translational modifications, conformation and oligomerization which may be more appropriate for protein antigens that normally are made by the vertebrate host cell; potentially long-lasting immune responses (perhaps due to long-term expression of antigen); and a ready ability to combine diverse

immunogens into a single preparation to decrease the number of vaccinations required.

This entry will focus on the immune responses generated by DNA vaccines and describe the mechanisms whereby the vaccines induce the immune response. For a review of the disease models in which clinical efficacy has been shown and immunogenicity demonstrated the reader is referred to Donnelly, Ulmer, Shiver and Liu, 1997.

DNA vaccine technology grew from an observation that *in vivo* injection of plasmid DNA encoding reporter genes resulted in the *in situ* production of protein, with muscle being the site that resulted in the greatest expression of protein. The DNA was simply injected as a saline solution without any requirement for special delivery agents such as the cationic lipids that are used for DNA transfection *in vitro*. An alternate means of delivery, and the ability of the expressed protein to induce antibody responses, were subsequently described using a biolistics delivery device known as a 'gene gun' which propelled DNA precipitated onto gold beads into the skin.

The initial demonstration that *in vivo* injection of DNA encoding an antigen could result in immune responses that were protective against viral challenge, and moreover in major histocompatibility complex (MHC) class I-restricted T cell responses (which were protective against cross-strain challenge), utilized plasmid DNA encoding an internal protein of influenza A virus injected intramuscularly in mice in saline. The generation of class I-restricted cytotoxic T lymphocytes (CTLs) was of particular interest, because such responses are thought to require the endogenous production of the protein antigen, so that the protein can enter the proteasome/TAP transporter processing pathway and the resulting epitope peptides can associate with MHC class I molecules in the endoplasmic reticulum. Because most exogenous proteins preferentially enter the MHC class II/endosomal pathway rather than this class I antigen-processing pathway, vaccines that are not live intracellularly growing organisms do not generally result in the induction of MHC class I-restricted CTLs. DNA vaccines thus provided a means of generating these CTLs without the use of a live replicating vector. The ability to generate CTLs

by providing intact proteins (rather than peptides), allows determinant selection to occur, thus broadening the potential applicability to a diverse population. The mechanisms whereby this occurs will be addressed below.

The ability of a DNA vector encoding an internal conserved protein, nucleoprotein (NP), to generate CTLs as well as cross-strain protection provides an example of the responses that have been observed in various other systems. Immunization with DNA encoding the NP of the 1934 H1N1 strain A/PR/8/34 protected mice from subsequent lethal challenge with the heterosubtypic 1968 H3N2 strain A/HK/68 (Figure 1). CTLs that recognized the MHC class I (H2K^d)-restricted epitope of NP were generated and were capable of lysing both peptide-pulsed and virally infected target cells. This was true for spleen cells specifically restimulated with antigen, as well as for spleen cells activated without antigen using only cytokine. Immunization with recombinant NP prepared in a baculovirus expression system did not produce MHC class I-restricted CTLs or cross-strain protection under these conditions. Transfer of restimulated splenocytes but not sera from immunized animals conferred the same cross-strain protection on recipient mice. Thus immunization with DNA encoding a conserved protein was shown to be capable of generating CTL responses which, as shown in this model, were effective at mediating protection against a different strain of virus, against which strain-specific antibodies would be ineffective.

More recently immunization with NPmut, a plasmid encoding a full-length mutant form of the same NP in which the anchor residues of the H2K^d-restricted 147–155 epitope were altered to prevent its binding to H2K^d, was observed to induce CTL directed against a recessive class I-restricted epitope. These CTLs were likewise protective against cross-strain challenge. Thus the DNA vaccine preserved the hier-

archical order of immunogenicity of the epitope peptides for the induction of the CTLs.

Since immunization with DNA encoding proteins results in protein synthesis in the definitive host, one would predict that such a protein would be similar or identical to the wild-type protein produced by viral infection. This is in contrast to chemically inactivated virus, in which surface proteins may be cross-linked, or recombinant proteins which may have different post-translational modifications, conformations or ability to oligomerize. While this appears to be an advantage for viral and tumor antigens, it may be an issue for bacterial and parasitic proteins as discussed below for *Mycobacterium tuberculosis*.

An example of the potential advantage of a DNA vaccine for generating native antigens for B cell responses is the hemagglutinin (HA) of influenza virus, which is the major surface glycoprotein of influenza against which virus-neutralizing antibodies are directed. Current influenza vaccines are grown in eggs, which may select for variants with HA sequences differing from the sequences of virus grown in mammalian cells, and are inactivated with formalin. These vaccines provide significant but highly strain-specific immunity. DNA vaccines could encode the exact sequence of mammalian isolates and avoid any chemical alterations, while providing the conformational epitopes needed for inducing protective antibodies. In fact, doses as low as 1 µg of DNA (given twice) provided complete protection against homologous challenge. The antibodies were similar to those induced by natural infection in the predominance of the IgG2a subtype. In contrast, immunization with inactivated virus yielded equivalent titers of IgG1 and IgG2a. Studies in primates have shown that DNA vaccines elicit HI antibody titers equal to or greater than those induced with inactivated whole or split virus.

Splenocytes from DNA vaccine-immunized ani-

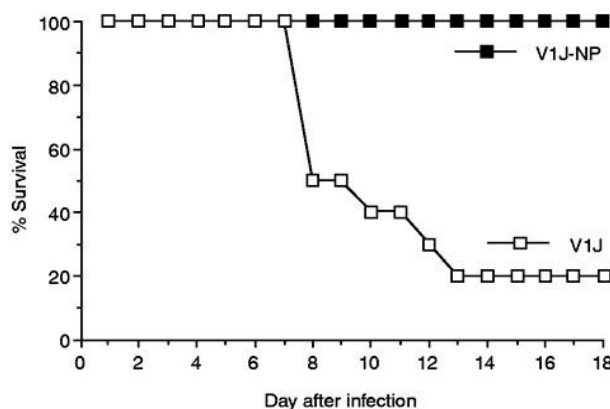


Figure 1 Cross-strain protective immune responses to influenza in mice immunized intramuscularly with NP DNA. Groups of 10 female BALB/c mice were injected at 4, 7 and 10 weeks of age with 100 µg of plasmid DNA encoding the NP from influenza A/PR/8/34 (H1N1) (solid squares), or with control vector (open squares). Intramuscular injections were given in both quadriceps muscles in a total volume of 100 µl. Mice were challenged with 200 TCID₅₀ of influenza A/HK/68 (H3N2) at 13 weeks of age. (Reproduced with permission from Montgomery D et al (1993) *DNA and Cell Biology* 12: 777–784.)

mals proliferated when re-exposed to antigen *in vitro*. In addition secretion of interleukin-2 (IL-2) and interferon γ (IFN γ) but not IL-4, were seen, consistent with a T_H1-like response. The predominance of IgG2a responses in mice following immunization with various DNA vaccines gives further evidence of the generation of T_H1-type helper responses.

These specific and protective immune responses have been seen in various other disease models. In a cottontail rabbit papillomavirus (CRPV) model, we have shown that immunization with DNA encoding the major capsid protein, L1, protected against wart formation following challenge with CRPV. In viral infection, L1 is directed to the cell's nucleus for assembly into capsids which are recognized by neutralizing antibodies. Hence, it was not clear that DNA immunization would yield the antigen in an accessible and appropriate conformational form. Yet, vaccination of rabbits with L1 DNA generated neutralizing antibodies which protected rabbits from development of warts upon challenge and which persisted undiminished in titer for 32 weeks or more. Transfer of immune serum prior to infection likewise protected the animals.

The ability of a vaccine to generate immune responses effective for pathogens that initially infect via mucosal surfaces may determine the effectiveness of such a vaccine. Immunization with DNA encoding gD and gB of herpes simplex virus type 2 (HSV-2) induced neutralizing antibodies against these envelope glycoproteins and protected guinea pigs in a mucosal challenge model.

DNA vaccines encoding proteins of *M. tuberculosis* have been shown to be effective for generating the desired cellular responses and protection from challenge. This model has been instructive, in that in this system the mycobacterial protein is made by the mammalian host cell rather than by the organism, and therefore the post-translational modifications of the protein have in some cases been different from those of the native protein. To avoid the glycosylation that was seen of one particular antigen, antigen 85C, selected point mutations were introduced to remove the N-linked glycosylation sites and thus to generate an antigen more like the native protein in its lack of glycosylation.

Although both intramuscular (i.m.) and intradermal (i.d.) immunization result in antibodies, i.m. immunization has been the only route resulting in significant cross-strain protection (for influenza) and has been the route yielding the highest levels of helper cell responses (for HIV env). Potential mechanisms for antigen presentation after i.m. DNA immunization include:

1. synthesis and presentation of antigen by the myocytes,
2. synthesis of antigen by the myocytes followed by uptake of some form of the protein by an antigen-presenting cell (such as a bone marrow-derived dendritic cell), or
3. the presence of DNA (either by direct uptake or transfer from myocytes) in antigen-presenting cells in addition to the myocytes.

Studies utilizing parental $\rightarrow$ F1 bone marrow chimeric mice have demonstrated that following i.m. immunization with NP DNA, all of the CTLs that were detected were restricted by the MHC class I haplotype of the bone marrow donor. Thus, only professional antigen-presenting cells (APCs) were able to prime the CTLs for an MHC class I-restricted response. Therefore, muscle cells may synthesize antigen, but do not present antigen for priming of CTLs after DNA immunization i.m. Conversely, it is not necessary to invoke direct transfection of APCs by the DNA since if myoblasts that are permanently transfected to express NP were transplanted into similar bone marrow chimeras, the CTLs were again restricted to the donor haplotype even if the myoblasts were of the other parental haplotype. Thus production of an antigen by myocytes is sufficient for the induction of CTLs, indicating that transfer of some form of antigen must occur, possibly the 'cross-priming' reported in other systems.

The plasmid DNA appears to also have adjuvant activity which may influence both the magnitude and the type of immune responses. For example, although immunization with recombinant NP resulted in the generation of comparable levels of IgG1 and IgG2a in mice, addition of noncoding plasmid DNA to the recombinant NP shifted the isotype of the antibodies to a predominance of IgG2a, as is seen in the case of immunization with NP DNA or following infection with live influenza virus. Addition of noncoding plasmid DNA to DNA coding for influenza HA also increased the titer of HA-specific antibody in nonhuman primates. The mechanism for this adjuvant effect of DNA is unclear. Certain motifs of the DNA base pairs (sequences containing CpG) that are more common in bacterial DNA may play a role since oligomers containing these motifs have been shown by various groups to be mitogenic for lymphocytes *in vitro*. Very preliminary data *in vivo* have yielded more complex results as to whether or not the presence of such motifs in the plasmid play any role in increasing immune responses to the protein encoded by the DNA.

A number of potential issues related to safety need to be more fully explored as the clinical utility of

DNA vaccines is being determined. Questions which must be answered include: does integration of the plasmid occur that may potentially lead to insertional mutagenesis? Does induction of pathogenic anti-DNA antibodies occur? Does immunologic tolerance develop if the production of antigen persists? Studies to date have failed to demonstrate integration, induction of pathogenic anti-DNA antibodies, or tolerance. Indeed, regarding the latter point, DNA vaccines have been seen to prime and boost well.

While the work presented here has focused upon viral and bacterial diseases, the same attributes may provide a useful prophylactic or immunotherapeutic approach to parasitic diseases or cancer.

In summary, DNA vaccines may provide a broadly applicable technology for generating protective cellular and antibody responses. Although a number of issues still need further exploration (e.g. safety, mechanisms of immunogenicity), DNA vaccines have given promising results in a number of preclinical studies and have provided a tool for studying mechanisms of antigen presentation and for antigen discovery for vaccines.

See also: Adjuvants; Antigen-presenting cells; Antigenic variation; Cytotoxic T lymphocytes; Dendritic cells; Vaccination, methods of administration; Vaccines; Viruses, immunity to.

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DOMAINS, IMMUNOGLOBULIN-TYPE

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The notion that immunoglobulin (Ig) chains are made up of homologous modular units arose in the mid-1960s, when the pioneering experiments of Porter, Nisonoff and Milstein had already revealed the periodic distribution of disulfide bridges along the polypeptide chains, and several groups were reporting the first partial amino acid sequences that confirmed the internal similarities in light and heavy chains. Taken together, these results suggested that Ig chains arose by successive duplications of a common ancestor gene. A repeated segment of about 110 amino acids, the Ig homology unit, was thus identified.

At the same time, a structural model of the antibody molecule was emerging from a wealth of biochemical and biophysical evidence. Tanford and coworkers proposed, in 1965, the now classic Y-shaped model of the IgG molecule consisting of three globular compact structures, the Fc and two Fab fragments. Ultrastructural support for this model was soon provided by Valentine and Green's electron micrographs of rabbit IgGs. Furthermore, the apparent size of the molecule was consistent with the first preliminary X-ray studies of immunoglobulin crystals, and small-angle X-ray scattering studies were compatible with a flexible Y-shaped model for the IgG molecule.

In this context the notion of homology unit evolved into the domain hypothesis. In 1969 Edelman and coworkers reported the first complete sequence of an Ig molecule and suggested that each repeated homology unit folds into a compact structure, the Ig-type domain. This hypothesis not only placed the domain at the evolutionary origin of immunoglobulins, but also recognized that different domains evolved to serve different biological functions. The Ig-type domain was therefore conceived to be a unit of sequence homology, molecular evolution, protein architecture and biological function.

The Ig fold in antibodies

The structural implications of the domain hypothesis were confirmed in 1973 by the first detailed X-ray diffraction studies of immunoglobulin fragments. The three-dimensional structures of a Bence Jones protein and of Fab fragments from myeloma proteins established that the postulated domains are indeed spatially separated and share a common pattern of polypeptide chain folding, the Ig fold. Since then, the

introduction of myeloma hybridization techniques for the production of monoclonal antibodies and the impressive advances in molecular biology and biophysical techniques have greatly stimulated further biochemical and structural studies of immunoglobulin fragments. Detailed three-dimensional structures are now known for a large number of antibody fragments and antigen-antibody complexes, as well as for non-Ig proteins bearing one or more Ig-type domains, thus providing a basis for the understanding of the important role of the Ig-type domain in biological recognition.

Basic structure of the Ig fold

Immunoglobulin domains fold into a globular, compact structure that fits into a parallelepiped of approximately $40 \times 25 \times 25 \text{ \AA}$ ($1 \text{ \AA} = 10^{-10} \text{ m}$). The basic folding motif consists of two layers of anti-parallel β sheets that surround an internal volume closely packed with hydrophobic side-chains. The overall shape of the domain can be described as a flattened cylinder (β barrel) or sandwich-like structure of two β -pleated sheets (Figure 1). The variable (V) and constant (C) subunits of immunoglobulins differ in the number and regularity of the β strands forming the bilayer: the C domains are built up from seven β strands arranged such that four strands form one β sheet and three strands form a second sheet, whereas the V domains contain nine β strands instead of seven. The two additional strands in V domains, inserted at the edge of one β sheet, are functionally important since they define the second hypervariable region directly involved in the formation of the antigen-recognition site.

The topology of a protein domain – that is the order in which the elements of secondary structure are connected to each other in the three-dimensional structure – is a distinctive feature that serves to establish a taxonomy of protein folding patterns. The topology of the Ig fold is quite simple (Figure 1) and is usually represented in the form of a 'Greek key barrel' (so called since the two-dimensional topological diagram is reminiscent of the motifs observed in old Greek pottery). As a result of this topology, there are at least two β strands, consecutive in the sequence but not contiguous in space, that are linked by a connecting loop crossing the top or bottom of the β barrel (the first hypervariable loop of V domains is one example). All Ig-type domains are topologically equivalent, and this unique pattern of linkages may be

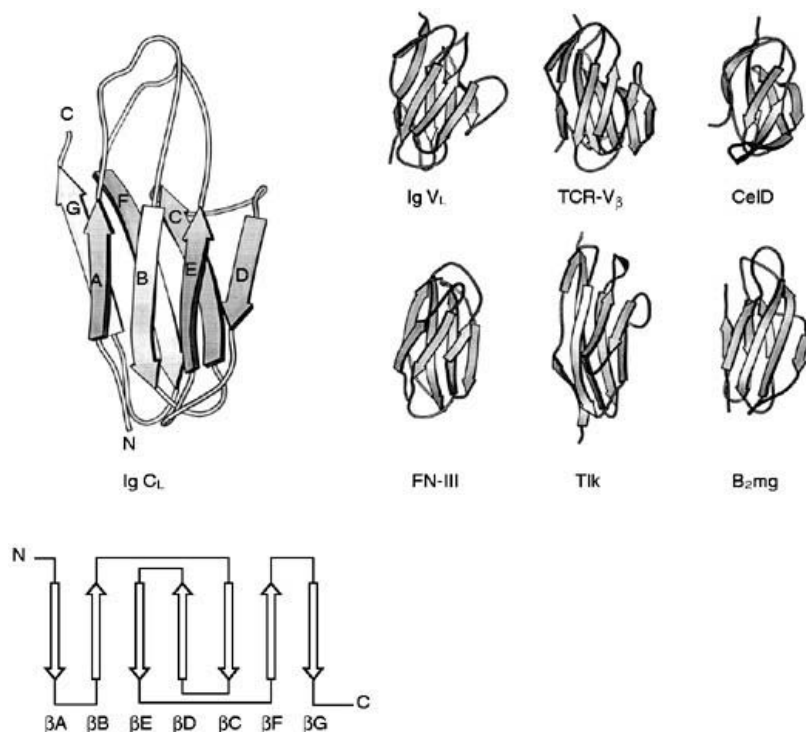


Figure 1 Structural diversity of Ig-type domains. The constant domain of antibodies (Ig C_L) represents the common core present in all Ig-like structures, composed of seven antiparallel β strands labeled A–F. The two-dimensional topological diagram (bottom, left) shows the connected Greek key motif of this structure. Also shown are selected examples of Ig-type domains from different subtypes: Ig V_L, variable domain of an antibody light chain; TCR-V _{β} , variable domain from a T cell receptor β chain; CelD, N-terminal domain of a bacterial cellulase; FN-III, fibronectin type III domain of tenascin, an extracellular matrix glycoprotein expressed during embryonic development; Tik, C-terminal domain of smooth muscle myosin light chain kinase; B₂mg, β_2 -microglobulin

considered as the ‘signature’ of the immunoglobulin fold.

The loops connecting the β strands are relatively short and, as a result, a majority of the residues of the domain are contained in the two β sheets. The strands exposed to solvent display the characteristic pattern of alternating polar–apolar residues, with the apolar side-chains extending towards the interior of the domain to form a hydrophobic core between the two β sheets. Most of the conserved residues in V and C domains of antibodies are found in this internal core, in particular two highly conserved cysteine residues which form an intramolecular disulfide bridge linking the two β sheets. Other core positions show extensive amino acid substitutions, but preserve the secondary and tertiary structures and, to a lesser extent, the hydrophobic character of the protein interior. At the two extremities of the domain structure, the β strands are connected by peptide loops of varying length and character. These connecting segments may form regular reverse turns or short α -helical stretches, but in most cases present an irregular structure.

Domain association

The Ig domains of antibodies are associated in pairs stabilized by noncovalent interactions. With the exception of the C_{H2} domains, Ig subunits interact over a large contact area and are tightly associated.

The variable domains associate to form V_H–V_L heterodimers in Fab fragments, or V_L–V_L homodimers in Bence Jones proteins, by face-to-face packing of the five-stranded β sheets. Two V domains are related by an approximate (heterodimer) or exact (homodimer) twofold axis of symmetry. Inter-subunit interactions are very extensive, with roughly 20% of the entire molecular surface of each domain buried upon dimerization. Highly conserved amino acid residues at the V_H–V_L interface allow the association of different pairs of light and heavy chains to form the antibody molecule.

The constant domains of antibodies and Bence Jones proteins also form closely packed dimers. As in V dimers, paired C domains are related by an approximate (C_{H1}–C_L) or exact (C_L–C_L, C_{H1}3–C_{H3}) twofold axis of symmetry, and the arrangement of subunits among all the C-type pairs studied is very similar. There is, however, a reversal of domain–domain interactions compared to V dimers: C dimers are formed by a close packing of the four-stranded β sheets which in V domains are exposed to solvent.

The antigen-recognition site of the antibody molecule involves the hypervariable loops which connect the β strands at the distal extremity of the V_H–V_L dimer. Functional differentiation of variable domains is accomplished through extensive genetic restructuring of this region (amino acid insertions, deletions and substitutions), which confers the antibody mol-

ecule with a potentially unlimited range of antigen binding specificities without significantly affecting the basic structural framework of the Ig domains.

The Ig-type domain in non-Ig molecules

First described in Ig molecules, the characteristic β -sheet fold of the Ig-type domain has also been found in a large number of proteins of diverse biological function. Many of these proteins share additional genetic and functional similarities with Ig domains, which suggests a common evolutionary origin, and are thus grouped together into the so-called Ig superfamily. The presence of one or more Ig-type domains in members of the Ig superfamily can be inferred by sequence homology, and has been demonstrated in various cases by crystallographic analysis (for example, Ig H and L chains, T cell receptor (TCR) α and β chains, major histocompatibility complex (MHC) class I and class II, surface antigens CD2, CD4, CD8, β_2 -microglobulin, neuroglian, tenascin). However, structural studies have also revealed the presence of Ig-like domains in proteins which bear no sequence homology with the Ig superfamily. Examples include proteins as diverse as chaperones (the protein PapD required for the assembly of pili in bacteria), enzymes (a bacterial cellulase and a fungal galactose oxidase), and viral proteins (the major envelope protein from a flavivirus), for which the presence of one or more Ig-type domains was unexpected prior to structure determination.

The structure of the Ig-type domain present similar

features in all these proteins, independently of any genetic or functional relationships. The Ig-type domains consist of a β barrel composed of seven to nine antiparallel β strands with the typical Greek key topology, and display a structurally conserved core that includes the central strands from the two β sheets. Also, the hydrophobic character of the internal space between the β sheets (mostly composed of aliphatic and aromatic side-chain residues) is maintained. In contrast, the length and regularity of the β strands as well as the length and structure of the connecting loops are extremely variable (Figure 1). Indeed, the Ig-domain topology can be achieved by quite different sequences and, as a result, no single interaction or amino acid position can be uniquely identified as the principal determinant of the structure. For example, the intramolecular disulfide bridge linking the two β sheets in antibody domains, early recognized as a hallmark of the Ig fold, is not necessary to determine the Ig-type domain structure. Even within the Ig superfamily, these cysteine residues can be found at different positions in the β sheets or may be completely absent.

As initially observed in antibodies, the Ig-type domains in other proteins often associate in pairs to form homo- or heterodimers. Noncovalent association of Ig-type domains usually occurs through the exposed faces of the β sheets, giving rise to compact globular dimers as shown in Figure 2 for the surface antigen CD8 and the Fab fragment from an Ig molecule. On the other hand, head-to-tail interactions predominate between consecutive Ig-type domains

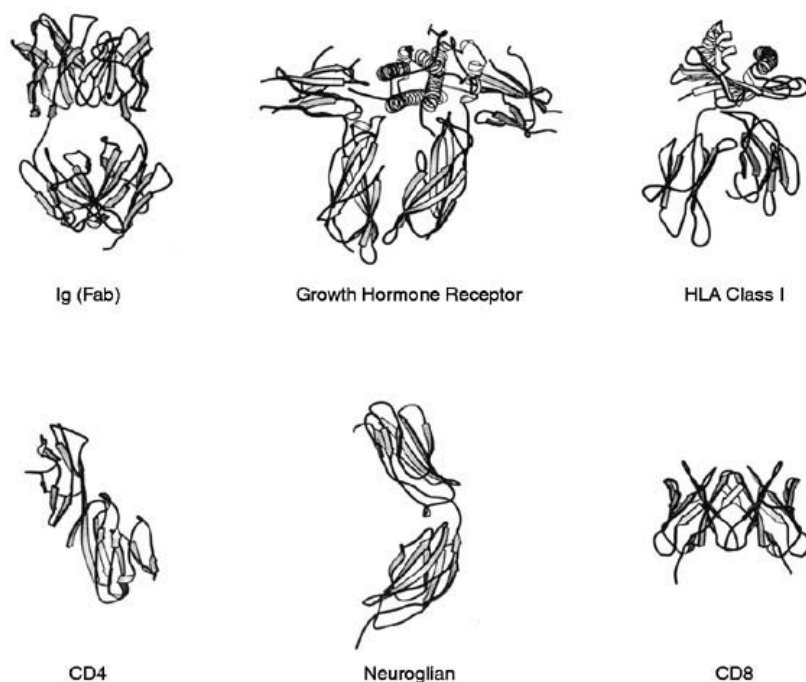


Figure 2 Different types of subunit association between Ig-type domains. The human growth hormone receptor is shown with its bound ligand. Only the two N-terminal domains of CD4 are shown. Neuroglian is a modular protein involved in neural cell-cell adhesion. The extracellular portion of neuroglian consists of 11 Ig-like domains, two of which are shown here.

along a single polypeptide chain (e.g. the CD4 co-receptor of T lymphocytes and neuroglian, a protein involved in cell–cell adhesion, shown in Figure 2). In these membrane-bound extracellular proteins containing several Ig-type repeats, head-to-tail interactions give rise to elongated quaternary structures that may serve, for example, as structural ‘spacers’ allowing the molecule to interact with a distant target such as another protein on a neighboring cell.

Ig-type domains occur in functionally diverse proteins that interact with a wide range of ligands, from metal ions to very large proteins. The available crystal structures reveal that every region of the domain surface can be used for interaction with other molecules. For example, the loop regions determine the ligand (antigen)-binding site in antibody V domains, whereas many other Ig-type domains interact via part of their β sheets. This functional differentiation of Ig-type domains is even possible within a single protein. In fibronectin, for example, the interaction with ligand may be mediated by a single loop (the RGD motif) or by other parts of the molecular surface, and possible ligands include carbohydrates, other Ig-like domains, or even a whole network of molecules. Quite often, the ligand-binding site is defined by a cleft between two consecutive Ig-type domains, as in neuroglian (Figure 2) where domain dimerization creates a metal ion-binding site. The chaperone PapD and the extracellular regions of the receptors for growth hormone (Figure 2) and interferon γ also use two consecutive Ig-type domains to bind their respective protein ligands.

Whether the result of the evolutionary divergence from a common ancestor gene or the convergence towards a particularly stable structure, the Ig-type domain represents a rich source of natural variation on a single structural theme, and additional studies

will undoubtedly reveal further unexpected features about its ubiquitous nature and functional diversity.

See also: Antigen-binding site; Bence Jones proteins; β_2 -microglobulin; CD2; CD4; CD8; Cytokine receptors; HLA class I; HLA class II; Immunoglobulin gene superfamily; Immunoglobulin structure; Antibody-antigen complexes, three-dimensional structures.

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DRUGS, ALLERGY TO

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Pharmacologically active agents administered to humans for diagnostic or therapeutic purposes can cause a wide range of immunologically mediated diseases. Drugs can act as immunogens that induce immune responses and then elicit immunopathologic reactions. In addition, a variety of agents used in human medicine can activate immune effector systems through pharmacologic rather than immunologic mechanisms. Opiates can cause mast cell

mediator release by activating cell surface opiate receptors. Aggregated immunoglobulin G (IgG) in heteroantisera can activate complement and thereby produce anaphylaxis. Some drugs can induce autoimmune syndromes in which the specific immune responses are directed toward self rather than drug-related determinants.

Allergic (immunologically mediated) reactions to drugs cause increased morbidity, and rarely fatal out-

comes, in individuals already sufficiently ill to warrant pharmacologic intervention. About 1–5% of hospitalized patients experience an allergic reaction to a medication. Approximately 10% of adults have experienced an allergic reaction to a drug. Identification of the offending agent, reversal of the immunopathologic processes, and introduction of effective alternative therapy can be very difficult challenges. The human and financial costs of these reactions are considerable. In fact, the morbidity and mortality induced by allergic reactions to drugs unambiguously make this one of the most important categories of immunologically mediated disease.

Inherent in drug therapy of humans is the administration of potential immunogens in standard doses and intervals in the context of somewhat similar clinical disorders. As relevant drug epitopes are defined in biochemical terms, primary and secondary immune responses can be studied in the context of current understanding of immunogenetics, immunoregulation and immunopathology. Studies of specific immunoglobulin and lymphocyte responses to drugs in relation to clinical drug allergic reactions have provided fundamental insights into IgE-mediated anaphylaxis, immune cytopenias and solid tissue inflammation. Drug therapy of humans permits both the systematic testing of hypotheses generated from basic immunologic knowledge and the generation of diverse new hypotheses to be assessed in model systems.

Drugs as antigens

The administration of insulin, heteroantisera and other macromolecules to humans frequently causes immune responses. For example, many diabetics treated with insulin, even recombinant human insulin, produce IgG to insulin. In most instances, the immune response to the complete antigens used in human medicine does not lead to clinically detectable immunopathologic reactions or to significant neutralization of function. Low molecular weight drugs, in contrast, usually are not capable of causing immune responses or eliciting immunopathologic reactions in their native states. Covalent linkage of a drug, or a reactive metabolite of a drug, to a macromolecule creates an epitope that can cause immunologic reactions. Landsteiner designated the covalent binding process 'haptentation' and the drug-derived epitope a 'hapten'.

Two principal forms of haptentation have been documented (Figures 1 and 2). β -Lactam antibiotics such as penicillin and a small number of other drugs used in clinical medicine are intrinsically reactive with proteins and, therefore, are capable of direct

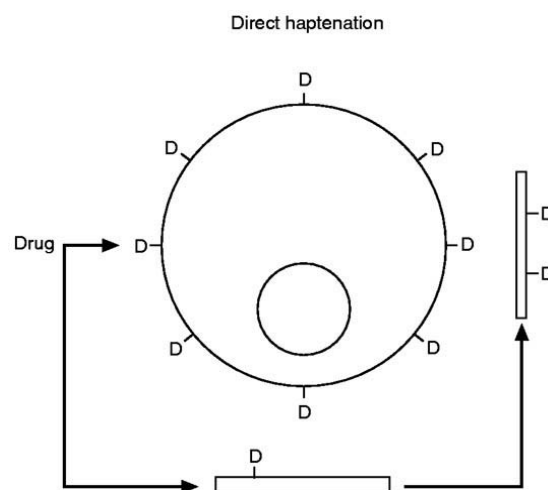


Figure 1 Haptentation of cells and extracellular molecules by intrinsically reactive compounds. β -Lactam antibiotics and other reactive agents rapidly form covalent bonds with cell surfaces, extracellular matrix, soluble proteins, amino acids and other molecules. A dynamic array of sparsely to densely substituted solid phases and a fluid phase with univalent and multivalent substituted carriers is formed.

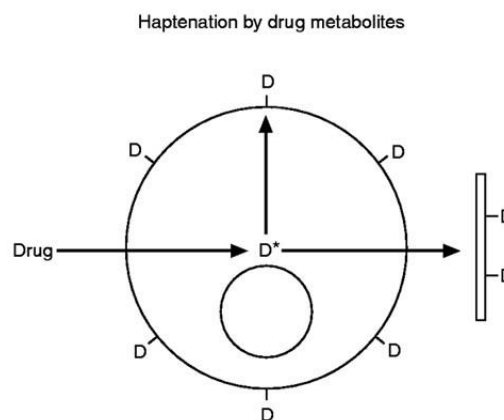


Figure 2 Haptentation of cells and extracellular molecules by molecules with little or no intrinsic reactivity. Metabolism of drugs (occurring primarily within hepatocytes, keratinocytes and other cells) can create reactive intermediates that can form covalent linkages to carrier molecules. Haptentation of molecules being synthesized in the cell at the time of drug metabolism can result in long-lasting surface expression of haptentated molecules and secretion of univalent and multivalent carriers. Reactive metabolites also may escape to the extracellular space to haptentate any suitable carrier.

haptentation (Figure 1). Cell surfaces, extracellular structural proteins and diverse soluble molecules become haptentated. Approximately 10% of the penicillin molecules administered become covalently bound to human cells, proteins and other body constituents. Individual cells can accumulate thousands of surface haptent determinants within minutes of

exposure to therapeutic concentrations of β -lactam antibiotics.

Most low molecular weight substances used in clinical medicine are not reactive with macromolecules. As depicted in **Figure 2**, however, metabolites of some medications can haptenate. Intracellular haptenation of proteins being synthesized at the time of drug exposure can lead to haptenation of molecules eventually expressed on the cell surface, or to release of haptenated cellular secretion products. Extracellular metabolism or release of reactive intermediates into the extracellular space also can lead to cell surface haptenation and haptenation of fixed and soluble extracellular molecules.

Thus, the generation of immunogenic haptens and of targets for immunopathologic reactions occurs by diverse pathways. A dynamic array of haptenated cell surfaces, haptenated fixed proteins such as collagen, soluble molecules bearing single hapten substituents (univalent carriers), and soluble molecules bearing multiple hapten substituents (multivalent carriers) appears during drug therapy.

Factors influencing reactions to drugs

Clearly the structures, metabolism and chemical properties of specific drugs and their metabolites have important influences on human immune responses and the expression of clinical allergic reactions. The dose, route, duration and frequency of therapy also can influence the expression of allergic reactions to drugs. In addition, active disease processes, such as infections, and a variety of individual patient factors influence responses.

The presentation of drug antigens during an infection can be influenced by products released from the infectious agent. Bacterial endotoxins markedly alter the local and systemic immunoregulatory environment. Lysis of bacteria just after the administration of microbicidal drugs abruptly increases the amount of free endotoxins and causes striking increases in the levels of tumor necrosis factor (TNF), interleukin 1 (IL-1) and other cytokines. Certain microbes release superantigens, supercarriers (proteins that when haptenated elicit very strong antihapten responses), and immunomodulatory proteins that can affect immune responses to drugs.

Several human factors also can influence the expression of drug allergy. While the full importance of HLA class I and class II genes in drug allergy is not clear, both class I and II associations have been detected for some allergic reactions to some drugs. Genetically determined differences in drug metabolism are known to influence susceptibility to reactions to some drugs, including isoniazid and hydrala-

zine. A familial propensity to allergic reactions to structurally diverse antimicrobial drugs has been reported.

Some individuals have a propensity to react to haptenating drugs. If a patient has had an allergic reaction to one antimicrobial drug, there is an approximately ninefold increased risk of reacting to another class of antimicrobial drug. Approximately 10% of adults have reacted to one class of antimicrobial drug and are in this high-risk category. The classes of agents causing these reactions are structurally diverse and the spectrum of immunopathologic reactions expressed is broad. The principal abnormality in these patients appears to be a consistent expression of an immunoregulatory environment during infections that favors immune responses to haptens presented during an infection. Overall, approximately one-half of allergic reactions to antimicrobial drugs occur in individuals who have reacted to different classes of antimicrobial drugs in the past.

Clinical reactions to drugs

Immune responses to drugs are necessary but not sufficient for clinical allergic reactions to drugs. If the fluid phase has an excess of univalent haptenated carriers over multivalent carriers, many immunopathologic reactions will not occur. Cross-linking of IgE and activation of mast cells will not occur. Immune complexes cannot form in univalent hapten excess. Direct cytotoxic reactions are inhibited by fluid phase determinants. The affinity and isotype arrays of the immunoglobulins expressed also influence the nature of a reaction.

Counter-regulatory responses and nonimmunologic factors exert important influences on clinical expression of immunopathologic reactions. Individuals vary considerably in mast cell releasability, cellular resistance to cytotoxic attack, handling of immune complexes and susceptibility to other forms of immune injury. Superimposed on these immunologic considerations are the toxic and pharmacologic effects of some drugs that can mimic or add to specific immune reactions.

Stratification of clinical allergic reactions to drugs according to the immunopathologic scheme of Gell and Coombs can be considered. Type I IgE-mediated immediate hypersensitivity reactions do occur in the forms of anaphylaxis, urticaria and angioedema, but some drugs can induce these reactions via complement activation or direct pharmacologic effects. Type II cytotoxic reactions occur, for example immune cytopenias, but most cell-destructive or tissue-inflammatory reactions to drugs are difficult to

distinguish from type III and in some cases type IV reactions. Adding to the complexity of using a pathophysiologic stratification scheme is the frequent concurrence of multiple immunopathologic mechanisms. Sulfonamides, cefaclor and other commonly used drugs can induce reactions that include urticaria, fever, joint inflammation and variable vascular and solid tissue disorders that defy this form of stratification.

While a pathophysiologic stratification is routinely attempted, clinical stratification is more widely used to guide diagnosis, therapy and prognosis (Table 1). This classification scheme permits discussion, investigation, diagnosis and rational management of reactions of probable but unknown immune mechanisms such as drug fever and the Stevens–Johnson syndrome.

As summarized in Table 1, the clinical manifestations of drug allergy are quite diverse. The known variations in sites and intensity of haptentation, in patterns of immune responses and in tissue responses to these forces make these diverse clinical phenomena expected.

Diagnosis of drug allergy

Allergic reactions to drugs must be distinguished from similar clinical phenomena of different causes. Two forms of immunologic diagnosis then must be made: identification of the specific cause of the reaction, and identification of the immunopathologic

mechanisms mediating the reactions. All possible causal agents are identified and then stratified according to known propensities to cause the specific pattern of clinical disease; the temporal relationships of drug exposures to the onset of the reaction; the effects of drug discontinuation; and the effects of rechallenge (if this has occurred).

A rapidly expanding array of immunodiagnostic tests has been developed for the detection of specific responses to antimicrobial drugs and selected other classes of drugs. These include tests for IgE (by immediate wheal and flare skin tests, radioallergo-sorbent-RAST tests and leukocyte histamine release tests), IgG, IgM and IgA (primarily by ELISA), and specific lymphocytes (lymphocyte transformation tests). The presence of IgE to a drug that was given shortly before the onset of anaphylaxis, urticaria or angioedema provides strong evidence of a causal role. The presence of IgG, IgM, IgA or specific lymphocyte responses are much less closely related to clinical allergic disease – these responses are detectable in many individuals who do not have overt immunopathologic reactions to the drug. Nevertheless, evidence of an immune response to a drug given in appropriate temporal relationship to a reaction supports a possible causal role. The combination of clinical and immunologic assessments usually can identify the cause of a reaction, permitting continued use of other classes of needed medications.

Recent basic research has permitted clinical assessment of some immune effector systems, greatly facilitating pathophysiologic diagnosis and monitoring. Assays for serum mast cell tryptase permit biochemical diagnosis of anaphylaxis, a major improvement over assays for histamine and prostaglandin D₂ or their metabolites which arise from several cellular sources in addition to mast cells. Assays for complement anaphylatoxins C3a, C4a and C5a as well as other complement activation markers permit assessment of classical and alternative pathway complement activation during allergic drug relations. Emerging biochemical, histochemical, and molecular biological assays for mediators, cytokines and immunologically relevant cell activation should allow progressively more precise identification of active effector systems and the impact of pharmacologic or immunologic interventions.

Management of drug allergy

Acute allergic reactions to drugs usually are treated by withdrawal of the offending drug and conventional pharmacologic interventions appropriate for the specific reaction pattern (Table 1). In some instances, when no effective alternative therapy is

Table 1 Clinical classification of allergic reactions to drugs

<i>Systemic reactions</i>
Anaphylaxis
Drug fever
Serum sickness-like reactions
Vasculitis syndromes
Drug-induced lupus erythematosus and other autoimmune syndromes
Pseudolymphoma reactions
Pseudoallergic reactions
<i>Tissue inflammation or destruction</i>
Cutaneous reactions
Urticaria and angioedema
Toxic epidermal neurolysis/Stevens–Johnson/erythema multiforme
Fixed drug eruption
Pleomorphic eruptions
Contact hypersensitivity
Cytopenias
Pneumonitis
Myocarditis
Hepatitis
Nephritis

available and when the reaction is restricted to a pruritic rash, the drug can be cautiously continued with pharmacologic suppression of the reaction.

Several procedures have been developed to avoid or minimize reactions in high-risk populations. Among antibiotic allergic patients, vigorous efforts are made to correct factors predisposing to infections; to document infection before therapy; to avoid classes of drugs associated with prior reactions; to screen with immunodiagnostic tests; and to administer the first dose in a course of therapy under medical supervision. When possible, initial doses are given orally.

Premedication with H_1 antihistamines and systemic glucocorticoids, coupled with the use of nearly isosmolar radiocontrast media (RCM), minimizes RCM reactions in high-risk patients. Similar pharmacologic premedication is used to suppress other pseudoallergic reactions such as reactions to aggregated immunoglobulin.

Patients with IgE sensitivity to drugs that are required for therapy (e.g. insulin, penicillin in some cases) can be desensitized over a period of hours. Initial doses, usually selected on the basis of skin test reactivity, typically are 1/10 000 of a conventional dose. Increasing doses are administered at 15–20 min intervals until a therapeutic dose is reached. Conventional therapy can then be used, with no lapses in therapy.

As assessed by wheal and flare skin test responses, antigen-specific mast cell desensitization occurs during this procedure. Mast cell reactivity and clinical sensitivity return within 1–3 days after discontinuation of the drug in most patients. The most widely held hypothesis is that the gradual introduction of antigen favors univalent haptentation, resulting in hapten inhibition and gradual cross-linking of mast cell surface IgE, resulting in avoidance of clinically significant mast cell mediator release.

See also: Anaphylatoxins; Anemia, drug induced

immune hemolytic; Mast cells; Cytokines; Endotoxin (lipopolysaccharide (LPS)); Hapten; Hypersensitivity reactions.

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ECHINOCOCCUS, INFECTION AND IMMUNITY

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The tapeworm parasites of the genus *Echinococcus* (Class Cestoda) are divided into four species. Two are restricted to central and south America (*E. oligarthus* and *E. vogeli*) and very little is known about their immunobiology. The other two species (*E. granulosus* and *E. multilocularis*) are important zoonoses and have been studied extensively. *E. granulosus* occurs worldwide and its distribution has been greatly assisted at various times in history through the translocation of domestic animals during periods of human colonization. *E. multilocularis* is found mainly in holarctic regions, but it also occurs further south in some regions of Europe and North America. The cycle of transmission of *Echinococcus* involves the interaction between carnivores (definitive hosts) and herbivores/omnivores (intermediate hosts), with each host type containing one of two morphologically distinct parasite stages.

Definitive hosts are usually domestic and wild canids, but in some parts of the world these may be domestic or wild felids. Definitive hosts are infected with the tapeworm stage of *Echinococcus*. These tapeworms reside in the small intestine, are small and may be present in huge numbers. Infections of over 100 000 worms are not uncommon in Australian wild dogs (dingoes and dingo/domestic dog hybrids).

Ungulates (sheep, cattle, pigs, deer, camels) are the most common intermediate host but rodents, macropod marsupials or humans may also become infected. Intermediate hosts are infected through accidental ingestion of eggs passed into the environment with the feces of infected definitive hosts. Following ingestion and hatching of the eggs, oncospheres are released into the intestine. The oncospheres move through the intestinal mucosa and enter the hepatic portal vessels, eventually locating in internal organs where they develop into hydatid cysts, usually in the liver and/or lungs. Cysts enlarge over the life of the host and protoscoleces, the next generation of tapeworms, develop from a germinal membrane lining the cysts. Hydatid cysts of *E. gran-*

ulosus are fluid-filled unilocular structures which in humans, if left untreated, can grow to a volume of several liters and become life threatening. Lesions of *E. multilocularis*, in contrast to the large unilocular hydatid cysts of *E. granulosus*, consist of masses of small interconnected cysts (multilocular or alveolar hydatid cysts) that spread throughout the affected organ. In humans multilocular hydatidosis is often inoperable and invariably fatal if untreated. Transmission of *Echinococcus* is completed when protoscoleces are ingested by carnivores when feeding on hydatid-infected organs.

Dogs and sheep are pivotal in the transmission of *E. granulosus* in domestic situations in many parts of the world. Determining the prevalence of infection in dogs is an important means of monitoring the progress of control programs. Detection of infection in dogs has traditionally relied on a highly unreliable method, namely purging with arecoline hydrobromide and examining the purged sample for worms.

Immunology

Recent major advances have been in the use of immunological methods to diagnose infection in definitive hosts and in the development of a recombinant vaccine, highly effective in protecting sheep from hydatid infection.

Antigens

Ovine hydatid cyst fluid from fertile cysts (containing protoscoleces), and nonfertile cysts with intact germinal membranes, is the most suitable source of antigen. The highest concentration of antigen occurs in cyst fluid from fertile cysts. The major parasite antigens contained in the cyst fluid are heat-labile and heat-stable lipoproteins referred to as antigen 5 and antigen B, respectively. Antibodies against these antigens are important for immunodiagnosis of hydatid infection in humans. Antigens of *E. multilocularis* have been obtained from somatic extracts of proto-

scolec. An immunodiagnostic antigen M_r 54 kDa, obtained by immunodepletion techniques and designated EM2, has been used effectively in serodiagnosis of human alveolar echinococcosis.

Two protein antigens of M_r 27 kDa and M_r 94 kDa, derived from excretory/secretory and somatic products of protoscolec, are recognized specifically by sera from *E. granulosus*-infected dogs. Oncosphere antigens of M_r 22, 30 and 37 kDa appear to be specific to *E. granulosus* and stage-specific to oncospheres.

Expressing antigens using recombinant DNA methods is a powerful tool for antigen characterization and producing antigen in sufficient amounts for practical applications such as immunodiagnosis and vaccination. The technology is currently limited to protein antigens. A range of potentially useful recombinant clones have been made by various workers using mRNA isolated from activated protoscolec of both *E. granulosus* and *E. multilocularis* and oncospheres of *E. granulosus*.

Cellular responses to infection

There are few data regarding cellular responses against *E. granulosus* infection in dogs. *In vitro* studies indicate infection depresses the blastogenesis of some lymphocyte populations, whilst stimulating others. Most infected dogs exhibit immediate-type skin reactions to hydatid cyst fluid, but there is no delayed response.

T cells and activated macrophages are thought to be key elements in the cellular immune response against *Echinococcus* in intermediate hosts. In sheep there is a marked neutrophilia 3–5 days after infection, followed by increased eosinophil, lymphocyte and monocytes levels 25–30 days post infection. Eosinophils, lymphocytes and monocytes accumulate in large numbers in the peritoneal cavity in mice infected with *E. multilocularis* and peritoneal cells from rats with heavy infections of *E. multilocularis* kill protoscolec.

Humoral responses to infection

Dogs infected with *E. granulosus* are the definitive host studied in most detail. They produce specific circulating antibodies (IgA, IgG, IgM) against *E. granulosus* and IgG antibodies are readily detectable in sera within 2 weeks of infection. Dogs infected with closely related *Taenia* species of tapeworm produce stage-specific serum antibodies (IgG) against excreted/secreted substances of scolec and oncospheres. These antibodies become undetectable within a few weeks after the worms are lost. Antibody (IgA) against *E. granulosus* is produced by the intestinal mucosa, and elevated levels of IgE, associa-

ted with *E. granulosus* infections, are present in some dogs.

Antibodies (IgA, IgE, IgG, IgM) are produced in intermediate hosts infected with *E. granulosus*. Most studies have been carried out in humans, sheep and rodents infected with *E. granulosus* or *E. multilocularis*. In sheep the serum antibody responses against *E. granulosus* infection are weak or undetectable, whilst in humans they are easily detected and important for diagnosis.

Immunodiagnosis

Definitive hosts

Two methods (both enzyme-linked immunosorbent assays, ELISAs) have been developed for the detection of *E. granulosus* tapeworms in dogs. One detects specific circulating antibody in serum and the other parasite products (coproantigens) in feces. The sensitivity of the antibody test with sera from dogs from *E. granulosus*-endemic areas is poor, with as many as 60% of infected dogs testing negative for specific IgG antibody. Sensitivity can be improved if sera are also tested for IgE and IgM antibodies. The problems with the sensitivity of the current system make the test of little practical value for diagnosing infection in individual dogs, but the test may have some value in screening populations of dogs for recent past or current infection with *E. granulosus*.

Coproantigens of *Echinococcus* can be readily detected in the feces of infected dogs and differentiated from those of the closely related *Taenia* species of tapeworms commonly found infecting dogs (often in association with *Echinococcus*). A slightly modified version of the coproantigen test detects infection with *E. multilocularis* in foxes. The coproantigen ELISA is a very useful test because it requires no sophisticated equipment, is uncomplicated to perform and large numbers of specimens can be processed quickly. The test can be used under field conditions and shows great promise as a practical and safer means of monitoring the prevalence of dogs infected with *E. granulosus* in hydatid control programs.

The polymerase chain reaction has been used successfully to detect DNA from *E. multilocularis* eggs in feces of foxes. The method has a high sensitivity and 100% specificity, with no cross-reactions with *E. granulosus*. However, a number of foxes designated negative at necropsy were DNA positive. These apparently negative foxes may have been infected with a few worms that were overlooked.

Intermediate hosts

Detection of hydatid infection in intermediate hosts can be unreliable. Most studies have been conducted using sheep and the lack of success of immunodetection of infection in this species has been attributed to the presence of cross-reacting antibodies against related taeniid cestodes commonly infecting sheep and the relatively low titer of specific antibody produced by sheep infected with *E. granulosus*.

A variety of immunological methods have been used in the detection of hydatid infection in humans. These methods provide a useful means for primary diagnosis and monitoring patients, post treatment, for persisting or recurrent infection. Immunodiagnosis relies mainly on the detection of circulating antibodies (IgG) or parasite antigens in serum. The techniques used most commonly include ELISAs, the indirect immunofluorescent antibody test (IFAT) and immunoprecipitation tests such as immunoelectrophoresis (IEP) and immunoblotting.

As with animal intermediate hosts, problems with diagnosis of cystic hydatidosis in humans may result from the presence of cross-reacting parasite antibodies in patients infected with heterologous helminth species (e.g. *Taenia solium*). There is also evidence of false-positive antibody reactions, unrelated to parasite infections, associated with malignancies, the presence of anti-P1 blood group antibodies and liver cirrhosis.

Evasive strategies by the organism

Mechanisms by which adult *E. granulosus* evade the immune response of the definitive host are incompletely understood. The immune system of the dog recognizes the presence of the parasites and produces specific circulating antibody (IgA, IgG, IgM) against them. In addition, mucosal IgA is produced in response to the secretions of the scoleces which are in intimate contact with the mucosa. This IgA has little apparent effect on the worms, as *E. granulosus* remain in the definitive host for several years with new infections being superimposed on existing infections. It would appear that the worms have the ability to suppress the cytotoxic and effector cell activity in the region of the scolex.

The strategies by which *E. granulosus* evades the immune system of the intermediate host are complex and also incompletely understood. The adaptability of the parasite to infect many different host species exemplifies its extraordinary adaptability. *E. granulosus* is thought to manipulate the immune system of the host with various factors such as cytotoxic substances produced by cysts, mitogenic substances produced by protoscoleces, immunosuppressive and

immunostimulatory cytokines and substances capable of stimulating lymphokine production or inactivating complement.

Concomitant immunity is important in ensuring the survival of *E. granulosus* in intermediate hosts. Concomitant immunity is the ability of established hydatid cysts to avoid the immune system of the intermediate host, whilst at the same time stimulating an effective immune response against subsequent infecting organisms. The process of concomitant immunity with *E. granulosus* is thought to be antibody mediated. The mechanism is directed against oncospheres and the process of killing is thought to be antibody-dependent, complement-mediated lysis of the parasite, with killing being enhanced by the presence of neutrophils.

Vaccination

Definitive hosts

Currently, it is not possible to induce complete host-protective immunity against infection with taeniid cestodes in dogs. Reports on induced immunity against *E. granulosus* in dogs are contradictory. Vaccination studies using somatic or *in vitro* products of protoscoleces, hydatid cyst fluid or somatic preparations of whole worms induce partial protection against infection in some dogs.

Intermediate hosts

Oncospheres of *E. granulosus* have been shown to be a potent source of host-protective antigens in sheep. Using recombinant DNA methods, antigen has been expressed in *Escherichia coli* and used to vaccinate sheep, inducing high levels of protection (>96%) against experimental infection with *E. granulosus*. Use of a successful recombinant vaccine capable of protecting sheep against infection will be a useful new strategy for the control of hydatid disease in domestic livestock and potentially also in humans. However, before a recombinant vaccine can be used effectively in programs to control *E. granulosus*, more research is required in areas such as determining the duration of immunity in sheep and other intermediate hosts, protection levels in young animals and effectiveness against naturally acquired infections. Using the same technology, it should also be possible to produce a similar recombinant vaccine against alveolar hydatidosis.

See also: Antibodies, detection of; Antigens; Antiserum; Canine immune system; Cell-mediated immunity; Cysticercosis; Enzyme labeling of antibodies and antigens; Enzyme-linked immunosorbent

assay (ELISA); Humoral immunity; IgA; IgD; IgE; IgG; IgM; Immunoassays; Immunodiffusion, single radial; Immunoelectrophoresis; Immunosuppression; Macrophage activation; Parasites, immunity to; Vaccines.

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ECZEMA

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Eczema as such does not exist. It is a vague description of a variety of dermatoses with widely differing etiology, pathology and clinical manifestations. 'Dermatitis' is often used as a synonym, meaning nothing more than inflammation of the skin. Eczema as a description of skin disease is therefore always used as part of a more precise, often binominal, diagnosis. Table 1 gives a representative overview, where the 'eczemas' are etiologically classified as being (mainly) of external, combined or internal origin.

The eczemas do form a group of diseases from a dermatological *morphologic* point of view. It is their polymorphism that brings them together. Morphologically, the eczemas are characterized by ill-defined

borders, redness or erythema, vesiculation in acute stages, lichenification in chronic stages, with various forms of papulation and plaque formation, scaling, and sometimes nodule formation. Secondary infection is a common complication. The eczemas are also histopathologically similar, with an epidermodermatitis (inflammation at the epidermal–dermal region), a mononuclear (lymphohistiocytic) infiltrate and accumulation of lymphocytes that infiltrate the epidermis. Quite characteristic is the epidermal intercellular edema (spongiosis), in acute stages leading to vesicle formation.

Eczema therefore is also seen as a particular and quite common reaction type of the skin, with acute and chronic inflammation, depending on the clinical stage, and with an influx of lymphocytes into the epidermal tissue. External factors, such as ultraviolet irradiation, toxic compounds, such as in irritant contact dermatitis and eczema cracquelatum, or even superficial infestations by bacteria (microbial eczema) or yeasts (seborrheic dermatitis), are now believed to have in common a particular response pattern of the epidermis. The skin epithelium is believed to serve as a proinflammatory organ, where widely differing stimuli lead to the induction of similar keratinocyte cytokine secretion patterns, primarily of proinflammatory cytokines such as interleukin 1 β (IL-1 β), IL-6, tumor necrosis factor α (TNF α) and IL-8. Some of these cytokines upregulate adhesion molecules on dermal endothelial cells, promoting influx of leukocytes. Also, others serve as chemotactic agents, attracting these cells to infiltrate the epidermis. In such a nonspecific way, a chronic inflammatory reaction pattern is the result of widely

Table 1 Classification of eczema/dermatitis

<i>Due to external influences</i>
Eczema solare
Irritant contact dermatitis
Eczema craquelé
Microbial eczema
Seborrheic dermatitis
<i>Due to combination of external and internal influences</i>
Allergic contact dermatitis
Photoallergic contact eczema
Atopic dermatitis
Nummular eczema
<i>Due to internal, systemic or constitutional influences</i>
Dyshidrotic eczema
Tyloitic eczema
Eczematous drug eruption

varying provocative factors to which the skin is externally exposed.

From a specific immunologic point of view, the most interesting and intensively studied types of eczema are allergic contact dermatitis and atopic dermatitis, which will be described in more detail.

Allergic contact dermatitis

An important characteristic of skin is that it is continuously exposed to environmental influences, such as ultraviolet irradiation. Numerous microorganisms and their degradation products, organic and inorganic chemicals, and natural and artificial compounds are continuously challenging the skin's epidermal barrier. This barrier is formed by the horny layer, which is composed of corneocytes (keratinocytes that entered a programmed cell death pathway leading to cellular structures filled with keratin proteins), interspersed with a bilaminar layer of lipids and water. This lipid bilayer is the major barrier for molecular compounds, and it is generally accepted that molecules with a molecular weight over 500 Da are unable to penetrate this barrier.

Small molecules, whatever their origin, do penetrate the barrier and the skin immune system is thus continuously exposed to a substantial amount of foreign compounds. In view of the enormous amount of different chemicals and haptens and the fact that visible or abnormal skin reactions to them are rather rare, it may safely be assumed that induction of 'tolerance' is the rule and immunologic responses the exception. The explanation of this phenomenon remains one of the most challenging fundamental questions of immunology.

Allergic contact dermatitis is one of the best studied forms of delayed-type hypersensitivity and it is the result of sensitization to these small molecular compounds, also described as haptens. Molecules with a molecular weight under 500 Da are assumed to be bound by intraepidermal Langerhans cells, the major cutaneous representative of the family of dendritic antigen-presenting cells (APCs). These haptens are transported by Langerhans cells to the skin draining lymph nodes, where, in the paracortical T cell areas, induction of immunity to a given hapten may occur. It is thought that expansion of hapten-specific T cells in the lymph nodes is followed by dissemination of these T cells in peripheral blood and subsequently, perhaps through skin-specific homing, into the skin. Rechallenge of the skin then leads to elicitation of an eczema-type response, with presentation of haptens to specific T cells in the skin compartment. Well-known examples of contact allergens

are nickel, chromium, cobalt and components of cosmetics such as preservatives and fragrances.

An essential question is why certain molecules, once an individual is regularly exposed to them, give high frequencies of subsequent contact allergy, while other compounds do not have such an effect. Dinitrochlorobenzene is an example of a chemical that is able to sensitize virtually every individual exposed to it. Such a compound has an almost 100% sensitization rate and is of course abandoned from the environment. The prevalence of nickel contact allergy is estimated to be 10% in European women. Finally, there are thousands of molecules to which sensitization has occurred, but so rare that they are only published as case reports.

The sensitizing potential of different compounds thus varies widely, which implies that the induction of clonal expansion of hapten-specific T cells is rather uncommon with most chemicals and standard with just a few molecules. The question is whether it is at the level of T cells where the distinction is made, or at the level of antigen presentation. Recent research has provided arguments for the assumption that it may be the APCs' biological behavior that is decisive.

When human skin is placed in organotypic culture (*ex vivo*, *in vitro*), these specimens can be kept in culture for several weeks. In this model, it is possible to expose the corneal surface of the skin samples to different chemicals, and a major advantage is that compounds that are only soluble in diluents that are extremely toxic in cellular models can safely be applied on the corneal layer's outside surface. Using this model, it was found that known contact allergens induce the migration of Langerhans cells into the basal layers of the epidermis, and subsequently in the dermal layer. Control compounds, such as diluents, toxic compounds (e.g. sodium laurylsulfate) and known 'tolerogens' did not result in this enhanced migration pattern of Langerhans cells.

Atopic dermatitis

Atopic eczema, also known as atopic dermatitis, is a very common entity. One out of every 20 children will have an episode of atopic dermatitis during one or more periods of their lives. The disease is part of a syndrome known as atopy. It is genetically determined but the precise gene(s) responsible have not yet been identified. Other diseases that form part of atopy are extrinsic allergic asthma, rhinitis allergica, conjunctivitis allergica and certain forms of allergic food reactions. Individual patients may have any combination of these clinical entities. In many cases, atopic dermatitis takes a chronic form, with

extremely itchy eczema lesions preferentially localized in the face, the neck, elbow folds and behind the knees. Stress may exacerbate the dermatitis, as may external irritants.

The complicated and multifactorial etiopathogenesis of atopic dermatitis has resulted in different models, including entirely psychosomatic and biochemical frameworks. The dermatological entity may, however, also be described almost entirely in immunologic terminology, including genetic, biochemical, external and psychosomatic influences. The primary and dominant abnormality in atopy is the preferential synthesis of immunoglobulin E (IgE) antibodies specific for a limited set of environmental antigens. Taking that as the central stage on which atopic dermatitis develops, all other factors implied may be taken into consideration.

Genes related to atopy thus far are those located on chromosomes 11q13 and 5q31.1–3. The abnormal gene at 11q13 might be coding for the β chain of the high-affinity receptor for IgE (Fc ϵ RI). However, linkage with 11q13 could not be confirmed when, instead of asthmatics, atopic dermatitis patients were taken as probands. On 5q31.1–3, a complex of genes related to IgE production in general is localized, and its association is more with total (increased) IgE levels than with atopy. Whatever the final outcome of atopy genetics, a relationship between the genes implied and immune function seems evident.

Biochemical abnormalities include disturbances in free fatty acid metabolism (8-6 desaturase activity is decreased) and increased phosphodiesterase enzyme activity, particularly in mononuclear leukocytes. Abnormal linoleic acid content of epidermis may explain disturbances in epidermal barrier function, leading to increased irritability and subsequent pro-inflammatory activity of epidermis, as explained above. Abnormal phosphodiesterase activity leads to increased cAMP levels, and may affect T cell regulation of IgE production. Biochemical abnormalities in atopy may thus be linked to abnormal immunologic and inflammatory responses.

The central immune abnormality is production of varying amounts of IgE to a limited set of environmental antigens. It is now known that IgE production by B cells is under control of T cells, of which the T_H2 subtype stimulates B cell class switching to IgE and the T_H1 subtype inhibits such an isotype switch. This T cell-directed IgE production is realized through soluble cytokines as well as membrane interaction (CD40/CD40 ligand) signals. T_H2

cells produce large amounts of IL-4, IL-5, IL-10 and IL-13 while T_H1 cells produce large amounts of IL-2, lymphotoxin and interferon γ (IFN γ). The first human T_H2 cells were found in atopy, and it is now clear that, in atopy, patients respond to the subset of environmental antigens by expansion of specific T_H2 cells, which subsequently help B cells switching to production of allergen-specific IgE.

With all this in mind, a model of the events in atopic dermatitis-involved skin can be described. External irritants lead to upregulation of adhesion molecules through cytokine production of keratinocytes. Nonspecific influx of T cells, among them allergen-specific T_H1 and T_H2 cells, is the result. Allergen-specific IgE, present on APCs, facilitates processing of minute amounts of allergens that have reached the skin, with subsequent expansion of immigrant T cells specific for them. Interaction of released cytokines with different subsets of immunocompetent cells leads to an inflammatory reaction and, clinically, to eczema.

Many questions remain in relation to the immunopathogenesis of atopic dermatitis, but the recent advances described here are already leading to the development of novel immunotherapeutic strategies.

See also: Allergens; Atopic allergy; Cell-mediated immunity; Contact hypersensitivity; Delayed-type hypersensitivity; IgE; Mast cells; Photoimmunology.

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EFFECTOR LYMPHOCYTES

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Lymphocytes participate in a broad array of immune functions, ranging from the regulation of the functions of various leukocytic subpopulations to the antigen-specific cytotoxicity of specific target cells. Antigen-activated lymphocytes which specifically lead to the elimination of the activating antigen have been termed 'effector lymphocytes'. Classically, effector lymphocytes were defined as activated T cells possessing highly specific receptors that react with, and have the ability to destroy, specific antigens or antigen-bearing target cells. Today, the term effector lymphocyte has a much broader meaning. More specifically, lymphocytes which have been activated by antigen or nonspecifically by cytokines act upon a certain target cell population by either modulating their biological function(s) or mediating their destruction. In some types of cell-mediated immunity, antigen-specific T cells or natural killer (NK) cells directly perform their effector function by mediating the activation or lysis of specific target cells. Antigen-activated T lymphocytes can also mediate effector functions indirectly through the secretion of cytokines which recruit and activate additional effector populations that are not specific for antigen, such as macrophages and NK cells, to the site of antigen deposition. Antigen specificity is conferred to non-specific effector cells by their activation in proximity to antigen-stimulated T cells. Many different lymphocytic populations have been shown to mediate effector functions. These cells include:

1. *T cytotoxic* (T_C) cells, which are typically $CD8^+$ T lymphocytes that are capable of destroying allogeneic cells and virally-infected target cells which recognize peptide fragments in the context of major histocompatibility complex (MHC) class I molecules on the target cell surface.
2. *T helper* (T_H) cells, which are typically $CD4^+$ T lymphocytes that potentiate immunoglobulin production and isotype switching by B cells, cooperate with cytotoxic T cells in the recognition of allografts and virally-infected target

cells, and release cytokines which are capable of activating macrophages and other cell types.

3. *Natural killer* (NK) cells, which are capable of killing a number of virally-infected, transformed and allogeneic target cells to which they have not been previously sensitized.
4. *Lymphokine-activated killer* (LAK) cells, which are either T or NK cells that have been activated by interleukin 2 (IL-2) or other cytokines to enhance cell-mediated cytotoxicity of tumor, allogeneic or virally-infected target cells.
5. *T suppressor* (T_S) cells, which are typically known as regulatory cells and have been shown by some laboratories to mediate certain effector cell functions.
6. *B lymphocytes*, which, in certain instances, may modulate the activation and function of antigen-specific T cells as well as secreting specific immunoglobulin which may indirectly mediate cell-mediated cytotoxicity.

Different types of cell-mediated immune reactions may result from effector T lymphocyte recognition of antigen or a specific target cell population. Delayed-type hypersensitivity (DTH) responses are mediated by antigen-activated $CD4^+$ T cells which secrete cytokines (i.e. tumor necrosis factor α and interferon γ) that activate the local vascular endothelial cell barrier to recruit monocytes and lymphocytes to the site of antigen challenge and stimulate recruited monocytes to become activated macrophages. In cytolytic responses mediated against virally-infected target cells or allografts, or in graft-versus-host disease, antigen-activated $CD8^+$ T cells differentiate into functional cytotoxic T cells (CTLs) which lyse target cells expressing the relevant peptide-MHC complexes. This CTL differentiation process requires the presence of activating cytokines secreted by antigen-activated $CD4^+$ T helper cells. In addition, NK and $CD8^+$ T cells stimulated by cytokines secreted by antigen-activated $CD4^+$ T cells differentiate into LAK cells which nonspecifically lyse target cells.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

See also: Cell-mediated immunity; Helper T lymphocytes; Cytotoxic T lymphocytes; Delayed-type hypersensitivity; Graft-versus-host reaction; Cell-mediated lysis; Lymphocytes; Lymphokine-activated killer (LAK) cells; Natural killer (NK) cells.

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ELECTRON MICROSCOPY, IMMUNOLOGICAL APPLICATIONS

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Historical background

The potential for employing labeled antibodies to localize antigens or antibodies in tissues at the microscopic level was first conceived by and realized through the experiments of Coons and colleagues in 1941 and 1942. These experiments described the first immunoconjugates wherein antibodies were chemically coupled to fluorescein and used to specifically bind target antigens in tissues. The resultant ‘microprecipitates’ were identified by their brilliant green color in a light microscope equipped with an ultraviolet light source and appropriate excitation and emission barrier filters for fluorescence microscopy. Novel fluorochromes such as rhodamine, Texas red and phycoerythrin have since been discovered that can be coupled to antibodies which, when excited by the appropriate wavelength of ultraviolet light, can be identified by their red color. The ability to couple fluorochromes with distinct emission spectra to antibodies specific for different epitopes has permitted dual-antigen localizations on the same sample. In addition to immunofluorescence microscopy, enzymes have also been coupled to antibodies and used to localize the antibody–antigen interaction. In immunoenzyme microscopy, the antibody-coupled enzyme converts a colorless chromogenic substrate into colored end-products at the site of the antibody–antigen reaction, which can then be visualized by conventional light microscopy.

With the advent of the transmission electron microscope and its greater resolving power, now in the range of 0.14 nm, valuable approaches complementary to those employed for the light micro-

scope were developed to localize specific antigen at the ultrastructural level. The first such immunoconjugates for electron microscopy utilized electron-dense molecules to replace the fluorochromes used in the ultraviolet microscope. Singer was the first, in 1959, to develop a method for coupling an electron-dense molecule, horse-spleen ferritin, to antibodies without a concomitant loss of immunobinding capacity. The experimental results of Rifkind and colleagues conclusively demonstrated in 1960 the utility of these electron-dense ferritin–antibody conjugates in the immunodetection of antigens in the electron microscope. These experiments formed the early foundation for the field of study now known as immunoelectron microscopy.

Principles of immunoelectron microscopy

Immunoelectron microscopy can be defined as any technique that uses antibodies, or molecules that interact with antibodies (for example, protein A or protein G), in conjunction with electron microscopy to localize ultrastructurally antigens or antibodies in cells and tissues. In addition, a number of other biological macromolecules whose specific ligand-binding properties are known can be used (for example, lectins). Immunoelectron microscopy was a term that was originally confined to studies that made use of the transmission electron microscope but now include those that use the scanning electron microscope.

In the transmission electron microscope, electrons serve as a pseudo-light source. High-energy electrons

emitted from a filament are accelerated and forced by electromagnets into a very fine coherent beam in the center of the microscope column. Because electrons are used, the microscope column and specimen area are maintained in a vacuum (10^{-6} – 10^{-8} Torr) to prevent the deflection of electrons by atmospheric gases. The electron beam is focused on to the sample. Here, the more electron-dense molecules in the sample under observation adsorb and scatter electrons, preventing their passage through the sample. Those electrons with enough energy to penetrate are transmitted, further focused, and projected on to a phosphor screen. The image is resolved on the phosphor screen due to the emission of photons in the area struck by the transmitted electrons. The image can be permanently recorded directly on photographic film sensitive to electrons or visualized with photographic cameras interfaced with the electron microscope viewing chamber and a computer to rapidly generate high-resolution computer processed images.

In preparation for transmission electron microscopy, samples are usually fixed with aldehydes (glutaraldehyde, formaldehyde or a combination of the two), post-aldehyde fixed in osmic acid, *en bloc* stained with a uranyl acetate solution, dehydrated with alcohols and embedded in a plastic resin. The embedded samples are thin sectioned with a glass or diamond knife and subsequently counterstained with heavy metals (uranium and lead salts) to further enhance the electron density of positively or negatively charged molecules in the sample. Specimens thus prepared have good contrast and can be used to observe and record the molecular and subcellular detail of cells and tissues. Alternatively, molecules may be adsorbed to a thin plastic film followed by embedding in an electron-dense stain or angular shadowing with evaporated metals.

The mechanical principles of operation for the scanning electron microscope are virtually the same as for transmission electron microscopes. The major difference is in the formation of the image. In scanning electron microscopes, the focused electrons are scanned over the surface of a sample, as implied by the name. Instead of transmitting electrons, secondary electrons emitted from the interaction of electron beam and sample are collected, electronically deciphered and multiplied, and projected on to a high-resolution cathode-ray tube. These images give a quasi three-dimensional impression of the structure under observation because of the great depth of field of the scanning electron microscope. The image is recorded on film using an ordinary camera focused on the cathode-ray tube. Samples for scanning electron microscopy must be fixed and dehydrated, as in transmission electron microscopy, but instead of

embedding the sample in a plastic resin, it is covered with a conductive coating of a fine metal, such as gold, by evaporation; no further processing is necessary.

As some examples of the utility of these techniques in ultrastructural research, transmission electron microscopy can reveal the structure of macromolecules adsorbed to a film, the surface detail of samples through surface replicas, or the internal structure of cells or infectious agents through thin sections. Scanning electron microscopes are primarily used for observing the surface detail of cells and tissues. The incorporation of immunoconjugates or other ligands into electron microscopic studies permits the high-resolution study of the antigenic composition of cell organelles and surfaces in concert with ultrastructural analyses. Singularly, or in combination, these two types of microscopic analysis have proved invaluable in the correlative study of morphologic structures and their antigenic composition.

Markers for immunoelectron microscopy

Native antibodies or their functional subunits have been used in the absence of a marker in some applications. However, most applications require a morphologic or electron-dense marker to visualize the antibody–antigen microprecipitation. Many marker systems have been developed for immunoelectron microscopy. The markers in these systems fall into several major categories: electron-dense biological molecules such as ferritin, colloidal-gold sols, enzymes that produce an electron-dense product, synthetic microspheres, and biological molecules with a distinct morphologic shape (hemocyanins, bacterial, animal and plant viruses, etc.). The choice of marker depends primarily on the application. A marker system which renders the microprecipitation site electron dense or morphologically recognizable is preferable for transmission electron microscopy. **Figure 1** shows a gallery of markers used in transmission immunoelectron microscopy to localize retroviral envelope glycoproteins.

Morphologically recognizable markers such as viruses, latex microspheres and hemocyanins have the greatest utility in scanning electron microscopy, where electron density plays less of a role because of the metallic coatings that are applied for imaging with secondary electrons. Moreover, the markers for scanning electron microscopy must be larger than those required for transmission electron microscopy and have a distinct and uniform shape and size to be easily resolved against the topographical features of the cell surface and the lower resolving power of

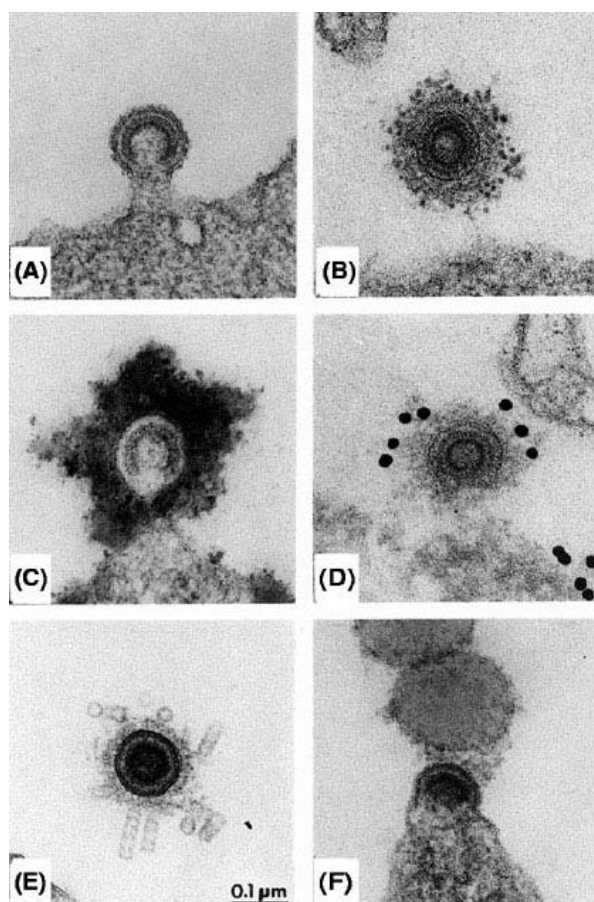


Figure 1 Gallery of indirect immunoelectron microscopic methods used to demonstrate the reactivity of an antiretrovirus envelope glycoprotein goat serum on the envelope of virus particles and the physical characteristics of the various markers as observed in the transmission electron microscope. (A) Virus-infected cells in which the primary serum was a normal goat serum, as used with any of the marker systems presented in (B–F). The primary serum in (B–F) was the antiretrovirus envelope glycoprotein goat serum; in each frame the primary antibody was localized by one or more secondary antibodies that brought an electron-dense or morphologically visible marker into the proximity of the microprecipitation. (B) Ferritin; (C) peroxidase; (D) colloidal gold; (E) unlabeled antibody hemocyanin; and (F) latex immunomethods. (Reproduced with permission from Gonda MA *et al* (1979) *Scanning Electron Microscopy*, vol 3. Illinois: SEM.)

scanning electron microscopes. Where correlative studies require the use of transmission and scanning electron microscopy, a marker resolvable by both transmitted and secondary electrons must be selected; hemocyanins and viruses are especially useful in this scenario. **Figure 2** shows the use of the hemocyanin marker in the localization of the surface glycoprotein of a retrovirus by scanning and transmission immunoelectron microscopy, for comparison.

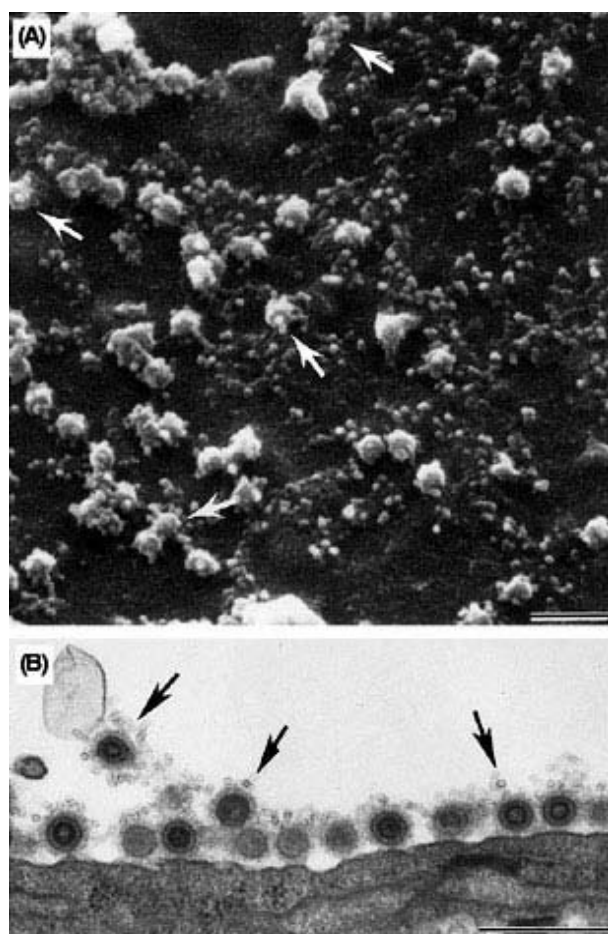


Figure 2 (A) Scanning electron micrograph of mouse mammary tumor virus-infected cells immunolabeled using specific antiserum to whole virus and the unlabeled antibody hemocyanin bridge technique (see **Figure 3h**). Virus particles (arrows) and discrete patches of cell membrane are labeled. Scale bar = 0.5 μ m. (Reproduced with permission from Gonda MA *et al* (1979) *Scanning Electron Microscopy*, vol 3. Illinois: SEM.) (B) Transmission electron micrograph of mouse mammary tumor virus-infected cells immunolabeled with antibodies specific to virus envelope proteins and the unlabeled antibody hemocyanin bridge technique (see **Figure 3h**) for comparison with images obtained by the scanning electron microscope in (A). Virions in all stages of morphogenesis and the cell surface are labeled intensely with hemocyanin (arrows). Scale bar = 0.5 μ m. (Reproduced with permission from Gonda MA *et al* (1981) *Scanning Electron Microscopy*, vol 2. Illinois: SEM.)

Immunoreagents

Polyclonal antisera that contain a mixture of antibody populations and monoclonal antibodies are the most commonly used reagents in immunoelectron microscopy where antigen (or antibody) is being localized. Immunoglobulin G (IgG) in serum fractions is most often preferred, unless the reaction considerations specify otherwise. Purity and specificity of polyclonal antisera can be improved by affinity purification steps. The benefits of monoclonal anti-

body use are well known. There are also several bacterial cell wall proteins, protein A and protein G, that bind the Fc portion of γ -globulin from various species and have proven useful in detecting antibody bound to antigen. Moreover, since protein A and G are proteins, they can be conjugated to markers useful in immunoelectron microscopy and applied to the detection of a variety of γ -globulin classes from multiple species. Other types of biological macromolecules (e.g. lectins, asialoglycoprotein, etc.) unrelated to antibodies, when conjugated to markers, can be used as ligands to localize ligand-receptor interactions because of their known specificity and affinity for target molecules. Two other macromolecules, streptavidin and biotin, have been used in immunconjugates because of their high affinity and specificity for each other. In this case, biotin is used to directly label the specific antibody and streptavidin is conjugated to the marker. The strong interaction between streptavidin with biotin brings the marker into contact with the bound antibody.

Antibodies can be coupled to the marker by various methods. The most common method, when two protein molecules are involved, is to conjugate the antibody to the marker chemically via a low molecular weight bifunctional coupling reagent (e.g. glutaraldehyde, metaxylylene di-isocyanate, thiocarbamide, etc.). However, in the case of highly charged metal sols, like colloidal gold, the electrostatic forces of this material can be used to irreversibly bind antibodies, protein A and G, or streptavidin molecules to the gold particulate.

Direct and indirect labeling approaches

There are two basic approaches to labeling target antigen with the immunoconjugate; they are the direct and indirect techniques (Figure 3). The direct method is the simplest and involves the use of specific antibody conjugated to the marker to localize its target antigen in a one-step procedure. In the indirect test, a two-step procedure is employed. An unlabeled-specific antibody, called the primary antibody, is used to bind to the target antigen. The unlabeled-specific antibody complex is localized with a marker-conjugated anti-immunoglobulin, called the secondary antibody. The indirect technique is sometimes called the 'sandwich technique'. In the case where an enzyme such as horseradish peroxidase is conjugated to the primary or secondary antibody, the immunoprecipitate is reacted with enzyme substrate and developer for contrast (see Figure 1C for an example of the indirect immunoperoxidase technique).

There are various themes of these two labeling techniques. One indirect labeling method worth

mentioning is the unlabeled antibody bridge method (Figure 3H), which involves four steps, but can be used in correlative transmission and scanning immunoelectron microscopy. Using the unlabeled antibody hemocyanin bridge as an example, unlabeled specific antibody (e.g. rabbit IgG) is first reacted with substrate. An unlabeled bridging antibody (rabbit-specific IgG), which was raised to the immunoglobulin species of the specific antibody, is then bound. A third antibody to marker, hemocyanin, made in the same species as the specific antibody, is then applied and bound by the free Fab arm of the bivalent bridging antibody (rabbit-specific IgG). Hemocyanin is then applied and bound by the free Fab arms of the marker-specific antibody (third antibody in the bridge). Figures 1E and 2 are examples of the unlabeled antibody hemocyanin bridge used to localize retroviral antigens.

Immunolabeling techniques

Immunolabeling techniques for transmission electron microscopy can be divided into three basic categories: no embedding, pre-embedding and postembedding. The no-embedding method is used primarily for immunostaining large biological molecules, such as proteins and nucleic acid-protein complexes, viruses, and frozen cryostat sections mounted on film-coated grids that need no further fixation or intramolecular supporting matrix. The pre-embedding technique is used for labeling cell surfaces (see Figures 1 and 2). This technique does not readily permit access to intracellular antigens. In the pre-embedding technique, unfixed or mildly fixed material is immunostained before the embedding and sectioning processes. In the postembedding method, the material is lightly fixed, embedded and thin sectioned. The thin sections are then immunostained. Figure 4 shows an application of the postembedding method in localizing an intracellular protein. This methodology is the most versatile in visualizing intracellular structures and associated antigens simultaneously and has overcome the many problems incurred by other methods. Since mild fixation and embedding are required in the postembedding thin-section immunostaining method, it should be noted that a number of potential problems can be encountered, including a reduction in immunogenicity, accessibility and concentration of target antigen, during the embedding process, and a loss of ultrastructural definition due to the mild fixation procedures.

All immunolabeling techniques for scanning electron microscopy are performed before dehydration and application of a conductive coating to reveal sur-

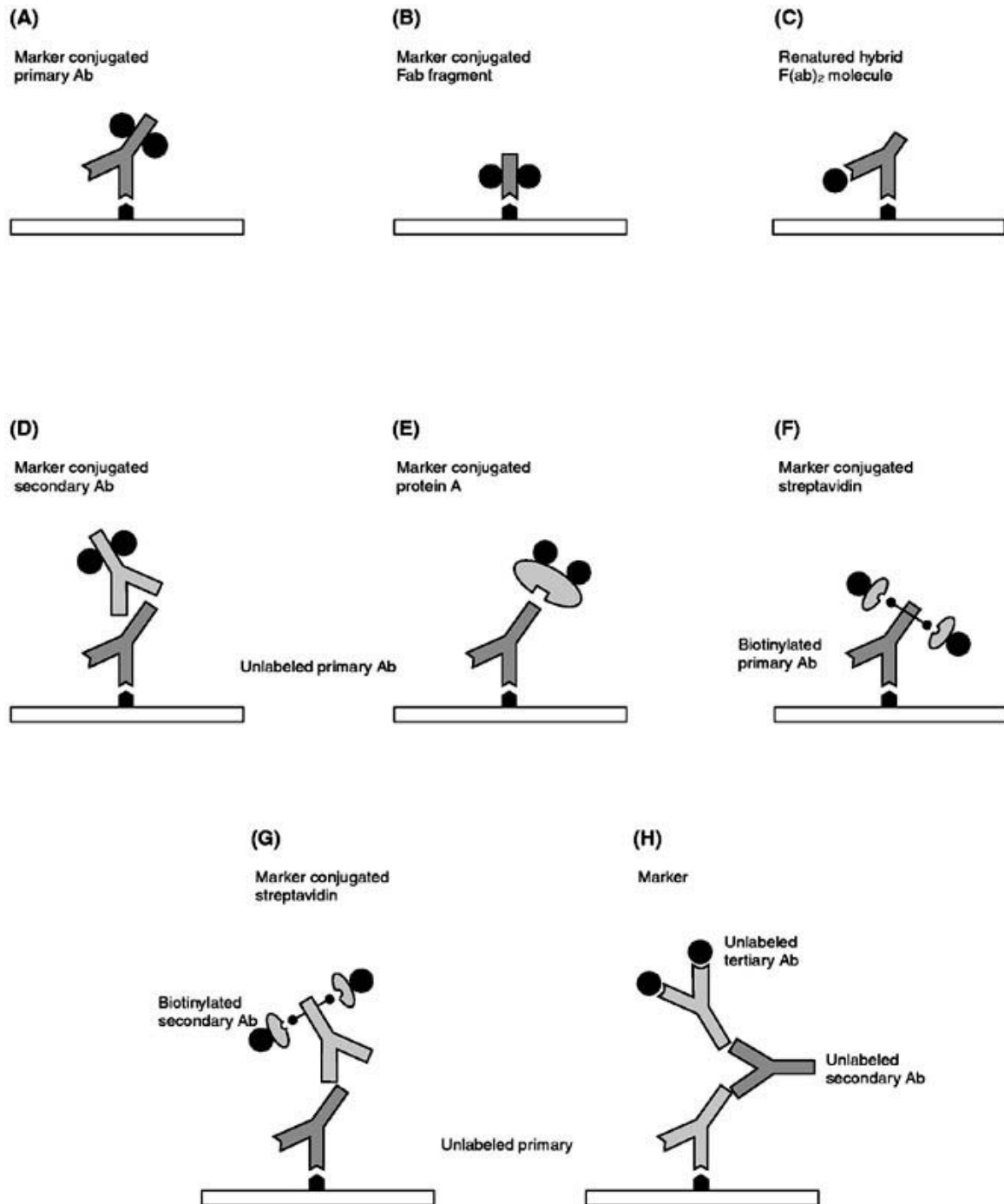


Figure 3 Direct (A–C) and indirect (D–H) immunologic labeling techniques used to localize antigens for immunoelectron microscopy. *Direct methods.* (A) Marker is conjugated to primary antibody (IgG) and (B) to Fab fragment. (C) Renatured hybrid $F(ab)_2$ fragment with dual specificity bridges target antigen and marker. *Indirect methods.* (D) Marker-conjugated secondary antibody (IgG) binds unlabeled primary antibody (IgG). (E) Marker-conjugated protein A binds unlabeled primary antibody (IgG). (F) Marker-conjugated streptavidin binds biotinylated primary antibody (IgG). (G) Biotinylated secondary antibody (IgG) bridges marker-conjugated streptavidin and unlabeled primary antibody (IgG). In the unlabeled antibody bridge method (H), unlabeled secondary antibody (IgG) bridges unlabeled tertiary antibody (IgG) to marker and unlabeled primary antibody (IgG) to target antigen; marker is bound by tertiary antibody (antibody to marker). (Reproduced with permission from Gonda MA (1994) *Immunochemistry*. New York: Marcel Dekker.)

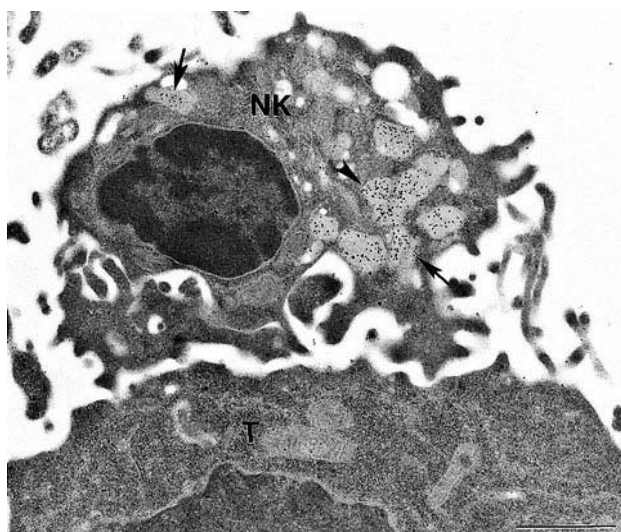


Figure 4 Electron micrograph of natural killer (NK) tumor target (T) cell conjugates 30 min after initial interaction. The post-embedding thin-section immunolabeling method was used to localize an intracellular pore-forming protein believed to have an active role in target cell lysis. Thin sections were labeled with monoclonal antibodies to pore-forming protein and bound antibodies were localized by the indirect immunogold method. The arrows in the NK cells depict the effector granules containing colloidal-gold-labeled pore-forming protein. Scale bar = 1.0 μm . (Micrograph contributed by J Ortaldo, K Nagashima and MA Gonda.)

face detail. Samples may be fixed or unfixed before the labeling. Often it is desirable to fix the sample mildly with dilute solutions of glutaraldehyde (0.1–0.25%) before labeling, especially where the labeling procedure may alter the surface architecture under investigation.

Future of immunoelectron microscopy

Applications of immunolabeling procedures can be found in many scientific disciplines involving ultra-structural studies of biological structure and function. The use of labeled immunological probes in conjunction with electron microscopy has made many contributions to our overall understanding of the antigen composition and structure of cells and tissues in various pathologic states. In addition, immunoelectron microscopy has helped to resolve the structure of pathogens and understand

pathogen–host interactions in various disease states. Although immunoelectron microscopy in the strictest sense implies that antibodies are used, there are a variety of other macromolecules that have been used in studies of receptor–ligand interactions. No doubt, with advances in molecular biology, the discovery, definition and availability of new ligands will continue at an increasing pace and be used in localization studies by electron microscopy. The use of immunoelectron microscopy, aided by highly specific monoclonal antibodies and genetically engineered probes, will continue to advance our knowledge of cellular and molecular structures and be an important tool in immunocytochemistry and histopathology.

See also: Affinity chromatography; Antibodies, detection of; Antiserum; Bacterial immunoglobulin-binding proteins; Enzyme labeling of antibodies and antigens; Fc receptors; Fluorochrome labeling; IgG; Immunocytochemistry and enzyme markers; Lectins; Monoclonal antibodies (mAbs).

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ELISPOT ASSAY

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Detection of soluble products secreted by cells of the immune system, particularly immunoglobulin (Ig) and cytokines, remains one of the cornerstones of immunological inquiry at both the clinical laboratory and research laboratory levels. The most sensitive methods for this purpose are those that detect secreted products of a single cell. The enzyme-linked immunospot (ELISPOT) assay is an ELISA-based technique that enables detection and enumeration of Ig- and cytokine-secreting cells at the single cell level. The assay is now applied to wide-ranging immunological investigation.

The immune system can very broadly be divided into two parts – cell-mediated immunity and humoral immunity. To some extent, each arm of the immune response is influenced by or dependent on the other. For example, CD4⁺ T lymphocytes (part of the cell-mediated immune system) respond to a viral infection by liberating factors (lymphokines or cytokines) which help B lymphocytes (broadly part of the humoral immune system) to differentiate into plasma cells. These cells secrete specific immunoglobulin (antibody) which may neutralize the virus. It is evident from this rather simplistic view of T–B lymphocyte interaction, that substances secreted by both cell types are central to the effective operation of the immune system. Thus, for many years, immunologists have been interested in quantifying and further characterizing these secreted molecules.

One of the most widely used *in vitro* assays for the detection of antibody in solution is the enzyme-linked immunosorbent assay (ELISA), first described in 1971. The ELISA utilizes the fact that many substances (such as protein antigens) nonspecifically adsorb to plastic surfaces and that specific antibody (for example in serum) can bind to this immobilized antigen and subsequently be detected by a second immunoglobulin-specific antibody which is coupled to an enzyme such as alkaline phosphatase or peroxidase. A substrate solution is added to this immobilized antigen–antibody complex which yields a soluble colored product in the presence of the enzyme. The intensity of the color of this solution is related to the amount of antibody bound to the antigen. From this, the concentration of antibody in the original serum sample can be determined.

The ELISA is not limited to the detection of antibody. Following the availability of monoclonal and

polyclonal antibodies specific for cytokines, ELISAs have now been developed for a range of factors secreted by cells such as T lymphocytes and macrophages. In this case, however, a cytokine-specific antibody rather than antigen is coated on to the plastic surface. The cytokine-containing solution (such as supernatant from *in vitro* activated T lymphocytes) is added followed by a second enzyme-labeled cytokine-specific antibody and substrate.

Notwithstanding the usefulness of assays such as ELISA for the detection of substances in solution, greater definition and sensitivity may be obtained by employing methods which enable one to detect secretion of immunoglobulin or cytokine at the single cell level. The first method to be described for the detection of single immunoglobulin secreting cells (ISCs) was the hemolytic plaque-forming cell (PFC) assay. This technique has served immunologists well since its description in 1963 but there are some circumstances in which the PFC assay is suboptimal in its sensitivity and specificity. Moreover, only limited success has been achieved with the PFC assay as a means of detecting cytokine secreting cells (CSCs).

The obvious advantages of the ELISA for the detection of low levels of antibody prompted the development of an ELISA-based system for the detection of ISCs and, more recently, for CSCs (Figure 1). The ELISPOT assay was developed by two groups and details of the technique published in 1983. This technique has now all but replaced the hemolytic PFC assay. The assay goes under a number of names which include those in the original descriptions (ELISA-plaque and ELISPOT assays) as well as SPOTELISA and ELISASPOT. However, the term ‘ELISPOT’ is now most commonly used and is widely recognized as defining a unique and distinct assay system. As illustrated in Figure 1, the assay is similar to a standard ELISA but with two important differences. First, cells are added rather than a solution and second, the substrate chosen for the ELISPOT yields an insoluble product. Thus, substrate precipitates at the site of enzyme activity which is localized at the point where antibody or cytokine was secreted. This ‘fingerprint’ of the secreting cell is revealed as a colored spot and can readily be counted.

As with the standard ELISA, there are a number of ways in which the basic method illustrated in

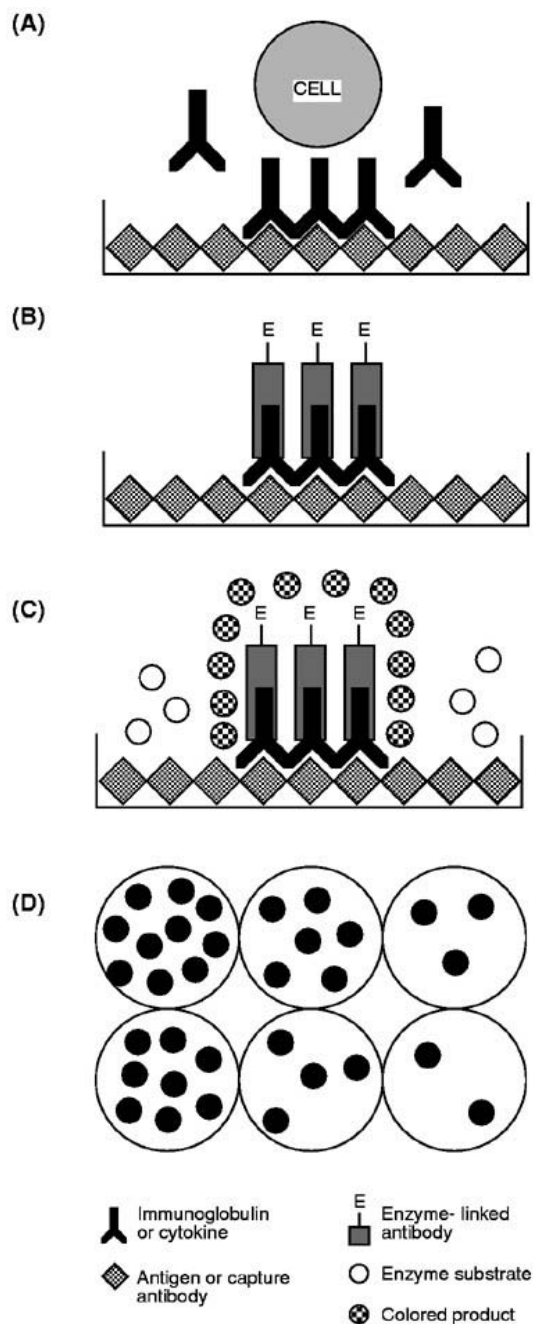


Figure 1 The ELISPOT assay. (A) Added cells secrete immunoglobulin or cytokine which binds locally to antigen or is captured by antibody bound to the plastic or nitrocellulose solid phase. (B) Cells are removed. Locally secreted product is detected by enzyme-linked antibody specific for immunoglobulin or cytokine. (C) The site of immunoglobulin or cytokine secretion is revealed by addition of enzyme substrate that yields an insoluble colored product. (D) Colored spots represent the secreted product of a single cell and can be counted by eye or under low magnification.

Figure 1 may be altered. For example, ELISPOT assays have now been performed using biotin-labeled antibody together with avidin-labeled enzyme. A further refinement is the use of antibody/enzyme/substrate combinations that enable detection of two distinct Ig isotypes simultaneously, by virtue of different colored substrate products.

One of the most important aspects to optimize in the ELISPOT assay is the solid phase which consists of the combination of vessel surface and coated antigen or antibody. Relatively high levels of some antigens, for example ovalbumin, are required when coating plastic surfaces while other molecules such as purified Ig effectively coat the plastic surface at concentrations 10–20-fold less than is required for ovalbumin. Capture antibody (i.e. coated Ig) is therefore a useful alternative and is mostly used when one wishes to detect ISCs of no particular antigenic specificity (total ISCs) or for the detection and enumeration of cells secreting factors such as cytokines.

For the detection of antigen-specific ISCs where antigen is only available in small amounts (for example, purified viruses) or does not bind readily to plastic, other modifications have been introduced. The first is the use of nitrocellulose rather than plastic in the floor of the assay vessel. Nitrocellulose has a much greater capacity to bind protein than plastic and so the efficiency of antigen binding is substantially increased. A second modification is the labeling of the antigen in such a way that binding to the plastic surface is unnecessary. For example, the plastic surface can be coated with avidin and biotinylated antigen added. Alternatively, it is possible to use antigen directly coupled to an enzyme. The plastic surface is coated with an isotype-specific capture antibody which binds all antibody of a particular isotype secreted by the added ISCs. Antigen specificity is achieved by adding the enzyme-coupled antigen which is bound only at the localized sites where antigen-specific immunoglobulin was secreted and captured.

The range of specificities of ISCs now detected in both experimental animal systems as well as in humans is considerable and varies from commonly used antigens such as ovalbumin and haptens to viruses and insulin and molecules of importance in autoimmunity such as thyroglobulin, myelin basic protein and rheumatoid factor. ISCs specific for particulate antigens including platelets, sheep erythrocytes and whole bacteria as well as purified bacterial antigens such as polysaccharide have also been enumerated. There is no apparent limitation on the antibody isotype that can be detected and the discriminating power of the ELISPOT assay means that ISCs

present at very low frequency (such as IgE-ISCs) can now be examined readily.

Successful detection of cells secreting a range of cytokines has been achieved, and includes those producing interleukin 1 α (IL-1 α), IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, interferon γ (IFN γ) and tumor necrosis factor (TNF). Direct comparisons between ELISA and ELISPOT assays for cytokine production by T cell clones *in vitro* has indicated that the ELISPOT assay for CSCs is between 10 and 200 times more sensitive than cytokine detection by ELISA performed on culture supernatant. CSCs directly *ex vivo* as well as after *in vitro* stimulation have been used successfully in the ELISPOT assay attesting to the versatility and utility of the technique.

In conclusion, the ELISPOT assay is a simple, versatile yet powerful technique for the detection and enumeration of cells liberating Ig or cytokine. The technique can be modified in a variety of ways to suit individual needs and circumstances and to optimize sensitivity. Its application to the detection of cells secreting molecules other than Ig or cytokine should also be considered. The detection of bone marrow-derived mast cells secreting mast cell protease II, is one published example of an alternative application for the ELISPOT assay.

See also: Antibodies, detection of; Cell-mediated immunity; Cytokine assays; Cytokines; Enzyme-linked immunosorbent assay (ELISA); Humoral immunity; Immunoassays; Plaque-forming cell (PFC) assays.

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EMBRYONIC ANTIGENS

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During embryonic and fetal life, unique proteins and carbohydrates are synthesized which are either absent or are produced only in very small amounts in normal adult individuals. Studies of these 'embryonic' and 'fetal' molecules have been crucial in the formulation of many concepts in the field of molecular genetics, and particularly in the identification of the processes responsible for switching on and off

genes during development. Interest in embryonic and fetal molecules also derives from their persistence or reappearance in certain disorders. Thus abnormalities involving the rate of synthesis of adult hemoglobin (Hb) chains (α or β) affect the regulation of embryonic and fetal Hb genes. For example, in fetuses with α -thalassemia, due to deletion of both the α -chain genes, hemoglobin, Bart's (γ_4), Hb H (β_4)

and embryonic Hb Portland ($\zeta_2\gamma_2$) continue to be produced.

Blood group antigens provide another example of fetal molecules that can be expressed in adults in various conditions. Red cells of almost all healthy adults carry the blood group antigen 'I' and very small amounts of 'i'. In contrast, fetal red cells react strongly with anti-i sera and weakly with anti-I. Using anti-i, increased agglutinability of red cells from adult subjects is seen in patients with thalassemia and hypoplastic anemia, while high levels of cold autoagglutinins with anti-i specificity are often detectable in patients with infectious mononucleosis.

Fetal proteins such as alphafetoprotein (α -fetoprotein, AFP) and carcinoembryonic antigen (CEA) are synthesized once again in large quantities in patients with selected tumors, and their estimation, by immunologic techniques, has proved to be a diagnostic and prognostic index of the disease.

Only a few fetal proteins of particular interest to immunologists, either because they are detected using immunologic techniques or for their presumed immunoregulatory function, will be described.

α -Fetoprotein

In 1956, human fetal sera were shown to contain a protein apparently absent in sera from normal adult individuals. A similar protein, termed α -fetoprotein (AFP) and shown to be structurally distinct from bovine fetuin, was later detected in all mammalian species investigated.

AFP appears early during human development; in fact, traces of this protein have been detected in 4-week-old embryos. The AFP plasma levels increase rapidly during the first trimester of gestation, reaching the highest values ($2\text{--}3\text{ mg ml}^{-1}$) around 12–16 weeks and later declining. In cord blood the AFP levels are usually below 0.2 mg ml^{-1} ; traces of the protein ($>20\text{ ng ml}^{-1}$) can be detected in sera from normal adults (Figure 1).

Human AFP has a molecular weight of approximately 66 kDa and a carbohydrate content near 3.5%. In all species investigated, AFP is structurally similar to albumin. In fact, a highly conserved pattern of gene organization, similar to the albumin gene, is evident. In humans, both the AFP and albumin genes, which comprise 15 exons separated by 14 intervening introns, map on chromosome 4.

Besides being present in plasma, AFP can be detected in most fetal fluids such as amniotic and cerebrospinal fluids (CSF) and urine. Small amounts of AFP cross the human placenta and increased levels (up to 500 ng ml^{-1}) of this protein are present in sera from normal pregnant women.

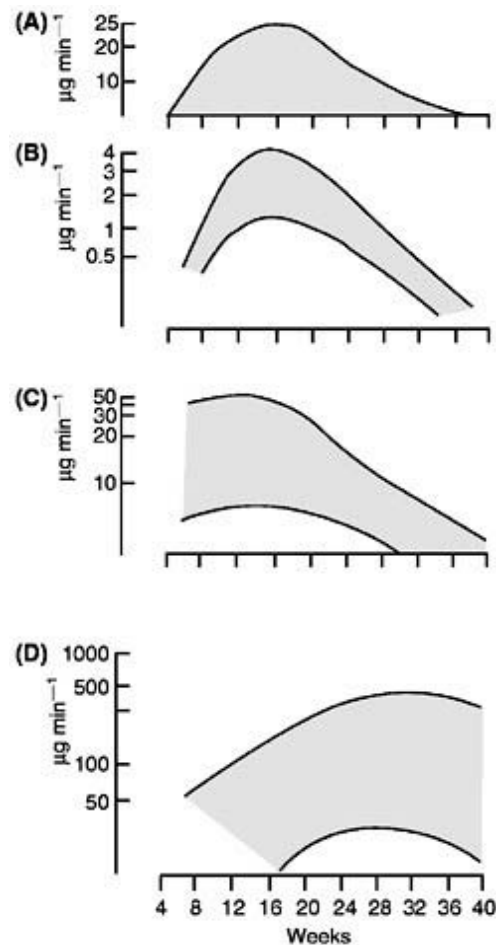


Figure 1 Synthesis of AFP in human fetal liver (A) compared with the levels of the fetal protein in fetal sera (B), amniotic fluid (C), and maternal sera (D). The concentration of AFP in maternal sera increases with gestation, due to the increased permeability of the placental barrier.

Abnormally high levels of AFP in amniotic fluid and maternal sera are associated with fetal neural tube defects (NTDs), such as anencephaly and open spina bifida. This results from the still immature blood–CSF barrier, which allows transfer of AFP from serum firstly into the CSF, and then into the amniotic cavity. Congenital abnormalities, other than NTDs, associated with high levels of AFP in amniotic fluid are shown in Table 1. On the other hand, abnormally low maternal serum levels of AFP during the first trimester of gestation are indicative of fetal chromosome trisomies (e.g. trisomy 21, the Down syndrome).

The discovery that the serum levels of AFP are greatly increased in patients with primary cancer of the liver has prompted a series of investigations which have documented the diagnostic and prognostic value of estimating the AFP plasma levels in such

Table 1 Some congenital abnormalities, other than neural tube defects, associated with abnormal values of α -fetoprotein

High levels in amniotic fluid:
hydrocephalus
congenital nephrosis
congenital esophageal atresia
exomphalos
Low levels in amniotic fluid:
Down syndrome

patients. Synthesis of AFP also increases during liver regeneration; thus abnormal levels of this protein suggest liver damage and repair. High levels of AFP have been detected in patients with teratocarcinoma and ataxia telangiectasia.

During the past 20 years, several investigators have focused their attention on the possible role of AFP as an immunosuppressive factor. Early reports suggested that AFP, partially purified from rodent and human fluids, would suppress the mitogenic effects of phytohemagglutinin, concanavalin A and lipopolysaccharide on spleen cells or lymphocytes, as well as inhibiting lymphocyte stimulation in the one-way mixed lymphocyte reaction.

Other investigators have not been able to confirm these findings nor to demonstrate the presence of specific AFP receptors on the surface of normal lymphocytes, as previously claimed. In addition, fetal mouse and human sera, containing high levels of AFP, have been repeatedly shown not to exhibit *in vitro* immunosuppression. In conclusion, the discrepancies of the experimental results and theoretical considerations do not support the claim that AFP has a major role as an immunosuppressive agent.

Carcinoembryonic antigen

Carcinoembryonic antigen (CEA), described by Gold and Freedman in 1965, was the first and most widely used plasma tumor marker. Like AFP, it belongs to the so-called 'oncofetal' antigens, which are normally found in fetal tissues and plasma but are present only in very small quantities in normal adult subjects.

It has been shown that CEA consists of a group of proteins of between 175 and 200 kDa, containing up to 50% carbohydrate, whose immune reactivity resides on the protein moieties. Early studies suggested that high levels of CEA are produced by fetal colon and colon carcinoma cells and that only small amounts of CEA are present in plasma from normal adult subjects (upper limit between 5 and 20 ng ml⁻¹; subsequent investigations have shown that abnormally high levels of CEA are also produced in patients with primary and secondary can-

cers of the liver, breast and ovarian tumors, and occasionally in patients with acute or chronic hepatic disease. Modest elevation of CEA has been detected in patients with colitis.

The general view is that CEA tests display adequate sensitivity but very poor specificity, and thus plasma estimation of this antigen is of little value in screening high-risk groups or asymptomatic individuals. Adding to the clinical problems, there are also technical difficulties associated with the immunologic techniques employed to estimate CEA plasma levels owing to interassay variability.

Common acute lymphoblastic leukemia antigen (CD10)

Originally identified on the surface of acute lymphoblastoid leukemia (ALL) cells and designated as common acute lymphoblastoid leukemia antigen (CALLA), the antigen has now been assigned the cluster of differentiation number 10 (CD10). CALLA was initially recognized using rabbit antiserum raised against ALL cells which had been precoated with rabbit antibodies against antigenic determinants present on normal lymphocytes. The rabbit anti-CALLA sera were found to produce a maximal reactivity against peripheral blood nucleated cells from ALL patients, and a minimal reactivity when tested against normal bone marrow cells or cells from patients with leukemias other than ALL. Using monoclonal antibodies, the distribution of CALLA⁺ cells has been further clarified. Thus very few CALLA⁺ cells are present in peripheral blood samples from normal individuals, while CALLA⁺ cells, coexpressing immunoglobulin M (IgM) molecules and CD19, are detectable in fetal bone marrow and peripheral blood samples. CALLA⁺ fetal bone marrow cells, cultured with interleukin 2 (IL-2) and irradiated feeder thymocytes can differentiate to CD2⁺, CD3⁺ cells expressing either CD4 or CD8. These findings are consistent with early data suggesting that CALLA molecules are expressed on B and T cells during early stages of fetal development. CALLA⁺ cells are detectable in a majority of patients with B-ALL, in a proportion of T-ALL patients and in some cell lines derived from T cell non-Hodgkin lymphoma (T-NHL). The antigen is expressed in about one-third of Ph⁺ chromosome-positive leukemia patients during blast crisis.

The molecular nature of CD10 has now been partially defined; cDNA clones have been isolated, sequenced and shown to control the synthesis of

a protein structurally identical to the human membrane-associated enzyme neutral endopeptidase (NEP 3.4.24.11).

The biological enzymatic role of CD10 molecules has not yet been clarified, but it has been suggested that CD10-NEP mediates chemotaxis and that the presence or absence of CD10-NEP might affect the clinical course of ALL in childhood. It seems, in fact, that CD10⁻ patients with ALL have shorter disease-free survival.

Elucidation of the biological function of CD10 molecules expressed on subpopulations of lymphocytes during fetal life and in patients with leukemia might only be obtained once the specific substrate of this enzyme has been identified.

Other 'fetal' antigens

Occasionally fetal antigens are claimed to be expressed on cancer cells, but in several instances these antigens are also present in some normal adult tissues and, accordingly, their presence in cancer cells is the result of an ectopic gene expression. 'Fetal' antigens have been claimed to be present in amniotic fluid, including several acid glycosphingolipids; however, the biological and biochemical properties, as well as the site of synthesis, of these factors are still uncertain. A fetal form of acetylcholine receptor (AChR) has been postulated to exist because maternal antibodies have been detected that specifically inhibit AChRs in the fetus and the newborn, but not in the mother, and are responsible for the syndrome termed arthrogryposis multiplex congenita.

Finally, it should be mentioned that immunologic methods have been found useful for localizing antigens to different parts of a tissue during adult or embryonic and fetal life. For example, immunohistochemical tests have shown that, during embryogenesis, lactase is present in different portions of the intestine to where it is found in the adult tissue. However, no structural differences of this enzyme have been observed.

Immunologic tests have also been successfully employed to evaluate quantitative differences of gene expression between adult and fetal cells. However, recently, the detection of mRNA has been more widely used to detect and quantify gene expression and the role that transcription factors play in terminal differentiation of stem cells into defined mature cell types.

Recently, antibodies against fetal Hb (Hb F) have been used to detect, and eventually isolate, fetal erythroblasts present in maternal peripheral blood samples. However, since a few erythroblasts are also

released in normal adults and, more frequently, during gestation, the fetal origin of such cells should be established by the detection of other fetal markers, such as the presence of a Y chromosome by fluorescent *in situ* hybridization if the fetus is male.

See also: Cold agglutinins; Leukemia; Lymphoma; Plasma; Tumor antigens.

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ENDOTHELIUM

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Endothelial cells have long been perceived as a 'sheet of nucleated cellophane', a 'passive' lining of vessels, endowed with negative properties, the most crucial one being that of representing a nonthrombogenic substrate for blood. As such, endothelial cells were viewed to participate in tissue reactions essentially as targets for injurious agents. In the last few years, endothelial cells have emerged as active participants in a variety of physiologic and pathologic processes. In particular, the ontogeny and function of white blood cells requires an intimate relationship with vascular endothelial cells. The endothelial cells are an important constituent of the hematopoietic microenvironment of the bone marrow and produce colony-stimulating factors (CSFs) which regulate hematopoiesis. The recirculation and homing of lymphocytes is accomplished via interaction with endothelial cell structures and these cells can activate specific immunity by presenting antigen. Finally, the effector arm of immunity, inflammatory reactions and hemostasis involve close interactions between leukocytes and vascular cells.

Endothelial cell-to-cell junctions

Circulating cells infiltrate into tissues by migrating through intercellular junctions. These organelles are formed by a complex network of transmembrane proteins linked to a well-developed plasmalemmal undercoat. One of the typical characteristics of endothelial junctions is their dynamic organization. Endothelial cells are able to change rapidly the architecture of the junctions to allow the passage of circulating blood cells. This effect, in most cases, is quickly reversible and the endothelium is able to disorganize/reorganize its intercellular junctions within minutes. Interendothelial junctions present a different degree of complexity along the vascular tree responding to different functional requirements. For instance, they are well organized and numerous in large arteries or in the blood vessels of the brain

where the control of permeability must be strict, whereas they are very primitive in the postcapillary venules, where cell extravasation and exchange of plasma constituents need to be particularly efficient. On the basis of morphologic and functional characteristics at least four types of junctions have been described in endothelial cells. These are: tight junctions, adherence junctions, gap junctions and syndesmos.

Although there is a great deal of information regarding the molecules that mediate leukocyte adhesion to the endothelium, the mechanisms by which leukocytes trigger the opening of endothelial cell junctions is still obscure. In many conditions the passage of leukocytes through endothelial junctions does not increase endothelial permeability *per se* or cause vascular damage.

Chemoattractants and adhesive molecules stimulate neutrophils to secrete oxygen-free radicals, lipid metabolites and proteases, each of which is a potential agonist of endothelial permeability. However, experimental evidence suggests that these reactive agents are not necessary for neutrophil extravasation. The question of how leukocytes pass through endothelial clefts remains open. An interesting possibility is that leukocyte adhesion to endothelial cells causes a cascade of events similar to that induced by soluble agonists of endothelial permeability. In particular, leukocyte ligation to endothelial adhesive molecules (such as selectins, VCAM-1 or ICAM-1) could generate intracellular signals similar to those caused by permeability-increasing agents. It has been found that endothelial cells respond to neutrophil contact and migration by increasing intracellular calcium and inhibitors of intracellular Ca^{2+} block neutrophil transmigration. In addition, ICAM-1 activation by specific antibodies leads to phosphorylation of the actin-binding protein cortactin. This or other signals could induce changes in cleft molecular organization (see above), leading to the opening of gaps between

endothelial cells. According to this hypothesis endothelial cells would not only play an important role in regulating leukocyte attachment to their surface but also actively modulate their extravasation.

Leukocytes might find preferential pathways for their passage through the interendothelial clefts. Tight junctions and adherence junctions comprise a system of discrete ion-selective pores rather than an absolute seal around the cells. In endothelial cells, the presence of areas of junctionless clefts that regulate the transendothelial transport of high molecular weight proteins has been described. Leukocytes might be directed to these pores through the concentration gradient of specific adhesive molecules such as CD31. Their passage would require them to squeeze through the pores, accompanied by rearrangement of the endothelial cell cytoskeleton organization around these structures.

There might be differences between the modalities of leukocyte extravasation for different types of vessels where the clefts present different levels of complexity. There might also be distinct mechanisms regulating the passage of the different types of leukocytes or of other types of cells.

Endothelial cell adhesion molecules

The recruitment of leukocytes into sites of inflammation involves a cascade of sequential events controlled by the interaction between adhesion molecules expressed by leukocytes and by the endothelium (Figure 1). This multistep process is a cell-to-cell adhesive reaction that involves specific binding of membrane receptors on one cell to counterreceptor structures on the other cell. Several adhesion receptors belonging to different families, including integrins and selectins, have been shown to participate in this mechanism. In the current model, selectins are implicated in the initial rolling, while adhesion receptors from the integrin family and the immunoglobulin superfamily are involved in the firm attachment, flattening and extravasation of leukocytes. Leukocytes

have to adhere to the endothelium before transmigrating, and it is difficult to distinguish between adhesion molecules, involved only in adherence, and proteins involved in the transmigration process. However, several studies have shown that intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), platelet endothelial cell adhesion molecule 1 (PECAM-1) and selectins are important for leukocyte diapedesis between endothelial cells.

Selectins

The selectin family comprises three proteins: E-selectin (CD62E), L-selectin (CD62L) and P-selectin (CD62P). They all contain a lectin domain, an epidermal growth factor domain, and a variable number of short consensus repeats of 60 amino acids present in the complement regulatory proteins. The three selectin genes are located on chromosome 1. E- and P-selectins are expressed on endothelial cells, while L-selectin expression is restricted to leukocytes. Selectins bind to carbohydrate ligands via their lectin domain. It has been shown that tetrasaccharides sialyl Lewis X and sialyl Lewis A have a ligand activity for all the three selectins. The role of selectins in leukocyte transmigration is still debated.

E-Selectin E-Selectin (CD62E) is a 115 kDa glycoprotein, only expressed on endothelial cells after activation by interleukin 1 (IL-1), tumor necrosis factor α (TNF α) or bacterial lipopolysaccharides. After endothelial cell stimulation, newly synthesized E-selectin is rapidly detected, with a maximal surface expression after 3–6 h and a return to basal levels within 24 h. This rapid downregulation, although not completely understood, has been explained by the release of a soluble form of E-selectin, and internalization of the molecule. This regulation of E-selectin expression might be crucial to control leukocyte accumulation in inflammatory responses. Several ligands for E-selectin have been identified on leukocytes, but have not yet been cloned. Monoclonal

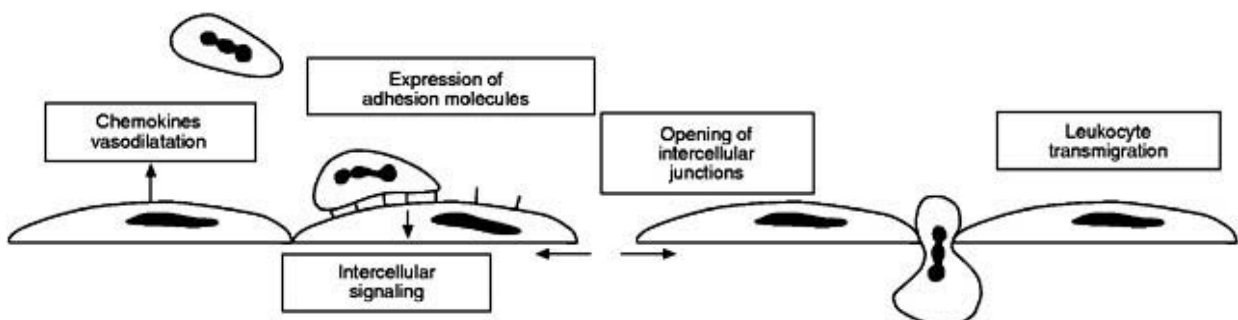


Figure 1 Leukocyte extravasation as a multistep process regulated by endothelium.

antibodies (mAbs) specific for E-selectin have been shown to inhibit leukocyte transmigration. It has been suggested that binding of leukocytes to E-selectin on activated endothelium upregulates CD11b (Mac-1) on the leukocytes, and induces an increased adhesion through ICAM-1/Mac-1 interaction.

P-Selectin P-Selectin (CD62P), previously termed PADGEM or GMP-140, is a single chain glycoprotein of 140 kDa expressed in platelets and endothelial cells. In platelets, P-selectin is stored in α granules, whereas in endothelial cells it is found in Weibel-Palade bodies. After activation, P-selectin is mobilized to the external plasma membrane within minutes. This increase in P-selectin expression is transient, and the protein is rapidly internalized inside the cell, where it is degraded or recycled. P-selectin is also upregulated transcriptionally by TNF α . Two ligands have been identified: the P-selectin glycoprotein ligand 1 (PSGL-1), expressed on various leukocytes, and a 120 kDa ligand expressed only on myeloid cells. P-selectin-deficient mice have been shown to be deficient in leukocyte extravasation.

ICAM-1

In general, interaction of leukocytes with ICAM-1 seems to be necessary for their extravasation. ICAM-1 (CD54) is a single-chain membrane glycoprotein of 80–115 kDa, with five immunoglobulin (Ig)-superfamily domains in its extracellular portion. ICAM-1 is moderately expressed on resting endothelial cells, but at sites of inflammation and immune responses the release of cytokines, such as TNF α , IL-1 or interferon γ (IFN γ), results in augmented cellular expression of ICAM-1. The expression of ICAM-1 has also been demonstrated on lymphocytes, monocytes and other nonhematopoietic cells, such as fibroblasts, epithelial cells and mucosal cells. ICAM-1 is a ligand for CD11a/CD18 (LFA-1) and for CD11b/CD18 (Mac-1). The primary binding site for CD11a/CD18 is located in the NH₂-terminal first Ig-like domain of ICAM-1, with domain 2 also involved in this interaction, while the binding site for CD11b/CD18 is in the third Ig-like domain. ICAM-1 also is a receptor for the major group of rhinoviruses and the malaria trophozoite *Plasmodium falciparum*; the binding sites for both ligands, though distinct from the LFA-1 binding site, are located in the first two domains of the ICAM-1 molecule. In addition, ICAM-1 is a receptor for CD43 and hyaluronan. The interaction between Mac-1/LFA-1 (CD11b/CD18) and endothelial ICAM-1 is a well-documented adhesion pathway, important in the adhesion and extravasation of leukocytes. It has

recently been shown that fibrinogen is a ligand for ICAM-1, and that fibrinogen binding to ICAM-1 results in enhanced adhesion of leukocytes to endothelial cell monolayers and an increase in their transendothelial migration. These results suggest that fibrinogen might act as a bridging molecule: for monocyte and polymorphonuclear leukocyte adhesion, it could bind to leukocyte CD-11b/CD18 and to endothelial cell ICAM-1; while for lymphocyte adhesion it could interact with two ICAM-1 molecules on opposing cells. This interaction between fibrinogen and ICAM-1 was inhibited by a commercially available mAb (LB2) specific for the first immunoglobulin domain of ICAM-1, suggesting that this domain is involved in the fibrinogen interaction with ICAM-1. However, the inhibition obtained with LB2 on fibrinogen-dependent adhesion was only partial, even at a high mAb concentration. This limited inhibition might reflect the fact that LB2 is not reacting with the exact binding site of fibrinogen on ICAM-1, but rather to a nearby site. Alternatively, an unidentified fibrinogen receptor present at the surface of endothelial cells could contribute to fibrinogen-mediated adhesion of leukocytes. Both fibrinogen-mediated leukocyte adhesion and transendothelial migration could be inhibited by a peptide from the fibrinogen γ chain.

VCAM-1

VCAM-1 (CD106) is a transmembrane glycoprotein of 110 kDa expressed only on cytokine-activated endothelium. A protein containing six Ig-superfamily domains was initially cloned (6D VCAM-1), but this form arises from alternative splicing of a gene which also encodes a seven Ig-superfamily domain form of VCAM-1 (7D VCAM-1), which is the dominant form on activated endothelium. VCAM-1 is a ligand for $\alpha_4\beta_1$ (VLA-4) and $\alpha_4\beta_7$ integrins. VLA-4 binds VCAM-1 through the first and the fourth Ig-like domain. Using mAbs, several studies have shown that VCAM-1 is involved in the transmigration of monocytes and eosinophils, but its involvement in lymphocyte transendothelial migration needs to be clarified.

PECAM-1

PECAM-1 (CD31) is a 130 kDa glycoprotein expressed on endothelial cells, platelets and some leukocytes. CD31 is constitutively expressed on endothelial cells, and its expression is not increased by cytokines. Molecular cloning studies have shown that CD31 is composed of six extracellular Ig-superfamily domains, a short transmembrane region, and a relatively long cytoplasmic tail of 118 amino acids containing potential sites for post-translational

modifications. Alternative splicing of the cytoplasmic tail can generate multiple CD31 isoforms that may differ in phosphorylation, cytoskeletal association and ligand affinity. CD31 is heavily glycosylated and glycosylation accounts for 40% of the mass of CD31. PECAM-1 appears to be able to interact both with itself in an homophilic interaction and with other molecules in an heterophilic interaction. In endothelial cells, CD31 is localized at intercellular junctions, and plays an important role in adhesion of endothelial cells. Its function might be regulated via phosphorylation of the cytoplasmic domain. PECAM-1 is directly involved in the process of leukocyte diapedesis between endothelial cells, as demonstrated by inhibition studies using PECAM-1-specific mAbs and soluble recombinant PECAM-1. Leukocytes blocked in transmigration by anti-PECAM-1 remained attached to the endothelium, clearly implicating PECAM-1 in diapedesis rather than in adhesion.

Cytokines and vascular endothelium

Endothelial cells and leukocytes interact closely in the activation and expression of immunity as well as in inflammatory reactions and hemostasis. Cytokines are important mediators of this interaction, endothelial cells being both a source and target for the action of polypeptide mediators.

Effects of cytokines on endothelial cells

Cytokines produced by activated lymphoid cells and mononuclear phagocytes elicit a complex spectrum of responses in vascular cells. These can be grouped into distinct sets of functions elicited by different cytokines (Figure 2).

IL-1 and TNF α IL-1 and TNF α induce the expression in endothelial cells of an activation program related to inflammation and thrombosis. The antithrombotic properties of endothelial cells

are profoundly altered by exposure to these inflammatory cytokines, which induce tissue-type procoagulant activity and suppress the cell surface anticoagulant activity mediated by the thrombomodulin–protein C pathway. They also induce the production of platelet-activating factor and augment production of an inhibitor of plasminogen activator, thus inhibiting the dissolution of fibrin polymers.

The production of the arachidonate metabolite prostacyclin (PGI₂) is stimulated by IL-1 and TNF α . Endothelial cell PGI₂ is involved in vasodilatation at sites of inflammation and cell-mediated immunity and in the hypotension associated with systemic administration of these mediators in experimental or therapeutic settings.

Endothelial cells treated with IL-1/TNF α play an active role in the recruitment of leukocytes from the blood compartment. While not directly chemotactic *per se*, IL-1 elicits leukocyte extravasation indirectly by changing the adhesive properties of endothelial cells and inducing production by these cells and tissue elements of leukocyte chemotactic and activating cytokines (CSFs; neutrophil activating protein/IL-8, active on polymorphs; monocyte chemotactic protein 1, active on mononuclear phagocytes).

Interferon γ As in other cell types, IFN γ augments major histocompatibility complex (MHC) class I, and induces MHC class II antigen expression in endothelial cells; it also augments lipopolysaccharide-induced production of IL-1. These changes render endothelial cells able to present antigen to T lymphocytes and thus to act as accessory cells. In the same perspective one can view the slow enhancement of ICAM-1 expression produced by this cytokine: ICAM-1, presumably by facilitating cell-to-cell interaction, can facilitate antigen presentation under conditions of low density of class II MHC. Thus, IFN γ activates a set of properties of endothelial cells related to accessory cell function, without inducing proinflammatory/prothrombotic or proliferative/migratory activities.

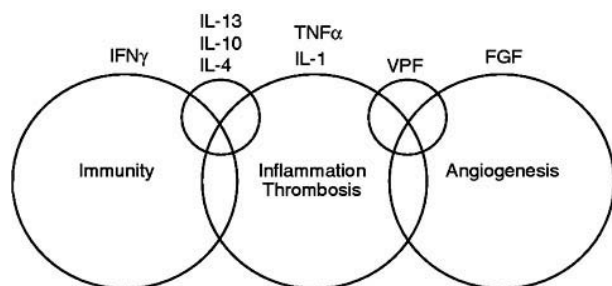


Figure 2 Functional programs activated by cytokines in endothelial cells. VPF, vascular permeability factor, also known as vascular endothelial growth factor; FGF, fibroblast growth factor.

Granulocyte and granulocyte-macrophage (GM) colony-stimulating factors (G-CSF, GM-CSF), transforming growth factor β (TGF β) and fibroblast growth factors (FGFs) These cytokines, which regulate growth and differentiation of hematopoietic cells (G-CSF and GM-CSF), mesenchymal cells (FGFs) or both (TGF β), induce proliferation and migration of endothelial cells. The induction of endothelial cell growth by TGF β is only observed in three-dimensional culture systems, with an inhibitory effect when cells are grown in monolayer. Consistent

with these *in vitro* activities, these cytokines induce angiogenesis *in vivo*.

IL-4 and IL-13 IL-4 and IL-13 selectively induce VCAM-1 and inhibit E-selectin and ICAM-1. These cytokines also amplify production of chemokines by endothelial cells. It is likely that these actions of IL-4 are important in the pathogenesis of the late-phase reaction which is associated with allergy.

Cytokines produced by endothelial cells

Endothelial cells produce cytokines that regulate the hematopoietic system, the proliferation and differentiation of T and B cells and the recruitment of leukocytes at sites of inflammation; endothelial cells are important producers of CSFs, including G-CSF, GM-CSF and M-CSF. Since G-CSF and GM-CSF act on endothelial cells themselves (see above), one can speculate on autocrine circuits involving endothelial cells and these cytokines, important for sustaining the hematopoietic microenvironment. Upon exposure to inflammatory stimuli (bacterial lipopolysaccharide, IL-1 and TNF α), endothelial cells produce IL-1 and IL-6. These are pleiotropic cytokines which affect the proliferation and differentiation of T and B cells and hematopoietic precursors and induce hepatocytes to produce acute-phase proteins. Thus by producing IL-1 and, at high levels, IL-6, endothelial cells take part in the regulation of specific immunity and acute phase responses. Finally, endothelial cells have the capacity to produce chemokines (MCP-1, RANTES and IL-8) which activate and induce chemotactic migration of leukocytes.

Overview

Endothelial cells are strategically located at the interface between circulating blood elements and tissues. It is therefore not surprising that these cells play an important active role in immunity, inflammation and hemostasis. Cytokine molecules are used as com-

munication signals in the complex bidirectional interaction between leukocytes and endothelial cells. Endothelial cells represent a source of cytokines and, at the same time, a target for the action of various of these polypeptide mediators. Cytokine-induced endothelial cell responses follow discrete patterns: a program related to inflammation and thrombosis is activated by IL-1 and TNF α , while IFN γ activates accessory cell functions. FGFs, G-CSF and GM-CSF elicit functions related to angiogenesis.

In the complexity of the activation patterns they can express, endothelial cells are reminiscent of mononuclear phagocytes: the long utilized term of 'reticuloendothelial system', although now largely abandoned, reflected the recognition of this similarity and interrelationship.

See also: Adhesion molecules; Chemokines; Colony stimulating factors; Cytokines; High endothelial venules; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Interferon γ ; Interleukin 1 and its receptors; Interleukin 4; Interleukin 13 and its receptor; Lymphocyte trafficking; Selectins (CD62-E/L/P); Transforming growth factor β (TGF β); Tumor necrosis factor α .

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ENDOTOXIN (LIPOPOLYSACCHARIDE (LPS))

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It is more than 100 years ago since it was recognized that the pathophysiological activities manifested during gram-negative infections are caused by components of the bacterial surface. Different observations at that time led to the recognition that the toxic principle present in the bacteria was a

firmly bound thermostable substance that was released after death and lysis of the bacterial cells. Hence the designation endotoxin in order to distinguish it from the heat-labile exotoxins which are released from live bacteria. The term endotoxin was first introduced at the beginning of the twentieth

century, most probably by Richard Pfeiffer, a pupil of Robert Koch.

Chemistry

Chemically, endotoxins are lipopolysaccharides (LPSs). They are macromolecules consisting of two covalently linked parts with contrasting chemical and physical properties: a hydrophilic polysaccharide which is subdivided into the O-polysaccharide and the core oligosaccharide, and a hydrophobic lipid region, the lipid A. Such complete LPSs, containing lipid A, core and O-polysaccharide are designated S (smooth)-form LPSs.

The O-polysaccharide is characterized by a high structural diversity among different bacterial genera and among the different serotypes of one and the same genus. It is built up of repeating oligosaccharide units whose structure and composition vary among the different LPSs and determine the O-serological specificity of the given LPS and of the parent bacterial strain. The core is more conservative in its structure and composition so that the relatively few core types identified so far are common to large groups of bacteria. In *Salmonella* the core is represented by an oligosaccharide containing D-glucose, D-galactose, N-acetyl-D-glucosamine, L-glycero-D-manno-heptose and 3-deoxy-D-manno-2-octulosonic acid (KDO). In addition, phosphate and ethanolamine are present. Lipid A is the most conservative part of the LPS molecule; its structure and composition are very similar among Enterobacteriaceae. In *Salmonella*, lipid A consists of a biphosphorylated disaccharide of D-glucosamine in β -1,6-linkage. The disaccharide carries amide- and ester-linked residues of 3-hydroxymyristic acid, which themselves may be esterified with lauric, myristic and palmitic acid. Other constituents frequently present in lipid A are phosphorylethanolamine and 4-amino-4-deoxy-L-arabinose.

The endotoxic activity of LPS resides in the lipid A part of the molecule, the polysaccharide being devoid of toxic properties. All three regions of the LPS, O-polysaccharide, core and lipid A are immunogenic, eliciting the formation of antibodies specific for structures in the respective region.

The presence of the core and of the O-repeating oligosaccharide units is a structural feature of many LPS of Enterobacteriaceae. In LPSs of other genera, however, the overall architecture of the molecule may vary considerably. The same applies to lipid A, whose structure and composition may be subject to large variations, as in the case of photosynthetic bacteria. Here, due to differences in lipid A structure, the LPSs of many photosynthetic bacteria are non-

toxic and may therefore not be designated as endotoxins.

Mutants have been isolated from many gram-negative bacteria – the so-called rough (R) mutants designated Ra to Re. Owing to biosynthetic defects they synthesize incomplete LPSs, lacking the O-polysaccharide, with the core being present in different degrees of completion. S-form bacteria also synthesize R-form LPS which is present in variable amounts in all S-form LPS preparations.

Isolation and heterogeneity

Two widely used methods for the isolation of S- and R-form LPSs in pure form are the aqueous phenol and the phenol chloroform petroleum ether methods, respectively. LPSs obtained by the latter method are essentially free of the usual contaminants (proteins). Purified LPSs are found to be highly heterogeneous. Analysis by sodium dodecyl sulfate or sodium deoxycholate-polyacrylamide gel electrophoresis results in the resolution of the LPSs in numerous bands in a ladder-like arrangement. This heterogeneity is mainly due to the presence of molecules of varying polysaccharide chain length (e.g. varying number of repeating units); LPSs also exhibit a microheterogeneity resulting from differences in the number of substituents such as phosphate ethanolamine, 4-amino-arabinose and acyl residues in the lipid A.

Biological properties

Endotoxins represent the pyrogens (fever-inducing agents) of gram-negative bacteria, pyrogenicity being probably the first activity of endotoxin recognized. Endotoxins are endowed with a large spectrum of biological activities that may be demonstrated *in vivo* and *in vitro*. Of these, pyrogenicity, leukopenia and leukocytosis, induction of the local, generalized Schwartzman phenomenon, and lethal shock are examples of classical endotoxic activities. A summary of the more important biological activities of endotoxin is given in Table 1.

Although endotoxins are so named after their toxic properties, they also exhibit a spectrum of beneficial activities. They induce nonspecific resistance to different infectious agents and to their own toxic effects. Endotoxins induce mitogenic stimulation and antibody synthesis and secretion in mouse B lymphocytes. The mitogenic activity of endotoxin for mouse B cells may be demonstrated both *in vivo* and *in vitro*. It is interesting that LPS exhibits no mitogenic activity for human or rabbit B lymphocytes *in vitro*.

Table 1 Biological activities of lipopolysaccharides and free lipid A

Pyrogenicity	Induction of nonspecific resistance to infection
Lethal toxicity in mice	Induction of tolerance to endotoxin
Leukopenia	Induction of early refractory state to temperature change
Leukocytosis	Adjuvant activity
Local Schwartzman reaction	Mitogenic activity for cells
Bone marrow necrosis	Tumor necrotic activity
Embryonic bone resorption	Macrophage activation
Complement activation	Induction of colony-stimulating factor
Depression of blood pressure	Induction of immunoglobulin G synthesis in newborn mice
Platelet aggregation	Induction of prostaglandin synthesis
Hageman factor activation	Induction of interferon production
Induction of plasminogen activator	Induction of tumor necrosis factor α and other cytokines
<i>Limulus</i> lysate gelation	Induction of mouse liver pyruvate kinase
Toxicity enhanced by growing tumors	Type C RNA virus release from mouse spleen cells
Toxicity enhanced by BCG, <i>P. acnes</i>	Helper activity for friend spleen focus-forming virus in mice
Toxicity enhanced by gram-negative infection	Inhibition of phosphoenolpyruvate carboxykinase
Toxicity enhanced by adrenalectomy	Hypothermia in mice
Toxicity enhanced by D-galactosamine	
Enhanced dermal reactivity to epinephrine (adrenaline)	

Mechanisms of action

The mechanisms by which endotoxin exerts its toxic activities remained unknown up to the middle of the 1980s. Today it is generally agreed that the biological activities of endotoxin are caused indirectly through the action of endogenous mediators induced after interaction of endotoxin with primary targets. Macrophages are probably the most important targets of endotoxin, playing a central position in the development of endotoxicity. Following interaction with endotoxin, macrophages are activated to produce a number of cytokines, such as tumor necrosis factor α (TNF α) and various interleukins (ILs). TNF α is a primary mediator of the lethal activity of endotoxin. Exogenous TNF α administered into experimental animals induces a lethal shock that is indistinguishable from that induced by endotoxin. In certain experimental models antibodies to TNF α inhibit the development of endotoxin shock.

Susceptibility to endotoxin

Sensitivity towards endotoxin is genetically determined – different animals showing an enormous variation in endotoxin susceptibility. Rabbits, dogs and swine are very sensitive, while mice, rats and guinea pigs are, in comparison, much less sensitive. Humans belong to the most endotoxin-sensitive mammals, an amount of 0.03 μg (per 70 kg body weight) of pure endotoxin administered intravenously sufficing to cause a marked leukopenia after 1 h, which is followed by leukocytosis 3 h later. Higher amounts (e.g.

0.1–0.3 μg per adult individual) elicit marked pyrogenic effects that may be accompanied by shivering, headaches, nausea and vomiting. The lethal amount of endotoxin for normal humans is estimated to be of the order of a few micrograms. Therefore, compared to mice, in which the LD₅₀ is in the range of 200 μg (per 15 g mouse), humans exhibit a several thousand-fold higher sensitivity to endotoxin.

Tolerance and sensitization to endotoxin

Experimental animals receiving minute amounts of endotoxin may become tolerant to a subsequent challenge with pyrogenic or lethal amounts of endotoxin. In humans an initial pyrogenic dose of endotoxin induces tolerance to the pyrogenic effect of subsequent endotoxin challenges carried out within short intervals (less than 10 days).

In contrast, normal sensitivity to endotoxin may be increased by treatment with live or killed gram-negative and -positive bacteria. Examples of these are BCG, *Propionibacterium acnes*, *Coxiella burnetii* and *Salmonella typhimurium*. Interferon γ (IFN γ) is an important mediator of the bacteria-induced sensitization to endotoxin. Sensitization to endotoxin is also induced by parasitic and viral infections. Also, hepatotoxic agents, such as D-galactosamine and α -amanitin, and various chemical agents, including actinomycin D, lead acetate and carbon tetrachloride enhance endotoxin activity. In addition, growing tumors, depletion of cortisone (in mice) by adrenalectomy or acute graft-versus-host reaction will lead to a higher sensitivity towards endotoxin.

LPS-binding proteins

It is interesting that LPS, due to its unique physico-chemical properties, exhibits a high affinity for cells of all kinds with which it interacts, regardless of whether these can be activated by LPS or not. LPS also interacts with various plasma and serum components and also with surfaces of all different kinds (glass, plastic, rubber, etc.).

A number of components of plasma or of cellular origin have been demonstrated to interact more specifically with purified LPS. These include, in addition to specific antibodies, high- and low-density lipoproteins, membrane and soluble CD14, LPS-binding protein, bactericidal permeability-inducing protein and others. The final role of these components for LPS activity, which in some cases has been shown to be inhibitory and in others enhancing, is still under investigation.

See also: Bacterial cell walls; Fever; Statistics, use in immunology; Toxins; Tumor necrosis factor α .

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ENHANCEMENT, IMMUNOLOGICAL

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The spectrum of antigens which any organism regards as self is not a fixed and immutable characteristic, but one which is subject to manipulation. This was first observed in nature by R.D. Owen in 1945, who discovered that cattle twins are life-long hematopoietic chimeras, as a consequence of vascular connections between the twin placentae and the exchange of hematopoietic stem cells *in utero*. This general phenomenon was reproduced experimentally in mice in 1953 by Billingham, Brent and Medawar. These observations stimulated great hopes that potent, antigen-specific approaches to immunosuppression, mimicking self-tolerance, would enable clinical transplantation to proceed on diverse fronts. In the event, contrary to all expectations in the 1950s, clinical transplantation has flourished using nonspecific immunosuppression.

Some definitions

Immunological enhancement, tolerance induction and related terms in transplantation are best brought under the single heading of donor-specific immunosuppression. This can be defined as immunosuppres-

sion which, directly or indirectly, inactivates those clones of lymphocytes responsible for the rejection of the particular graft a recipient receives. It is antigen-specific immunosuppression, and leaves untouched the bulk of the recipient's immune system for the defense against infection and the suppression of some tumors. Its great virtue is safety.

General principles

In recent years, major advances have been made in understanding the immunological mechanisms involved in immune nonreactivity to self, i.e. self-tolerance. Three broad mechanisms to prevent the action of potentially antiself-reactive lymphocyte clones appear possible: destruction (clonal deletion); continued survival but nonreactivity (clonal anergy); and suppression by regulatory T cells (clonal suppression). How these mechanisms relate to those involved in nonreactivity to grafted tissue is not certain. Where hematopoietic chimerism is induced, allowing bone marrow stem cells of donor type to seed the recipient thymus, the mechanisms of non-reactivity might be similar to those of self-tolerance.

However, in most clinically relevant situations, involving adult recipients of solid organ grafts, the predominant mechanism is likely to be that of T cell-mediated suppression.

A variety of additional factors, not applicable in self-tolerance, might also be operating in adult transplant recipients, e.g. altered immunogenicity of the graft, antibody-mediated suppression.

Traditionally, there have been two major approaches to donor-specific immunosuppression in transplantation:

1. *Antigen-induced suppression (active enhancement)* refers to specific immunosuppression induced by treatment of the recipient with preparations containing donor histocompatibility antigens.
2. *Antibody-induced suppression (passive enhancement)* refers to specific immunosuppression induced by treatment of the recipient with antibodies directed at donor histocompatibility antigens.

Over the past few years, there has been renewed interest in the potential of *anti-idiotypic immunity* for regulating immune responses. Very impressive work has been done on the suppression of experimental autoimmune encephalomyelitis (EAE) by immunizing with autologous T cell clones and even synthetic peptides corresponding to T cell receptor variable regions. However, whether or not these ideas can be translated in practice to the more complex area of immunosuppression in clinical transplantation remains to be seen.

The 'induction' and 'maintenance' phases

When considering donor-specific immunosuppression, one usually concentrates on the induction phase, that is, the period around the time of grafting when the recipient is being treated. During this phase, the immunological interactions in the recipient are likely to vary greatly, depending on the particular protocol used for donor-specific suppression. Several weeks or months after grafting, when active treatment has stopped and the graft has reached a more-or-less stable state, one enters the 'maintenance' phase. There is no precise distinction between the induction and maintenance phases, but it is nevertheless a useful concept to bear in mind. During the maintenance phase, the immunological mechanisms involved in graft acceptance are likely to be the same, irrespective of the precise protocols of immunosuppression (whether they be donor specific or nonspecific) used in the induction phase. The

presence of the graft is likely to be the dominant immunoregulatory factor.

Active enhancement

The first example of active enhancement was in fact published in 1906 by S Flexner and JW Jobling. At the turn of the century, there was much interest in the potential of vaccination as a therapeutic procedure. Flexner and Jobling immunized rats with a heat-killed transplantable sarcoma in the expectation that on rechallenge with live tumor the 'vaccination' would protect against tumor growth. However, the tumors in vaccinated rats grew much more rapidly than normally, a result which of course did not fit into any established theoretical framework of that era.

When considering active enhancement, a clear distinction must be drawn between experiments where neonatal animals are treated with donor bone marrow, and those where adult graft recipients are treated with various antigen preparations. In the neonatal situation, the treated animals become hematopoietic chimeras, and such chimerism, even if at low levels, is essential for maintaining the state of non-reactivity towards the donor strain. These experiments are unlikely to be relevant to donor-specific immunosuppression for organ allografts in adult animals, where classical hematopoietic chimerism does not occur.

The first experiments which successfully induced life-long acceptance of allografts in adult animals were performed by F Shapiro and colleagues in 1961. This group used active enhancement to suppress the rejection of skin grafts in mice. Since that time, a huge literature on this subject has been published, involving innumerable permutations and combinations of therapy. What has emerged is that active enhancement can be a very powerful approach to donor-specific immunosuppression. However, it should be remembered that exposure to antigen normally results in sensitization, i.e. a state of specific heightened immune reactivity. The factors regulating suppression versus sensitization following antigen exposure are poorly understood. It is this risk of unpredictable sensitization (leading to more vigorous and rapid graft rejection than normal) which has effectively precluded the clinical application of active enhancement to the present time.

Factors favoring suppression

From the mass of experiments on active enhancement, one can discern certain aspects of protocol which favor the development of specific immuno-

suppression, rather than sensitization. These are as follows:

1. *Route of administration.* The intravenous route is most favorable. In many studies, it has been demonstrated that identical treatments given intravenously and by other routes have resulted in suppression by the intravenous route, and sensitization by other routes (e.g. subcutaneous, intraperitoneal).
2. *Dose of antigen.* In general, the higher the dose of antigen, the more likely it is to induce immunosuppression.
3. *Timing of antigen exposure.* Multiple, closely spaced treatments are most effective. Where single treatments are given, a period of 1–2 weeks before grafting is the most favorable.
4. *Physical state of the antigen.* Donor histocompatibility antigens can be given in many ways, varying from whole living cells through to highly purified, water-soluble molecules. For many years, it was widely believed that genuinely water-soluble histocompatibility antigens would offer potent donor-specific immunosuppression, free from the risk of sensitization. However, it was not possible to test this idea until the recent availability of histocompatibility antigens lacking the hydrophobic transmembrane domain. Such water-soluble molecules are often ineffective for donor-specific suppression.
5. *Concurrent use of nonspecific immunosuppressive agents.* The use of active enhancement in conjunction with agents such as antilymphocyte serum, corticosteroids, azathioprine and cyclosporine has frequently been shown to result in better graft survival than that achieved with either agent alone.

The risk of sensitization

This is most obviously seen when antibodies develop to the histocompatibility antigens used in the active enhancement protocols. If these react with the graft donor, then, in the clinical situation, hyperacute rejection will occur at the time of grafting. Cellular sensitization, in the absence of antibodies, is likely to go unnoticed before grafting, but can give rise to early and vigorous rejection episodes which cannot be controlled by the immunosuppressive drugs currently in use.

Blood transfusions

In the late 1960s and early 1970s, clinical transplantation centers worldwide introduced policies of minimum transfusion, to try to decrease the potential risk of sensitization to histocompatibility antigens. How-

ever, kidney graft rejection in the transplant patients became more severe. In 1973, Opelz and Terasaki reported that random pregraft blood transfusions in fact markedly improved graft survival. By the late 1970s the pendulum had come full swing, and many centers would not transplant patients who had not previously been transfused. In many centers, there are now policies of deliberate blood transfusion, i.e. transfusions given not for anemia, but specifically for their beneficial effect on the survival of a subsequent graft.

The mechanism whereby blood transfusions are effective is unknown, but is likely to involve some form of active enhancement. The fact that the beneficial effect can be obtained from a single, random transfusion from a donor unrelated to the eventual kidney donor raises questions of specificity. However, given the large number of histocompatibility loci now known to exist, the probability that unrelated individuals might share one histocompatibility antigen is not insignificant. Moreover, many histocompatibility antigens have 'public' determinants, i.e. allodeterminants present on the products of many alleles.

Passive enhancement

The use of antibodies to donor histocompatibility antigens to suppress graft rejection was first demonstrated in tumor transplantation studies in the early 1950s. When passive enhancement was tested with skin grafts, its effect was noted to be weak. Graft survival was prolonged by only 2 or 3 days, and the clinical applicability of this approach was not considered of any great interest. However, the development of microsurgical techniques in the 1960s made possible the use of vascularized heart and kidney allografts in studies in inbred rodents. By the late 1960s, French and Batchelor in the UK and FP Stuart and his coworkers in the USA had established that passive enhancement was remarkably successful at suppressing kidney graft rejection in rats. Treatment of graft recipients with one or two injections of anti-donor antiserum at the time of grafting could suppress the rejection response completely, and result in life-long acceptance of major histocompatibility complex (MHC)-incompatible kidney grafts. This caused considerable excitement at the time, and the first attempts to reproduce this phenomenon in the clinic were reported in the early 1970s.

Mechanisms

The mechanisms whereby passive enhancement suppresses rejection is not clear. The most likely possibility is that the antidonor antibodies interact with

the interstitial dendritic cells (passenger leukocytes) of the graft and thereby prevent these highly immunogenic cells from stimulating the host's immune system.

Specificity of the enhancing antibodies

Antibodies to both class I and class II MHC antigens of the donor have been shown to be effective for passive enhancement. However, it is possible that anti-class II antibodies might be more effective.

The importance of the Fc region of enhancing antibody

There are many conflicting reports on the efficacy of the different classes and subclasses of antibody for passive enhancement. Overall, it is likely that immunoglobulin M (IgM) is not very effective and that the complement fixing classes (e.g. IgG2a in the mouse) are most effective. What is clear, however, is that F(ab')₂ fragments of enhancing sera are totally ineffective, and this is an important point to note.

Potency of passive enhancement

The high hopes for passive enhancement stemmed from studies with organ grafts in inbred rats. Even here, however, the efficacy varies markedly in different donor/recipient strain combinations. Several attempts at passive enhancement in larger experimental animals (dogs, primates) have been disappointing. Passive enhancement is clearly a relatively weak form of immunosuppression, but one that could make a contribution as part of a more complex protocol for immunosuppression.

The problem of hyperacute rejection

In humans and most large experimental animals, the presence at the time of grafting of antibodies to donor histocompatibility antigens results in immediate graft destruction ('hyperacute' rejection). This is caused by the interaction of the antibodies with the vascular endothelial cells of the graft, the fixation of complement and the consequent triggering of intravascular blood coagulation and vascular spasm. For reasons which are not entirely clear, this does not occur in most donor-recipient strain combinations studied in the rat. This has allowed passive enhancement to be studied in great detail in inbred rats.

However, the injection of antidonor antibodies in the clinical situation carries the risk of inducing hyperacute rejection. The early clinical studies attempted to overcome this problem by using F(ab')₂ and Fab fragments, because these preparations would not fix complement. However, it later became clear that such fragments are totally without enhancing activity. The use of noncomplement fixing IgG subclasses for passive enhancement is probably the only available option. In fact, noncomplement fixing mouse IgG1 monoclonal antibodies to polymorphic determinants of rat MHC antigens have been shown to be effective for passive enhancement, although large doses of antibody were required.

Future prospects

It has been demonstrated on several occasions that long-surviving patients develop at least an element of donor-specific nonreactivity (e.g. disappearance of cytotoxic T cell precursors against donor antigens). This demonstrates that antigen-specific downregulation of the immune system towards a graft is certainly possible in humans, and that what is required is to achieve this state at the time of grafting. The induction of donor-specific immunosuppression in clinical transplantation is probably the most important goal in transplantation research. When it is achieved, it will mean in essence that immunosuppression will be both safe and effective. This will not only greatly benefit existing clinical transplantation programs, but will broaden the use of transplantation as a therapeutic procedure for the replacement of diseased and defective tissues.

See also: Chimerism, hematopoietic; Graft rejection; Idiotypic network; Immunosuppression; Tolerance, peripheral; Transplantation.

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ENZYME LABELING OF ANTIBODIES AND ANTIGENS

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Based on the principle that an antibody reacts reversibly with its corresponding antigen in a highly specific way, several procedures have been developed in which antibodies or antigens labeled with appropriate tracers are used for the localization, detection or quantitation of substances of biological interest. The precursor of this type of procedure, immunofluorescence, based on the use of antibodies conjugated with fluorescein, was employed for the localization of cellular antigens, and was introduced by A Coons and collaborators in 1941. Since then, the techniques based on labeled antibodies or antigens have been diversified and developed. They now represent the most sensitive and widely used techniques. Radioelements, fluorochromes, heavy metals and enzymes, at this moment, constitute the most often used tracers.

Enzymes as tracers were introduced in 1966 by PK Nakane and GB Pierce in the USA and S Avrameas and J Uriel in France. The former group employed antibodies labeled with peroxidase for the immunocytochemical detection of cellular constituents, while antigen labeled with peroxidase or trypsin and antibodies labeled with peroxidase were used by the latter group for the detection of immune precipitates. Enzymes, because of their catalytic nature, offer the possibility of high amplification associated with either simple or sophisticated systems of detection and/or quantitation. This confers to immunoenzymatic procedures a flexibility of adaptation and, consequently, a wide range of applications. Thus at present various immunoenzymatic techniques are available and employed in such different fields as basic research, human and veterinary medicine, agriculture, and food and drug control. Such immunoenzymatic techniques are mainly employed either for the localization of tissue constituents, the detection of small amounts of antigen present in complex biological media, or the measurement of humoral and cellular components.

General principles

The basic principles underlying the various immunoenzymatic procedures are similar, if not identical, to those already developed for the other immunological procedures using labeled antibodies or antigens. In

immunoenzymatic procedures, however, enzyme-labeled antigens are used only occasionally and almost exclusively for the quantitation of small molecular weight substances. In all the other cases, enzyme-labeled antibodies are employed and the steps followed are more or less similar. Thus, as for immunofluorescence, in the enzyme-immunocytochemical localization of a tissue antigen an unlabeled antibody prepared in species A is usually first allowed to react with the cellular preparation. Then, after washings, an antibody, recognizing the immunoglobulins of species A, linked to an enzyme by means of either covalent bonds or biospecific covalent interactions, is added. Finally, after incubation and washings, the sites of enzyme activity are revealed by a cytochemical substrate, specific to the enzyme used as the tracer. Similarly, in order to detect an antigen in a complex biological medium, after an appropriate separation of the various constituents present in this medium, for example by electrophoresis in polyacrylamide gels, the antigen is immobilized on an insoluble phase, such as nitrocellulose or nylon, incubated with a specific unlabeled antibody and then the same sequence of steps, as above, are followed. For the localization or detection of antibodies, similar procedures are used. Thus, to the cellular or otherwise immobilized antibody, the antigen is added, allowed to react, then an enzyme-labeled antibody recognizing the antigen is added and finally the enzyme is revealed by an appropriate substrate.

Even if enzyme immunoassays enabling the quantitation of antigens (or antibodies) present some special features, the basic design and reagents used are similar.

From the above, it appears therefore that antibody, enzyme, and antibody-enzyme linkage are the most important elements of effective and sensitive immunoenzymatic assays.

Enzymes as labels

In principle any enzyme can be used as a label but, in practice these enzymes should express certain characteristics. Since all immunoenzymatic procedures are performed at room temperature, the first requirement is that they be stable under these

conditions. The enzymes used must be the purest possible, i.e. must have a high specific activity and act quickly on their substrate, i.e. have a high turnover number. Furthermore, the procedures used for their detection and/or measurement should be sensitive enough and, preferably, either simple to perform or amenable to automation. Finally, they should not lose a substantial part of their activity after their linkage to the antibodies, and should be available in relatively high amounts.

Horseradish peroxidase and calf intestine alkaline phosphatase, which fulfill more or less all the above conditions, are the enzymes of choice and are the most often used in immunoenzymatic techniques. *Escherichia coli* β -galactosidase and *Aspergillus niger* glucose oxidase are also employed, although to a lesser extent. Occasionally *E. coli* alkaline phosphatase, soy bean urease, bovine liver catalase, *Staphylococcus aureus* penicillinase, and *Electricus eel* acetylcholinesterase are also used.

Depending on the substrates available, these enzymes can be used for the localization (and detection) or the measurement of antigens (antibodies) or both. Localization and detection necessitate the use of specific cytochemical substrates yielding highly colored insoluble products. In contrast, measurement requires the use of specific substrates giving rise to soluble, precisely measurable products. For peroxidase, alkaline phosphatase and glucose oxidase, which enable the localization, detection and measurement of antigens (or antibodies), suitable substrates are available. All the other enzymes, because of the lack of adequate cytochemical substrates, have been found useful only in quantitative immunoassays.

Antibodies

As in all immunologic techniques using labeled compounds, the antibodies employed in immunoenzymatic procedures can be distinguished as unlabeled ones, i.e. those that take part in the first reaction with the antigen, and as enzyme-labeled antibodies that are added secondarily and, usually, in high excess. The sensitivity of these assays depends essentially on the avidity of the first, while their performance depends mainly on the qualities and characteristics of the enzyme-labeled antibody. Since high avidity antibodies are usually present in hyperimmunized animals, when possible it is preferable to use polyclonal and/or monoclonal antibodies derived from such animals. In the great majority of cases, an enzyme-labeled polyclonal antibody preparation recognizing immunoglobulins (usually a mixture of anti- μ , and anti- γ and anti- α), or less frequently a

given antigen, is used. The immunoglobulin G (IgG) fraction or the antibodies specifically isolated from the antiserum are bound to the enzyme. Although good results are obtained with labeled IgG, it is preferable, when possible, to use isolated antibodies; the antigen-specific staining is more intense and the non-specific background is weaker.

Labeling of antibodies with enzymes

Antibodies (or antigens) are labeled with enzymes either by covalent or by biospecific noncovalent linkages. To obtain covalent antibody-enzyme conjugates a number of cross-linking agents have been tried, but only glutaraldehyde, *m*-periodate and maleimide derivatives are currently used. Glutaraldehyde, reacting under mild conditions with the free amino groups of proteins, has been used for the preparation of almost all the enzyme-antibody conjugates applied in immunoenzymatic techniques. Treatment of peroxidase (or other polysaccharidic enzymes) with *m*-periodate results in the generation of active aldehyde groups in the peroxidase molecule which react with the free amino groups of antibodies (or antigens) added subsequently. Treatment of enzymes with maleimide (or pyridyl disulfide) derivatives introduces into these molecules active maleimide groups that react with subsequently added antibody molecules carrying thiol groups. All these coupling procedures give rise to heterogeneous populations of enzyme-antibody conjugates, but under certain experimental conditions homogeneous preparations of conjugates composed of one molecule of peroxidase coupled to one molecule of antibody are obtained using either glutaraldehyde or maleimide derivatives as the cross-linking agent.

Enzymes are also linked to antibodies (or antigens) using a number of specifically interacting systems, but only the antigen-antibody and the avidin (or streptavidin)-biotin systems are currently being used. The procedures based on the antigen-antibody reaction require an antibody recognizing the antigen and a second antibody recognizing the enzyme label, both prepared in species A, as well as an antibody prepared in species B and recognizing the immunoglobulins of species A. In a first step, the antibody is allowed to react with the antigen, then the anti-immunoglobulin antibody is added, followed by the addition of the antienzyme antibody, the enzyme and the substrate. These procedures are based on the principles that: 1) enzyme-antienzyme complexes are always catalytically active; 2) the anti-immunoglobulin antibody, added in excess, reacts with the antigen-bound antibody only by one of its two active sites and thus can operate as acceptor for the antienzyme antibody. These multistep protocols are

somewhat shortened by the prior formation of an enzyme/antienzyme soluble immune complex, the most commonly used being the peroxidase-antiperoxidase (PAP) procedure.

Two procedures are used for linking enzymes to antibodies on the basis of the strong interaction between avidin and biotin (dissociation constant 10^{-15} M). The first requires the preparation of antibodies labeled with biotin and an avidin-enzyme conjugate. Biotinylated antibody is allowed to react with the antigen and this step is followed by addition of enzyme-labeled avidin. In the second method antibodies labeled with biotin, native avidin and enzyme labeled with biotin are used. This protocol involves, in the first step, the interaction of the biotinylated antibody with the antigen; in the second step, the incubation with avidin, followed by the addition of biotin-labeled enzyme and substrate. This technique is based on the principle that avidin possesses four specific binding sites able to create bridges between two proteins labeled with biotin. This assay is somewhat shortened by the prior formation of an avidin-biotinylated-enzyme complex and is often called the ABC procedure. Streptavidin, yielding less background, is being more and more used instead of avidin. Its affinity for biotin is similar to that of avidin.

Localization of tissue antigens by enzyme immunocytochemistry

Specific cytochemical procedures for staining enzymes are available for both light and electron microscopy. At the light microscope level, mainly peroxidase but also alkaline phosphatase and occasionally glucose oxidase are used as labels. Because cytochemical staining procedures for enzymes give rise to reaction products of different colors, enzyme-labeled antibodies can be used for the simultaneous detection of two different cellular constituents. Alternatively, paired staining techniques combining autoradiography or immunofluorescence and immunoenzymatic staining are employed for the detection of two antigens. Detection of antigens by immunoenzymatic and immunofluorescence staining give comparable results both in localization and sensitivity.

At the electron microscope level, peroxidase is almost exclusively used as the label because 1) its cytochemical staining permits precise localization and identification of ultrastructural details, and 2) relatively low molecular weight peroxidase-antibody conjugates penetrate more easily into the interior of fixed cells.

Detection of antigens by enzyme cytochemical staining (immunoblotting type)

Like radiolabeled antibodies, enzyme-labeled antibodies are used for the visualization by means of enzymatic cytochemical staining of immune complexes formed in gels or present on various solid carriers. Enzyme cytochemical staining in immunoblot-type procedures, introduced by Towbin and colleagues in 1979, has been found particularly useful and is currently being used. Such procedures increase considerably (10- to 100-fold over conventional protein staining) the sensitivity of the detection of an immunoprecipitate. All the considerations concerning enzyme-immunocytochemical staining are also applicable to these procedures, but apparently better results are obtained with substrates giving rise to highly contrasting colors ranging from blue to black.

Quantitation of antigens by enzyme immunoassays

A number of procedures can be used for the precise measurement, by enzyme immunoassay, of minute amounts of antigens. Enzyme immunoassays, or in their abbreviated form EIA, are divided into homogeneous and heterogeneous tests. In homogeneous assays there is no need to separate the antigen-antibody immune complexes from free antigen and/or free antibody; however, such a separation is required in heterogeneous assays. The heterogeneous enzyme immunoassays are described in the entry entitled ELISA.

The homogeneous assays are based on antibody-mediated changes in enzyme activity. A specific antibody, by reacting with an antigen-enzyme conjugate, diminishes the capacity of the enzyme to interact with its substrate and, consequently, decreases the enzyme's activity; addition of free antigen proportionally restores the enzyme's activity. Satisfactory results are obtained in these assays only when the antigen is hapten and the enzyme label is either glucose-6-phosphate dehydrogenase or malate dehydrogenase. To date, the most widely used homogeneous enzyme immunoassay, known commercially as EMIT (enzyme-multiplied immunotechnique), is employed, essentially to test drugs. It is rapid and simple. However, its sensitivity is limited and therefore it is applicable only when ligands are present in relatively high concentrations. Several variants of this basic homogeneous enzyme immunoassay employing enzyme inhibitors, prosthetic enzyme groups, enzyme substrates and two enzymes

catalyzing consecutive reactions have been described. Although they are based on original and interesting principles, such procedures are only rarely employed, if at all.

See also: **Electron microscopy, immunological applications; Enzyme-linked immunosorbent assay (ELISA); Immunoassays; Immunocytochemistry and enzyme markers; Western blotting.**

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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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Yalow and Berson's development in 1959 of the radioimmunoassay based on the use of a new tool, i.e. an antigen labeled with a radioactive tracer, introduced the ability to measure a substance with both accuracy and high specificity. Although radioimmunoassay is highly sensitive and versatile and it has been applied with success in various fields, its further extensive development has been hampered by the inherent drawbacks encountered with radioisotopes. In order to overcome these difficulties, several alternative procedures based on the use of other marker substances, such as free radicals, chemiluminescent or fluorescent labels, bacteriophages and enzymes, were proposed. Among them, enzyme immunoassays, probably because of the high amplifying capacity of the enzyme marker and the relatively simple laboratory equipment they require, have been extensively developed during the past 15 years.

Enzyme immunoassays were first reported in 1971 by S. Avrameas and B. Guilbert in France, E. Engvall and P. Perlmann in Sweden, and B. van Weemen and H. Schuurs in Holland. They were employed for the quantitation of antigens and subsequently for the titration of antibodies. These enzyme immunoassays were based on principles similar to those of radioimmunoassays and, therefore, necessitated the use of an antigen or antibody immobilized on a solid phase, i.e. an immunoadsorbent, to separate the free antigen

or antibody from the antigen–antibody immune complex. These heterogeneous or solid-phase enzyme immunoassays were termed, by Engvall and Perlmann, enzyme-linked immunosorbent assay or ELISA. In 1972 Rubinstein and collaborators introduced enzyme-multiplied immunotechnique (EMIT), a homogeneous enzyme immunoassay, in which the immunological reaction and the measurement of the enzyme were performed in the same liquid medium without an immunoadsorbent.

Principles of heterogeneous enzyme immunoassays

The principles of ELISA, as already mentioned, are similar to those developed for quantitative radioimmunoassays but measurement of enzyme activity replaces radioactivity counting.

To quantify antigens by ELISA, both competitive and noncompetitive procedures are used. The first competitive assay, similar to the conventional radioimmunoassay, consists of incubating the antigen to be measured, a constant amount of the same antigen labeled with an enzyme, and a constant and limited amount of immobilized antibody. After washings, the enzyme activity associated with the solid phase is measured. Because the number of antibody-combining sites is less than the number of epitopes present on the unlabeled and labeled antigen,

competition occurs between them. Since the only variable is the amount of the unlabeled antigen, the enzyme retained by the immobilized antibody is inversely proportional to the amount of antigen to be determined. The second competitive assay, the immunometric procedure, is based on the use of a constant amount of immobilized antigen, and a constant and limited amount of enzyme-labeled antibody. The labeled antibody is first incubated with the antigen to be measured, the immobilized antigen is then added and allowed to incubate and, finally, after washings the enzyme associated with the solid phase is measured. As in the first procedure, the amount of the enzyme bound to the solid phase decreases with increasing concentrations of the antigen to be measured. The 'sandwich' assay is essentially the only noncompetitive procedure used in ELISA for the measurement of antigens. It involves the use of a constant amount of immobilized antibody and an excess of enzyme-labeled antibody possessing the same or similar specificities as the immobilized antibody. The antigen is incubated with the immobilized antibody then, after washings, the enzyme-labeled antibody is added and allowed to incubate and, after washings, the enzyme associated with the solid phase is measured; in this case, the enzyme activity is proportional to the amount of antigen added. When monoclonal antibodies are used this procedure can be shortened by adding simultaneously the antigen and the labeled antibody to the immobilized antibody.

In all the above procedures, under the same experimental conditions, calibration curves are prepared using varying amounts of antigen and, in a given sample, the unknown concentration of this antigen is determined by reference to these curves. The competitive assays are the only procedures enabling the measurement of haptens and other small molecules; the 'sandwich' assay, which requires that at least two epitopes be present on an antigen, cannot be used in such cases. The competitive assays are, however, generally less sensitive than the sandwich assay and more susceptible to variations due to interfering substances present in the samples. Furthermore, the non-competitive procedure is more suitable for routine work.

For the quantitation of antibodies the 'sandwich' noncompetitive procedure is most commonly used. This assay involves the use of a constant amount of immobilized antigen and an excess of enzyme-labeled anti-immunoglobulin antibody. The immobilized antigen is incubated with the antibody to be determined. After washings, the enzyme-labeled anti-immunoglobulin antibody is added, allowed to incubate and, after additional washings, the enzyme as-

sociated with the solid phase is measured. Antibody quantitation by this procedure gives satisfactory results in terms of both sensitivity and reproducibility and, for this reason, it has been applied extensively in various fields. However, because antibodies possess different affinities for a given antigen, unlike antigen measurement, antibody measurement in the strict sense is not possible. The values obtained reflect both the quantity and the affinity of the antibody and have to be considered in terms of titration rather than quantitation.

The heterogeneous enzyme immunoassays, like all immunological procedures, always involve, in addition to the specific antigen-antibody binding, various degrees of nonspecific binding. This ratio appears to be primarily dependent on the solid phase and on the properties of the enzyme conjugates.

Solid phase

Immobilization of antigen or antibody on a solid phase is obtained either by covalent binding or by adsorption through noncovalent interactions. Paper discs but also agarose, cellulose and polyacrylamide particles carrying various active chemical groups have been used for the covalent immobilization of antigens and antibodies. These types of immuno-adsorbent are, however, not often used in ELISA. In principle, covalent binding has the advantages of the irreversible binding of ligands and of the preparation of homogeneous immobilized antigen or antibody solid phases. In fact, when sensitive enzyme immunoassays are employed, both of these advantages do not appear to hold true.

Various supports have been tested for the immobilization of antigen and antibody by physical adsorption. Plastic carriers, such as beads, films, plates and tubes, are the most common and have proved the most successful. Adsorption to such surfaces, principally polystyrene, occurs mainly by hydrophobic interactions. Because antigen or antibody immobilization on plastic surfaces is very simple and plastic carriers are easy to handle, this type of immobilization has led to the development of enzyme immunoassays suitable for large-scale testing. Of these, the procedure making use of microtitration plates, introduced in ELISA by A. Voller and collaborators, has enabled the design of automatic devices which are now routinely used. A few years ago, the major disadvantages of the plastic surfaces were that the adsorbing capacity varied from one batch of plastic to another. In the plates now commercially available, this serious drawback is only occasionally encountered. Recently, active functional groups were introduced on the surface of the plastic and proteins

were covalently bound or proteins were adsorbed on to surfaces made more charged and hydrophilic after radiation treatment. The results obtained thus far are not conclusive as to whether such procedures can advantageously replace simple physical absorption on to an underivatized plastic support.

Enzyme conjugates

Biotin-labeled antibodies (or antigens) followed by enzyme-labeled avidin (or streptavidin) are often used in ELISA. However, in the majority of assays, conjugates prepared by covalently coupling enzymes to antibodies (or antigens) by glutaraldehyde, *m*-periodate or maleimide derivatives are used. All the enzyme-labeled antibody (or antigen) preparations, with the exception of peroxidase conjugates, are composed of a more or less heterogeneous population of conjugates, some of which are of high molecular weight. However, in ELISA procedures that involve no particular steric hindrance problems, high molecular weight conjugates containing a large number of enzyme molecules can be used advantageously, provided they do not enhance nonspecific adsorption.

The enzymes most often used in ELISA are horseradish peroxidase, calf intestine, alkaline phosphatase and *Escherichia coli* β -galactosidase. For peroxidase, several chromogens are employed, yielding various colors that allow its spectrophotometric measurement. The results obtained indicate that *o*-phenylene diamine (giving an orange color) is the chromogen that gives the most sensitive and reliable measurement. The fluorometric assays of peroxidase are neither as sensitive nor as reliable as those using chromogens. Alkaline phosphatase and β -galactosidase are easily and accurately measured spectrophotometrically using, respectively *p*-nitrophenyl phosphate and *o*-nitrophenyl- β -D-galactoside as substrates, while minute amounts of these enzymes are measured by spectrofluorimetry using 4-methylumbelliferylphosphate or 4-methylumbelliferyl- β -D-galactoside as substrates. Several other enzymes, for example, urease, glucose oxidase, acetylcholinesterase, penicillinase and catalase, have been proposed as labels in ELISA but they are rarely used.

Evaluation of several parameters influencing ELISA

In general, in ELISA, nonspecific adsorption can be reduced by including in the medium, during the incubation and washing steps, a nonionic detergent, such as Tween-20, either alone or supplemented with a protein, e.g. bovine serum albumin or gelatin. How-

ever, it must be realized that even under the best defined conditions, there will always be nonspecific adsorption of the enzyme conjugate by the coated solid phase.

The choice of the enzyme label depends essentially on how quickly the results are needed as well as on the number of samples to be examined. When a large number of samples has to be tested simultaneously, it is preferable to use enzymes for which stable substrates are available and therefore allowing reactions of long duration. The substrates of alkaline phosphatase and β -galactosidase are more stable than those used to measure peroxidase. Consequently, peroxidase is not well suited to ELISA requiring reactions of long duration but it is particularly effective in rapid heterogeneous enzyme immunoassays. Comparative assays performed using chromogenic substrates and conjugates of peroxidase, glucose oxidase, alkaline phosphatase and β -galactosidase have shown that, after establishing the optimal conditions, these conjugates had virtually identical sensitivities and were capable of measuring equally small quantities.

Enzymes used as labels can be measured using several highly sensitive procedures. Thus with light emission techniques, peroxidase can be detected at the femtogram level. Using 4-methylumbelliferyl- β -D-galactoside, the fluorogenic substrate of β -galactosidase, 5000 molecules of this enzyme in solution are easily measured. Similarly, alkaline phosphatase can be measured in minute amounts using either fluorogenic substrates or amplifying enzymatic cyclic systems. One would expect therefore that, by using such sensitive procedures for the measurement of enzymes, a considerable increase in sensitivity of ELISA would occur. However, when ELISA using either conventional chromogenic substrates or highly sensitive procedures of detection are first optimized and then compared in a given antigen-antibody system, usually no increase in sensitivity is noted or, sometimes, a maximum 5- to 10-fold increase is achieved. For example, when estradiol is measured by a competitive assay using β -galactosidase-labeled estradiol and either the chromogenic (*o*-nitrophenyl- β -D-galactoside) or the fluorogenic (4-methylumbelliferyl- β -D-galactoside) substrate of this enzyme, the detection limit is the same (100 pg ml⁻¹). However, in order to achieve this result, 18 h of incubation are needed with the chromogenic substrate, while 2 h are sufficient with the fluorogenic one. Thus although the intensity and the rapidity of the enzymatic response is improved by using highly sensitive measurement procedures, the detection limit, i.e. the lowest concentration of antigen (or antibody) that can be measured, remains essentially unchanged.

Therefore, it appears that in ELISA, and probably in all the immunological procedures based on the use of labeled substances, the limiting factor is not the detection or the measurement of the enzyme level. The limiting factor is the binding affinity (avidity) of the antibody which will determine the ultimate sensitivity of the heterogeneous enzyme immunoassays.

See also: Affinity; Autoantibodies, tests for; Enzyme labeling of antibodies and antigens; Enzyme-linked immunosorbent assay (ELISA); Immunoassays.

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EOSINOPHIL CHEMOTACTIC FACTORS

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The discovery of eosinophils by Paul Ehrlich in 1879 and the first description of the process of directed single cell locomotion (chemotaxis) by Wilhelm Pfeiffer in 1884 are closely related in time. Ehrlich also noted increases in blood and tissue eosinophils in a great variety of diseases such as parasitoses, asthma, malignant tumors, vesicular and diverse other dermatoses, and in postinfectious reactions ('the pink dawn of recuperation'), but not in acute bacterial infections.

Efforts to explain the increase of eosinophils in these diseases during the subsequent decades resulted in studies in which a great variety of agents were injected into animals. Substances that induced tissue eosinophilia were sensitized lymphoid tissue, proteins, carbohydrates, synthetic polymers, parasite extracts and histamine. The question as to whether these agents were chemotactic by themselves or only chemotaxogenic, i.e. inducing the production of chemotactic factors after injection into the tissue, could only be resolved with the availability of *in vitro* methods to measure chemotaxis (Boyden chambers) in 1962. The current model for eosinophil accumulation in tissues involves multiple separate steps: eosinopoiesis in the bone marrow, generation and action of chemoattractants, eosinophil adhesion to endothelial cells, and extravasation, tissue eosinophil survival and eosinophil priming and activation.

Source, function and chemical nature of eosinophil chemotactic factors

There are two main sources of eosinophil chemotactic factors: 1) blood and tissue fluid; and 2) a diverse number of cells. The main eosinophil chemotactic factors in the fluid compartment are the complement-derived anaphylatoxins C3a and C5a, and to a lesser extent the latter's cleavage product C5a-desarg (Table 1). Whereas both are highly potent chemotaxins for eosinophils, C5a also activates several other mononuclear cell types. Most eosinophil chemotactic factors derive from resident tissue or from peripheral blood cells. They include different chemical entities such as lipids, peptides and proteins (Table 1).

During initial studies, mast cells and basophils were intensely investigated for the release of eosinophilotactic agents because these cells are considered the main effector cells of allergic reactions. The factors isolated were mostly of low molecular weight and probably represented the eosinophilotactic activities of platelet-activating factor (PAF) and the eicosanoids. The low molecular weight eosinophilotactic activity generated *de novo* from neutrophils was later shown to represent primarily leukotriene B₄ (LTB₄) and related eicosanoids (Table 1). All these factors are also able to attract a number of cell types other than eosinophils.

Table 1 Generally accepted and well-defined eosinophil chemotactic factors and their target cells during *in vitro* chemotaxis

<i>Factor</i>	<i>Chemical nature</i>	<i>Migrating cells</i>
C3a	Peptide	Eosinophils
C5a, C5a-desarg	Peptide	Neutrophils, eosinophils, basophils, monocytes
Eotaxin	Protein	Eosinophils
RANTES	Protein	Eosinophils, basophils, monocytes, memory T cells, mast cells
MCP-3	Protein	Eosinophils, basophils
MCP-2	Protein	Eosinophils, basophils
MIP-1 α	Protein	Eosinophils, monocytes, T cells
IL-8	Protein	Neutrophils, eosinophils, T cells
IL-5	Protein	Eosinophils
LCF	Protein	Eosinophils, monocytes, CD4 ⁺ T cells
5- and 12-HETE	Eicosanoid	Neutrophils, eosinophils
LTB ₄ , 20-OH-LTB ₄ , 5-O-HETE, 5-O-ETE	Eicosanoid	Neutrophils, eosinophils, monocytes, fibroblasts
PAF	Ether phospholipid	Neutrophils, eosinophils, monocytes
N-formyl peptides	Peptide	Neutrophils, eosinophils

Subsequently, lymphocyte products with eosinophilotactic properties could be identified which might participate in recruitment of eosinophils to T cell-dependent hypersensitivity reactions *in vivo*. The T cell-derived lymphocyte chemoattractant factor (LCF) has been shown to stimulate potential migration *in vitro* not only of CD4⁺ lymphocytes and of monocytes, but also of eosinophils. A more selective eosinophil chemotactic factor is interleukin-5 (IL-5).

In older studies, a group of inflammatory mediators, the chemokine superfamily, which specifically regulated chemotaxis of certain leukocyte subgroups, was discovered. While most early reports characterized leukocyte-derived chemokines, it is becoming increasingly apparent that chemokines can be produced by a wide array of tissues. In this regard, the small molecular mass CC chemokines eotaxin, RANTES and monocyte chemotactic protein 3 (MCP-3), and to a lesser extent MCP-2 and macrophage inflammatory protein 1 α (MIP α), represent the most potent eosinophil (but not neutrophil) chemotactic cytokines described so far. Although IL-8, a C-X-C chemokine, has clearly been shown to be a potent neutrophil chemotactic agent, the *in vitro* effect of IL-8 on eosinophil chemotaxis is controversial. The direct IL-8-induced eosinophil chemotaxis, as reported from one laboratory, could only be reproduced by other groups after priming of the cells with IL-5.

Finally, extracts from some parasitic worms have been shown to attract eosinophils *in vitro*. Synthetic analogs of bacterial chemotactic factors, the N-formylated peptides, have only weak eosinophil chemotactic activities. It should be noted that most eosino-

phil chemotactic factors, in addition to inducing migration, also have numerous other important biological functions. With regard to the eosinophils, this includes eosinophil chemokinesis, degranulation or selective secretion, enhanced cytotoxicity and endothelial cell adhesion. IL-5 induces differentiation and proliferation of bone marrow eosinophils, activates eosinophils and promotes their survival.

***In vivo* eosinophil chemotaxis**

For IL-5 and the chemokine eotaxin, convincing evidence of *in vivo* activity as eosinophil chemotactic factors has been provided. Much higher concentrations of IL-5 were, however, required for eosinophil chemotaxis *in vivo* than *in vitro*. Neutralizing antibodies to IL-5 are very effective at inhibiting eosinophil accumulation in blood and tissues in experimental models of allergic inflammation and helminth infection. Furthermore, mice transgenic for IL-5 have extremely high numbers of circulating eosinophils, yet show an otherwise normal phenotype.

There are also interesting data on an *in vivo* synergism between IL-5 and eotaxin. Neither of the substances alone induced eosinophil accumulation after intradermal injection in guinea pigs. Low-dose intravenous IL-5, however, caused a rapid (within 1 h) and dramatic enhancing effect on eosinophil recruitment to sites of intradermal eotaxin injections. Thus, IL-5 acts systemically as a hormone to stimulate the release of a rapidly mobilizable pool of bone marrow eosinophils into the circulation and facilitates the eosinophil recruitment from blood microvessels by locally released eosinophil chemoattractants. This

seems to hold also for other chemoattractants. Subcutaneously injected PAF and LTB₄ mediate eosinophil accumulation only in the presence of an IL-5-induced eosinophilia as well.

An important postulate for a truly eosinophil chemotactic factor is its demonstration in normal and/or pathologically altered tissue. This has so far been achieved for C5a, PAF, eicosanoids, IL-5, IL-8, MIP-1 α , RANTES and eotaxin, although the isolation of these factors has not been restricted to eosinophilic tissue. Although some eosinophil-specific chemoattractants have so far been identified, the mechanism by which eosinophils preferentially migrate into tissues are largely unknown. Extravasation of eosinophils involves a number of cell surface adhesion molecules, predominantly selectins for primary tethering and members of the β_1 - and β_2 -integrin family for firm adhesion and transendothelial migration. There is much evidence that the interaction of VLA-4 ($\alpha_4\beta_1$ -integrin), expressed by eosinophils but not neutrophils, and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells is a major adhesion pathway allowing selective migration of eosinophils. Blocking VLA-4 *in vivo* has been shown to reduce eosinophil accumulation in some models of allergic inflammation. In addition, antibodies to CD11/CD18 or intercellular adhesion molecule 1 (ICAM-1) (β_2 -integrins) reduced eosinophil accumulation in guinea pig skin. Recent studies have also demonstrated that these adhesion processes can be controlled by chemokines. RANTES, but not MIP-1 α or IL-8, is capable of stimulating eosinophil transendothelial migration via VLA-4/VCAM-dependent mechanisms in cells primed with IL-5.

Modulation of eosinophil chemotaxis

As in all biological systems, stimulating factors of eosinophil chemotaxis can be opposed by down-modulating factors. Such agents can inactivate eosinophilic factors, block their interaction with the cell and its receptor, or interfere with the complex cellular mechanism necessary for locomotion. Mediator antagonists such as antibodies to IL-5, IL-4 and MIP-1 α are very effective at inhibiting eosino-

phil accumulation in experimental models of allergic inflammation.

Among therapeutic drugs, corticosteroids and the immunosuppressant cyclosporine are well known, potent, rapidly acting reducers of blood and tissue eosinophil levels. Sympathomimetics and phosphodiesterase inhibitors have a similar effect. More recently, second-generation histamine H₁-receptor antagonists (cetirizine, ketotifen) have been shown to have both *in vivo* and *in vitro* effects on eosinophil accumulation or migration. This effect is not shared by other H₁-receptor antagonists. Also, leukotriene CysLT₁-receptor antagonists and inhibitors of 5-lipoxygenase effectively suppress eosinophil recruitment in allergic inflammation.

In summary, many questions remain to be resolved in elucidating which factors govern the infiltration of eosinophils into tissue, their interplay with each other and with other factors, and their role in pathologic processes.

See also: Arachidonic acid and the leukotrienes; Chemotaxis; Eosinophils; Histamine; Interleukin 5 and its receptor; Mast cells; Platelet-activating factor (PAF).

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EOSINOPHILS

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Eosinophils are postmitotic leukocytes of the myeloid lineage. They are recognized easily in blood films or tissue sections by their large, regular, eosin-staining cytoplasmic granules and segmented nuclei. Although there are some species-to-species variations in morphology, eosinophils have been detected in every vertebrate that has been studied except the hagfish.

In normal human subjects, numbers of blood eosinophils range from approximately 100 to $700 \mu\text{L}^{-1}$, but can be elevated (in extreme cases by a factor of 100 or more) in association with allergies, asthma, parasitic diseases, chronic inflammation in a variety of tissues, certain neoplasms, use of L-tryptophan dietary supplements, and experimental immunotherapies of cancer or acquired immune deficiency syndrome (AIDS). From the bloodstream, eosinophils enter the margined pool and the tissues, where they exhibit their major impact on the pathophysiology of disease. At the local site of inflammation, eosinophils release toxic substances that may kill or damage infectious organisms and surrounding tissue. Eosinophils are also a major source of immunologic mediators that contribute to the development of the tissue lesion.

Cell structure, biochemistry and activation

The nucleus of the mature eosinophil is segmented, with an average lobe index of approximately 2.1. The cytoplasm of the eosinophil is filled abundantly with secretory granules. The large granules have a central crystalloid body and a surrounding matrix containing cationic proteins that are responsible for the binding of acidic dyes, such as eosin. Small granules can also be observed by electron microscopy. These have many of the same constituent substances as the large granules and may represent a precursor or remnant of the large granule rather than a separate class of granule.

The cytoplasm also contains lipid bodies, glycogen and prominent Golgi bodies, and large amounts of lysophospholipase. This enzyme can form crystals of a characteristic shape (bipyramidal needle), known as Charcot-Leyden crystals, which are occasionally found within the eosinophil and more commonly at the site of inflammation in tissues (Figure 1).

The eosinophil surface membrane displays receptors for immunologically active substances, including

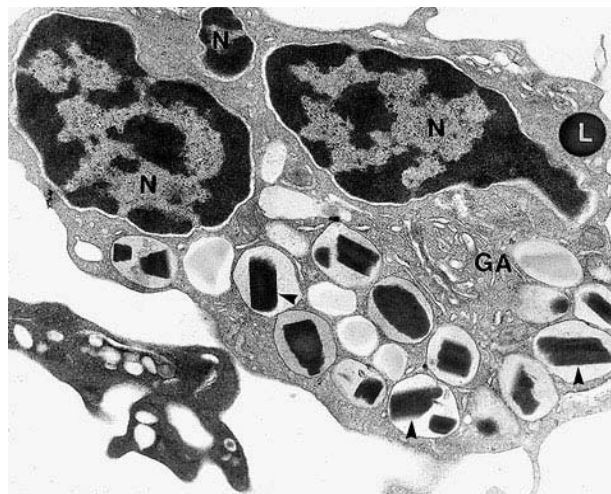


Figure 1 Transmission electron micrograph of normal human blood eosinophil. The characteristic features include a segmented nucleus (N), secretory granules with an electron-dense crystalloid core (arrowheads), a lipid globule (L) and a prominent Golgi apparatus (GA). Magnification $\times 20\,000$. (The figure was kindly provided by Dr Khwaja Siddiqui.)

C3a/C5a, C3b, iC3b, Fc γ (Fc γ RII), Fc ϵ (Fc ϵ RII), Fc α , granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), IL-5, tumor necrosis factor (TNF), and other cytokines, the chemokines RANTES, MIP-1 α , MIP-1 β , MCP-3 and eotaxin, mast cell-derived ECF-A tetrapeptides, and formyl-methionyl peptides. Other surface structures involved in immunologic functions include CD4, IL-2R (gp55), LFA-1 and 3, VLA-4 and HLA-DR antigens.

Stimulation of eosinophils through Fc or complement surface membrane receptors induces the directional release of secretory granule contents, the activation of a respiratory burst and associated release of oxygen metabolites, and the release of pharmacologically active lipid metabolites (Table 1).

Eosinophil secretory granules have a crystalline core composed of major basic protein (MBP), an 11 kDa cationic protein that is toxic to a variety of tissues and microorganisms. The matrix surrounding the core contains the structurally related eosinophil cationic protein (ECP, 18–22 kDa), eosinophil-derived neurotoxin (EDN, 18–19 kDa), the eosinophil peroxidase (EPO), elastase, collagenase, arylsulfatase B, β -glucuronidase, histaminase, phosphatases, phospholipase B and D, catalase, nonspecific esterases, and proteoglycans. The EPO is

Table 1 Functions of activated eosinophils

- *Phagocytosis of immune complexes and certain microorganisms* (e.g. *Escherichia coli*, *Candida albicans*, *Trypanosoma cruzi*)
- *Directional release of secretory granule substances*, including the toxic cationic proteins MBP, ECP, EDN, lysosomal enzymes and EPO
- *Release of toxic oxygen metabolites* including O_2 and H_2O_2 . In the presence of the peroxidase and halide ions, H_2O_2 is converted to hypohalous acid, preferentially HOBr
- *Release of pharmacologically active lipid metabolites*, including minor amounts of leukotriene B_4 , major amounts of leukotriene C_4 and D_4 , 15-HETE (15-hydroxyeicosatetraenoic acid), platelet activating factor, prostaglandins E_1 , E_2 and thromboxane B_2
- *Release of cytokines*, including IL-1, IL-2, IL-5, IL-6, IL-8, TNF and $TGF\alpha$

structurally different from the neutrophil myeloperoxidase and is relatively insensitive to cyanide.

The respiratory burst is associated with increased uptake of glucose and oxygen, hexose monophosphate shunt activity, the oxidation of NADPH, and the reduction of O_2 to O_2^- . O_2^- , in the presence of superoxide dismutase, is converted to H_2O_2 , which is converted to hypohalous acid, preferentially HOBr, in the presence of EPO and halide ions.

Activated eosinophils are important sources of the lipid metabolites leukotrienes C_4 and D_4 (slow reacting substance of anaphylaxis) and platelet activating factor, with a release of 100 ng 10^6 cells or more of each mediator measured in some experiments, quantities substantially greater than those released by mast cells.

Eosinophils are also important sources of multiple cytokine species, including IL-5, which can provide positive feedback on eosinophil development, and transforming growth factor α ($TGF\alpha$), which promotes tumor angiogenesis.

Hematopoiesis

Eosinophil development is predominantly in the bone marrow, but may be found at other sites (e.g. liver or lymphoid tissue) in certain human diseases, and in other mammalian species, particularly in embryos and neonates. The formation of cytoplasmic granules begins in the late myeloblast stage and continues through the myelocyte stage. Segmentation of the nucleus begins with indentation at the myelocyte stage and is complete in the mature bone marrow eosinophil.

Eosinophil development does not take place in experimental animals deprived of T cells, and it is reduced severely in patients with T cell deficiencies,

such as in AIDS. The critical T cell function is the production of the hematopoietic growth factors IL-3, GM-CSF and IL-5. These hematopoietins stimulate the growth and differentiation of distinct eosinophil precursor populations in respective order from most primitive to most differentiated, and it is thought that eosinophil development requires the sequential action of all three factors. IL-5, the eosinophil lineage-specific growth factor, is absolutely required for eosinophil development, and it has been detected in high concentrations in plasma of patients with eosinophilia.

The receptors for IL-3, GM-CSF and IL-5 are heterodimeric, with an α chain that confers ligand specificity and a common β chain that is involved in signal transduction. It is known that occupancy of the IL-5 receptor leads to activation of JAK2 kinase, MAP kinase and STAT5. Thus, these three signal transduction molecules may drive commitment to the eosinophil lineage.

Migration to tissues

Segmented mature eosinophils have a half-life of approximately 13 h in the blood after emergence from the bone marrow. The initial process in the exit from the bloodstream to the tissues is the adherence to the vascular endothelium. E-selectin, CD11b/18 and VLA-4 appear to be the most important adhesion molecules involved in this process, with VLA-4 exhibiting eosinophil versus neutrophil selectivity. This adherence can be enhanced by the presence of certain immunologic mediators, such as TNF, or by the eosinophil-selective chemokines RANTES, MCP-3 and eotaxin. Homing to the tissue site is induced by the chemokines or other chemotactic factors (Table 2).

Eosinophils in disease

Eosinophils are able to phagocytose immune complexes and microorganisms (e.g. *Escherichia coli*, *Candida albicans*, *Trypanosoma cruzi*), but these capabilities are generally not as great as those of other phagocytes. The ability to release toxic substances against nonphagocytosable surfaces is probably of greater importance. By this mechanism, eosinophils are able to kill certain metazoan parasites. There is also some evidence to suggest that eosinophil infiltration of tumors increases patient survival in certain types of cancer (and cures certain experimental cancers in mice).

However, the release of toxic substances has a major damaging effect on surrounding tissues at the site of eosinophil infiltration. The cationic granule substances (Table 1) are highly toxic to cells and

Table 2 Immunologic mediators that regulate eosinophil function

- *Eosinophil hematopoietins* (IL-3, GM-CSF, IL-5) – increased longevity in culture, increased cytotoxic function and leukotriene synthesis, reduced cell density, altered proteoglycan synthesis, processing of ECP to the secretory form. IL-5 is a chemoattractant
- *Tumor necrosis factor* – increased cytotoxic function, respiratory burst activities and adherence to vascular endothelium
- *Eosinophil activating factor* (monokine, 40 kDa) – increased cytotoxic function and leukotriene synthesis, processing of ECP to the secretory form
- *Eosinophil cytotoxicity-enhancing factors* (monokines, 10 kDa and 13 kDa, structurally related to thioredoxin) – increased cytotoxic function and leukotriene synthesis, processing of ECP to secretory form
- *Human interleukin 8* (lymphokine, 41 kDa) – chemotaxis and chemiluminescence
- *IL-1 α and β* – increase or decrease secretion of granule substances, depending on the method of costimulation
- *IL-2* – chemoattractant; administration to humans increases cytotoxic function, reduces cell density and induces processing of ECP to the secretory form
- *IL-4* – suppression of Fc γ R expression and IgG-triggered functions
- *TGF β* – suppression of hematopoiesis, induction of apoptosis
- *DF3 secretory epithelial cell mucin* – suppression of adherence to and triggering by IgG-coated targets
- *C3 β chain* – suppression of antibody-dependent cytotoxic function
- *Chemotactic factors and chemokines* – platelet-activating factor, leukotriene B $_4$, formyl-methionyl peptides, IL-5, IL-8, C3a and 5a, eosinophil chemotactic factor of anaphylaxis (mast cell-derived tetrapeptides), histamine, RANTES, eotaxin, MCP-3

tissues *in vitro*, and are probably responsible for damage that is seen at the site of inflammation *in vivo* (e.g. the desquamation of the tracheal epithelium with bronchial asthma or the progressive endomyocardial fibrosis with the idiopathic hypereosinophilic syndrome). Toxic oxygen metabolites and lysosomal enzymes are also thought to contribute to tissue injury.

Leukotrienes C $_4$ and D $_4$ are extremely potent inducers of smooth muscle contraction and inducers of microvascular leakage. Platelet activating factor is a chemoattractant and activator of several types of inflammatory cell. Thus, local release of these mediators may have diverse and pronounced physiologic effects, including the constriction of airways in bronchial asthma and the accumulation of fluid and inflammatory cells in tissues. The success of leukotriene inhibitors in asthma clinical trials supports the importance of the eosinophil (and the mast cell) in the pathogenesis of the disease.

Regulation of eosinophil function

The regulation of eosinophil functions by immunologic mediators is suggested by two lines of evidence. 1) Eosinophils from patients exhibiting an immune response and eosinophilia have enhanced functions as compared with normal eosinophils. These functions include chemotaxis, cytotoxicity, respiratory burst activities and arachidonic acid metabolism. Eosinophils from these patients also have a slightly increased longevity in culture and a reduced density. 2) *In vitro* treatment of purified eosinophils with defined immunologic mediators can enhance or suppress measurable responses to activating stimuli, increase longevity in culture, and alter cell density (Table 2). Thus, certain mediators can induce phenotypic changes in normal eosinophils similar to those observed in eosinophilic patients.

Of the mediators that are potential *in vivo* regulators of mature eosinophil functions, the best studied are IL-3, GM-CSF and IL-5. Where freshly isolated eosinophils undergo apoptosis within 48 h in culture, appropriate doses of these growth factors extend eosinophil survival to 2 weeks and beyond. Eosinophils cultured in this manner have greatly enhanced cytotoxic function and biosynthetic capabilities. Thus, the hematopoietins that stimulate the development of eosinophils from bone marrow precursors also regulate their postmitotic phenotype. Other mediators also have the potential to enhance or suppress functions of eosinophils (Table 2).

There is substantial evidence for the presence of eosinophil hematopoietins and other regulatory molecules in plasma or tissue samples from human subjects with eosinophilia. One study found that three patients with the idiopathic hypereosinophilia syndrome had very high levels of circulating IL-5. Multiple studies have shown high accumulations of these three hematopoietins in the airways of asthma patients. This information suggests that elevated levels of these regulatory mediators are an underlying cause of eosinophilia-associated diseases. It also suggests that therapy directed at the production of certain immunologic mediators may benefit the patient.

See also: Arachidonic acid and the leukotrienes; Cytokines; Eosinophil chemotactic factors; Leukemia inhibitory factor.

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EPITOPES

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An epitope is the part of an antigen that interacts with the antigen-specific receptor or antibody. Strictly speaking, an epitope is determined by the specificity of the clonally distributed receptor to which an antigen binds. However, the part of an antigen that can reproducibly elicit B cell or T cell responses is often referred to as an epitope without reference to the specificity defined by the particular receptor. Epitopes, which are often used interchangeably with antigenic sites or antigenic determinants, can be classified as B cell epitopes or T cell epitopes on the basis of the types of cellular responses they elicit.

B cell epitopes bind to specific receptors, which are membrane-bound immunoglobulins (mIgs), on the surfaces of B cells. These specific interactions lead to a series of events that stimulate the expansion of that particular B cell population. As a result of B cell activation, antibodies with the same binding specificities as those of the B cell receptors are produced. This is often referred to as the humoral immune response. In principle, any soluble molecules that can bind to B cell mIgs are potential B cell antigens. However, the specificities defined by the B cell receptors determine the efficiencies of these potential B cell epitopes.

T cell epitopes interact specifically with MHC (major histocompatibility complex) molecules and T cell receptors (TCRs). Thus, T cell antigenic determinants are defined by the specificities of both MHC and TCRs. MHC molecules (both class I and II) have specific binding sites for short peptide fragments. Protein antigens have to be processed by intracellular proteases before they can bind in the peptide-binding groove on MHC molecules. TCRs are structurally similar to mIgs and antibodies. The complementarity-determining regions (CDR) on TCRs physically contact the peptide–MHC complexes. The tripartite interactions involving peptide, MHC molecule, and TCR result in T cell activation by cytokine-mediated mechanisms. The outcome of T cell activation depends on the types of MHC molecules (hence, the antigen-presenting cells) and the types of T cells involved. Peptides bound to MHC class I molecules (which are found on most nucleated cells) typically interact with TCRs on the surface of CD8⁺ T cells (cytotoxic T cells). These interactions eventually lead to cell killing by the activated and expanded cytotoxic T cells. This process is often referred to as the cellular immune response. Peptides bound to MHC class II molecules, which are found

on antigen-presenting cells such as dendritic cells, B cells and macrophages, interact specifically with TCRs on the surface of CD4⁺ T cells (helper T cells). These interactions result in the activation of helper T cells, which then release cytokines to effect macrophage activation and/or B cell differentiation into antibody-producing plasma cells.

B cell epitopes

B cell epitopes interact directly with mIgs on the surfaces of B cells and no processing of the antigen is required for these interactions. As a result, B cell receptors can bind antigens in their native conformations. This is why antibodies raised against native proteins often recognize 'discontinuous epitopes', antigenic determinants composed of amino acids that are far apart in primary sequences but are in close proximity in the folded structures. By the same token, antibodies raised against denatured proteins or synthetic peptide antigens often recognize 'continuous (sequential) epitopes' and cannot recognize the corresponding proteins in their native conformations.

Antigens that can elicit B cell responses are quite diverse; they can be proteins, lipids, carbohydrates, or small molecules (e.g. arsenate, trinitrophenol). This is in contrast to most T cell epitopes which are usually short peptide fragments. The diverse nature of B cell antigens makes it difficult to predict B cell epitopes *a priori*.

Antigen binding to mIgs on B cells triggers a signaling cascade which leads to B cell activation. The activation of B cells may or may not require the help of T cells. Antigens that require the help of T cells for B cell activation are thymus-dependent (T-dependent) antigens. Antigens that can stimulate B cells without the help of T cells are thymus-independent (T-independent) antigens. The majority of protein antigens are T-dependent, while most of the T-independent antigens are polymeric molecules with repeated antigenic determinants. Examples of T-independent antigens include microbial products such as polysaccharides and polymeric proteins.

T-dependent B cell antigens first bind to mIgs on the surfaces of B cells. After binding, these antigens are internalized by receptor-mediated endocytosis or by nonspecific pinocytosis. While in the cell, these proteins remain in the vesicular compartments and are not mixed with proteins in the cytoplasm. These internalized proteins are processed by lysosomal proteases to liberate peptide fragments which then bind to MHC class II molecules. These peptide-MHC complexes then emerge on the surface of B cells where they interact with specific TCRs on the sur-

faces of T helper cells (CD4⁺ T cells). The MHC-peptide-TCR interactions result in cytokine release from T cells to direct B cell proliferation and maturation. The activated B cells eventually differentiate into plasma cells, which have greatly expanded cytoplasm and extensive endoplasmic reticulum and are wholly devoted to the production of antibodies. The antigenic determinants (B cell epitopes) that are involved in the initial binding to the B cell receptors are not usually the same as the determinants (T cell epitopes) that interact with MHC and TCRs. However, these B cell and T cell epitopes might be localized to overlapping regions on the antigen proteins.

T-independent B cell antigens activate B cells by cross-linking the surface receptors. Without the help of T cells, these B cells do not undergo immunoglobulin gene class switching and the antibodies thus generated are predominantly IgM. In contrast, antibody responses to T-dependent antigens usually mature to contain a greater proportion of IgG with a higher affinity for the antigen.

T cell epitopes

TCRs recognize peptide fragments complexed with MHC molecules (either class I or II). Over the years, several structures of peptide-MHC I complexes have been determined. Peptides bind to the MHC in the peptide-binding groove, which is about 30 Å long and 12 Å wide and is formed by two parallel α -helices, in an extended conformation. These structures have provided insights into how the peptide fragments interact with the MHC molecules, how polymorphism in MHC molecules affects immune responses, and how these complexes might interact with TCRs.

Each individual has only a limited number of different MHC molecules. However, these MHC molecules need to bind a large array of short peptide fragments (octapeptides or nonapeptides for MHC I molecules) with high affinities. Although tight binding usually demands extreme specificity in protein-ligand interactions, MHC molecules solve this apparent dilemma by interacting extensively with the ubiquitous backbone amides and the amino and carboxyl groups on the termini of the peptides. MHC molecules interact with only two amino acid side-chains (usually the second and the ninth amino acids starting from the N-terminus of the peptide fragments) from the bound peptides, leaving most amino acid side-chains available for interaction with TCRs. This simple mechanism of peptide-MHC interactions suggests that it might be possible to predict potential T cell epitopes from protein primary

sequences. Indeed, this has been demonstrated in several cases. This mode of MHC-peptide interaction (i.e. most amino acid side-chains available for TCR recognition) allows the immune system to respond to the vast array of antigens. The mammalian genome encodes around 5×10^7 amino acids in the expressed proteins. These diverse protein sequences require that at least six amino acid side-chains be displayed in a TCR-peptide-MHC complex in order to ensure specificity. This plus the two amino acid side-chains involved in MHC binding suggest that the fact that MHC I molecules have evolved to bind peptide fragments of eight or nine amino acids long might not be accidental.

The structures of TCR-peptide-MHC I complexes have recently been determined by X-ray crystallography. These structures confirm the prediction that the complementary-determining regions of TCRs interact simultaneously with the peptide fragments and MHC molecules in order to achieve the diverse specificities.

Class I and class II MHC molecules have different overall structures and are involved in different subsets of immune responses. Differences between peptide interactions with class I and class II molecules include the following. 1) The peptide-binding grooves of MHC II molecules are open on both ends, whereas those on MHC I are not. As a result, MHC II molecules can accommodate peptide fragments longer than nine amino acids (often around 14 amino acids). 2) Peptides can be bound with MHC I molecules in an arched conformation such that some amino acids are lifted from the floor of the peptide-binding groove. In contrast, peptides bound to MHC II molecules lay flat on the floor of the binding groove. Besides these minor differences, the structural basis for peptide binding to MHC II molecules and for TCR binding to peptide-MHC II complexes are probably similar to those involving MHC I molecules.

Since both MHC I and MHC II molecules bind short fragments of peptides, T cell epitopes are composed of short linear peptide sequences, i.e. are not dependent on native protein conformation. Hence, both native and denatured forms of the same protein should have the same T cell epitopes. The requirement for short peptide fragments demands that protein antigens be proteolytically processed before T cell epitopes are available.

Protein antigens of extracellular origin are internalized by receptor-mediated endocytosis or nonspecific pinocytosis. The internalized antigens remain in vesicular compartments, where they are processed by lysosomal proteases and eventually form complexes with class II MHC molecules. These

peptide-MHC II complexes then activate CD4⁺ T cells. CD4⁺ T cell activation in turn leads to stimulation of the antigen-presenting cells (APCs) such as B cells. In contrast, protein antigens of intracellular origins (such as cytoplasmic proteins and proteins which arise from viral infections) are probably processed by multisubunit proteasomes, although the involvement of other proteolytic machineries cannot be ruled out. The active site structures of proteasomes suggest that these enzymes are capable of cleaving proteins into eight or nine amino acid fragments in a sequence-independent manner, a property consistent with the notion that they are responsible for antigen processing. The processed intracellular antigen fragments then combine with MHC I molecules to effect CD8⁺ T cell activation and cellular immune responses.

See also: Affinity; Antibodies, specificity; Antibody-antigen intermolecular forces; Antigen-binding site; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Antigens; Hapten; Immunodominance; MHC restriction; Specificity; T cell receptor, recognition by; Vaccines.

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EPSTEIN-BARR VIRUS, INFECTION AND IMMUNITY

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Epstein-Barr virus (EBV) is an important human pathogen. It is carried by the vast majority of adults as a persistent and largely asymptomatic infection. However, it is the most common causative agent of infectious mononucleosis. It can also induce lymphoproliferative disease in immunosuppressed patients and is implicated in the pathogenesis of various malignant diseases.

Characteristics of EBV and its antigens

EBV is a member of the gamma-herpesvirus family. It is an enveloped virus of about 150–180 nm diameter with an icosahedral nucleocapsid containing a double-stranded DNA viral genome of about 172 000 base pairs. The nucleocapsid is composed of a major 160 kDa nonglycosylated polypeptide and a minor 125 kDa glycoprotein, both of which form part of the viral capsid antigen (VCA) complex. The virus lipoprotein envelope contains at least three virally-encoded glycoproteins, designated the membrane antigen (MA) complex. Two antigenically-related MA glycoproteins, gp340 and gp220, potentiate binding of the virus to a specific cell surface receptor molecule during the infection of target cells. The third MA glycoprotein, gp85, is involved in the fusion of bound virus with the host cell membrane. Following fusion, viral DNA is released into the cell and is transcribed and replicated in the nucleus, where it persists as multiple episomal copies.

The 140 kDa CD3d complement receptor molecule (CD21) is the virus receptor in the human host. CD21 expression is largely restricted to mature B lymphocytes, hence the predominant B cell tropism of EBV. However, the B lymphocyte is not the only cell type that is infectable with EBV, as has long been apparent from the association of EBV with undifferentiated nasopharyngeal carcinoma and other non-B cell malignancies (see below). Infection of primary epithelial cells and of immature thymocytes has been achieved *in vitro*, but these infections were demonstrated with a difficulty that contrasts with the ease and reproducibility with which B cells can be infected. EBV infection of resting B cells results in growth-transformation and the establishment of immortalized lymphoblastoid cell lines (LCLs) in which infection is largely nonproductive and only a

limited number of the viral genes are transcribed. The transformation-associated or so-called 'latent' viral proteins expressed in LCLs comprise six nuclear antigens (EBNAs, for EBV-induced nuclear antigens) and three membrane-associated proteins (LMPs, for Latent membrane proteins), whose functions are summarized in Table 1.

Some LCLs are partially permissive for virus production *in vitro*. In such lines, a minor subpopulation of cells express the BZLF-1 protein (encoded by a gene in the *Bam* *HI* *Z* restriction fragment of EBV, beginning with the first leftward reading frame), which is a transcriptional activator that can trigger a cascade of viral gene expression from more than 80 lytic cycle genes. The lytic cycle antigens were first identified using human sera from EBV-positive individuals which contained antibodies reactive in immunofluorescence with virus-producer cells. Historically, these antigens were classified into groups according to whether their expression was independent of DNA synthesis, i.e. early antigens (EAs), or blocked by inhibitors of DNA synthesis, i.e. late antigens. The EAs were further subdivided into diffuse (EA-D) or restricted (EA-R) components according to their cellular localization and their sensitivity to methanol fixation in immunofluorescence tests. The EA genes encode nonstructural proteins, which include transcriptional activators and enzymes. The late antigens are structural proteins of the EBV nucleocapsid (VCA) and lipoprotein envelope (MA).

Immune responses of the host to EBV infection

Persistent infection in the healthy host

An important feature of EBV shared by all herpesviruses is that following primary infection of the host the virus is carried as a persistent infection for life. More than 90% of all adults in all populations worldwide carry EBV as an asymptomatic infection. Infectious virus can be detected in the throat washings of nearly all healthy carriers. In addition, EBV-positive LCLs can be established spontaneously from cultures of circulating B lymphocytes. The virus is transmitted horizontally via salivary secretions, but current opinion is divided as to the target cell of primary infection. Until recently, dogma held that

Table 1 EBV-encoded proteins expressed in nonproductive infection of normal B cells *in vitro*

Viral gene product (alternative nomenclature) ^a		Functions
EBNA1		Essential for viral plasmid replication, mediated through binding to specific DNA sequences in <i>ori-p</i> . Transcriptional activator
EBNA2		Essential for B cell transformation. It is a transcriptional activator that associates with sequence-specific DNA-binding cellular factors such as RBP-J κ and PU.1. It activates viral gene expression (e.g. LMP1 and LMP2) as well as cellular gene expression (e.g. CD21 and CD23)
EBNA3A	(EBNA3)	Essential for B cell transformation. Binds RBP-J κ and antagonizes EBNA2/RBP-J κ function
EBNA3B	(EBNA4)	Not essential for B cell transformation. Binds RBP-J κ and antagonizes EBNA2/RBP-J κ function
EBNA3C	(EBNA6)	Essential for B cell transformation. Binds DNA and is a probable transcriptional repressor. It can also bind RBP-J κ transcription factor and antagonize EBNA2/RBP-J κ function
EBNA-LP	(EBNA5)	Not essential, but greatly enhances efficiency of B cell transformation. Cooperates with EBNA2 during primary infection of resting B cells to induce the G ₀ -G ₁ transition. There is also evidence that it interacts with Rb tumor-suppressor gene product
LMP1		Essential for B cell transformation, and oncogenic in rodent fibroblast assays. Can regulate cell growth, but its main functions are to enhance cell survival and to alter cell phenotype to increase the antigen-presenting capacity. Pleiotropic effects on cell are mediated in part by activation of the NF- κ B transcription factor
LMP2A	(TP1)	Not essential for B cell transformation, but may act to block switch from the latent state into lytic cycle. Substrate for <i>src</i> family tyrosine kinases, and can modulate transmembrane signal transduction.
LMP2B	(TP2)	Not essential for B cell transformation. LMP2B is an alternatively spliced gene product that is identical to LMP2A except that it lacks the amino-terminus kinase-interacting domains. Its function is unclear, but it may complex with and modulate LMP2A function

^aThere is no agreed standard nomenclature for the EBV-encoded proteins, and two versions are in common usage. Use of the nomenclature in parentheses is perhaps less widespread, being confined mainly to European research groups.

primary infection involved epithelial cells of the oropharynx and that these cells harbored a lytic infection from which a secondary latent infection of trafficking B cells led to virus persistence; in this scenario, persistence occurs in the B lymphoid compartment and subsequent secretion of virus in the oropharynx is postulated to derive from foci of secondarily-infected epithelial cells in lytic cycle. Recent re-examination of the available evidence has led people to question whether epithelial cells are involved at all in normal persistent infection. The alternative postulate argues that primary infection occurs in mucosal lymphocytes; these cells are both the reservoir for latent virus and also have the potential to be activated into lytic cycle, producing the virus particles detected in throat washings.

In both scenarios the site of latency is postulated to be the B lymphocyte. This certainly is supported by experimental evidence. Interestingly, reverse transcription polymerase chain reaction (RT-PCR) analysis of viral DNA expression in peripheral blood lymphocytes showed that both the pattern of latent gene expression (EBNA1 and LMP2 only) and the B cell

surface phenotype (CD23⁻ and CD80⁻) are distinct from that of EBV-transformed LCLs *in vitro*. It is very likely that EBV persists in a nondividing long-lived B cell which is effectively invisible to virus-specific cytotoxic T lymphocyte (CTL) responses, and which is sensitive to normal physiological controls over B cell development.

Figure 1 summarizes the essential aspects of EBV persistence and the immune responses that normally keep the pathogenic potential of the virus in check. While the virus apparently persists in a non-dividing and long-lived B cell in an immunologically silent state, the host mounts potent HLA-restricted CTL responses which are specifically directed against target peptides derived from those EBV latent genes expressed in growth-transformed LCLs, in particular EBNA3A, -3B and -3C. The potency and persistence of this CTL response suggests that reactivation of EBV-infected B cells to lymphoblastoid cells with a broader pattern of latent gene expression is a constant feature of the normal biology of EBV infection *in vivo*. The importance of this cellular immune response in controlling EBV-induced B lympho-

antibodies to VCAs and EA-R. Anti-EBNA responses are delayed by several weeks and may reflect the release of EBNA from latently-infected cells following the development of secondary potent EBV-specific CTL responses.

Of the various humoral responses, only the anti-MA antibodies are protective. Protection probably occurs at two levels: 1) by neutralizing infectious virus; and 2) by mediating antibody-dependent cellular cytotoxicity (ADCC) through binding to MA on the surface of lytically-infected cells.

Immune response of the host in EBV-associated diseases

Infectious mononucleosis Infectious mononucleosis (IM) occurs mainly in affluent Western societies where primary infection with EBV generally occurs at a later age. IM is a self-limiting lymphoproliferative disease that is characterized clinically by fever, sore throat, lymphadenopathy, hepatosplenomegaly and abnormal liver functions. Many of the manifestations in IM are probably a consequence of ineffective cellular immune responses to EBV-induced proliferation of B cells or due to inadvertent tissue damage by autoimmune processes. The majority of atypical lymphocytes in the blood of these patients are T cells, and elevated numbers of suppressor T cells, CTLs and natural killer (NK) cells are present. The cytotoxic response shows a broad specificity that is largely non-HLA-restricted, although a minor population of EBV-specific HLA-restricted CTLs is detectable and this subsequently dominates the cellular immune response as the disease resolves itself.

The serological picture in IM patients is distinct from that seen in asymptomatic primary infection in children. In addition to IgM antibodies to VCA, IM patients also show a transient (2–3 weeks) IgM heterophile antibody response of the Paul–Bunnell type. Furthermore, the IgG antibodies to EA are usually directed to the diffuse (EA-D) components rather than to the EA-R as in silent primary infection. The presence of IgG antibodies to EA-D may correlate with lymph node involvement, which is consistent with the elevated incidence of these antibodies in nasopharyngeal carcinoma, where invasion of draining lymph nodes commonly occurs, and their initial absence in Burkitt's lymphoma, when lymph nodes are not usually involved (see below). Unlike silent primary infection, where IgG antibodies to EA and VCA are transient, these antibodies may remain elevated in IM patients for up to 2 years after the clinical symptoms resolve themselves, which is indicative of increased levels of virus production. The antibody response to the gp340/220 MA components is

delayed, which is consistent with the relatively low virus-neutralizing titers observed in IM sera.

Burkitt's lymphoma Burkitt's lymphoma (BL) is a monoclonal B cell tumor and is the most common childhood tumor in equatorial regions of Africa and Papua New Guinea. In these high-incidence regions, EBV is found in all the tumor cells of at least 96% of BL patients. In addition to EBV, malaria and a specific chromosome translocation are cofactors in the development of BL. The chromosome translocation, which results in deregulated expression of the *c-myc* oncogene, is an essential feature of all BLs and is thought to arise as a rare accident of DNA rearrangements occurring during B cell development. Young children who are at greatest risk for developing BL suffer multiple bouts of clinical malaria each year, and during acute attacks their T cell immunity to EBV is demonstrably impaired. It has been suggested, therefore, that these periods of immunosuppression may lead temporarily to increased EBV-induced B cell proliferation which would increase the chance of chromosome aberrations. Alternatively, or additionally, since the malarial parasite is itself mitogenic, the translocation may arise during malaria-induced hyperproliferation of germinal centers. Whatever the precise mechanism of lymphomagenesis, an important feature of BL is the outgrowth of the tumor in the face of normal EBV-specific cytotoxic T cell responses between bouts of malaria.

Serologically, BL patients show elevated IgG antibody titers to VCA and to EA-R. Antibodies to EA-D frequently appear during late stages of the disease. Total anti-EBNA titers may be normal or elevated, but qualitatively there is a broader response so that antibodies to several of the EBNA in addition to EBNA1 may be detected. This characteristic pattern of antibody responses clearly does not reflect altered antigen load due to the pattern of EBV genome expression in the tumor cells themselves since they express only EBNA1 and none of the other latent or lytic genes, but presumably reflects unusual viral activity in normal, nonmalignant cells.

Lymphomas in immunosuppressed individuals

EBV-positive lymphoproliferative lesions of oligoclonal or monoclonal nature may arise in post-transplant patients undergoing cyclosporine immunosuppressive therapy. In contrast to BL, these lymphoproliferations do not have any consistent chromosome aberrations and their cellular and viral gene expression closely resembles that of cultured LCLs. The implication is that the tumors are primarily virus driven and are a direct consequence of

impaired cell-mediated immunity. Patients with Duncan's syndrome have a congenital defect in the ability to mount an EBV-specific cellular immune response, so that primary infection with EBV results in fatal lymphoproliferative disease driven by the virus. EBV-positive B cell tumors in acquired immune deficiency syndrome (AIDS) patients are usually similar to the lymphomas of post-transplant patients but, interestingly, some AIDS patients do develop classical BL-type tumors.

Nasopharyngeal carcinoma Undifferentiated carcinoma of the nasopharynx (NPC) is particularly common in Chinese populations in South-East Asia, where it accounts for nearly 20% of all malignant neoplasms. EBV is present in the tumor cells of all NPC patients, but the etiology of the disease is poorly understood. Genetic, dietary and environmental factors have all been implicated as cofactors along with EBV. Viral gene expression in the tumors is restricted to EBNA1 with or without LMP1 and LMP2. The CTL response to EBV-infected B cells is only marginally reduced in NPC patients relative to healthy controls.

A remarkable feature of the humoral response to EBV in NPC is the characteristic IgA response to lytic cycle antigens. Screening at-risk populations for serum IgA responses to VCA and EA allows the detection of early stages of NPC, at which point the tumor is particularly responsive to radiotherapy. Following eradication of the tumor, the IgA responses diminish and are therefore of prognostic value. IgG responses both to lytic cycle antigens and to the latent antigens are also elevated in NPC. As with BL, the characteristic pattern of antibody responses in NPC is not simply a consequence of antigen load due to viral gene expression in the tumor cells themselves, but must reflect other aspects of virus activity in these patients.

Other diseases associated with EBV EBV has been implicated in the pathogenesis of a number of other diseases, including: oral 'hairy' leukoplakia in AIDS patients, smooth muscle tumors in immunocompromised children, certain T cell lymphomas, Hodgkins disease, central nervous system lymphomas and Sjögrens syndrome.

Evasive strategies by the virus

In common with other persistent viruses, EBV has evolved strategies for evading immune responses so that it is never completely eliminated from the infected host. The hypotheses of EBV latency in healthy carriers have been greatly influenced by studies on EBV-positive BL tumors which are refractory to

CTL lysis. The primary mechanism of immune escape by these tumors is that expression of the immunodominant target antigens (EBNA3A, -3B and -3C) is repressed; EBNA1 is the only virally-encoded protein expressed in BL tumors, and this protein has a unique strategy for evading CTL responses. EBNA1 contains a large glycine/alanine-rich region that somehow prevents processing of EBNA1 target peptides for CTL recognition, but does not affect the ability of target peptides to be presented from other antigens in the same cell. In addition to the restricted pattern of viral gene expression, BL cells are also inherently impaired in endogenous antigen-presenting capability, owing to transcriptional downregulation of the TAP peptide transporter proteins and major histocompatibility complex (MHC) class I products. Finally, these tumors express low levels of cellular adhesion molecules that are involved in CTL:target cell interactions.

It has long been thought that the immune-evasion strategies of BL may not be unique to the malignant cells, but might also be a feature of virus latency in normal healthy carriers. In certain respects this hypothesis is supported by recent research. Thus, in healthy carriers RT-PCR analysis of circulating lymphocytes suggests that EBNA1 and LMP2 are the only virally-encoded proteins expressed. The detection of LMP2 is unexpected, and does pose a problem as LMP2 is a membrane protein that can provide HLA-A2-binding peptides that are transported to HLA in the endoplasmic reticulum in a TAP-independent manner, and CTL recognition of TAP-negative and LMP2-positive target cells has been demonstrated *in vitro*. However, there are several points that should be borne in mind. Firstly, the RT-PCR analysis does not reveal whether all the EBV-positive cells express LMP2, and neither does it indicate the level of expression. Secondly, LMP2 appears not to be an immunodominant target antigen for EBV-specific CTL. Thirdly, it may be relevant the latent EBV-carrying cells *in vivo* lack expression of the CD80 costimulatory molecule.

Given the potent transforming capacity of EBV, it is very important that strategies for immune evasion are balanced by mechanisms for eliminating excessive lymphoproliferation. In this respect it is notable that the LMP1 gene, whose expression is essential for EBV-induced growth transformation of resting B cells, has a major effect upon the immunogenicity of the target cell by upregulating TAP, HLA and cell surface accessory molecules. This ensures that EBV-induced proliferation of normal B cells is accompanied by a concomitant sensitization to cellular immune responses.

Antiviral treatment

The best documented antiviral reagent used to combat EBV is acyclovir, which inhibits the viral DNA polymerase but which has no effect on the latent viral genome. Acyclovir has been used successfully to treat the oral 'hairy' leukoplakia lesions in AIDS patients. However, trials with IM patients showed that, while detectable virus secretion in the oropharynx is abolished by acyclovir treatment, the numbers of latently-infected B cells in the circulation apparently remains unchanged. Most importantly, acyclovir treatment has no effect on the clinical course of IM. The use of interferon α in renal allograft recipients appears to have some effect upon virus secretion, but the effect is less marked than with acyclovir and is similarly ineffective clinically.

The development of a vaccine for EBV has so far concentrated on gp340 MA, either as purified glycoprotein or in recombinant adeno- or vaccinia viruses, with the aim of generating antibodies that are virus neutralizing and which might also be effective against cells in late lytic cycle. Animal trials using the cotton-top tamarin have been encouraging, although the mechanism of protection is unclear, as virus neutralizing antibodies are not always produced in vaccinated animals. Human trials with this vaccine are in progress. An alternative vaccine strategy involves the use of CTL epitope peptides directed against latently-infected cells. A 9-mer peptide, FLRGRAYGL, that is the immunodominant epitope of EBNA3A presented through HLA-B8, is currently being investigated.

Adoptive transfer of EBV-specific CTLs represents an alternative approach to restoring cellular immunity in immunosuppressed patients and may prove useful for the eradication of EBV-positive tumor cells. Rooney and colleagues have applied this strategy in bone marrow transplant recipients, where CTL infusions have reduced the viral load in patients

showing signs of EBV reactivation and resolved an immunoblastic lymphoma.

See also: Acquired immune deficiency syndrome (AIDS); Adhesion molecules; Antibody-dependent cellular cytotoxicity; Complement receptors; Heterophile antibodies; HLA class I; Immunosuppression; Malaria.

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ERYTHROCYTES

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Structure and shape of erythrocytes

The final stage of erythroid maturation is the circulating erythrocyte which performs the basic function of oxygen delivery. Normal human erythrocytes

appear as circular, flexible, biconcave discs, lacking a nucleus and most of the cytoplasmic organelles accompanying the erythroid precursors during their differentiation and maturation processes. These cells stain red-orange with a relatively pale centre in normally prepared smears (Giemsa staining), while they

show the characteristic biconcave shape (discocyte) by scanning electron microscopy (SEM).

Resting cells have a diameter of 7.5–8.3 μm . They are 1.7 μm thick (0.6 μm on dried blood films) and have an average volume of 83 μm^3 . When first formed and released from the bone marrow, the young erythrocyte is subjected to some surface remodeling, due to numerous events (mechanical fragmentation, immunologic reactions, chemical alterations) which may irreversibly alter the membrane structure. The progressive loss of membrane fragments, with subsequent reduction of the cell surface area, leads to the formation of a rigid spherocyte, unable to withstand continuous deformations required by circulatory stresses. The maintenance of the normal shape of the erythrocytes is dependent not only on the environment of the cell but also on its metabolic condition and age. Thus modification of the cellular shape appears as a dynamic process which allows the red cell to be stretched (up to one-twentieth of the cell diameter) when passing through circulatory obstructions or narrowed vessels, and to undergo, under physiologic conditions, different reversible changes.

The availability of three-dimensional morphologic features obtained by SEM has provided a careful analysis of a spectrum of red cell shape changes occurring in normal and pathologic conditions. A visual demonstration of the deformation processes of the red cells and of the mechanisms underlying the fragmentation and resealing of these membranes has been obtained from several human and experimental models. Under physiologic conditions the discocyte may undergo two distinct reversible changes, i.e. the *echinocytic* (spiculated) and the *stomatocytic* (cup-shaped) forms, which are strictly dependent on the pH of the medium and intrinsic metabolic rate of the cells. According to Bessis, these two basic features can be viewed as opposite phenomena, resulting from the exposure of the red cells to conditions or substances which are antagonistically active (Figure 1). The persistence of the action of transforming

agents is associated with a progressive deformation of these configurations, leading through morphologically recognizable stages (spheroechinocyte and spherostomatocyte) to a fully developed spherocytic appearance which is a nonreversible condition. Spherocytes are easily trapped by reticuloendothelial cells, mainly in the spleen. The mechanism of the reversible changes is possibly due to the differential expansion of either the outer or inner leaflet of the lipid bilayer. The expansion of the outer half is associated with membrane spiculation, whereas the expansion of the inner half leads to membrane invagination and cup-shaped cells.

Beside these physiologic (reversible) modifications of the red cell shape, some cleavage processes of the membrane or metabolic events may cause a persistent (irreversible) deformation of the cell surface. The variety of the morphologic features of distorted erythrocytes has led to a classification (according to an international Greek nomenclature), which reflects the prominent characteristics of the pathologic cells. The detection of these features offers a key for the interpretation of some underlying disorders in many patients (for example, the presence of acanthocytes in abetalipoproteinemia in liver alcoholic cirrhosis, of codocytes (target cells) in thalassemia, hemoglobinopathies or in hereditary lecithin-cholesterol acyltransferase (LCAT) deficiency, of keratocytes (helmet cells) and schizocytes in microangiopathic hemolytic anemia and disease associated with intravascular deposition of fibrin, of dacryocytes (tear drop cells) in myelofibrosis, of drepanocytes in sickle cell anemia.

Red cell membrane

Several functions of the red cells are closely related to different structures of the cell membrane. These structures are involved in most of the metabolic, antigenic, transporting and receptor activities of the erythrocytes.

Biochemical aspects

The red cell membrane is constituted by a *lipid bilayer* and by a submembraneous network of polypeptides (*membrane skeleton*) which confers upon the red cells the capacity to maintain its characteristic biconcave shape and the flexibility to change shape during passage through the circulation without apparent damage.

Lipids represent 50% of the membrane weight (Table 1). They are asymmetrically distributed across the bilayer plane as well as within each bilayer. This asymmetry is due to the action of an aminophospholipid translocase or 'flippase', possibly related to the

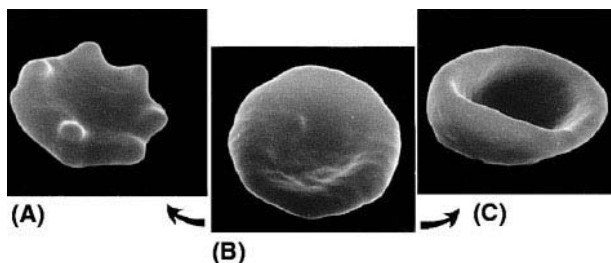


Figure 1 Echinocytic (A) and stomatocytic (C) cells represent distinct morphologic changes of the normal erythrocyte (discocyte) (B), possibly related to the selective expansion of the outer or inner leaflets of the lipid bilayer.

Table 1 Lipid composition of normal human red cell membrane

	Weight (%)	Approx. no molecules/ghost (million)	Outer half of bilayer (%)	Inner half of bilayer (%)
Phospholipids	62	250	~45	~55
Cholesterol	28.9	195	50	50
Glycolipids	6.6	10	100	0
Free fatty acids	2.2	20	?	?

multidrug resistance (*mdr*) gene product. Furthermore, surface-bound membrane proteins may contribute to the nonrandom topography of the lipid plane, leading to the so-called 'cis asymmetry'.

The red cell membrane contains 10–15 major polypeptides conventionally classified as 'integral' or 'peripheral' on the basis of their localization with respect to the lipid bilayer. They are usually identified by their mobility in SDS-PAGE (Figure 2). Most of the biochemical characteristics of these components and their functional properties are summarized in Table 2, see p. 838.

Many of these molecules interact with each other, participating in the building of the membrane *skeleton*, a network of proteins underlying the lipid bilayer membrane, which preserves the structural integrity and deformability of the human erythrocyte – both essential for its survival. This skeleton is a polygonal lattice created by the cross-linking of short *actin* filaments by long tetramers of *spectrin* (25–30% of membrane total protein), which maintains the cellular shape, regulates the lateral mobility and provides structural support for the lipid bilayer. Spectrin is composed of two polypeptide chains, α and β , that are associated in an antiparallel manner into fiber-like flexible heterodimers, approximately 100 nm long. Heterodimers associate head-to-head to form tetramers. The tails of tetramers interact at junctional complexes through the binding to F-actin filaments, leading to a polygonal array mostly composed of hexagons and pentagons. This last interaction is greatly strengthened by protein 4.1. The spectrin–actin–4.1 junction contains other associated proteins, *dematin*, the two polypeptides of *adducin*, *tropomyosin*, *tropomodulin*, that modify and regulate the interaction of the three core components. Protein 4.1 cross-links spectrin dimers with actin and with proteins in the overlying lipid bilayer.

The membrane skeleton is also connected to the integral proteins of the overlying lipid bilayer by *ankyrin* (Figure 2B). This molecule binds β spectrin near the self-association site and links it to the cytoplasmic portion of *band 3* (anion exchange protein 1, AE-1), which constitutes the major integral protein of the red cell, and represents the true anchor

for the membrane skeleton. *Pallidin* is a 77 kDa myristylated peripheral membrane protein that binds to band 3 and probably to ankyrin.

Glycophorins represent a complex family of integral membrane sialoglycoproteins. The obvious function of glycophorins is to provide a negative surface charge that red cells require to avoid cellular adhesion. In addition, they may participate in the transmembrane signalling and bear receptors for *Plasmodium falciparum*.

All the major erythrocyte proteins are affected by one or several types of post-translational modifications, such as proteolysis, oxidation, phosphorylation and fatty acid acylation, which may have profound effects on the function of the protein. Some membrane proteins use a lipid-containing anchor to be inserted in the membrane, just as do most non-glycoprotein membrane-bound polysaccharides. The anchor consists of a complex including phosphatidylinositol (GPI) or a glycolipid. The absence of GPI-linked proteins in paroxysmal nocturnal hemoglobinuria (PNH) is the result of a defect in the very early steps of the biosynthesis of the anchor. The PNH gene resides on the X chromosome.

Several forms of hereditary hemolytic anemias may exhibit some abnormalities in the interaction of the skeletal components. Molecular defects of spectrin and of protein 4.1 have been recognized in some patients with spherocytosis and elliptocytosis.

Antigenic aspects

Immunologic properties of the red cells are mainly related to the presence on their membrane of blood group antigen-bearing molecules (glycoproteins and glycosphingolipids) (Table 3, see p. 840). The most abundant sialoglycoproteins, glycophorin A (GPA) and GPB are integral membrane proteins, which, like band 3 glycoprotein, may extend beyond the surface of the cells with attachment of numerous carbohydrate chains. The external domain of GPA carries blood group M or N activity (depending on the amino acid residues at positions 1 and 5), while GPB carries blood group N activity as well as blood S or s antigen activity with respect to the amino acid substitutions at residue 29. GPC contains the Gerbich

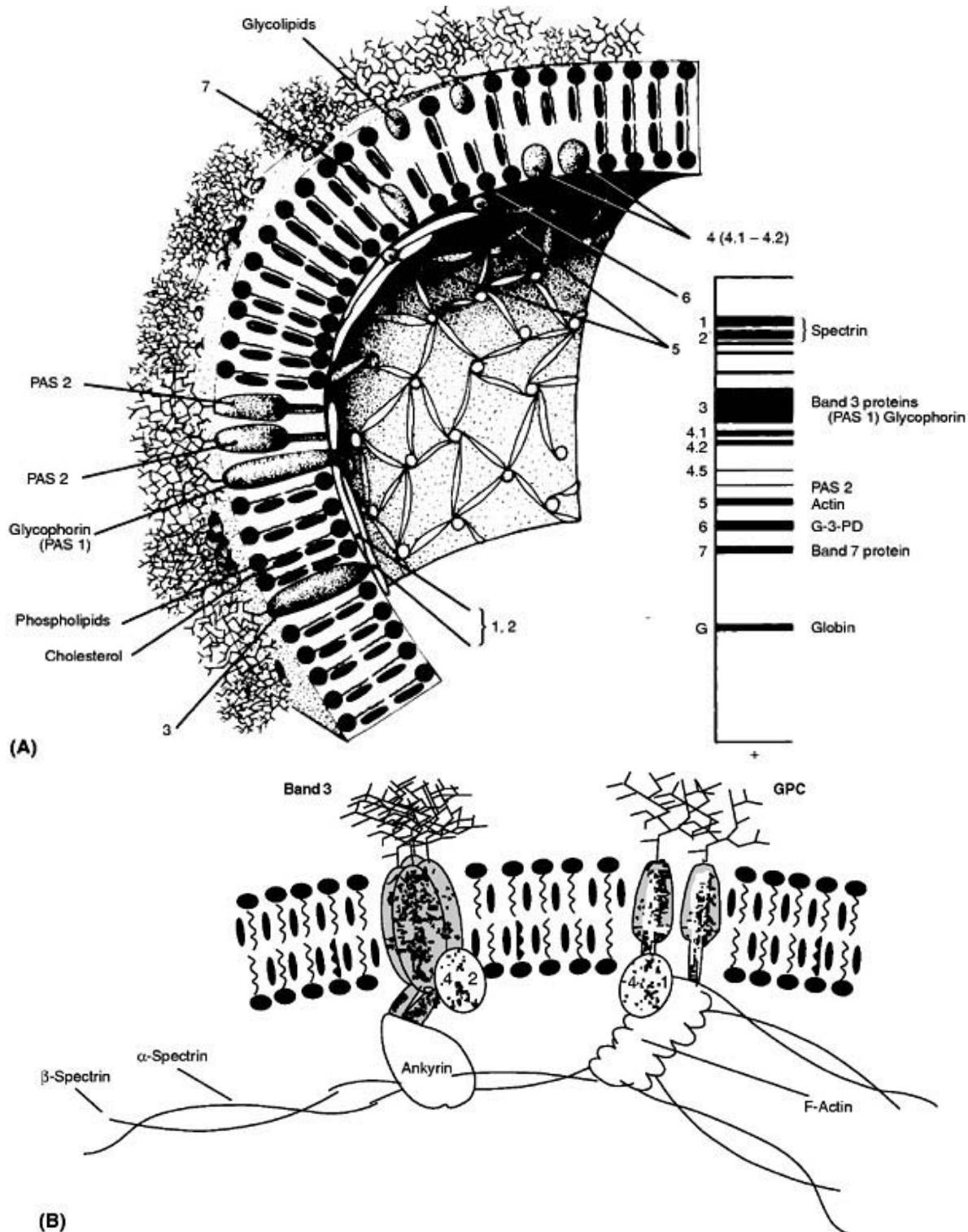


Figure 2 (A) Model of the human erythrocyte membrane showing the major membrane proteins, numbered according to their sequence in SDS-PAGE pattern after Coomassie blue staining (inset). 1–2: Spectrin; 3: band 3 protein(s); 4.1 and 4.2: band 4.1 protein and band 4.2 protein; 5: actin; 6: glyceraldehyde-3-phosphate dehydrogenase; 7: band 7 protein. Red cell membrane sialoglycoproteins (glycophorins), after separation by SDS-gel electrophoresis and staining with the periodic acid–Schiff base stain (PAS), appear to be associated with different ‘PAS regions’. The dimer of glycophorin A is associated with PAS 1 region and migrates within band 3. PAS 2 region is composed of three bands: the monomer of glycophorin A, the dimer of glycophorin B and glycophorin C. The monomer of glycophorin B is associated with PAS 3 region (not shown in the figure). (Adapted from Castoldi and Beutler (1988).) (B) Protein interactions at junctional complexes showing the associations between a spectrin tetramer and band 3, and between spectrin tail ends and GPC in the erythrocyte membrane.

blood group antigens. GPC binds protein 4.1, participating in the anchoring of the skeleton to the membrane. GPD is a shortened form of GPC arisen from an alternative initiation codon on the same RNA. Another member designated glycophorin E (GPE) has been identified in the course of molecular genetic studies.

Band 3 protein carries I, i, H, A and B specificities: the complexity of the carbohydrate chains is variable, as suggested by the fetal (linear) form of the i antigen and the adult (branched) form of the lactosaminoglycan of the I antigen. Furthermore a part of band 3 protein has been possibly associated with the major Rh determinant(s).

Rh polypeptides have been found to be a major fatty acylated membrane protein in red blood cells. The RH locus is composed of two homologous, closely linked genes per haploid genome, one encoding the D protein, the other encoding the CcEe protein(s). Most of the Rh polypeptide chain is hydrophobic and is predicted to reside between the leaflets of the phospholipid bilayer with 12 or 13 bilayer-spanning domains, while the short N-terminus and C-terminus are located intracellularly. They seem to be required for the correct transport or cell surface expression of certain glycoproteins. The absence of Rh antigens may lead to the loss of membrane phospholipid asymmetry and to the appearance of morphologic abnormalities.

An integral 93 kDa glycoprotein, structurally similar to zinc-neutral endopeptidases and related to McLeod antigens, identifies the Kell (K) group, while a 35–43 kDa glycoprotein, the carrier of the Duffy blood group Fy antigen, is involved in the invasion of red cells by *Plasmodium vivax*.

Some antigens are not synthesized by the erythroid cells, but they may be in some way acquired from the plasma, like the glycoproteins Chido (Ch) and Rogers (Rg). Ch and Rg substances are located on the C4d fragment of complement and are acquired by red cells as a consequence of complement activation *in vivo* with subsequent cellular attachment of C4d.

Blood group antigen deficiencies may be associated with some abnormal red cell shapes; for example, the absence of Gerbich blood group antigen(s) is, in some instances, associated with ellipsocytosis (Leach phenotype), Rh null phenotype with stomatocytosis, and McLeod phenotype with acanthocytosis. These features may support the contention that molecules bearing the antigenic determinants are important for maintaining the membrane properties and physiologic functions of the red cells.

Enzyme activities and energy metabolism

The metabolic activity of the red cells and fulfillment of their different functions is dependent on the utilization of glucose in the plasma as an almost exclusive source of energy. Glucose transfer into red cells is accomplished by a membrane carrier which combines with D-isomers of hexose (not fructose) without requiring insulin. The further steps of utilization of glucose involve two major metabolic pathways: the Embden–Meyerhof direct glycolytic pathway (by which the glucose is anaerobically catabolized to pyruvate or lactate) and the hexose monophosphate (HMP) shunt involving a direct oxidative pathway. While the erythrocytes utilize the anaerobic pathway for generating ATP, the HMP does not generate high-energy phosphate bonds. The latter provides NADPH which works as a substrate for the reduction of glutathione-containing disulfides in the erythrocytes, in response to oxidant stress.

A large number of enzymes of glucose metabolism are involved in these processes; however, some enzymatic activities may utilize other substrates (adenosine, inosine, fructose, mannose, galactose, dihydroxyacetone, lactate) different from glucose. These alternative energy sources of the red cells may be important during blood storage.

Red cells are endowed with a further series of enzymes such as carbonic anhydrase (which is considered an early marker of erythroid differentiation), catalase, acetylcholinesterase, aldehyde dehydrogenase, protein kinase activities and dipeptidases.

Ion pump and channels

The osmotic imbalance created by the presence of impermeant polyanions (hemoglobin, 2,3-diphosphoglycerate, glutathione) in the red cells requires a series of regulatory mechanisms, involving the control of small ions, in order to avoid cell swelling and subsequent hemolysis. The membrane of the red cell is easily permeable by plasma water and anions such as chloride (Cl^-) and bicarbonate (HCO_3^-), while cations such as Na^+ and K^+ have a very slow exchange through the membrane. Thus several transporting systems, which are responsible for maintaining a steady-state cation concentration, are constantly needed by the erythrocytes. One of the most important is represented by the Na^+ pump (Na^+/K^+ -ATPase complex) which utilizes ATP for exchanging Na^+ (3 mmol of Na^+ extruded for each 2 mmol of K^+ transported into the cell). Several hormones, such as aldosterone, thyroid hormone and insulin, appear to modulate the activity of the Na^+ pump. Furthermore, various derangements of the red cell Na^+ and K^+ transport pathway have been detected in hyper-

Table 2 Major membrane proteins of red cells

<i>Electroph. protein band</i>	<i>Designation</i>	<i>Mol. wt. (kDa)</i>	<i>Amino acids</i>	<i>Chromosome location</i>	<i>Gene size (Kb)</i>	<i>Approx copies/ghost</i>	<i>Function</i>	<i>Interactions</i>	<i>Localization</i>	<i>Assembly state/comments</i>	<i>Associated diseases</i>
1	α -spectrin	281	2429	1q22-q23	80	250 000	Membrane skeleton	Band 3, ankyrin	Peripheral	Associates to heterodimers, tetramers, oligomers	HE, HPP, HS
1	β -spectrin	246	2137	14q23-q24.1	>100						
2.1	Ankyrin	206	1880	8p11.2	>120	125 000	Link between proteins of skeleton and plasma membrane	Band 3, β -spectrin	Peripheral	Monomer; 2.2, 2.3 and 2.6 bands are ankyrin isoforms	HS
2.9	α -adducin	81	737	4p16.3		30 000	Links actin filaments	Spectrin, actin	Peripheral	Heterodimers, tetramers	
2.9	β -adducin	80	726			30 000	Links actin filaments	Spectrin, actin	Peripheral	Heterodimers, tetramers	
3	Anion exchange protein 1	102	911	17q12-q21	17	1 100 000	Anion transport	Ankyrin, Spectrin, Protein 4.1	Integral	Dimer or tetramer; divided in two structural domains	HS, SAO, HAC
4.1	Protein 4.1	66	588	1p33-p34.1	>250	200 000	Membrane skeleton	Spectrin, actin, GPC	Peripheral	Monomer; exists in a variety of isoforms	HE
4.2	Pallidin	77	691	5q15-q21	20	250 000	Links skeleton to lipid bilayer	Band 3, ankyrin	Peripheral	Dimer or trimer; homologous to transglutaminases, but without activity	HS
4.9	Dematin (α , β)	43 + 46	383	8p21.2-21.1		140 000	Actin-bundling protein	Actin, lipids	Peripheral	α_2 , β -trimer; C-terminal half similar to villin	

4.9	p55	53	466	Xq28	80 000		Protein 4.1, GPC	Peripheral	Dimer
5	β -actin	42	375	7pter-q22	>4 500 000		Spectrin, Protein 4.1	Peripheral	Double helical actin filaments which form vertices of hexagonal lattice
5	Tropomodulin	41	359	9q22	30 000		Tropomyosin, Actin	Peripheral	Monomer
6	GADPH	36	335	12q13	5 500 000		Band 3	Peripheral	Tetramer
7	Stomatin	32	288		12 288 000		Actin	Integral	HSt
7	Tropomyosin	28	239	1q31	70 000		Actin	Peripheral	Heterodimer
8	Protein 8	23						Peripheral	
GPA	Glycophorin A	14	131	4q31	>40 1 000 000		Negatively charged Phospholipids	Integral	Dimer; provides most of the negative surface charge
GPB	Glycophorin B	8	72	4q31	>30 200 000		Protein 4.1	Integral	None
GPC	Glycophorin C	14	128	2q14-q21	14 200 000		Membrane skeleton anchor	Integral	Heterodimer (C + D) HE
GPD	Glycophorin D	11	107	2q14-q21	14 200 000			Integral	Originated by alternative splicing of GPC mRNA
GPE	Glycophorin E	6	59	4q31	>30				Resembles GPB in structure

HE, Hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; HS, hereditary spherocytosis; SAO, South-East Asian ovalocytosis; Hac, hereditary achantocytosis.

Table 3 Blood group antigen systems according to International Society of Blood Transfusion (ISBT) terminology (1995)

<i>System name</i>	<i>Chromosomal location</i>	<i>Gene name</i>	<i>Molecule type</i>
ABO	9q34.1–q34.2	ABO	Polysaccharide
MNS	4q31	GYPA, GYPB, GYPE	Polysaccharide
Rh	1p34–36	RHD, RHCE	Protein
Lutheran	19q12–q13	LU	Protein
Kell	7q32–36	KEL	Protein
Lewis	19p13–3	FUT3	Polysaccharide
Duffy	1q22–q23	FY	Protein
Kidd	18q11–q12	JK	Protein
Diego	17q12–q21	AEI	Protein
Yt	7q22	ACHE	Protein
Xg	Xp22–32	XG	Protein
Scianna	1p36.2–p22.1	SC	Protein
Dombrock	Unknown	DO	Protein
Colton	7p14	AQP1	Unknown
Landsteiner–Wiener	19p13.2 cen	LW	Protein
Chido/Rodgers	6p21.3	C4A, C4B	Protein
Hh	19q13	FUT1	Polysaccharide
Kx McLeod	Xp 21	XK	Protein
Gerbich	2q14–q21	GYPC	Protein
Cromer	1q32	DAF	Protein
Knops	1q32	CRI	Protein
Indian	11p13	CD44	Protein

ACHE, acetylcholinesterase; AQP1, aquaporin 1, water transporter; FUT, fucosyltransferase; DAF, complement regulatory molecule-decay accelerating factor; CRI, C3b/C4b receptors.

tensive patients. Ca^{2+} fluxes in the red cells are regulated by another transport system constituted by an enzyme stimulated by calmodulin which improves its affinity for Ca^{2+} . Increase of Ca^{2+} sharply impairs the deformability of the erythrocyte. The Ca^{2+} pump activity appears to be influenced by thyroid hormone.

Band 3 is the conduit for the important chloride–bicarbonate exchange, but it is also the major binding site for a variety of enzymes. The 40 kDa N-terminal cytoplasmic domain binds and inhibits the glycolytic enzymes glucose-3-phosphate dehydrogenase (G3PDH), phosphoglycerate kinase (PGK), aldolase, and hemoglobin too, and modulates the binding of band 3 to protein 4.1, pallidin and ankyrin. The 52 kDa transmembrane domain is composed of 12 or 14 transmembrane helices which form the transport channel. The 1.2 million channels in each red cell can exchange 10^{10} – 10^{11} HCO_3^- and Cl^- anions s^{-1} . An anion moves by a ‘ping-pong’ mechanism in which an intracellular anion enters the transport channel and is translocated outward and released (outward conformation) until the penetration of the channel by an extracellular anion triggers the reverse cycle. The mechanism involves fairly large conformational changes.

Receptors

The red cell membrane is provided with several receptor activities. Some of them include receptors for hormones like insulin (not utilized for glucose transport) or thyroid hormones. In addition β -adrenergic receptors (possibly playing a role in the transport of Mg^{2+}) and acetylcholine receptors have been demonstrated. Erythrocytes bear a complement receptor (CR1) which is able to bind C4b molecules attached to the red cell membrane during complement activation. No transferrin receptors have been detected on the mature erythrocytes, these structures being lost at the reticulocyte stage. Putative receptors for prostaglandin E, parathyroid hormone, diphtheria toxin, ceruloplasmin and opiates have been reported.

Red cell aging and destruction

The average life span of circulating erythrocytes is about 120 days. Aging of the red cells is associated with a decrease of the enzymatic activities, a progressive increase of hemoglobin concentration and red cell viscosity due to loss of total cation and water content (‘dense red cells’), and structural modifications of the membrane with a reduction of the sur-

face:volume ratio, leading to the formation of a rigid spherocyte with enhanced osmotic fragility. These aspects occur in physiologic conditions, but similar features may be observed in various pathologic disorders associated with hemolytic processes.

Trapping of the senescent erythrocytes by reticulo-endothelial cells is the last event of the life of these cells. This phenomenon may be viewed as an immunologic process characterized by the exposure of hidden antigenic sites followed by the binding of naturally occurring autologous antibodies (immunoglobulin G) which trigger the phagocytosis. Exposure of epitopes of band 3 may represent one of the events closely associated with the senescence of the erythrocytes.

Several other damaging processes (oxidative, proteolytic) may intervene during the life of the circulating erythrocytes; some of these are operative concomitantly with the presence of molecular defects of the cytoskeleton or of the hemoglobin structure, accelerating the red cell clearance.

See also: ABO blood group system; Agglutination; Anemia, autoimmune hemolytic in human; Blood transfusion reactions; Bone marrow and hematopoiesis; Carbohydrate antigens; Complement receptors; Embryonic antigens; Hemolytic disease of the newborn; Protein separation techniques; Rh antigens; Spleen.

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ESCHERICHIA COLI, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Escherichia coli is a gram-negative, facultatively anaerobic, small bacillus (1–2 µm) in the family Enterobacteriaceae. The Enterobacteriaceae are a large and diverse group of gram-negative, nonspore-forming bacilli that ferment glucose with the production of acid and usually gas, are oxidase-negative, reduce nitrate to nitrite, and are usually catalase-positive. *E. coli* is peritrichous, meaning that flagella grow from many places on the cell surface. It also often contains a polysaccharide capsule. *E. coli* colonies produce a characteristic 'metallic green sheen' when cultured on eosin methylene blue agar.

E. coli is a member of the indigenous (i.e. normal) intestinal microflora of humans and animals and also is isolated from a variety of environmental sources. It is the bacterium most often isolated from clinical specimens sent to the medical laboratory. In fact, *E. coli* is the most common bacterial type isolated from hospitalized patients with bacteremia/septicemia. The genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* are collectively referred to as 'coliforms' because of their ability to ferment lactose. The isolation of coliforms from water and other sources by bacteriology laboratories is often used as an indicator of human and animal fecal contamination.

Serotypes (antigenic classification)

The major antigens of *E. coli* are the K, H and O antigens. K antigens are components of the polysaccharide capsule or pilus-like proteins, H antigens are located on flagella, and O antigens compose the outer polysaccharide chains linked to the core lipopolysaccharide (LPS) of the bacterial cell wall of gram-negative bacteria. O antigen specificity is determined by the sugar sequence of the polysaccharide chains. These heat-stable, somatic O antigens commonly identify the serogroup of an *E. coli* strain. More than 170 O antigen *E. coli* serogroups have been identified and there is a relationship between certain *E. coli* serogroups and *E. coli* virulence. For example, *E. coli* O55 serogroup is often associated with human disease.

H antigens (>50) identify the *E. coli* serotype. Thus, *E. coli* serotypes are specific combinations of O and H antigens. *E. coli* O157:H7 is the serotype most recently associated with hemorrhagic colitis, often caused by eating undercooked ground beef. K antigens identify particular *E. coli* strains by their capsule type. For example, *E. coli* K1 is the most common cause of bacteremia/meningitis in infants.

Most *E. coli* also produce heat-labile surface proteins seen microscopically as filamentous structures (called pili or fimbriae) different from flagella and antigenically unrelated to O, H or K antigens. Even with the introduction of new molecular biology methods of *E. coli* classification, serologic typing is still widely used as a convenient method for tracing outbreaks of *E. coli* diarrheal disease.

Virotypes (diarrhea-producing *E. coli*)

Recently, *E. coli* strains have been categorized on the basis of certain virulence factors (i.e. virotyping). This classification scheme is based on the production of certain *E. coli* toxins, patterns of *E. coli* adherence to particular host cells, the abilities of *E. coli* strains to invade the intestinal epithelium, and the patterns of intestinal mucosal pathology produced by *E. coli*. Virotyping therefore more directly relates various *E. coli* strains to the actual disease process than does serotyping. Currently, diarrhea-causing *E. coli* are divided into five virotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC). The various properties of these *E. coli* virotypes are presented in **Table 1**.

Enterotoxigenic *E. coli* (ETEC) produce a diarrheal disease similar to that caused by *Vibrio cholerae*, although less severe. Nonetheless, ETEC diarrhea when combined with malnutrition is a major cause of morbidity and mortality in infants and small children during the first 2 years of life in Third World countries. Transmission is through contaminated food and water. Thus, ETEC cause infant diarrhea in the Third World, traveler's diarrhea in both infants and adults traveling from developed to less developed countries, and food poisoning (i.e. gastroenteritis) in infants and adults in both

Table 1 Characteristics *E. coli* virotypes

	ETEC	EPEC	EHEC	EIEC	EAEC
Disease	Infant diarrhea Traveler's diarrhea Gastroenteritis (food poisoning)	Infant diarrhea Traveler's diarrhea Persistent diarrhea in children	Dysentery Hemorrhagic colitis Hemolytic uremic syndrome	Dysentery	Traveler's diarrhea Persistent diarrhea in children
Fever	Usually absent	Present	Absent	Present	Sometimes?
Toxins	LT and ST	No toxins	SLT	No toxins	ST ('EAST')
Primary site	Small intestine	Small intestine	Large intestine	Large intestine	Small intestine?
Invasiveness	Not invasive	Invasive	Not invasive	Invasive	Not invasive
Mucosa pathology	Intact mucosa	'Attaching and effacing' lesions 'Pedestals'	'Attaching and effacing' lesions	Necrosis and ulcerations similar to <i>Shigella</i>	Intact mucosa
Incidence	Common	Common	Rare	Very rare	Fairly rare?

developed and less developed countries. ETEC diarrhea is often misdiagnosed as a viral diarrhea since ETEC are not detected in stool samples by routine microbiology unless special instructions are given to the clinical laboratory.

ETEC produce several types of pili or fimbriae, such as type 1 pili and a number of specialized pili called colonization factor antigens (CFA), that act as mucosal adhesins enabling ETEC to colonize the small intestine. These CFA are plasmid-encoded. Recently, ETEC have been reported to possess a long polar pilus termed 'longus'. Like *V. cholerae*, ETEC adhere to the epithelium in the small intestine but do not usually penetrate the intestinal mucosa.

The pathogenesis of ETEC diarrhea involves the colonization of the small intestine by ETEC followed by the elaboration of diarrheagenic enterotoxins. On the intestinal mucosa, ETEC elaborate a heat-labile enterotoxin (LT) inactivated by heating at 100°C for 30 min and a heat-stable enterotoxin (ST). There are two subtypes of LT: plasmid-encoded LT-1 and chromosome-encoded LT-2. LT-1 shares a high degree of amino acid sequence identity with *V. cholerae* toxin and, like cholera toxin, is a classical AB toxin composed of one A (active) subunit and five B (binding) subunits. The B subunits bind to a specific ganglioside (G_{m1}) on the epithelial cells of the small intestine but are not internalized. The A subunit is subsequently activated by cleavage of a peptide bond and internalized. The A subunit catalyzes the ADP-ribosylation of membrane-bound adenylate cyclase resulting in increased cAMP and increased secretion of fluids and electrolytes. Thus, ETEC produces a watery noninflammatory diarrhea similar to cholera diarrhea.

ST toxins appear to be a group of small peptides ranging from 18 to 50 amino acids in length. ST-I binds to a glycoprotein receptor, thereby activating a membrane-bound guanylate cyclase. The increase

in cGMP increases the secretion of fluids and electrolytes to cause an LT-like watery diarrhea. ST-II appears to cause increased fluid secretion by a different mechanism and has been found so far only in porcine ETEC strains. The small ST peptides are nonimmunogenic.

Enteropathogenic *E. coli* (EPEC) cause only sporadic cases of diarrhea in developed countries but are a major cause of diarrhea in the Third World, especially in children less than 1 year of age. There are three stages in the pathogenesis of EPEC. In the first stage, EPEC attach to epithelial cells in the small intestine by a 'nonintimate' association facilitated by bundle-forming pili (BFP). The genes responsible for this adherence are located on a plasmid called EAF (EPEC adherence factor). EPEC form microcolonies on the intestinal mucosa during this stage. During the second stage, a signal transduction event is triggered in the host epithelial cell which activates host cell tyrosine kinases resulting in increased host cell intracellular calcium. In the third stage, a protein produced by EPEC called intimin allows EPEC to associate more closely with the host epithelial cells ('intimate' association). This association causes rearrangement of host cell actin resulting in deformation and destruction of epithelial microvilli ('effacement') and the formation of 'pedestal-like' structures of filamentous actin on which EPEC rests. This process is therefore called 'attaching and effacing' and the genes involved are designated eae. EPEC do not produce LT or ST and diarrhea is probably a consequence of the damaged intestinal epithelial cells no longer being able to absorb water effectively. It is unclear whether EPEC actually invade the intestinal mucosal barrier.

In developed countries, EPEC diarrhea is limited to occasional outbreaks in nurseries and day-care centers. In less developed countries, however, EPEC diarrhea occurs at a high incidence and a high

mortality, especially in children 6–12 months old. EPEC is also a recognized cause of chronic diarrhea of children in less developed countries.

Enterohemorrhagic *E. coli* (EHEC) possess powerful cytotoxins called Shiga-like toxins (SLT-I and SLT-II), since they are antigenically and functionally similar to the classical Shiga toxin of *Shigella dysenteriae*. SLT-I is identical to Shiga toxin whereas SLT-II is 56% homologous. SLT is an AB type toxin consisting of one A subunit and five B subunits. The B subunits bind to a membrane glycolipid while the A subunit is internalized and causes cell death by enzymatically modifying ribosomal RNA thereby preventing elongation factor 1-dependent binding of amino acyl tRNA.

EHEC can cause attaching and effacing lesions similar to EPEC, but the site of EHEC infection is the colon rather than the small intestine. EHEC SLT causes capillary thrombosis and associated inflammation of the colonic mucosa leading to hemorrhagic colitis. There is an acute inflammatory response and mucosal tissue destruction resulting in bloody diarrhea, the hallmark of hemorrhagic colitis. Symptoms usually resolve in 3–10 days.

E. coli O157:H7 is the EHEC serotype most often associated with hemorrhagic colitis and may produce larger amounts of SLT than other EHEC serotypes. *E. coli* O157:H7 hemorrhagic colitis was first recognized in 1982 and appears most prevalently in the USA in the Pacific Northwest, although cases do occur nationwide. Because of these hemorrhagic colitis outbreaks, hospital laboratories now test specifically for *E. coli* O157:H7. Most cases of *E. coli* O157:H7 have occurred following the ingestion of undercooked ground beef, although other contaminated sources also have been identified. Approximately 1% of apparently healthy cattle carry *E. coli* O157:H7 in their gastrointestinal tracts and meat becomes contaminated during slaughter and in the process of making ground beef. It is now recommended that ground beef be cooked at a temperature of 155°C.

Approximately 13% of patients with hemorrhagic colitis, most commonly children between 1 and 4 years of age, develop a sequelae called hemolytic uremic syndrome (HUS). EHEC toxins can cause direct renal endothelial damage and alter platelet function. The kidney damage may be irreversible and even lead to death. HUS is now the leading cause of acute renal failure in children.

Enteroinvasive *E. coli* (EIEC) is a newly recognized *E. coli* virotype (since 1971). EIEC cause an invasive dysenteric form of diarrheal disease, similar to that of *S. dysenteriae* but less severe. EIEC invade the mucosal epithelium of the colon and multiply, behav-

ing like an intracellular pathogen. No toxins have been identified. Fortunately, EIEC diarrhea is rare, occurring primarily in developed countries in children less than 5 years of age.

Enteraggative *E. coli* (EAEC) is the newest category of diarrhea-causing *E. coli* and, as the name implies, was first recognized because of the aggregation of EAEC *in vivo* on intestinal epithelium and also *in vitro* on certain tissue culture cells. The pathogenesis of EAEC is not understood, but EAEC appear to possess particular adhesins called GVVQP fimbriae, the name derived from the primary amino acid sequence. EAEC also produce a stable toxin called EAST (enteroaggative ST). EAEC may be a cause of so-called persistent diarrhea of infants and young children.

***E. coli* indigenous to the gastrointestinal tract**

E. coli is a member of the indigenous (i.e. normal) microflora (400–500 species) present in the gastrointestinal tract of humans and animals. Indigenous *E. coli* differ from the pathogenic ETEC, EPEC, EHEC, EIEC and EAEC virotypes discussed above. The indigenous *E. coli* are not usually responsible for intestinal infection or diarrhea, but may translocate across the intestinal barrier to cause extraintestinal infections in other tissues and even bacteremia/septicemia. Consequently, indigenous *E. coli* are 'opportunistic' pathogens and can cause disease depending upon increased populations of *E. coli* in the intestines ('intestinal overgrowth') and predisposing host factors, such as compromised immune defenses and increased intestinal permeability. Thus, *E. coli* septicemia in debilitated patients probably originates from the patient's own gastrointestinal tract rather than from *E. coli* contamination from the environment, even if entering via wound sites.

Attempts have been made to associate extraintestinal disease caused by indigenous *E. coli* with certain *E. coli* virulence factors, such as the production of hemolysin, capsule polysaccharide, colicin V, aerobactin, type 1 fimbriae, P fimbriae, and even the presence of certain adhesion operons (pap, sfa, afa, hly). However, it is not possible as yet to relate any of these putative *E. coli* virulence determinants or even a group of determinants with indigenous *E. coli* extraintestinal disease.

Immunity

Immunoglobulin M (IgM) and IgG serum antibodies are readily produced to *E. coli* O, H and K antigens.

More importantly, anti-*E. coli* secretory IgA decreases the adherence of diarrhea-producing *E. coli* in infant intestines. Thus, breastfed infants have a lower incidence of ETEC diarrhea than do bottlefed infants in less developed countries, demonstrating the importance of protective secretory IgA in breast milk (passive immunity).

Adults living in ETEC endemic areas also have a lower incidence of ETEC diarrhea than do travelers to these areas. Repeated episodes of ETEC diarrhea in less developed countries apparently stimulate secretory IgA specific for LT and colonization factor antigens (CFA). Therefore, adults in less developed countries acquire a certain degree of immunity and ETEC diarrhea in less developed countries is primarily a disease of infants and young children.

E. coli vaccines, primarily against ETEC, are presently under development utilizing a variety of approaches. The prophylactic administration of anti-*E. coli* serum antibodies to decrease *E. coli* septicemia in debilitated patients also is under intense investigation but with little success to date. Nonetheless, the immunologic prevention of *E. coli* diarrheal diseases and septicemia would be of tremendous

medical significance in both developed and less developed countries.

See also: Bacteria, immunity to; Bacterial cell walls; Endotoxin (lipopolysaccharide (LPS)); Gastrointestinal tract infections; Urinary tract infections.

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EXERCISE AND THE IMMUNE RESPONSE

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Exercise induces a variety of coordinated physiological and biochemical responses which dramatically alter the distribution and function of immunocompetent cells. Exercise-associated changes in hormone release, blood flow distribution, cardiac performance and substrate utilization have significant implications for the functioning of the immune system. For example, short, vigorous bouts of exercise (e.g. sprinting), sustained, strenuous exercise (e.g. marathon running), or intermittent, vigorous exercise (e.g. soccer) induce an immediate leukocytosis with white blood cell counts increasing anywhere from two- to eightfold above pre-exercise levels. Immunological changes which accompany exercise are of both fundamental and applied interest. Physiologically, exercise-associated changes in the behavior of immune cells parallel changes induced by other physical stressors, such as thermal injury, hemorrhagic shock, and surgery. From a public health perspective, exercise-induced immunosuppression (or immunoenhance-

ment) may impact on disease risk for some segments of the population.

Exercise and lymphocyte subpopulations

The changes in the types of lymphocytes in the circulation as a result of exercise have been well studied. The absolute number of CD3⁺, CD8⁺ and CD16⁺/CD56⁺ (natural killer, NK) cells increase during and immediately after exercise. B Lymphocytosis also occurs with acute exercise. These increases are transient and return to pre-exercise levels within a few hours of cessation of exercise. The ratio of CD4⁺CD8⁺ lymphocytes (helper/suppressor ratio) decreases immediately after exercise, largely because the CD8 count increases more than the CD4 count. Subject fitness level does little to modify this basic pattern and exercise intensity and duration are the key variables in modifying the extent of the lymphocyte subpopulations shifts. Following acute

exercise, the absolute concentration and *in vitro* cytolytic activity of CD16⁺/CD56⁺ cells increases dramatically. Following exhaustive physical activity, NK cell numbers in blood decrease and cytolytic activity declines. The absolute number of CD45RA⁺45RO⁺ lymphocytes (indicative of cellular activation) increases after endurance exercise.

Exercise and cytokines

Multiple forms of stress elicit an increase in interleukin (IL) concentrations in blood and it is, therefore, not surprising that exercise also induces cytokine release. IL-6, but not IL-1, has consistently been shown to be elevated in plasma after exercise. Blood levels of tumor necrosis factor α (TNF α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are virtually undetectable after exercise. The concentration of IL-2, an important regulator of T cell function, has been shown to decrease (relative to baseline) after exercise. Interferon α (IFN α) levels are elevated immediately after exercise at 70% of $\dot{V}O_{2\max}$. Urinary excretion of cytokines are probably more indicative of the impact of exercise since elevated blood levels of cytokines usually occur only after severe inflammatory challenge or extensive muscle damage. Elevated urinary concentrations of IFN γ , TNF α and IL-6 have been detected after completion of long-distance running in humans. Despite our understanding of changes in cytokine profiles with exercise, the physiological consequences of altered cytokine profiles in response to exercise are largely unknown.

Exercise and lymphocyte functions

Although exercise induces an increase in the absolute number of leukocytes and changes the lymphocyte subpopulation profile, the functional impact of exercise on immune effectors is equivocal. While there have been an increasing number of investigations in this area, the results are difficult to compare because of variations in protocol methodology, the confounding of exercise effects by circadian hormone patterns, and the heterogeneity in exercise capacity among subjects. These limitations notwithstanding, a number of general patterns emerge regarding exercise and lymphocyte responses.

The most well-characterized lymphocyte response to exercise is that of the cellular proliferation response to mitogens and antigens *in vitro*. The ability of lymphocytes to proliferate to classical T cell mitogens (phytohemagglutinin (PHA), concanavalin A (con A)) is decreased immediately after exercise but returns to baseline levels within 24 h; the

proliferative response to B cell mitogens (lipopolysaccharides, LPS) or mixed T-B cell mitogens (pokeweed mitogen, PWM) increases post-exercise. These proliferative changes do not appear to reflect altered function or activation of individual lymphocytes but are rather due to concurrent numerical changes in lymphocyte subsets. Natural killer (NK) cell (i.e. CD56⁺/CD16⁺) function is markedly affected by exercise above 60% $\dot{V}O_{2\max}$; *in vitro* cytolysis of ⁵¹Cr-labeled tumor cells is suppressed after intense exercise which is due to decreased numbers of NK cells in blood and prostaglandin-mediated suppression. The influence of a single bout of exercise on modulating antibody synthesis is minimal. In trained athletes, high-intensity endurance exercise is associated with a decrease in salivary IgA levels after exercise; levels of secretory IgA in nasal washings remain unchanged. In contrast, basal levels of serum IgG, IgM and IgA, and the capacity to produce antibody to tetanus toxoid antigen is not compromised by exercise.

Endocrine and other mediating mechanisms

Several factors influence the responses that are activated in response to exercise, including immunological experience and many biopsychosocial factors. Despite the diversity of these factors, a stereotypical set of neuroendocrine pathways are critically involved. The dramatic changes in fuel requirements, together with cardiovascular adjustments needed to accomplish the increased demand for muscle tissue oxygen supply, requires a strong regulation in order to maintain internal homeostasis during exercise. Changes in autonomic nervous activity and in hormone secretion are central in this respect. Muscular exercise is associated with an increase in the concentration of a number of stress hormones in the blood including epinephrine, norepinephrine, growth hormone, β -endorphins and cortisol; the concentration of insulin slightly decreases with exercise.

The leukocytosis of exercise (largely a lymphocytosis and neutrophilia) is a consequence of hemodynamic and endocrine effects of exercise mediated by the autonomic nervous system. Norepinephrine release has marked α -adrenergic effects including increased pulmonary blood flow and arteriole vasoconstriction in the viscera. The consequences of α -adrenoceptor stimulation with exercise on lymphoid parameters are twofold. First, although the noncirculating neutrophil and lymphocyte pools in the lungs are small, leukocytes become rapidly demargined with increased lung blood flow. Second, vasoconstriction in the splenic vascular beds lead to a large

initial movement of blood cells into the circulation. The importance of splenic reservoirs and subsequent demargination to exercise leukocytosis remains open since leukocyte number increases in splenectomized individuals as well. Concurrent stimulation of the adrenal medulla results in the release of epinephrine into the plasma with marked β -adrenergic effects. A prominent β -adrenergic effect on the immune system is that of decreased leukocyte–endothelial cell adherence in the viscera; this would also result in a net release of leukocytes from visceral tissues, despite a reduced blood flow to these regions. Taken together, the α - and β -adrenoceptor-mediated effects lead to an increase in circulating leukocytes (leukocytosis) that is characteristic of exercise.

Studies where hormones were infused, hormone receptors were blocked by drugs, or the hormone production was inhibited by epidural blockade in relation to physical stress contribute greatly to our understanding of the mechanisms of action of exercise on the immune system. Epinephrine and, to a lesser degree, norepinephrine are responsible for acute exercise effects on lymphocyte subpopulations. The increase in catecholamines and growth hormone mediate the acute effects on neutrophils, whereas cortisol exerts its effects within a time lag of at least 2 h. Cortisol contributes to the maintenance of lymphopenia and neutrocytosis after exercise of long duration. The role of the endorphins is less clear. β -Endorphin is derived from the same precursor molecule (pro-opiomelanocortin) as adrenocorticotropic hormone (ACTH) and has consistently been shown to enhance NK cell function *in vitro* at concentrations of 10^{-7} – 10^{-9} M; however, based on intervention studies, we do not believe that β -endorphin plays a major role in the immediate recruitment of leukocytes to the blood; β -endorphin may be important, however, in chronic training stress.

Substrate availability is another potential factor to consider in exercise–immune cell interactions. Glutamine is required for lymphocyte mitogenic responses and it has been suggested that skeletal muscle plays a regulatory role for the synthesis and release of glutamine. The so-called ‘glutamine hypothesis’ suggests that with intense or prolonged physical activity, the demand for glutamine by immune (and other) cells is greater than the anabolic capacity of skeletal muscles to produce glutamine; the consequence is a glutamine debt and immunosuppression. Experimental support for this hypothesis has been inconsistent.

Exercise and immunity

An important question is to what degree exercise-associated changes in immune function are of clinical significance, e.g. whether exercise influences the resistance to acute or chronic diseases that have an immunological component either in etiology or pathogenesis. Clinical relevance of exercise-mediated immunomodulation has been harder to demonstrate than the phenomenological aspects.

It has been known for about 100 years that exercise or training before exposure to infection decreases morbidity or mortality, whereas exercise during the infection has the opposite effect. Thus, acute exercise to exhaustion after infecting laboratory animals with anthrax bacilli or *Streptococcus pyogenes* resulted in increased mortality compared to sedentary controls. In contrast, repeated exposures to exercise prior to exposure with type 1 *Pneumococcus* was associated with reduced mortality in experimental animals relative to sedentary controls. Epidemiological studies suggest that the relative risk for upper respiratory tract infections (URTI) is increased during and for 1–2 weeks following overtraining or marathon-type exercise in endurance athletes. Since prolonged, intense exercise is also associated with some immune suppression, this has led to the ‘open window’ hypothesis (Figure 1). This hypothesis suggests that during the period immediately following heavy and prolonged training, host immunity is suppressed and risk of URTI is elevated. Testing of this hypothesis will require investigations with necessary methodological rigour (e.g. in selection of appropriate control groups) including adjustment for confounders (such as season and air temperature) and potential bias (such as selection and recall bias) which have limited the conclusions of existing studies in this area.

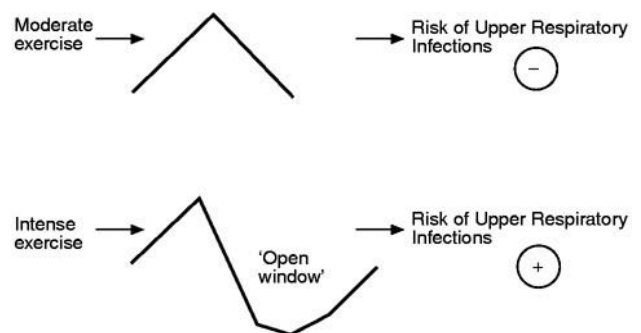


Figure 1 During moderate and intense exercise lymphocytes are recruited to the blood and the NK cell activity increases. Intense, prolonged exercise is followed by immunodepression (lymphopenia, suppressed NK cell activity, reduced production of secretory IgA) during which there is an ‘open window’ of opportunity for pathogens.

Although the empirical evidence concerning exercise and most types of cancer is limited, over 25 epidemiological studies have been conducted to investigate relationships between exercise and colon cancer; there are also a handful of animal studies on exercise and colon cancer. Despite varying designs, study populations, and measures of exercise, the vast majority demonstrate small, but consistent, increases in risk for colon cancer with physical inactivity. Far fewer studies on the effect of physical activity (or inactivity) on risk of breast and prostate cancer have been conducted; the limited results suggest the same magnitude and direction of association as observed with colon cancer (i.e. risk estimates ranging from 1.1 to 2.0 for physical inactivity). Endocrine rather than immunological mechanisms have been proposed for these findings, although further research is warranted.

Animal models point to the importance of timing of exercise and the intensity of exercise in modulating tumor growth. Early reports, in which rodents were exercised intensively prior to establishment of tumors, describe inhibition of tumor growth (sarcomas and carcinomas). Growth of an experimental hepatoma was decreased in rats given mild-to-moderate swim exercise after tumor implantation. Rat and mouse models suggest a potential role for exercise in controlling tumor metastasis. Baseline elevations in NK cell and macrophage cytolytic functions with endurance training have been linked to reduced experimental lung tumor burden and greater *in vivo* clearance of radiolabeled tumor cells.

There is no evidence to date that exercise in humans modifies the onset of autoimmune diseases. In animals, however, exercise before disease onset generally does not exacerbate the clinical signs of autoimmune disease, except for experimental autoimmune myocarditis.

Concluding comments

Despite limitations in the design and comparability of current exercise immunology research, it is apparent that physical exercise modifies a number of immune parameters. This is not unexpected since the

neuroendocrine changes accompanying exercise parallel those neuroendocrine changes which occur in response to other physical (and psychological) stressors. Thus, exercise immunology should be viewed as a specialized focus within the larger theme of neuroendocrine-immune interactions.

Many studies have described the impact of exercise on the immune system (for example, in 1993–1994 over 150 papers were published on exercise immunology). However, what remains unclear is the clinical impact of exercise-induced changes in immune function. While elite athletes may be at risk for more frequent or sustained episodes of immunosuppression, understanding the subtle and transient changes which accompany moderate exercise (and which have potentially far greater implications for population health) will require additional research efforts.

See also: Immune response; Neuroendocrine regulation of immunity.

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EXOCYTOSIS

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Exocytosis is a universal cellular process operative in plants, protozoa, invertebrates and vertebrates alike. It is essential for cellular differentiation and function, and intimately dependent on molecular constitution and cytomorphology. Originally, it referred narrowly to extracellular release of cell products and was termed *secretion*, *eccytosis* or *emiocytosis*. Commonly, secretion products are confined intracellularly within fluid or storage granule vesicles that fuse with the plasmalemma, and the term *exocytosis* can refer strictly to that ultimate vesicular extrusion step, or can be broadened to embody the entire functional anterograde path from site of synthesis to plasmalemmal fusion, irrespective of vesicular content(s) or membrane composition and whether granular products are released extracellularly in addition to membrane insertion into the plasmalemma.

Historical background

Cellular engulfment of solid particles via vesicles (phagocytosis) was first reported in the late 1800s. Vesicular fluid intake (pinocytosis) was described subsequently. These activities implied a large consumption of cell surface membrane, but biochemical studies in the 1950s failed to demonstrate commensurate lipid biosynthesis. This led to the concept of membrane recycling linking *endocytosis* (both phago- and pinocytosis) to *exocytosis*, respectively vesicular uptake or discharge of matter, whereby membrane segments are concomitantly respectively withdrawn from or added to the plasmalemma. Anterograde membrane flow (from cell center to plasmalemma) can originate from various organelles but ultimately homes in at specific domains of the cell surface. Membrane flow serves several functions and is involved in 1) membrane conservation, 2) endocytosis, 3) discharge from the cell of synthesized, metabolized or unmetabolizable materials, 4) transcellular transport, and 5) withdrawal from, or insertion into, the plasmalemma of lipids and integral membrane proteins, a quasisecretion process *didactically designated in this overview* as 'm(membrane)-exocytosis' to distinguish it from 's(cretion)-exocytosis', i.e. secretion/extrusion of vesicular serous or granular contents into the extracellular space. 'Exocytosis' normally refers to both of these variants. Although s-exocytosis can not occur independently of m-exocytosis, the converse can. The

term 'secretion' is more general; it does not necessarily involve vesicle-membrane fusion and presents semantic conundrums ascribable to different vocabularies of histophysiology and molecular biology; it includes 1) discharge of proenzymes from pancreatic acinar cells (merocrine secretion), 2) release of serous mixtures from sweat glands (eccrine secretion), 3) shedding of lipid from active mammary alveoli (apocrine secretion), and 4) spilling of contents upon cell death in sebaceous glands (holocrine secretion), but excludes kindred processes euphemistically called 1) 'externalization' of product-laden membrane evaginations (matrix components of cartilage and bone, some viruses, myoblast lectin), 2) 'expulsion' of nuclei from erythroblasts, or 3) 'shedding' of platelets by megakaryocytes.

Cytomorphologic infrastructure of exocytosis

Eukaryotic cells feature various membrane-delimited sacs embedded in a structured cytoplasmic matrix. These form a coherent set interconnected morphologically or via vesicular transport, thereby establishing a functionally continuous vectorial *cisternal space* (Figure 1), embedded within but topologically distinct from the cytoplasm, 'outside' the cell much as our digestive tract is topologically 'outside' our body. The cisternal space includes 1) the nuclear envelope, a perforated double-membraned sac continuous with 2) the endoplasmic reticulum, partly studded with ribosomes proximally (rough ER, RER), but not so distally (smooth ER, SER), 3) the Golgi apparatus, a set of flattened sacs shaped like soup plates nested within each other that, among other functions, encapsulates cell products into 4) membranous intracellular transport vesicles. Free cytosolic polyribosomes synthesize constitutional proteins (cytoskeletal elements, cytoplasmic enzymes, dedicated proteins such as hemoglobin). Proteins destined 'for export', i.e. m- and s-exocytosis, are synthesized by polyribosomes attached to the RER and are either anchored within the RER membrane or extruded *in toto* into the cisternal space, whence they are translocated through the Golgi apparatus for post-translational modification and/or packaging, then sorted in the trans-Golgi network into either exocytotic vesicles or lysosomes. Exocytotic vesicles with or without cargos of

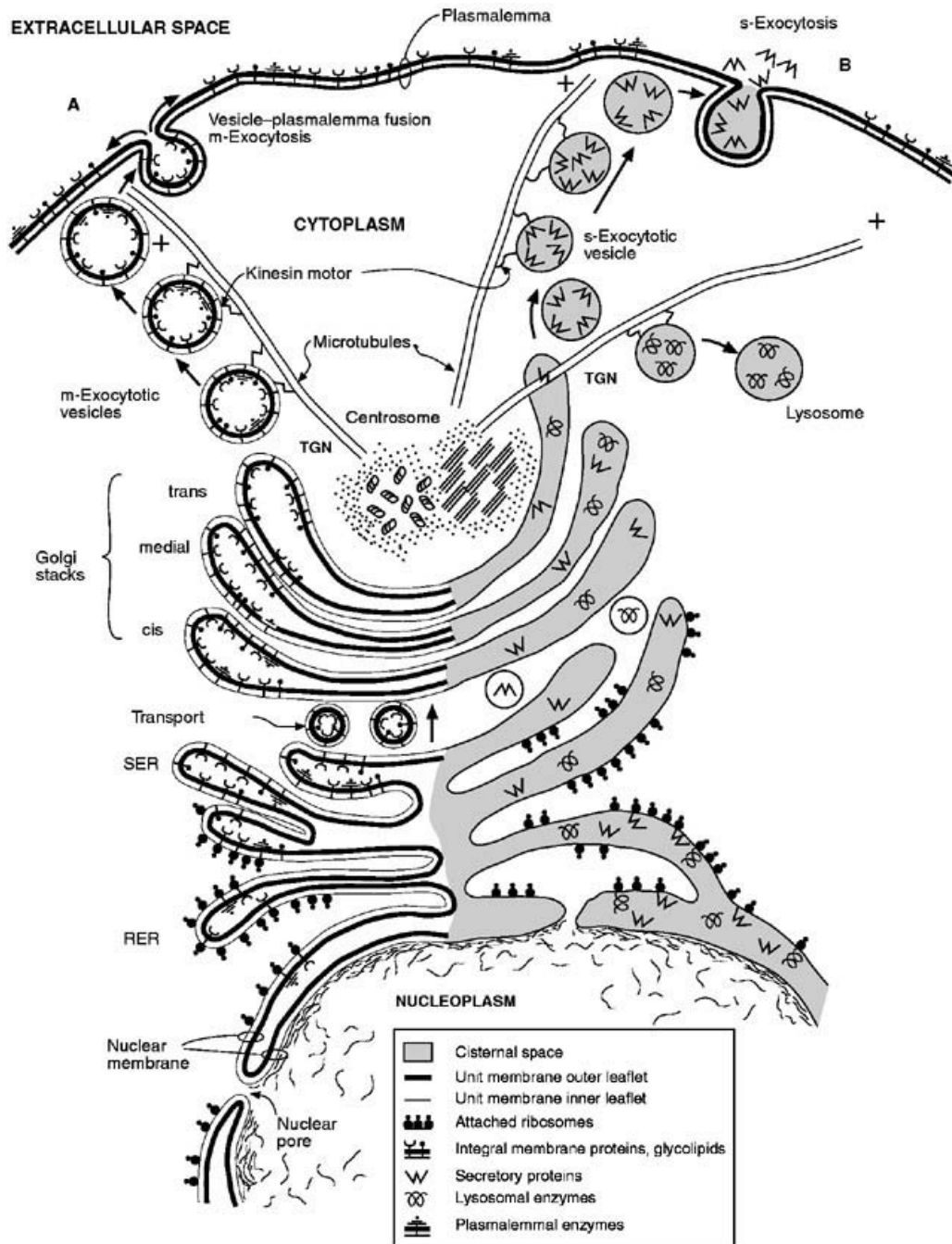


Figure 1 Exocytotic morphologic pathways for A, m-exocytotic insertion of integral membrane proteins (synaptic choline esterase, insulin or cholesterol receptors, cell attachments) and lipids (cholesterol, glyco- and phospholipids), or B, export/secretion (s-exocytosis) outside the cell. Pathway A is an obligate component of pathway B, but not vice versa. The left side of the diagram emphasizes consistency of polarity between inner and outer membrane leaflets, the right side continuity of the cisternal spaces. Integral membrane proteins (receptors, enzymes, ion gates, active transport) and glycolipids remain anchored within the lipid membranes (left side of diagram). The ultimate destination of most such proteins is the plasmalemma, although some associate with Golgi stacks, lysosomes, peroxisomes, etc. By contrast, proteins destined for release into the extracellular compartment are first injected *in toto* into the RER cisternal space and are ultimately conveyed, via contiguous segments of the cisternal space, to s-exocytotic vesicles, some propelled by cytoplasmic kinesin motor proteins to the plasmalemma along microtubules originating from the centrosome. When s-exocytotic vesicles/granules fuse with the cell surface, their membranes insert into the plasmalemma and their contents spill passively out of the cell. TGN, trans-Golgi network; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; +, growing ends of microtubules. Note that s- and m-endocytosis are one and the same process that here has been dissociated for didactic purposes to emphasize the overlapping though different end-points.

specific secretory products (peptide hormones, proenzymes, etc.) first bud off from the ER, join and traverse the Golgi, and bud off again from the trans-Golgi network. Exocytotic vesicular traffic within the cell is either undirected, with docking determined by chance contacts between associated complementary targeting and accepting molecules (Table 1) on vesicular and plasmalemmal membranes, or directed to appropriate cell surface domains by cytoskeletal fibers (microtubules, actin and intermediate filaments, each associated with helper proteins modulated by specific biochemical reactions (Figure 2). s-Exocytosis occurs 1) of zymogen granules by gastric chief cells at the apical surface, 2) of chylomicra by enterocytes, at the lateral membrane behind the junctional complexes that anchor epithelial cells to each other, restrict membrane flow between apical and basolateral domains, and insulate tissue spaces from lumina, and 3) of plasma components for transfer into tissues across endothelia, at the basal cell surface. Endocytic vacuoles can be translocated to primary lysosomes (Figure 3) where fusion of the two yields secondary lysosomes, thence back to the cell surface, a path characteristic of receptor-mediated phagocytosis; their receptors can be recycled. Polarized motor proteins, cytoplasmic dyneins and kinesins, translocate some vesicles along microtubules toward or away from the cell center, respectively, but other mechanisms may exist. Interference with microtubule assembly by colchicine impedes secretion of pancreatic zymogens, parathormone and fibroblast procollagen.

s-Exocytosis to the extracellular space

s-Exocytosis can be *regulated* or *constitutive*. Regulated exocytosis occurs on demand via secretagogues and signal transduction. Constitutive exocytosis is continuous; no secretagogue is involved, there are no storage vesicles, and the products are released apace upon synthesis. s-Exocytosed products of nonneuronal cells (digestive proenzymes, peptide hormones, immunoglobulins, chylomicra lipoproteins) originate in the RER, are carried by vesicles to and through the Golgi apparatus, then by vesicles or membrane-bounded granules to the plasmalemma, and are finally extruded by fusion of the vesicles with the cell membrane. For that entire transit they remain confined topologically within the cisternal space (Figure 1). In neurons some vesicles recycle and reload near the synapse. In pancreatic acini, fusion of progressively deeper granules with more peripheral ones, called *sequential* or *compound exocytosis*, forms extended extrusion channels, also said to occur in mast cells. Various agents elicit release of mast

cell mediators: Ca^{2+} ionophores, cross-linkers of immunoglobulin E (IgE) receptors on the cell surface by antigen, polycations or some lectins. Phagocytosis of negatively-charged thorium dioxide particles or plastic beads by rat serosal mast cells delivers them over time via endosomes to young, then mature granules, without causing degranulation. The metal is retained for months and migrates between granules, which change their staining properties as the cell ages. They have *in situ* enzymatic activity (and *ipso facto* function?), and their mediators have long half-lives. Polylysine causes degranulation. Cationic (polylysine-coated) beads breach the granular sac by a different path that bypasses endosome vesicles, and they contact mature granules directly without delay. All this argues for a transient or permanent membranous sac enfolding multiple granules in a way unrelated, if morphologically resembling, sequential exocytosis. Could IgE antigens act as *pathologic* mast cell secretagogues? This paradoxical dichotomy suggests that mast cell degranulation interferes with a primary intracellular function, whatever that might be, and that evidence for compound exocytosis must be interpreted cautiously.

At plasmalemmal docking sites, a series of biochemical signaling reactions involving G protein(s), enzymes, phosphoinositol metabolites, etc. may lead to transient shifts of ionic calcium into cells and/or between some intracellular compartments. These Ca^{2+} shifts may not be obligate steps for all cells or all s-exocytotic processes. As fusion of a vesicle with the cell membrane establishes continuity between vesicular contents and extracellular space, all the contents stored within are disgorged simultaneously (epinephrine, enkephalins, chromogranins, nucleotides, etc., by adrenal chromaffin cells), faster and in much higher concentrations than possible via active transport mechanisms. Vesicular pathways can couple endocytosis with s-exocytosis to transport material across a cell (endothelium, kidney tubules) by *transcytosis*. Though uptake at one surface may be selective, there need be no modification of the vesicular contents before s-exocytosis at another surface. Capillary endothelium transfers bulk plasma into tissue spaces, and selected substances across the blood-brain barrier. IgA and IgM are moved across enterocytes in the opposite direction, from basal to apical surfaces.

m-Exocytosis to the cell membrane

Membrane traffic is critical for defining cell type and function which depend on plasmalemmal

Table 1 Some identified components of the membrane fusion machinery in exocytosis and endocytosis^a

1. Vesicular coats -- shape membranes into vesicles	
Clathrin	Trimeric protein complexes that shape budding vesicles. Forms triskelion cages under coated pits, endocytic vesicles, and buds on trans-Golgi membranes
Coatomer	Coat protomer. Non-clathrin polypeptides condensations around shuttling cytoplasmic vesicles. At least seven COP components recognized; form stoichiometric complexes with ARF. Drives vesicle blistering of Golgi membranes
COP1 coats	Coat Proteins forming ARF-coatomer complexes around vesicles that shuttle between ER and cis-Golgi, and vice versa. Unstable; assembled by ARF-GTP
COP2 coats	As COP1, but stable. Assembled by ARF-GTP. Dissociated by hsp70 or SAR1 ATPases. Direct vesicles from ER to cis-Golgi
ARFs	ADP-Ribosylation Factors that regulate vesicular coat structures. These GTPases assemble with COPs to trigger formation of coatomers. Bind to Golgi membranes and cause vesicle budding or uncoating. ARFs are myristylated; they are cytosolic (water soluble) in GDP form, but membrane bound (water insoluble) in GTP form
AP1	Assembly Polypeptide. ARF-dependent complex vesicular protein coat promoting direct transport from trans-most Golgi cisterna to lysosomes. Associated with clathrin
AP2s	Family of Assembly Polypeptides. ARF-independent stable clathrin-associated protein complexes. Associated with coated pits, endocytotic vacuoles, and presynaptic plasmalemma of nerves
SAR proteins	ARF-like G-protein ATPases
Rab proteins	Family ($n = 30+$) of small (22–27 kDa) G proteins with GTPase activity; required for assembly of SNARE complexes. Associated with multiple stages of vesicular cytoplasmic traffic in exocytotic and endocytotic pathways. Regulate or trigger secretion (of enzymes, hormones, neurotransmitters, etc.) via stimulation or inhibition
Annexin XIII	Nonmembrane protein implicated in vesicle transport to apical plasmalemma
TAP (p115)	Protein needed for docking and fusion of transcytotic vesicles to apical cell membrane as well as transport of vesicles between Golgi stacks
2. Membrane targeting and docking	
v-SNAREs	Family of vesicular integral membrane coat proteins that act as destination codes with affinity for cognate SNAP receptors on target membranes. Effect docking with t-SNAREs
t-SNAREs	Family of SNAPs–NSF complexes. Integral membrane proteins that act as destination codes in receptive target membranes. Act as SNAPs receptors to effect docking of specific v-SNAREs
VAMPs	Family of Vesicle-Associated Membrane Proteins. Synaptosome-associated integral membrane protein of brain (synaptobrevin) involved in neurotransmitter release; associated with dense-core vesicles (loaded and recycled at trans-Golgi, in perikaryon) and with small vesicles (loaded and recycled at neuron terminal). Also present in gastric parietal cells, zymogen and chromaffin granules, and yeast
Syntaxins	Integral membrane proteins present in presynaptic plasmalemma, gastric parietal cells and yeasts
3. Membrane fusion	
NSFs	Family of NEM-Sensitive Fusion ATPase proteins. Complex with SNAPs and SNAREs to effect vesicle fusion. Associated with ER, Golgi, plasmalemma and endosomes.
SNAPs	Soluble NSF Attachment Proteins. Colocalize with NSF and SNAREs to elicit fusion of intracellular membranes in animals and yeasts. Associated with Golgi membranes
SNAP25	SyNaptosome-Associated Protein 25 kDa. A presynaptic peripheral membrane protein (structurally unrelated to SNAPs) involved in neurotransmitter release upon local Ca^{2+} influx into the nerve. Induces rapid axonal transport. In gastric parietal cells, elicits expression of H^{+}/K^{+} -ATPase and K^{+}/Cl^{-} channels on surface of extended microvilli to activate HCl secretion.
Synaptotagmins (p65)	Family of integral membrane proteins, primary Ca^{2+} receptors of presynaptic plasmalemma. Inhibit neurotransmitter release. Also implicated in recycling of dense-core neuropeptide vesicles and clathrin-coated small synaptic vesicles at nerve terminals. Interacts with AP2, syntaxin, calmodulin and phospholipids. Involved in both exocytosis and endocytosis. Synaptotagmins can effect both positive and negative regulation of neurotransmitters. Also present in fibroblasts
Synaptophysins	Family of integral membrane proteins of small synaptic vesicles. May enhance exocytosis. Nonneuronal homologs reported in fibroblasts and other cells

Table 1 Continued

4. Ancillary agents	
Nucleotides	GTP and ATP. Cytosolic energy sources for assembly and disassembly of coat proteins in conjunction with GTPases and ATPases. Induce conformational changes in specific molecules
Ca ²⁺ ion	Second messenger for many exocytotic and endocytotic processes, including release of neurotransmitters
c-AMP	Second messenger in many transduction signaling processes
Secretagogues	Primary agents (polypeptide hormones, catecholamines, etc.) that trigger exocytosis by cognate cells. Generally specific ligands for target cell receptors
Fatty acyl-CoA	Alter water/lipid solubility of G proteins and effects membrane detachment/attachment
Proton pumps	ATPases that acidify endosome-lysosome vesicles to activate lysosomal enzymes and dissociate ligands from receptors
Kinesins	Family of protein motors effecting anterograde translocation of transport vesicles along microtubules
Cytoplasmic dyneins	Family of protein motors effecting retrograde translocation of transport vesicles along microtubules
Shaperones	Proteins (e.g. hsp70) that control folding/unfolding of proteins in exocytotic processes

^aCurrent status of participating agents in intracellular vesicular membrane traffic: factual, putative and hypothetical. Entries are not necessarily operative in all cell types, and their specific properties/role(s) are not fully elucidated. The proteins involved in the highly specific and complex processes that lead to exocytosis are members of families of homologous proteins represented in all eukaryotic cells. The mechanisms of transport, docking, fusion and exocytosis are highly conserved from yeast to mammalia, albeit with some specializations and/or variations among cell types and species, with many isoforms present in given species, tissues, cells and even single vesicles. The table includes only a few of the plethora of reported agents. Many additional ones unquestionably will be characterized in this rapidly-developing research area.

differentiation and maintenance. It modulates up-/downregulation of surface receptors involved in signaling and thus actuates switches that control cell function. Cell membranes present domains of differing composition and functions; they assure epithelial polarity which, when disturbed, can lead to severe pathology. The plasmalemma is a mosaic of integral proteins floating in a bilayer of hydrophobic lipids. Intracellular membranes define various aqueous compartments within the cytoplasm. For m-exocytosis the proteins, synthesized by the RER, remain anchored in a polarized orientation within the lipid bilayer by means of hydrophobic sequences in their molecular backbone; they can diffuse laterally within the plane of the membrane, but can not flip-flop. Proteins that project into the cisternal space can be glycosylated or otherwise modified post-translationally. m-Exocytosis of vesicles budded off the trans-Golgi network is the vehicle for translocating these molecules from their site of synthesis to their site of function at the plasmalemma. It insures that their orientation in the membrane is retained during translocation to and fusion with specific domains of the cell membrane, in which they may then diffuse or aggregate laterally to form specialized functional sites (enzyme or transport proteins at the tip of enterocyte microvilli, ion channels, receptors, integrin binding sites for fibronectin) or components of cell-cell junctions (desmosomes, synapses, tight, gap and neuromuscular junctions). Lipids and glycolipids made by the ER-Golgi complex are translocated similarly. Membrane differentiation gives cells their individuality and many of their special functions. It is how cells develop functionally different apical,

lateral and basal surfaces, express specific surface molecules (ABO antigens, receptors) or recognition surface proteins (T lymphocytes).

Membrane flow and conservation

Membrane traffic is essential to the dynamic responsiveness of cells. Plasmalemmal turnover can be massive and very rapid. Because membrane augmentation by exocytosis is coupled with endocytosis, which withdraws membrane from the cell surface, an equilibrium is established and membrane components can be efficiently conserved, added, removed, replenished, changed or recycled. Extracellular ligands snagged by surface receptors can be drawn into the cytosol by endosomes that fuse with lysosomes and become acidified, thereby dissociating ligand from receptor. Dysjunction of the endosome-lysosome complex yields a digestive vesicle and a shuttling receptor-carrier vesicle that can rejoin the plasmalemma via m-exocytosis for another cycle (Figure 3). Normal topological relations are unaffected in those exchanges, as critical membrane polarity is retained (Figure 1). Lastly, massive fusion of exocytotic vesicles can effect cell shape changes (yeast shmooing (formation of pear-shaped cells during mating), microvilli extrusion in activated gastric parietal cells).

The above discussion clearly points to the central role of exocytosis in the very existence of living cells. DNA directs synthesis of cell constituents that establish cellular ultrastructure, but it is cell structure that, via exocytosis, executes the myriad processes that

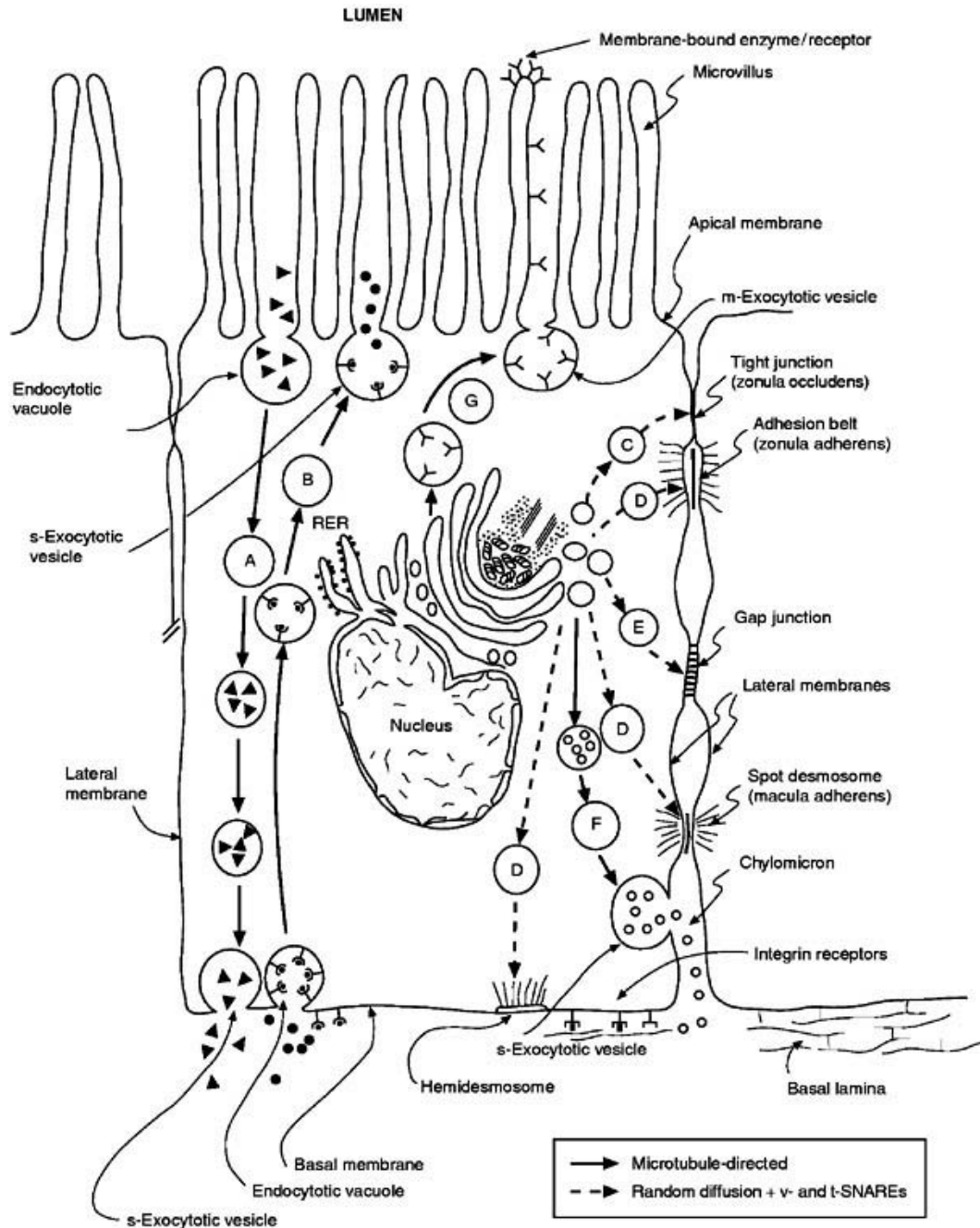


Figure 2 Targeting/capture of m-exocytotic and s-exocytotic vesicles to/by specialized cell membrane domains. A polarized epithelial cell is depicted, but many of the same features are found in other cell types (neurons, macrophages, lymphocytes, yeast). A, Transfer of substances across an epithelial cell (endothelium, kidney tubule) via a transcellular path from apical (endocytosis) to basal (s-exocytosis) domains; B, uptake of extracellular material (endocytosis) for transfer to luminal space (s-exocytosis) from basal to luminal domains (IgA, IgM across enterocyte); C, m-exocytosis of integral membrane protein (uncharacterized) to tight junction between lateral domains of abutting epithelial cell membranes; D, m-exocytosis of adhesion integral membrane proteins (CAMs, integrins) to form spot and belt desmosomes and hemidesmosomes at lateral and basal membrane domains; E, m-exocytosis of integral membrane proteins (connexin) to communicating (electrotonic) gap junctions in the lateral domain of abutting cells; F, m-exocytosis of receptors to basal membrane (binding to fibronectins, glycosaminoproteoglycans, intermediate filaments). Specialized cell junctions are transient in that they can be withdrawn and restored rapidly under appropriate conditions. The bimolecular unit membranes are drawn as a single line. Note that s- and m-endocytosis are one and the same process that here has been dissociated for didactic purposes to emphasize the overlapping though different end-points.

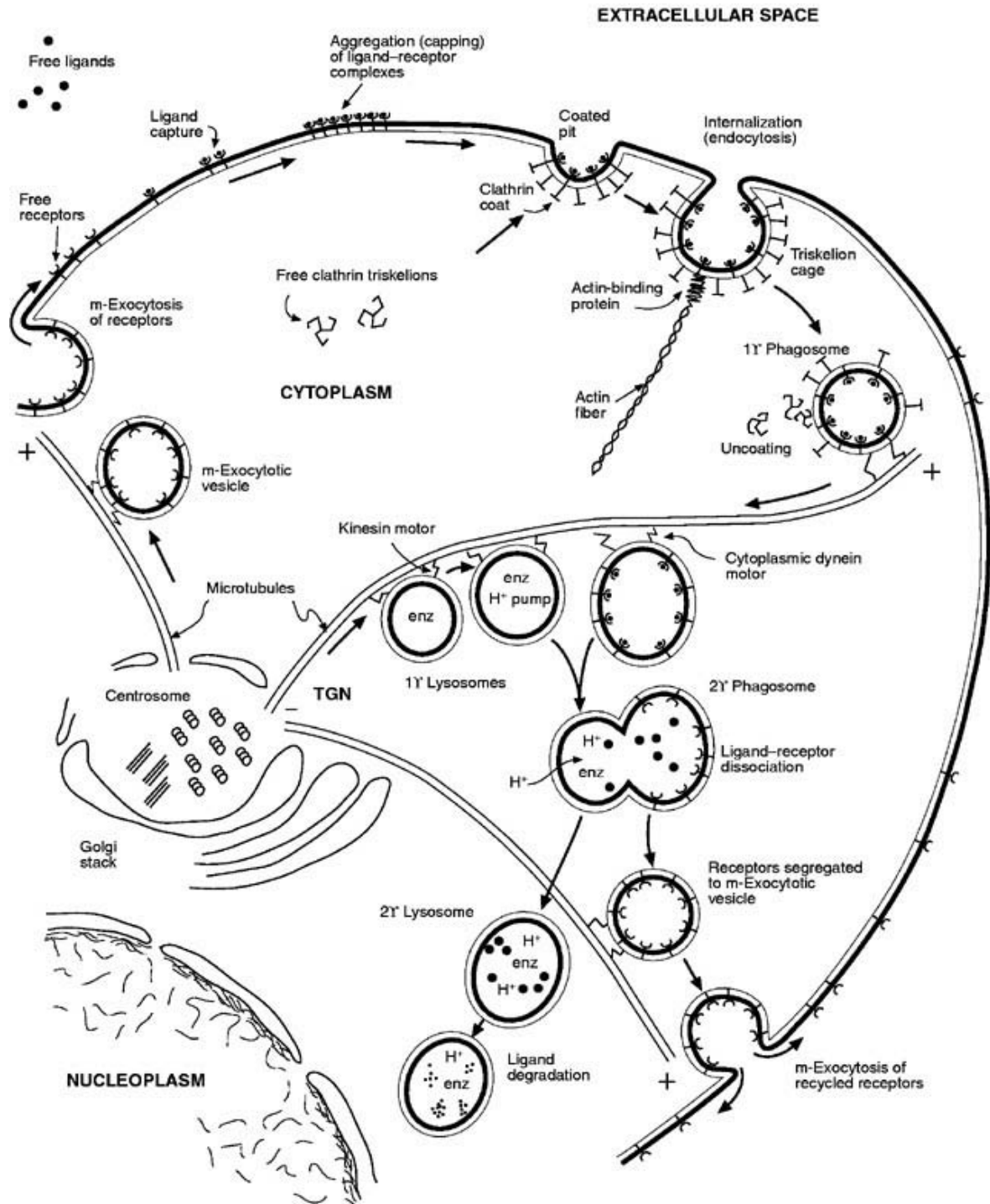


Figure 3 Endocytosis–exocytosis coupling, membrane conservation and receptor recycling. Integral membrane proteins such as receptors delivered to the plasma membrane by m-exocytosis (Figure 1) bind specific ligands, and the complexes then aggregate into clathrin triskelions-coated pits that are internalized by endocytosis of vesicles linked to and pulled off the plasmalemma by actin filaments. After internalization, the triskelion cage depolymerizes and the vesicle connects to microtubules via cytoplasmic dynein motor proteins that move it along retrogradely. The vesicles join and fuse with lysosomes shed off the trans-Golgi network and propelled anterogradely by kinesin motor proteins, also along microtubules. The fused vesicles become acidified by an ATP-driven proton pump, the receptor–ligand complex dissociates and the lysosome–vesicle organelle (secondary lysosome) reseparates into lysosome and vesicle moieties. The freed ligand, sequestered within the secondary lysosome, is degraded by acid hydrolases, while the receptors sequestered within the regenerated m-exocytotic vesicle membrane are translocated back to the plasmalemma for recycling. TGN, trans-Golgi network complex; H⁺, proton; enz, lysosomal enzymes; triskelions, clathrin trimers. Note that s- and m-endocytosis are one and the same process that here has been dissociated for didactic purposes to emphasize the overlapping though different end-points.

breathe life into the organism and assure survival of its DNA.

See also: Adhesion molecules; Antigens, cell surface; Atopic allergy; Capping, clustering, membrane microdomains and all surface dynamics; CD antigens; Cell-mediated immunity; Complement receptors; Degranulation; Fc receptors; Hypersensitivity reactions; Immunoglobulin, cell surface; Mast cells; Phagocytosis.

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EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system (CNS) which can be induced in susceptible strains of laboratory animals by immunization with CNS antigens. It is characterized clinically by ascending paralysis, and pathologically by perivascular mononuclear cell infiltration and demyelination in the brain and spinal cord. Although EAE is an experimental disease of laboratory animals, it was first observed in humans when, over a century ago,

it was reported that a small percentage of patients receiving Pasteur's rabies vaccine developed neuro-paralytic signs. The rabies virus vaccine was prepared in rabbit CNS tissue. That CNS antigens contaminating the vaccine preparations were probably responsible for the neurological side-effects of the vaccine was demonstrated in the 1930s, by Rivers and his colleagues, who reported that repeated intramuscular injection of extracts of normal rabbit brain (i.e. without rabies virus) induced pathologic changes

in the brains of monkeys; these changes resembled the CNS disease that occurred in humans subsequent to rabies vaccine treatment. With the development of Freund's adjuvant in the 1940s, it became possible to reproducibly elicit EAE in a majority of animals after a single injection of CNS extract emulsified in the adjuvant preparation.

Encephalitogenic antigens

Experimental evidence to support the hypothesis that CNS tissue contains organ-specific antigens capable of eliciting an immune response was first provided by Witebsky and Steinfeld in 1928. Thus, once the EAE model had emerged, a widespread search was initiated to identify the relevant organ-specific antigens responsible for this autoimmune disease. It is known today that two myelin structural proteins, myelin basic protein (MBP) and proteolipid protein (PLP), are the major encephalitogenic antigens (Figure 1). MBP, which constitutes 30% of the total myelin protein, was first reported to induce EAE by Marian Kies in the early 1960s. This antigen actually

consists of a series of differently charged isomers. The predominant form has a molecular weight of 18.5 kDa, and consists of approximately 169 amino acids. MBP is highly conserved, although minor structural variations occur from species to species. This variation influences the encephalitogenic activity of the MBP molecule in different animal species (Figure 1). Furthermore, in rodents, a smaller 14.5 kDa molecule is produced in addition to the 18.5 kDa MBP molecule, as a result of differential RNA splicing of the MBP gene.

The MBP molecule has been extensively studied, and the amino acid sequences of MBP derived from many species have been elucidated. Furthermore, the major encephalitogenic epitopes have been located, and synthetic peptides corresponding to these determinants have been prepared and shown to induce EAE in experimental animals. Of importance is the finding that different sequences are responsible for EAE in different species. Thus, the major encephalitogenic MBP epitope for the susceptible Lewis rat resides in the sequence delineated by amino acid residues 68–86, and a second, minor, epitope exists

Amino Acid Sequences of Major Encephalitogenic CNS Antigens

A. Myelin Basic Protein

Bov GP	AAQKRPSQR ASQKRPSQRH	SKYLASAST GSKYLATAST	MDHARHGFLP MDHARHGFLP	RHRDTGILDS RHRDTGILDS	LGRFFGSDRG IGRFFGSDRA
Bov GP	APKRGSGKDG APKRGSGKDS	HHAARTTHYG HHAARTTHYG	SLPQKAQ <u>GHR</u> SLPQKSQ R	PQDENPVVHF SQDENPVVHF	FKNIVTPRTP FKNIVTPRTP
Bov GP	PPSQGKGRGL PPSQGKGRGL	SLSRFSWGAE SLSRFSWGAE	GQKPGFGYGG GQKPGFGYGG	RASDYKSAHK RA DYKS K	GLKG HDAQG GFKGAHDAQG
Bov GP	TLSKIFKLGG TLSKIFKLGG	RDSRSGSPMARR RDSRSGSPMARR			

B. Bovine Proteolipid Protein

GLLECCARCL VGAPFASLVA TGLCFFGVAL FCGCGHEALT GTEKLIETYF SKNYQDYEYL
 INVIHAFQYV IYGTASFFFL YGALLAEGF YTTGAVRQIF GDYKTTICGK GLSATVTGGQ
 KGRGSRGQH Q AHS LERVCHC LGKWLGHPDK FVGITYALT V VWLLVFACSA
 VPVYIYFNTW TTCQSIAPS KTSASIGTLC ADARMYGVL P WNAFPGKVCG
 SNLLSICKTA EFQMTFHLF AAFVGAAATL VSLTTFMIAA TYNFAVLKLM GRGTFK

Figure 1 Amino acid sequences of major encephalitogenic CNS antigens. (A) The sequences of the 18.5 kDa isoforms of bovine (Bov) and guinea pig (GP) MBP molecules aligned for maximal homology. The N-terminal alanine residue is acetylated. Slight structural variations influence encephalitogenic activity in different animal species, e.g., Bov MBP is relatively nonencephalitogenic for Lewis rats because the addition of GH (underlined) in the dominant 68–86 epitope inhibits recognition by V β 8.2 positive autoreactive T cells. (B) The sequence of the bovine PLP molecule, which is highly conserved in all mammalian species. Single letter abbreviations for the amino acids are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

within MBP residues 87–99. In susceptible PL/J mice, the dominant encephalitogenic MBP epitope is located within the N-terminal end of the molecule, more specifically within residues 1–9. The response of Lewis rats and PL/J mice to MBP illustrates the species variability that exists with respect to encephalitogenic epitopes. This variation even extends to different strains within a species. Thus, whereas the PL/J mouse responds to MBP epitope 1–9, encephalitogenic epitopes for the susceptible SJL/J mouse strain are contained within MBP sequence 89–101 (Table 1).

Although MBP was the first encephalitogenic antigen to be characterized and studied extensively, the isolation of PLP predates MBP by over a decade. Folch and Lees first described PLP in 1951, and subsequently attributed encephalitogenic activity to this molecule. PLP constitutes 50% of the myelin protein. It is a 29.8 kDa molecule that consists of 276 amino acid residues, but is water insoluble. This characteristic has hampered the investigation of PLP, and for many years it was argued that the encephalitogenic activity ascribed to this molecule was due to contamination with MBP. However, recent studies with synthetic PLP peptides have clearly established that PLP is a potent encephalitogenic antigen for many species, including rats and mice. Several PLP epitopes have been located within the molecule, and strain variations have also been reported (Table 1).

Although PLP and MBP are the major encephalitogenic antigens, it has been reported that EAE can also be elicited by myelin oligodendrocyte glycoprotein (MOG), and it is conceivable that other CNS constituents will eventually also be shown to be capable of inducing autoimmune CNS disease.

Immunopathology of EAE

Philip Paterson made the important observation in 1960 that EAE can be transferred with viable lymphocytes from immunized donor animals, whereas attempts to transfer the disease with serum containing antibodies against CNS antigens have been

unsuccessful. These findings provided the first indication that cell-mediated immunity is important in the pathogenesis of this autoimmune disease. A major advance in our understanding of the nature of the effector mechanism underlying EAE came about when it was discovered by Panitch and McFarlin, and by Richert and his colleagues, that adoptive transfer could be dramatically enhanced if donor lymphocytes were activated *in vitro* with concanavalin A or MBP prior to transfer to recipients. Subsequently, we used this method to determine that the disease-inducing effector cells in the *in vitro* activated population are CD4⁺ T cells. A major advance came when Ben-Nun, Cohen and Wekerle first established long-term encephalitogenic rat T cell lines, thus permitting detailed analyses of the disease-inducing cell population. Today, many investigators are utilizing clones of encephalitogenic mouse and rat T cells to gain insight into the pathogenesis of autoimmune CNS disease. T cell clones reactive against MBP and PLP have also been isolated from multiple sclerosis (MS) patients, although T cells that react with these neuroantigens are reportedly also found in healthy individuals.

In susceptible rats, EAE is usually an acute disease, characterized by ascending paralysis from which the animal eventually recovers. After recovery, the rat is resistant to further attempts to reinduce the disease. The histopathologic features of EAE in the rat include meningeal, perivascular and parenchymal mononuclear cell infiltration in the CNS, with lesions prevalent in the spinal cord. Demyelination is not pronounced, perhaps because of the acute nature of the disease. A major advantage to the use of rats is the ease of induction and predictable time of onset and recovery from EAE with low doses of MBP in Freund's adjuvant.

EAE is more difficult to induce in mice, and ancillary adjuvants are often required, e.g., pertussis vaccine. In SJL/J and PL/J mice, a chronic relapsing form of EAE (CREAE) has been reported following immunization with neuroantigen and adjuvants, or after adoptive transfer of encephalitogenic T cells. CREAE resembles the exacerbating–remitting form of MS in its cyclical pattern, and demyelination is a prominent feature of both diseases. This makes CREAE a particularly attractive investigational model. CREAE has also been reported in rats, but is generally more difficult to induce than in mice.

The inflammatory lesions in both acute and relapsing forms of EAE contain CD4⁺ and CD8⁺ T cells and macrophages; B cells and plasma cells may also be present. Encephalitogenic T cells are present in the lesions. These can be isolated and have been shown to adoptively transfer EAE to recipients. However,

Table 1 Encephalitogenic myelin epitopes for rats and mice

Species (strain)	Encephalitogenic epitope
Rat (Lewis)	MBP 68–86 (dominant) MBP 87–99 (minor)
Mouse (PL/J)	MBP 1–9 (dominant) ^a PLP 43–64
Mouse (SJL/J)	MBP 89–101 (dominant) ^a PLP 43–64 and other PLP epitopes

^aMinor encephalitogenic epitopes have also been described.

only a small percentage of the cells present in the lesions in transferred EAE are of donor origin, suggesting that many of the infiltrating cells are 'bystander' cells which are recruited into the CNS.

Although the presence of encephalitogenic T cells in the CNS of animals with EAE directly implicates them in the pathogenesis of this disease, the mechanism by which they exert their immunopathologic effect remains to be elucidated. The CD4⁺ T cells that mediate EAE secrete the inflammatory cytokines interferon γ (IFN γ) and lymphotoxin (also known as tumor necrosis factor β), and thus appear to belong to the T_H1 subset which mediates delayed hypersensitivity reactions. Early events in the immunopathologic process include the upregulation, probably by IFN γ , of adhesion molecules and major histocompatibility complex (MHC) class II molecules on the cerebrovascular endothelial cells in the CNS. This presumably facilitates the attachment and penetration of activated encephalitogenic T cells into the CNS. Once these cells have crossed the blood-brain barrier, it is postulated that they recruit bystander cells and secrete cytokines (e.g. lymphotoxin), which promote inflammation. Recent evidence has also implicated prostaglandins, nitric oxide and chemokines in the pathogenesis of EAE. These molecules are produced by the inflammatory cells, e.g. macrophages, within the CNS lesions.

Genetics of EAE

In most species, a strong association exists between susceptibility to EAE and MHC haplotype. The response to a given encephalitogenic determinant is governed by the MHC class II gene products, and the T cell receptor (TCR) repertoire available to the animal (Table 2). For example, the response of PL/J mice to MBP 1–9 is restricted by MHC I-A^U, and the responding T cells preferentially use V α 2 or V α 4 and V β 8.2 in their TCR. SJL/J mice recognize two epitopes within MBP 89–101 in the context of I-A^S, and 50% of the responding T cells express the V β 17a TCR. Lewis rats recognize the dominant encephalitogenic epitope (MBP 68–86) in the context of

RT-1B^I (the rat equivalent of murine I-A), and the majority of encephalitogenic T cells express V α 2 and V β 8.2. In contrast, the response to MBP epitope 87–99 is restricted by RT-1D^I (i.e. the homolog of murine I-E), and the responding T cells express diverse V region gene products. Thus, it seems clear that susceptibility to EAE is governed by both MHC and TCR gene products which recognize defined MBP epitopes. Murine encephalitogenic responses to PLP determinants are also restricted by MHC class II, but Sobel and his colleagues have shown that TCR receptor usage is diverse.

Regulation of EAE

EAE has also served as an important model for the investigation of potential therapeutic strategies for human demyelinating diseases. Alvord and his colleagues reported in 1965 that EAE is inhibited in animals tolerized to MBP. This approach has been exploited over the years and, depending upon the tolerization protocol, the inhibitory effect can be attributed either to clonal anergy or active suppression. For example, clonal anergy can be induced in rats and mice fed high doses of MBP, or injected with MBP coupled to syngeneic spleen cells. In contrast, low doses of MBP, given orally, or preimmunization with MBP in incomplete adjuvant (which is nonencephalitogenic), gives rise to suppressor T cells that regulate EAE.

We, and others, have also demonstrated that suppressor T cells develop as Lewis rats spontaneously recover from EAE. These suppressor cells produce transforming growth factor β (TGF β), a cytokine that downregulates the secretion of inflammatory cytokines (i.e. IFN γ and lymphotoxin) by encephalitogenic T cells. Moreover, several studies have reported that EAE can be suppressed in rats and mice by treatment with TGF β . The disease has also been inhibited in rodents with interleukin 4 (IL-4) and IL-10, which are produced by the T_H2 subset of CD4⁺ T lymphocytes that provide T cell help for antibody-mediated immune responses. The suppressor T cells associated with recovery from EAE bear this pheno-

Table 2 T cell receptor usage and MHC class II restriction of encephalitogenic T cells

Species (strain)	MBP epitope	TCR usage ^a	MHC restriction
Rat (Lewis)	MBP 68–86 ^b	V β 8.2	RT-1B (I-A)
Rat (Lewis)	MBP 87–99	Variable	RT-1D (I-E)
Mouse (PL/J)	MBP 1–9	V β 8.2	I-A
Mouse (SJL/J)	MBP 89–101	V β 17a ^c	I-A

^aPredominant TCR expressed.

^bDominant epitope for Lewis rats.

^cExpressed by approximately 50% of responding T cells.

type, suggesting that suppression may reflect a switch from a T_H1 to a T_H2 response with a subsequent diversion from inflammatory T cells to helper T cell production.

However, it is likely that multiple mechanisms underlie the regulation of this autoimmune disease. For example, Pender and his colleagues have presented evidence that spontaneous recovery from EAE may also be due to the apoptotic elimination of encephalitogenic cells from the CNS, and others have reported that adrenal corticosteroids are involved in recovery. Recently, EAE has also been inhibited by treatment with antibodies against cytokines, adhesion molecules and chemokines. Thus, the autoimmune response can be modulated at many levels, from events associated with early T cell activation, to cytokine secretion by mature, activated effector cells.

Other therapeutic regimens are based on interfering with the antigen–MHC–TCR trimolecular complex that triggers the autoimmune response. These strategies have been directed against either the MHC class II molecules on the antigen-presenting cells, or the TCR of the encephalitogenic T cells. Success has been achieved by blocking MHC class II presentation of the encephalitogenic peptide using antibodies directed against MHC class II membrane antigens, and with TCR-specific antibodies which block presentation of the encephalitogenic epitope by the antigen-presenting cell. EAE can also be inhibited by immunizing animals with synthetic peptides corresponding to the CDR2 or CDR3 regions of the TCR expressed by the encephalitogenic T cells. Thus, Offner and Vandenbark were able to suppress EAE induced with the MBP 68–86 peptide by injecting Lewis rats with a peptide corresponding to V β 8.2 CDR2. Suppression is apparently due to an anti-idiotypic response directed against the V β 8.2 CDR2 region of the TCR. The ultimate objective of these various studies is to develop therapeutic measures that may eventually be suitable for the treatment of human demyelinating diseases.

Peripheral nerve disease

Experimental autoimmune encephalomyelitis is a

demyelinating disease of the CNS. In 1955, Waksman and Adams described an autoimmune disease of the peripheral nervous system, experimental autoimmune neuritis (EAN), which they induced in rabbits immunized with sciatic nerve tissue in adjuvant; EAN serves as a model for Guillain–Barré syndrome. Animals with EAN develop cellular immunity and antibodies to the P2 protein, an autoantigen found in peripheral nerve myelin. P2 is a basic protein containing 131 amino acid residues, which is structurally and antigenically distinct from the CNS MBP molecule. EAN has been transferred with CD4⁺ T cells from donors immunized with either P2 protein, or synthetic peptides corresponding to the P2 neuritogenic epitopes.

Investigations of EAE and EAN have provided insight into the mechanisms responsible for the induction of autoimmune tissue damage, and the regulation of immune responses directed against ‘self’ antigens. These studies will ultimately advance our understanding of mechanisms underlying human demyelinating diseases.

See also: Autoimmune diseases; Autoimmunity; Multiple sclerosis; Neurological autoimmune diseases; T cell receptor, $\alpha\beta$; T cell vaccination; Tolerance, peripheral.

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EXTRACELLULAR MATRIX

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In recent years we have seen an explosion in information relating to all aspects of our understanding of the extracellular matrix (ECM). The major constituents identified in the ECM are collagens, proteoglycans and glycoproteins. At present 20 different collagens have been described which are capable of generating a variety of supramolecular structures. Considerable progress has been made in delineating the protein families found in the proteoglycans, and in understanding the role of alternative splicing that is a hallmark of many of the matrix glycoproteins. All cells respond to instructions from the outside, and we are beginning to understand not only the particular roles of individual macromolecules in the matrix, but also how the matrix binds and facilitates the action of growth factors. The dynamic nature of matrix belies earlier concepts that its only role was to act as a stable structural material, and while we understand more about the structural properties of some of the macromolecules, we are only just beginning to appreciate how macromolecules interact with one another and with cells, and indeed what signals are generated as a consequence of this cellular interaction.

The ECM evolved in response to forces acting on multicellular organisms; it provides mechanical support and physical strength to tissues, organs and to the whole organism. This may take the form of a simple basement membrane supporting cells in a multicellular organism like hydra, to the more diverse connective tissues, skin, tendon and ligament found in humans. The tendency to think of the matrix purely as providing strength and support belies the profound influences that it has on the behavior and pattern of gene expression of cells with which it is associated.

In humans the ECMs are highly varied in appearance, structure and function. This diversity provides tissue with rope-like characteristics such as tendon, the shock-absorbing characteristics of cartilage to the stony hardness of bone and the transparent matrix of cornea. Despite this tremendous variation in appearance, there is a great similarity in the types of molecules found in the ECM. It is the variation in the relative amounts of the different types of matrix macromolecules and the way that they are organized in the matrix that results in the varied appearances of connective tissues.

Cells of mesenchymal origin produce the ECM; these can be fibroblasts, smooth muscle cells or more differentiated cells, such as chondrocytes and osteoblasts. The matrix produced may be considered as a composite of insoluble fibers and soluble polymers. The principal fibers are collagen and elastin, while the soluble molecules include proteoglycans and glycoproteins.

Fibrous proteins – collagen and elastin

The collagens constitute what is now known to be a highly specialized family of glycoproteins. At present, 20 genetically distinct types encoded by at least 30 genes have been described. All collagens contain a domain with a triple helical conformation and are integral components of the extracellular matrix.

In triple helical domains three polypeptide (α) chains, each with a left-handed conformation, wind round each other to form a right-handed superhelix. Every third residue is glycine and of the repeating triplet [Gly-X-Y] that makes up the chain, approximately 20% of X and Y are imino acids, proline and hydroxyproline. This triple helical domain varies in length for the different collagen types and can either be continuous or interrupted with nonhelical domains.

The molecular configuration and tissue distribution of some of the collagens are shown in Table 1. Attempts have been made to classify collagens, both with regard to their gene organization and supramolecular structure, and while some of the more recently described collagens cannot be categorized in this way, it does provide a useful guide as to their role in the ECM.

The bulk of the collagen exists as fibrillar collagens, types I, II, III, V and XI. In these assemblies, molecules pack in quarter-staggered arrays to produce banded fibrils and fibers which provide mechanical support to the tissue (Figure 1). In contrast the FACIT (fibril associated collagens with interrupted triple helices), types IX, XII and XIV, are associated with the fibrillar collagens and may serve as a molecular link between fibrillar collagens and other matrix molecules. A different form of assembly is found in basement membranes, where type IV collagen forms a three-dimensional network (Figure 1). In addition beaded microfilaments are produced from type VI collagen (Figure 1), antiparallel dimers

Table 1 The collagen family

Collagen types	Number of α -	Supramolecular form	Distribution
I	2	Large diameter banded fibrils	Widespread: skin, tendon, cornea, bone
II	1	Small diameter banded fibrils	Cartilage, vitreous
III	1	Small diameter banded fibrils	Widespread in association with type I
IV	6	Network	Basement membranes
V	3	Heterotypic fibrils with type I and III	Widespread
VI	3	5 nm microfibrils with 100 nm periodicity	Widespread
VII	3	Anchoring fibrils	Specialized epithelia
VIII	2	Hexagonal lattice	Descemet's membrane
IX	3	FACIT	Cartilage, vitreous
X	1	Hexagonal lattice	Hypertrophic cartilage
XI	3	Heterotypic fibrils with type II	Cartilage
XII	1	FACIT	Widespread
XIII	1	Protein not isolated, 3 COL domains	Endothelial cells, epidermis
XIV	1	FACIT	Skin, tendon, cartilage
XV	1	Protein not isolated, 9 COL domains	Placenta, pancreas
XVI	1	FACIT	Skin, lung, amnion
XVII	1	Protein not isolated, 13 COL domains	Hemidesmosomes
XVIII	1	Protein not isolated, 10 COL domains	Liver
XIX	1	FACIT?	Unknown

of type VII collagen form anchoring fibrils, and the short-chain collagens VIII and X form hexagonal lattices.

While collagen undoubtedly provides the tensile strength of tissues, the variety of supramolecular forms found in the collagen family clearly indicate other roles for this important class of matrix macromolecules. In contrast, the other major fibrous protein in the ECM, elastin, is a single gene product and is largely responsible for the ability of tissues to stretch and recoil.

Elastin has a high degree of elasticity and can deform to large extensions with small forces; it is therefore found in abundance in those tissues that undergo repeated stretching, such as lung and blood vessels. Elastin (70 kDa) has a high content of hydrophobic amino acids and is the major protein of elastic fibers that form a randomly oriented, interconnected fiber system in many tissues. Elastin deposition in tissues is preceded by deposition of microfibrillar aggregates (10–12 nm); amorphous elastin deposits are seen to increase in these bundles until true elastic fibers are seen. The relative proportion of microfibril to elastin declines with age, and mature elastic fibers have a sparse association of microfibrillar material. The principal component of the microfibrils is the glycoprotein fibrillin. Defects in fibrillin on human chromosome 15 have been shown to result in the Marfan syndrome, a condition

where the failure to deposit elastin correctly can result in aortic dissection.

Proteoglycans

Proteoglycans are a diverse family of molecules that are characterized by having one or more glycosaminoglycan (GAG) chains attached to a protein core. These molecules are found inside cells, intercalated into membranes, and in the ECM, and appear to be ubiquitously associated with animal cells. Heterogeneity is found at both the protein level and particularly at the GAG level, where variations in both the type of GAG chain and the extent of modification are found.

Characteristically the GAG chain is composed of a repeat disaccharide sequence in which one sugar is an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) and the other a uronic acid (galactose in one case). The main types of GAGs found in the matrix are shown in Table 2. As can be seen, the majority, chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and keratan sulfate (KS), are sulfated and are charged on each sugar residue, while hyaluronan (HA) is non-sulfated and charged only on alternate residues. In addition, hyaluronan is the only GAG chain that is not covalently attached to a protein core *in vivo*. The other GAG chains are attached to the protein core via a

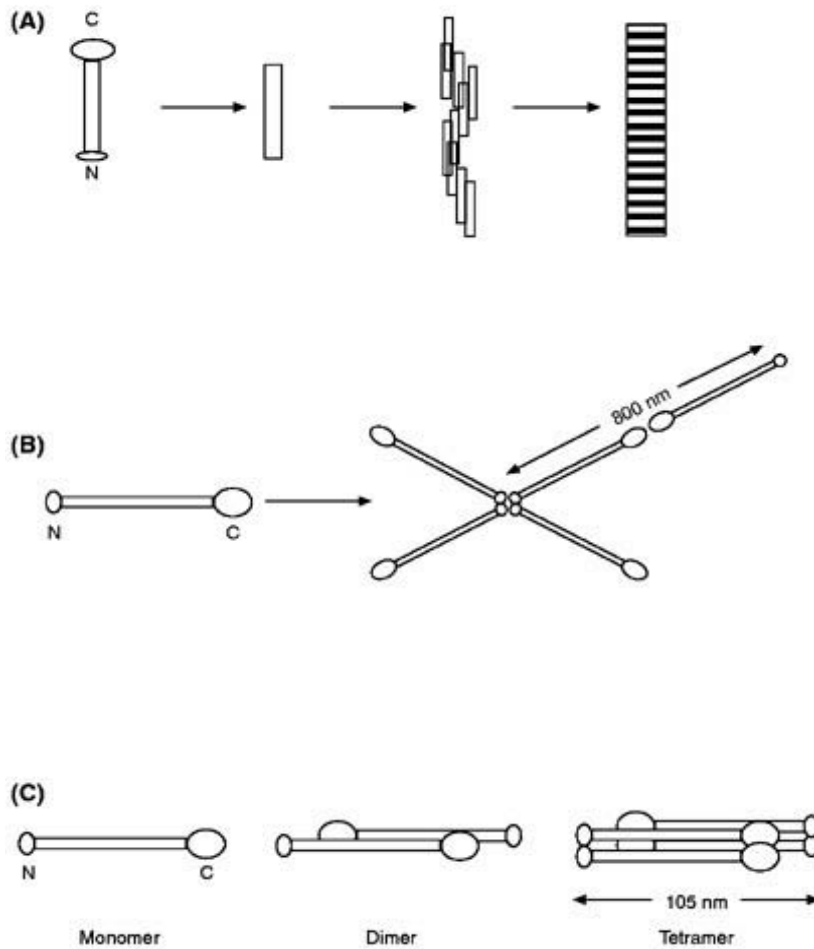


Figure 1 Diagrammatic representation of type I (A), IV (B) and VI (C) collagen molecules and their supramolecular arrangements. Triple helical and globular portions not drawn to size. Variation in the size of the triple helical portion of the molecule, and the size and nature of the N- and C-terminal globular ends, determines to a large part the way in which individual monomers can aggregate. Type I collagen forms 67 nm cross-banded fibers/fibrils by quarter-staggered arrays of individual monomers. Type IV collagen forms a flexible open network by association through both the N- and C-terminal ends of the molecule. Type VI collagen with a short triple helix and large globular extensions allows lateral association of two antiparallel monomers and two of these dimers associate in a parallel fashion with their ends in register. End-to-end association of the tetramers forms 105 nm beaded microfibrils.

Table 2 Glycosaminoglycans

GAG	Repeat disaccharide	Mol. wt. (kDa)
Hyaluronic acid	Glucuronic acid and <i>N</i> -acetylglucosamine	5–10 000
Chondroitin sulfate	Glucuronic acid and <i>N</i> -acetylgalactosamine (can be sulfated at 4 or 6)	5–50
Dermatan sulfate	Glucuronic acid (or iduronic acid) and <i>N</i> -acetylgalactosamine (sulfated at 4 or 6)	10–60
Keratan sulfate	Galactose and <i>N</i> -acetylglucosamine (sulfated at 6)	5–20
Heparan sulfate	Glucuronic acid (or iduronic acid) and <i>N</i> -acetylglucosamine. Extensively sulfated on both residues	5–10

xylose residue linkage to a specific serine residue in the protein core, with the exception that KS chains are attached to proteins via *N*- and *O*-linked glycosidic linkages.

Hyaluronan is very large and can have a molecular weight of up to 10 000 kDa. It assumes a randomly kinked, coil structure which occupies a large solution volume and endows solutions with high viscosity. These molecules can self-associate and form networks; in addition, a number of proteoglycans can form supramolecular aggregates with HA. It therefore has an important role in the development and function of many tissues.

The advent of cloning and sequencing techniques has greatly increased the understanding of the protein core of proteoglycans and now a number of the protein cores can be classified into families on the basis of their domain structure. In the case of the small matrix proteoglycans (decorin, biglycan, fibromodulin) the characteristic features of the protein core are the leucine-rich repeats, the presence of which accounts for some of the protein-protein

interactions that these small proteoglycans exhibit. The large aggregating proteoglycans, aggrecan (found in cartilage) and versican (found in skin) also share similarities in the protein core, principal of which is the hyaluronate-binding region at the N-terminus. A similar domain is also found in the cell surface proteoglycan CD44.

The highly acidic and hydrophilic GAG chains might be expected to dominate the functional properties of these molecules, and while much of the tissue hydration caused by these molecules is attributable to this part of the molecule, specific interactions are mediated by both the GAG chains and the protein core. These include regulating protease activity, binding growth factors and mediating interactions with other matrix components.

Interactions and hydration of proteoglycans are key determinants of articular cartilage function, where the ability to withstand compressive forces serves to protect the underlying bone. In cartilage, proteoglycans, such as aggrecan, form a supra-molecular aggregate with hyaluronan and this interaction is stabilized by link protein (Figure 2). This helps maintain a high concentration of proteoglycans in cartilage, which is important in determining the hydration of the tissue. Collagen fibers provide the scaffold that resists tensile forces, while the proteoglycan aggregates provide a hydrated, viscous gel that absorbs compressive forces.

Glycoproteins

The collagen family of proteins contain a triple helical portion in their structure, and the proteoglycans

all contain GAG chains attached to a protein core. Matrix glycoproteins, however, apart from containing O-linked and/or N-linked sugars, appear to have little in common with one another. Functionally, the ability to interact with other matrix molecules and with cells appears to be the common feature of this class of molecule, and a number have been described as 'adhesive glycoproteins'.

Many of the matrix glycoproteins contain distinct and functionally active protein domains that prescribe interactions with the cell surface as well as other matrix molecules. In addition, a number of the molecules form oligomers, and families of molecules are produced by alternative splicing. Many possess short aspartate-containing sequences such as RGD, which mediate cellular adhesion through the integrin family of membrane receptors.

Fibronectin is found in the ECM and blood plasma; it contains two nonidentical subunits joined by disulfide bonds and is the best characterized of the adhesive glycoproteins. The domain structure of one of the subunits is shown in Figure 3, and different domains are specialized for binding extracellular matrix molecules of bacterial or eukaryotic membrane receptors. Different fibronectin isoforms arise by alternative splicing of the transcript of a single gene. Its principal functions appear to be in cellular migration during development and wound healing, regulation of cell growth and differentiation, and hemostasis and thrombosis.

Many mesenchymal cells preferentially use fibronectin to adhere to; in contrast epithelial and endothelial cells use the glycoprotein laminin. Laminins are present in all basement membranes and have

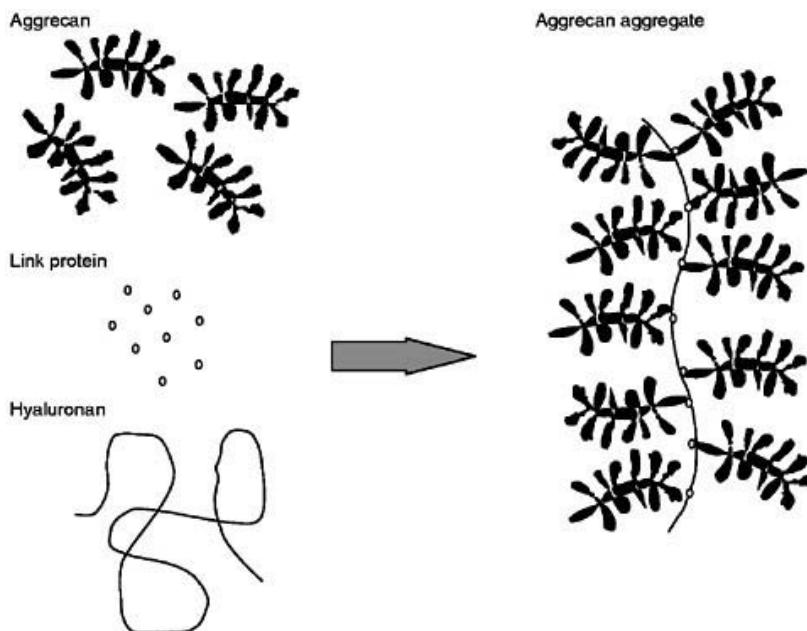


Figure 2 Aggrecan organization in cartilage. In cartilage the large proteoglycan aggrecan interacts with the GAG hyaluronan, this association is stabilized by the glycoprotein link protein. The aggrecan aggregate may have a molecular weight approaching 1 million kDa.

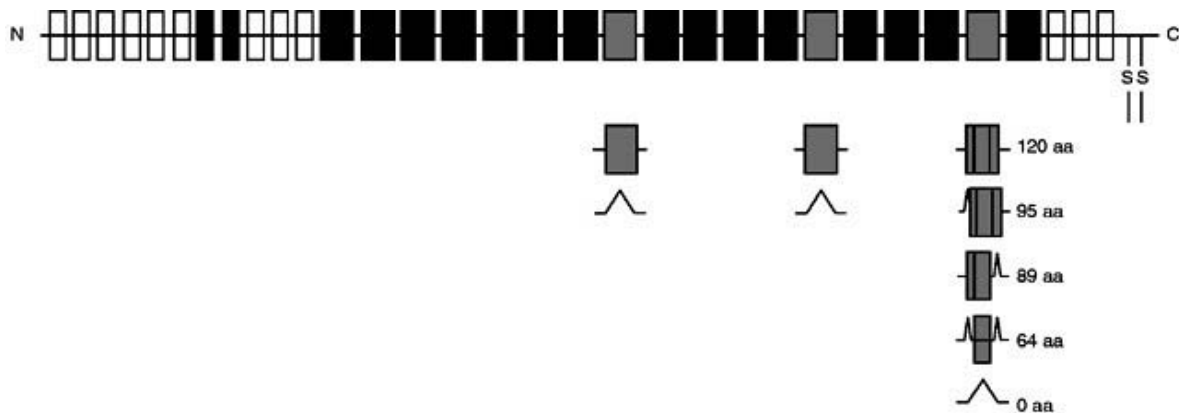


Figure 3 Domain structure of one of the subunits of fibronectin. FN type I repeats (□), FN type II repeats (■) and the FN type III repeats (▨) are shown. The three type III repeats that undergo alternative splicing are indicated. The number of additional amino acids (aa) encoded by splice variants of the third FN type III segment are indicated.

major influences on adhesion, growth, migration and differentiation of cells. Laminins are composed of three chains (α , β and γ) with molecular weights of 140–400 kDa. At present eight genetically distinct laminin chains and seven different assemblies are known. Chain association occurs through an α -helical coiled coil near the C-terminus of each chain. The specific binding of laminins to perlecan, entactin, type IV collagen and other basement membrane components suggests that laminins play a crucial role in the supramolecular organization and assembly of basement membranes.

The type III repeat of fibronectin containing the RGDS cell-binding motif is found in a number of matrix glycoproteins, including thrombospondin and tenascin, and is probably a recurring motif for cell binding. Another common domain in matrix glycoproteins is the epidermal growth factor (EGF) repeat. In addition to laminin, entactin, thrombospondin and tenascin, it is found in the proteoglycans aggrecan and versican. These repeats may mediate two functions: first, facilitate protein–protein interactions; and second, interact with EGF receptors and control cellular activity. The occurrence of multiple domains in the glycoproteins suggests multiple functions, and during evolution this has been achieved by exon shuffling, which has produced the variety of matrix glycoproteins.

Matrix turnover

In most adult tissues turnover of matrix is slow. There are, however, situations such as in embryonic and postnatal development, and in the postpartum uterus, where there is considerable tissue removal and replacement. The structural fibers, in general,

have a longer half-life than the ground substance. In the case of elastin in aorta it has been suggested that, under normal circumstances, once laid down it lasts through life.

Resorption of matrix components is achieved by resident cells as well as infiltrating cells, such as neutrophils and macrophages. In general both extracellular and intracellular mechanisms are thought to play a part, although current thinking is that the initial step is an extracellular proteolytic cleavage.

Matrix macromolecules can be degraded by four classes of proteinases, and their relative roles *in vivo* may vary depending on the situation. The cysteine and aspartic proteinases function optimally at acid pH, and while there may be local acidic environments and some of the cysteine proteinases have been found extracellularly, they are probably major determinants of degradation of phagocytosed material. Circulating plasminogen activator and plasmin are key serine proteinases in regulation of turnover. The elastase and cathepsin G of polymorphonuclear leukocytes and the tryptase and chymase of mast cells, are all serine proteinases capable of matrix degradation.

The matrix metalloproteinases (MMPs) are a family of enzymes which bind metals, are secreted in an inactive form and require extracellular activation. There are at least nine members of the MMP gene family, which share extensive sequence homology, but differ in their substrate specificity (Table 3). Regulation of activity is determined intracellularly by transcriptional control, and extracellularly by activation and the presence of specific inhibitors. Inhibitors like α_2 -macroglobulin bind and trap active enzymes; the tissue inhibitors of metalloproteinases (TIMPs) form noncovalent complexes with active

Table 3 Matrix metalloproteinase family

Enzyme	Mol. wt. (kDa)	Substrate
MMP1*	52–57	Fibrillar collagen
MMP8*	75	Fibrillar collagen
MMP3†	55–60	PG core, fibronectin, laminin, collagen IV
MMP10†	55–60	PG core, fibronectin, collagen IV
MMP2‡	72	Gelatin, collagen IV, V, IX, X and elastin
MMP9‡	92	Gelatin, collagen IV, V, elastin

*Interstitial collagenases.

†Stromelysins.

‡Gelatinases.

A number of other members of the family have been described and recent work shows the presence of a membrane-bound matrix metalloproteinase on some tumor cells.

forms of MMPs and in some cases with the latent enzymes. TIMP-1 and TIMP-2 have been described and there may be other members of this family.

Cell matrix receptors

Cells respond to their environment. This can be seen in a variety of morphogenetic effects, cell attachment, spreading, differentiation, etc., and in recent years it has become increasingly apparent that the ECM can regulate the expression of a variety of genes which control cellular activity. These effects are mediated by a variety of cell surface receptors, including integrins, proteoglycans and selectins.

Integrins, a family of cell surface heterodimers, are receptors for a number of matrix molecules. While there are about 14 α and 8 β subunits, only about 20 different dimer combinations have been identified. Ligands appear to bind to multiple integrins, and individual integrin receptors appear to bind many different ligands, indicating that redundancy may be a key feature of this type of interaction. Movement and shape changes seen in cells indicate that integrins associate with intracellular cytoskeletal elements. Ligand binding to integrins may also involve signal transduction and gene regulation. This, coupled with the presence of growth factor receptors, provides a mechanism for cellular response to changing matrix conditions.

See also: Adhesion molecules; Fibronectin; Integrins; Microenvironment; Rheumatological disorders.

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EYE, AUTOIMMUNE DISEASE

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The eye is subject to a number of sight-threatening inflammatory disorders of an immune-mediated nature that can affect different parts of the ocular anatomy. The uveitic entities affecting the back of the eye are particularly likely to affect vision adversely, and, in fact, blindness is a not infrequent outcome. Some uveitic diseases in humans are disorders confined only to the eye, whereas others are part of a more generalized, systemic, syndrome. Because the eye is an immunologically privileged organ, special considerations apply to the establishment and maintenance, as well as the potential breakdown, of self-tolerance to eye-specific components. Animal models of ocular autoimmunity induced with eye-derived antigens are being used to study these questions, and are providing valuable insights into the basic mechanisms of, and potential immunotherapeutic approaches to, immunologically mediated eye disease.

Autoimmune and immune-mediated eye diseases in humans

Although strong associations have been noted between many uveitic diseases and HLA type, indicating an immunogenetic component to disease etiology, the extent to which autoimmunity in the strict sense plays a role in human disease is still controversial. The reason for this is that in most cases the eliciting antigens have not yet been positively identified. One exception is phacoanaphylaxis, caused by an autoimmune response to lens proteins, usually following trauma and disruption of the lens capsule. Lens-specific antibodies and complement activation appear to be intimately involved in the disorder, but it is still unknown whether a significant cell-mediated component exists. In animal models of lens-induced uveitis (LIU), a primarily humoral mechanism of disease has been demonstrated.

In posterior uveitic disease, sympathetic ophthalmia (SO) has for a long time been considered the prototypic ocular autoimmune disease. As in phacoanaphylaxis, pathogenesis can be linked to a discrete inducing event, i.e. a penetrating trauma to one eye. Characteristic of SO is the subsequent development, weeks to months later, of an acute inflammation in the contralateral (sympathizing) eye, which has not been injured. This has been attributed to the development of an autoimmune response to

the ocular antigens released into the bloodstream from the injured (exciting) eye. It is unclear why SO develops in only a small proportion of the cases of ocular trauma, and what the predisposing factors are. Genetic susceptibility and a concomitant adjuvant effect due to infection have been proposed as possible facilitating mechanisms. Additional posterior and intermediate uveitic diseases for which an autoimmune component(s) has been postulated include birdshot retinochoroidopathy, and systemic diseases with ocular involvement, such as Behçet's disease and Vogt-Koyanagi-Harada syndrome. Many patients suffering from these diseases display strong immunologic responses to retinal proteins. Of particular note, a number of studies that demonstrated specific antibody and lymphocyte responses to the retinal soluble antigen (S-Ag) in birdshot retinochoroidopathy patients and in other uveitis patients. Although it is difficult to determine to what extent these responses might play a role in the etiology and/or pathogenesis of their disease, the S-Ag is one of several retinal proteins which cause experimental autoimmune uveoretinitis in laboratory animals (see below). Antigens that might be involved in anterior uveitic disease affecting the iris and ciliary body are also yet to be identified, but at least one type of anterior uveitis (the Reiter syndrome) has been linked to the presence of anti-*Klebsiella* responses in the affected patients (see below).

Animal models of uveitis, elicited by immunization with eye-derived proteins, have been developed and serve as research tools that have yielded invaluable insights into the mechanisms and therapy of these sight-threatening ocular inflammatory diseases (Table 1).

Is there tolerance to ocular antigens?

Central to the understanding of the mechanisms of ocular autoimmunity is the special localization of the ocular antigens in relation to the immune system. It has long been known that the eye is an 'immunologically privileged' organ, such that histoincompatible tissue grafts placed in the anterior chamber of the eye are not rejected. The reason for this immune privilege has been attributed mainly to the fact that there is no lymphatic drainage of the interior of the globe, and to the existence of a blood-ocular barrier

Table 1 Animal models of autoimmune diseases affecting the eye

<i>Model</i>	<i>Animal species</i>	<i>Main site of inflammation</i>	<i>Eliciting antigen(s)</i>	<i>Primary immunologic mechanism</i>	<i>Human disease(s) represented</i>
Lens-induced uveitis (LIU)	Rat, rabbit, guinea pig	Panuveitis	Lens proteins	Humoral	Phacoanaphylaxis
Experimental autoimmune anterior uveitis (EAAU)*	Rat	Anterior (iris and ciliary body)	Melanin proteins	Cell mediated	Anterior uveitis
Experimental autoimmune uveoretinitis (EAU)	Mouse, rat, guinea pig, rabbit, monkey	Posterior (retina and choroid)	Photoreceptor proteins	Cell mediated	Intermediate and posterior uveitis (sympathetic ophthalmia, birdshot retinochoroidopathy, and others)

*Also known as melanin protein-induced uveitis (EMIU).

which prevents free egress of lymphocytes from the circulation into the ocular tissues.

Current knowledge postulates that most lymphocytes capable of recognizing tissue-specific antigens are eliminated by negative selection in the thymus during ontogeny. Those that escape deletion must be controlled in the periphery by other tolerance-inducing mechanisms, such as clonal anergy or deletion due to encounter with tissue-specific antigen in the absence of accessory signals, or active suppression by regulatory cells. It is unclear, however, to what extent any of these mechanisms are operative in the case of ocular antigens. The separation between the immune system and the eye occurs early in ontogeny, and although recent work shows that messenger RNA encoding some retinal antigens can be detected in the thymus by the sensitive method of polymerase chain reaction (PCR), detectable levels of retinal proteins are not readily demonstrable in the thymus. Antibodies and lymphocytes that recognize ocular antigens are detectable in the blood of healthy human and animal subjects who show no evidence of autoimmunity. This supports the notion that there is neither clonal deletion nor inactivation of these cells. Disruption of the blood-retinal barrier during trauma or inflammation can give access to lymphocytes, which are not tolerant, into the eye. Antigen may be presented either locally (major histocompatibility complex (MHC) class II-expressing cells are present in the inflamed eye) or peripherally (antigen which escapes under the conjunctiva will be drained into regional lymph nodes). How, then, is ocular nonaggression maintained? And what are the circumstances leading to its breakdown, resulting in autoimmune uveitis?

Insights gained from animal models of ocular autoimmunity

The questions outlined above are being studied in animal models of ocular autoimmunity (Table 1). The best studied is experimental autoimmune uveoretinitis (EAU), which serves as a model for human posterior uveitic disease that is most likely to result in impairment of vision. EAU can be induced in various species of rodents and in primates by any of several defined retinal antigens injected in emulsion with complete Freund's adjuvant (CFA). A number of uveitogenic proteins derived from the photoreceptor cell layer have been identified (Table 2). EAU is characterized by destruction of the photoreceptor cells of the retina, where the eliciting antigen(s) are located (Figure 1), and is usually accompanied by autoimmune inflammation of the pineal gland ('third eye'), which shares ocular-specific antigens with the retina.

A dictum which clearly emerges from studies in animal models is that mere exposure to an ocular antigen is insufficient to elicit ocular autoimmunity. Use of adjuvants is mandatory to elicit uveitis in experimental animals, and a concomitant microbial infection has been implicated in cases of SO in humans. Bacterial components and toxins are particularly efficient in promoting induction of EAU, possibly because of their effects in promoting a T_H1 -type response to the uveitogen. Depending on the species, the adjuvant effect can be provided by mycobacterial components (CFA), and/or by products of gram-negative bacteria such as *Bordetella pertussis* toxin or *Klebsiella pneumoniae* capsular lipopolysaccharide. Some studies indicate that microbial

Table 2 Uveitogenic photoreceptor-derived proteins

Protein	Mol. wt. (kDa)	Biological function	Location
Retinal soluble antigen (S-Ag, arrestin)	48	Phototransduction cascade	Intracellular
Interphotoreceptor retinoid-binding protein (IRBP)	140	Transport of vitamin A derivatives between the photoreceptor and the retinal pigment epithelium	Extracellular
Rhodopsin (and its illuminated form, opsin)	40	Rod visual pigment	Intracellular
Recoverin	40	Calcium-binding protein	Intracellular
Phosducin	33	Phosphoprotein	Intracellular

components may do more than simply provide an adjuvant effect. Sequence similarities have been found between a uveitopathogenic site of S-Ag, *Escherichia coli* protein and yeast histone C3. Moreover, rats immunized with synthetic peptides containing these microbial sequences develop typical EAU and demonstrate lymphocyte responses cross-reactive with the corresponding peptide sequence derived from S-Ag. These findings suggest that under some circumstances molecular mimicry might play a role in induction of ocular autoimmunity. In this context, of note are the reports that, in human anterior uveitis associated with rheumatoid disease and HLA-B27, a gram-negative bacterial infection precedes the onset of symptoms. A homology of six amino acids was found between HLA-B27 and *K. pneumoniae* nitrogenase, with antibodies against this residue being found in a high proportion of the patients.

A central role for T cells in human uveitis is indicated by the dramatic response of some of these diseases to the T cell targeting agent cyclosporine. The immunopathogenic mechanisms of EAU, as studied in both rats and mice, indicate a central involvement of T lymphocytes in this experimental disorder. Thus, EAU can be adoptively transferred with long-term T cell lines and clones, and is suppressed by therapy that targets T cells, including cyclosporine, monoclonal antibodies to T cell markers, and interleukin 2 (IL-2) receptor targeting therapy with monoclonal antibodies and chimeric toxins. A number of uveitogenic epitopes for rats and mice have now been elucidated, and research aimed at characterizing the T cell receptors of uveitogenic cells is under way. Analyses of the lymphokine profile of uveitogenic T cell lines and clones have uniformly indicated that the pathogenic lymphocyte produces high levels of the lymphokine interferon γ (IFN γ), or in some cases both IFN γ and IL-4, characteristic of a T_H1-type or a T_H0-type cell. In contrast, some types of protective responses (e.g. tolerance induced by oral administration of antigen) are characterized by a concomitant induction of IL-10, IL-4 and trans-

forming growth factor β (TGF β), suggesting that the putative regulatory lymphocyte may be of the T_H2 phenotype. Thus, immunotherapy designed to direct an ongoing autoimmune response towards the T_H2 differentiation pathway could potentially divert the response from a pathogenic to a nonpathogenic one. This basic research may eventually lead to the development of novel therapeutic strategies, targeting the specific lymphocytes and processes involved in ocular autoimmunity.

Similarly to human uveitis, EAU shows a strong genetic association. In the mouse EAU model, immunogenetic studies done in congenic strains indicate that susceptibility is connected to the MHC haplotype, presumably due to recognition of the appropriate (pathogenic) epitopes. However, quantitative aspects of the disease are strongly modulated by background, non-MHC genes, whose effects can be sufficiently strong to render the bearer of a 'susceptible' MHC haplotype resistant to disease. The mechanisms for most of these genetic effects remain obscure, but at least one is connected to a propensity to mount a T_H1-dominated immune response. The susceptible genetic background in both rats (Lewis) and mice (C57/BL) is one that supports development of an IFN γ -dominated response to the uveitogen, whereas resistant strains tend to mount an IFN γ -low (but not necessarily an IL-4-high, i.e. T_H2-dominated) response. Thus, while susceptibility may depend on a dominant T_H1 response, it appears that resistance may be achieved through more than one regulatory pathway. It is unclear at this time whether these findings have a parallel in genetic susceptibility and resistance to ocular autoimmunity in humans.

Although a number of studies in the EAU model have indicated a role for regulatory (T_H2-type?) cells in downregulation of disease, it is still an open question whether such regulatory cells exist before the onset of ocular autoimmunity and have a role in its prevention. It was believed in the past that because the immune system never comes in contact with ocular antigens, development of such active regulation is both unlikely and unnecessary. It is clear

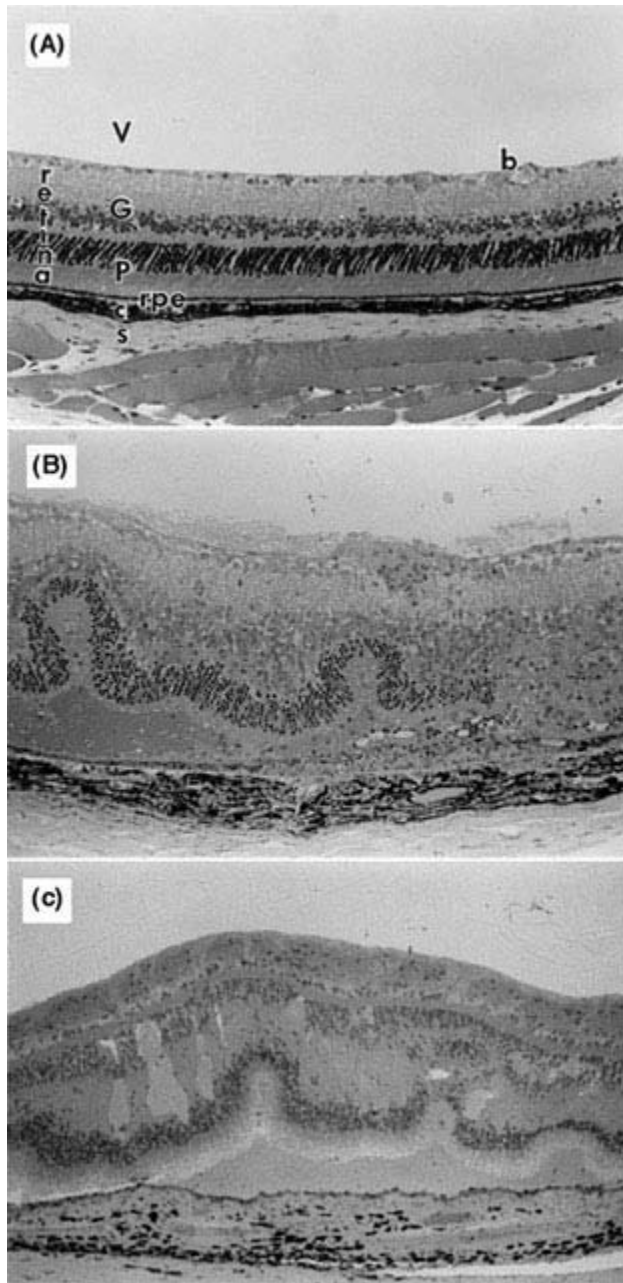


Figure 1 Histopathology of experimental and clinical uveitis. (A). Section through a healthy mouse eye. Note ordered structure of tissue layers and absence of inflammation. V: vitreous body; G: ganglion cell layer (cell bodies and nuclei); P: photoreceptor cell layer (nuclei and outer segments); rpe: retinal pigment epithelium; c: choroid; s: sclera. b: retinal blood vessel. (B). Section through a mouse eye with experimental autoimmune uveitis induced with IRBP. Note disrupted ocular architecture: cells and debris in the vitreous, vasculitis, retinal folds and retinal detachment, subretinal hemorrhage, subretinal granuloma, disrupted retinal pigment epithelium, infiltrated and thickened choroid. (C). Section through a human eye with uveitic disease. This patient has ocular sarcoid. Note similarity in histological appearance to experimental uveitis in the mouse.

today that this view is no longer tenable. Recent work has shown that by using the sensitive PCR technique, messenger RNA for ocular antigens is detectable in the thymus. The proteins themselves, however, are not readily detectable by conventional laboratory techniques and it is not clear whether their level of expression (if indeed they are expressed) is sufficient to contribute to induction of tolerance. However, easily detectable levels of uveitogenic proteins such as S-Ag and interphotoreceptor retinoid-binding protein (IRBP) are present in the pineal gland (third eye), which has no blood–organ barrier. Furthermore, numerous studies indicated that antigens placed in the anterior chamber of the eye (AC) do reach and are recognized by the immune system. In some cases (depending on the antigen), a deviant form of immunity is elicited, appropriately known as anterior chamber-associated immune deviation (ACAID). ACAID is characterized by suppressed delayed-type hypersensitivity responses, together with normal, or supernormal, induction of cytotoxic T lymphocytes and (noncomplement-binding) antibodies. Some researchers suggest that ocular antigens escaping from the AC into the circulation through the trabecular meshwork (facilitated by inflammation?) may naturally elicit an ACAID-like response, which would prevent, rather than promote, induction of uveitis. Under experimental conditions, ACAID induced to a uveitogenic retinal antigen can protect mice and rats from EAU induced by a subsequent immunization with the same antigen. It is unclear, however, whether a natural equivalent of this experimental phenomenon exists and plays a role in preventing ocular autoimmune disease.

Irrespective of the existence of putative systemic protective responses, the eye has several locally acting mechanisms that might help to curb autoimmune responses if and when the integrity of the blood–retinal barrier is breached. These mechanisms are also believed to contribute to ocular immune privilege. The ocular fluids contain immunosuppressive factors and cytokines, among them various neuropeptides and TGF β . More recently, high levels of Fas ligand were identified in ocular tissues. Lymphocytes infiltrating the AC underwent a Fas-mediated programmed cell death by apoptosis, and were prevented from causing tissue damage. In addition, ocular resident cells are able to suppress activation and clonal expansion of uveitogenic T cells through what is apparently a Fas-unrelated mechanism. For example, retinal glial cells (Müller cells) and ciliary body cells were shown to suppress activation and clonal expansion of uveitogenic T lymphocytes in an *in vitro* system derived from the rat EAU model, and chemical depletion of retinal Müller cells caused a

normally EAU-resistant rat strain to develop disease. Interestingly, the potent lymphocyte inhibitory function of these ocular resident cells was found to mask an underlying capacity to act as antigen-presenting cells. It is an intriguing notion that locally acting suppression mechanisms of this type might have evolved to prevent organ-resident cells, with the potential to present autoantigens, from initiating and perpetuating autoimmunity.

In summary, currently available evidence points to the notion that central mechanisms involving the thymus may have a limited role in maintaining a state of functional tolerance to ocular antigens. That ocular autoimmunity is the exception, rather than the rule, may be attributable to the effectiveness of the blood-ocular barrier and the various local and peripheral immunosuppressive mechanisms in curtailing immunologic processes that would lead to ocular autoimmune disease.

See also: Adjuvants; Autoimmune diseases; Autoimmunity; Eye infections; Privileged sites.

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EYE INFECTIONS

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Strategies of infection and host defense mechanisms

Pathogens reach the eye by two routes. They infect the eye directly through the conjunctiva and cornea, or they reach the eye through the vascular, lymphatic or nervous systems following infection elsewhere. The front of the eye protects itself from infection in many ways, and few organisms can infect the intact front surface. An eye with a damaged front surface is, however, far more susceptible to infection. Pathogens which reach the avascular tissues of the eye are partially isolated from host defense mechanisms. They can multiply more quickly and cause far more damage in these tissues than in vascularized tissue. Infection of the eye can compromise vision and lead to blindness. The common ocular pathogens can be

divided into two groups according to whether they cause short-term or long-term infections.

Short-term infections

Viruses are the commonest causes of short-term infections and they infect the eye directly. It seems likely that host immune responses terminate infection. These infections do not usually cause long-term damage to the eye.

Picornaviruses cause acute hemorrhagic conjunctivitis, which is common in tropical and subtropical areas of the world with high humidity and high population densities. There have been two pandemics. Enterovirus 70 is the commonest cause, but coxsackie virus A24, poliovirus and adenoviruses are also associated with this disease.

Adenoviruses cause acute conjunctivitis and epidemic keratoconjunctivitis. They are the commonest cause of ocular infection in developed countries. Many adenovirus types have been isolated from the eyes of patients, and types 3, 7 and 10 predominate in the UK. Adenoviruses also cause ocular disease in developing countries: they have been isolated from patients with acute hemorrhagic conjunctivitis (see above) and they were the commonest cause of acute conjunctivitis in a study in Pakistan, where virtually all the isolates were type 8.

Ocular infection with enteroviruses and adenoviruses evokes the production of specific antibodies. Antibodies against enteroviruses can neutralize virus infectivity, and antibodies against adenoviruses can fix complement. The roles of these antibodies in disease are not known.

Measles virus affects the conjunctiva and cornea of most, if not all patients with measles. This usually resolves without damage to the eye. In parts of Africa, however, many children with measles subsequently develop corneal ulceration and postmeasles blindness. Although the roles of immune responses in measles are well defined, their importance in the development of postmeasles blindness is not known. Measles infection can reduce tear flow, and children who are severely ill are unable to close their eyelids, so that their eyes become more susceptible to secondary infection. Malnutrition and measles virus infection both cause immunosuppression. Vitamin A deficiency can lead to xerophthalmia, and traditional eye medicines can damage the eye. In some areas of Africa, children with measles often develop corneal ulcers caused by herpes simplex virus. This may result from direct infection of the eye or from reactivation of existing infection (see below).

Long-term infections

Ocular pathogens establish long-term infections by direct infection of the eye or indirectly following dissemination of infection from elsewhere in the host. These pathogens survive or evade the host immune response, damage the eye and affect sight.

Herpes simplex virus

Herpes simplex virus type 1 (HSV-1) is associated with a range of eye diseases known as herpes simplex virus ophthalmia (HSVO). It causes conjunctivitis and lesions in the skin surrounding the eye, and ophthalmologists commonly describe this as primary HSVO. It also causes corneal ulceration, vascularization of the cornea, uveitis and glaucoma, and consequent loss of vision. Ophthalmologists describe this as recurrent HSVO. Studies of patients with HSVO

have shown that the terms primary and recurrent are inaccurate and misleading. It has therefore been proposed that HSVO should be described by its clinical features.

A life-long infection is established by HSV-1 in the sensory ganglia where it remains isolated from the host immune system. Occasional forays along nerves to the eye produce active HSVO, and HSV-1 can be isolated from the eyes for about a week. This suggests that host defense mechanisms terminate the active phase. Active corneal disease can however last for months; HSV-1 can survive for long periods in the cornea and has been isolated from corneas during quiescence. Patients with HSVO develop humoral and cellular immune responses to HSV.

Experimental infections in animals have shown that different clinical forms of HSVO result from differences in genetic factors of host animals and also differences between strains of HSV-1. The ability of HSV-1 to cause severe corneal disease, to establish latent infection in the eye and to multiply in isolated trigeminal ganglia infected *in vitro* differs in different strains of mice. Susceptibility to corneal disease is determined by alleles of the immunoglobulin heavy chain locus. Paradoxically, the major functional immunological difference is that susceptible mice have a much higher proportion of T helper lymphocytes in the corneal periphery than resistant mice. Different strains of HSV-1 produce different forms of HSVO. Strains isolated from patients with conjunctivitis caused conjunctivitis and strains from patients with corneal disease caused corneal disease. Strains isolated from patients with chronic stromal disease produced larger amounts of soluble precursor glycoprotein D than strains isolated from patients with other forms of HSVO.

Studies in rabbits and mice showed that previous ocular or dermal infection, immunization with inactivated virus, subunit vaccines and human hyperimmune gamma globulin all conferred a degree of protective immunity against subsequent ocular infection. Immunization of mice with HSV glycoprotein D prevented the development of stromal keratitis and corneal vascularization, and spread of infection to the trigeminal ganglia and the central nervous system. These protective effects were associated with raised levels of neutralizing antibodies and complement-dependent cytotoxicity.

Trachoma

Chlamydia trachomatis is an obligate intracellular bacterium which infects mucosal epithelia. Serovars A, B, Ba and C cause trachoma, an important cause of preventable blindness in developing countries. Infection is transmitted directly from eye to eye. In

contrast, serovars D to K are a common cause of genital infection in developed countries and occasionally infect eyes, but they do not cause trachoma.

Patients with trachoma have immune responses to the agent. Tears and blood contain antibodies which reduce the infectivity of *C. trachomatis*. Skin-testing for cell-mediated responses produced negative results in children with trachoma in The Gambia, but adults with severe trachoma in South Africa had positive reactions. Immunohistochemical studies of conjunctival biopsies obtained from patients with trachoma show that the inflammatory cell infiltrate contains B and T lymphocytes and that epithelial cells have MHC class II antigens (HLA-DR) as well as class I on their surfaces.

When strains of *C. trachomatis* isolated from patients with trachoma were inoculated into the eyes of blind human volunteers and nonhuman primates, they did not produce trachoma. They produced disease resembling that found in developed countries. Studies in animals (subhuman primates infected with *C. trachomatis*; guinea pigs infected with guinea pig inclusion conjunctivitis agent, a strain of *C. psittaci*) showed that antibodies and cell-mediated responses developed during infection and terminated infection. Features of trachoma, i.e. chronic conjunctivitis, corneal vascularization, scarring of the palpebral conjunctivae and subsequent distortion of the eye-lids developed after repeated episodes of infection. In guinea pigs, repeated ocular infection produced partial microbiological immunity which comprised two components. The short-term local component increased the amount of agent required to initiate reinfection and was probably mediated by secretory IgA in the eye. The long-term systemic component limited the growth of the agent following reinfection and was probably mediated by T lymphocytes. It paralleled the delayed-type hypersensitivity skin reaction to the agent, was associated with tissue damage and was suppressed by cyclosporine.

Staphylococcal eye diseases

Staphylococcus aureus causes acute and chronic conjunctivitis, corneal ulcers and blepharitis. Patients with staphylococcal eye diseases were skin-tested with staphylococcal antigens to see if hypersensitivity reactions played a role in the disease process. Patients with corneal disease were more likely to have enhanced delayed-type skin responses to killed whole cells of *S. aureus* and *S. epidermidis* than those with blepharitis. Experimental infections in rabbits have demonstrated that immune responses contribute to pathology. Ocular infection only produced

corneal disease and blepharitis when it was preceded by immunization with staphylococcal antigens.

Leprosy

Mycobacterium leprae causes leprosy, which is common in many developing countries. It is divided into four clinical forms. Patients with lepromatous leprosy have disseminated infection associated with poor cellular immune responses. They often develop major ocular complications and blindness. The mechanisms which protect the front surface of the eye are compromised because of damage to the facial and trigeminal nerves caused by *M. leprae*. This leads to lid abnormalities and abolition of the blink and corneal reflexes, which render the eye more susceptible to secondary infection. The iris is damaged by acute and chronic iritis. Acute iritis is part of a generalized reaction, known as the 'lepra' reaction, which is caused by the deposition of immune complexes in the iris. Acute iritis is similar to other forms of acute nonleptous iritis, and can lead to glaucoma and cataract. Chronic iritis is caused by *M. leprae* growing in the iris, causing iris atrophy. This is the commonest cause of visual impairment.

Onchocerciasis

Onchocerciasis or river blindness is caused by the filarial nematode *Onchocerca volvulus*, which is transmitted by several species of blackflies of the genus *Simulium*. Infected blackflies transmit larvae to humans by biting them. Larvae migrate to subcutaneous tissues where they become mature adults. Gravid females produce large numbers of microfilariae which migrate throughout the body. Over a period of many years, microfilariae migrate into the ocular tissues and die, causing small inflammatory lesions which leave scars. The accumulation of small scars in the cornea and the retina cause blindness. Live microfilariae are difficult to see in the cornea because they do not stimulate an inflammatory response. In contrast, dead microfilariae evoke punctate keratitis; small lesions which consist of dead microfilariae surrounded by lymphocytes, eosinophils and local edema.

Drugs which kill microfilariae cause a marked inflammatory response known as the 'Mazotti reaction' and concurrent treatment with anti-inflammatory drugs is required to reduce the severity of this reaction. The drugs suramin and diethylcarbamazine cause severe reactions, increasing corneal and retinal lesions and damaging the optic nerve, causing blindness. Ivermectin is currently being tested on patients and it has significant advantages over the other two drugs. In clinical trials, the number of microfilariae in the anterior chamber of the eye was reduced,

patients had the same or improved visual acuity and the Mazotti reaction was less marked. Ivermectin does not enter the eye, so that ocular tissues are spared the damaging and potentially blinding reaction to dead microfilariae.

See also: Adenovirus, infection and immunity; Chlamydia, infection and immunity; Eye, autoimmune disease; Herpes simplex virus, infection and immunity; Mycobacteria, infection and immunity; Onchocerciasis; Paramyxoviruses, infection and immunity; Picornavirus, infection and immunity; Staphylococcus, infection and immunity.

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FAS (CD95) AND FAS LIGAND

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Apoptotic elimination of cells is an important mechanism for both homeostatic regulation of the immune system and ensuring that autoreactive and unwanted lymphocytes are removed. One means of achieving this control is mediated via the cognate

interactions between Fas (APO-1/CD95) and Fas ligand (FasL). FasL binds to its receptor and in many instances this results in a cascade of events leading to the death of the target cell within hours. The prime role of the Fas/FasL apoptotic pathway

appears to be in peripheral deletion of autoreactive lymphocytes and limiting the extent of an immune response by clonal downsizing. Dysregulation of the Fas/FasL apoptotic pathway may contribute to certain disease states, including autoimmune disorders, tumor progression and immunodeficiency.

Fas

Fas was independently identified by two laboratories using monoclonal antibodies (anti-Fas and anti-APO-1) to a cell surface molecule that induced apoptosis in certain cell lines. The subsequent cloning of Fas demonstrated it to be a member of the tumor necrosis factor (TNF) receptor (TNF-R) family of membrane proteins. This receptor family includes the two TNF-Rs (p55 and p75), CD27, CD30, CD40, 4-1BB, OX-40, LT β R, human DR3 (death receptor 3)/WSL-1 and DR-4 (TRAIL receptor), human HVEM (herpesvirus entry mediator), and chicken CAR1 (receptor for cytopathic avian leukosis-sarcoma viruses). Members of this family of receptors share 20–25% amino acid identity and contain varying numbers of cysteine-rich extracellular domains. Cloning and mapping studies revealed that the *lpr* (lymphoproliferation) mouse mutation, which manifests itself as hypergammaglobulinemia, autoantibody production and the accumulation of CD3⁺CD4⁺CD8[−] lymphocytes in secondary lymphoid tissues, is a recessive mutation in Fas. The predominant function of Fas is to transduce apoptotic signals and Fas shares a homologous 70 amino acid 'death domain' sequence in its cytoplasmic region with the p55 TNF-R and with the more recently identified apoptotic receptors DR-3/WSL-1, DR-4 and CAR1. The death domain is distantly related to the *Drosophila* suicide gene reaper and is essential for transducing apoptotic signals. Fas is expressed by many cell types in the immune system but simple expression of Fas does not correlate well with susceptibility to Fas-mediated apoptosis. Whether a cell will undergo apoptosis upon ligation of Fas appears to be primarily dependent upon the activation state of the cell and by other signals that the cell has received that regulate apoptosis in a positive or negative manner (discussed further below).

Fas ligand (FasL)

The structure of Fas and its homology with the other members of the TNF-R family suggested that it was a receptor for an unknown cytokine. FasL was first identified using a bioassay that demonstrated that a CD8⁺ cytotoxic T lymphocyte (CTL) hybridoma cell line could induce lysis of Fas⁺ but not Fas[−] target

cells. The cDNA for FasL was subsequently cloned and shown to encode a type II transmembrane protein (i.e. its C-terminus is extracellular) of approximately 40 kDa that is part of the TNF family of ligands. The TNF ligand family includes TNF, LT α , LT β , CD27L, CD30L, CD40L, 4-1BBL, OX40L and TRAIL (TNF-related apoptosis-inducing ligand). Mice that are homozygous for the *gld* (generalized lymphoproliferative disease) mutation have an autoimmune phenotype virtually identical to that of mice homozygous for the *lpr* (Fas) mutation. Studies have demonstrated that this disease process is the result of a point mutation in the gene encoding FasL. FasL forms homotrimers of subunits that complement the trimeric receptor complex structure. FasL is expressed by both CD4⁺ and CD8⁺ T cells, and is predominantly expressed by the T_H1 subset of CD4⁺ T cells. FasL has also been reported to be expressed by neutrophils and human immunodeficiency virus (HIV)-infected or CD4 cross-linked macrophages and certain tumors and immune-privileged tissues (see below). Like TNF, membrane FasL is cleaved by a metalloprotease into a soluble form that is biologically active and thus can mediate its effect both locally and systemically.

Table 1 lists the expression and functions of Fas/FasL.

Fas apoptosis signaling pathway

The mechanism by which ligation of Fas on a cell surface leads to apoptosis is an area of current

Table 1 Expression and function of Fas/FasL

	FasL	Fas	Biological function
Naive T cells	−	±	Activation
Activated T cells	+	+	Apoptosis (AICD)
NK cells	+	−	Target cell apoptosis
slg activated B cells	−	+	Activation
CD40L activated B cells	−	+	Apoptosis
Self reactive (anergic) B cells	−	+	Apoptosis
Neutrophils	+	+	Apoptosis
Eosinophils	−	+	?
Monocytes	−	+	?
Activated macrophages	±	+	Apoptosis
Retina/cornea	+	−	Immune privilege
Sertoli cells (testes)	+	−	Immune privilege
Hepatocytes	−	+	Apoptosis/hepatitis
Normal thyrocytes	+	−	Immune privilege?
HT thyrocytes	+	+	HT pathogenesis
Certain tumors	+	±	Immune escape

interest. Receptor trimerization appears to be critical to Fas-mediated signalling because pentameric immunoglobulin M (IgM) Fas-specific antibodies or Fas-specific antibodies that aggregate in solution (such as IgG3 isotype) act as potent Fas agonists. In contrast, dimeric Fas-specific antibodies (e.g. IgG1 isotype) do not signal through Fas unless they are cross-linked. In fact, dimeric Fas-specific antibodies can act as potent antagonists of FasL-induced apoptosis. The signalling cascade mediated by Fas has several features in common with that mediated by the p55 TNF-R. Both pathways utilize interleukin 1 β (IL-1 β)-converting enzyme (ICE)-like proteases, recently designated caspases ('c' for cysteine protease and 'aspase' referring to their ability to cleave after aspartic acid), as the effector components of the apoptotic pathway. The caspase genes are homologous to the ced-3 gene which is responsible for programmed cell death in the nematode *C. elegans*.

Although it has been clear for some time that caspases are responsible for the effector phase of apoptosis, the initial events that ultimately result in activation of these proteases have only recently begun to be elucidated. The currently available data indicate that binding of FasL to Fas induces trimerization of Fas and subsequent association of the cytoplasmic protein FADD/MORT1 (Figure 1). FADD is comprised of two domains, a C-terminal death domain (DD) that noncovalently associates with the DD of the cytoplasmic region of Fas, and a death effector domain (DED) at its N-terminus. Caspase-8 (FLICE/MACH/Mch5) then binds to FADD via their respective DEDs which results in the cleavage and release of the p20 and p10 subunits of caspase-8 and the formation of the active enzyme. Caspase-8 then mediates the production of active caspase-3 (CPP32/Yama/apopain) from its inactive pro-form and caspase-3, in turn, mediates proteolytic cleavage of poly-ADP ribose polymerase (PARP) and ultimately chromosomal DNA degradation. Caspase-6 (Mch2) and caspase-7 (Mch3) are also activated via the Fas apoptotic signalling pathway. Caspase-6 has been found to proteolytically degrade lamin A, whereas caspase-7 both cleaves PARP and contributes to the protease cascade via cleavage of procaspase-6. It seems unlikely that cleavage of a single substrate is sufficient to result in a lethal event to the cell. However, there appears to be a coordinated cascade of proteolytic events that are induced subsequent to the ligation of Fas, and it seems quite likely that it is the concerted cleavage of a variety of substrates that leads to the apoptotic death of the cell.

Why do some cells succumb to Fas-mediated death while others that express equivalent amounts of Fas resist apoptosis or instead become activated upon lig-

ation of Fas? The answer may lie in negative regulators of apoptosis. Bcl-2, which is a mammalian homolog of the *C. elegans* gene ced-9, is a member of a family of proteins that regulate apoptosis upstream of the caspases. Although it is well documented that Bcl-2 prevents apoptosis of cells triggered by a number of stimuli, including cytokine deprivation and chemotherapeutic drugs, its role in inhibiting Fas-mediated apoptosis is unclear. Interestingly, studies in which Bcl-2 transgenic mice were crossed with Fas deficient *lpr/lpr* mice have indicated that Bcl-2 regulation of apoptosis is largely independent of the Fas apoptotic pathway. Recent studies have indicated that Bcl-xL, which is another member of the Bcl-2/Ced-9 family, may be critical in inhibiting Fas-mediated apoptosis of B cells, whereas Fas-mediated apoptosis of T cells does not appear to be inhibited by either Bcl-xL or Bcl-2. CrmA, which is a cowpox virus protein that inhibits caspase activity, inhibits Fas-mediated death of T cells, though a mammalian homolog of this protein has not as yet been identified.

Immune function and regulation

Although Fas is expressed by many tissues and cell types, the expression of FasL is relatively restricted. Both CD8⁺ and CD4⁺ T cells, natural killer (NK) cells and lymphokine-activated killer (LAK) cells all express functional FasL and are capable of inducing apoptosis in Fas-expressing target cells. However, in spite of its expression by cytotoxic effector cells, in most instances FasL does not appear to play a critical role in CTL killing. Analysis of perforin gene deficient mice and mice mutated in the Fas pathway (*lpr/lpr* mice) has indicated that CTL killing of target cells is mediated primarily via the perforin/granzyme pathway. In accordance, mice that are perforin deficient are susceptible to many viral infections, whereas Fas-deficient mice mount a normal immune response to most pathogens.

T cells that recognize self antigens are eliminated primarily in the thymus by the process of negative selection. Interestingly, although Fas is expressed in the thymus and thymic subpopulations are susceptible to Fas-induced apoptosis, Fas does not appear to play a critical role in thymic T cell deletion. In particular, analysis of *lpr/lpr* and *gld/gld* mice has indicated that the Fas/FasL apoptotic pathway is not critical for T cell receptor (TCR) repertoire selection in the thymus. Instead, evidence has accumulated that Fas plays a pivotal role in the inactivation and removal of self reactive T cells in the periphery that have escaped thymic deletion. These peripheral self reactive cells are thought to die through the process

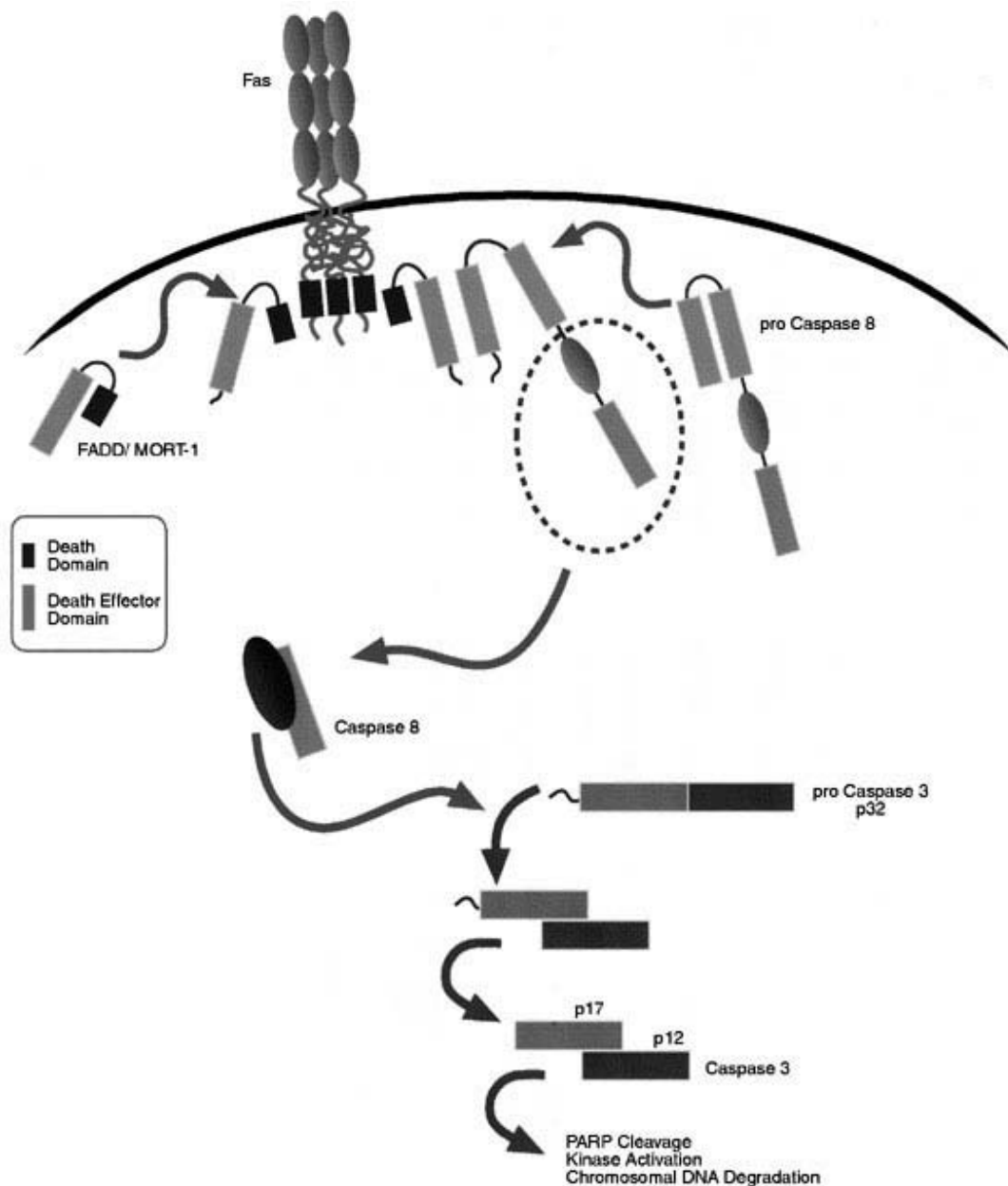


Figure 1 Fas apoptotic signaling pathway. Binding of trimeric FasL to its cognate receptor results in a coordinated cascade of cytoplasmic events leading to the activation of a series of cysteine proteases and ultimate death of the cell.

of activation-induced cell death (AICD) whereby previously activated T cells that are stimulated through the TCR undergo apoptosis. In this regard, the process of AICD in T cell clones and hybridomas is blocked by antagonists of Fas. Moreover, *lpr/lpr* and *gld/gld* mice are deficient in AICD as a result of mutations in Fas and FasL, respectively, thereby demonstrating a pivotal role for Fas/FasL interactions in this process. Finally, Fas/FasL interactions have been demonstrated to mediate peripheral but

not thymic deletion of antigen specific T cells in TCR transgenic mice.

Fas has also been implicated in regulating the duration of an immune response. Following a primary immune response, the vast majority of lymphocytes are eliminated by apoptosis (clonal downsizing) and only a small percentage survive to differentiate into effector and memory cells. Fas appears to play a critical role in clonal downsizing because mice that are defective in the Fas/FasL (*lpr/lpr* and *gld/gld*)

mice) pathway accumulate lymphocytes over time, resulting in splenomegaly and lymphadenopathy (Figure 2).

As mentioned earlier, expression of Fas does not always correlate with susceptibility to Fas-induced apoptosis. Most naive T cells either express Fas or can be induced to express Fas rapidly upon activation, though these cells do not die upon signaling via Fas. Instead, ligation of Fas on these T cells in the presence of T cell receptor cross-linking results in enhanced proliferation, increased expression of activation markers and increased cytokine production. Signaling via Fas in other cells, such as certain B cell lines and the HT-29 colon carcinoma cell line, can also result in activation and cytokine secretion rather than apoptosis. Upon continued stimulation, however, T cells become progressively susceptible to induction of apoptosis subsequent to cross-linking of Fas by either multimeric Fas-specific antibodies or FasL. Thus, the activation state of the cell appears to be a crucial determinant of whether

ligation of Fas results in T cell costimulation or apoptosis. In particular, cells in G_0 and G_1 are generally resistant to apoptosis, whereas they become sensitive to apoptotic stimuli as they enter the S phase of the cell cycle.

Apoptosis is also an important means of eliminating both self reactive and inappropriately activated B cells. B cells can be induced to express Fas and, depending upon the activation signals received, can become susceptible to Fas-induced apoptosis. For example, B cells activated by CD40 ligand (CD40L) in the absence of ligation of cell surface Ig become susceptible to Fas-mediated apoptosis. This may represent a mechanism for elimination of B cells that have been activated in a 'bystander' manner. In addition, B cells that have become desensitized by chronic stimulation with self antigens also become susceptible to Fas-mediated apoptosis and this may represent a means of eliminating 'anergic' B cells. In contrast, B cells that have been activated through their surface Ig receptor in conjunction with CD40L express Fas but are not susceptible to Fas-mediated apoptosis, and this appears to be mediated through the upregulation of Bcl-xL. Moreover, evidence from the study of antigen-specific B cells in Ig transgenic mice has indicated that Fas may play a role in conjunction with CD40L in the expansion of antigen-stimulated B cells, supporting the notion that ligation of Fas can transduce either apoptotic or activation signals and that the ultimate outcome depends upon other signals received by the responding cell.

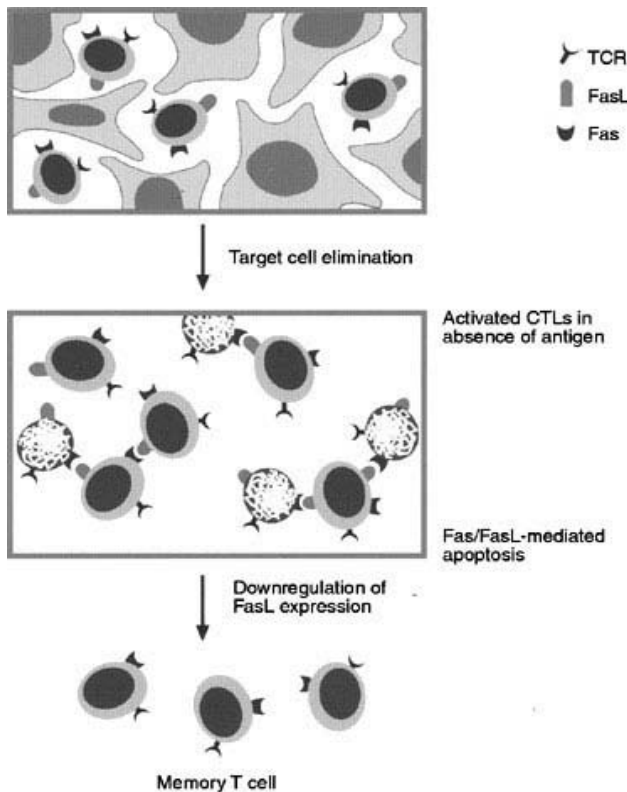


Figure 2 Role of Fas and FasL in clonal downsizing of an immune response via activation-induced cell death (AICD). As target cells are eliminated, activated T cells expressing both Fas and FasL interact with one another, promoting clonal downsizing via AICD. Subsequently, FasL is downregulated following elimination of antigen and the remaining cells go on to form memory cells. (Reproduced with permission from Elsevier Science Ltd and Lynch *et al* (1995).)

Dysregulation of the Fas/FasL apoptotic pathway and pathological consequences

Natural mutations in the Fas and Fas ligand genes have provided insight into the role of these molecules in the immune system. As noted earlier, the autosomal recessive genes *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) are spontaneously derived mutations in mice that result in the development of progressive lymphadenopathy and splenomegaly. Mice homozygous for the *lpr* gene express very little Fas due to a mutation that interferes with expression of the full-length Fas protein. The phenotype of *gld/gld* mice results from a point mutation in the FasL gene that alters the ability of the translated protein to bind to its cognate receptor, Fas. Both mutant mouse strains have a phenotype characterized by hypergammaglobulinemia, auto-antibody production, glomerulonephritis, arthritis and vasculitis. Significantly, a functional human equivalent of the *lpr/lpr* mouse exists. Children with autoimmune lymphoproliferative syndrome (ALPS) have a phenotype that is very similar to the *lpr/lpr*

mouse, including a profound lymphadenopathy characterized by the expansion of CD3⁺CD4⁺CD8⁻ lymphocytes and hypergammaglobulinemia. This syndrome is the result of mutations in the Fas gene that result in premature truncations of Fas translation in a dominant interfering manner and a subsequent defect in Fas-mediated T cell apoptosis.

Just as deficiency in the Fas/FasL apoptotic pathway leads to lymphoproliferation (or more correctly lymphoaccumulation) and autoimmune disease, overexpression of this pathway can result in the unwanted elimination of cells. The finding that the loss of CD4⁺ T cells in individuals infected with HIV is due to inappropriate apoptosis has prompted many studies on the potential role of Fas in this process. Not only has Fas been shown to be upregulated in T cells from HIV patients but these T cells also show increased susceptibility to Fas-mediated apoptosis. Cross-linking of CD4 on T cells by immune complexes comprised of HIV gp120 and gp120-specific antibodies results in susceptibility to Fas-mediated cell death. Moreover, infection of macrophages with HIV or cross-linking of CD4 on macrophages induces expression of FasL and these cells can then induce apoptosis of activated T cells that have increased expression of Fas and become susceptible to Fas-mediated apoptosis. Thus, HIV-infected macrophages may be the prime mediators of apoptosis of bystander T cells in HIV⁺ patients progressing toward AIDS. Fas may also be involved in the progressive loss of CD4⁺ T cells in patients with idiopathic CD4⁺ T lymphocytopenia (ICL). T cells from these patients not only express elevated levels of Fas, but also constitutively express FasL (whereas T cells from normal donors do not) and have an increased rate of spontaneous T cell apoptosis *in vitro*.

A role for dysregulation of the Fas/FasL pathway has also been suggested in the progression of both hepatitis and Hashimoto's thyroiditis (HT). Although the role of Fas/FasL in these diseases differs mechanistically, together they illustrate how critical regulation of this apoptotic pathway is to prevent tissue pathology. Although FasL is constitutively expressed by thyrocytes, under normal conditions Fas is not. In HT, however, the data suggest that IL-1 upregulates expression of Fas and increases susceptibility to Fas-mediated apoptosis in thyrocytes. The coexpression of Fas and FasL in HT results in apoptotic loss of thyrocytes. On the other hand, hepatocytes constitutively express Fas and therefore infectious agents such as hepatitis B virus and *Propionibacterium acnes* that induce infiltration of FasL-expressing T cells into the liver can induce apoptosis of hepatocytes. Hepatitis in animal models

can be blocked by a soluble form of Fas and thus inhibitors of Fas may have potential in the treatment of diseases where Fas-mediated apoptosis results in disease progression.

Role of Fas/FasL in immune privilege and immune escape

Under normal conditions, transplantation of major histocompatibility complex (MHC)-mismatched tissues results in their rejection by a strong T cell response specific for alloantigens. There are, however, certain 'immune privileged' tissues, such as Sertoli cells in testis and corneal and retinal pigment epithelium in the eye, that are not rejected upon transplantation into an MHC-disparate host. Recent studies have demonstrated that these tissues avoid rejection because they constitutively express FasL. Thus, Sertoli cells from a normal mouse are not rejected upon transplantation into an MHC-mismatched host, whereas testis grafts from gld/gld mice (that have nonfunctional FasL) are immunologically rejected. It is thought that as the T cells that recognize the transplanted tissue become activated, they increase expression of Fas and become susceptible to Fas-mediated apoptosis and are killed by FasL expressed by the transplanted tissue. This raises the possibility that engineering tissues for transplantation to express FasL may help prevent allograft rejection. Indeed, cotransplantation of islets of Langerhans with myoblasts expressing FasL was found to prevent rejection of the graft and delayed onset of hypoglycemia in a mouse model for diabetes. Immune-privileged sites can also mediate induction of systemic T cell tolerance via Fas/FasL interactions. Thus, injection of antigen into the anterior chamber of the eye results in systemic tolerance to the antigen that requires that the eye be FasL⁺ and the lymphoid cells be Fas⁺.

A similar mechanism of elimination of activated T cells by constitutive expression of FasL may provide a means of escape from the immune system by tumor cells. Certain tumors, including a proportion of melanomas, colon cancer cells and hepatocellular carcinomas, have been demonstrated to constitutively express FasL and appear to use this as a means of evading the T cell immune response by eliminating tumor-reactive T cells as they become activated.

It has become clear in recent years that tight regulation of the Fas/FasL apoptotic pathway is critical not only in maintaining immune system homeostasis but also in modulating host responses to pathogens and tumor cells. Disruption of this pathway by cytokines, pathogens and carcinogens can clearly result in disease progression. Understanding of these

processes presents the opportunity for the development of novel therapies for certain diseases and manipulation of the immune response for induction of tolerance or immunity.

See also: Apoptosis; CD40 and its ligand; Tumor necrosis factor α ; TNF receptors.

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FASCIOLIASIS

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Fascioliasis is a geographically widespread parasitic disease which is of economic importance because it lowers the productivity of livestock. Recently the disease has also been shown to be prevalent and clinically significant in humans in Latin America. The flat, leaf-shaped worms which cause this disease are digenetic trematodes of the genera *Fasciola* or *Fascioloides*. The common liver fluke, *Fasciola hepatica* (Figure 1), is considered here as it is the most prevalent and important species.

Characteristics of the organism and its antigens

Fasciola hepatica has an indirect life cycle. Many mammals, including sheep, cattle, rodents, marsupials and humans, can act as definitive hosts. Adult liver flukes, which are about 10 mm wide and 25 mm long, live in the bile duct, feeding on blood, bile and epithelial cells. The hermaphroditic adults reproduce sexually and release eggs which are carried in the bile

to the intestine. Eggs pass out with the feces and, if deposited in water, embryonate to form ciliated miracidia which hatch out and then swim until they find a snail of a suitable species. Asexual reproduction takes place in the snail to produce hundreds of cercariae. The cercariae burst out of the snail and, with the aid of a tail, swim until they reach vegetation, on which they rapidly encyst. The infective, metacercarial cysts have a sticky, multilayered protective casing. Mammals become infected by eating the lush but contaminated vegetation growing at the edge of the water.

Within the definitive host the metacercariae excyst in the small intestine to release juvenile flukes. The juvenile fluke moves through the intestinal wall, enters the peritoneal cavity and then penetrates the liver. Lobes of the liver closest to the duodenum are most heavily infected. The fluke grows rapidly as it migrates through the liver parenchyma, secreting proteolytic enzymes and feeding on liver tissue. Liver migration causes extensive damage with necrotic

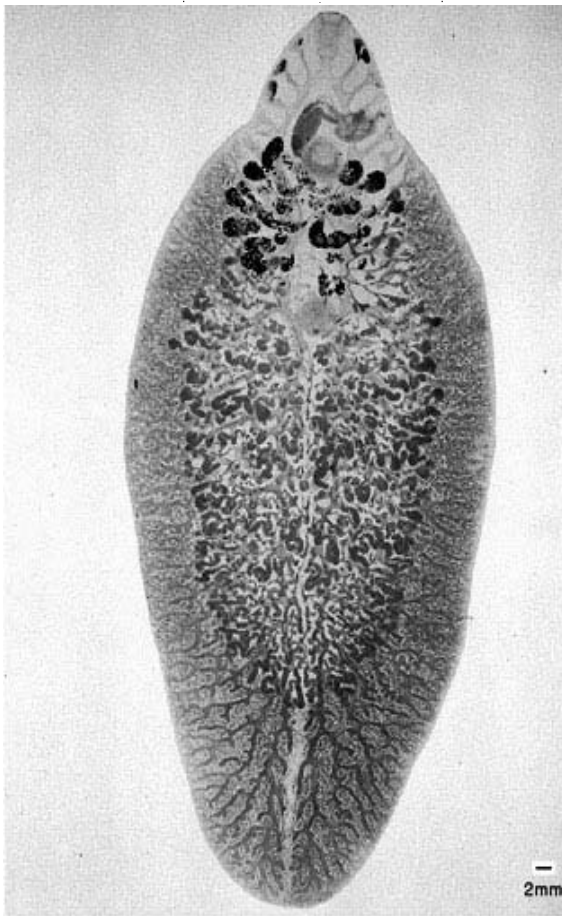


Figure 1 Adult *Fasciola hepatica*.

tracks forming in the wake of the fluke. This is called the acute phase of infection and lasts from 3 to 7 weeks, depending on the species of host. The chronic phase begins when the juvenile fluke penetrates the wall of the bile duct and develops into a sexually mature adult. If the host is not reinfected, the liver parenchyma is repaired but the adult worms persist for many years, causing host morbidity and mortality.

A complex array of antigens are expressed during the migration and development of *F. hepatica*. Some are unique to specific developmental stages but others are common to all stages. Antigens are released from the alimentary canal and the tegument. Since liver flukes do not have an anus they must periodically regurgitate their gut contents so that they can continue feeding. Within the tegument there are electron dense secretory bodies, characteristic of the stage of the fluke, which secrete their contents on to the tegument. Tegumental antigens are released when the polyanionic outer layer, or glycocalyx, is shed. The host is also exposed to somatic antigens as typically only a small proportion of the metacerca-

riae establish as adult flukes in the bile duct. Somatic antigens will be released when flukes are damaged or dead.

Immune responses of the host

In the acute phase of a primary infection a pronounced inflammatory response occurs in the liver soon after infection. The tracks left by the fluke and the surrounding necrotic areas are infiltrated by neutrophils, eosinophils, lymphocytes (mainly CD4⁺ T cells in sheep), macrophages and hepatocytes. In all host species a prominent eosinophilia also occurs in the peritoneal cavity and the blood. There is, however, no cellular response around the juvenile flukes themselves. In the chronic phase of a primary infection many macrophages and fibroblasts are present in damaged areas. The liver becomes fibrotic. In sheep the fibrotic strands contain many CD8⁺ and $\gamma\delta$ -TCR⁺ T cells. The larger bile ducts become hyperplastic and infiltrated by many lymphocytes and eosinophils. In cattle a granulomatous reaction leads to calcification of the bile duct which may cause the loss of worms.

Primary infection in some host species, including cattle, rats and possibly humans, results in the development of resistance to reinfection, whereas in other species, including sheep and mice, this does not occur. The mechanisms of resistance appear to be different in cattle and rats. In secondary infections in cattle, incoming juvenile flukes are killed in the liver. Fibrosis of the liver is more severe in cattle than in other species and is thought to slow or prevent the migration of juvenile flukes, making them more vulnerable to immunological attack. In secondary infections in rats, juvenile flukes are killed before they reach the liver. Most are killed in the intestinal mucosa, even in nude rats. In transmission electron micrographs of intestinal tissues, tegumental damage to juvenile flukes was only noticeable near attached eosinophils. *In vitro* eosinophils, as well as the reactive oxygen intermediates and major basic protein they release, are toxic to juvenile flukes. Since an eosinophilia develops in T cell-deficient rodents infected with *F. hepatica*, the release of toxic products from eosinophils may kill juvenile flukes in the intestinal mucosa. In rats, any flukes which manage to pass through the intestinal mucosa are killed in the peritoneal cavity by T cell-dependent mechanisms which have not been fully elucidated but probably involve immunoglobulin IgG2a (IgG2a) and perhaps also eosinophils.

Sheep acquire large fluke burdens as they do not develop resistance to reinfection, and in subsequent infections inflammatory responses in the liver are

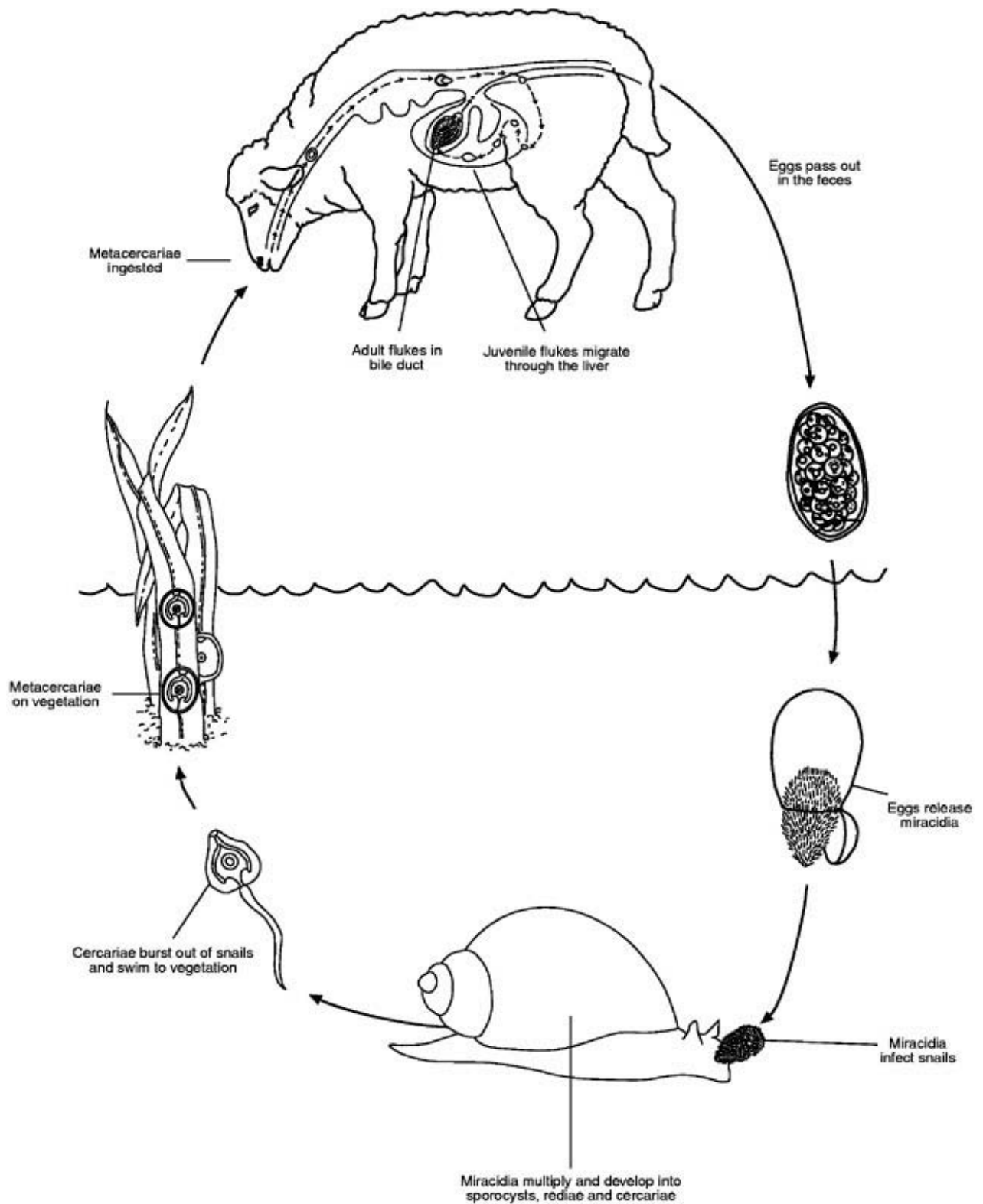


Figure 2 Life cycle of *Fasciola hepatica*.

similar to, but more pronounced, than those which occur in a primary infection. Sera from infected sheep recognize many of the *F. hepatica* antigens detected by sera from infected rats and can passively transfer resistance to naive rats. Overall, this suggests that deficiencies of cellular immune responses may account for the failure of sheep to develop resistance to reinfection (Figure 2).

Evasive strategies by the organism

Release of molecules from *F. hepatica* is thought to limit the effectiveness of the host's immune responses. Flukes secrete cathepsin proteases which cleave immunoglobulins and thus prevent attachment of host effector cells. Molecules which are toxic to host cells and thus immunosuppressive are also secreted. Juvenile flukes are also thought to be protected because they move from site to site, frequently shedding their glycocalyx as they do so.

Vaccines

Recent vaccine trials have produced promising and interesting results. Three purified *F. hepatica* antigens have potential as both immunodiagnostic and immunizing agents. The antigens are Fh12 (a 12 kDa *Fasciola/Schistosoma* cross-reactive antigen), glutathione S-transferase (GST) and cathepsin protease. The protective capacity of each of these has been tested in cattle or sheep. Cattle immunized with Fh12 had on average 55% fewer flukes than nonimmunized controls. Fh12 is a fatty acid-binding protein which is involved in intracellular transport of long-chain fatty acids and acyl CoA esters. Immunization with native *F. hepatica* GST had very variable effects, ranging from no effect at all to lowering fluke burdens by more than 90%. Cattle were more protected than sheep and the choice of adjuvant was found to be critical and to vary with host species. Reasons for the variability and the mechanism by which immune responses to *F. hepatica* GST lower fluke burdens are unclear. Vaccination of sheep with native cathepsin proteases had no effect on fluke burdens but fairly consistently lowered egg production by about 70%. The small number of eggs produced had low viability. These observations are consistent with cathepsin proteases being important in the formation

of the egg shell. Overall these vaccine trials have shown that sheep do not have an inherent inability to develop resistance to *F. hepatica* and have identified a number of antigens which induce protective immune responses by different mechanisms. This suggests that immunization of both cattle and sheep with a mixture of purified antigens could consistently and severely limit *F. hepatica* infections, which would be commercially useful.

See also: Eosinophils; Fibroblasts; Gastrointestinal tract infections; Parasites, immunity to; Schistosomiasis.

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FATTY ACIDS (DIETARY) AND THE IMMUNE SYSTEM

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The nutritional status of the host can influence the immunological defense systems. Among the nutrients that affect the host, dietary lipids exert potent and pervasive effects on both the antigen-specific and nonspecific immune functions. Polyunsaturated fatty acids (PUFAs) have been employed successfully as preventive and therapeutic modalities to control the pathologic processes of several diseases in clinical trials and experimental animal models. Recent studies have addressed a large spectrum of issues related to the effects of dietary manipulations of n-3 PUFAs, principally linolenic acid (18:3) from plants and docosahexaenoate (22:6) from fish and algae, and n-6 PUFAs, principally linoleic acid (18:2) from plants and arachidonic acid (20:4) from animals and fungi. Studies in humans, rodents and other experimental animals have been reported on rheumatoid arthritis and other rheumatic diseases; coronary artery disease; immunosuppression after trauma; complications from infections; chronic fatigue syndrome; immune functions in the elderly; carcinogenesis; transplanted grafts and autoimmune diseases; alteration of tumorigenesis by modulation of macrophage function; antiviral activities against a thymic lymphoma-inducing retrovirus; changes in renal disease, inflammatory and immune-related disorders; and dermatological conditions.

The effects of dietary fatty acids on immune responses are related to 1) total and fat-derived energy intake, 2) production of myriad eicosanoids, and 3) changes in the composition of cell membranes. Some of the important dietary fatty acid effects on the immune system and their modes of action are summarized herein.

Total and fat-derived energy intake

Moderate calorie restriction prevents or delays the development of age-associated disease such as breast cancer and renal disease in rodents. Similar dietary manipulations may also alter the risk and rise of life-shortening diseases in humans. Consumption of a low calorie diet containing only 5% corn oil increases expression of interleukin-2 receptor, and enhances cell-mediated immune responses in rats. Age-associated diseases, particularly autoimmune diseases with viral etiology, appear to be intensified

by increased intakes of vegetable oils or *trans*-fatty acids from hydrogenated oils. In the USA, 35–40% of the total calories are from dietary fat. Although consumption of saturated fat, which has been associated with increased cardiovascular disease, has decreased during the past 50 years, consumption of both monounsaturated and PUFAs of n-6 origin has either increased or been substituted for saturated fats.

It has been suggested that intake of excess linoleic acid is a factor increasing the incidence of many chronic diseases, including cancer and thrombotic diseases. Many researchers have concluded that this reflects an imbalance of n-6 PUFAs versus n-3 PUFAs in diets. Excess intake of linoleic acid and changes in the essential fatty acid balance of diets may tend to predispose certain individuals to hyperreactivity to various allergens.

Increased n-6 PUFA intake, principally as vegetable oils, and decreased antioxidant intake may enhance the production of free radicals. Free radicals can decrease antioxidant enzyme mRNAs, and the resulting decreased levels of antioxidant enzymes can further affect immune function by decreasing production of anti-inflammatory cytokines such as interleukin 2 and tumor growth factor β mRNA levels. Cytokines are biologically-active peptides produced by immune cells. Since a high percentage of dietary PUFA can have a negative effect on the immune system, it is recommended that total fat intake be decreased so that 7–10% of dietary energy is consumed in the form of PUFAs and saturated fatty acids and 10–15% of monounsaturated fatty acids.

Eicosanoids

Eicosanoids are produced by the enzymatic oxygenation of n-6 and n-3 PUFAs. Eicosanoids are biologic mediators with myriad effects on immune cells. The n-3 PUFAs, especially eicosapentaenoic acid, competitively inhibit the utilization of the n-6 PUFA, arachidonic acid. Through the cyclo-oxygenase and lipoxygenase pathways, n-3 PUFAs are substrates for the production of biologically active products (thromboxane A₃ and leukotriene B₅ from eicosapentaenoic acid) that do not exert the same intensity of

action as those derived from n-6 PUFAs (for example, prostaglandin E₂ (PGE₂) and leukotriene B₄ from arachidonic acid). The overproduction of eicosanoids contributes to pathogenesis of chronic and acute inflammatory, autoimmune, atherosclerotic and neoplastic diseases. Feeding a fish oil (n-3 PUFA) diet restores splenic T cell blastogenesis, reduces splenic cell secretion of PGE₂ and alleviates splenic suppressor activity. In contrast, feeding a corn-oil diet (n-6 PUFA) to tumor-bearing mice suppresses cell-mediated immunity, with a concurrent increase in PGE₂ production.

Interactions between PUFAs and the immune system are influenced by the rate of their conversion to, and the efficacy of, the various eicosanoids. The rate of eicosanoid synthesis is determined by PUFA turnover, which depends on the activity of phospholipases and dietary alterations that affect the availability of n-6 and n-3 PUFAs in the phospholipid pool. Dietary n-3 PUFAs decrease the severity of certain autoimmune, inflammatory and atherosclerotic diseases by decreasing the production of n-6 PUFA-derived eicosanoids and cytokines.

The elevated levels of eicosanoids associated with shock and trauma can also induce immunosuppression in humans. The immunomodulatory mechanisms involved in providing such patients with an enteral diet containing n-3 PUFAs are controversial, however, most information available indicates that dietary supplementation with n-3 PUFAs may prevent immunosuppression after trauma. Rheumatoid arthritis patients taking dietary fish oil supplements exhibit improvements, such as a decreased number of tender joints. In short-term clinical studies, n-3 PUFAs seem to diminish cyclosporine-induced nephrotoxicity and the attendant complication of hypertension, to inhibit inflammatory and atherogenic mechanisms in lupus nephritis, and to preserve renal function and reduce proteinuria in immunoglobulin A nephropathy.

Feeding high levels of dietary PUFAs to mice and rats suppresses the lymphoproliferative response to polyclonal mitogens. Substitution of fish oil for olive oil as a vehicle for the administration of cyclosporine reduces cyclosporine-induced and eicosanoid-mediated acute nephrotoxicity in experimental animals. Concurrent administration of fish oil with a subtherapeutic dose of cyclosporine prolongs graft survival. Lymphocytes and macrophages derived from animals fed n-3 PUFAs as linolenic acid or fish oil markedly inhibits the mixed lymphocyte reaction, an indicator of graft survival. An allogeneic mixed lymphocyte reaction response in mice fed n-3 PUFAs as fish oil was less than the response of animals fed equal amounts of n-6 PUFAs.

Recent work suggests that alterations in the immune system caused by dietary fat may affect tumorigenesis. Studies have focused on dietary fat modulation of macrophage function because that cell plays a pivotal role in many immune responses, including antitumor activity. One possible mechanism by which dietary fat affects macrophages is altered signal transduction, which, in turn, could alter gene regulation and macrophage function. Arachidonic acid is a mediator of signal transduction through the protein kinase C pathway. In addition, arachidonic acid effects the transcription of a variety of genes. Interferon-associated arachidonic acid hydrolysis from membrane phospholipids induces transcription of the interferon-stimulated response element, and inhibition of the prostaglandin synthase and 5-lipoxygenase pathways enhances this signal.

Membranes

Changes in the physical properties of membranes with aging or dietary lipids may influence the sensitivity of immune cells to antigenic stimuli. Dietary fatty acids can modulate the biosynthesis of eicosanoids from arachidonic acid by increasing the ratio of n-3:n-6 PUFAs in the membrane phospholipids and by modulating the biosynthesis of eicosanoids via the cyclo-oxygenase and lipoxygenase metabolic pathways. The rate of uptake and conversion of linoleic acid to arachidonic acid is age dependent and is genetically controlled. The availability of the direct precursor fatty acid is a limiting factor in eicosanoid biosynthesis. Biological responses similar to those seen with eicosanoids can be modified by manipulation of the composition of dietary lipids that influence the fatty acid composition of various tissues, including immune competent cells (e.g. macrophages, platelets, T cells, B cells, etc.). The n-3:n-6 PUFA ratio of various tissue phospholipids correlates with the n-3:n-6 PUFA ratio of the diet. An increased incorporation of n-3 PUFAs increases the n-3:n-6 fatty acid ratio in platelets and macrophages. Linoleic acid is the sole fatty acid required to support proliferation and maturation of immunoglobulin-secreting cells. Suppression of B cell functions may be caused by displacement of linoleic acid and arachidonic acid by n-3 PUFAs. Enhanced B cell functions in animals fed unsaturated fatty acids and corn oil could be due to an increase in the incorporation of linoleic acid and arachidonic acid in the membrane phospholipids.

The lipid composition of cell membranes plays a pivotal role in the mediation of the primary and secondary immune responses against an antigenic challenge or to an infection. Ongoing studies that are

showing how dietary fatty acids can profoundly affect immune functions may provide a basis for future therapeutic intervention.

See also: **Nutrition and the immune system.**

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Fc RECEPTORS

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Fc receptors (FcRs) are molecules expressed on the surface of a variety of cells that recognize and bind the Fc region of certain immunoglobulin (Ig) classes and subclasses. Hematopoietic cell FcRs specific for IgG (FcγR), IgA (FcαR) or IgE (FcεR) have been well characterized and their genes have been cloned (Figure 1). Some data suggest the existence of FcRs for IgM and IgD on leukocytes, although structural entities for such receptors have not been well defined. In addition, two epithelial cell FcRs have been well characterized: FcRn which mediates both IgG transport across the placenta and IgG uptake by neonatal intestinal epithelium, and the pIgR (poly Ig receptor, also known as secretory component) which transports IgA into mucosal secretions.

The high- and medium-affinity hematopoietic cell receptors for IgG (FcγRIa and FcγRIIIa, respectively) and the high-affinity receptor for IgE (FcεRI) bind monomeric Ig cytophilically, 'arming' the effector cell for subsequent interaction with antigen. All other FcRs appear to react only with Ig-coated (opsonized) cells or with antigen–antibody (Ag–Ab) complexes. Recognition is of the native Fc structure, not of new Fc region epitopes that might result from formation of Ag–Ab complexes. Rather, the increased avidity of Ag–Ab complexes for FcR interaction results from the multivalent interactions of numerous cell-associated Fc receptors with an array of Ig Fc regions associated with the antigen.

The initiation of most biological events upon ligand binding to FcR requires receptor cross-linking. Even for high-affinity receptors, binding of monomeric Ig is insufficient to transmit an activation signal, although recycling of high-affinity receptors to which monomeric ligand has bound probably does occur. The nature of the biological response initiated by cross-linking FcRs depends on the class and subclass of FcR and the cell type on which the FcR is displayed. This response ranges from very rapid degranulation upon cross-linking of FcεRI on mast cells, to inflammatory mediator release and/or killing of the Ig-coated pathogens by antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis following engagement of FcγR, FcαR, or FcεR on many types of effector cells, to regulation of B cell activation by both cell surface and soluble forms of FcR.

Comparable receptors exist in many animal systems, but are generally less well characterized than the human FcR. The following discussion therefore focuses on the biochemical and biological properties of human FcR.

Fc receptors for IgG (FcγR)

The FcγRs, by triggering phagocytosis, ADCC, and release of mediators, represent the primary effector molecules involved in the removal of IgG-coated pathogens by monocytes, macrophages, neutrophils

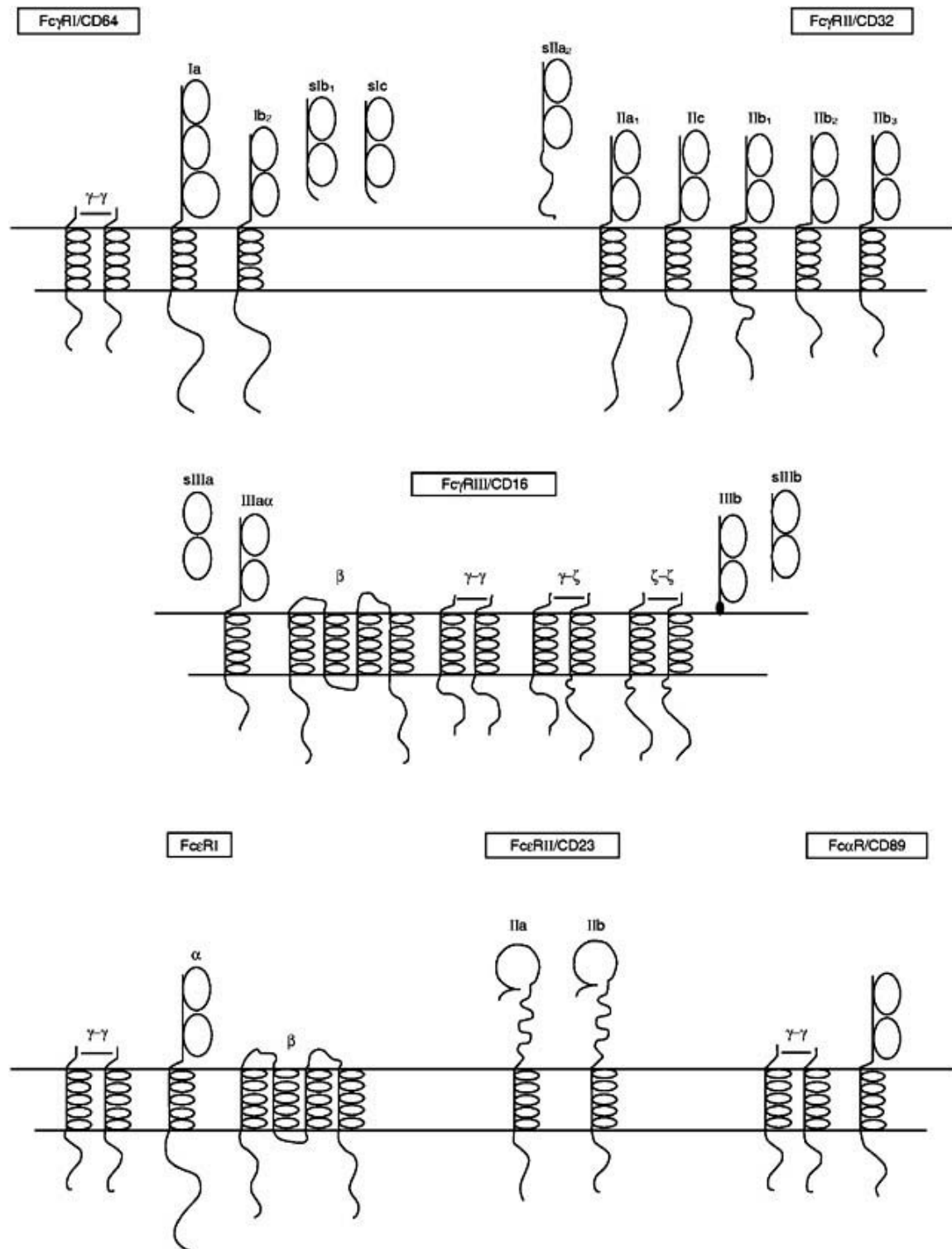


Figure 1 Fc receptor structures. Each of the Fcγ receptors, the FcαR and the α chain of FcεRI are members of the immunoglobulin gene superfamily. FcεRII is a member of the C-type animal lectin family.

and eosinophils. On large granular lymphocytes (LGL)/natural killer (NK) cells, these receptors mediate efficient non-major histocompatibility complex(MHC)-restricted cytotoxicity of virus infected and tumor cells. Platelets respond to FcγR cross-linking by mediator release and expression of adhesion-related activation antigens, resulting in platelet aggregation. B cell FcγRs are involved in the regulation of cell activation and immunoglobulin production. In addition to these beneficial effects, the FcγRs are also involved in autoimmune diseases. IgG autoantibody-coated red cells, platelets, etc., are removed through interactions with FcγRs on tissue macrophages.

Distribution and properties of FcγR (Table 1)

FcγRIa, the only isoform of CD64 that has been characterized at the protein level, is a 72 kDa glycoprotein which is recognized by monoclonal antibodies (mAbs) 32, 22, 197 and 10.1. Although FcγRIb2 mRNA is coexpressed with the FcγRIa message, corresponding proteins have not been identified for this or the FcγRIb1 and FcγRIc transcripts. FcγRI is the receptor with the highest affinity for monomeric IgG, having an equilibrium dissociation constant (K_d) of 10^{-8} to 10^{-9} M for human IgG1 and IgG3, and mouse IgG2a and IgG3. FcγRI is therefore occupied by IgG under physiologic conditions. It is expressed constitutively by only monocytes, macrophages and dendritic cells, but cytokines can induce its expression on granulocytes. FcγRIa requires associated γ chains for stable expression and activation of phagocytic signaling, but can appar-

ently mediate Ig uptake in the absence of associated molecules.

FcγRII (CD32) is a family of related 40 kDa glycoproteins identified by several mAbs, including IV.3, KB-61 and AT-10. Different FcγRII isoforms are found on different hematopoietic cells, as well as on endothelium. All forms of FcγRII have low affinity for monovalent human IgG, and appear to bind only immune complexes and opsonized particles. The cytoplasmic domains of members of the CD32 family contain typical ITAMs (immunoreceptor tyrosine-based activation motifs) or ITIMs (immunoreceptor tyrosine-based inhibitor motifs) which enhance or inhibit effector functions, respectively.

FcγRIII (CD16) was originally identified as a 50–70 kDa glycoprotein which is recognized by various mAbs including 3G8, Gran1 and B73.1. Two isoforms of FcγRIII exist. On neutrophils, the isoform designated FcγRIIIb has low affinity for IgG, lacks a cytoplasmic domain, and is bound to the membrane through a phosphatidylinositol glycan (PIG) linkage. On macrophages and LGL/NK cells, the isoform designated FcγRIIIa is a transmembrane molecule containing a typical cytoplasmic domain. The latter has an intermediate affinity for IgG ($K_d \sim 3 \times 10^{-7}$ M) and is expressed in association with signaling molecules including ζ and γ chains.

Genes encoding the FcγR

Eight different genes coding for human FcγR have been defined, and are transcribed into at least 12 different mRNAs. Along with the FcεRIα gene, all are localized to the long arm of chromosome 1, band

Table 1 Properties of the Fc receptors

Receptor	Mol. wt (kDa)	Affinity	CD	Distribution	Associated molecules
FcγRI	72	High	64	Monocytes, macrophages, dendritic cells, neutrophils ^a	γ chain
FcγRIIa	40	Low	32	Monocytes, macrophages, granulocytes, platelets, endothelial cells	γ chain
FcγRIIb	40	Low	32	B Cells, monocytes, macrophages	None described
FcγRIIc	40	Low	32	Monocytes, macrophages, granulocytes, platelets	None described
FcγRIIIa	50–70 ^b	Medium	16	Macrophages, LGL/NK	γ chain ζ chain
FcγRIIIb	50–70 ^b	Low	16	Neutrophils	None described
FcαR	55–100 ^b	Medium	89	Monocytes, macrophages, neutrophils, eosinophils	γ chain
FcεRI	α,45; β,33; γ,8	High	ND	Mast cells, basophils, dendritic cells, eosinophils	β chain γ chain
FcεRII	45	Low	23	B Cells, T cells, eosinophils, platelets, monocytes, macrophages	None described

ND, No CD assigned.

^aNeutrophil CD64 is very low; increased by IFNγ and G-CSF.

^bMolecular weight varies due to differential glycosylation.

q23–24. Expressed proteins have been identified for six different Fc γ R isoforms (Fc γ RIa, IIa, IIb1, IIb2, IIIa and IIIb); it is unknown in which cells, if any, the remaining predicted mRNAs are expressed. Sequence homology indicates that all are members of the Ig gene superfamily. The Fc γ RII and III molecules have two extracellular Ig-like domains, while Fc γ RI has a third extracellular domain which imparts its higher affinity for IgG. Allelic variants of the Fc γ RIIIa and Fc γ RIIIb genes have been described which result in polymorphisms in expression and function of these receptors, and have been associated with altered susceptibility to infectious and immune complex diseases.

Modulation of Fc γ R expression

Although strict measurement of the number of receptors per cell has not been made for many cell types, estimates based on quantitative flow cytometry have been obtained. Fc γ RI is almost exclusively restricted to monocytes (15 000–40 000 per cell), macrophages (>50 000 per cell) and dendritic cells (3000–20 000 per cell). Less than 1000 Fc γ RI sites are found on freshly isolated granulocytes of healthy subjects, while there are 5000–20 000 receptors per cell when neutrophils are cultured with interferon γ (IFN γ) or are obtained from people who have bacterial infections. IFN γ also causes a 5- to 10-fold increase in the number of Fc γ RI on monocytes, monocyte-derived macrophages, alveolar macrophages, peritoneal macrophages, dendritic cells and human myeloid cell lines. Granulocyte colony-stimulating factor (G-CSF) and interleukin 10 (IL-10) also induce increased Fc γ RI expression, but to a lesser extent than IFN γ . Certain other cytokines, such as IL-4 and IL-13, decrease Fc γ RI expression.

Different Fc γ RII isoforms are expressed on the surface of virtually all hematopoietic cells except erythrocytes. Fc γ RIIIa is found in similar numbers (20 000–40 000 sites per cell) on monocytes, macrophages and neutrophils. Its expression on myeloid cells is increased by granulocyte-macrophage colony-stimulating factor (GM-CSF) and inhibited by IL-4 and IL-13. Fc γ RIIIa is probably the sole Fc γ R on human platelets, whereas Fc γ RIIIb isoforms predominate on B lymphocytes.

Fc γ RIIIb is expressed at high levels (100 000–200 000 sites per cell) only on human neutrophils, and is shed or internalized in response to neutrophil activation or exposure to tumor necrosis factor α (TNF α). The Fc γ RIIIa isoform is expressed by only a small subset of human monocytes, whereas macrophages and LGL/NK cells express high levels of this receptor. Culture of monocytes, particularly in the presence of exogenous transforming growth factor β

(TGF β), increases expression of Fc γ RIIIa. Although its expression does not appear to be modulated by IL-2 and IFN γ , these cytokines enhance macrophage cytotoxic activity triggered via Fc γ RIIIa.

The Fc receptor γ chain is essential for phagocytosis and ADCC triggered through Fc γ RI, and can also participate in signal transduction following engagement of Fc γ RII and Fc γ RIIIa. IFN γ induces higher expression of γ chain, and this may account for some of the enhanced functions of leukocytes following activation by IFN γ .

Functional properties of Fc γ R

Mononuclear phagocytes Fc γ RI, Fc γ RII and Fc γ RIII all act as trigger molecules for phagocytosis and ADCC, the latter requiring engagement of adhesion molecules (e.g. LFA-1) for maximal efficiency. IFN γ enhances monocyte-mediated killing through Fc γ RI, especially when limited antibody is available on the target. In contrast, IFN γ does not enhance monocyte expression of Fc γ RII or Fc γ RIII. Although little Fc γ RIII is expressed on freshly isolated monocytes, this receptor is induced when monocytes mature into macrophages. Monocytes cultured for even short periods mediate lysis of tumor cells through Fc γ RIII. In addition, antigen-specific T cell activation is markedly enhanced when the antigen is targeted to either Fc γ RI or Fc γ RII on monocytes or dendritic cells.

Neutrophils Freshly isolated neutrophils do not lyse nucleated targets through Fc γ R. However, when cultured with IFN γ , they mediate killing through Fc γ RI and Fc γ RII. Killing through Fc γ RI correlates with the induction of this receptor on the neutrophil by IFN γ , whereas, IFN γ renders neutrophils capable of cytotoxicity via Fc γ RII by inducing changes which potentiate cytotoxic mechanisms, without increasing Fc γ RII expression. Neutrophil-mediated cytolysis of red blood cells occurs through Fc γ RII and Fc γ RIII, and incubation with IFN γ enhances cytotoxicity through Fc γ RII but not Fc γ RIII. GM-CSF also significantly enhances killing by neutrophils through Fc γ RII, again without affecting expression of Fc γ RII. Fc γ RIII on neutrophils does not mediate cytotoxicity of tumor targets. These observations suggest that erythroid and tumor targets are killed through different pathways, and that triggering of Fc γ RI or Fc γ RII on neutrophils activates lytic mechanisms distinct from those initiated via Fc γ RIII.

Eosinophils Although freshly isolated eosinophils do not kill tumor targets, after activation with GM-CSF they mediate killing through Fc γ RII but not Fc γ RIII, even though both Fc γ Rs are expressed on

these cells. Thus, like neutrophils, eosinophils require lymphokine activation to perform ADCC of tumors through Fc γ RII. Fc γ Rs have also been shown to mediate ADCC of parasites by eosinophils after activation by GM-CSF, TNF or IL-5.

Large granular lymphocytes and NK cells LGL/NK cells very efficiently mediate ADCC of tumor cells through Fc γ RIIIa. This contrasts with the inability of Fc γ RIIIb on neutrophils to mediate tumor cell killing, but is consistent with the cytolytic ability of macrophage Fc γ RIIIa. This difference correlates with the presence of transmembrane and cytoplasmic domains and/or association with γ or ζ chains, which occurs for Fc γ RIIIa only, and appears to be required for signaling pathways resulting in lysis of tumor cells.

U937, HL-60 and THP-1 cells Myeloid cells representing various steps in monocyte differentiation have facilitated evaluation of the functional maturation of the different Fc γ Rs. Although these cell lines express Fc γ RI and Fc γ RII, and are induced to express much larger numbers of Fc γ RI by IFN γ , they do not lyse tumor targets through Fc γ Rs, even after treatment with cytokines. They are, however, able to mediate ADCC of red cells through Fc γ RI and Fc γ RII after IFN γ or G-CSF treatment.

Fc γ Rs in infectious disease

In addition to their role as cytotoxic trigger molecules which mediate killing of opsonized tumors and pathogens, Fc γ R have been shown, under certain conditions, to mediate enhanced infection by certain infectious agents. For example, Fc γ Rs play a major role in dengue virus infection of human mononuclear phagocytes and the pathogenesis of the disease caused by this virus, as the rate of infection is markedly enhanced when these cells encounter antibody-coated virus. Human immunodeficiency virus type 1 (HIV-1) infection of monocytes and macrophages is also enhanced by opsonization of the virus with very small amounts of antibody. It would appear however, based on studies using bispecific antibodies, that Fc γ Rs are not infectivity receptors for HIV, but rather increase cell contact with the virus, enhancing its ability to interact with CD4 on the cell and in this way contribute to enhanced infectivity. Such findings must obviously be taken into account in strategies for the development of vaccines for these and other intracellular pathogens.

Fc receptors for IgE (Fc ϵ R)

IgE-binding proteins subserve a variety of functions. The type I Fc ϵ R, a multimeric complex once thought to be exclusively expressed by mast cells and basophils, has more recently been identified on eosinophils, monocytes, Langerhans cells and blood dendritic cells. Thus, in addition to playing a central role in the release of inflammatory mediators in atopic disease, it may also facilitate antigen uptake by antigen-presenting cells. The low-affinity type II Fc ϵ R (CD23), which bears no homology to Fc ϵ RI, is found on B cells, eosinophils, monocytes, Langerhans cells, platelets and probably T cells. Fc ϵ RII is a B cell differentiation antigen and also a trigger molecule for IgE-mediated lysis of parasites by myeloid cells. Soluble Fc ϵ RII is found in the blood, and appears to influence B cell differentiation and monocyte migration.

Distribution and properties of Fc ϵ R

Fc ϵ RI is highly expressed (>100 000 sites per cell) on mast cells and basophils, where it consists of a heterotetrameric complex containing one α , one β and two γ polypeptide chains (Figure 1). It appears at lower levels on a variety of other cells (Table 1), where its association with accessory molecules may be different. It binds the C ϵ 2 and C ϵ 3 domains of the Fc region of IgE with high affinity ($K_d = 10^{-10}$ M) via the glycosylated α chain of the receptor. The γ chains are essential for efficient and stable expression of the receptor on the cell surface, and are also required for signal transduction.

Fc ϵ RII is a single-chain glycoprotein (Figure 1) which exists in two forms (Fc ϵ RIIa and Fc ϵ RIIb) that differ only in their cytoplasmic tails. Fc ϵ RIIa appears to be restricted to B lymphocytes, while Fc ϵ RIIb has been found on B cells, T cells, eosinophils, platelets, monocytes, macrophages, Langerhans cells and human myeloid cell lines such as U-937. The C ϵ 3 domain of IgE is involved in binding to this receptor. A soluble form of Fc ϵ RII, termed IgE-binding factor (IgE-BF) or soluble Fc ϵ RII/CD23 (sCD23), appears to be released from CD23⁺ cells by an autocatalytic mechanism, although alternative transcripts encoding for a soluble CD23 have been defined.

Genes encoding Fc ϵ R

The cDNA for the α subunit of Fc ϵ RI encodes a polypeptide with structural similarity to the Fc γ receptors. There are two immunoglobulin-related extracellular domains, a transmembrane domain containing eight residues that are identical to those of Fc γ RIIIa, and a relatively unique cytoplasmic domain consisting of 31 amino acids. The cDNA for

the β subunit predicts four transmembrane domains and no homology to the immunoglobulin gene superfamily. The cDNA for the γ subunit indicates homology with the ζ chain which is expressed both in association with Fc γ RIIIa on large granular lymphocytes and with the T cell receptor (TCR) complex on T cells. The γ chain consists of five extracellular amino acids, a single transmembrane domain and a 42 residue cytoplasmic tail.

The two forms of Fc ϵ RII (Fc ϵ RIIa and I Ib) arise from alternative splicing of a single gene. Both molecules consist of 321 amino acids with the C-terminus on the outside of the plasma membrane, no leader sequence, and a single transmembrane domain. The I Ia and I Ib forms differ only at seven residues in the N-terminus, and share apparently identical extracellular C-terminal domains.

Modulation of Fc ϵ R

Fc ϵ RI is uniformly distributed throughout the plasma membrane, but is rapidly aggregated and internalized upon cross-linking of receptor-bound IgE. The expression of Fc ϵ RII is increased by elevated IgE levels, apparently by protection from degradation to the soluble form. IL-4 and IFN γ upregulate CD23 expression by increasing Fc ϵ RII synthesis, while IFN α suppresses Fc ϵ RII expression.

Functional properties of Fc ϵ R

Fc ϵ RI is expressed on antigen-presenting cells, such as monocytes and Langerhans cells, are able to enhance antigen presentation to T cells. The cross-linking of Fc ϵ RI on mast cells and basophils, in contrast, triggers phosphoinositide hydrolysis, mobilization of cytoplasmic calcium, influx of extracellular calcium, explosive release of granular components such as histamine, and synthesis of potent inflammatory mediators, such as leukotrienes and platelet-activating factor. A large number of cytokines are also released in response to Fc ϵ RI triggering, including IL-1, IL-3, IL-4, IL-5, IL-6, TNF α , IFN γ , GM-CSF, macrophage inflammatory protein 1 α (MIP1 α) and MIP1 β . These may contribute markedly to the inflammatory reaction associated with mast cell degranulation.

Fc ϵ RII on macrophages, eosinophils and platelets can mediate cytotoxicity and/or phagocytosis of IgE-coated particles. In addition, aggregated IgE triggers release of reactive oxygen metabolites and other inflammatory mediators from Fc ϵ RII-bearing cells. Thus, on some cell populations this receptor may be involved in inflammation and/or removal of IgE-coated agents. On other cells (e.g. B cells, Langerhans cells), Fc ϵ RII may be involved in modulation of the isotype of humoral immune responses and

enhancement of antigen presentation. Moreover, soluble Fc ϵ RII appears to play a role in B cell growth and differentiation, and in association with IL-4, enhances IgE synthesis.

Fc receptors for IgA (Fc α R)

IgA fulfills a critical protective role against the constant environmental insults encountered at mucosal surfaces. Although the mechanism of protection is not well understood, the IgA-mediated mucosal immune response may be at least partially manifest through binding of IgA-coated targets to Fc receptors for IgA on effector cells. Monocytes, macrophages, neutrophils, eosinophils and myeloid cell lines express cell surface IgA Fc receptors (Fc α R; CD89) consisting of a 50–70 kDa molecule which, like Fc γ RI, Fc γ RIIIa and Fc ϵ RI, associates with the signal-transducing γ chain. The IgA-binding chain contains a 206 amino acid extracellular domain including six potential sites for N-linked glycosylation, a feature that, along with O-glycosylation, accounts for the significantly higher molecular weight observed for native Fc α R. The extracellular region is followed by a 19 residue transmembrane region and an intracytoplasmic region of 41 amino acids. Sequence analysis indicates that Fc α R is a member of the immunoglobulin superfamily and is homologous to other known Fc receptors (Figure 1). Binding of monomeric IgA probably occurs under physiological conditions, but binding *per se* does not trigger function. Rather, cell signaling for phagocytosis, ADCC, degranulation, and/or inflammatory mediator release requires receptor cross-linking.

Various cytokines play a role in regulating Fc α R expression and function by monocytes, macrophages and neutrophils. LPS, in addition to TNF α and IL-1, which are produced in response to LPS, strongly upregulate monocyte Fc α R expression, as does GM-CSF. Increases in Fc α R-specific mRNA are also detectable following monocyte stimulation with either TNF α , IL-1, GM-CSF or LPS. IFN γ , in contrast, downmodulates both surface expression of Fc α R and intracellular Fc α R-specific mRNA levels. These results indicate that Fc α R on monocytes are modulated by endotoxin and an array of cytokines distinct from those that regulate expression of FcRs for IgG.

Summary

The distinct human FcR classes perform discrete tasks, with their function largely dictated by the receptor isoform and the cell type on which they are displayed. Receptor signaling is mediated both

through phosphorylation domains in the cytoplasmic tail of the ligand-binding α chain (e.g. Fc γ RII), and via associated molecules such as γ - or ζ -chains (e.g. Fc γ RI, Fc γ RII, Fc γ RIIIa, Fc ϵ RI and Fc α R). FcRs have in common the requirement for receptor cross-linking to trigger most cell functions, with the probable exception of endocytosis. Cytokines modulate both FcR expression and function, but sometimes in opposite directions. In some cases, FcR function is enhanced without any overt change in receptor expression (e.g. in response to G-CSF or GM-CSF). It seems likely that the specific tissue microenvironment which dictates the overall molecular interactions of the FcR-bearing cell plays a crucial role in the ultimate level of function that is triggered by engagement of FcR.

See also: Antibody-dependent cellular cytotoxicity; Basophils; Mast cells; Cytokines; Degranulation; Eosinophils; IgA; IgE; IgG; Immune complexes; Mononuclear phagocyte system; Natural killer (NK) cells; Neutrophils; Phagocytosis.

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FELINE IMMUNE SYSTEM

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The immune system of the cat possesses no unique structural elements and it is safe to assume that in its basic features it conforms to the pattern seen in other placental mammals. Nevertheless, the feline immune system does show some interesting and unique differences, described here.

Lymphoid tissues

Cats, like ruminants and pigs, possess a population of pulmonary intravascular macrophages. These cells are considerably more important than the Kupffer cells in removing particulates from the bloodstream. Thus 86% of injected streptococci were removed in the lungs as opposed to 14% in the livers of cats. The presence of this population of cells may explain

why cats are more susceptible than other mammals to septic shock mediated by macrophage-derived tumor necrosis factor (TNF). The lymph nodes, spleen, tonsils, Peyer's patches, bone marrow and thymus are of comparable structure to that seen in other mammals. The thymus appears in fetal kittens at 28 days after conception.

Feline lymphocytes

About 40–45% of feline peripheral blood lymphocytes are identifiable as B cells. The ontogeny of B cell development is similar to that seen in other animals. Pre-B cells have been identified at 42 days after conception in the fetal kitten liver. Subsequently, they

are found in the spleen and bone marrow. Adult levels of B cells are reached in the kitten by 12 weeks of age.

About 32–41% of peripheral lymphocytes are identifiable as T cells. Feline T cells also form E rosettes with red blood cells from guinea pig, mouse and rat. Guinea pig erythrocyte receptors appear to be the most satisfactory T cell markers and are found on both differentiated and undifferentiated T cells. Both T helper and T suppressor cell activities have been identified in the cat. About 20% of feline peripheral blood lymphocytes lack both T and B cell markers. These null cells, found both in immune and nonimmune animals, can destroy tumor cells or virus-infected cells. They are thus assumed to be natural killer (NK) cells. NK cell activity against herpes simplex-infected target cells has been demonstrated in cats.

The mitogenic responses to phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) are primarily a property of T cells, although feline lymphocytes respond relatively poorly to PHA and lipopolysaccharide in comparison to other mammals. Feline analogs of CD4 and CD8 have been identified and several monoclonal antibodies to undefined lymphocyte cell membrane antigens have been described. fCD molecules that have been identified include CD5, CD9, CD10, CD18 (LFA- β chain) and CD45R. Such evidence as is available suggests that these molecules are very similar to their equivalents in mice and humans.

Feline cytokines

The molecular weight of feline interleukin 1 (IL-1) ranges from 15 to 20 kDa. As in other species, it occurs in three different isoforms. IL-1 is released in large quantities from peritoneal macrophages in cats infected with the coronavirus of feline infectious peritonitis and may play a part in the development of the lesions in that disease. It is also released by lipopolysaccharide-treated alveolar macrophages.

Feline IL-2, released by the action of Con A on lymphocytes, has a molecular weight of 16 kDa. It supports the proliferation of Con-A-activated feline peripheral blood lymphocytes. IL-2 production is significantly decreased in cells from cats infected with feline leukemia virus (FeLV). IL-2-containing cell supernatants can stimulate the development of cytotoxic activity (LAK) in peripheral blood lymphocytes from normal cats. The target in this case was the feline lymphoblast cell line FL74, which continuously produces FeLV.

Feline IL-6, derived from Con-A-stimulated splenocytes, has a molecular weight of 30–40 kDa. Its physicochemical properties are somewhat different from the human and mouse analogs.

Interferons α , β and γ have been characterized and resemble those in other species. Interferon produced *in vitro* by Newcastle disease virus-stimulated Crandall feline kidney cells will make the cells resistant to invasion by FeLV and vesicular stomatitis virus (VSV).

Feline immunoglobulins

Cats probably possess all the immunoglobulin (Ig) isotypes seen in other species, although neither IgE nor IgD have been formally identified. Adult cat sera, colostrum whey, tears and nasal secretions contain IgG, IgM and IgA. Electrophoretic analysis suggests that there are at least three IgG isotypes – G1, G2 and G3 – and preliminary evidence suggests the existence of a fourth. A reagenic antibody obtained from a cat infected with the microfilariae of *Brugia pahangi* has been characterized and had properties compatible with IgE. Given the recent evidence that some mammals, pigs for example, have no δ chain genes and cannot make any IgD, it is not unlikely that cats may also lack this isotype. Lambda (λ) is the predominant light chain type in the cat, accounting for 80–90% of light chains. In common with other mammals with placentas impermeable to immunoglobulins, female cats are obliged to transfer antibodies to their offspring through colostrum. As a result, IgG, IgM and IgA are present in colostrum whey in high concentrations (Table 1). As lactation proceeds and immunoglobulin levels drop, IgG remains the major immunoglobulin class in cat milk. IgM and IgA are the predominant immunoglobulins in cat bile. IgA is the predominant immunoglobulin in intestinal and respiratory mucus and in tears. As in other species, cat secretory IgA is a J chain-linked dimer. Immunoglobulins cannot be detected in the urine of adult cats but may be found in the urine of suckling kittens. This may reflect the excretion of small immunoglobulin fragments absorbed by the intestine from colostrum.

While IgE has been functionally identified in the cat by methods such as passive cutaneous anaphylaxis, it has not been physicochemically characterized. Feline IgE is weakly reactive with human and bovine anti-IgE, as measured by the ability of feline atopic serum to block an ELISA test for bovine IgE.

Table 1 Serum immunoglobulins in the cat (mg ml⁻¹)

Source	IgG	IgM	IgA
Serum	7.2–19.7	0.8–1.9	0.7–2.8
Colostrum	44.0	0.6	3.4
Milk	1.5	0	0.2

Cell-mediated immunity

Cats infected with tuberculosis respond poorly to intradermally injected tuberculin and, as a result, the tuberculin test is unsatisfactory in this species. They do not respond with delayed hypersensitivity reactions to proteins such as bovine serum albumin. Nevertheless, cats can develop a good delayed skin response to dinitrochlorobenzene, to viral antigens and to BCG vaccine, although these delayed hypersensitivity reactions are not as intense or as consistent as in other species, such as the guinea pig. Despite this lack of skin reactivity, the granulomatous reactions to tuberculosis in the cat are indistinguishable from those seen in other mammals.

Feline major histocompatibility complex (MHC) molecules

The feline histocompatibility (FLA) system has been unusually difficult to study because the conventional approach of generating lymphocytotoxic antibodies by transfusion, multiple births or skin grafting does not work well. (Only about 25% of cats rejecting allografts produce lymphocytotoxic antibodies.) It may also be difficult to produce substantial mixed lymphocyte culture reactivity between some unrelated feline cells. Although it is possible that this may be due to a lack of FLA polymorphism, it is more likely to be due to a relatively low level of MHC expression on cat mononuclear cells. Thus genetic analysis indicates that the FLA system contains 10–20 class I gene loci, of which only two are expressed. Cats also possess five class II gene loci, of which only three are expressed. Feline class I antigens display 81–82% sequence identity with the human and 73–79% identity with the mouse homologs. Analysis of the histocompatibility antigens of other felids suggests that their MHC has a similar structure. Cat class I and class II MHC genes are located on chromosome B2.

The lack of MHC polymorphism in the cat may help to account for the ease of bone marrow transplantation in this species. The success rate of bone marrow allografts in cats previously treated with X-radiation or cyclosporine is high and graft-versus-host disease is not a major problem.

Feline complement

Cats possess all the major complement components at levels comparable to those in other species of mammal. Cat complement is, however, not as hemolytic as rabbit, dog or guinea pig complement when using rabbit antibodies and sheep erythrocytes as antigens.

Feline hypersensitivity

The lungs are the primary target organ for anaphylactic shock in the cat, and serotonin release from mast cells is probably the most important mediator of anaphylaxis. Flea-bite dermatitis is the most common allergic reaction in cat skin, whereas allergic rhinitis is rarely encountered. Food hypersensitivity has been described in the cat.

Feline blood groups and hemolytic disease

There are only two feline blood group antigens, called A and B. The prevalence of each of these two antigens varies according to geographical region. Thus, in the USA, 99% of cats are group A and only 1% are group B. In contrast, in Australia, 73% of cats have group A erythrocytes, while the rest are group B. Blood group B tends to be found in more exotic breeds of cats rather than in the common domestic shorthair. Very rare AB (0.4%) cats have been described. About 20% of group A cats possess natural anti-B with titers ranging from 16 to 512. Group B cats invariably contain anti-A at high titer. Agglutination and hemolysis can be used for feline blood typing.

Neonatal isoerythrolysis has been recognized in the cat. It may be induced artificially by immunization of queens with vaccines containing feline blood components. It has also been reported to occur spontaneously when sires of blood group A are bred repeatedly to females of blood type B. The antibodies are transferred from the mothers to the kittens through their colostrum. These then cause massive erythrocyte destruction and profound anemia.

Autoimmunity in the cat

Several spontaneous autoimmune diseases have been described in the cat, including hyperthyroidism, hemolytic anemia, thrombocytopenic purpura, pemphigus vulgaris, pemphigus foliaceus, systemic lupus erythematosus, myasthenia gravis and arthritis.

Feline hyperthyroidism is recognized as an important clinical entity in older cats. The presence of autoantibodies against thyroid microsomes has been demonstrated by immunofluorescence in about one-third of these cases, while lymphoid infiltration is found in another third. Autoimmune hemolytic anemia, while usually occurring spontaneously, may also develop secondary to FeLV infection. An autoimmune thrombocytopenia has also been reported to be associated with FeLV infection.

Immunodeficiencies in the cat

Primary immunodeficiencies are rarely recorded in the cat. Thymic hypoplasia and lymphopenia have been reported in a Siberian tiger, while 'nude' cats – thymic aplasia and hypotrichosis – have been recorded in a litter of kittens. These kittens had no thymus, and showed lymphocyte depletion in T cell areas of the lymph nodes, spleen and Peyer's patches. Their most common congenital defect in immune function is the Chédiak–Higashi syndrome. This is classically seen in some Persian cats. Affected animals show defects in pigmentation with a characteristic pale coat color and red tapetal light reflection. Their leukocytes contain enlarged lysosomal granules but increased susceptibility to infections has not been observed. Of considerably greater importance as a cause of feline disease is failure of passive transfer of colostral immunoglobulins. This results in the development of severe and intractable bacterial infections in newborn kittens. The most important cause of secondary immunodeficiency in the cat is FeLV infection. Despite its name, this virus usually causes severe immunodeficiency in infected animals. Kittens born to FeLV-infected queens may show thymic atrophy and depressed allograft responses, while infected adult cats may develop a panleukopenia. As a result, they die due to fulminating bacterial infections. Many cats with chronic bacterial or fungal infections such as pneumonias, abscesses and stomatitis are found to be FeLV infected. Feline panleukopenia, due to a parvovirus, also causes an acquired immunodeficiency syndrome with severe leukopenia and lethal secondary infections.

Feline immunodeficiency virus

Feline immunodeficiency virus (FIV) is a widespread lentivirus that infects domestic and wild cats. Infection with this virus results in progressive depletion of CD4⁺ T lymphocytes. The resulting disease in many respects resembles human acquired immune deficiency syndrome (AIDS) and is an important model of the human disease. FIV replicates preferentially in feline T lymphoblastoid cells and causes a characteristic cytopathic effect *in vitro*. The virus receptor is probably not mediated through fCD4 (which is expressed on only a subset of T cells) but through fCD9. Infection is also associated with a marked, polyclonal expansion of B cells and of a subset of fCD8⁺ cells. As a result the CD4:CD8 ratio can be reduced to as low as 1.6, compared with a normal ratio of 3.3. Kittens artificially infected with

FIV initially exhibit few clinical signs, most notably anemia, lymphopenia, neutropenia and lymphadenopathy. Eventually a terminal AIDS-like illness develops. Natural FIV infections result in death as a result of secondary infections of the oral cavity, upper respiratory tract, gastrointestinal tract, skin or urinary system. Neurologic signs have also been described. Epidemiologic analysis suggests that FIV is mainly transmitted between cats by biting.

Gammopathies

Plasmacytomas, although uncommon, are well recognized in the cat. The great majority of these tumors secrete immunoglobulins of the IgG isotype, but IgA- and IgM-secreting myelomas have also been described. In some cases these plasmacytomas may be associated with the deposition of immunoglobulin-associated amyloidosis.

See also: CD antigens; Cytokines; Leukemia viruses.

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FETAL CALF SERUM

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Historical background

The beginning of cell culture techniques during the first decade of this century was marked by the finding that biologically derived fluids are required to maintain cells *in vitro*. Various supplements were used successfully, varying widely from tissue extracts, spinal and amniotic fluids, lymph and milk to plasma and serum. Later, substantial improvement was brought about by basal nutritive solutions (media) containing defined concentrations of salts, amino acids, sugars and vitamins, which, however, required supplementation by biologicals. The use of supplemented basal media is still a mainstay in cell culture techniques.

Rationale for use of fetal bovine serum

Serum is most commonly used as a cell culture supplement because it is readily available and relatively stable in composition. As most sera of adult animals contain naturally occurring heterophilic antibodies, sera free of relevant antibodies are desirable. In this context, the serum of unborn calves (FCS, fetal calf serum; FBS, fetal bovine serum) is advantageous because it is naturally devoid of maternal antibodies if collected properly. In cattle, there is no transplacental transfer of antibodies, which are transferred enterally in large amounts via the colostrum (low levels of antibodies of fetal origin, like antiviral antibodies, are often present in fetal calf serum). If free of antibodies, newborn calf serum is like fetal calf serum. In many culture systems, e.g. cultured human cells, homologous serum compatible for relevant blood group antibodies can be used with equal or better success than fetal calf serum.

Production process

Growing demand for fetal calf serum has fostered commercial preparation and supply. Today, use of commercial products is the rule. Processed fetal calf serum is also available (removal of lipids, γ -globulins, steroid hormones or low molecular weight components by dialysis, etc.).

Preparation

There is no firm, standardized procedure for preparation.

Suppliers usually buy the raw material from brokers; it stems mainly from large cattle ranches in the Americas, Australia, southern Africa or Europe. In the slaughterhouse, the blood of fetal calves is generally gathered by heart puncture. If collection is not performed under sterile, nonpyrogenic conditions, the contamination with bacterial pyrogens is frequent. Some manufacturers use closed, aseptic conditions and offer special products. Small pools of 4.5 l serum (from approximately ten calves) are shipped frozen to the manufacturers, who perform some quality control tests, homogenize sera into large pools of up to 2000 l and filtrate them before bottling. Depending on the pore size used (generally 0.1 μ m, sometimes 0.04 μ m), even mycoplasmas may be removed by this procedure.

Quality assurance

Codes for quality controls, including checks for viral contamination, exist only in some countries. As a result the quality may vary between different countries. The supplier will usually run a series of tests for quality control (Table 1). Many manufacturers currently use the Limulus assay for detection of pyrogens. This is, however, not necessarily sufficient to detect all biologically relevant endotoxins. Assays using monokine production (interleukin 1, tumor necrosis factor and interleukin 6) as indicator have been proposed for screening for functionally active endotoxins and proved to be superior to *in vivo* tests like the rabbit pyrogenicity test.

Generally, the users have to evaluate different batches of fetal calf serum to find the most suitable one for their special purpose. Preservation of cell culture characteristics, cell growth curve (lag period, doubling time, saturation density), plating efficiency and cloning efficiency are some frequently used criteria. Even within one batch of fetal calf serum,

Table 1 Quality controls for fetal calf serum

Content of
Proteins, glucose, fructose, minerals
Lack of contamination by
<i>Mycoplasma</i> , bacteria, endotoxins
Bovine viruses (e.g. bovine viral diarrhea virus, BVDV)
Antibodies, hemoglobin
Growth support of cell lines

differences of characteristics can occur if pooled sera were not homogenized completely before bottling.

Applications

Use in cell culture in cellular biology research and in the pharmaceutical industry is the major application for fetal calf serum. Culture supplement is usually 1–20% v/v fetal calf serum in the basal culture medium. Most users treat fetal calf serum for 30 min at 56°C in order to inactivate heat-labile complement components. Supplementation by fetal calf serum can provide cells with factors in physiologically balanced form (Table 2) which are necessary for cell survival, growth, differentiation and other cellular functions. On the other hand, potential cytotoxicity of serum should be considered because it is not the physiological fluid of the environment of most cells *in vivo*. The content of functionally active endotoxins (see above) can also have a critical influence, as this agent can modulate the growth and function of many cells either directly or by inducing cytokines. Contaminating bacterial substances can be antigenic and mitogenic.

Biohazards

Large pools of fetal calf serum may be contaminated with bovine pathogens, when one or a few calves car-

ried infectious agents. This may cause a biohazard if further processing cannot remove the pathogens, such as bovine viruses or possibly even the causative agent of bovine spongiform encephalopathy. It has been shown that bovine viruses are present in many commercial batches of fetal calf serum and may infect a variety of cell lines from several species. Obviously, bovine pathogens can be introduced into pharmaceuticals if fetal calf serum or derivatives thereof are used during the pharmaceutical production process.

Substitutes and future prospects

Much knowledge has been gathered on the relevant ingredients of biological cell culture supplements. Presently, there is no formula at hand which could generally replace poorly defined culture supplementation by biologicals like fetal calf serum. Hence, serum-free cell culture is still limited to defined culture conditions with adjustments to each cell population or cell line. The applications of serum-free culture techniques are certainly increasing and may eventually replace the use of fetal calf serum in many areas. These techniques provide a better control of culture conditions, can be free of pathogens and may reduce batch-to-batch variations to a minimum. This development will be accelerated by increasing sophistication of available formulas, which include mixtures of hormones, growth factors, transport proteins/albumin and attachment factors (hormonally defined media). Other substitutes such as conditioned media derived from cell culture supernatants and low protein or low serum media may themselves contain fetal calf serum or derivatives thereof.

Often fetal calf serum cannot be replaced owing to the lack of an adequate, convenient and economic alternative – despite its high costs. Fetal calf serum is therefore likely to remain a ubiquitous agent in laboratory-scale biology and immunology, as well as in industrial-scale biotechnology, for many years.

See also: Colony stimulating factors; Cytokine receptors; Endotoxin (lipopolysaccharide (LPS)); Humoral immunity; Leukocyte culture; Mixed lymphocyte reaction (MLR); Natural antibodies; Organ culture of lymphoid cells.

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Table 2 Fetal calf serum factors relevant to cell culture

Biologically active substances
Cytokines, hormones (e.g. insulin), vitamins
Growth factors released during coagulation (e.g. platelet-derived growth factor, PDGF; transforming growth factor β , TGF β)
Extracellular biomatrix proteins
Attachment factors, spreading factors
Adhesion glycoproteins (fibronectin)
Transport proteins
Binding proteins (e.g. transferrin)
Lipids complexed to a carrier (albumin, lipoproteins)
Protection factors
pH buffer
Protease inhibitors
Radical scavengers (glutathione)
Viscosity of protein solution
Trace elements
Fe ²⁺ , Zn ²⁺ , Cu ²⁺ , Mn ²⁺ , Co ²⁺
Nutrients
Peptides, polyamines, lipids, sugars
Sporadic exogenous contaminants
Bacterial substances, viruses
Cellular products induced by exogenous materials
Interleukins from macrophages and lymphocytes
Proteolytic enzymes from granulocytes

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FETUS AS ALLOGRAFT

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Modern considerations of the haplo-nonidentical fetus as an allograft in outbred pregnancy have developed from initial postulates, put forward by Medawar, Brent and Billingham in 1953, that were derived from their work on transplantation tolerance. In these, they proposed that 1) the conceptus may not be immunogenic, 2) pregnancy may alter the immune response, 3) the uterus may be an immunologically privileged site, and 4) the placenta may be an effective immunological barrier between mother and fetus. They were also influenced by the earlier work in 1945 of RD Owen, who demonstrated transplantation tolerance between dizygotic twin cattle (who share a common placenta and blood supply).

In 1958, Woodruff showed that pregnant female rats could react immunologically against fetal tissue removed from one embryo and grafted to the thigh muscle, whereas the remaining intrauterine embryos were unaffected. This demonstrated that the 'transplantation privilege' normally offered to the fetoplacental unit in pregnancy depends on intrauterine mechanisms. However, several later studies in experimental animals have shown that tissue transplants (e.g. skin grafts) placed at intrauterine sites in hormonally primed or pregnant recipients are not exempt from immune rejection, although a degree of prolongation of graft survival times can occasionally occur. Thus, the pregnant uterus is not an immunologically privileged site. In addition, it is clear that pregnancies will normally survive in maternal hosts that have high levels of pre-existing alloimmunity from either natural immunization (previous pregnancy or blood transfusion) or experimental immunization with fetal or paternal cells.

The modern focus of attention on the fetus as an allograft has now been directed more toward the specialized features of fetal trophoblastic cells that appear to confer transplantation protection allowing

viviparous reproduction. Trophoblast is the first cell type to differentiate following fertilization and forms the lining cell of the placenta and extraembryonic membranes encasing the conceptus; it represents the entire fetal tissue interface with maternal blood and decidualized endometrial tissue. In humans, the outermost trophoblastic layer of the chorionic villous placenta is syncytiotrophoblast, a continuous cell layer without intercellular junctions, that has a mononuclear cytotrophoblast layer immediately below within the villous structure. It is through syncytiotrophoblast that the fetus 'eats, breathes and survives', and molecular expression by this cell reflects its wide variety of essential biological functions. There is a separate population of invasive extravillous mononuclear cytotrophoblast that migrate into the decidualized endometrium and also proliferate laterally to form the chorionic membrane; in early pregnancy, these extravillous cytotrophoblast cells display many of the cell biological characteristics of tumor cells in their proliferative and invasive properties, which largely cease after the first trimester of pregnancy. This entry will concentrate largely on human pregnancy, since there are numerous anatomic differences in placentation between species, but will also refer to observations in other species when appropriate.

Passive resistance of trophoblast to cytotoxicity

It is now clear that fetal trophoblast utilizes several mechanisms which may critically protect it from maternal cytotoxic attack. First, human placental villous cytotrophoblast and syncytiotrophoblast do not express any classical polymorphic class I or II major histocompatibility complex (MHC) antigens (e.g. HLA-A, -B, -DQ or -DR), and it appears that constitutive HLA expression may not be induced by any of the known exogenous upregulators (e.g.

interferon γ , IFN γ). Classical MHC antigens are thus not expressed throughout gestation, from the trophoectoderm of the preimplantation blastocyst to the syncytiotrophoblast of the term placenta. The genetic mechanisms of gene expression and control in syncytiotrophoblast, a true cellular syncytium, are difficult to envisage. However, there would appear to be little doubt that the lack of expression of classical class I MHC molecules at this site in humans is of central importance in protecting the fetal tissue interface from cytotoxic attack by maternal T cells or allo-antibody. There may also be other, more subtle implications, since syncytiotrophoblast can express endogenous retroviral particles; these are likely to be involved with syncytial cell formation, but could also strongly influence expression of other genes. In rodent pregnancies, in contrast, there is evidence of placental expression of some classical class I MHC antigens; the immunobiological implications of this are still far from clear.

Recent studies have shown comprehensively that the extravillous cytotrophoblast population in humans selectively expresses the nonclassical class I MHC antigen, HLA-G, which has limited genetic polymorphism; the HLA-G gene lacks an intact interferon-stimulated response element (ISRE) for upregulation. To date, HLA-G expression has not been noted in adult tissues and this class I MHC locus may have evolved for a specialized immunogenetic function in pregnancy. HLA-G protein expression in fetal tissues other than trophoblast remains controversial, notably in fetal thymus, where positive selection of HLA-G-restricted T cells could possibly occur. There has been speculation that HLA-G might serve in cytotrophoblast invasion and proliferation in endometrial tissue, or as a passive cell surface class I MHC molecule protecting this cytotrophoblast population from MHC-nonrestricted natural killer (NK) cell attack (see below) without presenting a classical class I MHC antigen that could act as a target for MHC-restricted T cell cytotoxicity. An opposing argument is that trophoblastic HLA-G expression may be a redundant evolutionary relic. This could be supported by observations that β_2 -microglobulin-deficient mice, obtained by homologous recombination in germ-line genes and which do not express constitutive class I MHC molecules, are capable of reproducing readily and with normal litter size; however, these mice lack functional CD8⁺ cytotoxic T cells and would not have the ability to distinguish self from nonself.

The final mechanism that trophoblast employs throughout gestation to protect itself from maternal cytotoxicity is to express a high level of complement regulatory proteins on its surface, notably membrane

cofactor protein (MCP: CD46), decay accelerating factor (DAF: CD55) and membrane attack complex inhibitory factor (CD59). The former is of particular interest since, for many years, a polymorphic cell surface antigen system termed trophoblast-leukocyte common (or cross-reactive) antigen (TLX antigen) attracted attention from reproductive immunologists as a potentially immunoregulatory cell surface polymorphic antigen expressed by both fetal trophoblast and maternal immunocompetent cells. It is now recognized that this TLX antigen is identical to CD46; its strong expression by all trophoblast subpopulations serves to protect this cell type from complement-mediated damage consequent to either maternal antibody attack or local tissue restructuring and hemostatic alterations that may promote persistent low-level local complement activation.

Active maternal immunoregulatory responses

A central creed in reproductive immunology is that there is active maternal immune recognition of pregnancy (i.e. of fetal trophoblast) that leads to cellular, antibody or cytokine responses which protect the fetal allograft. Taking antibody responses first, there has been much evidence that so-called blocking antibodies (which impair *in vitro* lymphocyte response assays) can be found in normal pregnancy sera and placental eluates. However, the data often are not convincing that these systemic effects can always be ascribed to specific antibody, rather than other serum factors, and that they occur in all successful pregnancies. For example, agammaglobulinemic women do not suffer undue problems of pregnancy failure other than indirectly following their susceptibility to infection. It is probable that the blocking antibody phenomenon is an effect rather than a cause of the immunologic success of outbred pregnancy.

Of more interest may be local cellular adaptations within the maternal endometrium and placental bed site. It is now recognized that mature CD3-positive T cells and, particularly B cells are comparatively rare at these sites. The predominant cell types are: 1) activated class II MHC antigen-positive macrophages (which serve as an immediate defense to infection at these vital tissue sites in pregnancy, but may also be providing nonspecific immunosuppressive mediators such as PGE₂); and 2) CD3⁺ CD16⁺ CD56^{bright} granulated lymphocytes (endometrial granulated lymphocytes, eGL). These latter cells have attracted much attention regarding their apparent novel phenotype and function. They may be hormonally regulated in that they occur in endometrial

tissue in significant numbers during the secretory rather than proliferative phase of the cycle, and are normally lost by menstruation. In pregnancy, their numbers increase still further in first-trimester endometrium (or, in ectopic pregnancies, in any decidualized tissue), but most CD3⁺ eGL have been lost by term. What is their function? Their phenotype most closely resembles that of NK cells and pre-NK cells, although most CD3⁺ eGL may not proliferate rapidly in response to interleukin 2 (IL-2) and do not express other NK cell molecules (e.g. CD16 or CD57). One postulate is that they are a form of NK cell in arrested maturation, perhaps because of persistent expression of HLA-G on the target invasive cytotrophoblast?

In this context, it could be relevant that several rodent models of recurring fetal loss in pregnancy appear to operate via a final mechanism involving attack by NK cells of macrophage-derived products against the fetoplacental unit. Unfortunately, clear immunopathological evidence for cellular immune damage at the fetal tissue interface in cases of human recurrent spontaneous abortion has been difficult to obtain. In susceptible rodent immunogenetic breeding combinations, several other potent immunomodulators can cause fetal loss (e.g. lipopolysaccharide treatment). Hence, examples of pregnancy failure in rodents may reflect endogenous genetic factors together with exogenous pathogens, endotoxins, or environmental stimuli. Nevertheless, scid/beige double mutant mice (that lack T cell, B cell and NK cell function) reproduce normally, although these mice do still bear a granulated lymphoid cell type in endometrial tissue. In the absence of environmental factors, an intact immune system may not be necessary for reproductive success.

More recent attention has been directed toward cytokine cross-talk that may occur at the fetomaternal tissue interface in pregnancy. One hypothesis has been that fetal trophoblast antigens (e.g. HLA-G) or other products might stimulate maternal lymphocytes in endometrial tissue to produce cytokines and growth factors that act in a paracrine manner beneficial to trophoblast growth and differentiation (the so-called immunotrophism hypothesis). Although the relatively low numbers of mature T cells in endometrial tissues could limit this effect, it is also well established that decidualized endometrial tissue extracts are particularly potent in demonstrating immunosuppressive effects. It is now clear that other cell types in this tissue are highly active in cytokine secretion (e.g. eGL and glandular epithelial cells). For example, there is evidence for the significant production of the colony stimulating factors (CSFs), tumor necrosis factor α (TNF α), IL-6 and trans-

forming growth factor β (TGF β) within decidual tissue. Similarly, fetal syncytiotrophoblast would appear to be excessively endowed with growth factor receptors, including those for the CSFs, IFN γ , TNF α , TGF β , epidermal growth factor and transferrin. Thus, a complex interactive cytokine network may be operative within uteroplacental tissue that provides both immunosuppressive and growth-promoting signals.

Based on the above considerations, it had been proposed that certain cases of unexplained recurrent spontaneous abortion in humans could result from a failure of active maternal immune adaptation to the fetal allograft in pregnancy and might be assisted by deliberate prior immunization with paternal or third-party cells to promote these missing protective responses. However, the most recent information now suggests that there is no strong conceptual framework for this hypothesis acting at a systemic level, and that alloimmunization in such cases may not always significantly improve subsequent successful pregnancy outcome rates in fully controlled clinical efficacy studies.

Fetal protection from prenatally transferred maternal IgG antibody

In man and certain other species, maternal immunoglobulin G (IgG) is selectively transferred prenatally into the fetal circulation following initial recognition by specific transporting Fc γ receptors on placental syncytiotrophoblast. This provides transient passive immunity to assist postnatal immune protection following parturition and release from the sterile intrauterine environment into pathogen-rich environments at a time prior to development of neonatal immunocompetence. Significant maternal IgG transfer occurs in humans from around the 20th–22nd week of gestation. However, together with beneficial antimicrobial immunity, deleterious maternal autoantibodies and fetal-specific alloantibodies could also potentially be transferred into fetal circulation. Hence, elegant mechanisms have evolved to protect the fetus from adverse maternal IgG antibody, notably maternal anti-HLA alloantibody (which may have developed from a previous pregnancy) and that would be expected to have catastrophic effects on the developing fetal immune system if allowed to penetrate further than extraembryonic tissue.

It is now recognized that maternal HLA-specific alloantibody with specificity for the fetal HLA type will be efficiently bound by nontrophoblastic cells bearing fetal HLA antigens (i.e. macrophages, fibroblasts, endothelium) within the villous mesenchyme of placental tissue, and will not reach the fetal circu-

lation. Similarly, any other maternal antibody to a fetal antigen expressed within placental tissues will be sequestered by binding to an antigen-expressing cell or by binding as a soluble immune complex to high avidity Fc γ receptor-bearing macrophages and fetal stem vessel endothelium that are abundant in these extraembryonic tissues; the latter have specificity solely for complexed rather than monomeric IgG. Hence, these potentially deleterious maternal antibodies are trapped by the placental 'sink' or 'sponge'. Indeed, many immunohistological studies have drawn attention to the extensive IgG and complement deposition within term placental villous tissues – essentially, the placenta needs only to survive this immunopathologic onslaught for up to 5 months, unlike other body organs.

The exceptions that escape placental trapping of adverse maternal IgG antibodies are also of interest. For example, maternal IgG rhesus (D)-specific or platelet-specific antibodies will meet their cognate antigen first on fetal erythrocytes or platelets, respectively, within placental stem vessels. Hence, erythrolysis or thrombocytopenia will occur *prior* to trapping of immune complexes on fetal Fc γ receptor-positive placental vessel endothelium. In addition, maternal organ-specific autoantibodies reactive with autoantigens not represented within the placenta will not be sequestered and retained at this site. Thus, for example, maternal IgG thyroid-specific autoantibodies can reach the fetal thyroid and can sometimes directly cause transient symptoms of autoimmune thyroid disease during the neonatal period of persistence of passively transferred maternal IgG autoantibody.

Conclusions

Our understanding of the immunobiology of the fetal allograft has progressed considerably over the past 40 years. Current attention in human pregnancy is focused on the probable specialized role of selective HLA-G expression at the fetal tissue interface and on elucidating the function of the numerous maternal

CD3-negative granulated lymphocytes that can be found in the surrounding decidualized endometrial tissue, together with the complex cytokine networks that may exist within these tissues. It is possible that all of the components and mechanisms contributing to the success of the fetal allograft in immunocompetent pregnant hosts have now been identified, even if not all of the details are yet known. However, it is impossible to test this hypothesis in humans, and difficult to test in experimental animals. Hence, further novel approaches to unraveling the immunobiological strategies employed for successful viviparity can be expected.

See also: Alloantigens; Cytokines; Cytotoxic T lymphocytes; Graft rejection; Hemolytic disease of the newborn; Maternal antibodies; MHC, functions of; Natural killer (NK) cells.

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FEVER

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Since the writings of Hippocrates, fever has been almost universally recognized as a manifestation of disease. A linkage between the two was formally established by the demonstration that filtrates of

autogenous pus could cause fever when inoculated into rats; however, at the time this observation was made, the pyrogenic activity of bacterial endotoxins was unsuspected. The existence of an endogenous

pyrogen was definitively established in 1953, when Bennett and Beeson showed that leukocytes contain a factor that induces fever in the absence of infection. Subsequently, a moiety with activity similar to that of the leukocyte pyrogen was found in the serum of animals that developed fevers after inoculation of various microbial agents. The bacterial and endogenous pyrogens were not identical, as the former disappeared rapidly from the circulation, whereas titers of endogenous pyrogen increased, and showed a good correlation with the course of the ensuing fever. The induction of fever in conjunction with hypersensitivity responses, and the passive transfer of a fever-inducing substance in the serum of sensitized animals, clearly confirmed the endogenous origin of the pyrogen. Other organisms, such as gram-positive bacteria and viruses, can also induce fevers, the kinetics of which differ in some respects from those induced by bacterial endotoxin.

Endogenous pyrogens

Leukocytes, and macrophages in particular, produce a variety of cytokines that often exhibit similar or overlapping biological activities. Many of these have been shown to induce fever either directly or by the induction of other endogenous pyrogens. Although the use of recombinant material has clarified the specific functions and pyrogenic activities of the various species, their interrelationships are very complex, as they can induce their own – and each other's – production both *in vitro* and *in vivo*. The mechanisms that regulate these interactions have not been unraveled, but may involve inhibitory effects of prostaglandins on the synthesis of endogenous pyrogens. The major pyrogenic cytokines are briefly described below.

Interleukin 1

Although several cytokines have now been shown to induce fever *in vivo*, interleukin 1 (IL-1) is most likely to represent the molecule(s) originally described as the endogenous pyrogen. Two genes have been identified that code for two different forms of IL-1: IL-1 α and IL-1 β . Although the two forms of IL-1 exhibit only limited homology (<27%), both are translated as 31 kDa precursor peptides. These peptides lack a cleavage sequence, implying that an enzymatic step is required for processing of the precursor molecule to its mature form. Consequently, a proportion of the translated protein remains associated with the cell as the high molecular weight precursor. The biological activities of IL-1 α and IL-1 β are for the most part identical, implying that the short regions of sequence homology represent active sites common

to both forms. Analysis of recombinant IL-1 molecules has shown that both a 24.5 kDa and a 17.3 kDa peptide evoke monophasic fevers when injected into rabbits; however, the smaller peptide is approximately 100 times more active than the larger. This is consistent with the concept that a large, relatively inactive precursor is processed to a smaller, biologically active form. However, there may be more than one active moiety, as a 4 kDa peptide has also been shown to cause fever.

Tumor necrosis factor

Tumor necrosis factor (TNF) is a macrophage product that produces a classical endogenous pyrogen fever in rabbits and mice. Its biological activity is similar to that of IL-1, in that 50–200 ng kg⁻¹ of either factor induces comparable febrile responses. However, injection of larger quantities evokes a second peak, and there is evidence that TNF induces IL-1 both *in vitro* and *in vivo*. Another cytokine, lymphotoxin, shows considerable homology with TNF, and this substance also induces IL-1, and produces fever in animals.

Interferons

Interferons (IFNs) were originally described as antiviral agents, but they have been found to possess a wide range of biological activities, including pyrogenicity. However, substantially more recombinant human (rh) IFN α than rhTNF or rhIL-1 is required to induce a fever in rabbits, although this difference can perhaps be attributed to the species specificity exhibited by the interferons. IFN β and IFN γ are both pyrogenic for humans, though somewhat less so than IFN α . IFN α and IFN β show considerable amino acid homology, but IFN γ is quite distinct, and the mechanism of fever induction by the latter may also be different.

Interleukin 2

Interleukin 2 (IL-2) is known to cause fever in humans. Its half-life in the peripheral circulation is very brief, so it probably acts by stimulating the production or release of other endogenous pyrogens or pyrogenic prostaglandins.

Macrophage inflammatory protein 1 (MIP-1)

This cytokine has been cloned and sequenced, and is composed of two distinct but highly homologous proteins, the separate functions of which have not yet been characterized. MIP-1 is secreted in response to endotoxin, elicits localized inflammatory responses, is chemotactic for human neutrophils, and activates these cells for an oxidative burst. It also

induces a monophasic fever of rapid onset, approximately equal in magnitude to that induced by rhTNF or rhIL-1. However, the fever induced by MIP-1 is not blocked by cyclo-oxygenase inhibitors, so it is apparently initiated by a pathway that does not involve synthesis of prostaglandins.

Mechanism of fever induction

The control of body temperature resides in the hypothalamus, which is a primitive part of the brainstem that regulates certain bodily functions, such as sleep, and that also secretes a number of neuroendocrine-releasing factors. The anterior hypothalamus contains thermosensitive neurons, whose response is affected both by the temperature of the blood and by neural connections with peripheral temperature receptors. Provided that the mechanisms governing heat production and heat loss are intact, fever results from a resetting of the thermoregulatory centre in the hypothalamus to a higher level.

The crucial step in the induction of fever appears to involve increases in the production of arachidonic acid metabolites, especially prostaglandin E_2 (PGE_2). Several different lines of evidence favour a role for PGE_2 as the neural mediator in the febrile response. Antipyretics such as ibuprofen and indomethacin are potent inhibitors of prostaglandin synthesis; cytokines such as IL-1, TNF, and IFN α all stimulate PGE_2 production from hypothalamic tissue *in vitro*; and injection of PGE_2 directly into the hypothalamus induces fever within minutes. Thus, current concepts envisage the febrile response being initiated by the production and release of endogenous pyrogens as a consequence of an infection, an inflammatory

response, or an immunologically mediated disease. The pyrogenic immunomodulators increase the level of PGE_2 in the blood, and this mediator, as well as endogenous pyrogens such as IL-1 and TNF, act on the hypothalamus to induce fever. Brain regions sensitive to the direct effect of endogenous pyrogen are restricted to regions near the organum vasculosum laminae terminalis (OVLT), whereas the nucleus Broca ventralis, preoptic area, and the anterior and ventromedial hypothalamus, are able to respond directly to injections of small doses of prostaglandins E_2 and F_2 . The ventromedial region of the hypothalamus is the area most sensitive to PGE_2 , as measured by the induction of fever in rabbits.

The different sites of action of the different mediators show that there exist two separate pathways for induction of fever. First of all, prostaglandins synthesized outside the blood-brain barrier act on multiple sites in the central nervous system to induce rapid-onset fever. In contrast, although the endogenous pyrogens have the effect of stimulating release of arachidonic acid metabolites from the hypothalamus *in vitro*, there is no direct evidence that they cross the blood-brain barrier. Their actions are probably concentrated in the vicinity of the OVLT, inducing the synthesis and release of pyrogenic prostaglandins that produce a secondary peak of fever (Figure 1). There is no evidence that prostaglandins can, of themselves, act as neurotransmitters; but they are known to increase levels of cyclic AMP, which can perform this function. Prostaglandins certainly play a central part in the induction of fever, but there exists at least one prostaglandin-independent pathway, as demonstrated by the pyrogenic action of MIP-1. The relative importance *in vivo* of

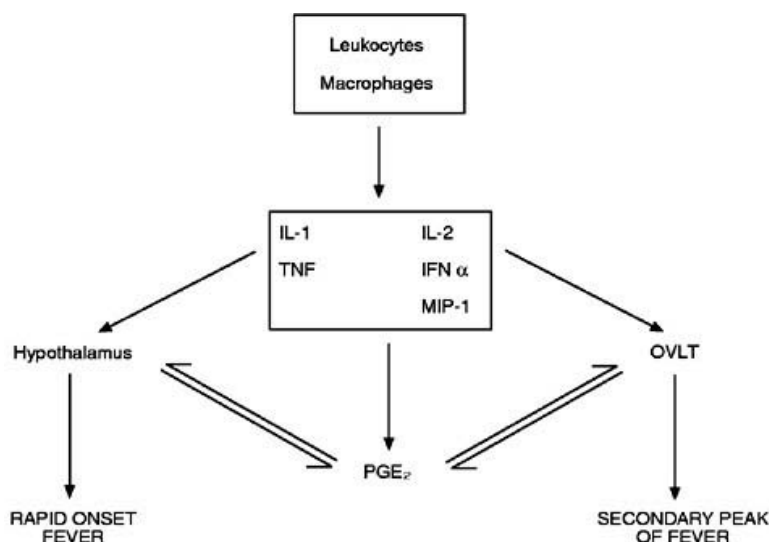


Figure 1 Schematic representation of pathways leading to the induction of fever.

each of these pathways, and their relationship, remain to be elucidated. Just as the pyrogenic cytokines can act both individually and in concert to induce fever, there similarly exists a number of endogenous antipyretics that can limit the magnitude of the febrile response, and thus prevent thermal damage. The ability of IL-1 to stimulate production of prostaglandins can be blocked by α -melanotropin-stimulating hormone; and arginine vasopressin, adrenocorticotrophic hormone and corticotropin-releasing factor can all act to alter the firing rate of neurons involved in thermoregulation.

The complexity of the pathways involved in the induction of fever are also reflected in changes in the response with age. The febrile response of kittens to endotoxin is poor during the first 6 weeks of life, and a comparable weakness in human neonates can be inferred from the failure of lymphocytes from cord blood to show enhanced T cell proliferative response at elevated temperatures. The febrile response to bacterial pyrogens also weakens with age, and micro-injection of PGE₁ into the anterior hypothalamus, as well as central administration of endogenous pyrogen, both induce responses that are significantly weaker in older animals. However, it appears that at least some of the differences can be attributed to age-related changes in vasoconstrictor responses in the peripheral vasculature.

Function of fever

The powerful mediators and complex pharmacologic pathways involved in the induction of fever indicate that the febrile process has the potential to modulate many different bodily functions. However, since fever has traditionally been associated with infection or disease, the central question is whether it is advantageous to the host. This point has been contentious, but the weight of evidence strongly suggests that fever is an adaptive response to infection.

The beneficial effects of fever are most striking in the poikilothermic vertebrates, which regulate their body temperature by behavioral means. When injected with *Aeromonas hydrophila*, a natural pathogen, the desert iguana (*Dipsosaurus dorsalis*) actively seeks to raise its body temperature, and animals permitted to maintain an elevated temperature show a marked decrease in mortality. Experiments in homeothermic animals have suggested that elevated temperatures enhance host resistance and increase survival; however, in some of these studies, growth of the organisms used was inhibited at febrile temperatures. Nevertheless, many different host defense mechanisms are modulated or augmented at temperatures corresponding to mild-to-moderate fevers

in humans. The bactericidal capacity of polymorphonuclear leukocytes is increased, as is the production of cytokines such as leukocyte migration inhibition factor (LIF) and interferon. The human lymphocyte proliferative response to polyclonal T cell mitogens and to allogeneic cells is increased; and in the mouse, both the efficiency of generation of antiviral cytotoxic T cells and the levels of activation of T helper cells in the antibody response against sheep red blood cells are enhanced. Although some of these effects can be attributed to an increase in the metabolic activity of the cells, the effect of fever on immune responses is not simple, as the responses of B cells are not affected; and at elevated temperatures, cytokines such as interferon inhibit both B and natural killer (NK) cell responses, but potentiate T cell responses. Thus, the data generally indicate that one consequence of fever may be a preferential augmentation of T cell responses, which are crucial to recovery from initial infections with viruses and bacteria.

The concept that fever is beneficial to the host is strengthened by observations that bacterial infection increases the duration of slow-wave sleep, and that activation of the immune system with accompanying fever alters sleep patterns. Many of the endogenous pyrogens, such as IL-1, TNF and the interferons, are also somnogenic, although the pathways involved in fever induction and sleep regulation are not identical. Therefore, the effect of cytokines and microbial products on hypothalamic and neural function may represent a coordinated physiologic response that allows the body to marshal all of its resources to overcome the infectious agent.

See also: Cytokines; Endotoxin (lipopolysaccharide (LPS)); Interferon α ; Interferon β ; Interferon γ ; Interleukin 1 and its receptors; Interleukin 2; Prostaglandins; Tumor necrosis factor α ; Lymphotoxin.

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FIBROBLASTS

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Fibroblasts are cells of mesodermal origin present in all forms of connective tissue. They are the least specialized of the connective tissue cell types. The primary role of fibroblasts is the production of proteins and polysaccharides that are secreted into the extracellular space of connective tissues to form the extracellular matrix. Appropriate combinations of these fibroblast-derived synthetic products endow each form of connective tissue with suitable physical and mechanical characteristics. The composition of the extracellular matrix produced by fibroblasts is markedly influenced by local microenvironmental factors. Some fibroblasts retain the capacity to differentiate into more specialized types of connective tissue cells, including chondrocytes, osteocytes, adipocytes and smooth muscle cells. These multipotential fibroblasts are also referred to as mesenchymal cells.

Fibroblasts also function as accessory cells in many immune and inflammatory responses. Fibroblasts can produce or respond to a wide variety of cytokines, and these mediators allow fibroblasts and leukocytes to cooperate during complex processes such as wound healing. Fibroblasts are capable of modifying their output of extracellular matrix components in response to mediators released from other cell types. Some human chronic inflammatory diseases ultimately develop into disabling fibrotic disorders, demonstrating how persistent activation of the immune system can lead to severe perturbations in fibroblast function. The constitutive production of class I major histocompatibility complex (MHC) molecules and the inducible expression of class II MHC molecules permit fibroblasts to participate in antigen presentation to T cells, although with considerably diminished efficiency relative to professional antigen-presenting cells. Cross-talk between fibroblasts and cells of the immune system occurs on a routine basis and contributes to the maintenance of homeostasis in mammalian connective tissues.

Principal fibroblast structural proteins – collagens and elastin

Production of fibrous proteins in the collagen family is a major function provided by most types of fibroblasts. At least 19 distinct types of collagen exist; they can be classified into fibrillar collagens, fibril-associated collagens (typically associated with the surface of fibrillar collagens) and network-forming collagens (involved in the formation of flat mesh-like structures such as basement membrane). Fibroblasts produce multiple types of collagen, including the fibrillar collagens I and III, which together account for 90% of the total collagen in the connective tissue of skin (Figure 1).

Type I collagen is the predominant form of collagen in skin and bone. It serves as a prototype for the structural organization of fibrillar collagens. The basic polypeptide subunit of a collagen molecule is an α chain which is composed of short globular N-terminal and C-terminal propeptides flanking a long central domain. Multiple post-translational modifications of α chains facilitate the folding of three α chains into a triple helical procollagen molecule. There are at least 25 distinct genes encoding collagen α chains scattered throughout the genome. Specific types of collagen can be assembled from trimers of the same α chain, or from two or three different α chains. For example, type I procollagen is formed by the assembly of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain. A prominent feature of the central portion of all fibrillar collagens is the existence of three amino acid repeats with a glycine in every third position (Gly-

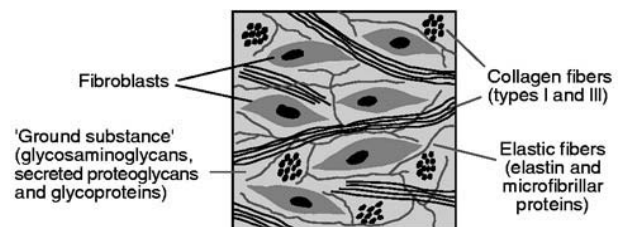


Figure 1 Major components of the extracellular matrix synthesized by fibroblasts within dermal connective tissue.

X-Y sequence). The X and Y positions can be filled by any amino acid, but many of the X and Y positions are occupied by prolines. During the biosynthesis of procollagen molecules, a subset of proline and lysine residues are hydroxylated to generate hydroxyproline and hydroxylysine residues. The hydroxylysines are further modified by specific glycosylation events. These specialized amino acids participate in intrachain and interchain hydrogen bonding that stabilizes the triple helix. After secretion, procollagen molecules are converted into collagens by the cleavage of N-terminal and C-terminal propeptides. Mature extracellular collagen molecules formed from three α chains associate with other collagen molecules to form collagen fibrils measuring 10–300 nm in diameter. These fibrils can in turn assemble into still larger bundles, termed collagen fibers (up to several microns in diameter).

The fibril-associated and network-forming collagens differ structurally from the fibrillar collagens. In fibril-associated collagens (e.g. types IX and XII), the triple helical stretches are interrupted with non-helical domains and the propeptides are not removed from the procollagen molecules. The resulting collagen molecules are more flexible and associate with molecules of fibrillar collagen rather than with each other. Network-forming collagens are exemplified by type IV collagen, a major component of the basement membrane. The Gly-X-Y repeats in type IV collagen are interspersed with numerous non-collagenous domains, permitting the collagen monomers to assemble into a net-like structure.

The role of fibroblasts in generating collagenous structures is not limited to synthesis of the structural proteins. Fibroblasts also have the capacity to organize and shape collagenous matrices by binding to collagen molecules through cell surface receptors and exerting mechanical force on this substrate. Through this action, fibroblasts can engineer collagen into flat sheets or rope-like strands, depending on the specific type of collagen and its tissue of origin. The major cell surface receptors for collagen on fibroblasts involved in these processes are β_1 -integrins. These integrins serve as a link between the proteins of the extracellular matrix and the intracellular actin cytoskeleton. Fibroblasts also remodel connective tissues through the production of multiple metalloproteinase enzymes that digest excess matrix material. Balanced activity of the matrix-generating synthetic machinery of fibroblasts and their metalloproteinase enzymes allows fine tuning of the extracellular matrix content within connective tissues.

Collagen fibers provide strength to connective tissues, but resiliency and flexibility are dependent on the presence of elastic fibers. Elastic fibers are made

up of a core of cross-linked tropoelastin monomers associated with microfibrillar proteins to form microfibrils measuring about 10 nm in diameter. The cross-links between individual elastin polypeptides occur via lysines that are modified and covalently linked to form the specialized amino acids desmosine and isodesmosine. The proteins associated with elastin in elastic fibers include the fibrillins (fibrillin 1 and fibrillin 2), microfibril-associated glycoprotein (MAGP) and associated microfibril protein (AMP). The microfibrillar proteins assemble in the extracellular matrix before the addition of elastin and provide the foundation on which tropoelastin molecules are subsequently deposited. The presence of elastic fibers enables tissues to recover to their original dimensions without injury after the application of a stretch force. Connective tissues rich in elastic fibers are found in the dermis of skin, the lung and the walls of large arteries.

Glycosaminoglycans and proteoglycans

Glycosaminoglycans are polysaccharide molecules that provide the extracellular matrix with additional physical properties not provided by structural proteins alone. These glycosaminoglycans include hyaluronan, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and keratan sulfate. All of the glycosaminoglycans consist of multiple copies of a basic disaccharide repeat. Most of these molecules (with the exception of hyaluronan) are covalently linked to serine residues on core proteins through a link tetrasaccharide to form proteoglycans. Examples of proteoglycans made by fibroblasts and present in the extracellular matrix include decorin, biglycan and fibromodulin. Glycosaminoglycans and the proteoglycans they form are large negatively-charged molecules that attract cations and water molecules, leading to the formation of a hydrated gel. These molecules comprise the 'ground substance' in connective tissues that provides support while permitting cell migration and diffusion of nutrients and soluble signaling molecules within the extracellular matrix. Some glycosaminoglycans produced by fibroblasts are not secreted, but instead associate with the cell surface (e.g. CD44, betaglycan and syndecan-2).

An important additional function provided by many proteoglycans is binding of growth factors and cytokines. Attachment of these soluble mediators to proteoglycans can influence their activity in several ways. For proteins in the fibroblast growth factor (FGF) family, association with proteoglycans containing heparin or heparan sulfate greatly enhances their biological activity. Many of the chemokines, a family of structurally and genetically related cyto-

kines that are chemotactic for subsets of leukocytes, also display significant affinity for proteoglycans containing heparin and heparan sulfate. Immobilization of chemokines on to proteoglycans of the extracellular matrix allows establishment of solid-phase gradients that can direct the migration of leukocytes traveling through tissue. Other cytokines with significant affinity for heparin and heparan sulfate are granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) and IL-7. In some cases, binding of a cytokine to proteoglycans can inhibit rather than enhance its biological activity. For example, binding of transforming growth factor β 1 (TGF β 1) to the core protein of decorin or the related proteoglycans biglycan and fibromodulin leads to a loss of TGF β 1 bioactivity.

Adhesive proteins derived from fibroblasts

Not all fibroblast products incorporated into the extracellular matrix serve a primary structural function. Fibroblasts are one cellular source of fibronectin, a 440 kDa glycoprotein that is widely distributed within the extracellular matrix. Fibronectin facilitates cell adhesion to the matrix and cell migration through the matrix. Fibronectin has multiple functional domains which interact with proteins and proteoglycans of the extracellular matrix or with integrins on cell surfaces. Differential splicing of fibronectin RNA allows production of multiple isoforms of the protein that differ in the number and type of functional domains present. The complement of fibronectin isoforms that are synthesized changes during development and varies between different tissues. Laminin is an adhesive protein, produced by some fibroblasts, that is typically restricted to the basement membrane, where it interacts closely with type IV collagen. Laminin within the basement membrane provides a crucial substrate for attachment of epithelial cells.

Growth factors for fibroblasts

Multiple polypeptide growth factors stimulate the growth of fibroblasts. Most of these growth factors have mitogenic effects on several cell types, rather than being restricted in their action to fibroblasts. Platelet-derived growth factor (PDGF) is liberated from platelets that are activated following formation of a blood clot. It provides an initial stimulus for fibroblast proliferation following wounding and is also chemotactic for fibroblasts. The FGF family is a group of nine proteins with common structural fea-

tures. Of the FGFs, FGF-1 (acidic FGF) and FGF-2 (basic FGF) are the most widely expressed. FGF-1, FGF-2 and FGF-9 lack a typical signal sequence, suggesting that specialized mechanisms exist for the export of these proteins from cells. FGF-7, also known as keratinocyte growth factor (KGF), is a member of the FGF family with mitogenic effects that are restricted to epithelial cells. Epidermal growth factor (EGF) receptor ligands, including EGF and TGF α , also stimulate fibroblast growth.

Modulation of extracellular matrix production by fibroblasts

Fibroblasts regulate their secretion of extracellular matrix products in response to several types of signals. Proinflammatory cytokines are important positive and negative regulators of the production of collagen, elastin, proteoglycans and the metalloproteinases that are responsible for degradation and remodeling of the extracellular matrix. TGF β 1 and IL-1 are both strong activators of the transcription of collagen genes in fibroblasts. TGF β 1 also contributes to fibrogenesis by inhibiting metalloproteinase synthesis and stimulating the production of protease inhibitors that inhibit metalloproteinase activity. IL-4 stimulates the transcription of collagen and fibronectin in fibroblasts. In contrast, collagen production by fibroblasts is inhibited by other cytokines, including tumor necrosis factor α (TNF α) and interferon γ (IFN γ). Both TNF α and IL-1 enhance the synthesis of metalloproteinases by fibroblasts. Several human diseases characterized by chronic immune-mediated injury to a specific organ eventually progress to fibrosis of the affected organ; examples include scleroderma, idiopathic pulmonary fibrosis and chronic glomerulonephritis. In these diseases, combinations of proinflammatory cytokines that stimulate a net excess of extracellular matrix production by fibroblasts contribute to the development of pathologic fibrosis.

The composition of extracellular matrix produced by fibroblasts can also be influenced by environmental factors such as ultraviolet irradiation. A prominent abnormality in chronically photodamaged skin is solar elastosis, characterized by replacement of normal elastic and collagen fibers with abnormal elastic fibers. This ultraviolet light-induced pattern of injury results from the combination of chronic ultraviolet induction of matrix-degrading metalloproteinase enzymes and relative overproduction of elastin and fibrillins by dermal fibroblasts.

Cytokine production by fibroblasts

Most cytokine production by fibroblasts is not constitutive, but occurs following activation by agonists such as primary cytokines (i.e. IL-1 and TNF α), bacterial products (e.g. lipopolysaccharide) or T cell-derived cytokines (e.g. IL-17). Many of the cytokines that fibroblasts produce are also made by a wide variety of other cell types. These cytokines include IL-1 receptor antagonist (IL-1Ra), interleukin-6 (IL-6), interleukin-11 (IL-11), interleukin-15 (IL-15), GM-CSF, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and leukemia inhibitory factor (LIF). Fibroblasts can also be induced to make chemokines that are members of the α -chemokine family, of which IL-8 is the prototype, or the β -chemokine family, typified by monocyte chemoattractant protein 1 (MCP-1). Fibroblasts are a major paracrine source of KGF for epithelial cells such as epidermal keratinocytes. Other growth factors fibroblasts can be induced to synthesize are FGF-2 and hepatocyte growth factor (HGF). IL-1 and TNF α stimulation of fibroblasts also increases the activity of cyclo-oxygenase, leading to enhanced production of eicosanoids such as prostaglandin E₂. Even activated fibroblasts generally make only a subset of the cytokines listed in Table 1. The repertoire of cytokines elabor-

ated by fibroblasts at any given point in time is dependent on the combination of activating and inhibitory signals present in the local environment.

Fibroblasts and tissue repair

Fibroblasts play a central role in restoring the integrity of injured tissue. Soon after wounding, multiple molecules with chemotactic activity for fibroblasts are generated at the wound site. These include fragments of fibrin and fibronectin as well as several proteins released from platelet granules (PDGF, platelet factor 4 and β -thromboglobulin). Platelet factor 4 and β -thromboglobulin are both α -chemokines that are also chemotactic for neutrophils. Guided by their integrin receptors, fibroblasts migrate into the provisional wound matrix containing fibrin, fibronectin and vitronectin. The granulation tissue that forms in the first few days at a wound site contains numerous fibroblasts that are actively dividing under the influence of several fibroblast growth factors, including FGF-2, TGF α and PDGF. Fibroblasts in granulation tissue produce a rich collagenous matrix, including collagens I and III, that provides strength to the wound site. During the early stages of wound healing, fibroblasts produce more collagen III as a fraction of total collagen production than under normal resting conditions. Later on in the healing of a wound, some of this type III collagen is progressively replaced by type I collagen. Fibroblasts at wound sites have prominent cytoplasmic actin and myosin fibers and are sometimes referred to as myofibroblasts. The cytoskeletal bundles of actin and myosin in these myofibroblasts are associated with enhanced contractile function during the contraction phase of wound healing.

Concluding remarks

Fibroblasts either produce or respond to a substantial fraction of the mediators that participate in immune and inflammatory processes. The presence of fibroblasts and related connective tissue cells in nearly all organs and tissues ensures that these cell types have the opportunity to function as accessory cells influencing certain interactions between leukocyte populations. Fibroblast-generated extracellular matrix is also an important regulator of cell-cell interactions and signaling occurring via soluble mediators.

See also: Chemokines; Cytokines; Extracellular matrix; Fibronectin; Integrins; Transforming growth factor β (TGF β).

Table 1 Cytokines produced by stimulated fibroblasts

<i>Cytokine</i>	<i>Stimuli reported to elicit production</i>
TNF α	Ultraviolet B irradiation, lipopolysaccharide (LPS)
IL-1Ra	IL-1, TNF α
IL-6	IL-1, TNF α , IL-17
IL-11	IL-1, TGF β
IL-15	Ultraviolet B irradiation
C-X-C Chemokines ^a	
IL-8	IL-1, TNF α , IL-17
GCP-2	LPS
KC/Gro α	IL-1, TNF α , PDGF
C-C Chemokines ^a	
MCP-1	IL-1, TNF α , PDGF, LPS
RANTES	IL-1, TNF α
LIF	IL-1, TNF α
G-CSF	IL-1, TNF α , IL-17
M-CSF	IL-1
GM-CSF	IL-1
KGF (FGF-7)	IL-1, IL-6, TGF α , PDGF
FGF-2	IL-1
HGF	IL-1, TNF α

^aFibroblasts make multiple types of C-X-C (α) and C-C (β) chemokines. Not all of these molecules have been extensively studied, so this table lists only a few well-characterized members of the two major chemokine families that are known to be made by fibroblasts.

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FIBRONECTIN

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Fibronectin (FN) is a ubiquitous extracellular glycoprotein endowed with many biological activities (Figure 1); it is present in a soluble form in plasma and in an insoluble form in the extracellular matrix (ECM). FN interacts with collagen, heparin, fibrin, and several cell surface receptors of the integrin family. These interactions play important roles in cell adhesion and migration, wound healing, hemostasis and thrombosis, inflammation and immune response, tumor invasion and embryonic differentiation.

Fibronectin structure and assembly

FN is a dimeric glycoprotein with a monomer molecular weight of 220–250 kDa composed almost entirely of three types of modules, known as types I (45 amino acids), II (60 amino acids) and III (90 amino acids). This glycoprotein is organized into proteolysis-resistant functional domains which contain binding sites for integrins, ECM and plasma

proteins (Figure 2). Interaction of FN with integrins is mainly mediated by the Arg-Gly-Asp (RGD) sequence that is incorporated in a stable protein scaffold provided by the tenth type III module. Additional residues (Pro-His-Ser-Arg-Asn) in the ninth type III module, known as the synergy region, are also involved in this interaction. In the same ninth type III module a binding site for $\alpha_{IIb}\beta_3$ -integrin is also present. Another major cell-adhesive domain is termed IIICS (type III connecting segment). This domain contains three important integrin-binding sites: Glu-Ile-Leu-Asp-Val-Pro-Ser (EILDVPS) in the CS1 region, Arg-Ala-Pro-Ser (REDV) in the CSS region, and Ile-Asp-Ala-Pro-Ser (IDAPS) in the adjacent heparin-binding domain (Figure 2).

FN matrices are deposited in a temporally and spatially defined pattern and comprise both newly synthesized cellular and soluble FNs. All five type I modules present in the N-terminal are required for FN assembly into the fibrillar matrix. The ninth type I domain in the collagen-binding region, and the first type III domain also appear to form a functional unit that is involved in FN assembly. This process is mediated by integrins including $\alpha_5\beta_1$ and $\alpha_v\beta_3$.

FN is encoded by a single gene whose pre-mRNA can be alternatively spliced in three different regions. Different isoforms of FN result from alternative splicing of EDA and EDB modules and mostly of a region termed V (variable) or IIICS. Multiple alternative splicing occurs in this region and generates 20 potentially different forms of FN.

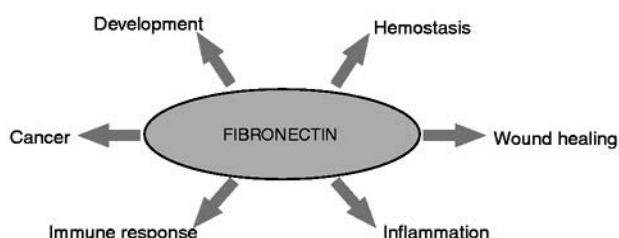


Figure 1 Fibronectin is involved in many biological processes.

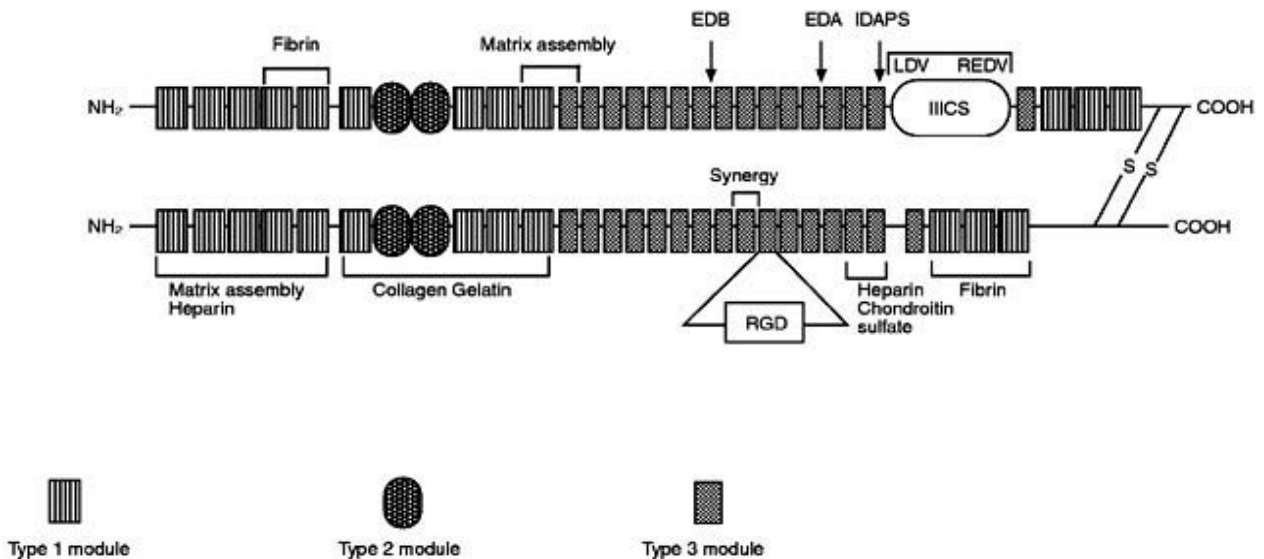


Figure 2 FN structure and domain organization. The modular structure of FN shows the position of the types 1, 2 and 3 modules. The positions of the alternatively spliced regions EDA, EDB and IIICS, which contain integrin-binding sites, are shown. The ninth type III module contains the synergy region. The tenth type III module contains the RGD sequence involved in integrin binding. The IDAPS sequence located near the carboxyl terminus of the heparin-binding domain of FN is involved in integrin binding. The other binding sites with the counterpart ligands are indicated by square brackets.

Fibronectin expression and distribution

FN is present in all body fluids and most abundantly in plasma (330–350 $\mu\text{g ml}^{-1}$). It is also widely distributed in the basement membranes and in the connective tissue matrices throughout the body, where it is expressed together with other ECM proteins. Cellular FN is mainly synthesized by fibroblasts and tissue macrophages. Chondrocytes, myoblasts, endothelial cells, astrocytes, Schwann cells, hepatocytes, kidney cells and keratinocytes also produce FN. Resting platelets, neutrophils and monocytes do not express surface FN, but they produce and release it upon activation during hemostasis and inflammation; T lymphocytes and natural killer (NK) cells constitutively synthesize and secrete FN.

Fibronectin ligands

FN interacts with a large number of molecules and ECM components including collagens, fibrin, fibrinogen, proteoglycans, and factor VIII via specific binding sites (Figure 2). FN specifically interacts with collagens (most strongly with collagen type III) and gelatin. FN binding to fibrin occurs on at least two different sites and plays an important role in the formation of blood clots and cryoprecipitates in the plasma. FN also interacts with and is a substrate for factor VIII. Factor VIII cross-links FN to fibrin during blood clotting and induces cross-linking of FN to collagen and bacteria. FN interaction with heparin

involves two specific sites in the C- and N-termini. FN interaction with gangliosides, phospholipids, C1q complement component, acetylcholinesterase and thrombospondin have been reported. Cell adhesion to FN is mostly mediated by cell surface receptors belonging to the integrin family. Integrins represent a large group of heterodimeric receptors that are of central importance in cell surface interactions between many cell types and a variety of ECM proteins including FN. Each integrin is composed of α and β subunits which are membrane glycoproteins with a large extracellular domain, a single membrane-spanning segment and a short cytoplasmic portion. Divalent cations regulate the association of α and β subunits and ligand binding. The extracellular portion of integrins binds to ligands, whereas the cytoplasmic domain interacts with cytoskeletal elements. A general model of the integrin structure is presented in Figure 3.

The RGD sequence specifically binds to $\alpha_5\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, and $\alpha_6\beta_6$. The $\alpha_{IIb}\beta_3$ -integrin can also interact with an 11 residue peptide present in the ninth type III module. The IIICS domain, with the adhesive sequences EILDVPS in the CS1 region and REDV in the CS5 region, and the adjacent heparin-binding domain, with the IDAPS sequence, provide the binding region for $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins. The FN-binding integrins and FN recognition sequences are shown in Figure 4.

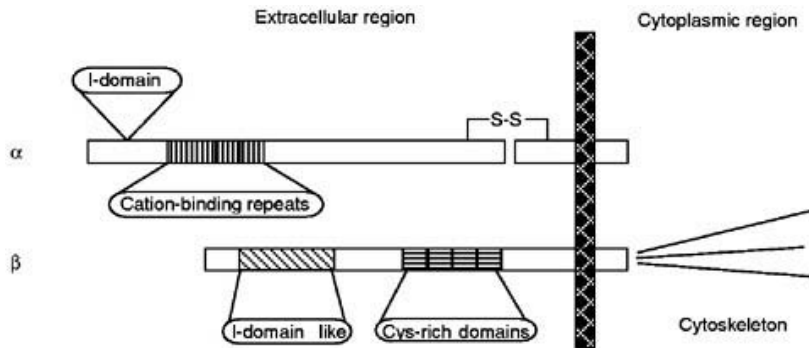


Figure 3 Integrin structure and domain organization. The N-terminal sequences of α and β subunits contribute to the ligand-binding site. The extracellular portion of the α subunit is characterized by the presence of a region which binds divalent cations. The I-domain is present only in some α subunits. The α subunits without an I-domain undergo intracellular proteolytic processing. The resulting heavy and light chains remain bound by a disulfide bridge. The β subunit is characterized by four cysteine-rich repeats and by an I-domain-like sequence.

Fibronectin and signaling

Engagement of FN receptors by FN or specific antibodies initiates biochemical signaling events within the cell important for regulating different cell functions such as migration, adhesion, proliferation, differentiation, apoptosis and specific gene expression. Recently, a variety of potential signaling mediators activated by FN interaction with integrins have been identified in many cell types: they include the focal protein tyrosine kinase FAK (pp125^{FAK}), the serine-threonine kinases PKC and MAPK, the adapter molecules SHC and GRB-2, the GTP-binding proteins p21 RAS and RHO, the phosphatidylinositol 3-kinase (PI3K). FN binding can also trigger other events, such as elevation of intracellular pH and Ca^{2+} transients. The FN signaling pathways are shown in Figure 5.

Fibronectin and development

Mutations of FN and FN-binding integrin genes in the mouse have provided exciting insights into their function during morphogenesis. Null mutations in either FN or the integrin α_5 gene lead to embryonic lethality. FN-null embryos develop abnormalities

shortly after gastrulation. Defects are found in mesodermal derivatives along the anterior-posterior axis and in the yolk sac vasculature. Mutant embryos lack notochord and somites along the entire axis, although precursors for these tissues are present.

Fibronectin, cell growth and apoptosis

Cellular interaction with FN promotes cell cycle progression and proliferation by affecting cyclins and immediate early response genes. Clustering of $\alpha_5\beta_1$ fibronectin receptor induces an increase in CDK-2 and CDC-2-dependent kinase activity accompanied by hyperphosphorylation of the retinoblastoma protein, Rb. In addition, cell adhesion to FN results in a rapid induction of *c-fos* and *c-myc* transcripts. FN is also involved in cell survival and protection from apoptosis in many cell types, including epithelial and endothelial cells and leukocytes. In human endothelial cells FN or FN-binding integrins, which trigger the RAS-MAPK pathway via SHC, arrest apoptotic cell death even in absence of mitogens; in Chinese hamster ovary (CHO) cells increased expression of Bcl-2 gene, which is a potent antagonist of apoptosis, is specifically induced by $\alpha_5\beta_1$ ligation; adhesion to FN via $\alpha_4\beta_1$ prolongs cosinophil

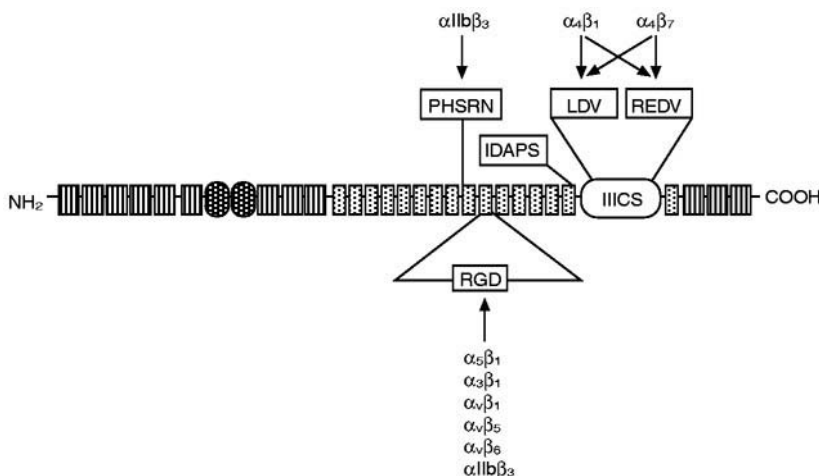


Figure 4 Integrin-binding sites and recognition sequences on FN. The RGD sequence in the tenth type III module mediates binding to the indicated integrins. The PHSRN sequence, in the ninth type III module, mediates binding to the $\alpha_{11b}\beta_3$ integrin. The LDV and REDV sequences in the alternatively spliced IIICS bind $\alpha_4\beta_1$ and $\alpha_4\beta_7$ -integrins. The IDAPS sequence located near the carboxyl terminus of the heparin-binding domain of FN is also recognized by the $\alpha_4\beta_1$ integrin.

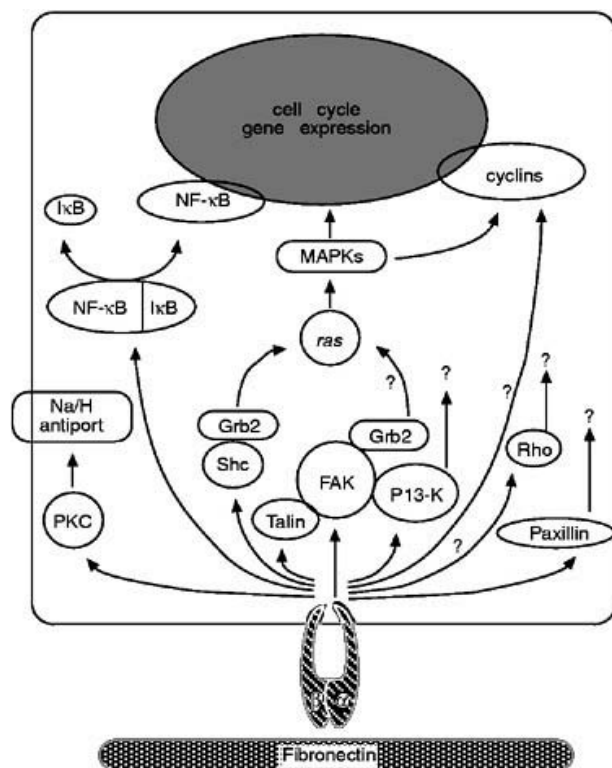


Figure 5 FN signaling pathways. The major intracellular signaling pathways activated by FN via integrin ligation are indicated.

survival by triggering production of cytokines. In some cases, however, leukocytes can respond to FN by undergoing apoptosis rather than proliferating or surviving. Growth of myeloid leukemia and several hematopoietic cells is suppressed by seeding on FN which triggers apoptosis by interaction with $\alpha_5\beta_1$.

Fibronectin and the immune system

Many studies indicate that FN plays an important role in the regulation of lymphocyte activation, differentiation and function. Induction of thymocyte differentiation depends on FN interaction with both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ -integrins. Enhancement of T cell receptor (TCR)-mediated T cell activation occurs following either ligand-induced or antibody-mediated cross-linking of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ -integrins. FN costimulates CD4⁺ T cell proliferation, expression of activation markers, including CD25 and CD71, and interleukin 2 (IL-2) production. Interestingly, regulation of IL-2 gene expression is related to the ability of FN to induce the expression of AP-1 transcription factor. FN also potentiates the cytotoxic activity of CD8⁺ T lymphocytes and NK cells. In cytotoxic T cells, the costimulatory activity has been associated with the enhancement of TCR-induced inositol

1,4,5-triphosphate (IP₃) generation whereas in NK cells it is associated with the potentiation of CD16-triggered calcium flux. In B cells, FN interaction with $\alpha_4\beta_1$ affects the maturation of bone marrow cells into immunoglobulin-secreting cells.

Fibronectin, inflammation and phagocytosis

During inflammation, leukocytes extravasate into the tissue stroma where they produce a variety of cytokines that trigger the inflammatory response. It is well established that FN induces the expression of several cytokine genes, including IL-1 β and CSF-1 in monocytes, and of specific protease genes in synovial fibroblasts. Numerous studies have reported that phagocytes and microorganisms such as staphylococci, streptococci, gram-negative bacteria and *Candida albicans* interact with FN and that FN promotes phagocytosis of various particles and microorganisms. FN does not act as a classical opsonin (i.e. IgG1, C3b) but rather functions as an enhancer of phagocytosis mediated by other receptors on phagocytic cells. Indeed, C3b receptors and Fc receptors appear to be activated for phagocytosis by occupation of FN receptors by FN on the same cell. In addition, FN acts as a costimulus with cytokines for induction of the respiratory burst in neutrophils.

FN may play an important role for activation of phagocytes in extravascular locations such as wound beds and interstitial spaces where they are attached to the ECM.

Fibronectin and hemostasis

FN participates in the interaction of platelets with the fibrin matrix during clot retraction and in physiologically important processes including clot dissolution, clearance of fibrin debris, and clot formation on wound healing. Platelets contain an intracellular pool of FN, which is located in α granules and is released upon activation with thrombin or collagen. The $\alpha IIb\beta_3$ -integrin which binds to both cell-associated and released FN is expressed on the platelet cell surface. FN plays a relevant role in the primary response of hemostasis by mediating platelet adhesion and spreading on subendothelial basement membranes, and in platelet-platelet interaction cooperating with fibrinogen and thrombospondin. FN also interacts with fibrin, is incorporated into clots and is cross-linked to the fibrin by factor VIII.

Fibronectin, wound healing and fibrosis

The healing of epidermal wounds is a good model to analyze the role of FN. When a wound is made in the skin, the first detectable event is the formation of a fibrin-FN clot which promotes the migration of epidermal cells to cover the wound. Moreover, FN synthesis in the underlying and surrounding dermis and FN-binding integrins on the keratinocytes become activated. Meanwhile, in the granulation tissue which contains fibroblasts and phagocytes, FN, produced also by the immigrating cells, promotes fibroblast migration and fibrillogenesis of collagen type I. The FN role in the neovascularization process is less clear, but several studies suggest its involvement in migration and proliferation of endothelial cells. Furthermore, FN is a chemotactic factor for many cells and it is produced by activated neutrophils and macrophages. The possibility arises that cells recruited and activated in response to FN produce more FN, thus leading to ECM accumulation and fibrosis.

Fibronectin and cancer

Tumor cells often show cell type-specific changes in FN receptor expression and function, associated either with malignant transformation or with tumor progression and metastasis. A loss of $\alpha_5\beta_1$ expression has been found in several human and experimental tumors and an inverse relationship between malignant behavior and $\alpha_5\beta_1$ levels has been described for some tumor cell lines. In addition, overexpression of $\alpha_5\beta_1$ -integrin in CHO cells results in reduced migration *in vitro* and loss of tumorigenicity *in vivo* of transfected cells as compared with parental cells expressing low levels of FN receptors. $\alpha_5\beta_1$ /FN matrix interactions are also implicated in the control of metastasis as coinjection of tumor cells with RGD-containing peptide or antibodies to $\alpha_5\beta_1$ results in inhibition of experimental tumor spreading.

See also: **Adhesion molecules; Apoptosis; Clotting**

system; Extracellular matrix; Integrins; Phagocytosis; Platelets; T lymphocyte activation; Transgenic animals.

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FILARIASIS, LYMPHATIC

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Lymphatic filariasis is caused by thread-like nematodes of three filarial species, *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. While *W. bancrofti* is responsible for infections in more than 90% of the

estimated 90 million individuals infected, *B. malayi* accounts for all but 40 000–50 000 of the infections in the remaining 10 million. Infection is established only after a mosquito vector transmits infective

larvae during a blood meal. These infective larvae molt twice and mature over a period of 6 months to 2 years, finally becoming mature adults that reside within the host's lymphatic vessels. The adult females continuously produce microfilariae that circulate in the blood, often with a periodicity that is nocturnal. These microfilariae cannot develop further until they are ingested by a mosquito. In the mosquito, they complete their life cycle by developing over a 2 week period into mature third stage (L3) larvae, again capable of being infectious for humans. The parasites are long lived. The average lifespan of an adult is estimated to range from 5 to 15 years, while microfilariae have a lifespan of 3–12 months. Because of their long period of survival and their inability to replicate in the definitive (human) host, these parasites have developed complex and specialised mechanisms to evade killing by the host immune effector mechanisms.

Clinical manifestations in all three lymphotic filariases are quite similar but also wide ranging. Although the most characteristic are those reflecting the consequences of lymphatic dysfunction, damage or inflammation (e.g. elephantiasis, hydrocele, retrograde adenolymphangitis), the most common manifestation is an asymptomatic state characterized by high levels of circulating microfilariae associated with a relatively downregulated cellular immune response. A much rarer condition, tropical pulmonary eosinophilia, appears to be a result of specific immunological hypersensitivity to the parasite, characterized by pulmonary interstitial inflammation with marked peripheral blood eosinophilia and extraordinary elevations of serum immunoglobulin E (IgE). Further, there are individuals clearly exposed to the parasite but who have no obvious signs of infection. This 'endemic normal' group is likely to be a heterogeneous one ranging from those harboring cryptic or occult infections to those who may be solidly immune.

Better definition of the parasitologic status of all individuals (infected and uninfected) living in filarial-endemic regions has been made by the use of specific tests for circulating parasite antigen (for *W. bancrofti*), IgG4 filarial-specific antibodies (for both bancroftian and brugian filarial infection) – felt to reflect active infection – and the use of high resolution ultrasonography to detect the living adult worms *in situ* (generally in the scrotal lymphatics of males).

Immunological features

In response to filarial infection, high levels of parasite-specific antibodies are produced, although the

distribution of immunoglobulin isotypes varies considerably depending on the clinical expression of infection. Asymptomatic microfilaremic individuals have high levels of filarial-specific IgG4, whereas those with chronic lymphatic obstruction have predominantly IgG1, IgG2 and IgG3. Antifilarial IgE levels are elevated in most infected groups, with the highest levels in those with tropical pulmonary eosinophilia. As the IgE and IgG4 antibodies are often directed at the same antigenic determinants and are regulated by similar cytokines (interleukins IL-4 and IL-13), the balance between IgG4 and IgE within a given individual has been postulated as being important in preventing allergic manifestations in these infections or in mediating parasite clearance and/or pathology.

Lymphocytes from individuals with asymptomatic microfilaremia generally display little or no proliferative response to parasite antigen, responses that contrast significantly to those seen in microfilarial-negative individuals with chronic lymphatic disease and those termed endemic normals. This lack of T cell reactivity in microfilaremic individuals has been shown to be parasite antigen-specific, in that proliferative responses to nonparasite antigens and mitogens remain intact. Analyses of parasite antigen-driven cytokine responses (based on mRNA levels, protein expression and cytokine-producing T cell frequencies) have shown that the asymptomatic microfilaremic individuals have a relative inability to produce interferon γ (IFN γ) while retaining the capacity to produce IL-4 and IL-5; those with chronic lymphatic pathology (particularly those who are negative for circulating parasite antigen) make IFN γ , IL-4 and IL-5 in response to the parasite antigen.

IL-10 appears to play a significant role in modulating the production of IFN γ in the microfilaremic state, as neutralizing IL-10 antibodies enhance both lymphocyte proliferation and IFN γ production in peripheral blood mononuclear cells from asymptomatic microfilaremic individuals, and IL-10 production, both spontaneously *ex vivo* and in response to parasite antigen, is markedly enhanced in cells from microfilaremic individuals.

In animal models of filarial infection (where time of infection can be ascertained), parasite-specific unresponsiveness develops at the time microfilariae appear in the bloodstream, suggesting that this stage of the parasite is involved in the modulation of the cellular immune system. Further, in *in vitro* studies using human cells stimulated with stage-specific antigen preparations, stages containing microfilariae (Mf and adult females) have been shown to preferentially

downregulate the cellular (IFN γ , proliferation) responses.

A number of mechanisms apart from the down-regulation of the immune response by immunoregulatory cytokines (e.g. IL-10) have been proposed for the diminished or absent proliferative and IFN γ response toward parasite antigens seen in the asymptomatic microfilaremic individual. Although there is some evidence for a role of host genetic factors (particularly certain HLA class I and class II alleles) in determining the nature of the immune response to parasite antigens, studies in endemic populations have shown that prenatal exposure to the microfilarial stage antigens occurs in filarial-endemic regions and can result in long-term 'anergy' to filarial antigens once naturally infected.

Determinants of pathology

While the nature (quality, quantity) of the immune response has been proposed as a major determinant of disease in lymphatic filariasis, based on differences in measurable immune responses among infected but clinically distinct populations, the parasite itself (or parasite-derived products) has also been implicated as another important determinant of pathology. Indeed, in various immunodeficient strains of mice (athymic nude, SCID) severe lymphatic dilatation occurs in the absence of a mature T and B cell response. Interestingly, with immune reconstitution of these filarial-infected immunodeficient mice, the lymphatics become markedly obstructed, suggesting that there are two pathologic phases in lymphatic filariasis – one parasite-mediated and the other immunologically-mediated. Further, there is evidence that much of the severely deforming manifestations of lymphatic filariasis may be caused by bacterial and fungal infections superimposed on poorly functioning lymphatics.

Protective immunity

There is evidence for protective immunity to filarial

parasite. Foremost is the observation in filariasis that the adult worm burden generally increases through childhood and adolescence, but then plateaus or falls in adulthood. This observation can be explained by the acquisition of so-called concomitant immunity where an individual develops resistance to new infection while harboring adult parasites.

In filarial infection, a small group of individuals appear to be infection free despite long-term residence in areas of high endemicity, the so-called 'putative immune' (PI) or 'endemic normal' group. However, such individuals with sterile immunity are not commonly identified.

Models of filarial infections in susceptible animals (e.g. jirds infected with *B. malayi*) provide the most compelling evidence that protective immunity can be induced by attenuated larvae or by repeated infections. Indeed, partial protection has been induced with defined antigens such as paramyosin. Nevertheless, the effector mechanisms underlying this immunity have not been determined nor have universal targets of immune attack. Thus, vaccine development for lymphatic filarial infections is in its infancy.

See also: **Parasites, immunity to.**

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FILOVIRUSES (MARBURG VIRUS AND EBOLA VIRUS), INFECTION AND IMMUNITY

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Filoviruses cause fulminating hemorrhagic fever with severe shock syndrome and high mortality in humans and nonhuman primates. Marburg virus, the prototype of these agents, was discovered during an outbreak in Europe in 1967. Ebola virus was first isolated in 1976 as the causative agent of two simultaneous outbreaks in southern Sudan and northern Zaire. Re-emergence, particularly of Ebola virus, has since been observed, with irregular intervals. Filovirus infections are of zoonotic nature, although the natural reservoir is not known. The epidemiological characteristics of identified outbreaks are summarized in Table 1.

Characteristics of the organism and its antigens

Filoviruses are a family of nonsegmented negative-stranded RNA viruses, with Marburg virus and Ebola virus constituting two different species. Particles are filamentous with a variable length (up to 14 000 nm) and a uniform diameter (80 nm) (Figure 1).

Inside an envelope there is a nucleocapsid containing the RNA genome (19 kb) and four internal proteins. These are the L-protein catalyzing transcription and replication, the phosphoprotein VP35 that

Table 1 Outbreaks of filoviral hemorrhagic fevers

Location	Year	Virus/subtype	Cases (% mortality)	Epidemiology
Germany/Yugoslavia	1967	Marburg	32 (23)	Imported monkeys from Uganda source of most human infections
Zimbabwe	1975	Marburg	3 (33)	Unknown origin; index case infected in Zimbabwe; secondary cases were infected in South Africa
Southern Sudan	1976	Ebola/Sudan	284 (53)	Unknown origin; spread mainly by close contact; nosocomial transmission and infection of medical staff
Northern Zaire	1976	Ebola/Zaire	318 (88)	Unknown origin; spread by close contact and by use of contaminated needles and syringes in hospitals
Tandala, Zaire	1977	Ebola/Zaire	1 (100)	Unknown origin; single case in missionary hospital; other cases may have occurred nearby
Southern Sudan	1979	Ebola/Sudan	34 (65)	Unknown origin; recurrent outbreak at the same site as the 1976 outbreak
Kenya	1980	Marburg	2 (50)	Unknown origin; index case infected in western Kenya died, but physician secondarily infected survived
Kenya	1987	Marburg	1 (100)	Unknown origin; expatriate traveling in western Kenya
USA	1989–90	Ebola/Reston	4 (0)	Introduction of virus with imported monkeys from the Philippines; four humans asymptotically infected
Italy	1992	Ebola/Reston	0 (0)	Introduction of virus with imported monkeys from the Philippines; no human infections associated
Ivory Coast	1994	Ebola/Ivory Coast	1 (0)	Contact with chimpanzees; single case
Gabon	1994	Ebola/Zaire	Unknown	Unknown
Kikwit, Zaire	1995	Ebola/Zaire	315 (77)	Unknown origin; course of outbreak as in 1976
Gabon	1996	Ebola/Zaire	50 (50)	Outbreak was linked to the butchering, transport and preparation for consumption of a dead chimpanzee
USA	1996	Ebola/Reston	0 (0)	Introduction of virus with imported monkeys from the Philippines; no human infections associated
Gabon	1996–97	Ebola/Zaire	60 (75)	Uncommon origin: index case was a hunter

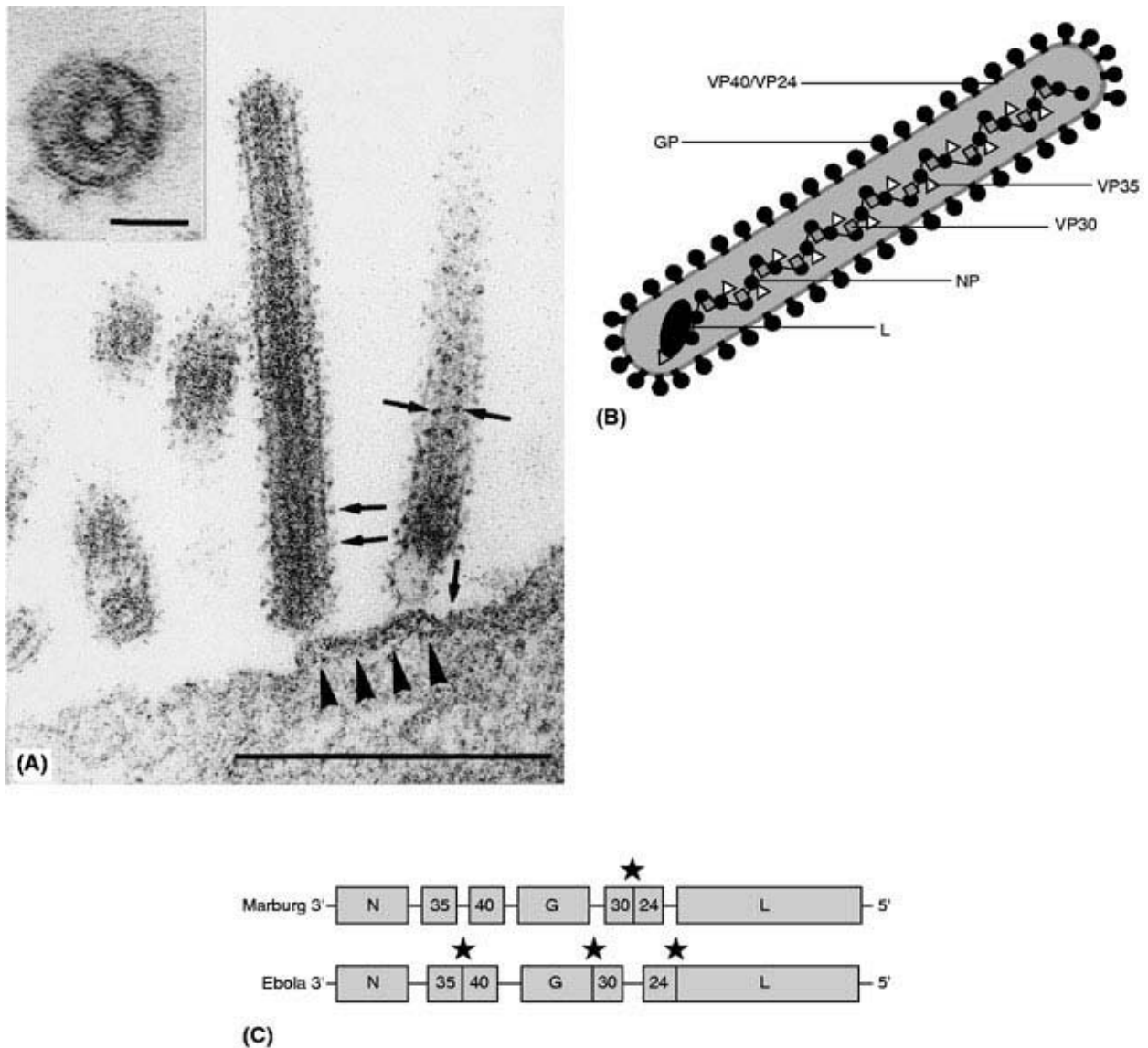


Figure 1 Characteristics of filoviral particles. (A) Electron micrograph. Budding of Marburg virus particles from the plasma membrane of infected primary cultures of human endothelial cells. Particles consist of a nucleocapsid surrounded by a membrane in which spikes are inserted (arrows). The nucleocapsid contains a central channel (inset). The plasma membrane of infected cells is often thickened at locations where budding occurs (arrowheads). Ultrathin section – bar 0.5 μm ; bar inset, 50 nm. (B) Four proteins are involved in the nucleocapsid formation; polymerase or large (L) protein (ellipse), nucleoprotein (NP) ($\bullet$), virion structural protein (VP) 30 ($\square$) and VP35 (Δ). The glycoprotein (GP) is a transmembrane protein and anchored with the carboxy-terminal part in the virion membrane. Homotrimers of GP form the spikes on the virion surface (arrows in (A)). VP40 and VP24 are membrane-associated proteins. (C) Genome organization of filoviruses. Filoviral genomes consist of a single, negative-stranded, linear RNA molecule. Differences in organization between viruses of the Marburg and Ebola species are indicated. *, Position of gene overlap; Ebola, isolate Mayinga, subtype Zaire; G, glycoprotein gene; L, polymerase (L) gene; Marburg, isolate Musoke; N, nucleoprotein gene; 24/30/35/40, virion structural protein (VP) genes.

may function as a cofactor to the L-protein, the major nucleoprotein NP encapsidating the genome, and VP30 that may be a second nucleoprotein. The envelope consists of a lipid membrane derived from the plasma membrane of the host cell and a transmembrane glycoprotein GP which forms the spikes at the surface of the virion. There are two additional

membrane-associated proteins, the matrix proteins VP40 and VP24 of unknown function. Replication and transcription of predominantly monocistronic mRNA occur in the cytoplasm, as is the case with most nonsegmented negative-stranded RNA viruses. Full length GP of Ebola virus is expressed from two open reading frames by a mechanism of transcrip-

tional editing. A nonedited transcript of the GP gene yields a small soluble glycoprotein. Synthesis of Marburg virus GP does not require such an editing mechanism.

Clinical syndrome and pathogenesis

Transmission by intimate contact with skin and secretions of infected patients is the main route of filovirus infection for humans. After 4–16 days of incubation there is an abrupt onset of disease with fever, headache, anorexia and myalgia, followed by nausea, vomiting, sore throat, abdominal pain and diarrhea. At their first clinical examination, patients usually show already signs of severe illness with dehydration, apathy and desorientation, and may develop a characteristic nonpruritic, maculopapular centripetal rash. Hemorrhagic manifestations develop during the peak of the illness. Bleeding into the gastrointestinal tract is most prominent, along with petechiae and hemorrhages from puncture wounds and mucous membranes. Death occurs between days 7 and 16 after onset of disease and is usually associated with severe hemorrhages and hypovolemic shock. Mortality ranges between 20% (Marburg) and almost 90% (Ebola-Zaire). With Ebola-Reston, there is no evidence for human pathogenicity.

Pathophysiological changes that make filovirus infections so devastating are just beginning to be unraveled. Clinical and biochemical findings support the anatomical observations of extensive liver involvement, renal damage, changes in vascular permeability, including endothelial damage, and activation of the clotting cascade. The visceral organ necrosis is a consequence of virus replication in parenchymal cells. However, no organ, not even liver, shows sufficient damage to account for death. The role of disseminated intravascular coagulation (DIC) in pathogenesis is still controversial, as a laboratory confirmation of DIC in human infections has never been demonstrated.

Laboratory parameters in the crucial early stage of filovirus hemorrhagic fever (HF), such as high AST/ALT (aspartate aminotransferase/alanine aminotransferase) ratio, normal bilirubin levels, and marked lymphopenia followed by a dramatic neutrophilia with left shift, suggest extrahepatic targets in the infection. As with some other HFs (hemorrhagic fever with renal syndrome (HFRS), dengue HF, Lassa fever), fluid distribution problems and platelet abnormalities are dominant clinical manifestations indicating dysfunction or damage of endothelial cells and platelets. At postmortem examination there is little monocyte/macrophage infiltration in sites of

parenchymal necrosis, suggesting that a dysfunction of white blood cells, such as macrophages, also occurs. Morphological studies on Ebola-Reston infected monkeys from the 1989 epizootic and monkeys experimentally infected with Ebola-Zaire showed that monocytes/macrophages and fibroblasts may be the preferred sites of virus replication in early stages, whereas other cell types may become involved as the disease progresses. Human monocyte/macrophages in culture are also sensitive to infection, resulting in massive production of infectious virus and cell lysis. Although, the studies on infected non-human primates did not identify endothelial cells as sites of massive virus replication, *in vitro* studies and autopsy observations of human cases clearly demonstrated that endothelial cells of human origin are suitable targets for virus replication. Here, infection leads to cell lysis, indicating that damage to endothelial cells may be an important pathophysiological parameter during infection.

Besides evidence for direct vascular involvement in infected hosts, the role of active mediator molecules in the pathogenesis of the disorders needs to be discussed. Although the source of these mediators during filovirus infections is still unknown, candidate cells exist. In addition to the endothelium, which has yet to be examined for production and secretion of such mediators, the common denominator remains the monocyte/macrophage, a cell type shown to be a major site of virus replication *in vivo* and *in vitro*. Monocytes/macrophages are known as a pivotal source of different proteases, H_2O_2 , and mediators such as tumor necrosis factor α (TNF α), interleukin 6 and 8, and platelet-derived growth factor (PDGF). TNF α can result in secondary activation of mediators with important protective as well as deleterious effects. Recently it has been demonstrated that supernatants of filovirus-infected monocyte/macrophage cultures are capable of increasing para-endothelial permeability in an *in vitro* model. Examination for mediators in those supernatants revealed increased levels of secreted TNF α . These data support the concept of a mediator-induced vascular instability, and thus increased permeability, as a key mechanism for the development of shock syndrome seen in severe and fatal cases. Thus the syndrome may be comparable to shock symptomatic in response to various endogenous and exogenous mediators. The bleeding tendency could be due to endothelial damage caused directly by virus replication as well as indirectly by cytokine-mediated processes. The onset of the bleeding tendency is supported by the loss of the integrity of the endothelium, as demonstrated in tissue and organ culture and infected animals. The hemorrhage occurs later in

infection and could be due to extended damage which cannot be repaired by wound-healing mechanisms. The combination of viral replication in endothelial cells and virus-induced cytokine release from monocytes/macrophages may also promote a distinct proinflammatory endothelial phenotype that then triggers the coagulation cascade. A model of these pathophysiological events is shown in Figure 2.

Immune responses of the host

Fatal filovirus infections usually end with high viremia and no evidence of an effective immune response. In monkeys infected with Ebola–Reston virus nonprotective antibodies have been observed shortly before death. Altogether, however, the data available to date do not support an important role of neutralizing antibodies in virus clearance. Since circulating monocytes/macrophages are primary target cells in filovirus infections, and since the extensive disruption of the parafollicular regions in spleen and lymph nodes results in the destruction of the antigen-presenting dendritic cells, cellular immunity appears also to be affected during filoviral HF. In addition to these cytolytic effects, there are several other mechanisms by which filoviruses may interfere with the immune system. Firstly, the high carbohydrate content of Marburg and Ebola virus GP may suppress its immune reactivity. Secondly, the non-structural glycoprotein that is secreted from cells infected with Ebola virus may interfere with the

virus-directed immune response. Thirdly, a fragment of spike GP which is also secreted in large amounts from infected cells may have similar effects. Fourthly, filovirus GP displays a sequence motif homologous to an immunosuppressive domain observed in retroviral glycoproteins. Altogether, there is now a large body of evidence indicating that filoviruses induce immunosuppression in the infected host, which appears to be a major factor for the rapid generalization and the severity of the disease.

Vaccines

Although neutralizing antibody titers in human convalescent sera can barely, if at all, be detected in laboratory tests, there are anecdotal case reports suggesting the potential benefit of passive immunization against Ebola virus infection. Furthermore, recent reports from the 1995 outbreak in Zaire, about effective treatment of acutely ill patients with whole blood transfusions from convalescent donors, suggest that quantities of antibodies, predicted to be marginally effective in laboratory tests, may still be protective. A study in which Ebola virus-infected monkeys have been treated with equine hyperimmune serum indicated that passively acquired antibody can also have a beneficial effect in reducing the viral burden. Effective treatment of human patients, however, may require antibodies with higher specific activities and more favorable pharmacokinetic

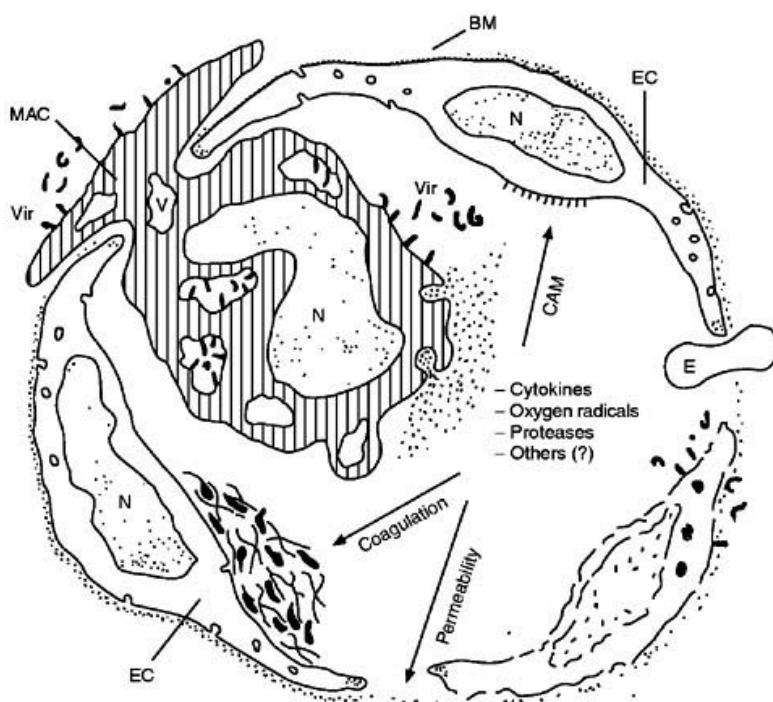


Figure 2 Possible role of macrophages and endothelial cells in the development of hemorrhagic fever caused by filoviruses. EC, endothelial cell; MAC, macrophage; Vir, virus particle; CAM, cell adhesion molecule; E, erythrocyte; BM, basement membrane; N, nucleus; V, vacuole.

properties than the presently available equine immunoglobulin.

There is experimental evidence that active immunization employing killed virus and recombinant Ebola and Marburg virus glycoproteins is partially successful in animals, suggesting that this may be a feasible strategy for eliciting protective immunity. At present, however, vaccines for human application are not available. It should also be mentioned in this context that filoviruses are relatively resistant to the *in vitro* antiviral action of interferon, and prophylactic interferon administration to monkeys has failed to increase survival or reduce virus titers, although a slight prolongation of time to death was observed. Finally, no antiviral drugs are available for filovirus treatment either.

See also: **Paramyxoviruses, infection and immunity;**

Rhabdovirus, infection and immunity; Viruses, immunity to.

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FISH IMMUNE SYSTEM

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It is important to realize that among the Vertebrata, the class Pisces has within it the oldest and most numerous species. The most recent and evolutionarily advanced group of fish, and the one of most commercial importance, is the teleosts (bony fish), which first appeared in the late Devonian Age, 300 million years ago, and rose to prominence 70 million years ago. Now they are the most numerous with over 20 000 extant species (compared with the 4500 species of mammals) occupying very diverse aquatic habitats. It is to be expected, therefore, that interspecies differences will exist in the immune system of fish. Most of the available information appertains to the teleosts, and in particular to a few species of commercial importance in aquaculture. Differences among these species are not considerable and this entry presents a generalized picture of the teleosts only, there being no attempt to draw attention to anatomical, cellular or molecular differences which exist between teleosts and other groups of fish for which data exist, e.g. the hagfish and lamprey (Agnatha) or the sharks and rays (Chondrichthyes).

Functional anatomy of the fish immune system

The primary and secondary lymphoid tissues of fish

are located in the thymus, kidney and spleen (**Figure 1**). Of course, the whole integument of fish, including the skin, is a mucous membrane and contains immune cells and secretions. The gills are important in antigen uptake (see Vaccination of Fish below). Fish do not possess lymph nodes or organized gut-associated lymphoid tissues, although the gut and gill do contain lymphoid and antibody secreting cells.

The *thymus* is a paired organ located dorsolaterally in the gill chamber. In ontogeny it is the first organ to become lymphoid and arises within the epithelium, external to the basement membrane, between the gill arches. In young fish it is covered externally only by a single layer of epithelial cells. With age, the thymus becomes surrounded by a thin connective tissue capsule which gradually invades the lymphoid tissue in a slow process of involution. In most species the thymus retains a very superficial position.

In structure and function the fish thymus is similar to that of mammals except in some details. It is composed mainly of lymphocytes in various stages of development and division and a few epithelial-type cells and macrophages are also present. However, differentiation into cortex and medulla and presence of Hassell's corpuscles are not marked. Blood vessels within the organ possess a specialized endothelium

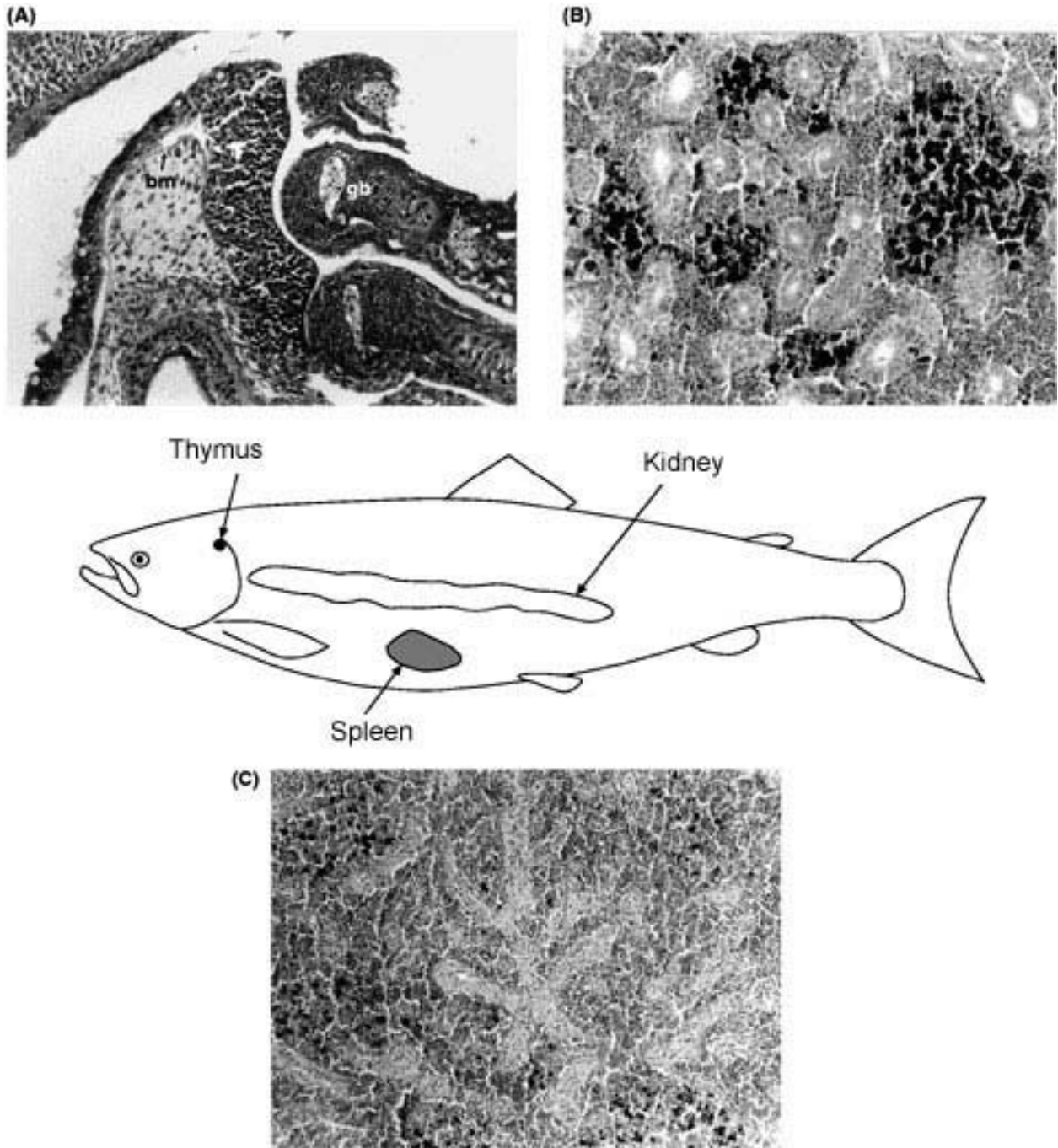


Figure 1 Lymphoid organs of teleost fish. (A) The thymus insert is that of Atlantic salmon eight days pre-hatch. The thymocytes develop within the pharyngeal epithelium, external to the basement membrane (bm). The organ is covered externally by a single layer of squamous epithelium. gb, primary gill bars. (B) The kidney insert is that of the plaice, a flatfish, showing the melanomacrophage centers and kidney tubules surrounded by hematopoietic tissue. (C) The spleen depicts the branching ellipsoid system within the splenic pulp.

with tight junctions. There is considerable evidence to indicate that the fish thymus, as in higher vertebrates, is a primary lymphoid organ. Such evidence includes the exclusion of foreign particles and anti-

gens present in the circulation, the exclusion of circulating lymphocytes, the high mitotic rate of thymocytes, which is presumably antigen independent, the absence of antibody-producing cells, the migration

of thymocytes to other lymphoid organs and the fact that the relative size of the organ is greatest in young fish. Data appertaining to lymphocyte subpopulations indicate that the thymocytes of fish are similar in many respects to the T cell lineage in mammals (see below). Unfortunately the crucial experiments of early thymectomy to show ablation of T cell function have proved difficult to perform in fish as the organ contains lymphocytes at or soon after hatching.

The *kidney* is the major hematopoietic and immunologically reactive organ in teleost fish. The organ is located ventral to the spinal chord and extends the full length of the peritoneal cavity. The anterior tip (so-called 'head kidney' or pronephros) is constituted entirely of hematopoietic and lymphoreticular tissues, which, in the rest of the organ (opisthonephros), occupy the intertubular spaces. The hematopoietic tissue bears close resemblance to the bone marrow of higher vertebrates but differs in having a highly active reticuloendothelial system and is the major antibody-producing tissue of fish. In this respect it has functional similarity to mammalian lymph nodes. However, there is no anatomical differentiation of the lymphoid tissue and no primary or secondary lymphoid follicles in fish. Within the hematopoietic tissue are many pigment-containing cells, termed melanomacrophages. These macrophages contain melanosomes and to a greater or lesser degree ceroid and lipofuscin.

In primitive teleosts (e.g. salmonids) the pigment cells are scattered throughout the tissue, while in advanced species (e.g. flatfish and cyprinids) they form aggregates, termed melanomacrophage centers, which possess a delicate reticulin fiber capsule and contain other cell types, particularly lymphocytes and pyroninophilic cells. Foreign materials (e.g. carbon particles) in the circulation are actively phagocytosed by cells of the reticuloendothelial system lining the hematopoietic sinuses. These phagocytes then migrate and aggregate within melanomacrophage centers.

The *spleen* of fish is mainly composed of red pulp sinuses, while the white pulp is poorly differentiated, forming a cuff of tissue surrounding melanomacrophage centers, similar to those in the kidney and the extensive ellipsoid system. The latter comprises the whole of the splenic arterial capillary network, the vessels of which are surrounded by a thick sheath of reticulin fibres, embedded in which are numerous macrophages which phagocytose foreign particulate matter in the blood and, like the reticuloendothelial cells of the kidney, then migrate and accumulate in the melanomacrophage centers. Following antigen stimulation, antibody-producing cells

appear in the spleen but are less numerous than in the kidney tissue.

The origin and function of melanomacrophage centers in fish kidney and spleen is far from clear but the available information suggests they may be primitive analogs of germinal centers in higher vertebrates. Following antigen stimulation, clusters of pyroninophilic cells appear in the kidney hematopoietic tissue and the splenic ellipsoid walls and it is thought these clusters develop into melanomacrophage centers. The latter are also focal points for the aggregation of replete macrophages, and studies on lymphocyte recirculation in fish have demonstrated that small lymphocytes also migrate to the centers. Furthermore, immune complexes are trapped by reticulin fibers in the spleen and kidney, especially within the splenic ellipsoids and melanomacrophage centers, where they are retained for several months. The function of germinal centers in mammals is concerned with immunologic memory and the finding that priming fish with immune complexes, besides resulting in immediate trapping within ellipsoids and melanomacrophage centers, also induces stronger immunologic memory than priming with antigen alone, suggests that the ellipsoid and melanomacrophage system of fish serves a similar function.

The role of melanin associated with phagocytes in fish is not clear but a possible explanation is that as fish tissue lipids are highly unsaturated they are particularly susceptible to oxidation by free radical reactions initiated by phagocytic cells. The melanosomes present in highly phagocytic tissues in fish may function to quench the free radical chain reactions and protect the cells from severe damage.

Lymphocyte subpopulations in fish

While convincing evidence now exists for lymphocyte heterogeneity in fish, the extent to which functional diversity among lymphocyte populations have evolved in fish is still not clear. What can be said is that a T and B lymphocyte dichotomy basically similar to that of higher vertebrates has evolved in fish. The evidence supporting this view rests upon studies of surface markers, mitogen and carrier-hapten responses, and cytokine production.

The presence of surface immunoglobulin (sIg) molecules, detected by labelled antisera to serum Ig, is a characteristic of mammalian B lymphocytes. In fish, such techniques stain all lymphocytes, including thymocytes. However, studies using monoclonal instead of polyclonal antibodies specific to serum Ig stain only about 30% of peripheral lymphocytes and do not stain thymocytes. The polyclonal antibody staining of fish thymocytes is thought to be due to carbo-

hydrate moieties on thymocyte membranes and serum Ig which cross-react. In combination with other data (see below), it seems likely that the fish B-like lymphocyte antigen receptor is sIg, as in higher vertebrates. The fish T-like lymphocyte antigen receptor is still uncharacterized but recent genetic studies have indicated that gene fragments coding for molecules homologous to mammalian T cell receptor β chains occur in the goldfish and salmonids and strongly suggest that the nature of the T cell receptor in fish is basically similar to that in all other vertebrates.

The use of monoclonal antibodies in conjunction with mitogen or carrier-hapten responses provide compelling evidence for the presence of T and B lymphocyte types in fish. Examples of such evidence include the correlation between the presence of sIg and responsiveness to lipopolysaccharides (LPS; a B cell mitogen) and the lack of sIg with responsiveness to concanavalin A (Con A, a T cell mitogen). A monoclonal antibody has been produced which reacts with channel catfish sIg⁻ but not sIg⁺ blood lymphocytes. The proportions of these cell types in the blood were 46 and 38% respectively. This monoclonal stained virtually all thymocytes. The population of peripheral blood lymphocytes reacting with this monoclonal antibody was also shown to respond to con A stimulation as well as act as T helper cells but not antibody-producing cells in *in vitro* primary hapten-specific antibody responses. Thus, this monoclonal antibody appears to react with cells possessing typical T lymphocyte functions. Conversely, the population of peripheral lymphocytes not reacting with this monoclonal but reacting positively with a monoclonal to serum Ig were capable, after separation by panning, of *in vitro* antibody production to the T-independent antigen TNP-LPS, but not to the T-dependent antigen TNP-KLH. Responsiveness to the latter was restored by the sIg⁺ population. The nature of the marker on sIg⁻ cells reacting with the above monoclonal has not been characterized but molecular weight studies and the fact that it also cross-reacts with certain brain cells suggests it may be similar to mammalian Thy-2.

Studies on separated sIg⁺ and sIg⁻ blood lymphocytes in trout have shown the latter, but not the former, to be capable of producing a factor with macrophage-activating and interferon activity.

Thus, with the use of monoclonal antibodies fish lymphocytes may be separated into distinct subpopulations with functions homologous to mammalian T and B cells.

Concerning the origin of T and B lymphocytes in fish, as virtually all thymocytes possess characteristics of T lymphocytes and none of B lymphocytes,

it seems likely that the thymus in fish is the principal source of T lymphocytes, as in higher vertebrates. Analysis of other lymphoid organs (i.e. kidney and spleen) show there to be a mixed population of T and B cells but both organs possess large quantities of blood which contains both T and B cells. However, as the only organ containing significant amounts of lymphopoietic tissue in teleost fish is the kidney hematopoietic tissue, and this is also the most important source of antibody-producing cells, it must be more than likely that the kidney is the source of B lymphocytes.

Other leukocytes and related cell types

Monocytes and *macrophages* are morphologically and cytochemically similar to their mammalian counterparts. Macrophages are widespread in tissues, including the gills, where they are thought to play an important role in vaccine uptake (see below); they form an extensive reticuloendothelial system in the kidney and spleen and, in some species, in the atrium of the heart. However, teleost fish do not possess Kupffer cells in the liver. Monocytes and macrophages are characterized by their nonspecific esterase staining and avid phagocytic activity. The latter is enhanced by complement and specific antibody, indicating the presence of opsonin receptors on these cell types. Their role in immune responses in fish as antigen-presenting cells and a source of interleukins is not yet well characterized. Channel catfish monocytes have been shown to function as accessory cells required for blood T lymphocytes to respond to the mitogen Con A. Furthermore, this function of monocytes could be replaced by the supernatant obtained from monocyte cultures stimulated with LPS, indicating the production of a 'monokine'. Channel catfish monocytes also perform an essential accessory cell function in the *in vitro* primary antibody response of peripheral blood lymphocytes to both T-independent (TNP-LPS) and T-dependent (TNP-KLH) antigens. In these studies the role of the monocyte in stimulation of antibody-producing cells was replaceable by monocyte culture supernatants, suggesting that other cell types (possibly B lymphocytes) could effectively 'present' antigen in the presence of the monokines.

Fish macrophages can also be activated *in vitro* by exposure to supernatants from mitogen-stimulated lymphocytes, indicating the responsiveness of macrophages to interleukins.

Granulocytes are numerous in the circulation of fish and play an important role in inflammatory responses. The neutrophil is the most common type of granulocyte and is well characterized, bearing

close similarity in cytochemistry to mammalian neutrophils. It is phagocytic but not avidly so and its role in defense against bacterial infection may be to secrete antimicrobial enzymes rather than perform intracellular killing. Granulocytes with the morphological and staining characteristics of eosinophils and basophils are not common in teleost fishes and, where they do occur, nothing is known about their function.

Mast cells with precisely the same characteristics as in mammals do not appear to exist in fish. However, a granulocytic cell with a connective tissue distribution similar to that of mast cells in mammals does exist and it is believed to be the analog of mast cells. This cell possesses eosinophilic or PAS-positive granules and ultrastructurally resembles mast cells. They have been termed eosinophilic granular cells (EGCs). *In vivo* degranulation can be induced by certain bacterial toxins as well as typical mast cell degranulating agents such as compound 48/80 and Con A. Degranulation is associated with darkening in skin color of the fish, some loss of equilibrium, swelling and vasodilatation of the intestine and a return to normal by 24 hours. Degranulation is a noncytotoxic event with regeneration of granules by 48 hours. The physiologic changes associated with degranulation were blocked by prior treatment with the antihistamine drugs promethazine and cimetidine but, unexpectedly, so was the degranulation of the EGCs. In mammals, antihistamines block histamine receptors on target cells but do not inhibit degranulation of mast cells induced by 48/80 or con A.

Natural cytotoxic cells (NCCs) have been identified in a wide range of teleost species but they have not been well characterized and may represent a heterogeneous population of cells. They have cytotoxic effects on a wide range of tissue culture cells, especially those infected by virus, and activity against protozoan parasites has also been demonstrated, suggesting these cells play an important role in defense. NCCs are found in the peripheral blood and in some species have been demonstrated also in the thymus, kidney and spleen. Their activity can be enhanced in the presence of phytohemagglutinin. NCCs are non-adherent to nylon wool, and ultrastructural studies indicate the involvement of at least two cell types which resemble monocytes and lymphocytes morphologically.

The immune response

The basic elements of the humoral and cell-mediated immunity are present in fish but many parameters of these responses are not as well developed as in higher vertebrates. An important aspect of immune respon-

siveness in fish is temperature dependency. Different species of fish inhabit waters with very different natural temperature ranges and their physiologic responses are well adapted to these ranges. At temperatures low in the natural range a particular species of fish will respond immunologically very slowly, if at all, to an antigenic stimulus. Thus at 4°C the immune response of trout (a temperate water species) is very slow, while at 12°C the response is maximal. In contrast, the warm water carp has an optimal response at 22°C and is virtually immunosuppressed at 12°C.

Fish produce only a single class of immunoglobulin (Ig), which resembles mammalian IgM in molecular structure. However, while mammalian serum IgM is most frequently a pentameric molecule, that in the serum of teleosts is tetrameric and, in some species, monomeric. A secretory form of IgM has been characterized from the bile and mucus of skin and gut in a few species where it occurs in a dimeric form. These and other data indicate that local (secretory) and systemic responses exist in fish. Evidence for structural heterogeneity in fish IgM heavy and light chains is growing: for example, the existence of two forms of L chains which parallel the κ and λ chains of higher vertebrates and the different molecular masses of H chains among serum, bile and mucus Ig. Recent studies with monoclonal antibodies have revealed the existence of three forms of H chain in catfish serum Ig. Fish IgM appears to be capable of performing all of the functions for which the different mammalian Ig isotypes show specialization, e.g. complement activation and opsonization, and it is possible that these functions in fish may be carried out by different specialized subclasses of IgM. The kinetics of the *antibody response* in fish are similar to those of higher vertebrates but the lag phase is somewhat longer (10–15 days) and peak titers may take up to 5–6 weeks to be reached. Antibody persists for fairly long periods, particularly in primitive species such as trout, where persistence for up to 1 year has been reported for antibodies to certain bacterial antigens.

The correlates of cell-mediated immunity are present in fish – for example, mixed leukocyte reactions, macrophage migration inhibition, interleukin production inducing macrophage activation, delayed type hypersensitivity and acute skin rejection responses. Allogeneic restriction of antigen presentation and the existence of MHC genes in fish have been demonstrated.

Evidence for lymphokine production in fish is well established and while no fish cytokines have yet been purified, evidence indicates the existence of several types, for example interferon γ (IFN γ), interleukin 1

(IL-1), IL-2, colony-stimulating factor (CSF), transforming growth factor β (TGF β) and tumor necrosis factor.

Immune memory has been demonstrated in fish for both humoral and cell-mediated immunity. However, the magnitude of antibody titers reached in the secondary response as compared with the primary response in fish is generally lower than in mammals (about 10-fold higher, compared with 100-fold in mammals). Of course, fish differ from higher vertebrates in that there is no shift of antibody isotype from IgM to IgG. Typically, memory induction takes longer to be established in fish (3–6 months) than in mammals and persists for 8–12 months after primary stimulation. Induction of memory appears to be temperature dependent. Generally speaking, immune responses in fish which involve the stimulation of T lymphocyte functions appear to require temperatures fairly high in the natural range of a particular species, while antibody production itself can occur at lower temperatures. Some doubt has been expressed as to the ability of temperate species, such as trout, to mount a significant secondary antibody response and it may be that these species rely instead upon prolonged production of antibody to provide long-term protection.

Immune tolerance has been demonstrated in several species of fish. The carp anti-BSA response has been suppressed by intravenous injection of soluble BSA. The induction of tolerance at high temperature was dependent upon the route of injection (intravenous) and not on the dose, while at low temperature it depended both on the intravenous route and high dose. Tolerance in these experiments lasted at least 16 months. Evidence also exists for induction of short-term tolerance by exposing very young fish to certain antigens.

Vaccination of fish

Since the great expansion of fish farming, which began in the early 1970s, there has been much interest in vaccinating fish against the principal bacterial and viral diseases. Effective vaccines have been developed to many fish diseases but only a few have achieved commercial production. The cost constraints on commercial application principally relate to the size of the fish requiring protection and the methods available for effective administration of a vaccine. There are three methods of vaccinating fish: injection, immersion and oral. Many vaccines are only effectively delivered by injection and this method is impractical for small fish (under 15 g). Presently there are only a few vaccines available commercially. These are against important bacterial dis-

eases of farmed salmonid fish, i.e. enteric redmouth (ERM) caused by *Yersinia ruckeri*, vibriosis caused by *Vibrio anguillarum*, cold-water vibriosis caused by *Vibrio salmonicida*, and furunculosis caused by *Aeromonas salmonicida*. These vaccines are effectively delivered by both injection and immersion administration.

Injection vaccination appears to be the most effective means of stimulating protective immunity. The method allows the use of adjuvants but it has a number of disadvantages: it requires fish to be handled and anaesthetized, which are stressful events; it is impractical for small fish; and it is labour and time consuming. Nevertheless, when the disease risks are high injection vaccination is cost effective and frequently practised. Recently, injection machines have been marketed.

Immersion vaccination has proved very useful for a number of bacterial vaccines. These vaccines are formalin-killed broth cultures which are diluted in water and the fish are immunized simply by immersing them, with the use of a hand net, for up to 1 min in the vaccine. It is believed that the vaccine is taken up mainly through the gills. The technique is very useful for vaccinating small fish with the minimum of handling.

Oral vaccination is the method of choice because it does not require the fish to be handled and in this respect is the only method which could be applied to extensive fish farming where the fish are not handled until slaughter. Unfortunately, this method has not proved to be very efficacious. Nevertheless, there is still much interest in the method because administration of vaccines by anal intubation appear to be very effective. Recent studies have demonstrated that, in carp and trout, the hind intestine is capable of antigen absorption, which results in effective stimulation of protective immunity; thus, interest is now centered upon microencapsulation of oral vaccines to protect the antigens during their passage through the anterior gut and to release them in the hind gut.

There is a growing interest in using recombinant DNA technology to achieve cheap production of viral and parasite antigens for use in vaccinating fish. There is still very little known concerning the nature of the protective antigens or the protective immune response to most vaccines used in aquaculture: the future of fish vaccination will probably depend very largely upon identifying the nature of these so as to optimize the production of vaccines and their effective administration. Fish farming is rapidly becoming a major industry in many countries of the world and there will be a growing need for vaccines to control disease in a wide range of fish species in the future.

See also: Immunoglobulin, evolution of; Innate immunity; Phylogeny of the immune response.

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FLAVIVIRUS, INFECTION AND IMMUNITY

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The family Flaviviridae includes 69 viruses, the majority of which are transmitted by mosquitoes and ticks. Some are zoonotic agents without known arthropod vectors. More than half of these arboviruses have been associated with human disease. The viruses are distributed worldwide in many distinct ecological patterns, and cause three principal syndromes in humans: febrile illness, encephalitis and hemorrhagic fever. The most important viruses are yellow fever (YF), dengue (DEN), Japanese encephalitis (JE) and tickborne encephalitis (TBE). Flaviviruses are enveloped RNA viruses containing a single linear RNA molecule of approximately 10 000 nucleotides, encoding three structural and at least seven nonstructural proteins (Figure 1). The genome is encapsidated by a core protein and enclosed within a lipid envelope containing the M and E proteins. The E protein is critical for infection and immunity, as it contains cellular receptor-binding determinants, a membrane fusion activity and epitopes for neutralizing antibodies.

Characteristics of flaviviruses

Flaviviruses have been traditionally classified on the basis of antigenic cross-reactivity of the E protein, including both neutralization and hemagglutinating properties. Group-reactive, serocomplex-reactive and type-specific determinants have been described, although these underrepresent the antigenic relation-

ships of many viruses. Eight different antigenic groups can be defined (Table 1).

YF, the prototype of the flavivirus family, is antigenically distinct and classified with 16 other viruses which are serologically unrelated to defined groups. YF is a zoonotic disease which causes hemorrhagic fever in subsaharan Africa and tropical regions of South America. Up to a few hundred cases in South America, and several thousand cases in Africa have been reported annually.

JE virus, the prototype of the JE serogroup, is distributed throughout many countries of Asia, and is responsible for 30 000–40 000 cases annually in China, with an associated mortality rate of approximately 30%. It is the most important arthropod-transmitted viral encephalitis worldwide. Other JE serocomplex viruses are also important human pathogens.

The tickborne encephalitis (TBE) virus complex includes 14 viruses, of which eight are known human pathogens. TBE occurs in regions of Europe and the former Soviet Union where the ixodid tick vector is prevalent and is a serious disease with variable mortality depending on the subtype of virus and age of onset in clinical cases. Two viruses within the TBE complex cause hemorrhagic fever.

The dengue (DEN) antigenic complex includes four serotypes associated with dengue fever (DF), and dengue hemorrhagic fever/shock syndrome (DHF/DSS). Together these cause large epidemics

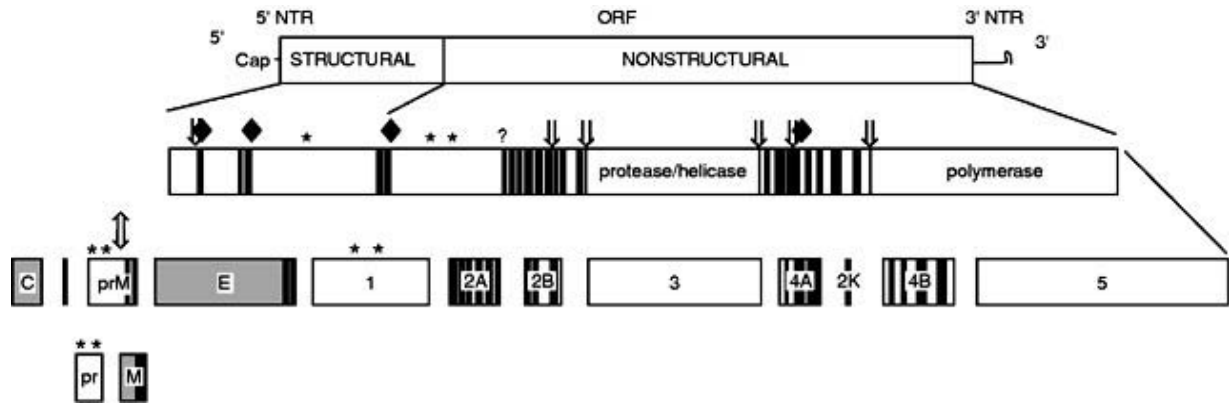


Figure 1 Structure of the flavivirus genome and processing of the viral polyprotein. At top is the organization of the genome common to all family members. Cap, 5' cap structure; 5' NTR and 3' NTR, untranslated regions of the genome; ORF, open reading frame. Symbols indicate proteases involved in processing viral proteins as follows: solid diamonds, host signalase; single open arrows, viral protease; question mark, uncharacterized host endoplasmic reticulum protease; double open arrows, host Golgi protease. Asterisks indicate *N*-linked glycans on prM and NS1. The viral structural proteins (C, capsid; prM, precursor to M; E, envelope) are shaded light, and nonstructural proteins (numbered 1–5) are unshaded. Hydrophobic regions of the polyprotein are shaded dark. (From Fields *Virology*, 3rd edn (1996), Lippincott-Raven Press, Philadelphia, courtesy of Dr Charles M Rice.)

Table 1 Antigenic groups of the flaviviruses

Group	Members	Principal viruses	Arthropod vector	Disease in humans	Significance ^a	Vaccine available
Tickborne encephalitis (TBE)	12	Russian spring summer (RSSE); Central European (CEE); Omsk hemorrhagic fever (OHF); Kyasanur Forest disease (KFD)	Ticks	Encephalitis (RSSE, CEE); hemorrhagic fever (OHF, KFD)	Moderate	TBE, KSD
Rio Bravo (RB)	6	Rio Bravo (RB)	None	Febrile illness (RB)	Minor	
Japanese encephalitis (JE)	10	JE; St Louis (SLE); Murray Valley (MVE); West Nile (WN); Kunjin (KUN)	Mosquito	Encephalitis (JE, SLE, MVE, KUN); febrile illness (WN)	JE: major; others: moderate	JE
Tyulenyi	3	Tyulenyi	Tick	No		
Ntaya	5	Ntaya	Mosquito	No		
Uganda	4	Uganda, Brazil (B), Edge Hill (EH)	Mosquito	Febrile illness (B, EH)	Minor	
Dengue (DEN)	4	DEN serotypes 1–4	Mosquito	DF; DHF/DSS	Major	No ^b
Modoc	5	Modoc (M)	None	Aseptic meningitis (M)	Minor	
Ungrouped	17	Yellow fever (YF) Rocio (R), Zika (Z), Wesselsbron (W), Ilheus (I)	Mosquito (YF, R, Z, W, I)	Hemorrhagic fever (YF); encephalitis (R); febrile illness (Z, W, I)	YF: major; others: moderate-minor	YF

Data in part from Calisher *et al* (1989) *Journal of General Virology* 70: 37–43, and Karabatsos N (ed) (1985) *International Catalog of Arthropod-borne Viruses*, 3rd edn, San Antonio: American Society for Tropical Medicine and Hygiene.

^aBased on number of human cases reported/estimated; as in Monath TP (1986) *Pathobiology of the flaviviruses*. In: Schlesinger S and Schlesinger MJ (eds) *The Togaviridae and the Flaviviridae*, pp 375–440. New York: Plenum Press.

^bDengue vaccine is under development (see text).

throughout tropical regions of Asia, Africa, Australia and Central and South America. Estimates of the annual global disease burden include up to 100 million cases of DF and 250 000 cases of DHF. Primary DF is an acute self-limited febrile illness which induces protection against homologous but not heterologous serotypes. Sequential infections can pro-

duce severe disease (DHF/DSS). The incidence of DHF has risen dramatically in Asia, and it now poses a serious public health threat in the Western Hemisphere due to introduction of multiple serotypes and re-establishment of the *Aedes aegypti* vector throughout this region.

Nucleotide sequence comparisons of flavivirus E

proteins have been used to construct phylogenetic trees which provide a more uniform classification scheme than those based on serology, as well as insight into virus evolution. Flaviviruses may have originated in central Africa from a rudimentary insect virus, the cell-fusing agent, several thousand years ago. Westward spread into the Southeast Asian basin and across the former Soviet Union and Europe was accompanied by emergence of many encephalitic viruses of the tickborne and mosquito-borne groups. Emergence of hemorrhagic fever caused by YF and DEN probably occurred within the last 500 years, with adaptation of these viruses to lymphoid cells and changes in their animal reservoirs and mosquito vectors. Nucleotide sequence comparisons have also revealed geographic variation of DEN, JE, and YF viruses. The relationship of genotypic differences to epidemiology and pathogenesis is under investigation, particularly for DEN for which a serotype 2 strain has been associated with recurrent outbreaks of DHF in the Americas.

Host factors can affect susceptibility to flavivirus infection. Resistance to flaviviruses in mice is associated with an autosomal dominant allele (the *Flv* locus) which restricts viral replication by an unknown mechanism. This may involve cellular proteins which bind to stem-loop structures in the 3' untranslated region of viral RNA. Other host factors relevant to disease susceptibility include the haplotype-restricted immune responses observed in mouse models and the association of certain HLA alleles with occurrence of severe dengue infection among humans. Virus-specific proteins involved in virulence have been inferred from nucleotide sequence com-

parisons of virulent and attenuated strains, such as the Asibi strain of YF and its vaccine derivative, YF17D. The most-well characterized is the viral envelope (E) protein. Determinants within other regions of the genome, including the 5' and 3' untranslated regions, and the prM and NS1 proteins can also affect virulence properties. Whether these act by direct effects on viral replication or by affecting interactions with host factors requires further study.

Solution of the three-dimensional structure of the E protein for TBE virus has provided a model for all flavivirus E proteins. This protein is oriented on the virion surface as flat elongated dimers, each subunit of which contains three distinct domains (Figure 2). Epitopes for neutralizing antibodies are distributed widely over the protein, and are predominantly conformation dependent. Domain I (central domain) contains a conserved glycosylation site and is connected to the long looped structure of domain II (dimerization domain) by a hinge region. The distal end of domain II contains a conserved sequence proposed to be a fusion peptide. Fusion is associated with low pH-induced conformational changes within domains I and II that lead to reorganization of subunits as a trimer and changes in epitopes for neutralizing antibodies within these domains. Domain III has the fold of an immunoglobulin constant domain and contains a putative cellular receptor attachment site. The mechanisms of antibody neutralization probably involve interference with either the dimer-trimer transition, or virus-cell interactions governed by domain III. Amino acid residues which affect virulence have been studied with monoclonal

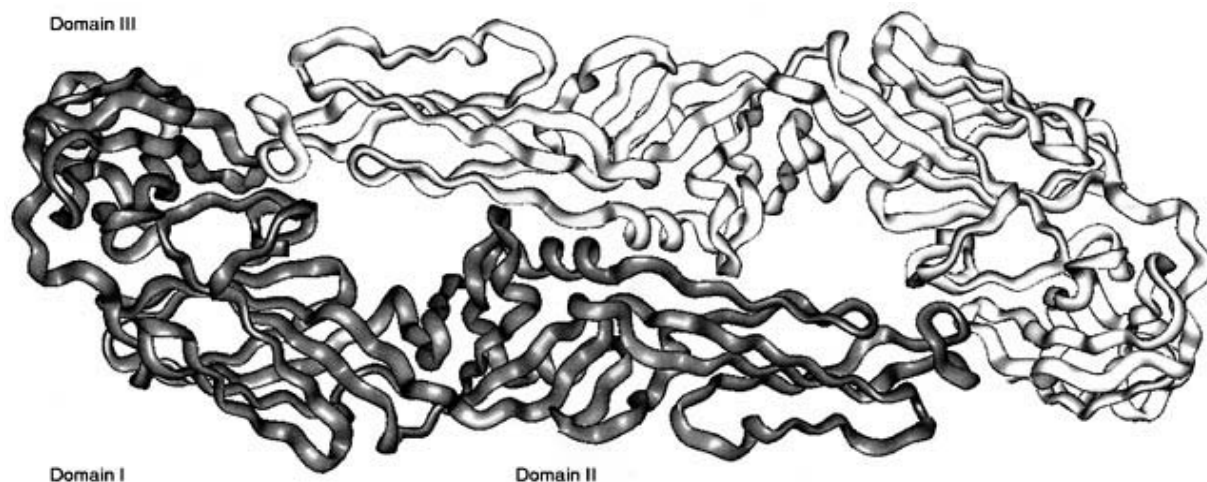


Figure 2 Structure of the flavivirus E (envelope) protein. Shown is the ribbon diagram of the model derived from the X-ray crystallographic structure of the soluble fragment of the TBE envelope glycoprotein (Rey *et al* (1995) *Nature* 375: 291–298). The figure depicts the antiparallel arrangement of two individual subunits (shaded light or dark) of the dimer as would be seen from a top view. Domains are as referred to in the text. (Courtesy of Dr Franz X Heinz.)

antibody neutralization-escape mutants, host-restricted mutants, virulence revertants and viruses engineered with molecular clones. Such residues cluster to specific sites within the E protein, which include the lateral surface of domain III (cellular receptor binding domain), the external surface of domain I, the domain I–II interface (hinge region), the distal loop of domain II (fusion peptide), and the adjacent conserved N-linked carbohydrate attachment site. It is presumed that substitutions at these sites affect early virus–cell interactions during entry and uncoating.

Immune responses to flaviviruses

Immune responses to flaviviruses have been investigated in both encephalitic and hemorrhagic models of disease. Although the mouse has been used for studies of the encephalitic viruses, there is no animal model for dengue and studies of YF pathogenesis require nonhuman primates. Understanding of these latter two diseases is therefore limited. In mice, virus entry into the central nervous system (CNS) probably occurs by hematogenous dissemination, based on correlations between viremia and the onset of encephalitis and the observation that damage to the blood–brain barrier facilitates neuroinvasion. Such damage has been demonstrated in dengue infection of mice. Susceptibility to encephalitis in mice is age dependent and may involve both immaturity of the blood–brain barrier in young animals and a greater sensitivity of developing neurons to virus-induced cell death. Although flaviviruses are typically cytopathic viruses in mammalian cells, both DEN and JE can induce apoptosis in certain cell types *in vitro*. The relationship of this phenomenon to pathogenesis in animal models and natural infections requires further study.

Adoptive transfer, immune abrogation/depletion experiments and studies in immunodeficient animals have been used to infer the roles of immune responses against flaviviruses. Important virus–host interactions have been described at the level of non-specific host defense systems. Macrophages are involved in clearance of viremia, but antiviral functions and susceptibility to infection may depend on their activation state and levels of virus-specific antibodies. This is particularly relevant during dengue infection (discussed below). *In vitro*, TBE virus-infected macrophages have been shown to be impaired in antiviral effector systems such as production of nitric oxide, which has been reported to have antiviral activity against flaviviruses. Impairment of natural killer (NK) cell function is associated with West Nile (WN) virus infection and may result from induction of class I antigen expression on

infected cells. This effect has been proposed as a viral strategy for early immune system escape and subsequent cytotoxic T lymphocyte (CTL)-mediated release of virus from infected cells.

Virus-specific antibody responses have been clearly shown to provide protection against flavivirus disease. Antibodies to the E protein are protective in a variety of animal models, and are probably the major component of immunity in natural infections. Prior flavivirus infections are believed to ameliorate or protect from subsequent infections with heterologous viruses by stimulating group-specific antibodies. This may partially explain the failure of YF to cause disease in Asia, where dengue virus is widely prevalent. The flavivirus NS1 protein also induces protective antibodies. Active immunization with NS1 or passive administration of antibody can provide varying levels of protection. NS1 is found as cell surface and secreted forms, including oligomers which contain multiple conformation-dependent epitopes. Protection by anti-NS1 immunoglobulin G (IgG) is dependent on an intact Fc portion and is governed by antibody isotype, but the mechanism is not known. Cell surface NS1 can mediate complement-dependent lysis of virus-infected cells, although no requirement for complement during protection *in vivo* has been demonstrated. Antibody-dependent cellular cytotoxicity (ADCC) has been observed for dengue virus and is proposed as a possible mechanism.

The functions of specific T cells during protection against encephalitis remain unclear and the balance of antibody versus cell-mediated immune responses may influence the occurrence of disease within the CNS. T cells adoptively transferred into unimmunized mice can protect against lethal encephalitis, and both nude and SCID mice are susceptible to flavivirus strains which are nonpathogenic in normal animals. This suggests that cellular immunity is required for control of infection. Although both NK cells and virus-specific cytotoxic lymphocytes have been recovered from WN-infected mouse brain, their cellular targets and antiviral functions within the CNS have not been directly demonstrated. A requirement for both CD4⁺ and CD8⁺ T lymphocytes for protection against JE in mice has been reported, but the effector functions of these T cells *in vivo* are also not known. This is a relevant question because of the possibility of flavivirus persistence, which may occur in animal infections and has been suspected in some cases of chronic CNS disease in humans. Both CD4⁺ and CD8⁺ T cells have been detected in human brain during JE infection, and both IgG and immunoglobulin/virus complexes have been found in human cerebrospinal fluid. This suggests that neu-

tralization by virus-specific antibody is an important protective mechanism within the CNS, but cellular immune responses may also be required for virus clearance.

Molecular determinants of virus-specific immunity have been best studied for members of the JE serogroup (WN, JE, MVE and KUN) and the DEN viruses. Flavivirus-specific CTLs have been demonstrated in many mouse models, and cross-reactivity of such responses against related flaviviruses has repeatedly been observed. Antigenic determinants involved in CD8⁺ CTL activation and lysis have been studied in mice using KUN virus. Determinants within the nonstructural proteins NS3 and NS4A/4B induced the strongest responses, many of which were cross-reactive with the related virus WN. Although few overall determinants are involved, the fine specificity of responses is markedly haplotype restricted. Despite cross-reactivity at the level of target cell recognition, this does not necessarily apply to the induction of memory CTL responses in response to heterologous viruses. The implications of these findings for cross-protection versus induction of immunopathology during sequential flavivirus infections have been emphasized. Studies on the molecular basis for murine CD4⁺ class II-restricted responses for KUN revealed a predominance of reactivity only to the structural and NS5 proteins. Haplotype differences in both the levels of responses and the efficiency of different antigen-presenting cells for induction of T cell proliferation have also been described. If these observations apply to human T cells, then it is likely that detailed investigation of the HLA allele-specific responses to flavivirus antigens will be needed to fully understand the pathogenesis of encephalitis and guide the development of novel vaccines.

Virus-specific immune responses to dengue infection have been studied in mice and humans. Dengue is thought to be an immunopathologic disease (Figure 3), with its most severe form, dengue hemorrhagic fever and shock syndrome, dependent upon antibody-enhanced infection of mononuclear phagocytes involving Fc and possibly complement receptors. Cross-reactive antibodies induced by prior infection with a heterologous serotype are believed to enhance secondary dengue infections when present at subneutralizing levels, leading to a large virus burden in the monocyte/macrophage. Primary dengue infection also sensitizes serotype cross-reactive memory T cells for activation during the secondary infection, resulting in the release of inflammatory cytokines which promote the development of a capillary leak syndrome. In mice, CD4⁺ and CD8⁺ T cells having a variety of specificities (serotype-specific, serotype and flavivirus cross-reactive) are generated in

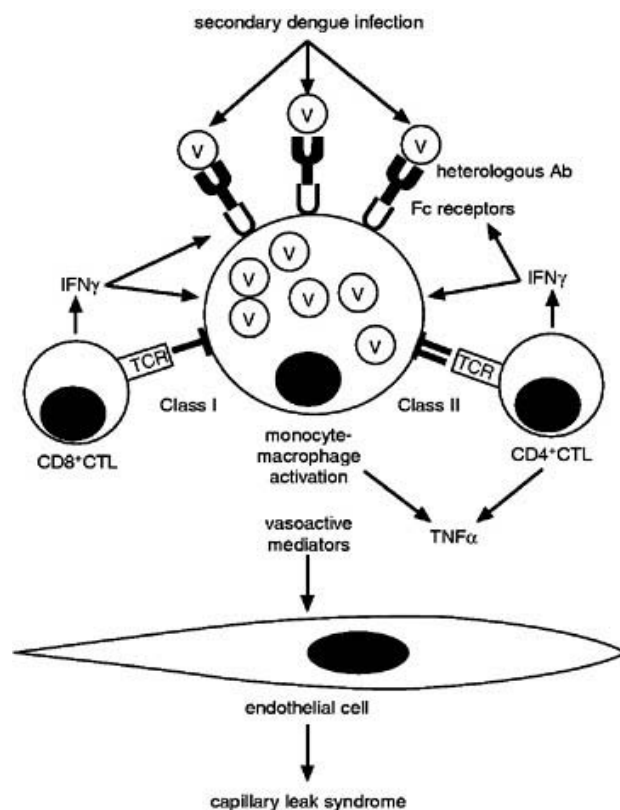


Figure 3 Model of the immunopathogenesis of dengue hemorrhagic fever and shock syndrome (DHF/DSS). Antibody-dependent enhancement of secondary dengue virus (V) infection by heterologous cross-reactive subneutralizing antibody results in a high virus burden in the critical target cell, the monocyte/macrophage. Interferon γ production by CD4⁺ and CD8⁺ memory T lymphocytes induces Fc receptor and MHC antigen expression in conjunction with macrophage activation, augmenting virus infection and antigen presentation (APC). Lysis of macrophages occurs by virus-specific cytotoxic T lymphocytes (CTL) restricted by both MHC class I and II antigens. Production of TNF α by T cells and macrophages, together with other pro-inflammatory cytokines, contributes to the capillary leak and hemorrhagic diathesis that are characteristic of severe dengue infection. (Based on studies reviewed in Kurane I and Ennis F (1992) Immunity and immunopathology in dengue virus infections. *Seminars in Immunology* 4: 121–127.)

response to dengue infection. Similar findings have been obtained with human T cells. Both dengue virus-specific CD4⁺CD8[−] and CD4[−]CD8⁺ cytotoxic T cell responses capable of lysing virus-infected cells have also been demonstrated. Although many viral proteins contain CTL epitopes, NS3 in particular encodes immunodominant epitopes which can drive cross-reactive CTL responses. CD4⁺ T lymphocytes can also be stimulated with dengue antigen to produce interferon γ (IFN γ). The model which incorporates all of these findings proposes that IFN γ up-regulates Fc receptor and MHC antigen expression on activated monocytes/macrophages, increasing

viral burden and antigen presentation, and leading to cytotoxic lysis by dengue-sensitized memory T cells. The multiple serotype cross-reactivities and numerous T cell epitopes demonstrated in humans supports the concept that memory responses are primed for extensive activation during secondary infections. Elevated markers of T cell activation have been shown during early dengue infection in humans, including sCD8, sCD4, sIL-2, and sTNF α receptors, and IFN γ . The source of the proinflammatory cytokines and the role of the T cell responses in providing protection rather than deleterious effects during dengue pathogenesis remain important questions.

Vaccines against flaviviruses

Vaccination against flaviviruses will remain an important public health measure because it is unlikely that these viruses can be eradicated from their animal reservoirs or arthropod vectors. Vaccine for yellow fever (YF17D strain) was the first available live-attenuated vaccine for this family. Derived by serial passage of its virulent parent in cell culture and eggs, YF17D is a safe, highly effective vaccine capable of inducing long-lasting protection. Yellow fever vaccination has been proposed as part of the World Health Organization (WHO) expanded program of immunization to control recurrent epidemics in Africa.

JE, DEN and TBE are also major targets for vaccine development. For TBE, a highly purified formalin-inactivated vaccine is available, which is very effective and has few side-effects. An immunoglobulin preparation against TBE is also used in Europe for pre- and postexposure prophylaxis. Products which have been used for vaccination against JE include both inactivated and live-attenuated viruses. The Biken vaccine, which has been available since the 1960s, is an inactivated preparation purified from mouse brain. Other inactivated vaccines have been developed and used locally in Asian countries. The JE SA14-14-2 vaccine is a live-attenuated virus used extensively for human vaccination within China, but is not currently approved by the WHO.

Vaccine development for DEN has been a major priority for many years but remains problematic because high levels of neutralizing antibodies against all four serotypes are required to prevent the occurrence of DHF/DSS. The most promising candidate is a live-attenuated tetravalent vaccine developed in Thailand and currently undergoing evaluation for human use. Alternatives, such as engineered cDNA clones encoding attenuated viruses, subunit vaccines incorporating the E or NS1 proteins, recombinant vector systems and subviral particles incorporating

the prM and E proteins are under investigation. T helper epitopes capable of priming antibody responses have been identified within flavivirus E proteins, suggesting that synthetic peptides may also have value as vaccines or vaccine adjuncts.

DNA vaccination for flaviviruses has not been widely studied but protective immunity generated by recombinant plasmids encoding the prM and E proteins has been reported. There is a definite need to further investigate the cellular and molecular requirements for induction of solid protective immunity against these viruses in order to understand to what extent these various approaches will be useful. Although only a handful of flaviviruses are now targeted for vaccine development, emergence of new ones in the future may change this situation.

See also: Antibody-dependent cellular cytotoxicity; Cell-mediated immunity; Helper T lymphocytes; Cytokines; Cytotoxic T lymphocytes; Epitopes; Fc receptors; Immunopathology; Interferon γ ; T lymphocytes; Viruses, immunity to.

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FLOW CYTOMETRY

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History

By the time the prototype of the fluorescence-activated cell sorter (FACS) went into operation (at Stanford in the early 1970s), immunofluorescence studies with the light microscope had already demonstrated that lymphocytes could be broadly subdivided according to whether they expressed surface immunoglobulin (Ig) or surface Thy-1. These visually-defined subpopulations, which we now know as B cells and T cells, were presumed to be responsible for different lymphocyte functions; however, the lack of methods for cleanly separating one subpopulation from the other confounded attempts to establish this point.

During the next few years, while the FACS was undergoing its initial metamorphosis into a commercially-available single-laser instrument, a variety

of physical and immunocytotoxic methods were developed that could selectively deplete B cells, T cells or T cell subpopulations from a cell suspension; for example, passage through nylon wool, passage through an affinity column, or complement-dependent killing with antibodies to distinctive cell surface molecules. These depletion methods have proven extremely useful; however, they are mainly restricted to depleting unwanted cell types from a population and thus do not provide for the specific isolation of subpopulations, which is necessary for definitively characterizing their functions.

FACS-based cell isolation methods, in contrast, are well suited to this task, as they provide the potential for sorting and testing viable subpopulations. A significant advantage over mechanical separation methods is that the populations isolated by FACS are usually highly pure. Whereas mechanical separations usually result in purities of 60–90% (with the

remaining cells being unwanted subsets), single FACS-separation runs can achieve over 99% purity – with re-sorting resulting in essentially 100% purity. In addition, the electronic control of the sorting in FACS gives the researcher the ability to sort only a single, viable cell – the quickest and most efficient cell cloning technology extant.

The major advantage of FACS-based separations, however, resides in the multiparametric nature of the measurement. A wide array of characteristics can be used, individually or in combination, to define subpopulations. For example, cell size, surface phenotype, mitotic stage, and intracellular levels of enzymes and metabolites. Thus, as the capabilities of this newly-developed instrument became known, it found increasing use in studies of the functional and developmental relationships among subpopulations of lymphocytes, as well as other cells.

In the last decade, the power of the FACS has been utilized for a much greater range of samples than leukocytes. All kinds of unicellular organisms, from bacteria to yeast to paramecia, have been separated by FACS. This technology has also been applied to the separation of subcellular particles: for instance, sorting endosomes, lysosomes or mitochondria. In addition, the FACS has been used to separate individual eukaryotic chromosomes – a technique that has been pivotal in the establishment of chromosome-specific DNA libraries, an important tool in the human genome project.

In recent years, the application of FACS to biology has taken another significant turn – that of single-cell functional characterization. A variety of assays have been developed to quantitate the functional capacity of individual cells by flow cytometry. These include assays for proliferation or cell cycle progression (e.g. total DNA, RNA and protein contents, as well as number of divisions measured by dyes permanently retained by DNA or cellular membranes), activation (e.g. expression of activation markers on the surface of cells), cytokine expression, apoptosis, calcium mobilization, acidification of internal compartments, changes in reduction–oxidation potential, metabolic activity and various enzymatic activities.

The power of these assays by FACS comes through two unique capabilities. First, since the assay is single-cell based, the resulting measurements provide a view of the *distribution* of activities within a population, rather than an average across the entire population. Since few biological processes are homogeneous across all cells, this view can be critical in assessing the true functional nature of cells. Second, the functional assays can be combined with standard immunofluorescence assays to phenotype the cells;

thus, the functional quantitation can be restricted to any desired subpopulation of cells.

While such functional assays are still reserved primarily for the basic researcher, more and more of them are finding clinical utility. As the instrumentation and data analysis becomes routinely automated, these assays progress into clinical settings, such as prognosis in human immunodeficiency virus (HIV) disease. Over the next 10 years, many of these single-cell FACS-based functional assays will become routine in the clinic because they provide information that cannot be obtained by standard bulk assays.

Elucidation of lymphocyte subpopulations by FACS

Much of the early work centered around the characterization of B cell phenotypes, functions and precursor–progeny relationships. The first significant FACS studies in these areas, published in 1972, demonstrated that antigen-binding cells are precursors of cells that secrete antibodies reactive with the bound antigen. Other studies, begun shortly thereafter, demonstrated that the surface Ig molecules expressed on B cells are restricted to individual allotypes and isotypes and that these surface Ig molecules reflect the allotype and isotype commitment of the cell and its antibody-producing (plasma cell) progeny.

By 1976, sorting and transfer studies with Ig allotype congenic mice placed this basic description of ‘allelic exclusion’ in its current context by showing that all Ig heavy chains expressed by an individual B cell or its progeny are encoded on the same Ig heavy chain chromosome (haplotype). These findings, which provided the operational and theoretical framework for a large series of subsequent B cell development studies, also laid the groundwork for current molecular and FACS studies defining the genetic organization of the Ig heavy chain (IgH) chromosome region and the mechanisms involved in IgH gene rearrangements and class (isotype) switching.

FACS contributions to T cell studies during this period were less dramatic, largely because of difficulties encountered in obtaining antibodies (and hence FACS reagents) that specifically detected individual cell surface antigens. At this time, antibodies for use in FACS (and other) studies were prepared from conventional antisera, which typically contain a mixture of antibodies reactive with the target antigens and an abundance of irrelevant Ig that can stick nonspecifically to cells. Anti-Ig reagents, such as those used in the B cell studies cited above, were relatively easy to prepare because Ig-specific antibodies can be bound to (and eluted from) secreted Ig

coupled to an insoluble matrix. Specific reagents for detecting surface determinants other than Ig, however, were substantially more difficult to produce, as they could not readily be isolated by binding and elution methods. By and large, these reagents had to be produced from antisera (or fractionated Ig) from which contaminant antibodies were removed by absorption with appropriate cells. Thus, FACS T cell work proceeded slowly until the late 1970s, when monoclonal antibodies were introduced as FACS staining reagents.

The development of monoclonal reagents for detecting the major T cell surface determinants in mouse and humans went hand in hand with the development of FACS methods for identifying and sorting the major T cell subpopulations. In essence, the availability of highly specific and easily purified antibodies that could be readily coupled with fluorochromes made it possible to use the quantitative expression of cell surface antigens to recognize lymphocyte (and other) subpopulations and to sort these subpopulations to chart their functions.

Initial studies with this monoclonal-based methodology defined the murine Ly-1 (CD5) and Lyt-2 (CD8) lymphocyte surface antigens and characterized the expression of these antigens on T cell subpopulations in mouse spleen, lymph node and thymus. These FACS characterizations were used to discover monoclonal antibodies that detected the human Leu-1 (CD5) and Leu-2a (CD8) homologs of the murine genes (Figure 1). In addition, these early studies used these (and other) monoclonal antibodies to identify and characterize the so-called 'helper/inducer' and 'suppressor/cytotoxic' subsets of human T cells.

At present, nearly all FACS studies characterizing the expression of cell surface antigens are conducted with monoclonal antibody reagents. The shift to this virtually exclusive use of these reagents occurred extremely rapidly and was accompanied by an equally rapid expansion in FACS utilization, particularly in studies of human and murine lymphocytes. Surface phenotypes for helper and suppressor T cell subsets were further defined and new subpopulations within these subsets were identified. B cell FACS studies also prospered and marked advances were made in clinical FACS application. In one striking example, leukemia phenotyping opened the way to a rationalization of treatment protocols that substantially improved patient survival. In another dramatic instance, measurement of helper and suppressor subpopulation frequencies in normal human adults laid the groundwork for the current widespread use of such measurements as an index of disease pro-

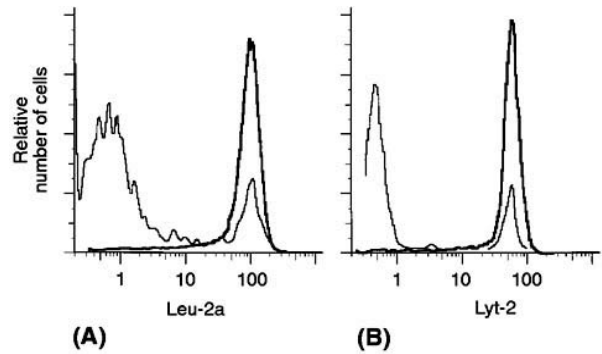


Figure 1 Two-parameter FACS: Leu-2a and Lyt-2 expression on (A) human and (B) mouse lymphoid cells. Early single-color FACS analysis, in conjunction with biochemical studies, demonstrated that human Leu-2a, which is found only on suppressor/cytotoxic cells, is expressed in a very similar pattern as the murine Lyt-2 surface antigen. The Leu-2a antigen was detected by indirect staining with SK1 monoclonal antibody supernatant followed by fluorescein-conjugated goat antimouse IgG1. The Lyt-2 antigen on mouse cells was detected by direct staining with fluorescein-conjugated 53-6.7 monoclonal antibody. Both of the determinants are found on 80–90% of the thymus cells (light lines) and 20–40% of the spleen T cells (dark lines). These antigens are now known as CD8.

gression in acquired immune deficiency syndrome (AIDS).

Multiparameter FACS studies

Methods for labeling antibodies with different fluorochromes (e.g. fluorescein and rhodamine) were developed while the FACS was still in its infancy. Fluorescence microscope studies demonstrated that these 'differently-colored' antibodies could be independently detected on the same cell and thus could be used to distinguish subpopulations on the basis of qualitative or semiquantitative correlations in the expression of two or more surface antigens. In essence, these studies showed that lymphocyte subpopulations distinguished initially by the expression (or nonexpression) of one surface marker could be further subdivided according to the expression (or nonexpression) of a second marker, etc.

Developing an array of fluorescent dyes for reagent labeling and efficient FACS systems that could capitalize on the availability of differently-colored antibody reagents produced with these dyes took some time. Early FACS instruments were equipped with a basic two-color capability that allowed quantitative evaluation of the expression of pairs of cell surface markers on individual cells; however, these initial multiparameter studies were severely restricted by the limited dye combinations, the single-laser FACS instruments and the simplistic computer support available at the time. In fact, studies with these early

instruments basically provided a tantalizing glimpse of how valuable multiparameter FACS methods could be; relatively little was actually accomplished with these methods until the introduction of the dual-laser FACS, the phycobiliproteins (phycoerythrin (PE) and allophycocyanin (APC)) and the sophisticated FACS software that together characterize the multiparameter analysis and sorting methods in use today.

Strictly speaking, multiparameter FACS studies

(defined as the measurement of more than one parameter per cell) began with the prototype FACS, which was built with two sensors: one for fluorescence, and the other for low-angle light scatter ('forward scatter'). The forward scatter measurement on this initial instrument was intended solely to signal the arrival of a cell-sized object in the light path; however, studies with cell populations from lymphoid organs soon showed that the forward scatter signal could also be used as a meaningful measure of

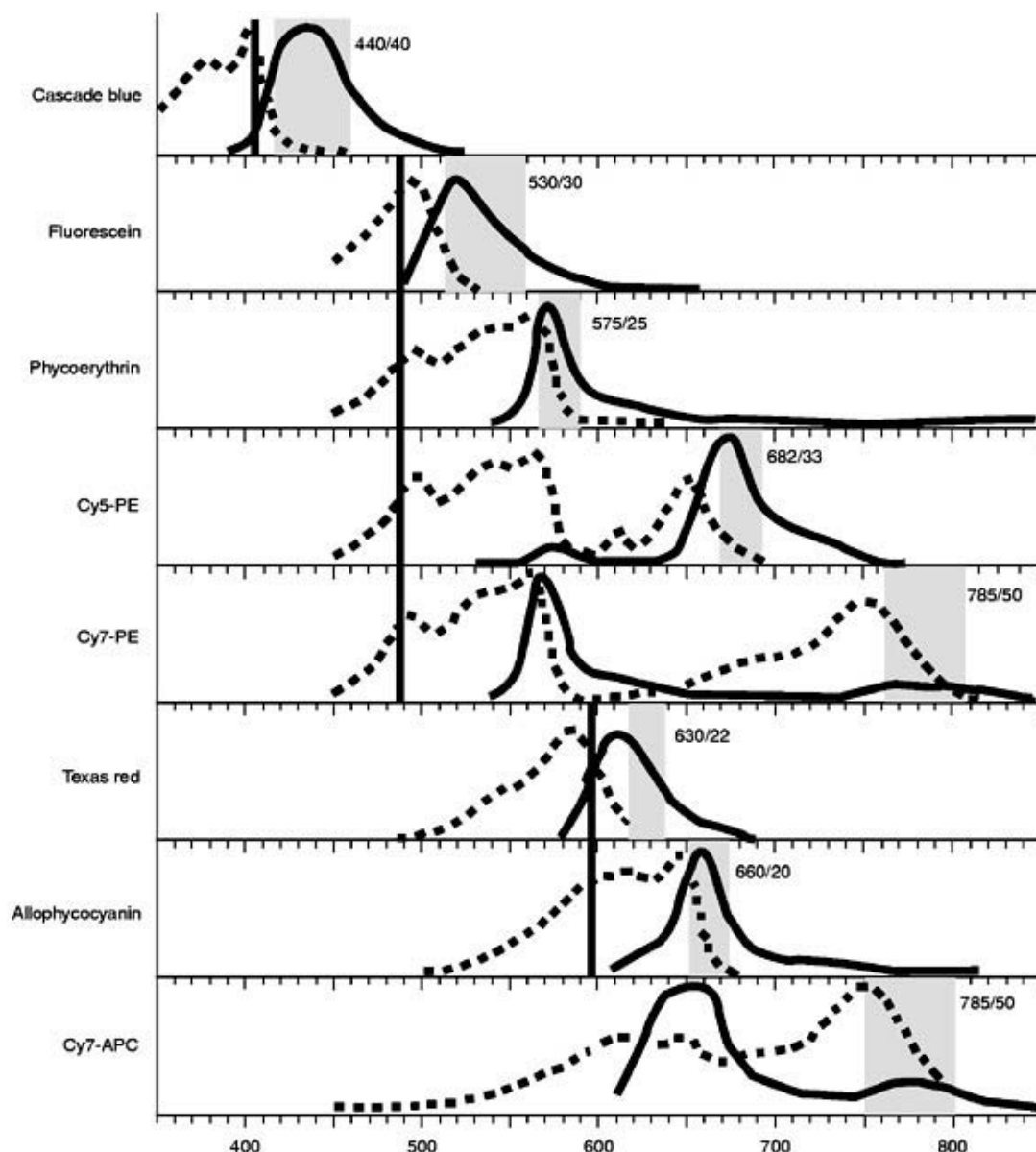


Figure 2 Fluorescence spectra of molecules conjugated to antibody reagents. The excitation and emission spectra of eight fluorescent molecules that can be conjugated to immunoglobulins and used in immunofluorescence studies are shown. All eight can be simultaneously and independently measured. Dashed lines, excitation spectra; solid lines, emission spectra. Spectra are uncorrected for detector sensitivity and are scaled for presentation purposes. Also shown are the excitation lines from the 406 nm krypton line, the 488 nm argon line, and the 595 nm dye laser line. Finally, the stippled boxes represent the transmission areas for each of the filters used to detect that fluorescent molecule by FACS.

cell size and could distinguish, for instance, lymphocytes from monocytes. In addition, the forward scatter signals were shown to be useful for discriminating live cells from dead cells, and for excluding ('gating out') dead cells during analysis and sorting. Thus, as the FACS came into use initially for biological studies, two parameters – forward scatter and fluorescence – were routinely measured and recorded for individual cells.

The introduction of methods for measuring two additional parameters – large-angle light scatter ('side scatter') and a second fluorescence color – moved FACS measurements closer to the current definition of multiparameter studies, i.e. those in which at least two fluorescence measurements and one light scatter measurement are taken per cell. Most flow cytometers are now capable of measuring forward and side scatter as well as at least three fluorescence colors. State of the art instrumentation at Stanford is capable of detecting the two scatter signals as well as eight different, independently-quantifiable fluorescence measurements. The advance to such ten-parameter, three-laser instrumentation has required development efforts at a multitude of levels: 1) chemistry – the continuing development of fluorescent dyes with spectra suitable for flow cytometry (e.g. excitation by commonly avail-

able lasers and emission spectra that do not overlap excessively) has made such advances possible (Figure 2, see p. 935); 2) optics – the construction and optimization of a complex array of filters and photomultiplier tubes that can separate the emitted light into its components and accurately measure those components; 3) electronics – the simultaneous collection and processing of ten or more analog signals, digitization, decisions for sorting, as well as distribution to various computer elements for data storage and analysis; and 4) computer software – analysis of this highly multidimensional data requires 'expert' systems software which can automate many of the tasks that are beyond the capacity of the human mind.

Power of multiparameter analysis

The power of these multiparametric analyses is evident from the unsuspected subdivisions their use has revealed in biological systems. Early on, the division of the two major T cell subsets (helper/inducer versus suppressor/cytotoxic) was shown to be possible by the simultaneous measurement of CD4 (expressed on

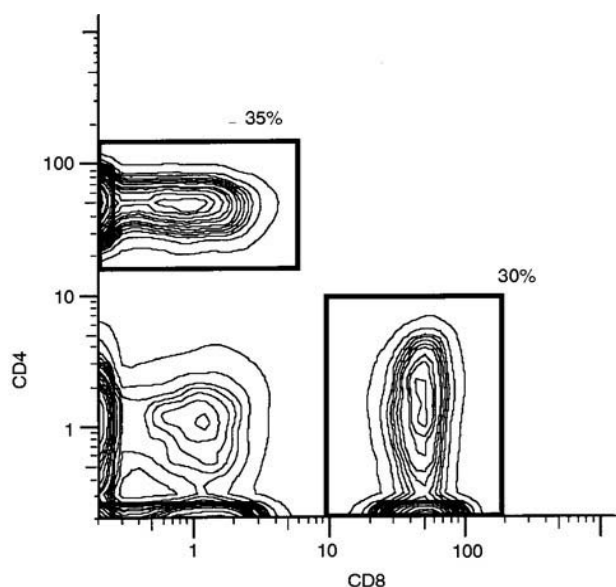


Figure 3 Four-parameter FACS: T cell analysis of a mouse lymph node. Two-color FACS analysis is routinely used to quantitate helper/inducer T cells and cytotoxic/suppressor T cells in mouse and humans. The 5% probability plots, which are also shown in other figures, contain an equal number of cells between each pair of contour levels. In this figure, three types of cell can be distinguished: those expressing CD4 (helper/inducer); CD8 (cytotoxic/suppressor); or neither (B cells, NK cells and other leukocytes).

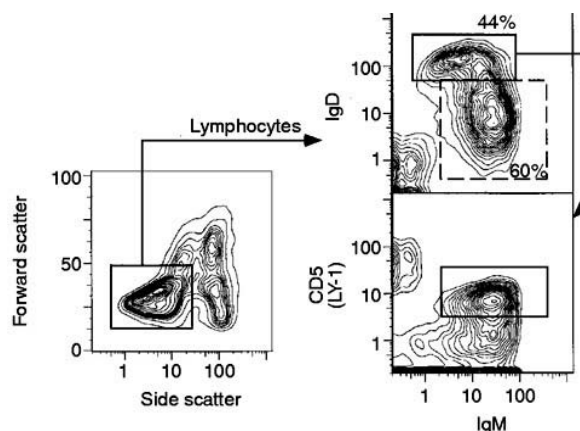


Figure 4 Six-parameter FACS: analysis of murine peritoneal cavity cells. Dual laser FACS analysis permits the simultaneous measurement of six or more parameters. The left plot shows the forward versus side scatter profile. The data, which are recorded in list mode, can be 'gated' for live lymphocytes as shown (i.e. only those cells which fall within the box drawn on the forward scatter versus side scatter plot are viewed in subsequent analyses). Cell surface staining profiles in the right panels do not include dead cells or large live cells such as macrophages. The cell surface analysis of the peritoneal cavity reveals an abundance of Ly-1 lineage B cells. The upper panel shows the characteristic IgM versus IgD phenotype: conventional B cells are indicated by the dashed box and the sum of the CD5⁺ and CD5⁻ Ly-1 B cell lineages are indicated by the solid box. The right bottom plot, which is gated as shown by the solid box in the upper panel, distinguishes the CD5⁻ Ly-1 lineage B cells (solid box) from the CD5⁺ 'sister' Ly-1 B cells (i.e. by the expression of CD5). Percentages are reported in terms of the total live lymphocytes, as determined by the forward and side scatter gates. (Courtesy of Dr Aaron Kantor.)

the helper/inducer) and CD8 (expressed on the suppressor/cytotoxic) populations (Figure 3). These distinctions are relatively easy to make, being primarily 'positive' versus 'negative' in terms of expression of a surface molecule. However, many lymphocyte subsets cannot be distinguished so easily. B cells, for example, were thought to be relatively homogeneous. However, initial multiparameter FACS studies conducted with a dual-laser FACS sys-

tem in our laboratory revealed a series of murine B cell subpopulations and lineages with distinctive functions, localizing behavior, developmental patterns, mitogen and lymphokine responsiveness, etc. Furthermore, potentially homologous B cell subpopulations with similar FACS and functional phenotypes have been identified in rat and in humans.

None of the B cell subpopulations/lineages are as yet recognizable by the presence, absence or surface

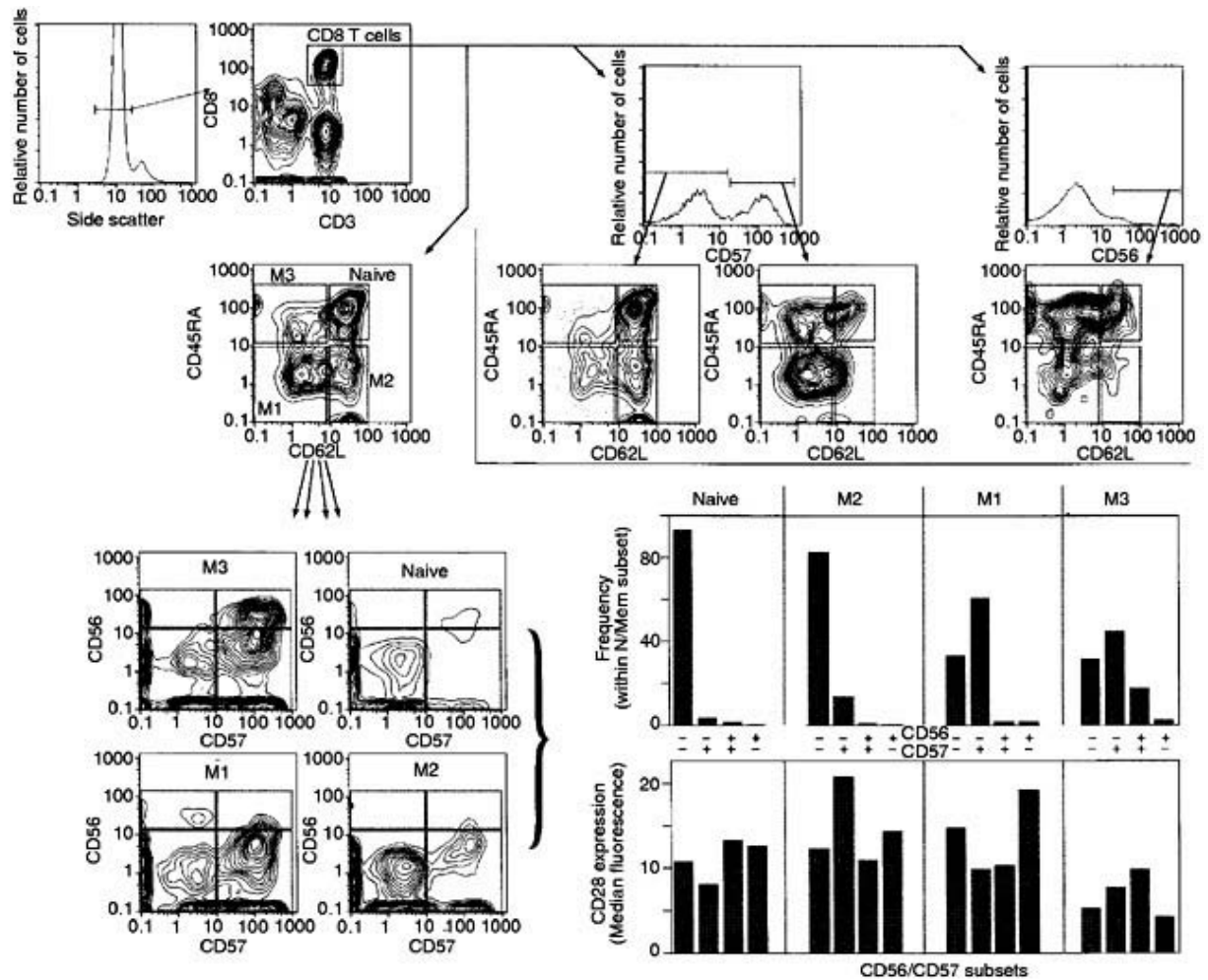


Figure 5 Nine-parameter FACS: analysis of human peripheral CD8 T cells. The complex heterogeneity of peripheral CD8 T cells in humans is exemplified by a seven-color staining together with forward and side scatter collection. PBMC were simultaneously stained with CD3, CD8, CD28, CD45RA, CD56, CD57 and CD62L. Shown is progressive 'gating' or restriction of the sample by these parameters. Lymphocytes are selected based on the size parameter (top left histogram); CD8 T cells are selected from this subset by coexpression of CD3 and CD8. The second line of plots shows CD45RA versus CD62L expression for (left to right): total CD8 T cells CD57⁻ CD8 cells, CD57⁺ CD8 cells, and CD56⁺ CD8 cells. Based on functional studies, 'naive' versus 'memory' CD8 T cells can be distinguished by CD45RA and CD62L, as shown in the leftmost panel of this series. CD56⁺ and CD57⁻ cells, which are themselves functionally distinct memory subsets, have heterogeneous expression of these markers – some of these cells fall into the 'naive' gate. Thus, unique identification of naive CD8 T cells requires the simultaneous measurement of (at least) six parameters: CD3⁺, CD8⁺, CD45RA⁺, CD62L⁺, CD56⁻, CD57⁻. Another evaluation of the expression of CD56 and CD57 on the naive and memory subsets is shown in the four plots on the lower left. The bar charts represent analyses of these 16 subsets: the frequency within the naive and memory (N/Mem) subsets (upper) and the relative expression of CD28 on cells of each subset (lower). Data sets such as this single sample are so complex that it becomes impossible to satisfactorily analyze by conventional methods (i.e. 'by hand'). Computer programs which can provide automated 'expert'-type analysis combined with nonparametric cluster algorithms will become invaluable, if not necessary, analysis tools.

level of an individual surface marker. Distinguishing these subpopulations requires measurement of at least two surface molecules (detected by differently-colored antibodies) (Figure 4, see p. 936). In fact, some subpopulations are even difficult to distinguish with three fluorescence and two scatter measurements. Thus, although B cell surface marker expression had been studied extensively in single-color analyses, the introduction of multiparameter FACS methods opened an entirely new perspective on the heterogeneity within the overall B cell population.

Nowhere is the power of multiparametric analysis more evident than in studies of peripheral T cell subsets in humans. Similar to the case in mouse, it was recognized early that T cells could be divided into helper/inducer and suppressor/cytotoxic subsets by the surface antigens CD4 and CD8, respectively. However, each one of these subsets is richly heterogeneous, and can be further subdivided by more than a dozen other antigens. The functional roles of these different subsets is poorly understood, primarily because studies have been performed so far using only three- or four-color separations, which does not result in truly homogeneous populations.

Figure 5 (see p. 937), for example, shows how human CD8 T cells can be subdivided into a number of different subsets based on the simultaneous quantitation of six different antigens. The need for this differentiation is exemplified by recent studies of immunopathogenesis in HIV disease (AIDS). Until the early 1990s, the understanding of the changes in the T cell compartment accompanying progression of this disease was rather simplistic: CD4 T cells declined continuously; CD8 T cells initially expanded, stayed high until very late in disease, and then declined somewhat.

However, there were dozens of publications detailing many changes within the CD8 T cell compartment based on two- or three-color approaches (i.e. using one or two colors to identify uniquely the CD8 T cells, and then measuring a single other parameter for those cells). These include increases in HLA-DR, CD38 and CD57 (taken as indications of activation); or decreases in CD26, CD28, CD31, CD45RA, CD62L, and increases in CD45RO, CD11b (taken as indications of a change in the state of differentiation). However, these changes by themselves were usually relatively minor.

Multiparametric analysis began to reveal that, in fact, there are major and consistent changes in the CD8 T cell population that accompany HIV disease – changes that could not be observed by the two-color approaches. For example, naive CD8 T cells are virtually obliterated during progression of

HIV disease. This subset of CD8 T cells is required for any new immune response; it is identified by the coexpression of CD26L and CD45RA. The loss of this important subset was missed for many years because many memory T cells express one or the other (but not both) of these markers; thus, three- or four-color phenotyping was required to reveal this loss. Indeed, when CD8 T cells are carefully phenotyped, it became evident that all 'normal' CD8 T cells were disappearing during HIV progression – exactly like CD4 T cells. The expansion of the bulk population was due entirely to the presence of activated CD8 T cells. Such studies profoundly impact the views of immunopathogenesis of disease.

Multiparametric studies in the near future will resolve much of the confusion surrounding the immunopathogenesis of diseases. These studies will reveal those populations that it is most relevant to quantitate. In addition, the sorting capability of the FACS allows the isolation of pure and homogeneous subsets of cells on which functional studies can be

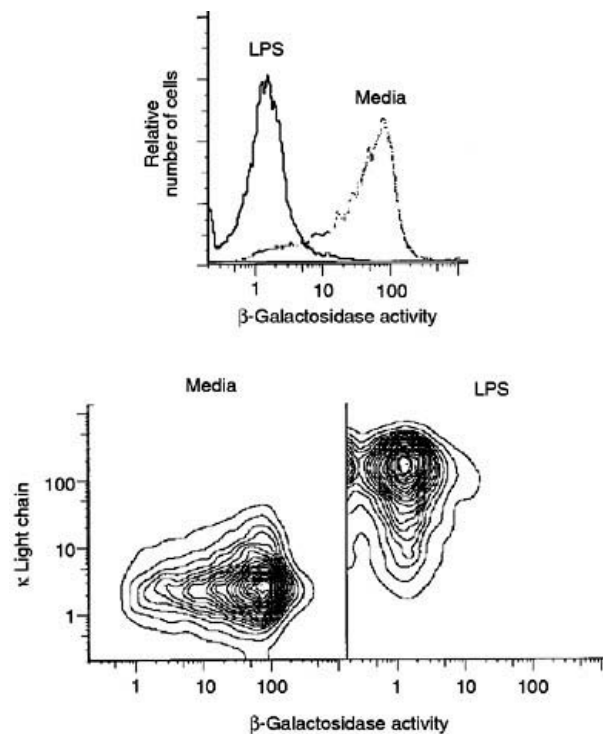


Figure 6 Analysis of intracellular gene expression. A B-cell line was generated, as a model of differentiation, which upon stimulation expresses κ light chain and turns off the expression of DNA elements controlling the artificially introduced bacterial *lacZ* gene. The histogram (top) demonstrates that *lacZ* (*E. coli* β -galactosidase) expression is reduced following lipopolysaccharide (LPS) stimulation. The β -galactosidase enzyme activity is measured by introducing a fluorogenic substrate, fluorescein digalactoside, into the cells. The contour plots demonstrate a concomitant increase in κ light chain expression, as detected by surface immunofluorescence. (Courtesy of Dr William Kerr.)

performed. These studies are necessary for understanding the pathogenic consequences of changes in the representation of fine lymphocyte subsets.

Molecular and functional studies

More and more frequently, the FACS is used in a wide variety of immunologically relevant molecular studies. These range from measuring changes in the concentration of intracellular small molecules (physiological state or signal transduction events) to quantitating gene expression to chart activation or differentiation events.

In the late 1980s, the FACS molecular tool kit was extended by the development of a technique for measuring the commonly-used reporter gene *lacZ* (β -galactosidase) within the intracellular compartment of mammalian (as well as bacterial and insect) cells. Depending on how it is introduced, this gene can be used either as a marker for lineage and migration

studies or as an insertion probe that can reveal differentiation-dependent enhancer and promoter sites (Figure 6, see p. 938). Furthermore, when introduced as a reporter gene under the control of particular promoter and enhancer elements, it can be used to investigate the mechanisms that regulate gene expression or provide a quantitative indicator of shifts in the activation or differentiation status of individual cells (Figure 7).

Recent developments using inherently fluorescent proteins have provided another tool in the arsenal of FACS-based reporter gene studies. The first of these proteins, termed green fluorescent protein (GFP), has spectral characteristics marginally suitable for quantitation by flow cytometry. An enormous effort has gone into the generation of mutants of the original GFP which have not only increased brightness, but different spectral properties as well. Currently, there are two versions of the GFP which can be simultaneously and independently quantitated. This brings

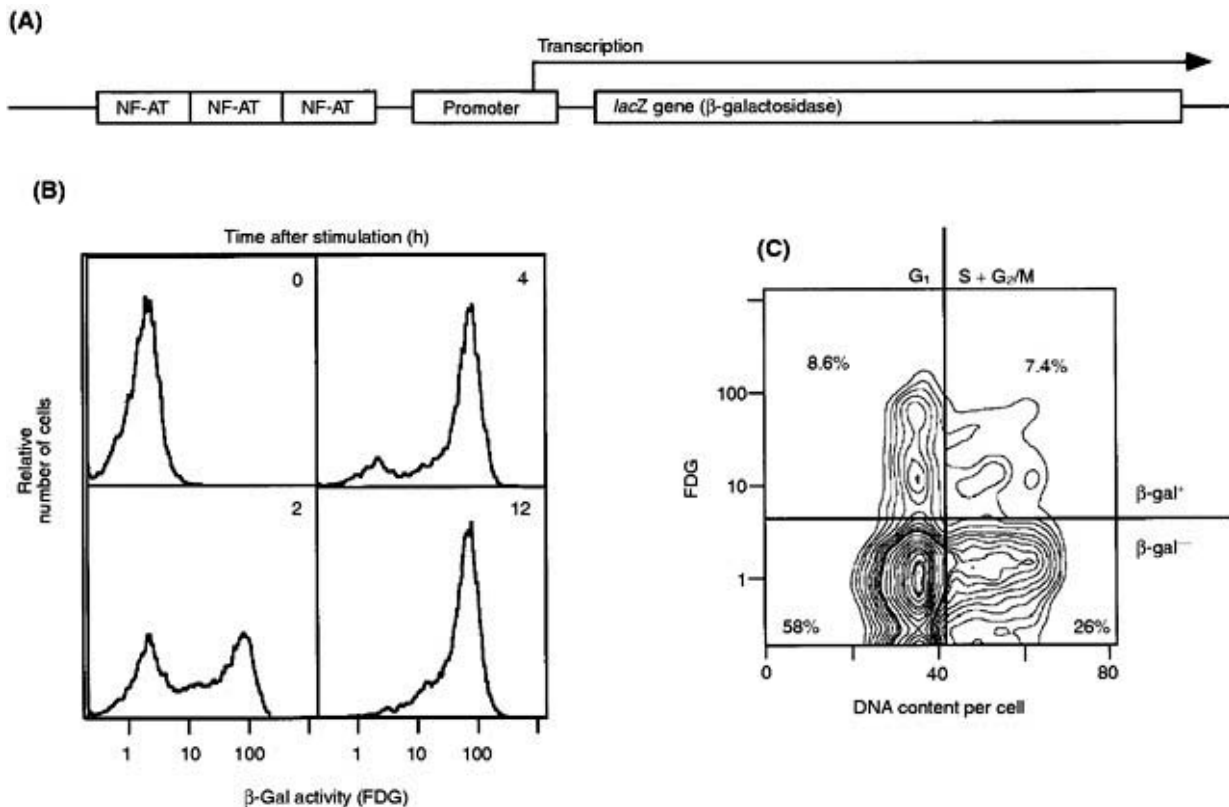


Figure 7 Correlation of gene expression with cell cycle progression. (A) The β -galactosidase gene expression in a Jurkat T cell line is controlled by DNA enhancer elements bound by a nuclear transcription factor termed NF-AT. Stimulation of T cells results in a signal transduction cascade, including the activation of this nuclear factor. (B) After stimulation for various periods of time up to 12 h, the fraction of cells expressing β -galactosidase activity (and thus having activated NF-AT) increases. β -Galactosidase activity is measured using the fluorogenic substrate fluorescein di-galactoside (FDG). The activation signal is a threshold event: after activation, no further increase in the production of β -galactosidase occurs (compare the positive cells at 2 h with those at 12 h). (C) Simultaneous quantitation of the activation of NF-AT (as revealed by β -galactosidase activity) and cell cycle progression (as revealed by the DNA content of cells) after 2 h of stimulation. Note that cells that have activated NF-AT are more likely to already be in cell cycle progression (S + G₂/M) than unactivated cells. (Courtesy of Dr Stephen Fiering.)

to gene expression analysis the power of multiparameter FACS; that is, the quantitation of different gene elements within individual cells, providing a correlated distribution of expression of those genes in a population (for example, see Figure 8).

The FACS is also a powerful tool for measuring the physiological state of cells. A commonly measured physiological response is the cytoplasmic calcium concentration. Calcium is mobilized from internal stores as well as the extracellular milieu in response to mitogenic signals. Thus, measuring the change in the internal calcium concentration after signaling is an important indicator of the responsiveness of cells, and a predictor of their ultimate proliferative capacity. Figure 9 shows an example of such an experiment. This experiment demonstrates that peripheral blood T cells have a heterogeneous responsiveness in terms of the ability to flux calcium – a heterogeneity which is only evident by combining this experiment with antibody stains to subdivide the populations.

It is very common in flow cytometric analysis to quantitate live versus dead cells. In general, this is most easily accomplished using propidium iodide (PI). PI binds avidly to DNA, but is excluded from DNA in live cells because it cannot cross the mem-

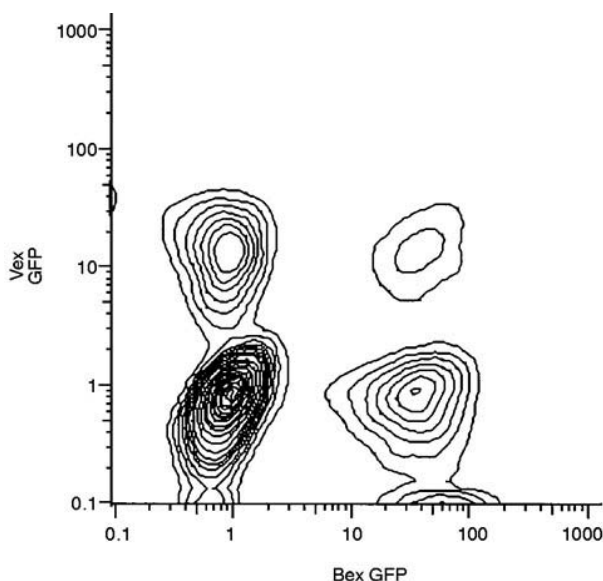


Figure 8 Simultaneous detection of two mutants of green fluorescent protein. Two different mutants (Bex and Vex) of the inherently-fluorescent GFP were selected so that they could be simultaneously and independently measured. In this experiment, cells were infected with a mixture of two different viruses, each expressing one of the mutant GFPs. Most cells were uninfected (expressing neither GFP); some cells were singly infected; and some cells were infected by both viruses (and show expression of both the Bex and Vex mutant GFPs). (Courtesy of Dr Michael Anderson.)

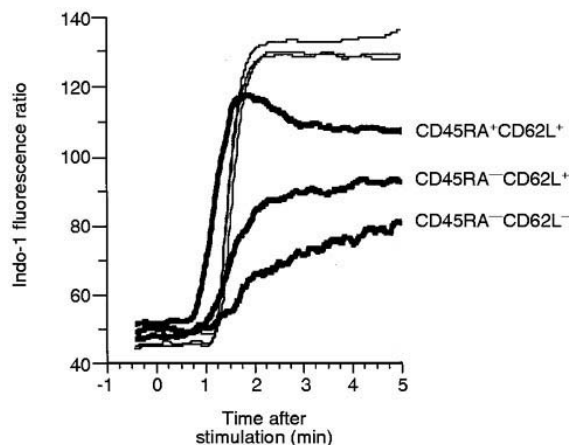


Figure 9 T cell signal transduction measured by calcium translocation. T cells isolated from a healthy individual were 'loaded' with Indo-1, a fluorescent molecule whose spectrum depends on calcium concentration. By measuring and calculating the ratio of two different Indo-1 fluorescences, one which declines and one which increases with increased calcium concentration, an estimate of intracellular calcium content can be made. In this experiment, T cells were also surface stained to determine their differentiation state. They were then stimulated through the T cell receptor, the primary signal for activation of these cells. The figure shows the relative calcium content after stimulating CD4 T cells with antibody to CD3 (dark lines). Cells that coexpress CD45RA and CD62L (and are 'naive' T cells) have a greater capacity to translocate calcium into the cytoplasm, compared with the memory subsets. This observation correlates with the greater capacity of naive T cells to proliferate after such a stimulation. The heterogeneous calcium flux is due to a difference in the CD3 signal transduction pathway: when the cells are stimulated with low concentrations of ionomycin (light lines), the three subsets have indistinguishable calcium mobilization profiles.

brane. Dead cells, on the other hand, have permeable membranes that readily allow PI to enter and bind to the DNA. Thus, inclusion of a small amount of PI in all samples allows dead cells to be excluded from further analysis (or at least separately enumerated). Recently, much interest has focused on pathways by which cells die, in particular that of programmed cell death (or apoptosis). Several assays exist for the quantitation of apoptosis; such an assay is shown in Figure 10. In this example, the cells that have been signaled to die are shown to progress through several distinct stages.

In addition to these examples, assays that measure intracellular pH, reduction-oxidation (redox) potential, mitochondrial activity, RNA content, membrane area, membrane potential and endocytic activity have been developed for use with the multiparametric paradigm of flow cytometry. Changes in these measurements reveal signal transduction events, stress responses, mitogenic responses, etc. – important parameters of the functional capacity of cells. While most of these are used primarily in basic

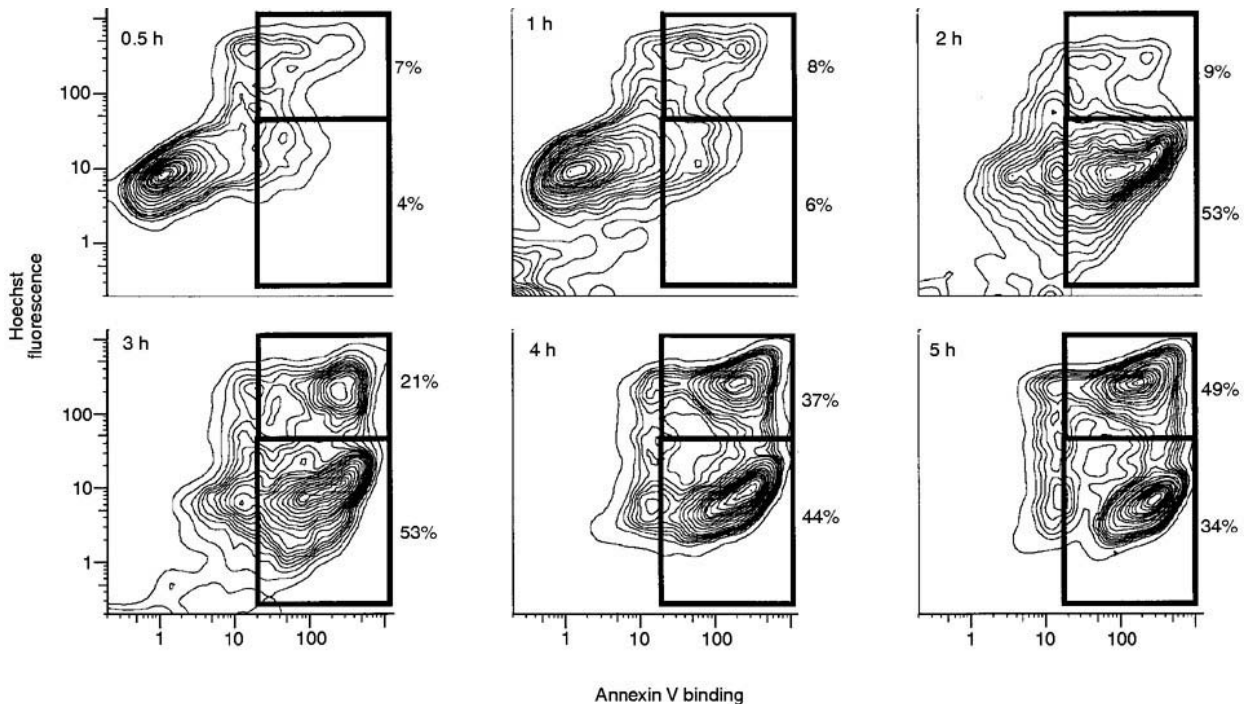


Figure 10 Discrimination of dying and apoptotic cells. Annexin V is a protein which binds to phosphatidylserine (PTS). PTS is normally not expressed on the outside of cellular membranes, but only on the inside. When cells commit to apoptosis (programmed cell death), one of the earliest events is the loss of this membrane asymmetry in PTS expression. Using fluorescently-conjugated Annexin V, cells early in the apoptosis commitment phase can be identified. During later stages of cell death, the chromatin condenses and binds Hoechst 33342 to a very high degree (note this use in distinction to **Figure 7**, where quantitative binding reveals DNA content); in addition, the Hoechst dye itself is no longer actively removed from the cells. In this figure, Jurkat T cells were induced to apoptosis; after various times they were assayed for Annexin V binding and Hoechst 33342 uptake. There is a clear progression of cells from double-negative, to Annexin V-positive (apoptosis-committed), to double-positive (dead). (Courtesy of Dr Peter Katsikis.)

research, some assays are beginning to find clinical relevance.

For example, oxidative stress has been shown to accompany HIV disease. A FACS assay which can measure intracellular glutathione (the major intracellular antioxidant) showed that lymphocytes in persons with HIV disease are depleted for glutathione – and that this depletion was a predictor of survival in advanced stage disease (**Figure 11**, see p. 942). The combination of surface immunophenotyping and a physiological measurement therefore provides a powerful, clinically relevant parameter. **Figure 11** also shows an example of the *in vivo* quantitation of gene expression of FACS. In this case, the number of CD38 molecules per CD8 T cell (i.e. proportional to the fluorescence of cells stained with phycoerythrin-conjugated anti-CD38 monoclonal antibody) also provides a significant predictor of progression to death in AIDS. Using these two parameters simultaneously is the most significant predictor of lifespan in AIDS patients known to date – far more significant than CD4 T cell counts, the most commonly used surrogate marker.

Clinical relevance has also been ascribed to the quantitative determination of DNA content in lymphocytes. In this case, lymphocyte membranes are disrupted to allow free entry of dyes such as Hoechst 33342 or PI. Quantitative fluorescence measurements easily reveal 2N or 4N DNA content (akin to the example in **Figure 7**). Clinical relevance comes from the observation that many leukemias and lymphomas have aberrant DNA content (aneuploidy). There is an extensive literature on typing such cancers based on DNA quantification.

Finally, the FACS can be used to perform powerful functional assays. FACS-based assays exist which can measure activation, proliferation, cytotoxic activity, apoptosis and cytokine production. Often, the FACS assays are considerably more sensitive than bulk assays. For instance, cytokine production assays can be performed in cell populations stimulated for only 6 h (for example, see **Figure 12**, see p. 942). Bulk assays, which measure cytokine production in supernatant, are technically easier to perform but yield considerably less information, together with greater potential artifact. Bulk assays for cytokine

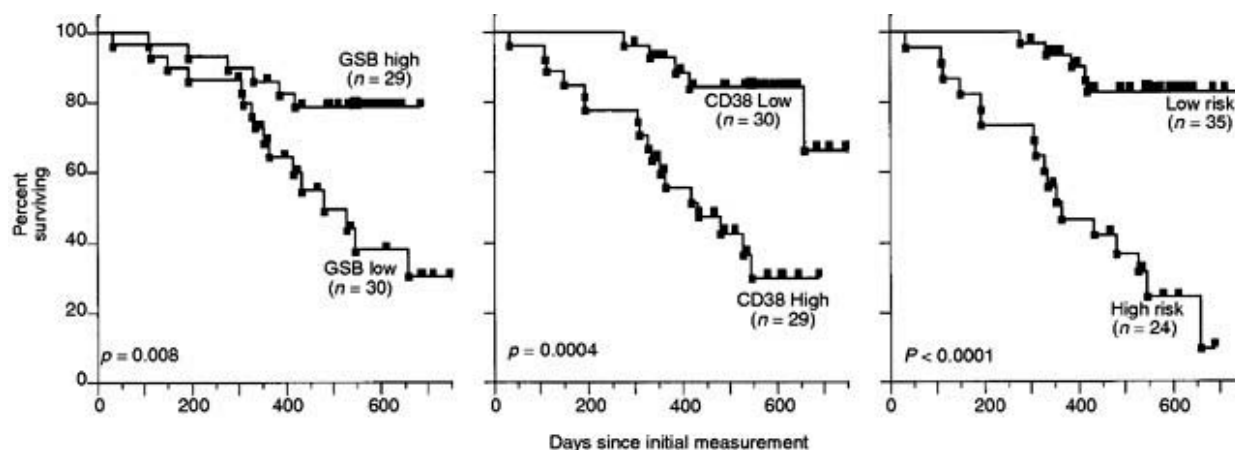


Figure 11 FACS-measured quantities are powerful prognostic variables in AIDS. Shown are Kaplan–Meier survival plots, which predict the survival of a group of individuals based on a measurement made at an initial time (day 0). (Left) The amount of glutathione (GSB) (the major intracellular antioxidant) was measured in CD4 T cells in 59 HIV-infected adults with advanced disease. Those with high glutathione were far more likely to survive 2 years than those with low glutathione. (Middle) The same individuals were divided on the basis of CD38 expression on CD8 T cells. Quantitating the absolute expression of this molecule, which may reflect the activation state of the immune system, also provides a powerful prognostic of death. (Right) The glutathione measurement and CD38 measurement were correlated to provide an index of individuals who are at high risk for progression to death (i.e. low glutathione and high CD38) versus those who are at low risk. The combination of these two parameters provides the most powerful prognostic value for predicting survival in AIDS patients.

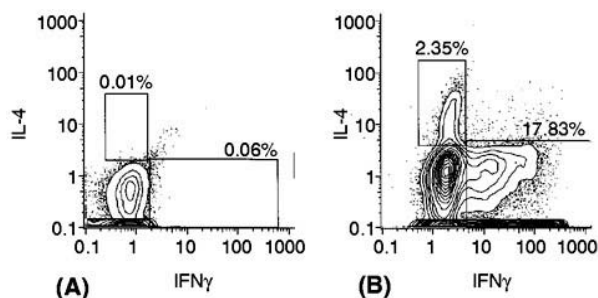


Figure 12 Cytokine profiles of human peripheral T cells. Human T cells were either resting (A) or stimulated with phorbol ester and ionomycin – a powerful activating combination (B). After 6 h, cells were stained for the presence of intracellular cytokines (i.e. the cytokines that are just synthesized and are about to be secreted for effector functions). Essentially no resting T cells are synthesizing cytokines; stimulated T cells will make either interleukin 4 (IL-4) or interferon γ (IFN γ) but very rarely both. Note that a bulk measurement of cytokine production would reveal that both cytokines are made in the population, but would not demonstrate the exclusivity of production on a cell-by-cell basis.

production are typically performed after at least 24 h of culture and provide no information about the distribution of cytokine expression among the cells (e.g. are all cells making the same amount of cytokine, or are only a small fraction of the cells each making a lot?). In the example of **Figure 12**, it would be impossible to deduce from a bulk assay that some cells make only IL-4, while others make only IFN γ .

Again, the real utility of the FACS for making functional assays comes with the combination of

these assays with each other or with immunophenotyping. Thus, not only is it possible to measure how many cells make a particular cytokine, but also what the phenotype of those cells is, and, for instance, what their proliferative capacity is.

Summary

Flow cytometry is a platform upon which dozens of very different, highly-specialized assays can be performed, and provides the capability for performing many of these assays simultaneously. It is this multifactorial nature of the analysis which makes the FACS a unique tool in immunobiology today. The extremely high information density of the data derived from FACS assays is an absolute requirement to begin to elucidate the extremely complex immune system. FACS technology continues to evolve at a fast pace, as it has for a quarter of a century. This evolution includes a continuous infusion of applications into the clinical environment – indeed, the power of FACS for clinical diagnosis and prognosis is already evident from the fact that virtually every modern hospital has at least one of these instruments. The assays being developed in basic research laboratories today, such as those described above, will become routine clinical assays in hospital settings over the next decade.

See also: B lymphocytes; CD antigens; Cell separation techniques; Fluorochrome labeling; T lymphocytes.

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FLUOROCHROME LABELING

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Fluorochrome-labeled reagents are an important tool in the analysis of cell surface antigens, both for use in fluorescence microscopy and in flow cytometry. The development of the hybridoma technique for the generation of monoclonal antibodies that can then be employed as fluorescent reagents has greatly aided the delineations of numerous functional subpopulations now known to constitute the immune system.

Furthermore, the workhorses of microscopy, fluorescein isothiocyanate (FITC, fluorescing green) and tetramethyl rhodamine isothiocyanate (TRITC, fluorescing red) have been joined in recent years by new dyes, the photosynthetic accessory proteins known as phycobiliproteins, possessing intense fluorescence and useful excitation/emission spectra. The techniques used for generating fluorescent

reagents using small organic dyes are relatively simple, while those employed for coupling large proteins such as phycobiliproteins are more involved.

Labeling with FITC, rhodamine or biotin

Fluorescein (green) and rhodamine (red) have the advantage of ease in labeling, which involves simply mixing an activated dye reagent with the antibody of interest. In microscopy, filter sets have been developed that permit rapid sequential observation of a double-labeled sample for fluorescein and then rhodamine fluorescence. For microscopy (typically carried out using a mercury arc lamp), fluorescein is not optimally excited, whereas rhodamine is well matched to the arc excitation and so is commonly employed where highest sensitivity is required. In flow cytometry, where lasers are typically used as the source of excitation, fluorescein is optimally excited by the 488 nm line of an argon ion laser and so is usually the dye of choice. Antibodies are typically labeled using the isothiocyanate derivative of fluorescein, whereas rhodamine coupling using the sulfonyl chloride form may be more effective.

The biotin/avidin system has seen extensive application in immunofluorescence analysis. One advantage of this approach is the amplification achieved which permits greater sensitivity in analysis of determinants expressed at low levels. In addition, certain dyes tend to inactivate antibodies (especially monoclonal antibodies) and these can often be utilized more effectively as biotin conjugates. This is particularly true for rhodamine conjugation.

Several years ago, the addition of a second laser on commercial flow cytometry systems introduced correlated two-color analysis. A krypton laser was used to excite x-rhodamine or an argon ion/rhodamine 6G dye laser was used to excite the sulforhodamine dye 'Texas Red'. These systems gave independent signals, both optimized for the indi-

vidual dye, and the latter system in particular permitted very high-quality dual-staining measurements. The obvious disadvantages of this approach were the requirements for a complex, expensive set-up in order to use the fluorescein/Texas Red combination. Such set-ups are now primarily restricted for use in three- or four-color analysis. Some of the more common labels employed in immunofluorescence analysis (from 1 to 4 color) are listed in Table 1.

Regardless of the label employed, certain common features emerge in using fluorochrome-labeled antibodies. One important precaution is the use of control reagents to discriminate between specific and nonspecific staining. Probably the best control is a monoclonal (or polyclonal) antibody of the same isotype prepared in the same way as the specific reagent. Another consideration is that staining can be carried out either with directly labeled reagents or else in multiple incubations (separated by washes) where only the final step is labeled. Multiple steps, while time-consuming, may lead to amplification of staining and so to better separation of labeled and unlabeled populations compared with direct reagents. On the other hand, it is progressively more difficult to carry out dual- or triple-color analysis using second-step reagents (where the second step may react with *all* of the first-step antibodies). Furthermore, care should be taken that directly labeled reagents have enough fluorochrome to achieve resolution, but not so much as to render the antibody 'sticky'. This ratio of fluorochrome : protein must be determined empirically and may be different for flow cytometry as compared with fluorescence microscopy. A final important point is to centrifuge reagents immediately prior to use at very high speed (top speed of a microfuge or 'Airfuge') to eliminate aggregated material from the staining solution, since such aggregates often contribute disproportionately to non-specific background staining.

Table 1 Reagents used in fluorescence labeling

Fluorochrome	Excitation ^a	Emission ^a	Coupling chemistry
Fluorescein	490	520	Isothiocyanate
Rhodamine	520–550	567	Isothiocyanate
Rhodamine ('Lissamine')	520–550	567	Sulfonyl chloride
Sulforhodamine ('Texas Red')	595	615	Sulfonyl chloride
Biotin	N/A	N/A	Succinimide ester
Phycoerythrin (PE)	480–560	570	GMBS/2-IT or DTT/GMBS
Phycocyanin	580–620	640	Same as for PE
Allophycocyanin	600–650	680	Same as for PE

^aApproximate excitation and emission wavelengths given in nm.

Phycobiliproteins

In laser-based flow cytometry, while fluorescein is well matched to the 488 nm argon ion laser line, rhodamine is suboptimally excited by the 514.5 nm argon ion laser line. Furthermore, the wavelengths of fluorescein and rhodamine emissions overlap considerably, not a problem for sequential measurements, but severely limiting simultaneous (correlated) measurements. While careful filtering and electronic compensation have permitted derivation of independent signals from the fluorescein/rhodamine pair, compromises necessitated in accommodating both dyes degraded sensitivity for both stains compared with either one used individually. Thus, the use of dual staining for correlated measurements in flow cytometry was initially restricted to dual-laser instruments.

This difficulty was overcome by the application of phycoerythrin, one of the family of phycobiliproteins, as a fluorescent dye. Phycobiliproteins are found in red algae and cyanobacteria where they serve to transfer energy from blue wavelength light to the chlorophyll photosynthetic system (which is not excited well in the blue). These proteins are well suited as fluorescent dyes because they possess many fluorescent groups (bilins) of several distinct types per protein. This results in a broad excitation range (but with narrow emission) and a very large extinction coefficient compared with small organic fluorochromes.

Phycobiliproteins are divided into three major groups: the phycoerythrins (PEs) with a molecular weight of 240 kDa, having major excitation peaks between 490 and 560 nm and emission at 570 nm; the phycocyanins (PCs) with a molecular weight of 240 kDa, having good excitation from 580 to 620 nm and emission at 640 nm; and the allophycocyanins (APCs) with a molecular weight of 110 kDa, having good excitation between 600 and 650 nm and emission at 680 nm. The orange fluorescence of PE can be excited with reasonable efficiency by the 488 nm line of the argon ion laser, and thus PE can serve as the second dye together with fluorescein in dual-label staining. Phycobiliproteins are available commercially from several sources or, for the more adventurous, can be purified from red algae (for PE) or cyanobacteria (for PC and APC) by simple chromatographic procedures.

Labeling antibody (Ab) with phycobiliprotein (PbP) is more difficult than with small organic reagents such as FITC. Probably the most reliable approach is to use a heterobifunctional cross-linking reagent such as succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) or *N*- γ -maleimidobutyryloxy-succinimide (GMBS). Both of these reagents

carry maleimide groups (reactive with free thiols) and succinimide ester groups (reactive with primary amines). Thus one can couple GMBS to lysine groups on the Ab and introduce free sulfhydryl groups into the PbP with a reagent known as 2-iminothiolane (2-IT). After labeling, the proteins are then mixed (using a 1.5- to 2-fold molar excess of PbP:Ab) and a complex is allowed to form. Small complexes of 1:1 PbP:Ab appear to be best for staining, since they show little nonspecific background. Often the complex does not require separation (this can be tested by staining before attempting separation), but if needed it can be chromatographed on a small hydroxyapatite column or fractionated by gel filtration. An alternative labeling approach that works for certain 'problem' antibodies where the other method fails is to label the PbP with cross-linking reagent and then mix it with Ab that has been mildly reduced (breaking interchain disulfide bonds and generating free sulfhydryl groups) with dithiothreitol (DTT).

Fluorescence microscopy

Fluorescein and rhodamine reagents can be employed individually or simultaneously with appropriate optics. Surface immunofluorescence staining can be performed either on cells in suspension (as outlined in the section on 'Flow cytometry' below) or on frozen tissue sections. After staining, cells are often then fixed with 2% fresh paraformaldehyde (15 min at room temperature) and can then be stored at 4°C for up to 2 weeks. Internal staining is also possible. Cells are applied to a microscope slide either as a 'smear' or, alternatively, by use of a device known as a cytocentrifuge which usually gives a more dense and uniform distribution (particularly important if cells are limiting). The slides are dried and then permeabilized with acetone. Staining is performed in humidified chambers to prevent evaporation, typically for 30 min at room temperature. After washing, stained preparations are mounted in glycerol/PBS containing an antifading compound (commercially available). A major difficulty encountered with fluorescence microscopy (particularly photography) is bleaching, which is somewhat less of a problem with rhodamine than with fluorescein. Finally, it is possible to view Texas Red labeling using rhodamine excitation/emission filters and in dual labeling with fluorescein it often shows better specific labeling than conventional rhodamine. One should also note that dual staining with PE (viewed either with rhodamine optics or specialized PE excitation/emission filters) has seen increasing application in the past few years.

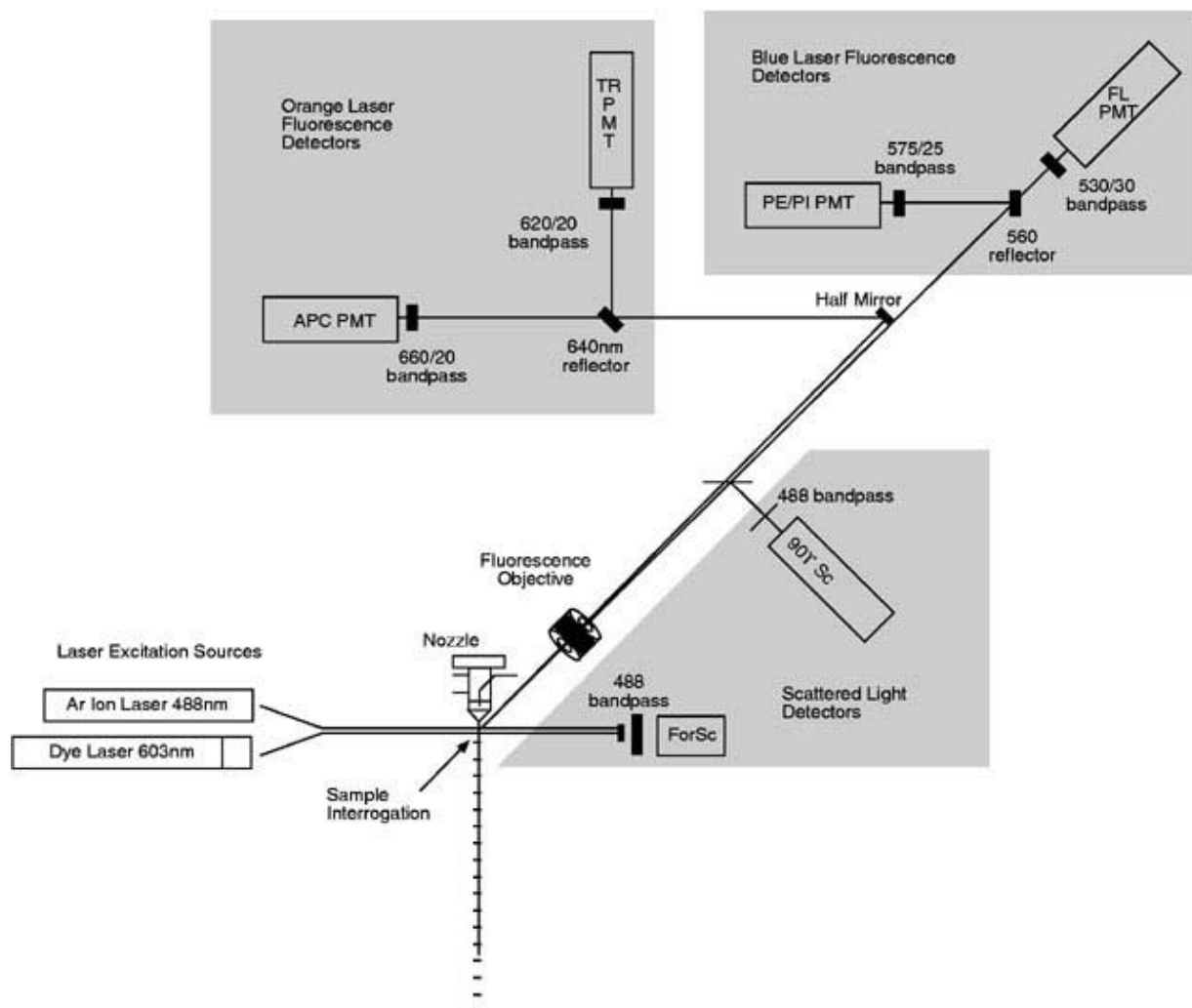


Figure 1 Six-parameter flow cytometry optical system for multicolor immunofluorescence analysis. Cell fluorescence excited by the blue (488 nm) laser is divided into green (FL) and orange (PE) signals, while fluorescence excited by the orange (603 nm) laser is reflected by a mirror and divided into near red (TR) and far red (APC) signals. Blue light scattered at small forward angles (ForSc) and at right angles (90° Sc) is also measured in this system, providing information on cell size and internal granularity.

Flow cytometry: two-color/single-laser

As mentioned above, dual staining in flow cytometry presented a real challenge, one which was not met by the fluorescein/rhodamine combination. Dual-laser systems, especially in combination with a dye laser, gave high-quality correlated immunofluorescence data with fluorescein and Texas Red, but at the cost of optical and electronic complexity and at considerable added expense compared with single-laser instruments. PE is now employed routinely together with fluorescein on a single-laser instrument for two-color correlated analysis. The broad excitation spectrum, large extinction coefficient, and high quantum yield of PE make it a reasonable dye to use in combination with fluorescein at 488 nm excitation. As shown by Figure 1 (blue laser fluorescence), the fluorescences are divided by a

560 nm longpass dichroic reflector, suitably filtered, and then the analog signals are compensated to correct for the small overlap of PE in the fluorescein (FL) channel and the somewhat larger overlap of fluorescein in the PE channel. Data quality compares favorably with two-color staining carried out on a dual-laser system.

Cell suspensions are stained on ice either using directly labeled reagents in a single step or indirectly in multiple incubations separated by washes. Incubations are usually 15–30 min and 2–3 wash steps are probably sufficient to eliminate unbound reagent. Staining medium can be a simple balanced salt solution or even modified cell culture medium and typically contains 5–10% serum (acting as carrier protein) and 0.1% NaN₃ (to prevent capping). If the biotin/avidin two-step staining procedure is employed, it is imperative that the staining medium

does not contain biotin. Dual staining requires that single-label controls also be included to verify that compensation is correct and that there is no interaction between reagents.

Flow cytometry: four-color/dual-laser

Phycobiliproteins also offer the possibility of carrying out three- or four-color correlated immunofluorescence measurements. As shown in Figure 1, a half-mirror splits the fluorescence signals from the two laser spots. Fluorescein and phycoerythrin fluorescences, excited by the 488 nm (blue) laser, are detected and compensated as described above for a single-laser system. Texas Red (TR) and allophycocyanin fluorescences, excited by a tunable dye laser operating at 603 nm (orange), are split by a longpass dichroic reflector at 640 nm, filtered further and then compensated for the somewhat greater overlaps in the TR/APC fluorescence emissions (as compared with the FL/PE combination). This design permits either four immunofluorescence measurements or three immunofluorescence measurements together with propidium iodide (PI) dead cell gating (to eliminate dead cells).

Three- and four-color immunofluorescence have opened up a new era in flow cytometry analysis. Very small populations of functionally distinct lymphoid cells can now be discriminated on the basis of the correlated expression of three or four cell surface antigens. In addition, it is possible to include control staining reagents together with specific reagents and

so determine specific staining on a cell-by-cell basis. Finally, the increased fluorescence signal derivable from phycobiliprotein reagents has eliminated the need for the amplification possible with second-step staining. Thus, these dyes now permit rapid simple quantitation of determinants recognized by monoclonal reagents, even those expressed at very low levels on rare populations in complex mixtures of cells.

See also: Cell separation techniques; Flow cytometry; Immunoassays; Immunocytochemistry and enzyme markers.

Further reading

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FOOD ALLERGY

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Untoward reactions to foods – especially cow's milk – were recognized in ancient times by Hippocrates and Galea. However, the first thorough clinical inquiries were undertaken at the beginning of the twentieth century, when chronic diarrhea and anaphylactic-type reactions were observed in babies fed with cow's milk. Later, other types of clinical manifestations were described, e.g. urticaria, eczema, anemia and various respiratory tract and gastrointestinal problems, but the concept of immunologically mediated clinical reactions appeared only progressively, with the discovery of the immunoglobulins and an improved method for the detection of specific antibodies.

More recently, the discovery of IgE immunoglobulins by Ishizaka has considerably increased our understanding of immediate type I hypersensitivity. Having a more accurate means of measuring IgE-mediated reactions, it soon became evident that only a modest part of all the intolerances due to food could be verified by these new *in vivo* and *in vitro* procedures. Consequently, the concept of false food allergy (pseudoallergy) gained in importance and now, besides IgE-mediated reactions, a large variety of mechanisms has been proposed and sometimes verified by appropriate techniques.

The following classification of adverse reactions to food is suggested.

1. Toxic reactions (favism, mushrooms, etc.).
2. Immunologic mechanisms: IgE, IgG and T cell mediated.
3. Food intolerance or pseudoallergy: pharmacologic, enzymatic and undefined reactions.

Disorders associated with food allergens

Gastrointestinal tract

The variety of intolerance reactions due to foods is great. In infancy and early childhood, cow's milk and soya are the major causes of allergic reactions, followed by cereals, eggs and fish. The age of onset is highly variable, from the first days of life (cow's milk) to the age of 2 years. In 90% of cases, clinical intolerance disappears after the age of 3 years. The most common clinical picture is of vomiting, diarrhea and abdominal pain, usually appearing within minutes, or at the most 1–2 h, after ingestion of the food. The period of sensitization is usually short; in some cases, the reaction may occur at the first exposure, probably as a result of previous sensitization by breast milk.

Among the quick-onset reactions, swelling of the lips and tongue, often associated with urticaria and angioedema, are the most common; however, respiratory manifestations also frequently occur, mainly in the form of asthma, cough and stuffy nose. A family history of atopy is often present in such infants and these types of clinical reaction usually correspond to IgE-mediated hypersensitivity, which can be analyzed by means of prick tests and the determination of specific antibodies. Among the slow-onset reactions, food-sensitive enteropathies are the most frequent and also the most difficult to diagnose, especially because of the late onset of the reactions after the ingestion of the incriminated food (sometimes 1–3 days) and the numerous other causes which show a similar clinical picture.

In addition to cow's milk and wheat, which represent more than 80% of all food-sensitive enteropathies in infants, many other foods have been shown on occasion to be the offending agents among older age groups. The major symptoms are diarrhea, abdominal pain, pallor, lethargy, vomiting and loss of weight. Allergic reaction to food is not necessarily evident, and other types of gastroenteropathies need to be excluded. When not recognized early enough, the disease may develop several accompanying symptoms, such as occult intestinal hemorrhage, protein-losing enteropathy, transient gluten intolerance, IgA deficiency and fat malabsorption, among others.

This later-onset gastroenteropathy usually starts during the first 6–8 months of life, when the physi-

ology and the immune competence of the gut are still immature. According to Ferguson, it seems likely that the enteropathy may be due to a type IV hypersensitivity T cell-mediated reaction.

There are no gastrointestinal symptoms due to food allergy which are specific to teenage and adult patients, despite some characteristic differences. For instance, food-induced enteropathy is unusual at this age; on the other hand, gastric and colic symptoms are very frequent. Cow's milk allergy is less prominent as a major allergen. According to large epidemiologic studies, celery, fish, seafood and eggs are the most frequently responsible food allergens among these age groups.

On the other hand, although almost unknown in infancy, among adults reactions to food additives and other chemicals, i.e. false food reactions, are more likely than to the foods themselves. The colon represents one of the major target organs for these various offending agents.

Respiratory tract

Food allergy and intolerance are important triggering factors of asthma. The true frequency is difficult to establish because, in most cases, other allergen families also play a role. Asthma due to a single food allergen is rather infrequent.

According to several clinical studies, the frequency of food-mediated asthma varies from 0.3 to 8.4% of asthma in patients aged under 15 years of age and is somewhat less frequent in adults. It is of interest to note that in studies where correct provocation tests have been undertaken, the percentage of true food-induced asthma is much lower than in those studies in which the diagnosis was made only by means of skin tests and/or the determination of specific IgE. In some cases, the route of sensitization is clearly gastrointestinal (flour, egg, vegetables); in other cases, inhaled allergens are the triggering factors (smell of fish, metabisulfites, garlic, etc.). As mentioned above, food-induced asthma is in most cases only part of a more complex clinical picture. Eczema is a frequent event, particularly in infants; however, urticaria and nasal problems, among other symptoms, constitute a multifocal hypersensitivity syndrome. The diagnosis is difficult because of the coexistence of true allergens with other triggering agents, acting through various mechanisms.

Acute or chronic allergic rhinitis due to food is a problem well known to all allergists; however, its true incidence is a matter of debate. According to various studies it varies from 2 to 25% of all rhinitis.

Additionally, in chronic rhinitis, there is almost always a hyperactive component that can be ascertained following nasal challenge with histamine.

Therefore, the presence of sulfite, nonorganic dusts, and a variety of additives and chemicals may add their effects to those of the suspected food. In such situations, the diagnosis, even with the aid of a provocation test, will often remain inconclusive.

According to various reports, secretory otitis media, particularly in its chronic form, may possess an allergic component in some cases. This is especially true where there exists a known allergy to foods with respiratory and gastrointestinal symptoms. The verification of such a problem is usually indirect, e.g. a clinical improvement being observed following an exclusion diet. According to some studies, up to 80% of patients with secretory otitis media also have an allergic rhinitis; therefore investigation of the rhinitis can eventually help to identify the food component of the otitis.

It is a common observation that some foods can initiate aphthous ulceration in the mouth; however, the true mechanism of these local inflammations is still unknown.

Skin

Eczema, urticaria, vasculitis and angioedema are the major skin reactions observed in food allergy. Urticaria, with its typical histamine-like wheal and flare reaction, is probably the most common reaction of adults to food allergy. About half of these patients develop angioedema simultaneously; angioedema may sometimes be present without urticaria.

Urticaria can be an acute isolated reaction, episodic or chronic. A form of contact urticaria also exists. In most cases, acute urticaria seems to be an IgE-mediated reaction. On the other hand, for chronic and contact urticaria, pharmacologic as well as immunologic mechanisms are involved. Investigation of a chronic urticaria is, unfortunately, deceiving. In no more than 20–30% of these cases can a single specific etiological factor be identified. Drugs such as penicillin and aspirin are the most frequent causative factors. As far as foods are concerned, the testing of patients and the application of elimination diets result in a poor recovery, at most a few per cent. The chances of success are certainly greater among atopic individuals. However, among such patients it is worth looking for histamine-releasing agents present in the food or analyzing the diet for a high histamine content (cheese, seafood, wine, salami, etc.). Also, a strict 2 week additive-free (coloring, preservative agents) diet is sometimes beneficial.

Allergic vasculitis, which is a type III immune complex-mediated reaction, is characterized by a local perivascular inflammation and purpura. Up to now only a few cases have been reported, with food as

the etiological agent. The foods that have been implicated include egg, milk, chocolate, tomato, shellfish, wheat, port and tartrazine.

Atopic eczema is largely a disease of children. This clinical form is rather easy to distinguish from other types of eczema, such as contact eczema. The overall incidence of eczema in early childhood is 10–15%. In only about 2–5% of these cases does the disease persist into adult life. Serum IgE levels are usually elevated – a sure sign of atopy – and other focal allergies such as gastrointestinal problems, asthma and hay fever are often present. However, despite this close association, the role of IgE antibodies in the genesis of eczema is still debatable.

Nevertheless, numerous studies have clearly demonstrated the role of foods (especially milk and eggs) in many cases of atopic eczema. In these clinical trials, many of them double-blind, 10–25% of patients were shown to respond to one particular food. The overall response rate, however, tended to be lower among adults than among children. Also, a lack of correlation has been observed in some cases between the response to diet and the presence of specific IgE antibodies to the food tested.

In this area, most studies have focused on the major allergenic foods, i.e. cow's milk, egg and wheat. In more recent clinical trials, an elemental and oligoantigenic diet has been introduced. Several authors have reported that, beside cow's milk and egg, other foods such as citrus fruit, fish, nuts, tomatoes, lamb, chicken, and colorings and preservatives may be responsible for the exacerbation of eczema among children. Unfortunately, few similar studies have been undertaken with adults.

In conclusion, atopic eczema is a complex clinical problem, the pathogenicity of which is still poorly understood. The following can be used in the management of this disease.

1. This disease is most often observed in atopic individuals.
2. Despite some controversial results, breastfeeding seems to protect infants or delay the onset of the disease.
3. Atopic eczema in breastfed infants may reflect the transmission by human milk of antigens derived from the mother's diet.
4. Specific IgE antibodies to foodstuffs does not necessarily mean a causal relationship with that food.
5. Many foods other than cow's milk have to be considered in the etiology of the disease.
6. Small molecular weight substances such as colorings and preservatives may precipitate attacks of atopic eczema.

7. Important antigens may appear during digestive degradation in the gastrointestinal tract.

Central nervous system

Food-induced migraine is an old problem. As early as 1873, Lieving reported a number of patients whose migraine was due to wine and burnt pastry. Following this study, many researchers reported a relationship between the intake of particular foods and beverages and attacks of migraine. Since then, more sophisticated methods have been introduced to show that there is, for instance, a relationship between the results of specific IgE antibody and skin tests and nontolerated foods, as demonstrated by means of elimination diets and provocation tests.

Patients with food-related migraine represent a subgroup among migrainous individuals. Indeed, they almost all exhibit a variety of symptoms associated with their migraine. Among these symptoms, fatigue and depression are common, as are conjunctivitis, rhinitis, angioedema, urticaria, asthma, irritable bowel syndrome and aching muscles and joints. Frequently the foods (often several foods simultaneously) responsible for the migraine may also play a role in the genesis of the associated symptoms.

Besides IgE antibody-related migraines, there are several biochemical factors associated with the provocation of migraine. Substances such as histamine, tyramine, phenylethylamine, monosodium glutamate, sulfites and phenolic compounds may be associated with attacks of migraine, and the list seems to be growing. Therefore, the diets of migraine patients – especially if they are members of atopic families – should be investigated thoroughly.

When considering a specific food, it is important to appreciate that not only may it induce an IgE-dependent reaction, but it may also act as a non-specific histamine liberator. Furthermore, several allergenic foods are rich in histamine, which may explain some of the clinical problems. Finally, several food constituents in combination could release allergenic mediators. The hyperkinetic syndrome in children has been studied repeatedly, and it has been shown that there is usually a relationship with the diet; however, there is no evidence as to which mechanisms are involved. At present, the best way of identifying which foods are involved is to use an oligoantigenic diet followed by sequential reintroduction of the foods.

Food anaphylaxis

Food anaphylaxis is a severe, sometimes life-threatening generalized reaction. Peanut allergy is the best known example.

The major foods which may induce anaphylaxis are shellfish (crustacea and molluscs), fish, egg, tree nuts, celery and black pepper. These food allergies are often lifelong and reactions can be elicited with minute amounts of the allergen. In most of those cases, oral provocation tests are too dangerous to be performed. Food-dependent, exercise-induced anaphylaxis is also rather frequent in these patients.

Food allergens, additives and chemicals

By carefully reviewing the specialized publications on food allergy, it appears that any food can be potentially allergenic. However, this potential is largely related to ethnic and cultural feeding habits: it is not surprising that allergy to cow's milk or fish is frequent in populations consuming large amounts of these foods. However, with the standardization of feeding habits in the world, exotic or unusual foods may develop their allergenic potential. Kiwi fruit were almost unknown in Europe 15 years ago, but now they have become a frequent allergen. Therefore, common sense dictates that the eating habits of a patient who is suspected of being allergic to a certain food(s) should be carefully noted.

Indeed, the existence of another focal allergy is of major importance. For instance, the knowledge of cross-reactivity between inhaled and ingested allergens demonstrates clearly the frequency of this problem. As an example, the cross-antigenicity between birch pollen and several fruits (apple, pear, peach, cherries, etc.) and vegetables (celery, carrots, parsley, etc.) or the relationship between celery and several spices represent a very complex mosaic of allergenic relationships. This means that unrelated food families might have common allergenic determinants. This does not facilitate the approach of the clinical diagnosis, but at least, for the interpretation of the results, it is essential to be aware of this complexity. However, for some food families for which cross-reactivity is commonly demonstrated *in vitro* or by skin testing, oral challenge results indicate that clinically relevant food allergy is usually specific for one item. This is especially the case for legumes and cereal grain allergy, for which dietary restrictions of the entire food family is rarely needed.

Several other mechanisms may be implicated, the understanding of which is progressing slowly. We are living in an environment which is largely under the influence of chemical substances. According to a recent German study, 140 000 chemicals are now part of our daily life; a number of these may be present in nutrients, either accidentally or in relation to the normal practice of the food industry. Very little information is available about tolerance to most

of these substances. For instance, almost 3000 food additives are used throughout the world, only a few of which have been studied in any detail.

Diagnosis and mechanisms

The major foods responsible for allergic reactions may be related to age as well as specific ethnic and local practices. Food allergens in infants are usually limited to cow's milk, eggs, fish, cereal and soya. In older children the diet is more or less the same as that of their parents; it therefore includes a larger variety of potential allergens. Some of the major criteria used for the diagnosis of food allergy are presented in Table 1.

One of the major steps toward a correct diagnosis is the elimination of the suspected food. If the patient's history does not suggest a specific food, there are two possible approaches:

1. To eliminate successively families of food (milk products, eggs, meat, vegetables, fruits, sweets). The test period should be of at least 2 weeks duration, which is usually sufficient for the symptoms to disappear. Another family of food should be eliminated if no changes are observed.
2. An alternative approach is to introduce a 2–3 week – sometimes longer in eczema patients – oligoallergenic diet, which should include rarely-consumed food such as exotic fruit (no citrus fruit) or cooked vegetables when there is no poliosis present.

There is no standard oligoallergenic diet. It has to be composed according to the feeding habits of each patient. Oral challenge with suspected foods should

be used with caution and under strict medical control because of the risk of an anaphylactic reaction. The best practice is to start with a small amount of food and increase it progressively, over 2–3 days, up to the usual amount. The diagnostic criteria, such as examination of the skin, presence of cough or dyspnea, diarrhea or vomiting or other gastrointestinal tract symptoms, should be carefully observed. A determination of blood eosinophilia is often of great value. A double-blind study is certainly the most objective way of obtaining indisputable results; however, one cannot ignore the difficulties of presenting a large amount of food in an unrecognizable form.

In patients to be challenged, a previous investigation should be performed with the aid of skin tests and the determination of food-specific IgE antibodies (Table 2). However, the food allergens are in most cases crude extracts of whole food, thus increasing the risk of cross-antigenicity and the development of nonspecific inflammatory reactions to skin tests. Besides, there is also a limitation to both the quality and quantity of the allergen coupled to the solid-phase material used for the *in vitro* identification of specific IgE. Therefore, even in atopic individuals, the predictive value of tests for IgE antibodies to foods appears to be rather limited for standard clinical use. Furthermore, types III and IV immunologically mediated hypersensitivities are not infrequent. Unfortunately, we have no reliable laboratory procedures to establish the diagnosis of these types of reactions, with the exception of the lymphocyte proliferation test applicable in the case of a delayed response following oral provocation.

False food allergy (or pseudoallergy)

These types of reaction outnumber the true immunologically mediated ones. Various clinical syndromes are also known to be induced by such reactions, for example chronic urticaria, anaphylactic shock, intermittent diarrhea and irritable bowel syndrome, migraines, rhinitis and asthma. Table 3 lists some of the substances and mechanisms implicated in false food reactions.

Table 1 Major criteria for the diagnosis of food allergy

1. Food allergy is most frequent among atopic individuals, in the presence of IgA deficiency, and in individuals suffering from chronic gastrointestinal problems
2. To some extent, all tissues and organs can be affected by allergenic reactions
3. Nasal, respiratory tract and skin problems can be present without any evidence of gastrointestinal tract involvement
4. The threshold of sensitivity to one specific food is highly variable
5. When the suspected food allergen has been eliminated, the symptoms should disappear
6. A positive elimination diet does not have any significance as far as the mechanism is concerned
7. Following provocation tests, a quick onset of symptoms often means an anaphylactic type of reaction, which usually needs only a small amount of allergenic food
8. Delayed reactions, such as atopic eczema, respond to a provocation test with a delay of 8–24 h

Table 2 Investigations for the diagnosis of food allergy

1. Skin tests (prick) with, where possible, purified allergens
2. Specific circulating IgE antibodies.
3. Double-blind placebo-controlled food challenge
4. Tests to be applied, eventually, before and after oral provocation:
 - Determination of fecal specific IgE antibodies
 - Intestinal permeability test
 - Circulating histamine release test

Table 3 Biologically active substances and mechanisms playing a role in false food reactions

Substances	Type of activity
Histamine	Present in large amounts in some foods; activates specific mediators
Histidine	Present in large amounts in some foods; precursor of histamine
Tyramine	Present in large amounts in some foods; causes pulsatile headache and hypertension
Phenylethylamine	Present in some food; causes migraine
Benzoate	Present in some foods and benzoic acid; causes asthma and rhinitis
Sodium bisulfite	Preservative; destroys vitamin B ₁ ; causes gastrointestinal symptoms
Sulfur dioxide and metabisulfites	Preservatives; cause gastrointestinal tract symptoms, headache, asthma; destroy vitamins B ₆ and B ₁
Monosodium glutamate	Flavor-enhancing agent; causes asthma and the Chinese restaurant syndrome
Food colorings	Especially yellow no. 5 and no. 6; cause asthma and other allergic reactions
Aspirin	One of many possible triggering factors inducing asthma, urticaria and angioedema

The diagnosis of false food allergy is difficult. Amine intolerance, which is frequent in atopic individuals, can eventually be suspected when analyzing the patient's diet. It may coexist with true IgE-mediated reactions. The diagnosis of intolerance to food additives is even more complicated. Besides a thorough clinical history and an analysis of the diet, the first approach is to introduce an additive-free diet, something that is not necessarily simple. Alcohol and coffee, which seem to augment the resorption of small molecular weight substances, should be discarded. Fortunately, additives which are used in small concentrations allow the practice of double-blind oral or sublingual provocation tests in capsules. Three to four concentrations of each substance are usually applied at 2 or 3 day intervals.

Patients who have repeatedly received antibiotics or have a diet particularly rich in sugars and fat might show an invasion of their gastrointestinal tract by *Candida albicans* or *Geotrichum candidum*. This infestation seems to favor allergy as well as pseudo-allergic reactions to foods, and should be treated.

Treatment

One of the major tasks in the management of food allergy is the avoidance of the responsible foods. In practice, this approach is not as simple as it seems. Often, allergy specialists are confronted with patients who have had a poor diet (from a nutritional point of view) for a long time, simply because foods have progressively been eliminated from their diet by their physician on purely subjective grounds. Therefore, it is worth the clinician spending time in controlling the elimination-reintroduction procedure in order to identify for certain the offending foods. Further, the dose-response is highly variable from patient to

patient, several of whom can tolerate a small amount of the food to which they are sensitive, an amount which can be determined by means of provocation tests. The new diet has to be appetizing and complete as far as nutrients, vitamins and minerals are concerned. For this specific aspect, the skill of a dietician is of major importance.

Drug therapy directed against the patient's symptoms is required, whatever the local organ involved. This is especially important in the case of chronic gastrointestinal and colic disturbances.

Drugs

A number of studies have shown that allergic reaction to foods can be ameliorated or prevented by sodium cromoglycate (SCG). This drug seems to be efficient in IgE-mediated reactions as well as in the case of histamine-releasing foods. There is sometimes intolerance to the drug itself. The best way of minimizing this problem is to start the treatment at low doses, increasing it daily to efficient levels by 10–15 days. However, the experience of several clinicians is that after several months of treatment the effect of SCG often decreases and sometimes disappears completely; therefore this therapeutic approach can only exceptionally be considered as a long-term treatment.

The best way of managing the food allergy problem is to introduce the correct elimination diet. If followed for a long period of time (more than 1 year), a relative tolerance to the offending food is frequently observed.

Hyposensitization

This therapeutic approach has never really been evaluated by allergologists, and in most cases there is no real indication for such a treatment. However,

there are two different situations in which such a treatment can be useful, even life-saving:

1. Anaphylactic shock to egg, celery, nuts, fish, milk, etc. is rather frequent in adult patients. Recently, ten such patients were hospitalized and a rush-desensitization procedure was undertaken. The treatment was continued at home. After 3 months of treatment, the offending foods could be tolerated by each patient.
2. Allergy to milk in infants is frequently complicated by an allergy to soya. We suggest an oral hyposensitization regimen beginning with 1:10 000 or 1:100 000 diluted cow's milk. After 3 months of increasing the oral dose daily, 80% of treated children are able to tolerate a glass of milk.

Thus this type of treatment seems to produce a long-lasting tolerance in most patients and is relatively easy to apply.

Conclusions

Allergic reactions and intolerance to food, additives and other chemicals now represent a fascinating and motivating field of research. Despite the fact that knowledge is lacking in several areas of this complex domain, it is actually possible to improve the life of a large number of individuals by simply applying good

clinical practice and a few simple investigative procedures.

See also: Allergens; Asthma; Atopic allergy; Eczema; Histamine; Hypersensitivity reactions; IgE.

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FORSSMAN ANTIGEN

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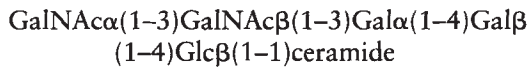
The Forssman antigen was named after the Swedish pathologist John F. Forssman (1868–1947) who reported, in 1911, that antibodies produced in rabbits against organ homogenates from guinea pigs reacted with sheep erythrocytes, causing hemolysis. The Forssman antigen is thus an example of a heterophile antigen or hapten which occurs in several species. In 1936 Forssman antigen was recognized by F. E. Brunius as a hexosamine-containing lipid. The determinant was later identified as the GalNAc α (1–3)GalNAc β (1–R) disaccharide group. The complete structure of a Forssman-specific glycosphingolipid from horse spleen was published in 1971 by B. Sidiqui and S. I. Hakomori. Forssman specificity was described in many animal species, plants and bacteria. In some species, for example the mouse, Forssman antigen is a developmental and differentiation

antigen. Although it is mostly expressed on the cell surface, there are examples of expression inside cells. Antibodies against Forssman antigen can be used to induce shock in guinea pigs.

Structure, conformation, isolation and detection

Although the concept of the Forssman determinant may have varied over the years since its discovery, originally being more or less synonymous with hemolytic antisheep erythrocyte specificity, the now accepted determinant is a terminal GalNAc α (1–3)GalNAc β (1–R) disaccharide group. This determinant can be linked to a glycosphingolipid residue, as is usually the case in animal cells. The most frequent

structure of such a glycosphingolipid is a member of the so-called globo series:



Information about the nonlinear conformation came from nuclear magnetic resonance. Isolation of Forssman-reactive glycosphingolipids can be achieved by conventional extraction and purification methods in use for glycosphingolipids. Forssman pentasaccharide has also become available through organic synthesis. Various assays for detecting Forssman antigen or Forssman-specific antibodies have been described, ranging from sheep erythrocyte agglutination inhibition to radioimmunoassays or liposome lysis assays. A number of monoclonal antibodies specifically detect the Forssman determinant without cross-reactivity with blood group A or globoside.

Species distribution, cross-reacting antigens from bacteria or parasites

Only certain animal species express Forssman antigen (Table 1). In humans, approximately 20–30% of individuals possess the antigen. Which particular cells are Forssman positive depends on the species.

Table 1 Occurrence of Forssman antigen in tissues from various animal species

Species	Forssman positive
Carp	Yes
Cat	Yes
Cattle	No
Chicken	Yes
Dog	Yes
Frog	No
Goat	Yes
Goose	No
Guinea pig	Yes
Hamster	Yes
Horse	Yes
Human	20–30%
Mouse	Yes
Musk shrew	Yes
Pig	No
Pigeon	Yes
Quail, Japanese	Yes
Rabbit	No
Rat	No
Sheep	Yes
Turtle	Yes

Some strains of gram-negative (*Shigella*) as well as gram-positive (*Pneumococcus*) bacteria carry Forssman specificity. In pneumococci Forssman antigenicity is associated with lipoteichoic acid. Passively acquired Forssman antigen was also found in amastigotes of *Leishmania* proliferating in Forssman-positive murine macrophages; however, here, as in virus grown in Forssman-positive host cell cultures, the antigen was derived from the host cell.

Cellular distribution, role as developmental and differentiation antigen

The function of Forssman-specific cell surface carbohydrate determinants for animal cells, if any, is not entirely clear. However, data from different experimental systems suggest that these carbohydrate structures may play a role in cell adherence and recognition. Thus Forssman determinants appear at certain stages in murine embryonic development, they are involved in laminin adhesion of canine kidney cells, they may play a role in the formation of hematopoietic foci in mouse bone marrow, and they are serological markers for subpopulations of murine mast cells or murine macrophages residing in defined areas of the spleen (Figure 1). At least in macrophages expression of Forssman antigen can be modulated by cytokines.

Forssman glycosphingolipid as a receptor for bacterial adhesins

Bacteria have acquired means of specifically adhering to target host cells via fimbriae. For example, uropathogenic *Escherichia coli* adhere to glycolipid receptors on epithelial cells. The receptor for class III adhesins from such bacteria is the Forssman glycosphingolipid. Other adhesins prefer the underlying globoside structure.

Forssman specificity and disease

Forssman-specific antibodies have been found to be associated with autoimmune diseases such as Graves' disease and Hashimoto's disease. The role of Forssman-specific antibodies in patients with Guillain-Barré syndrome is under discussion. Forssman determinants are also expressed in certain human cancer tissues – for example, in adenocarcinoma of the stomach and colon in individuals whose tissues are normally Forssman negative.

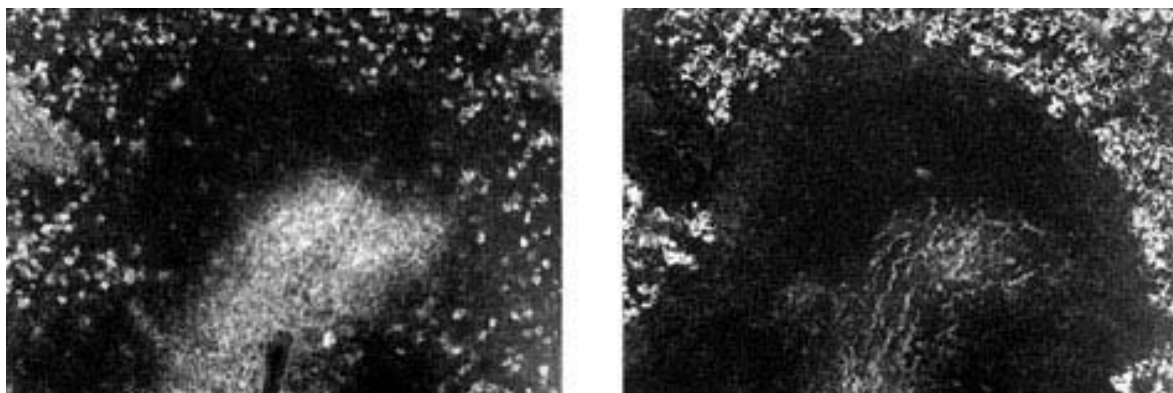


Figure 1 Immunostaining of Forssman-positive macrophages in the red pulp (left) and Thy-1-positive T cells (right) in adjacent sections of a murine spleen. Note the faint Forssman-positive immunostaining of reticular cells in the T cell area.

Forssman shock

Systemic Forssman shock ensues when Forssman-specific antibodies are given intravenously to guinea pigs whose endothelial cells of the lung and kidney are Forssman positive. A complement-dependent reaction leads to bronchioconstriction and pulmonary endothelial lesions. The reaction is used as an animal model to test various drugs against anaphylaxis, thromboxane-mediated bronchioconstriction or thromboembolism.

See also: ABO blood group system; Carbohydrate antigens; *Escherichia coli*, infection and immunity; Heterophile antibodies; Leishmaniasis; *Shigella*, infection and immunity.

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FRANCISELLA, INFECTION AND IMMUNITY

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Tularemia is a zoonotic disease caused by the facultative intracellular bacterium *Francisella tularensis*. There are two predominating forms of the disease: ulceroglandular tularemia which is vector-borne or caused by contact with infected animals, and respiratory tularemia which results from inhalation of contaminated dust. *F. tularensis* occurs in two main biovars: var. *tularensis*, and var. *palaeartica*. *F. tularensis* var. *tularensis* also called type A, is isolated only in North America. It is associated with ticks and rabbits and is highly virulent in humans. Before antibiotic treatment was introduced, mortality was about 10%. *F. tularensis* var. *palaeartica* (type B), which occurs in Asia, Europe, and to a much lesser extent also in North America, is associated with mosquitoes and rodents. It is less virulent in humans. The mortality was less than 1% in preantibiotic times. Biovar type A can be separated from type B by its ability to form acids from glycerol and to split citrulline into ornithine, carbon dioxide and ammonia. As judged from immunodiffusion studies, the two biovars have a similar antigen composition.

Characteristics of the organism and its antigens

F. tularensis is a small coccoid or ellipsoid nonmotile gram-negative bacterium. It has a capsule which can be dissociated from the cell wall by incubation of the bacteria in hypertonic NaCl.

It grows poorly on most ordinary culture media. Growth is, however, well supported on blood agar enriched with L-cysteine and glucose. Chocolate agar, such as used for the isolation of gonococci, also maintains the growth of *F. tularensis*. *F. tularensis* is strictly aerobic, weakly catalase-positive, and oxidase-negative. Carbohydrates are weakly catabolized with the production of acid but no gas. The organism has a characteristic pattern of cellular fatty acids, including long-chain (C20–C26) acids and the hydroxy acids 2-hydroxydecanoate, 3-hydroxyhexadecanoate and 3-hydroxyoctadecanoate.

F. tularensis seems to be unique among clinically important gram-negative bacteria in so far as more than 50% (wt/vol) of its capsule and cell wall are lipids. Accordingly, ether-water extraction is an efficient way to release antigen. The reactivity of immune serum with such an extract is due mainly to carbohydrate determinants, whereas determinants responsible for immunospecific lymphocyte stimu-

lation reside in proteins of the extract. Antibodies to membrane proteins of *F. tularensis* can be demonstrated in immune serum by the immunoblotting technique.

F. tularensis is strongly agglutinated by commercially available antisera. By immunodiffusion of sonicated *F. tularensis* against hyperimmune serum, several antigenic determinants are disclosed. Minor cross-reactions are found with *Brucella*, *Yersinia* and *Proteus*. The only strongly cross-reacting species seems to be *Francisella philomiragia*.

Proteins of *F. tularensis* able to induce cell-mediated immunity reside mainly in the bacterial membranes. According to *in vitro* studies, several membrane polypeptides of the vaccine strain *F. tularensis* LVS induce a T cell response in LVS-vaccinated individuals as well as in individuals previously undergoing tularemia. Experiments with T cell clones suggest that there is a heterogeneity of T cell determinants among these polypeptides.

Immune responses of the host

A strong antibody response to *F. tularensis* is mounted during infection. In diagnostic work, this response is generally demonstrated by use of agglutination techniques, although ELISA can also be applied. Irrespective of technique, the antibodies can be demonstrated at the end of the 2nd or during the 3rd week of disease. The antibodies endure for several years after recovery from tularemia. It should be noted that immunoglobulin M (IgM) antibodies do not appear earlier than IgG antibodies and that IgM antibodies may be present several years after recovery from tularemia. The possible role of antibodies in host defense to *F. tularensis* is largely unknown.

A cell-mediated immune response also develops during tularemia, as demonstrated by the delayed-hypersensitivity test or by the *in vitro* stimulation of T cells. An immunospecific response can usually be demonstrated within 2 weeks of onset of disease, and thereafter during more than two decades. Similar to other intracellular parasites, cell-mediated immunity toward *F. tularensis* is a prerequisite of host protection.

Evasive strategies by the organism

F. tularensis possesses an abundant capsule, which affords complete protection against complement-

induced lysis and gives resistance to intracellular killing by polymorphonuclear leukocytes. This resistance is a virulence trait, which is lost in the attenuated live vaccine strain *F. tularensis* LVS. LVS is efficiently killed by polymorphonuclear leukocytes due to strictly oxygen-dependent mechanisms. Both a wild strain of *F. tularensis* and LVS induce a respiratory burst in the phagocytes during endocytosis, but only LVS is susceptible to the hypochloric acid and hydrogen peroxide produced as a result of the burst. The actual virulence factor has not been identified.

There is no evidence to indicate that potent toxins are produced by *F. tularensis*. Similar to other facultative intracellular bacteria, *F. tularensis* seems to cause disease by a rapid proliferation in mononuclear phagocytes. A fulminating disease is prevented by evoking an effective T cell-mediated immune response before the bacterial burden becomes too large.

Vaccines

Host protection against tularemia is induced by natural infection or by vaccination with viable attenuated bacteria but not by vaccination with killed bacteria. Live tularemia vaccines were developed in the USSR during the Second World War. In 1956, a mixture of attenuated type B strains of *F. tularensis* was transferred from the Gamaleya Institute in Moscow to Fort Detrick, Maryland. A strain of suitable virulence was isolated, tested for safety and efficacy in humans and introduced as the live vaccine strain *F. tularensis* LVS. According to a retrospective study on the occurrence of laboratory-acquired type A tulare-

mia at Fort Detrick, LVS affords effective protection against respiratory tularemia and modifies signs and symptoms of the ulceroglandular form of the disease. Thus, an attenuated vaccine strain originating from *F. tularensis* type B affords protection against virulent type A organisms, indicating that the two biovars share determinants important to induce T cell-mediated immunity.

See also: Attenuated organisms as vaccines; Bacteria, immunity to: Bacterial cell walls.

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FUNGI, IMMUNITY TO

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The fungi are nonmotile eukaryotes that possess a cell wall and reproduce by means of spores. Over 100 000 species of fungi have been described, but only 100–200 regularly cause disease in humans. Most fungi are saprophytes rather than obligate parasites, human infection being an incidental event in the life cycle of those organisms capable of growing at body temperature in the environment provided by human tissues. However, there are many exceptions to this generalization: *Candida albicans*

colonizes mucocutaneous and gastrointestinal surfaces and is a part of the normal human flora; the dermatophytes are a group of specialized fungi causing localized infections of skin, nails and hair by virtue of their ability to utilize keratin; and the dimorphic fungi grow as saprophytic molds in soil or vegetation but can transform at 37 °C into parasitic yeast forms well-adapted for survival in susceptible mammalian hosts. In addition to causing localized and systemic infections, fungi can trigger immune-

mediated hypersensitivity reactions that can present as asthma, extrinsic allergic alveolitis, or allergic bronchopulmonary aspergillosis.

The medically important fungi are a very diverse group providing a formidable challenge to the immune system. Moreover, even within species, variations in morphology with associated changes in surface antigens are common and not confined to the dimorphic fungi. For example, in susceptible hosts, inhaled *Aspergillus* conidia shed waxy surface coats, swell and exhibit new surface antigens, then germinate to form hyphal filaments with further distinct antigens. These surface changes evoke distinct host humoral and cellular responses. Within tissues, *C. albicans* may grow as yeast-like blastoconidia, pseudohyphae and hyphae that differ in surface antigen expression. Moreover, *C. albicans* and other fungi can undergo heritable high-frequency switching of surface antigenic phenotypes *in vivo*.

A broad array of host defense mechanisms have evolved to protect humans against fungal invasion. Even the most pathogenic of the dimorphic species, *Coccidioides immitis* and *Histoplasma capsulatum*, usually induce only asymptomatic or self-limited infections in persons with intact host defenses. The majority of serious fungal infections occur in persons with defects in one or more of these defenses. Awareness of the type of compromise afflicting a host enables the clinician to predict which mycoses are likely to occur (Table 1). Conversely, a patient not known to be immunocompromised who presents

with a disseminated fungal infection usually warrants investigation for an underlying immunodeficiency. Cell-mediated immunity appears to be especially important for host defense against *Cryptococcus neoformans*, and the endemic dimorphic fungi, *C. immitis*, *H. capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*. Protection against *Pneumocystis carinii*, which has been recently classified as a fungus, is also dependent upon intact cell-mediated immunity. Protection against invasive candidiasis, aspergillosis and mucormycosis is more dependent on intact neutrophil function.

Non-specific immunity

A number of nonspecific host factors form an important first line of defense against fungal invasion. These include the mechanical barrier provided by the skin and mucous membranes, competition for nutrients from the normal indigenous bacterial flora, and the mucociliary clearance system of the respiratory tract. The importance of these factors is illustrated by the association of disseminated candidiasis with the disruption of mechanical barriers by burns, surgical wounds or intravenous catheters and with the inhibition of normal bacterial flora by broad-spectrum antibiotics. In serum, chelation of iron and other essential heavy metals restricts the growth of many fungi. The association of mucormycosis with therapy with the iron-chelator deferoxamine is thought to be due to the ability of the causative

Table 1 Etiological agents of systemic fungal infections associated with specific predisposing factors

Predisposing factor	Etiological agent(s)
Traumatized skin and mucosal surfaces	<i>Candida</i> species
Ketoacidosis	Agents of mucormycosis
Deferoxamine therapy	Agents of mucormycosis
Neutropenia	<i>Candida</i> species (disseminated disease) <i>Aspergillus</i> species Agents of mucormycosis <i>Pseudoallescheria boydii</i> <i>Trichosporon</i> species <i>Fusarium</i> species
Chronic granulomatous disease	<i>Aspergillus</i> species <i>Candida albicans</i> (disseminated disease)
Impaired cell-mediated immunity	<i>Candida</i> species (mucocutaneous disease) <i>Cryptococcus neoformans</i> <i>Histoplasma capsulatum</i> <i>Coccidioides immitis</i> <i>Paracoccidioides brasiliensis</i> <i>Blastomyces dermatitidis</i> <i>Penicillium marneffei</i> <i>Pneumocystis carinii</i>

fungi to use iron-saturated deferoxamine as a siderophore. Similarly, ketoacidosis may predispose patients to mucormycosis by making iron more readily available to the fungus. In addition, incompletely characterized serum factors, distinct from complement, have been reported to inhibit the growth of *Cryptococcus neoformans*, *C. albicans* and *Rhizopus oryzae*.

Complement

Unlike 'serum-sensitive' bacteria and viruses, fungi are resistant to lysis by the terminal components of the complement system, even in the presence of specific antibody. However, complement components are important opsonins facilitating the binding and ingestion of fungi by phagocytic cells. Fungi are generally potent activators of the alternative complement system. C3 fragments have been shown to be deposited on the surface of a number of pathogenic species. For *C. neoformans*, nearly all of the C3 is present as iC3b, while for *A. fumigatus* and *C. albicans*, both C3b and iC3b are present on the fungal surface. Activation of complement by fungi has also been shown to generate the potent chemoattractant C5a, which serves to recruit inflammatory cells to sites of infection.

Neutrophils

Neutrophils are of paramount importance in protection against a number of mycoses. In particular, disseminated candidiasis and invasive aspergillosis are not uncommonly seen in neutropenic patients. *In vitro*, neutrophils can kill the hyphae of *C. albicans* and *A. fumigatus*. Hyphae of both fungi stimulate neutrophils to undergo a respiratory burst and degranulate. The oxidants generated (e.g. hydrogen peroxide and hypochlorous acid) and granule products released (e.g. defensins) are fungicidal. The importance of neutrophils in defense against *C. albicans*, *A. fumigatus*, and other catalase-positive fungi is also shown by the frequency of these mycoses (20% in one large series) in patients with chronic granulomatous disease (CGD). Neutrophils from these patients are defective in their ability to generate microbicidal oxidants and are unable to kill *A. fumigatus* hyphae. *In vivo* administration of recombinant interferon γ (IFN γ) to CGD patients has been shown to significantly reduce the incidence of serious infections. Neutrophils from IFN γ -treated CGD patients acquire the capacity to damage *A. fumigatus* hyphae.

Monocytes and resident macrophages

The antifungal activities of monocytes and resident

macrophages have been extensively studied. Macrophages from different species and different anatomic sites vary in their capacity to inhibit and kill fungi. For example, activated murine peritoneal macrophages have potent antifungal effects on *C. neoformans*. However, investigators have been mostly unsuccessful demonstrating antifungal activity of activated human macrophages. The L-arginine-dependent generation of nitric oxide by activated murine macrophages appears to be at least partly responsible for antifungal effects. Generation of microbicidal concentrations of nitric oxide has been difficult to demonstrate in human macrophages, which may help to explain why antifungal effects seen in mouse macrophages have not always been reproduced in human cells.

For most fungi that cause systemic disease, initial exposure occurs by inhalation of airborne spores or yeast cells. Bronchoalveolar macrophages are thought to constitute a particularly important component of the immune response to these fungi. In several studies bronchoalveolar macrophages have been found to have greater antifungal activity than, for example, peritoneal macrophages. *In vitro*, bronchoalveolar macrophages can kill the spores but not the hyphae of *A. fumigatus*. Neutrophils, on the other hand, cannot kill ungerminated spores but can kill hyphae. *In vivo*, two lines of phagocyte defense may combine to prevent the establishment of infection. Spores that are not killed by resident bronchoalveolar macrophages will be susceptible to attack by recruited neutrophils. Bronchoalveolar macrophages have also been shown to prevent the phase transition of *B. dermatitidis* conidia (the inhaled form) into invasive yeasts, illustrating another mechanism whereby the establishment of infection may be prevented. Recently, some progress has been made in identifying the ligands and receptors involved in the binding of fungi by macrophages (Table 2, Figure 1).

Natural killer cells

Natural killer (NK) cells have been shown to inhibit the growth of *C. neoformans* and *P. brasiliensis* *in vitro*. In some, but not all, murine models of cryptococcosis, NK cells were also found to contribute to clearance of the organism. However, in mice infected with *H. capsulatum*, no significant role for NK cells in host defense could be demonstrated. Recently, human T lymphocytes have been shown to bind to and inhibit the growth of *C. neoformans* and *C. albicans* *in vitro*. The role *in vivo* of this direct antifungal activity of nonspecific T cells has yet to be determined.

Table 2 Examples of receptors and ligands involved in the binding of pathogenic fungi to macrophages

Pathogen	Ligand(s)	Receptor(s)
<i>H. capsulatum</i>	Unknown	CD18 (β chain of CR3, ^a CR4, and LFA-1)
<i>B. dermatitidis</i>	WI-1, a cell wall protein	CR3 and CD14
<i>C. neoformans</i>	Deposited iC3b ^b	CR1, CR3, CR4
<i>C. albicans</i>	Cell wall mannoproteins and β -glucans	Mannose and β -glucan receptors
<i>A. fumigatus</i>	Conidial cell wall polysaccharides	Mannose and/or β -glucan receptors

^aCR, complement receptor.

^bRecognition of *C. neoformans* by macrophages generally requires complement activation. Binding of opsonized organisms via complement and Fc receptors is an additional mechanism common to many other fungi.

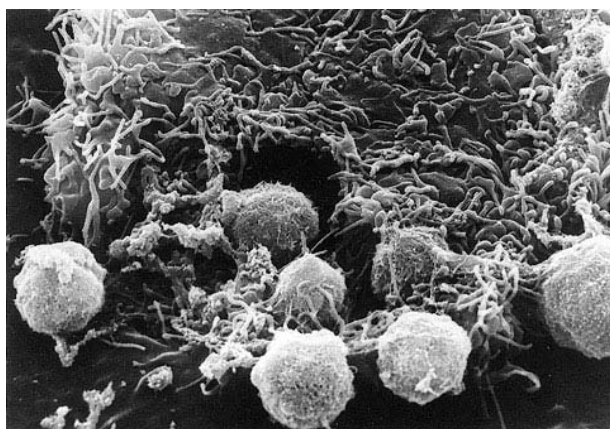


Figure 1 Scanning electron micrograph of a human monocyte-derived macrophage binding and ingesting serum-opsonized *Cryptococcus neoformans*. Micrograph by Robert Liss (with permission) from material prepared by Stuart Levitz and Abdul Tabuni.

Specific immunity

Antibody

Although it is difficult to draw definite conclusions, for most of the mycoses there is little evidence to suggest that specific antibodies are critical to host defense. In marked contrast to patients with impaired cell-mediated immunity, those with hypogammaglobulinemia are not particularly predisposed to the development of invasive fungal infections. The principal role of antibody may be to enhance binding of fungi to phagocytic cells via Fc receptors. In the case of cryptococcosis, specific antibody enhances the activity of most effector cell populations in *in vitro* systems and passive administration of antibody has been shown to be beneficial in animal models of infection. Some evidence from mouse models also suggests a role for humoral immunity in resistance to systemic candidiasis. Specific antibodies are also important in the pathogenesis of allergic responses to inhaled fungi. For example, IgE-mediated immediate hypersensitivity reactions to fungal allergens may play a part in some asthmatic attacks and precipitat-

ing antibodies to fungal antigens are responsible for some of the manifestations of extrinsic allergic alveolitis through the formation of immune complexes.

Specific cell-mediated immunity

Specific cell-mediated immunity is critical for a protective immune response to *C. neoformans* and the dimorphic fungi, and is involved in protection against dermatophyte infections. Cell-mediated immunity, rather than intact neutrophil function, is also of primary importance for protection against mucocutaneous candidiasis. These mycoses are seen with increased frequency in patients with the acquired immune deficiency syndrome (AIDS), lymphomas, sarcoidosis, and in those taking immunosuppressive medications such as corticosteroids, cyclophosphamide, or azathioprine. The high incidence of fungal infections in human immunodeficiency virus (HIV)-infected patients is particularly striking. HIV infects CD4⁺ lymphocytes and macrophages, the two cell types whose interactions are central to the cell-mediated immune response.

Development of a specific cell-mediated immune response to these fungi is thought to require antigen-presenting cells (generally mononuclear phagocytes), to process and present fungal antigen(s) to T lymphocytes (generally CD4⁺ helper cells). CD4⁺ lymphocytes expressing antigen-specific receptors recognize antigen in association with major histocompatibility complex (MHC) class II molecules, proliferate and release cytokines (e.g. IFN γ and IL-2) that activate effector cells to inhibit or kill the fungus. Studies of mouse models of candidiasis and histoplasmosis suggest that protection may be associated with a T_H1 type response stimulated by the production of IL-12 by antigen-presenting cells and characterized by high levels of IFN γ and IL-2, and low levels of IL-4, IL-5, and IL-10. In addition to CD4⁺ T cells, CD8⁺ T cells may be involved in the immune response to some fungi. In mice infected intratracheally with *C. neoformans*, CD8⁺ cells contribute to clearance of the organism. CD8⁺ T cells

have also been shown to be involved in the generation and expression of delayed-type hypersensitivity to *C. neoformans* in mice.

Activated macrophages are important effector cells, but cytokines generated in the course of a specific cell-mediated immune response may also enhance the antifungal activity of natural killer cells, nonspecific T lymphocytes and neutrophils. Enhanced activity against *C. albicans*, *B. dermatitidis*, *C. neoformans*, *H. capsulatum* and *C. immitis* has been demonstrated following incubation of murine macrophages with IFN γ . Interleukin 2 activates murine lymph node cells and human peripheral blood lymphocytes to kill *C. albicans* and *C. neoformans*, respectively. Other cytokines, including tumor necrosis factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-12, which may be produced during the course of a cell-mediated immune response, also have been reported to activate effector cells to inhibit or kill fungal targets. Cytokines that have suppressive effects, including IL-10 and transforming growth factor β , may also be generated in response to fungal challenge. When produced in appropriate amounts, these cytokines may be important in limiting damage to host tissues. Nevertheless, many of the clinical manifestations of the mycoses result from the inflammatory response to fungal antigens. Mediastinal fibrosis, a rare complication of infection with *H. capsulatum* thought to result from an overexuberant cell-mediated immune response, is an extreme example.

Vaccination and immunotherapy

The development of vaccines against the important systemic fungal infections is under intense investigation. A vaccine consisting of formaldehyde-killed spherules of *C. immitis* was protective in a mouse model of coccidioidomycosis, but no efficacy was demonstrated in a large trial of susceptible persons in the endemic area. Local reactions limited the dose that could be given. Immunization with subcellular fractions or purified antigens is likely to be better tolerated and suitable antigens are under development for both *C. immitis* and *H. capsulatum*. Cryptococcal glucuronoxylomannan, the major component of the polysaccharide capsule of *C. neoformans*, has been linked to tetanus toxoid and used to immunize mice. Conjugation with tetanus toxoid enhanced immunogenicity and conferred T cell-dependent properties to glucuronoxylomannan.

Most serious fungal infections, however, occur in significantly immunocompromised patients who are likely to have a suboptimal response to immunization and/or a suboptimal response to infection

despite prior immunization. It will need to be shown for each patient group and each antigen that active immunization can confer protection in these circumstances.

The defects in host immunity associated with serious fungal infections are being defined and some progress has been made in attempts to boost or reconstitute the immune response. Examples include IFN γ in patients with chronic granulomatous disease and granulocyte colony-stimulating factor and GM-CSF in neutropenic patients. In the case of cryptococcosis, monoclonal antibodies to capsular glucuronoxylomannan have been shown to confer protection when passively administered to mice infected with *C. neoformans*. This, despite the fact that specific antibodies appear to play a limited role in normal host defense against *C. neoformans*. Increased understanding of the complex network of cytokines regulating the cellular immune response has raised additional therapeutic possibilities. Soluble IL-4 receptor and antibody against IL-10 have been shown to be beneficial in murine candidiasis and IL-12 has been shown to be beneficial in animals models of cryptococcosis and histoplasmosis. Nevertheless, current treatment for serious fungal infections depends largely on antifungal drugs. Much additional experimental and clinical work is needed to define the role of adjunctive immunotherapy.

See also: **Acquired immune deficiency syndrome (AIDS); Allergens; Candida, infection and immunity; Coccidioides, infection and immunity; Cryptococcus, infection and immunity; Dermatological infections; Histoplasma, infection and immunity; Opportunistic infections; Pneumocystis carinii, infection and immunity.**

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FUSOBACTERIUM, INFECTION AND IMMUNITY

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Fusobacterium spp. are gram-negative, nonsporulating, nonmotile and, generally, moderately long filamentous organisms, sometimes slightly elongated spindle-shaped rods, that belong to the family Bacteroidaceae. They are weak fermenters or unable to ferment sugar. The ability of *Fusobacterium* spp. to produce *N*-butyric acid without isobutyric or isovaleric acids differentiates them from the other Bacteroidaceae. There are currently 12 known species of *Fusobacterium*. The species seen most often in clinical infections include *F. nucleatum* (currently divided into four subspecies), *F. necrophorum*, *F. gonidiaformans*, *F. naviforme*, *F. mortiferum*, *F. necrogenes*, *F. ulcerans*, *F. russii* and *F. varium*. Of these, *F. nucleatum* is best able to produce the enzyme β -lactamase.

Fusobacterium spp. are part of the normal oral, gastrointestinal and female genital tract flora; these sites, therefore, are the most common origin of infections that involve these organisms. The organisms are often recovered mixed with other anaerobes and aerobes in polymicrobial infections that are often synergistic.

Fusobacterium spp. produce lipopolysaccharide, endotoxin, neutrophil-cytotoxic substances and DNAase, which have all been associated with virulence. *F. nucleatum* can adhere to lymphocytes and facilitate their activity. The adherence is believed to be mediated by lecithin-like ligands. It can be inhibited by *N*-acetylglactosamine, lactose and α - and β -methylgalactoside. *F. nucleatum* also has an immunosuppressive effect on human peripheral blood lymphocytes. Such an effect may enhance the pathogenicity of the organisms as well as other copathogens.

As with other anaerobic bacteria, the ability of *Fusobacterium* spp. to induce subcutaneous abscesses correlates with the presence of a mucopolysaccharide layer in the cell wall. The ability to produce subcutaneous abscesses in experimental animals could, therefore, be an indication of the organism's virulence, as has been noticed with other anaerobic strains.

Factors associated with infections involving *Fusobacterium* spp. include break-up of the normal cutaneous and mucosal defenses, as may occur in Epstein-Barr virus pharyngitis, tissue injury caused by surgery or trauma, and impaired blood supply caused by microvascular disease. Moreover, the production of proteolytic enzymes by *Fusobacterium* spp. may allow early invasion of regional veins, even without tissue necrosis. Furthermore, the low oxidation-reduction potential and oxygen-free environment of devitalized tissue allows growth of these organisms.

Support for the pathogenic role of *Fusobacterium* spp. may be derived from *in vivo* studies that have demonstrated their synergistic potential with other anaerobic and aerobic bacteria. With few exceptions, the presence of *Fusobacterium* spp. enhanced the growth of other aerobic and anaerobic bacteria. Bacterial synergy was demonstrated between *Fusobacterium* spp. and anaerobic cocci, *Staphylococcus aureus* and *Pseudomonas aeruginosa* by means of a subcutaneous abscess model in mice. In this model, *Fusobacterium* spp. enhanced the formation of abscesses and mortality when inoculated with the other organisms.

The spectrum of infections in which *Fusobacterium* spp. play a pathogenic role include bacteremia, head and neck infections (such as chronic otitis media, sinusitis and mastoiditis, peritonsillar and retropharyngeal abscesses, Vincent's angina, gingival and dental infections), pulmonary infections (aspiration pneumonia, lung abscesses and empyema), intracranial infections (meningitis and intracranial abscesses), gastrointestinal infections (peritonitis, hepatic and abdominal abscesses), osteomyelitis, urogenital (prostatic and female genital abscesses, amnionitis) as well as skin and soft tissue infections, especially around the oropharyngeal area.

Fusobacterium spp. have been associated with single or polymicrobial bacteremia, of primarily oropharyngeal origin, that may accompany cervical

septic thrombophlebitis, necrotizing pneumonia or metastatic infection, known as Lemierre's disease.

F. nucleatum (currently divided into four subspecies) is the predominant fusobacterium found in the oral flora and in human infections. It has been associated with dental plaques, necrotizing gingivitis, acute ulcerative gingivitis (Vincent's angina) and periodontitis. It is also the major fusobacterium recovered from all head and neck infections and their sequelae.

F. nucleatum and related species can be recovered in a variety of mixed infection in animals, including bovine pyometra, necrobacillosis of the jaw in macropods and feline abscesses following cat bites.

Elevated antibodies to protein antigens of *F. nucleatum* were found in patients with periodontal disease, peritonsillar cellulitis and abscesses, infectious mononucleosis and acute streptococcal, nonstreptococcal and recurrent tonsillitis. It is possible that the increase in antibodies to these outer membrane proteins – which may contribute to bacterial attachment – may signify a potential direct or indirect pathogenic role for this organism in these infections.

F. necrophorum (currently divided into two subspecies) is the second most common pathogen in humans. It is found in pharyngeal and tonsillar infection associated with bacteremia, otitis, pneumonia, liver and colonic infections. It has a variety of virulence factors that makes it a potentially greater pathogen than other fusobacteria. These include a leucocidin, hemolysin, a hemagglutinin, a lipase, a DNAase, a proteinase and a lipopolysaccharide endotoxin.

F. necrophorum (divided into two biovars, A and B) is part of the normal gastrointestinal flora of herbivores and other species, and can cause endogenous infection in these hosts as well as in humans. Necrobiosis in humans (generally due to biovar B) is a throat infection followed by systemic spread and metastatic abscesses. Necrobacillosis infections in animals (generally due to biovar A) are usually polymicrobial and include coagulative necrosis and abscesses and can cause mortality. Necrobacillosis generally occurs in the oral cavity, liver and foot. Bovine hepatic necrosis is hematogenous, originating from minimal lesions and foot rot. Necrobacillosis can affect wild and domestic animals.

Necrobacillosis is often a synergistic infection. *In*

vivo synergy between *F. necrophorum* and *Actinomyces pyogenes*, *Escherichia coli* and *Staph. aureus* was demonstrated in mice, and growth stimulation between *F. necrophorum* and *A. pyogenes* was found *in vitro*.

Delayed-type hypersensitivity to *F. necrophorum* could be induced in mice, and leukotoxin-specific antibodies can be detected in cattle with induced or naturally-acquired hepatic abscess due to this organism. Antibodies to *F. necrophorum* can be detected after infection using passive hemagglutination and enzyme-linked immunosorbent assays. However, protection against reinfection is not always achieved after natural infection or vaccination with a toxoid prepared from the cytoplasmic fraction of *F. necrophorum*. Even utilization of an extreme method of immunization, by producing a severe subcutaneous *F. necrophorum* infection followed by curative therapy with metronidazole, produced only slight protection. That *F. necrophorum* is naturally a weak immunogenic pathogen is therefore plausible.

See also: Bacteria, immunity to; Bacterial cell walls; *Bacteroides*, infection and immunity.

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GAMMAGLOBULIN

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Gammaglobulin is a somewhat ambiguous term that still remains in common usage despite attempts to substitute 'immunoglobulin' in its place. In general terms, 'gammaglobulin' is a class of plasma proteins composed almost entirely of immunoglobulins (Igs), the proteins that function as antibodies. These plasma proteins are characterized by a very slow electrophoretic mobility in an alkaline buffer (Figure 1). 'Gammaglobulin' is also used as a clinical name, given to the immunoglobulin concentrates that are given parenterally to humans for immunoprophylaxis against infectious disease, or for passive antibody replacement in humoral immunodeficiency disease.

The term 'gammaglobulin' has deep roots in the history of immunology and the evolution of immunoprophylaxis. The globulins were those proteins found to be soluble in dilute salt solution and precipitated at half saturation with ammonium sulfate, in distinction from the albumins, which require complete saturation with ammonium sulfate in order to precipitate them. Typical globulins were first believed to be insoluble in water in the absence of salt, but the globulins were later divided into two

parts, the 'euglobulins' (mostly IgM) of which this feature is true, and the 'pseudoglobulins' (mostly IgG) which dissolve even in the absence of salt.

The electrophoretic separation of plasma into components of various charge, including the α , β and γ fractions, was first performed by Tiselius, who gave these names to the fractions in 1937. Two years later, Tiselius and Kabat identified antibody in the slowest moving proteins, the γ fraction. Ethanol precipitation of the gammaglobulin proteins from pooled human serum, introduced in 1944 by Cohn and colleagues, became a standard method of isolating almost pure gammaglobulin (called fraction II). Figure 2 shows the plasma fraction scheme that was developed. After fraction II was found to contain most of the antibodies present in serum, it became an important prophylactic agent given parenterally to humans to protect against certain infectious diseases. Later, this immunoglobulin concentrate was introduced as a means of reconstitution for immunodeficient patients who had no antibody production of their own.

Numerous physical and chemical studies on the properties of the gammaglobulin molecules were

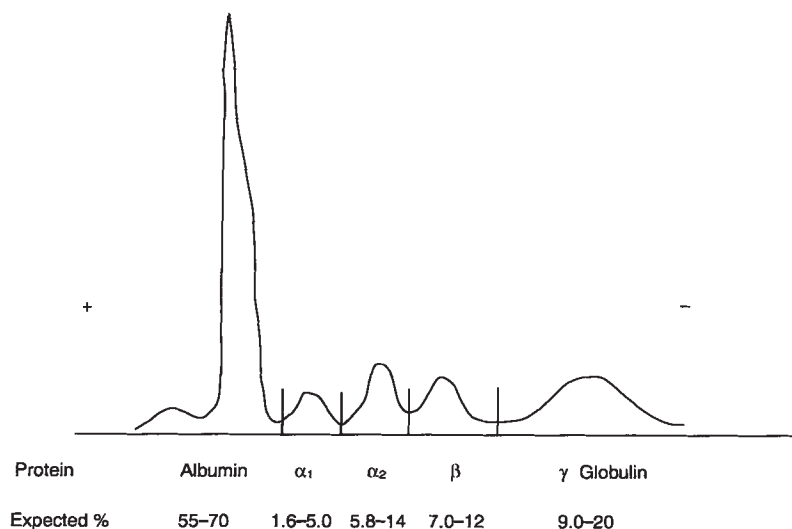


Figure 1 Serum protein electrophoresis. Typical conditions for separation on paper or other suitable support include a 0.1 M sodium diethylbarbital buffer (pH 8.6, potential gradient 6 V cm⁻¹) over a time period of 250 min.

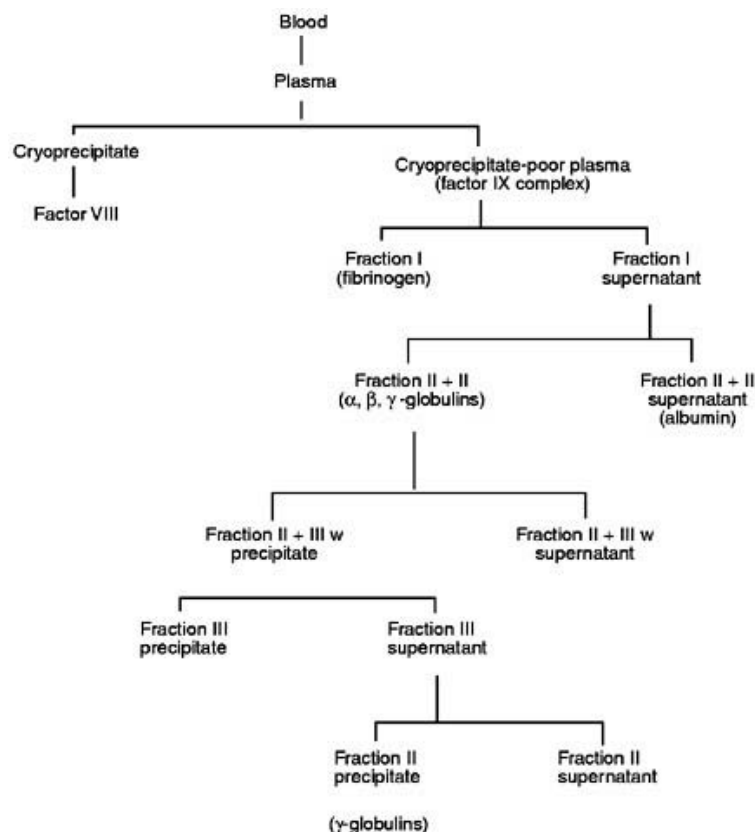


Figure 2 Cohn–Oncley alcohol fractionation. Plasma fractionation procedures yield a gammaglobulin concentrate (fraction II).

undertaken between 1948 and 1955 following the discovery that myeloma proteins were immunoglobulin molecules. IgG and IgM were described first, followed by IgA (1960), IgD (1965) and IgE (1966). In 1964, following the discovery that animal IgG protein could be subdivided, it was found that human IgG was composed of four classes: IgG1, IgG2, IgG3 and IgG4.

Gammaglobulin preparations

Commercial preparations of gammaglobulin, usually obtained from pooled plasma from 500–2000 or more donors by the Cohn–Oncley process, contains approximately 85% IgG, 10% IgM, 5% IgA and traces of other plasma proteins. The immunoglobulin concentrates contain the majority of antibodies of the IgG class present in the starting plasma. Two main forms of these immunoglobulin concentrates are available: a 15% solution intended for intramuscular (or subcutaneous) use, and lyophilized powders or liquids intended for intravenous use. The former often contains $8\text{--}20\ \mu\text{g ml}^{-1}$ thiomersal, as the solution is packaged in multidose vials; it also contains immunoglobulin aggregates which make it unacceptable for intravenous use. The lyophilized powders

and liquids for intravenous use contain no preservatives.

Intravenous infusion of gammaglobulin concentrates (initially the fraction II of the Cohn–Oncley process) was first attempted in the 1950s. Unfortunately, these infusions resulted in severe reactions in a number of instances, probably because of complement activation due to the infusion of aggregates. However, over time, various methods of modifying the immunoglobulin solutions were devised to produce products suitable for intravenous use. These methods included: enzymatic treatment of immunoglobulin; addition of chemical groups to prevent aggregation; reduction, sulfonation, or reduction followed by alkylation; addition of stabilizers (maltose, sucrose, albumin, glycine, mannitol); ion exchange column filtration; and precipitation of aggregates by polyethylene glycol.

The best method of preparation of intravenous immunoglobulin has not been established, and no comparative trials have been performed to compare the types available. Any method that produces an immunoglobulin concentrate containing intact and unmodified immunoglobulin in a form suitable for intravenous administration might be potentially satisfactory. Since enzyme-treated or reduced and

alkylated immunoglobulin has reduced effector functions in terms of opsonic capacity or fixation of complement, these preparative methods are no longer favored. There is also a general consensus that these concentrates should contain IgG subclasses in the approximate proportions present in normal plasma and that no preservatives be added.

Gammaglobulin for prophylaxis and disease prevention

The use of intramuscular gammaglobulin as a prophylaxis against infectious disease in persons exposed to hepatitis A has established therapeutic benefit. Intramuscular immunoglobulin is also recommended for nonimmune women exposed to rubella in early pregnancy, and for infants or immunosuppressed persons exposed to measles. Specific high-titered preparations of gammaglobulin are also available for prevention of disease following exposure to chicken pox (for immunosuppressed individuals) or following suspected inoculation with rabies virus or tetanus organisms.

Gammaglobulin has also been used for antibody replacement in patients with congenital immunodeficiency diseases such as X-linked agammaglobulinemia (Bruton type), common variable immunodeficiency, and other antibody deficiency syndromes in which B cells do not secrete adequate amounts of immunoglobulin. Intramuscular immunoglobulin replacement therapy was introduced in 1952; the generally recommended dose was $25 \text{ mg kg}^{-1} \text{ week}^{-1}$, usually given at biweekly intervals.

In the last decade the use of intravenous immunoglobulin has largely replaced the use of intramuscular immunoglobulin for treatment of immunodeficiency diseases, as the amount of immunoglobulin which can be easily administered is much greater when delivered by the intravenous route. Additionally, studies have shown that patients with primary immunodeficiency disease benefit from the larger doses, and that the serum levels of IgG can be normalized. The dosage range for intravenous immunoglobulin in antibody deficiency is generally $200\text{--}400 \text{ mg kg}^{-1}$ given at 2–4 week intervals.

Safety of gammaglobulin

A major concern surrounding the infusion of blood and blood products is the potential for the transmission of viruses. Although there was previously a risk of infection with hepatitis C for patients who

received factor VIII and factor IX concentrates, human immunoglobulin concentrates have not historically been noted for the transmission of hepatitis. However, there have been a few cases of hepatitis on record that were traceable to injections of intramuscular immunoglobulins, and there have also been instances of outbreaks of hepatitis C in patients who received intravenous immunoglobulins. Most recently, intravenous immunoglobulin concentrates of one manufacturer produced hepatitis C in more than 100 patients in the USA in 1993–1994, with a number of affected individuals also being reported in other countries. Since in each case the starting immunoglobulin is the same as for intramuscular immunoglobulin, it has remained a mystery how intravenous immunoglobulin could acquire infectivity during manufacturing. Various viral inactivation steps have now been incorporated into the processing of most immunoglobulin concentrates, with no cases of hepatitis being reported when such procedures have been employed.

Also of concern has been the theoretical possibility that immunoglobulin concentrates could transmit the human immunodeficiency virus (HIV) virus; however, there have been no documented cases of HIV transmission via any immunoglobulin concentrates. Thus it appears unlikely that immunoglobulin concentrates have any potential for transmitting this virus. Purposeful contamination of plasma with the virus and subsequent testing of residual infectivity at various points along the preparation cycle shows that several steps in the scheme remove infectivity.

See also: IgA; IgD; IgE; IgG; Immunodeficiency, primary; Immunoelectrophoresis; Immunoglobulin structure; Plasma; Plasmapheresis; Protein separation techniques.

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GASTROINTESTINAL TRACT INFECTIONS

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Gastrointestinal (GI) infectious diseases are widespread in developing areas of the world and are also a disturbing problem in industrial regions, despite greater emphasis on sanitary measures to provide safe food handling and water distribution. Diarrheal disease is the most common acute infectious disease in the world and responsible for considerable mortality in developing countries. Microorganisms associated with human GI infections include various species of bacteria, fungi, viruses and protozoa. Helminths also cause diarrheal disease after infecting the GI tract. The acquisition of diarrheal diseases depends upon both broad and specific aspects of the characteristics of individuals and on their socioeconomic environment and activities. Age is a determining factor for susceptibility to infection by some of these organisms. For some enteric pathogens, very young individuals are particularly at risk. For other pathogens the elderly are most at risk and for others both age groups are readily affected. However, regardless of age, severe immunocompromised patients, including those with the acquired immune deficiency syndrome (AIDS), are particularly susceptible to secondary infections, including diarrheal disease. As might be expected, pathogenic mechanisms that affect the intestine and result in diarrhea vary with the organism or group of organisms.

The normal intestinal flora consists of a wide variety of distinctive species of microorganisms that coexist in a complex ecosystem specifically adapted to the environmental conditions found in the GI tract. This flora maintains a symbiotic relationship with the host, is remarkably stable and can play a competitive role in preventing colonization of the GI tract by invading pathogens. In order to colonize the GI tract and cause infections, pathogens must be able to overcome or circumvent defense mechanisms of the host. The most important in this regard is integrity of the mucosal epithelium. Once the flora can penetrate broken mucosal epithelium, even some normally nonpathogenic flora can reach the deep tissues and disseminate through the bloodstream to other organs. The acidity of the stomach represents a significant deterrent. Other nonspecific host defense factors include the relative rapid motility of the normal gut which acts to deter bacterial colonization by removal of microorganisms. Expulsion of the microorganism by peristalsis is aided by the mucosal layer

which also serves as a diffusion barrier, limiting bacterial attachment to the mucosal surface. Specific as well as nonspecific mucosal immunity is a major area of interest to both microbiologists and immunologists, and appears to play a major role in preventing infection. For example, secretion of specific secretory immunoglobulin A (IgA) antibody is important in preventing adherence of microorganisms and replication of the invading pathogen at the mucosal level.

Bacterial infections

Mechanisms that affect the intestine and result in diarrhea vary with the bacteria involved. Diarrhea usually occurs in individuals harboring bacteria that produce toxins or other products capable of affecting intestinal epithelial cells directly and thereby alter their secretory function. Watery, noninflammatory diarrhea occurs with cholera and rotoviral disease. *Clostridium difficile* and enterotoxigenic *Escherichia coli* infections, among others. Disease caused by microorganisms such as *Shigella*, *Salmonella enteritidis*, and enteroinvasive *E. coli* usually involve the effects of exotoxins produced by the microorganism which affect the epithelium, resulting in inflammatory diarrheal disease. However, some gastrointestinal pathogens penetrate more deeply into tissues than others.

It is widely acknowledged that GI infection with various bacterial pathogens may be serious. Many are capable of overcoming protective host factors, although some bacteria that cause disease in the intestine are considered normal flora, but some strains acquire virulence determinants that mediate diarrheal disease. For example, enterotoxic strains of *E. coli* colonize the small intestine through attachment structures such as fimbriae or pili and elaborate thermolabile and thermostable toxins. These can result in net fluid and electrolyte secretion into the lumen of the small intestine. Also, enteroinvasive *E. coli* strains invade and proliferate within epithelial cells of the colon and cause a dysentery-like disease similar to that produced by *Shigella*, the classic agent of bacillary dysentery. Dysentery caused by this organism is a major health problem among infants worldwide. *Escherichia coli* strains that do not invade colonic epithelial cells can cause a hemorrhagic colitis, which is now considered an important health problem in institutions, including

nursing homes, daycare centers, schools, etc. Such enteropathogenic *E. coli* are also important causes of infant diarrhea in developing countries. These organisms colonize the ileum rather than the colon, are noninvasive, and associated with a distinctive histopathology of the microvillus membrane. Knowledge regarding the pathogenesis of some enteric diseases caused by microorganisms has within the last decade advanced markedly at both the cellular and molecular levels. For example, the mode of action of toxins, virulence factors such as adhesions, nutritional requirements of the invading microorganism, the mechanism of invasion, effects on eukaryotic cell signaling and stimulation of the inflammatory response by induction and actions of cytokines are now known in some depth and shown to be important factors.

The host response to cholera and cholera toxins has provided a model for studying similar aspects of molecular pathogenesis of other bacterial enteric diseases. It is now known there are virulence factors for *V. cholera* that enable colonization of the intestine and some molecular aspects of genetic control of these factors are now understood. The *E. coli* strains associated with diarrheal disease, including those which are enterotoxigenic and enteropathogenic, cause pathogenic responses similar to cholera but in a less severe form. It is known that cholera toxin enhances intracellular adenylate cyclase activity while *E. coli* enterotoxin increases guanylate cyclase activities. Nevertheless, in both cases the net result is increased secretion of water electrolytes but the electrolyte affected differs as well as the extent of the responses induced. Furthermore, enterotoxigenic *E. coli* toxins are cytokines that directly affect intestinal epithelial cells. The result is an inflammatory diarrhea rather than a watery noninflammatory one. In this pathogenesis the disease resembles shigellosis rather than cholera. Other *E. coli* strains produce hemorrhagic diarrhea. Thus it is obvious that within this one species various mechanisms of action on the human gastrointestinal tract have developed. It is important to note, however, that most strains of *E. coli* are not pathogenic and many are members of the human indigenous intestinal microflora and can participate in protecting the host from infection by a typical pathogen. Genetic control of the various virulence factors in different strains of one bacterial species or among groups of species may be plasmid or chromosomal in origin, or both.

Other invasive enteropathogens that have been studied in terms of their ability to cause diarrhea include *Yersinia* and *Campylobacter*. *Campylobacter* is recognized as an important and common cause of GI infection and a species of this bacterium, now

known as *Helicobacter pylori*, but first classified in the *Campylobacter* genus, is now known to be an important cause of gastritis and peptic ulcers.

Viral infections

Viruses are also known to cause GI infections, including diarrhea. Among the most important viral agents are the rotoviruses, as well as the Norwalk agent. Such viruses infect small intestinal enterocytes at the villi. Villus cells absorb electrolytes and their destruction leads to net secretion of fluid into the lumen. Rotoviruses are known to be a major cause of epidemic diarrhea in infants and young children. The Norwalk agent is responsible for epidemic outbreaks among schoolchildren and adults. Despite controversies about mechanisms of immunity against viral gastroenteritis, effective rotovirus vaccines based on serospecific immune protection against the disease are being developed. Combined polyvalent vaccines, as well as genetic constructs containing epitopes of major human rotovirus serotypes, are currently being evaluated as protective immunogens in field trials.

Parasitic infections

Various gastrointestinal parasites, including protozoa such as *Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium* spp., are important causes of gastrointestinal diseases. *Giardia* is known to infect the small intestine of all age groups and produces a mild but annoying diarrhea. The mechanism of infection is quite complex and not well understood, even though this parasite is not invasive. *E. histolytica* is often found in asymptomatic individuals but has the capacity to produce a chronic or dysentery-like diarrhea. This organism can penetrate the intestinal tissue causing ulceration of the intestinal epithelium and may spread to other tissues and organs. Serious infections by *Cryptosporidium* are usually considered rare but these parasites are an important cause of diarrhea and colitis in humans in both developing and developed countries and this parasite is an important disease-causing organism in patients with acquired immunodeficiencies such as AIDS.

Multicellular helminths or worms can colonize the human intestinal tract and often may be important disease-inducing organisms. Among the helminths are the roundworms such as nematodes, the tapeworms, i.e. cestodes, and the trematodes. Many intestinal helminths infect an individual following penetration through the skin or by ingestion. Pathogenesis includes occlusion of the intestinal lumen, tissue invasion, chronic inflammation and mal-

nutrition. Many helminths evade the host immune response by both subtle and complex mechanisms.

Both cellular and humoral immunity develop to protozoa including single cell parasites and amoebas, as well as intestinal helminths. However, such immunity is usually not effective in preventing colonization or disease. IgE responses of the host are thought to be protective and this immunoglobulin class is believed to have possibly evolved mainly in response to intestinal protozoa and helminthic infections.

Vaccine development

Although successful and useful vaccines have not yet been developed except for a few enteric bacteria or viruses, it is expected that by use of molecular techniques to study aspects of active immunization it will be possible to prepare vaccines that are more effective and have fewer side-effects for various human populations. Progress is being made in 1) the development and identification of specific epitopes for protective antigens from bacteria and viruses which cause GI infections, 2) understanding the effective interactions among mucosal, humoral and cellular immune functions which provide GI tract protective barriers, and 3) the development of purified antigenic molecules from several GI tract pathogens, especially bacteria and their exotoxins, modified to eliminate or decrease toxic properties.

See also: Campylobacter, infection and immunity; Cryptosporidiosis; Escherichia coli, infection and immunity; IgA; Parasites, immunity to; Salmonella, infection and immunity; Shigella, infection and immunity; Vibrio cholerae, infection and immunity; Yersinia, infection and immunity.

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GENE CONVERSION

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There are two main ways by which nucleotide sequences on a DNA molecule can be recombined to give a sequence derived from two different parental sources. They are called gene conversion (conversion) and crossing-over.

A cross-over is a reciprocal exchange such that all sequences on one side of the cross-over are derived from one parental source and all sequences on the other side of the cross-over are derived from the other parental molecule. The other molecule produced from the interaction has the other reciprocal set of information. Thus a cross-over redistributes sequences as if two similar molecules broke at homologous positions and then rejoined, each part of

one molecule with each part of the other. In contrast, conversion is a nonreciprocal transfer of DNA base sequence from one molecule, replacing the homologous sequence on another molecule. Conversion is strongly correlated with crossing-over between markers flanking the site of the conversion, giving rise to the idea that the two represent different consequences of the same recombination event.

Both conversion and crossing-over can occur between homologous sequences on separate DNA molecules, between sister molecules after replication and between homologous sequences on the same molecule (intramolecular events).

Gene conversion serves more than one role in biology. One of its roles is in meiotic recombination and the formation of gametes, whereby conversion is probably involved in the interaction of homologous chromatids that leads to the formation of cross-overs. These cross-overs are visible cytologically as the chiasmata that hold bivalents together on the first meiotic metaphase plate, thus ensuring the orderly separation of homologous chromosomes to opposite spindle poles at anaphase. Conversion events may also be responsible for the initial steps of chromosome synapsis.

A major role of conversion in somatic cells is repair of damage to DNA. In yeast cells, damage to both nucleotide chains (DNA strands) in the same region is repaired by the formation of a double-strand gap which can be filled by copying both strands of a homologous sequence elsewhere in the nucleus. In vertebrate cells also, double-strand breaks are recombinogenic. However, vertebrate cells (and to a lesser extent, yeast cells) have an alternative mechanism for repair of double-strand breaks in DNA via nonsequence-specific end-to-end joining.

A third biological role of conversion is in cell differentiation. This process is best understood at the molecular level in the phenomenon of mating-type switching in yeasts, whereby the genes determining one mating-type are replaced by previously nonexpressed genes of the complementary mating-type by a process of conversion similar to the mechanism of double-strand gap repair. Conversion-like processes in differentiation figure prominently in immunology, both in the ability of some pathogens to change the antigenic properties of their cell surface and in the use of conversion in generation of antibody diversity.

In simpler organisms, there are at least two mechanisms of gene conversion. These are double-strand gap filling, as seen in DNA repair and yeast mating-type switching, and repair of mismatches in heteroduplex DNA, as seen in meiotic conversion. The two following sections describe models that can serve as examples for thinking about these two mechanisms. Most, if not all, recombination events may involve both mechanisms at differing levels of importance.

Formation and correction of heteroduplex DNA

The 1975 model of meiotic recombination of Meselson and Radding may be used to illustrate mechanisms of heteroduplex DNA formation. As shown in Figure 1, the model postulates that the event is initiated by a nick in one nucleotide strand. The 3' end at the nick may then serve as a primer for DNA polymerase (shaded circle labeled 'pol'), which dis-

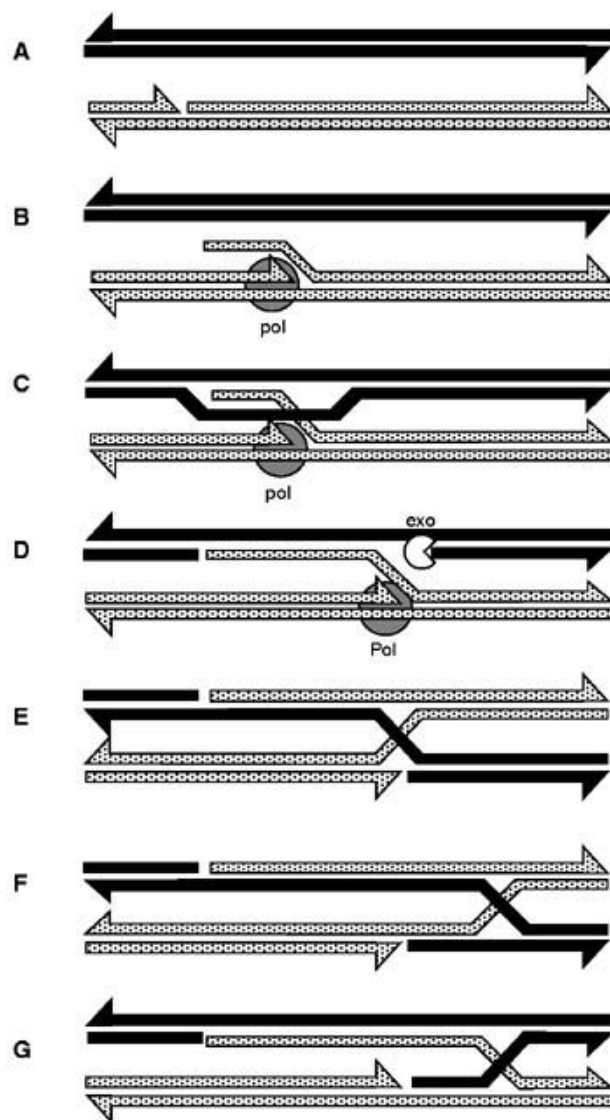


Figure 1 The Meselson and Radding model for recombination. Arrowed ends represent 3' ends, nonarrowed 5' ends.

places one strand of DNA, forming a loose 5' tail (Figure 1B). Single-strand DNA, and especially single-strand ends, are likely to be recombinogenic because they form a substrate for enzymes that catalyze the invasion and uptake of single-strand DNA into homologous duplex DNA, displacing the like strand from the invaded molecule. The classic example of this activity is that of RecA protein from *Escherichia coli*. Similar activities have now been reported in several prokaryotic and eukaryotic systems. Invasion by the single-stranded tail leads to the formation of a D loop (Figure 1C), in which there is a length of heteroduplex DNA and a loop of the displaced strand. *In vitro*, the length of homology RecA required is only 50 base pairs. Some mis-

matched bases can be accommodated within RecA-promoted heteroduplex.

The Meselson and Radding model postulated that further extension of this length of heteroduplex would occur if the loop were nicked to allow entry of an exonuclease. Concerted action of the polymerase and exonuclease (notched circle labeled 'exo') would allow continued strand displacement and uptake (Figure 1D). The length of heteroduplex formed by this process is described as asymmetric, as it occurs in one chromatid only. The asymmetric extension would cease if the polymerase molecule dropped off the DNA.

The structure shown in Figure 1E is an isomer of that seen in Figure 1D. This isomerization is expected to occur spontaneously and frequently. The structure shown in Figure 1E is known as a Holliday junction. It has the property that it can move by diffusion. More recently, proteins that actively push or branch-migrate such junctions have been shown to assist recombination in bacteria. If such branch-migration is to the right in this diagram, a length of symmetrical heteroduplex is formed (Figure 1F).

If a point of heterozygosity falls into a length of heteroduplex, a mismatched base pair will occur. Most such mismatches will be subject to mismatch repair, which is an excision repair process in which one genotype is excised and replaced by the other to give homoduplex DNA. The change of genotype produced by an invasion to form heteroduplex converts one strand of the duplex. Subsequent mismatch repair, removing the invaded molecule's original sequence, can lead to conversion of both strands.

The Meselson and Radding model postulates that the Holliday junction will be resolved by the action of an endonuclease (cutting at the crossed strands in Figure 1), with a cross-over resulting from resolution of the structure when it is in one isomeric form (Figure 1F) and not when it is in the other (Figure 1G). Holliday junction resolving endonucleases have been found in microbes.

Double-strand break repair

Figure 2 shows an example of a model for recombinational repair of double-strand breaks in DNA. The two interacting molecules, or lengths of homology within molecules, are shown together in Figure 2A. Figure 2B shows the result of processing of the broken ends by polar single-strand exonucleases. The single-strand tails, in this diagram, are presumed to have 3' ends. Both tails may invade the homologous sequence forming two D loops (Figure 2C). The 3' ends may prime DNA synthesis, so that both nucleotide strands of the undamaged molecule are copied

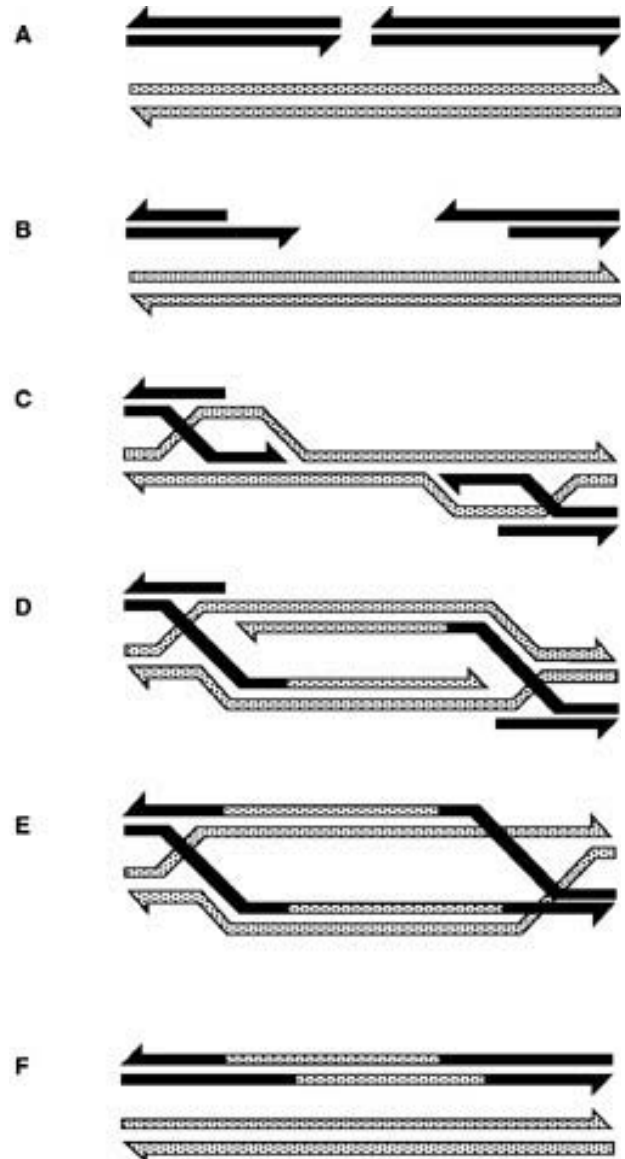


Figure 2 A double-strand-break repair model for recombination. Arrowed ends represent 3' ends, non-arrowed 5' ends.

(Figure 2D). Ligation of the remaining nicks produces a structure with a double Holliday junction (Figure 2E). If it had been the 5' tails that invaded, the 3' ends are still in a position to prime the synthesis of both new nucleotide strands. Hence this scheme can be applied to either or both polarities of single-strand uptake.

The structure shown in Figure 2E must now be resolved into two separate molecules such as those shown in Figure 2F. Three ways by which this could happen have been suggested. First, the Holliday junctions may be cleaved by an endonuclease. This may allow the event to be a cross-over or not, according to the isomeric form of the two Holliday junctions at the time of cleavage. Second, if the structure has

not yet been ligated, the new strands can be separated from the old by helicase activity. Reannealing then gives two DNA molecules, one of which contains a new duplex segment. Third, the action of topoisomerases may unwind the strands between the two Holliday junctions so that the two junctions approach each other, eventually merge and disappear. The last two mechanisms predict that the outcome will not be a cross-over. Either could explain why the proportion of repair conversions that include a cross-over is low.

By any method of resolution, the overall effect of double-strand break repair is that the damaged information has been replaced by copying an undamaged homologous sequence. This nonreciprocal transfer of information constitutes a mechanism of conversion. The model offered in **Figure 2** also includes lengths of heteroduplex DNA, so that within the double-strand break repair mechanism there is also room for conversion based on heteroduplex formation and mismatch repair.

Whether the operation of recombinational repair is dependent on the formation of double-strand discontinuities is unknown. Such events might be initiated by any single-stranded region or by unbroken regions. There may be many different configurations of recombination events occurring in response to varying circumstances. Conversely, it has been suggested that meiotic recombination events, which clearly show conversion by heteroduplex formation, were initiated by double-strand cutting.

Ectopic recombination

Ectopic recombination is recombination between homologous sequences that are not at the same position on homologous chromosomes. Ectopic recombination in yeasts occurs with a frequency comparable with the frequency of recombination in the correct homologous position. Thus one supposes that gross topological constraints do not affect the uptake of recombinogenic nucleotide strands into homologous sequences and that they will become integrated into any sequence that is sufficiently similar and extensive.

That the interacting sequences are ectopically placed has little or no impact on the occurrence of conversion between them. However, resolution of ectopic conversion into a cross-over can have significant consequences which respect to genome structure. Crossing-over between nonhomologous chromosomes leads to translocation and, in some cases, to inviable acentric or dicentric chromosomes. Out of phase crossing-over among repeated sequences causes changes in the number of repeats. Intramol-

ecular ectopic crossing-over gives rise to an inversion of the intervening sequences if the ectopic homology is inverted, and to a deletion, in the form of a ring, if the homology is in direct orientation.

Fidelity of conversion

The context in which concepts of conversion are related to immunological phenomena (at least in chicken and sheep B cell diversification) apparently requires that the conversion mechanism involved is imprecise. Almost all the experiences of those working with recombination in meiotic cells, somatic cells or prokaryotes is that conversion is extremely precise. The ends of the converted length are correctly placed so that there is neither addition nor deletion and no novel substitution mutations are found within the converted length. Exceptions of which we are aware include detection of low frequency mutations associated with recombination in *E. coli* and yeast, which have been attributed to errors during DNA synthesis associated with recombination (discussed below). However, mammalian ligase activity does make nonsequence-specific joints and so could account for the observed imprecision. Conversely, since this apparent imprecision was deduced by sequencing recipients of conversion and potential donor sequences in chicken, it is possible that those changes attributed to nontemplated mutations arise by precise conversion from pseudogenes or small partial gene homologies in as yet unsequenced regions. A third possibility is that vertebrate cells may also make DNA synthesis errors associated with recombination.

How similar must sequences be to recombine?

In any model for conversion or crossing-over, base pairing is the mechanism by which two molecules are held in precise enough register to recombine. *In vitro*, the *E. coli* protein, RecA, will promote base pairing of sequences differing up to 30% in nucleotide composition. Paradoxically, *E. coli* recombination *in vivo* exerts a tighter stringency on the extent to which two sequences may differ and still recombine; sequences sharing as much as 80% similarity do not enjoy homologous recombination.

How do cells discriminate against recombining partially homologous (homeologous) sequences? Because RecA can form homeologous heteroduplexes *in vitro*, M. Radman postulated that the barrier to homeologous recombination might occur after base pairing of the recombining DNA strands. Radman proposed that mismatch repair proteins, which

recognize and bind to mismatched bases, might be responsible for preventing homeologous recombination by blocking the processing of the heteroduplex, by destroying it, or by 'unzipping' it. Support for this hypothesis has come from the finding that sequences sharing 85% similarity will recombine in mutant cells that lack mismatch repair. In addition, mismatch repair proteins have been shown to block RecA-promoted strand exchange between homeologous DNAs *in vitro*.

Do cells regulate the stringency of their homology requirements for recombination? If cells could turn their mismatch repair systems on and off, they could control the degree of sequence similarity required for conversion and crossing-over. In the first edition of this encyclopedia, we suggested that such a mechanism may allow conversion by partially homologous pseudogenes. In chicken B cells, the 'mutations' that give rise to novel immunoglobulin chains include replacements, conversions, of the previous sequence by pseudogene sequences resident elsewhere in the genome. B cells may be able to recombine ectopic homeologous sequences because they are differentiated into a state of relaxed stringency of homologous recombination. According to this proposal, this differentiation is effected by shut-down of normal cellular mismatch repair. There is now good evidence that mismatch repair proteins are downregulated in *E. coli*. They decrease during a recombination-dependent, but nontemplated, mutation process called 'adaptive' mutation.

Recombination and mutation

The recombinational mechanism for mutation discussed above also accounts for other cases of high-level mutagenesis induced by physiological stress and for activation of otherwise cryptic genes under states requiring function of those genes. Physiological induction of both phenomena has been documented in bacteria and yeast ('adaptive' mutations). At least some of the bacterial adaptive mutations form using a recombination-dependent mechanism that is unlike diversification of V genes in chicken and sheep in that the mutations are nontemplated. The bacterial recombination-dependent mutation mechanism includes formation of DNA double-strand breaks, recombination, DNA polymerase errors which are hypothesized to occur during synthesis associated with recombination, and also a downregulation of

mismatch repair such that the polymerase errors persist to become mutations. Also recently, a direct association of apparently nontemplated mutagenesis with recombined DNA in yeast has been reported. In the bacteria, a special physiological state of active mutation and recombination seems to occur. This emerging picture suggests the likelihood of multiple roles of recombination in the genetic changes important to immunological phenomena and illustrates the multiple levels at which cells can regulate their ability diversify when it is beneficial to do so.

See also: Avian immune system; Diversity, generation of; Immunoglobulin, evolution of; Somatic mutation.

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GENE THERAPY

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Regarding possible relationships between genetic dysfunctions and pathologies, the goal of gene therapy is to promote a therapeutic activity by transferring new genetic information either into affected cells or tissues, or into accessory cells. Treatment of inherited pathologies or some acquired diseases, such as cancers, degenerative syndromes or viral infections, could be envisaged with this new strategy (Figure 1).

Gene therapy and immunology are highly interactive fields. First, genetic mutations responsible for some immune deficiencies or disorders have now been well characterized and could benefit from the use of a therapeutic gene transfer. Second, in patients with cancers, manipulation of tumor cells and/or immunocompetent cells in order to induce immunogenicity and trigger or increase an immune response could be a way to control proliferation of malignant cells.

Gene transfer tools

Numerous approaches to transferring new genetic information into target cells have been tested. Among them, viral vectors have been extensively used, taking advantage of the natural ability of genome viruses to cross the cell membrane and express exogenous coding sequences in eucaryotic cells. The most widely used viral vectors derive from retroviruses, adenoviruses, adeno-associated viruses (AAV) or poxviruses and are obtained by replacing viral sequences coding for trans-active proteins responsible for viral replication (i.e. gag, pol and env for retroviral vectors) with the gene(s) of interest. Expression of the transgene(s) can be controlled by viral elements and/or exogenous promoters. Transgene-containing defective viral particles are produced by trans-complementing cells engineered to constitutively express viral proteins absent from vectors (packaging cell lines). Retroviral vectors, and in some instances AAV-derived vectors, in contrast to adenoviral vectors, promote integration of the viral construct in the target cell genome and vertical transmission of the new genetic information. In the same way, to overcome some of the natural barriers associated with these vectors, several additional viral systems are now under development. Baculovirus, human immunodeficiency virus, herpes simplex virus, foamy virus, influenza virus, hepatitis B virus,

Sidbis/Semliki forest virus, spumavirus and simian virus 40 derived vectors can be foreseen as interesting candidates for transferring genetic information. Similarly, naked DNA, either alone or associated with lipidic particles, proteins or defective viral elements, represents an alternative to viral delivery of a transgene. In these vectors, genes of interest, placed under control of an eukaryotic promoter, are included in a plasmid backbone with a bacterial replication origin and an antibiotic resistance expression unit necessary for large-scale production. Naked DNA vectors have already been considered for clinical trials and rapid evolution in this field is anticipated.

Triggering immune reaction against cancers

Antigen presentation, cytokine-mediated cell activation and receptor–ligand interactions are interactive pathways able to induce normal immune responses. Failure of the immune system to direct a therapeutically efficient immune reaction against tumor cells can be due to insufficient effector cell activation. Experimentation and clinical trials based on these concepts, alone or in combination, have been developed to induce antitumor responses by manipulating either tumor or effector cells (Table 1).

Tumor antigen

A number of antigens encoded by genes of the MAGE, BAGE and GAGE families can be recognized by cytotoxic T lymphocytes (CTLs) in a wide range of tumors. These antigens are not expressed in normal tissues and are presented to the T cell receptor in the context of HLA. Other tumor antigens result from mutations or recombinations of ubiquitous genes such as p53, HLA-A2 or CASP-8. One can hypothesize that different or increased presentation of these antigens by the tumor cells themselves, or by antigen-presenting cells (APCs) such as monocytes-macrophages or dendritic cells can activate the immune reaction directed against cancer cells. Thus, increased immunogenicity of tumor cells has been monitored through a direct pulse of antigenic peptides or transfer of sequences coding for target antigens into tumor cells or into APCs. This approach parallels DNA vaccine models dedicated to infectious diseases.

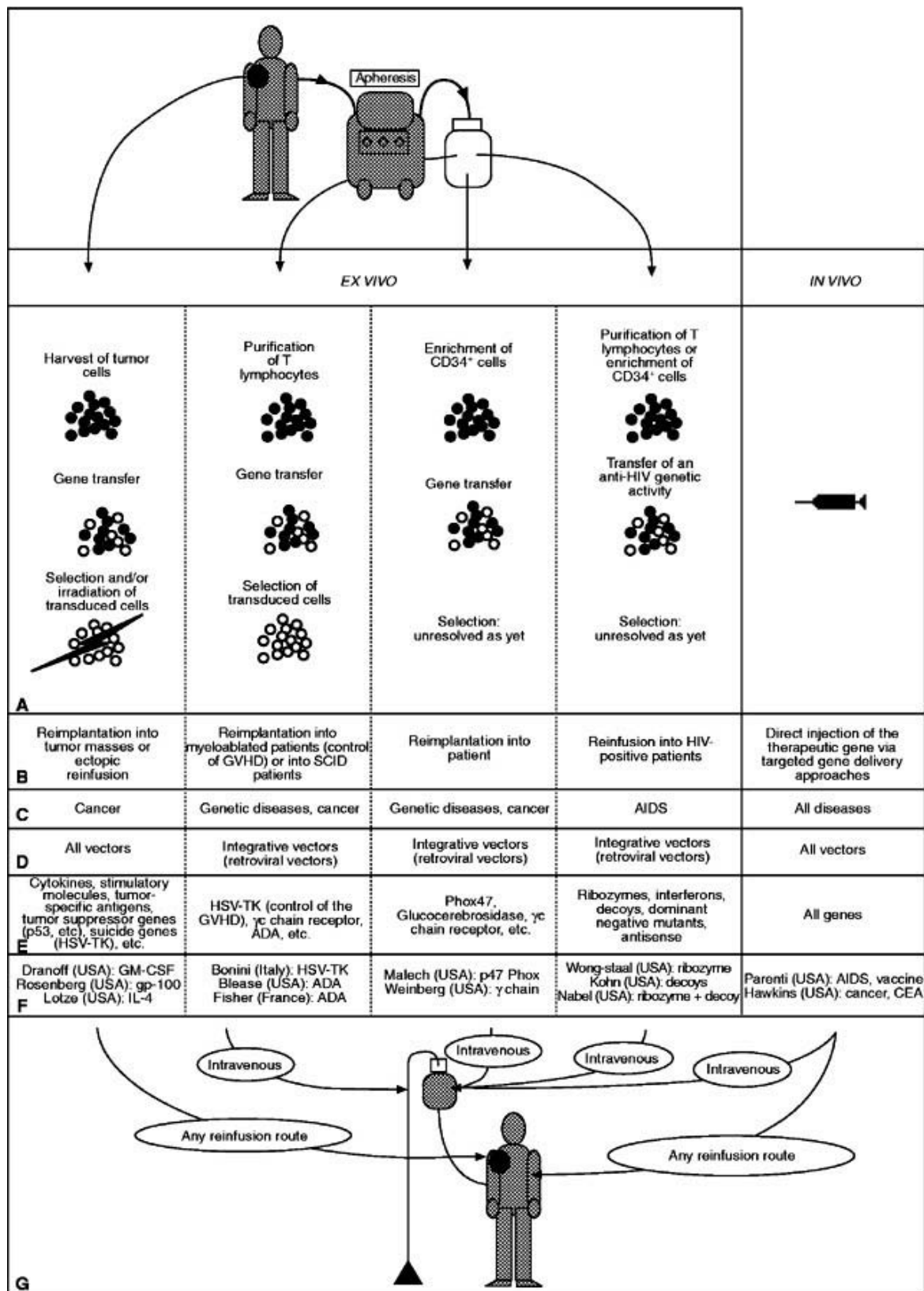


Figure 1 Gene therapy and immunology: an overview of the principles. (A) Overall manipulation of harvested cells; (B) status of the patient; (C) target diseases; (D) types of vector; (E) examples of transgenes of interest; (F) examples of ongoing clinical trials from **Tables 1** and **2**; (G) reinfusion routes.

Table 1 Cancer gene therapy clinical trials and immunology

<i>Gene</i>	<i>Cancer</i>	<i>First author</i>	<i>Vector</i>	<i>Comments</i>
HLA-B7/ β_2 M	Mesothelioma	Chang (USA)	Plasmids	IT (IJ); IV
	Colorectal	Rubin (USA)	Plasmids	IT (IJ); IV
	Melanoma, mesothelioma	Hersh (USA)	Plasmids	IT (IJ); IV
	Melanoma, renal	Fox (USA)	Plasmids	T (TC)
CEA	Cancer	Hawkins (USA)	Poxvirus	IM (IJ); M (TC); IV
	Breast, lung, colorectal	Hamilton (USA)	Poxvirus	ID (IJ); IV
	Breast, lung, colorectal	McAnemy (USA)	Poxvirus	ID (IJ); IV
	Breast, lung, colorectal	Saleh (USA)	Poxvirus	ID (IJ); IV
CEA + HBsAg	Colorectal	Curiel (USA)	Plasmids	IM (IJ); M (TC); IV
IL-2	Melanoma, renal	Gansbacher (USA)	Retrovirus	SC (IJ); Allo tum (TC)
	Melanoma	Mertelsmann (Germany)	Plasmids	SC (IJ); Allo fb (TC)
	Melanoma	Osanto (Netherlands)		SC (IJ); Allo tum (TC)
	Mesothelioma	Hersh (USA)	Plasmids	IT (IJ); IV
B7 (CD80)	Breast	Urba (USA)	Plasmids	Allo tum (TC)
	SCLC	Antonia (USA)	Plasmids	
	Melanoma	Sznol (USA)	Plasmids	
CD	Col	Crystal (USA)	Adenovirus	
antiTGF β_2	Glioblastoma	Black (USA)	Plasmids	SC (IJ); Auto tum (TC)
anti IGF1	Glioblastoma	Ilan (USA)	Plasmids	SC (IJ)
IFN β	Glioblastoma	Yoshida (Japan)		
GM-CSF	Melanoma	Dranoff (USA)	Retrovirus	Auto tum (TC)
	Melanoma	Rankin (Netherlands)	Retrovirus	Auto tum (TC)
	Melanoma, Src	Mahvi (USA)	Plasmids	SC (IJ); Auto tum (TC)
	Prostate, renal	Simons (USA)	Plasmids	Auto tum (TC)
IL-7	Melanoma	Economou (USA)	Retrovirus	SC (IJ); Allo tum (TC)
HLA-B7	Melanoma	Hersh (USA)	Plasmids	IT (TJ)
IFN γ	Melanoma, mesothelioma	Nemunaitis (USA)	Retrovirus	Auto tum (TC)
	Melanoma	Seigler (USA)	Retrovirus	
	Neuroblastoma	Rosenblatt (USA)	Retrovirus	
MART-1	Melanoma	Rosenberg (USA)	Adenovirus	IV (IJ)
gp100	Melanoma	Rosenberg (USA)	Poxvirus	IM (IJ); IV
			Adenovirus	SC (IJ); Auto tum (TC)
			Poxvirus	IM (IJ); IV
MAGE-1	Melanoma	Rosenberg (USA)	Poxvirus	ID (IJ); IV
IL-12	Melanoma, head and neck	Lotze (USA)		IP (IJ); Fib (TC)

Table 1 Continued

<i>Gene</i>	<i>Cancer</i>	<i>First author</i>	<i>Vector</i>	<i>Comments</i>
Ab anti-erbB2	Ovarian	Curiel (USA)	Adenovirus	IP (IJ); IV
TK/B7	Ovarian	Freeman (USA)	Plasmids	SC (IJ); Allo line (TC)
IFN γ /IL-2	Prostate	Gansbacher (USA)	Retrovirus	Allo tum (TC)
Idiotypic Ig	Leukemia	Hawkins (UK)	Plasmids	IM (IJ)
PSA	Prostate	Chen (USA)	Plasmids	SC (IJ); IV
	Prostate	Kufe (USA)	Plasmids	ID (IJ); IV
IL-4	Cancer	Lotze (USA)	Retrovirus	SC (IJ); Fib + Auto tum
	Glioblastoma	Bozik (USA)	Retrovirus	(TC)
	Melanoma	Parmiani (Italy)	Retrovirus	SC (IJ); Auto tum (TC) Allo tum (TC)
TNF/NeoR	Cancer	Rosenberg (USA)	Retrovirus	TIL (TC)
TNF/NeoR	Cancer	Rosenberg (USA)	Retrovirus	Auto tum (TC)
HLA-B7/IL-2	Cancer	Rosenberg (USA)	Retrovirus	Auto tum (TC)
	Cancer	Parmiani (Italy)	Retrovirus	Allo tum (TC)
	Renal	Lindeman (Germany)	Plasmids	ID (IJ); Allo tum (TC)
HSV-TK	Graft-versus-host-disease	Tiberghien (France)	Retrovirus	ItV (IJ); T (TC)
		Bordignon (Italy)	Retrovirus	ItV (IJ); T (TC)

(IJ), *Injection procedure*: IM, intramuscular; ID, intradermal; SC, subcutaneous; IT, intratumoral *in vivo* approach; ItV, intravenous. (TC), *Target cells*: M, muscle cells; TIL, tumor infiltrating lymphocytes; Auto tum, autologous tumor cells; Allo tum, allogeneic tumor cells; Fib, fibroblasts; Allo line, allogeneic cell line; T, T lymphocyte; Allo fb, allogeneic fibroblasts; SCLC, small cell lung cancer; Col, colon cancer; Src, sarcoma; IV, *in vivo* gene transfer approach.

Source: TMC Development. T. Marcel and D. Grausz; Dec 1996.

Cytokine gene transfer

Most of the cytokines do not have a direct effect on the tumor cells but can induce a marked antitumor effect *in vivo* by initiating or potentiating specific and nonspecific host responses against tumors. Use of cytokines in this context, such as interleukin 2 (IL-2), IL-4, interferon α (IFN α) and IFN γ , has already proved efficient in the treatment of some cancers. However, lack of or low activity of systemic delivery of cytokines and toxic side-effects were observed. Therefore, transfer and expression of a cytokine gene into tumor cells would allow *in situ* delivery of the product and should direct cellular responses against the tumor. Many animal studies have demonstrated that production of cytokines, such as IL-2, IL-4, IL-7, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNs or tumor necrosis factor α (TNF α), by transduced tumor cells elicits a host antitumor response and, in some cases, a memory response against a rechallenge with the same tumor. Clinical trials based on these approaches validated

by animal studies have already been developed. Although feasibility and absence of toxic effects have been demonstrated, until now no clear therapeutic benefit has been observed and only a few objective clinical responses have been reported.

Use of effector cell costimulatory molecules

Interaction of the CD28 receptor and its ligands B7 (CD80, CD86) produces a synergistic second signal for T cell activation and cytokine production. It has been determined that absence or low expression of B7 molecules on tumor cells could prevent T cell-mediated killing and that re-expression of this molecule at the surface of tumor cells could improve recognition and activate immune-mediated killing. Successful animal models have been developed. Assuming the possibility of teaching tumor cells to 'present themselves' in patients, Nabel and colleagues injected plasmid DNA coding for B7 directly into melanoma tumor cell masses and observed a correlation between expression of the transduced B7

gene and presence of specific T cells in tumor masses *in vivo*. Expression of CD95 ligand (Fas ligand) on tumor cells could also induce rejection of tumor cells by activating neutrophils.

Dealing with GVHD and GVL effect

A lower percentage of leukemia relapse is observed when allogeneic transplantation is associated with graft-versus-host disease (GVHD) which leads to the concept of a graft-versus-leukemia (GVL) effect. GVHD and GVL are probably mediated by the same subsets of T lymphocytes and the difficulty lies in establishing a balance between the GVL effect and the GVHD, which is associated with high morbidity and mortality.

Retroviral transfer of a suicide gene, such as the herpes simplex virus-thymidine kinase gene (HSV-TK), whose product induces sensitivity to ganciclovir, into donor T lymphocytes raises the possibility of controlling the GVHD. Reimplanted in combination with the stem cell graft, or at the time of relapse, ganciclovir-sensitive allogeneic T lymphocytes can induce a GVL effect and, if necessary, can be eliminated by injection of the drug if toxic side-effects endanger the patient's life. Considering the proliferative status of activated T lymphocytes, persistence of the suicide gene in the T lymphocyte is promoted by retroviral vector integration. Phase I clinical trials are now in progress.

Immune deficiencies or immune disorders and gene therapy

Inherited immunodeficiency diseases

Severe combined immunodeficiency diseases (SCIDs) were found to be associated with classical genetic defects. Lack of expression of adenosine deaminase (ADA), γ c cytokine receptor chain gene, p47 phox protein (in chronic granulomatous disease), Zap70 kinase and WASP protein (in Wiskott-Aldrich syndrome), could be restored by gene transfer and expression in appropriate target cells of a correct version of the gene involved in the pathology (Table 2).

Acquired immune deficiency syndrome

Acquired immune deficiency syndrome (AIDS), which results from the consequences of reactivation and spread of the human immunodeficiency virus (HIV), can be considered as an acquired genetic disease, assuming that HIV acts as a deleterious complex gene. Several blockades of the replication cycle have thus been proposed and include expression of antisenses, negative dominant mutated viral proteins, expression of protein fixation site decoys to compete

with natural sites present on the provirus, regulated production of IFN α and IFN β able to interact with viral production, intracellular synthesis of neutralizing antibodies against viral proteins, and expression of ribozymes able to specifically cleave RNA viral genomes. In addition, gene therapy vaccine approaches are now under development. Even if T lymphocytes were first proposed as target cells, it appears that difficulties in genetically modifying this cell population *ex vivo* when derived from HIV-positive patients dictated the consideration of hematopoietic progenitors as target cells and retroviral vectors as gene transfer tools to promote therapeutic activity in cell lineages involved in HIV infection. This approach would have the significant advantage of allowing therapeutic information to persist during the lifespan of the patient and of expressing the transgene of interest in dendritic cells and macrophages as well as in CD4⁺ T lymphocytes (Table 2).

Autoimmune diseases

Autoimmune disease is mediated by cellular and/or humoral responses self antigens expressed in normal cells or tissues. Induction of T cell tolerance specific for self proteins involved in autoimmune disease can prevent pathologic effects. This has been accomplished experimentally to treat induced immune-mediated diabetes by gene transfer and expression of autoantigens in bone marrow cells reinfused into animals. In addition, TNF α and IL-1 are known to be key mediators secondary to the leukocytic infiltration in rheumatoid arthritis. Blocking of IL-1 deleterious effects has already been demonstrated using either *ex vivo* transfer of IL-1 receptor antagonist gene in synovial cells prior to reinfusion into the knees of an antigen-induced arthritis rabbit model, or direct *in vivo* adenoviral transfer of soluble type I TNF α receptor or IL-1 receptor-IgG (immunoglobulin G) fusion gene. Clinical trials are now in progress (Table 2).

Animal models: a step forward and a trap

Gene therapy would not exist without animal models. Many biological concepts underlying gene therapy, and especially those using immunological tools, arise from animal studies. Impressive eradication of tumors by activation of cellular responses mediated by effectors such as CTLs, natural killer cells, macrophages, eosinophils and neutrophils were observed in animals reinfused with cells expressing transduced genes for cytokines or other stimulatory molecules. Conversely, use of the same concepts in human clinical trials has given relatively poor results in terms of

Table 2 Gene therapy clinical trials for inherited or acquired immune disorders and immunology

<i>Gene</i>	<i>Disease^a</i>	<i>First author</i>	<i>Vector^b</i>	<i>Comments^c</i>
p47 Phox	CGD	Malech (USA)	Retrovirus	CD34 ⁺ cells (TC)
ADA	SCID	Fischer (France)	Retrovirus	ItV (IJ); T (TC);
		Levinsky (UK)	Retrovirus	ItV (IJ); T (TC)
		Sakiyama (Japan)	Retrovirus	ItV (IJ); T (TC)
		Seeger (CH)	Retrovirus	
		Blaese (USA)	Retrovirus	ItV (IJ); T (TC)
		Bordignon (Italy)	Retrovirus	ItV (IJ); CD34 ⁺ cells, T (TC)
γ c chain	X-linked SCID	Weinberg (USA)	Retrovirus	ItV (IJ); CD34 ⁺ cells (TC)
IL-1 R antagonist	Rheumatoid arthritis	Evans (USA)	Retrovirus	Synoviocytes (TC)
Decoy	AIDS	Gilbert (USA)	Retrovirus	ItV (IJ); T (TC)
		Kohn (USA)	Retrovirus	ItV (IJ); CD34 ⁺ cells (TC)
Ribozyme	AIDS	Rosenblatt (USA)	Retrovirus	ItV (IJ); CD34 ⁺ cells (TC)
		Symonds (USA)	Retrovirus	
		Wong-Staal (USA)	Retrovirus	ItV (IJ); T (TC)
		Kohn (USA)	Retrovirus	ItV (IJ); CD34 ⁺ cells (TC)
Antigen (vaccine approach)	AIDS	Conant (USA)	Retrovirus	IM (IJ); E+R (AP); IV
		Galpin (USA)	Retrovirus	IM (IJ); E+R (AP); IV
		Galpin (USA)	Retrovirus	IM (IJ); E+Ta (AP); IV
		Haubrich (USA)	Retrovirus	IM (IJ); E+R (AP); IV
		Lutny (CH)	Plasmids	
		Weiner (USA)	Plasmids	E+R (AP), IV
Antigen + decoy	AIDS	Nabel (USA)	Plasmids	ItV (IJ); T (TC)
		Nabel (USA)	Retrovirus	
Intracellular immunization	AIDS	Pomerantz (USA)	Retrovirus	ItV (IJ); T (TC) anti-rev single-chain variable fragment
		Marasco (USA)	Retrovirus	
PNP	PNP	MacIvor (USA)	Plasmids	ItV (IJ); IV

CGD, chronic granulomatous disease; AP, antigenic protein; E, env; R, rev; Ta, tat; CH, Switzerland; PNP, purine nucleoside phosphorylase deficiency and see footnote to **Table 1**.

Source: TMC Development. T. Marcel and D. Grausz; Dec 1996

therapeutic effect. These differences mainly arise from the differences in health status between end-stage patients and animals bearing freshly implanted tumor cells, and the possibility that immunological pathways used differ between animal and human. Technical problems, such as transduction efficiency, expression of the transgene in the majority of tumor cells and large scale procedures with human material, have still to be resolved.

Although toxicity of a transgene product or inefficiency of a treatment could be checked with animal models, strategy developed in an animal model directly transposed to a clinical trial should be considered very carefully. In addition, for cancer gene

therapy animal models with naturally occurring tumors could be more relevant to the human context.

Immune reactivity against vector proteins and transgene products

Gene therapy will probably require multiple injection of vectors or of genetically modified cells, which emphasizes the possibility of immune-mediated inactivation of the treatment. Retroviral, AAV and plasmid vectors seem to be insufficiently antigen to induce an immune reaction by themselves. It is worth noting that major concerns have been raised about immune reaction against viral proteins encoded by

the remaining genome of adenoviral vectors, and work is now in progress to construct vectors devoid of viral sequences (gutless vectors) or which are less immunogenic. Immune reactions against heterologous transgene products, which can synergize with immune reactivity directed against viral vector protein, have already been described for all types of vectors.

Perspectives

The therapeutic benefit which would arise from gene therapy in treatment of cancers or immune disorders depends on both technical improvement and evolution in immunological concepts. Transduction efficiency and long-term expression of the transgene have already been emphasized as key problems in gene therapy procedures. Development of new vector strategies, targeting of cells to be transduced and control of expression of the transgene, using tissue specific promoters or inducible constructs, are now under investigation in order to overcome some aspects of these problems. In addition, full characterization of the cytokine network and cytokine functions in the human system would help to better define possible therapeutic utilities of some molecules in cancer therapy, and develop more therapeutically efficient combinations between the disease to be treated and the transgene used. In addition, even though most of the treatments to date have been performed by *ex vivo* genetic modification of the target cells, important efforts are under development to deliver new genetic information *in vivo*, thus emphasizing the need for cellular and molecular targeting in order to induce new functions restricted to the target cells.

See also: Acquired immune deficiency syndrome (AIDS); Adenovirus, infection and immunity; Antigen-presenting cells; Autoimmune disease, induced experimental models; Autoimmune diseases; Chronic granulomatous disease; Graft-versus-host reaction; Human immunodeficiency viruses; Immunotherapy of tumors; Liposomes; Retroviral vectors; Tumors, immune response to; Tumors, immunological escape of; Vaccination, methods of administration.

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GENETIC ANALYSIS AT THE MOLECULAR LEVEL

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With any genetic approach, one major requirement is to develop genetically homogeneous collections of organisms (e.g. mouse strains), cells (e.g. clones of T cells or B cells), or DNA molecules. DNA cloning techniques first relied on bacterial genetics (e.g. transducing phages) and then on genetic engineering methods. The latter were initially perceived as a breakthrough for the production of foreign protein by microorganisms, but it soon became obvious that they offered a quasi-general solution to the amazingly difficult problem of gene purification. The concomitant development of DNA sequencing in 1975 allowed the determination of the sequence of a purified gene, i.e. its structure, or more precisely, its chemical formula. Ten years later (1985), an *in vitro* cloning procedure was discovered. The polymerase chain reaction (PCR) enables amplification of tiny amounts (actually down to one or a few molecules) of DNA millions of times in just hours, thus allowing minute samples of DNA to be analyzed. Purified genes can be mutated *in vitro*, at random or specifically, thanks to synthetic oligonucleotides. Mutated or not, they can be introduced into living cells in culture by transformation or transfection, or used in the generation of transgenic animals. Targeted genes can also be disrupted in embryonic stem (ES) cells by homologous DNA recombination techniques. The resulting mutant ES cells can then be introduced into blastocysts of normal mice to generate chimeric embryos, which may develop into chimeric animals. Homozygous animals for the mutation are obtained by selective breeding over several generations. More recently, using different recombination systems, it has become possible to inactivate genes in a tissue-specific or inducible manner. Thus, a wide battery of methods and techniques is now available for genetic

analysis at the molecular level. The structural and functional analyses of genes can be bridged in a variety of ways. These powerful tools have been, and will go on being used productively in immunology. Techniques and protocols have rapidly been formalized into simple recipes and the necessary reagents and instruments have been commercialized quickly, such that many of these molecular analyses have become a matter of routine in a large number of laboratories. The following overview of these molecular approaches is illustrated by a few selected examples.

Analysis of gene structure

For the analysis of gene structure, cloning is mostly performed in *Escherichia coli*, where a variety of sophisticated cloning vectors facilitate cloning, sub-cloning and sequencing. Difficulties in the isolation of new genes usually arise when probes are lacking and 1) genes are poorly expressed (because the mRNA is rare and the corresponding cDNA is difficult to isolate, while the information on the protein product is scarce and hard to gather), 2) genes are exceedingly large (the gene responsible for Duchenne muscular dystrophy is over 2000 kb long), and 3) large-scale cloning and sequencing is involved. However many of these problems have been overcome by the use of PCR and other technologies embedded within the genome mapping and sequencing approaches. New instruments and the appropriate software are actively being developed in the frame of 'human genome' projects. A huge mass of information is being deposited in databanks. The entire repertoire of variable gene segments of antibodies and T cell receptors will soon be available in several

species (see below), with the additional prospect of comparing the corresponding loci in various species or various individuals of the same species.

Analysis of gene expression

Studies of the expression of endogenous genes can be performed in a variety of ways. Messenger RNAs can be assayed by hybridization with probes in northern blot experiments or with reverse transcription polymerase chain reaction (RT-PCR). Other sophisticated assays, useful to discriminate between alternatively spliced mRNA from the same gene, or mRNAs produced by close members of a multigene family, involve RNases and/or S1 nuclease to detect mismatches. Carefully controlled hybridization with oligonucleotides can detect a simple mismatch (and can also be used in northern blots, as well as in Southern or dot blots to analyze genomic DNA). When the sequence studied is already known, specific oligonucleotides can be derived and PCR techniques used to identify different messenger RNAs, mutations or small deletions (by direct sequence of the PCR product, digestion with restriction enzymes, hybridization of product with a specific probe, single-strand conformational polymorphism, etc.). *In situ* hybridization (with a sensitivity of about 10 copies per cell) and *in situ* PCR (for detection of low copy events, point mutations, etc.) open many possibilities in oncogenesis, embryology, RNA trafficking and detection of viral diseases.

Of course, the gene product itself can be assayed by its activity or by its specific binding to appropriate ligands such as antibodies. Detection of gene expression by molecular biology techniques associated with the detection of other gene products by flow cytometry analysis may be useful in some cases. For example, flow cytometry analysis followed by cell sorting and single-cell PCR of rearranged immunoglobulin or TCR genes allows analysis of antigen specific T and B cell repertoires in the course of immunization. In the same way, the phenotype of virus-infected cells (by human immunodeficiency virus (HIV), for example) can be determined by a combination of the intracellular PCR technique with fluorescent labeled oligonucleotides and flow cytometry analyses. This approach also applies to cytokines and other genes of immunological interest.

Apart from the mere observation of physiological or pathological situations, gene expression studies are widely used in structure-function relationship analyses, which require that the gene possibly altered or mutated is introduced into a living cell or organism (purely *in vitro* systems are most of the time inadequate). There are multiple empirical ways of

introducing DNA into cells. The most usual one is the calcium phosphate technique. Fusions with bacterial protoplasts, red blood cell ghosts and lipid vesicles are also used. Electroporation, as well as cationic liposome-mediated transfection (lipofection) are seemingly better controlled. Microinjection requires skill, and is not appropriate for large-scale experiments. Infection with viruses is another route. Thus, recombinant vaccinia viruses will express the recombinant protein in many different cell types, but in 2–3 days cells die from infection. Retroviral vectors for gene transfer have been more difficult to handle than was initially anticipated. Nevertheless, they do fulfill the requirements for efficient gene transfer without irrelevant alteration of the cell properties, and constitute useful tools. They have already been used in animals to transfer genes into different permanently dividing tissues such as the hematopoietic tissue or the liver. Immunization of animals with naked DNA (usually into the muscle) or formulated DNA is a relatively new means of assessing the immunogenicity of gene sequences.

The generation of transgenic animals has become a routine operation. However, it is more difficult to make transgenic mice in a homozygous congenic background which is necessary in many experiments of immunological interest. In such gene transfer experiments, the transferred DNA is added to a pre-existing genome and integrates more or less at random. Even when it carries homologies with resident genes, it is usually not substituted to them. Furthermore, the level of expression and the tissue specificity of the injected construct are not efficiently controlled. Animals often display unexpected phenotypes but these can turn out to be quite useful. For example, the ubiquitously expressed hydroxymethyl CoA reductase promoter yields levels of expression which vary even in animals derived from the same founder.

The powerful gene targeting technology has first been used to destroy specific genes in so called knockout mice. In its early version homologous recombination between exogenous DNA and its endogenous homolog in pluripotent mouse embryonic stem cells was used to disrupt the latter by insertion of a neomycin resistance gene. However, the insertion of a *neo^r* gene sometimes disturbs the surrounding chromosomal organization and gene expression. To overcome these problems, a 'cleaner' way to create mutations has recently been described. This method is based on the DNA-site specific recombination of the Cre/loxP system from the bacteriophage P1 or the yeast recombination system FLP/FRT. Two loxP (or FRT) sequences are introduced in predefined positions around the targeted

sequence by homologous recombination. The targeted sequence can then be deleted by recombination mediated by Cre/loxP (or FLP/FRT). This approach has several advantages since the mutated gene locus does not contain the selection marker and genes can be inactivated in a tissue-specific or inducible manner. Recombinant mice containing Cre/loxP sequences around one defined gene can be bred with other mice transgenic for the Cre enzyme gene under the control of a tissue-specific or inducible promoter (see **Figure 1**). Since lymphocyte differentiation and activation are processes characterized by complex but coordinated programs involving many molecules, targeted gene disruption appears to be a tool of choice to study the unique role played by each one among the others. This approach has so far revealed the somewhat unexpected plasticity of the immune system to adapt and compensate for the loss of key molecules. As the list of genetically modified mouse strains is growing on a monthly basis, no doubt more surprises will be forthcoming in our understanding of lymphocyte development and function in host defense.

Analysis of gene regulation

Another body of methods and techniques has been developed to analyze the mechanisms by which isolated genes are regulated, particularly at the transcriptional level. A usual scheme involves the deletion analysis of the promoter region attached to a reporter gene coding for an easily assayable product (e.g. chloramphenicol acetyl transferase, β -galactosidase, luciferase, peptide tags as ligands for specific antibodies, etc.). Once important regions are identified, the promoter is chopped into pieces and scanned for elements which positively or negatively modulate its activity, such as enhancer or enhancer-like sequences. Often, the putative important elements are polymerized *in vitro* (sometimes in the form of synthetic oligonucleotides) and assayed *in vivo* in appropriate expression vectors, or as competitors. Then starts the hunt for DNA-binding proteins, first with the less sophisticated, but artifact-prone, band shift (gel retardation) assays, followed by DNA footprints and methylation interference experiments, in which contact residues between the

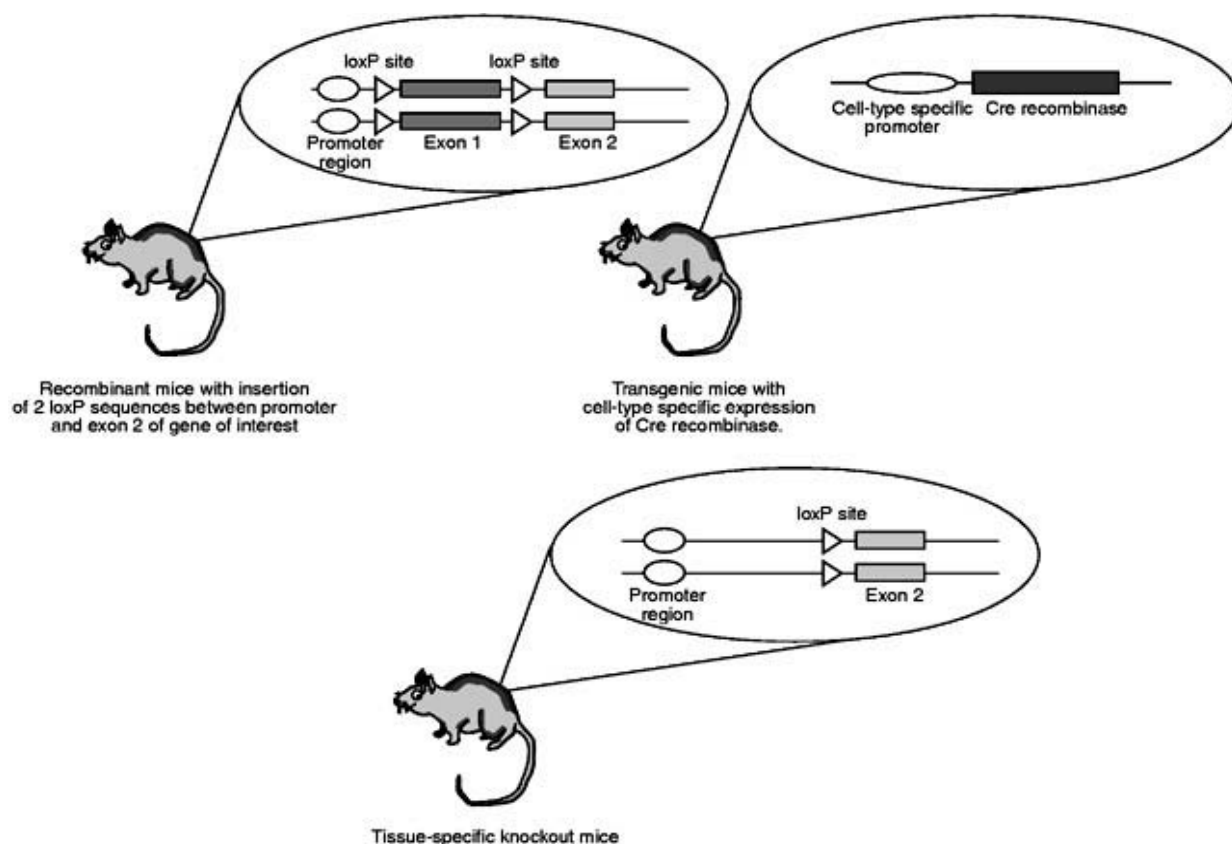


Figure 1 Generation of tissue-specific knockout mice based on the Cre/loxP DNA site-specific recombination system from bacteriophage P1.

protein factor and the DNA sequence are identified. Next, the DNA binding protein can be purified by a combination of conventional and affinity chromatography techniques (using the polymerized DNA sequence coupled to a column). Ultimately, the purified protein can be microsequenced, allowing the deduction of 'back-translated' probe or primers and the corresponding gene may be cloned.

An alternative strategy employs libraries of cDNAs cloned in *E. coli* expression vectors such as λ gt11 and an *in situ* assay allowing the detection, in the phage plaques, of specific binding of a labeled antibody raised against the protein of interest, or of labeled DNA sequence representing the binding site of the protein. When successful, this approach is much faster, but the identification of the encoded protein can remain elusive. The cloned factors can then be studied in many ways, and the synthesis of altered forms (truncated, mutated, fused, etc.) in *E. coli* or *in vitro* (e.g. in T7 RNA polymerase-based expression systems, involving cell-free translation procedures) is the next avenue of exploration.

The double hybrid system in yeast is a useful way to detect proteins capable of interacting with a known factor and results in the immediate availability of the cloned genes for these interacting proteins. One hybrid consists of the DNA-binding domain of yeast transcriptional activator proteins GAL4 or LEX A fused to the known protein; the other hybrid consists of the GAL4 or LEX A activation domain fused to protein sequences encoded by a library of genomic DNA fragments. Interaction between the known factor and a protein encoded by one of the family plasmids leads to transcriptional activation of a reporter gene containing a binding site for GAL4 or LEX A.

An important and difficult task is to compare the factors, in the course of their characterization, to others, cloning being the only safe way to decide whether factors are identical or different. Another essential question is to understand the relationships between DNA-binding proteins which attach to the same promoter sequence. Most human and mouse promoters contain sites that can bind several different factors (some of them even bind to each other), and the spectrum of bound proteins may vary in different cells and tissues, depending upon external stimuli. It should not be forgotten that regulatory elements can be found far upstream of genes, within genes, or downstream, and that many possibilities for post-transcriptional regulation have been found. It is difficult to assess the *in vivo* significance of one or a set of element(s) recognized as important *in vitro*, and the use of transgenic or knockout mice then becomes necessary.

Large-scale production of proteins

Another facet of molecular genetics is the ability to produce proteins in relatively large amounts from a cloned gene. Since this may be of obvious medical and economic interest, considerable efforts have been invested in a variety of production systems. The most popular include *E. coli* and yeast transformed with appropriate plasmids (expression often being inducible), insect cells infected with recombinant baculoviruses, and mammalian cells transfected with genes together with selectable (and sometimes amplifiable) markers, or infected with recombinant viruses (such as retroviruses and vaccinia viruses). Molecules of immunological interest have also been produced in plants (e.g. tobacco). The level of expression is most of the time unpredictable and the field remains very empirical. Numerous parameters are involved, including the rate of degradation of the foreign protein, its solubility or insolubility (cf. the formation of inclusion bodies), etc. Solubility can be manipulated by the choice of vector, cloning sites, host cells, or, in the case of bacterial expression, by cloning the desired sequence downstream of a bacterial signal sequence (like *pelB*) for potential periplasmic localization. Production of a fusion protein can also help in improving solubility of the recombinant molecule, and results in easier purification (an oligohistidine domain can be added for purification over nickel column, a peptide tag for immunological detection and affinity purification, etc.) A protease cleavage site (thrombin, factor Xa, enterokinase) introduced between the sequence of interest and the tag, may allow removal of the upstream domain by treatment of the fusion protein with a protease.

Accordingly, different proteins are expressed at widely different levels, and even minor changes can result in differences of orders of magnitude in their expression. (Vaccinia viruses are, perhaps, one of the most reproducible systems.) Contrary to initial beliefs, the problems often lie with RNA structure and splicing (aberrant splicing is a frequent source of problems) and/or translation (initiation, propagation and, perhaps, codon usage) while the efficiency of transcription is often not limiting. One should keep in mind that a genetically engineered protein may display minor differences (mostly due to post-translational modifications) to the natural one. These differences may be functionally important *in vitro* or *in vivo* (e.g. the rate of *in vivo* clearance of recombinant nonglycosylated interferon γ (IFN γ) is higher).

Expression of cloned genes is essential to overcome the problems associated with the purification of trace amounts of products of immunological importance. This holds true for interleukins and intracellular

regulatory factors, some of which have been produced in sufficiently large amounts for biological, biochemical, crystallographic studies or clinical trials. Another dimension has been added with the expression of antibodies and other polymorphic molecules in microorganisms (either as spontaneously assembled segments, or as single-chain molecules in which the variable regions are held together with a spacer sequence, single-chain TCR and MHC molecules have also been produced). Combining PCR technologies with the expression of light and heavy antibody chains fused to phage coat protein (fusion to pIII leading to 3–5 copies located at the tip of each phage or fusion to pVIII giving 2500 copies per phage), it is now possible to generate combinatorial libraries of antibody specificities with diversity in excess of 10^{10} , either from the mouse (immunized or naive) or human (usually naive) B cell repertoire. With 3 or 4 rounds of selection through column or plate-coated antigen, following amplification in *E. coli*, phages expressing a desired specificity can be isolated (differential selection using cells is more difficult and limits the use of the technology). One can imagine that these libraries are large or almost large enough to allow selection of any specificity which one may need. Micromolar antibody affinities which are often obtained are too low for most practical applications but affinity maturation can be mimicked *in vitro* using error-prone PCR or mutagenesis, followed by selection for higher affinity.

Repertoire-selection technology has also been used to select peptides. Random peptide libraries are constructed by ligation of a degenerate oligonucleotide insert to a chosen display vector. Once created, epitope libraries are universal reagents that can potentially be used to identify ligands for any available receptor (antibodies, MHC molecules, concanavalin A, etc.)

The specificity of molecular genetics in immunology

The above survey could apply to several fields of biology (developmental biology, neurobiology, etc.). To a certain extent, immunology brings about a number of specific problems, some of which are summarized below.

As in all fields of biology, and thanks to molecular methods, the 'natural history', or descriptive aspects, of immunology are expanding very rapidly at the gene level. It so happens that the immune system involves the largest of all known multi- and super-multigene families. Thus, sequence determinations, comparisons and analyses within a given species, and between different species, constitute an extremely

interesting and valuable body of data for evolutionary and polymorphism studies. Correlatively, structural analogies at the protein level permit computer-aided protein modeling to be used in a relatively robust way. This was illustrated by work on antibodies and MHC molecules in which, from a small number of known crystallographic structures, relatively accurate protein models were derived.

Second, the immune system is the only system so far to display such high polymorphism and diversity. This relates to the dynamics of the large multigene families (MHC genes, V regions of antibodies and TCRs) and of the selective forces involved. Furthermore, the combinatorial recombination of antibodies and TCRs raises the question of potential, actual or available repertoire at any given time in a given individual. Molecular genetics (e.g. PCR) is now providing a powerful support to the study of T cell repertoires, previously hampered by the difficulty in obtaining clonotypic reagents. With a combination of PCR reactions involving C_{β} , V_{β} and J_{β} primers, and size measurement providing the lengths of the CDR3-like regions of β chains, one can identify T cell repertoires from amongst 2000 clonotypes. Quantitative PCR has been greatly improved and is very helpful for measuring, for example, interleukin transcripts in biological samples, including human biopsies.

Finally, thanks to the progress made in cloning (animals, cells and genes), the immune system now appears as the most complex system for which the most detailed description of the constitutive elements is available. It is common to emphasize the reductionist attitude of molecular biology which, by essence, looks at a single element (gene or protein) at a time. It is interesting to perceive how, since 1976 (the cloning of an immunoglobulin gene by Tonegawa), genetic analysis at the molecular level has penetrated a field initially dominated by systemic studies. It is fascinating to observe how molecular studies have produced enough knowledge to begin drawing integrated views of the molecular elements, which it is becoming possible to try to match with their systemic behaviors.

See also: Northern blotting; Polymerase chain reaction (PCR) amplification; Retroviral vectors; Southern blotting; Transgenic animals.

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GENETIC ANALYSIS AT THE PHENOTYPIC LEVEL

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The words 'genotype' and 'phenotype' were first used in 1911 by a Danish botanist, W Johannsen, to elucidate the relationship between the genetic material and its resulting product, the phenotype. With the advent and application of sophisticated molecular techniques, the concept of genotype (gene) has become more clear. An individual's *genotype* refers to its genetic constitution: either the whole genome, which consists of full DNA content on its chromosomes; or, more specifically, a single gene, which is a single functional section of DNA on one specific chromosome. The term *phenotype* is, however, more complicated: it not only represents the expression of the genotype into biochemical, physiologic, structural and physical traits, but also depends on the interaction between the genotype and environmental factors in which the organism develops. In comparison to the individual's genotype, which is transmitted to daughter cells either through somatic (mitosis) or germ (meiosis) cell divisions, a given phenotype may or may not be directly inherited.

Some phenotypes, like monogenic (single-gene) traits, are largely genetically controlled; other phenotypes, like multifactorial traits, are the result of both polygenic (multiple genes) and environmental interactions. While the monogenically controlled phenotypes remain largely unaffected by environmental changes the multifactorial phenotypes are subject to changes over time depending on the environment to which organisms are exposed.

The molecular flow of information determines the nature of an individual's phenotype and proceeds from DNA to RNA and then to protein. In order to transfer its genetic constituent (genotype) to a specific gene product (protein), a portion of DNA (gene) is first transcribed into a messenger RNA, which by acting as a template translates the genetic information into a specific protein molecule. The basic unit

of the genetic code or information is a codon, which consists of three nucleotides, that code for a single amino acid. There are 20 different kinds of amino acids, which are the building blocks of the polypeptide chain(s) of a protein. The structure of protein is also influenced by post-translational changes, enabling it to be processed further.

Structural abnormalities in proteins occur due to mutations in corresponding genes. Mutations, which are inheritable, can either be minute or gross. The most common mutations are of the former type and referred to as point mutations, causing a single base change in the codon, that may change the amino acid sequence in the polypeptide chain and result in a variant protein phenotype. If structural changes occur in the functional domains of a protein vital to the organism's normal physiology, these mutations may lead to a clinically abnormal phenotype. However, most of the mutations, as they occur frequently in the population, are neutral because phenotypic changes due to mutations are equally functional. The existence of two or more common and different forms of either structural, biochemical, physiologic or physical phenotypic traits in the population is termed polymorphism. The basis of these multiple phenotypic forms of a given trait is the existence of multiple alleles at the relevant gene locus. If both parents contribute identical alleles, an individual is said to be homozygous; if the alleles are different, it is called heterozygous.

In the following sections, the concepts of genetic analysis at the phenotypic level is reviewed briefly using a few selected examples with the emphasis being placed on genetically determined variation in the structure of proteins and the role of such variation in affecting monogenic and polygenic phenotypic traits and human diseases.

Phenotypic variation in the structure of proteins

The concept of one gene—one polypeptide implies that the synthesis of each polypeptide chain of a given protein is controlled by a single gene. Thus, the structure (phenotype) of a protein is directly under genetic control. In protein structural studies, the term phenotype often refers to genetically controlled different structural forms of a protein. Analysis of genetically determined phenotypic variability in protein structure became possible with the advent of various electrophoretic techniques. Early work on protein polymorphism focused mainly on enzymes in humans (Harris) and *Drosophila*, a fruit fly (Lewontin and Hubby). Using these conventional methods of electrophoresis, Harris estimated that approximately one-third of all possible amino acid substitutions in the polypeptide chain of proteins could be detected. However, the early methods were not sensitive enough to detect phenotypic differences in molecules having similar or identical molecular size and conformation but different isoelectric points (a point at which protein carries no net electrical charge).

In the early 1980s, the application of the isoelectric focusing (IEF) technique made it possible to detect minute differences of isoelectric points between various protein isoforms, thus allowing the observation of previously undetectable phenotypic variation. In addition to the charged amino acid substitutions, the IEF technique can also detect some neutral amino acid substitutions in the protein structure. Approximately 40% of mutations resulting from single-base substitution would lead to electrophoretically detectable changes in charged amino acids, while another 40–60% would involve neutral-to-neutral amino acid substitutions. In addition to the progress in separating proteins, advanced protein detection methods have also played a major role in observing protein phenotypes. The development of immunoblotting or western blotting techniques has replaced most other detection methods because of their sensitivity and versatility. Today, there are about 100 plasma proteins and red cell enzymes that show electrophoretically detectable polymorphisms in humans.

Alleles coding for protein phenotypes are expressed in a codominant fashion because both the homozygous and heterozygous forms are distinguishable and both pairs of alleles are fully expressed in heterozygotes. In such cases, the genotype can be inferred directly from the visible phenotypic patterns on the electrophoretic gels. Most of these protein polymorphisms are neutral because they do not

appear to have drastic effects on an individual's physical or biological functions.

Phenotypic expression of a single gene

In addition to the common polymorphisms not associated with any obvious phenotypic abnormalities, there are several examples of phenotypic disorders associated with genotypic changes in a single gene. These disorders are commonly referred to as single-gene or monogenic disorders. In these disorders, phenotypic variability is primarily determined at the corresponding gene locus and the effect of other loci and environmental factors, although they may be present, is not the major determinant. These single-gene phenotypes (disorders) are also known as *Mendelian* traits because they segregate in families in observation of Mendel's laws of inheritance. The inheritance and expression of these traits depend on whether the given gene is on an autosomal chromosome or on the X chromosome, and whether the expression is *dominant* or *recessive*. An allele is *dominant* when it is expressed to yield a clinical phenotype in the heterozygous state, and *recessive* when expressed only in the homozygous state. However, at the molecular level, neither of the alleles are dominant nor recessive because the gene product (protein) of both alleles is expressed in a codominant fashion. Therefore, strictly speaking, it is the phenotypic expression of alleles which is either dominant or recessive. The majority of single-gene defects involved in the manifestation of human clinical phenotypes are rare in the general population. Clinical phenotypes in these disorders result from structural and biochemical defects in proteins, such as enzymes, hormones, cell receptors, etc. By way of example, the following is a brief description of some of the common monogenic disorders.

Familial hypercholesterolemia is an autosomal dominant trait that is characterized by elevated low-density lipoprotein (LDL), cholesterol, tendon xanthomas (cholesterol-containing tumors), and premature coronary heart disease. The molecular basis of this disease is mutations in the gene coding for the LDL receptor. The LDL receptor protein, located on the surface of most body cells, plays a pivotal role in the removal of LDL cholesterol from the blood circulation. Due to mutations in the LDL receptor genes, the production and function of LDL receptors are drastically altered leading to the accumulation of LDL cholesterol in the circulation. In heterozygotes, with a frequency of about 1 in 500 in most populations, the number of receptors is reduced to almost a half. In rare homozygous cases (1:1 000 000), the number of LDL receptors is reduced drastically and

the cholesterol level can rise up to 10 times its normal level.

Other relatively common autosomal dominant disorders include Huntington disease, adult polycystic kidney disease, von Willebrand disease, and neurofibromatosis 1.

Sickle-cell anemia is a classic example of the relationship between the genotype, gene product (protein), and the phenotype of the disease. The disease is caused by a single point mutation in the DNA that codes for the β -hemoglobin chain (human hemoglobin is composed of two α chains, each containing 141 amino acids and two β -polypeptide chains, each with 146 amino acids). The sickle cell mutation results in the replacement of a normally occurring amino acid glutamic acid by valine at the sixth position in the β chain. This single amino acid substitution is the only difference in primary structure between normal and the sickle cell hemoglobins and this difference can easily be detected electrophoretically due to their different migration positions on the gel. Since sickle-cell anemia is a recessive disorder, individuals with one copy of the sickle-cell gene are phenotypically normal but are classified as carriers; while affected individuals carry both copies of the defective gene. Sickle-cell anemia is manifested by severe hemolytic anemia due to gross abnormalities in the shape and function of red blood cells. The disease is more common in Blacks, but also occurs in India and in the Mediterranean area. Among US Blacks, the carrier frequency is about 1 in 10 and the frequency of affected individuals is about 1 in 400.

An individual having abnormal hemoglobin may yet experience several other phenotypic disorders, like liver, heart, lung, brain, skeletal and gastrointestinal damage, because of the prime importance of hemoglobin in supplying oxygen to various parts of the body. At least 25 different major phenotypic disorders are known which are the direct result of abnormal expression of a single gene due to a single-point mutation leading to the replacement of one amino acid in the hemoglobin molecule. Such genes having multiple effects on several phenotypic traits are referred to as being *pleiotropic*.

Cystic fibrosis (CF) is the most common autosomal recessive disorder in populations of European ancestry with a carrier frequency of about 1 in 25 and a disease frequency of about 1 in 2500 live births. The major clinical symptoms of the CF phenotype include exocrine insufficiency of the pancreas, chronic lung disease, and elevated concentration of sweat electrolytes. The basic biochemical defect causing the CF phenotype was unknown until relatively recently when the CF gene was cloned. Analysis of the sequence of the complementary DNA (DNA synthe-

sized from a messenger RNA template) of the CF gene predicts a protein containing 1480 amino acids and termed the cystic fibrosis transmembrane conductance regulator (CFTR). Approximately 70% of the mutations in CF patients are due to the loss of a single specific codon (three nucleotides) in the CF gene on chromosome 7. Deletion of this codon results in the loss of a single amino acid, the phenylalanine at position 508 in the CFTR. As with sickle-cell disease, the change of a single amino acid in the protein structure accounts for most CF cases, indicating that the CF phenotype may not result from the complete loss of the CFTR function.

Common examples of autosomal recessive disorders include phenylketonuria, several types of glycosphingolipidoses and mucopolysaccharidoses. Similarly, several X-linked phenotypic disorders due to single-gene defects are known: common ones include glucose-6-phosphate dehydrogenase deficiency, hemophilia A, Duchenne muscular dystrophy, and color blindness.

In addition to these clinically associated phenotypes, there are large numbers of other genetically determined phenotypes whose appearance or expressions are not associated with any disorders, e.g. eye color, hair texture, attached and free ear lobes, appearance of dimples and freckles, hairs on the back of finger, and cleft chin. All these phenotypes are due to autosomal dominant inheritance, and they do not have harmful effects but account for some interindividual variation.

Phenotypic expression due to multifactorial inheritance

Most human phenotypic traits and diseases are not determined by a single-gene effect; rather they are caused by the interaction of genes and the environment, and are thus known as multifactorial conditions. The inheritance of multifactorial traits (also known as polygenic traits because of the involvement of multiple genes in determining a single trait) account for much of the normal variation (e.g. height, weight, skin color and intelligence), and many common diseases, including cardiovascular disease, diabetes mellitus, hypertension and congenital anomalies. In contrast to single-gene traits which segregate in families, familial recurrences of multifactorial traits are difficult to predict because multiple genes are involved in the expression of a phenotypic trait and the effects of genes are modulated by different environmental factors. The exact number of genes involved in determining a given multifactorial trait is unknown; but each gene contributes a small but additive effect which may combine with other

environmental factors for their final impact on the phenotype.

The genetic and environmental basis of cardiovascular disease (CVD) is one of the most extensively studied of any of the common chronic diseases in humans. There are several candidate genes whose protein products are involved in the metabolic pathways of normal cardiovascular function; and variation in these gene products may determine in individual's susceptibility or resistance to CVD. Epidemiologic studies have also identified several environmental factors putting people at risk of developing CVD, including imprudent diet, obesity and smoking. Almost half of the candidate genes are structurally polymorphic and this genetic variation can interact with the above-mentioned environmental risk factors in determining an individual's susceptibility to the disease. For example, elevated level of cholesterol is a major risk factor for CVD and about 50% of interindividual variation in cholesterol level is under genetic control. If a person inherits a group of so-called susceptible genes whose cumulative effects are to increase the cholesterol level, and if that person also consumes a highly saturated fat diet rich in cholesterol, it is very likely that they will develop the phenotype of CVD much earlier than another person who has an identical genetic profile but does not eat a saturated fat diet. In such traits as CVD, genetic impact depends on environmental exposure to determine the final phenotype.

Similarly, there are several examples of monogenic and polygenic immunological diseases which can be summarized as follows. HLA, the major histocompatibility complex, comprises multiple closely linked genes, which are highly polymorphic, and inheritance of a certain group of alleles at these loci is found to be associated with specific diseases in response to environmental changes. Some diseases associated with specific HLA alleles include type 1 insulin-dependent diabetes mellitus, ankylosing spondylitis and hemochromatosis, and several autoimmune diseases. Similarly, genetic predisposition to several immune complex diseases are associated with com-

plement component deficiencies. The mode of inheritance of most of these complement deficiencies is autosomal recessive. Some examples of other inherited immunological diseases include: ataxia telangiectasia, immunologic amnesia and severe combined immune deficiency diseases which are autosomal recessive disorders; and hypogammaglobulinemias and chronic granulomatous disease which are X-linked recessive traits.

See also: Genetic analysis at the molecular level; Immunodeficiency, primary; Insulin-dependent diabetes mellitus, human; Isoelectric focusing; MHC disease associations; Relative risk; Western blotting.

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GERM-FREE ANIMALS

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Usually, all animals are associated with complex communities of microorganisms. In these animals, it is not possible to study the role of the microflora or parts of it. Germ-free animals have been developed to study the animal without interference from the microflora and to study the effects of certain floral elements.

Although the entire body surface and all the mucous membranes harbor microorganisms, most interest has centered around the intestinal microflora. A comprehensive term encompassing all forms of known host-contaminant association is 'gnotobiotic' or, for the discipline in general, 'gnotobiology'. This word has been derived from the Greek *gnotos* (known) and *biota* (all living vegetable and animal organisms). Gnotobiotic animals are derived by aseptic cesarean section or sterile hatching of eggs, which are reared and continuously maintained by germ-free techniques under isolator conditions and in which the composition of any associated fauna and flora is fully defined by accepted current methodology. Germ-free animals are gnotobiotic animals free from all demonstrable associated forms of life, including bacteria, viruses, fungi, protozoa, and other saprophytic or parasitic forms. If germ-free animals are associated with defined microorganisms, then these gnotobionts are termed monoassociated, diassociated, etc., depending on the number of microbial strains given.

The origin of the germ-free concept goes back to 1885, when Pasteur stated that life would probably become impossible if animals were deprived of their normal flora. A supporter of Pasteur's ideas at the turn of the century was Schottelius, who contributed much to the methods of rearing germ-free chickens. However, his animals were fed inadequate diets and were therefore unsuitable to study the effects of the missing flora. In 1886, Nencki was the first to attack Pasteur on theoretical grounds. About 10 years later, Nuttal and Thierfelder were the first to produce germ-free mammals. Based on the knowledge gained by these early workers, steel units for the maintenance of germ-free rodents were developed in 1946 by Reyniers and colleagues and plastic isolators by Trexler and Reynolds in 1957. Both types of housing methods are still in use on a large scale today.

Characteristics of germ-free animals

Germ-free animals not only differ from conventional animals by their absence of bacterial flora, but also by their lack of systemic responses to that flora. On autopsy of germ-free animals, most details appear to be normal. However, when the relative weights of freshly excised organs are compared, certain characteristics become apparent. In various animal species, parts of the digestive tract, notably the small intestine, its associated lymph nodes and in some instances the liver, show a lower weight in the germ-free group. Other organs, such as the heart and the brain, show little or no difference between the germ-free and normal stock. Thus, in terms of weight, the organs of the animals fall into two distinct groups: one harbors an abundant flora in normal life and shows reduced weight in the germ-free animals; the other group of organs that is normally remote from bacteria shows practically no difference between the germ-free and normal stock. A striking observation in germ-free mammals is the enlarged cecum (4–6-fold increase in weight). This cecal enlargement does not appear to impair the animal's health particularly, but indirectly the extended cecum can give rise to intestinal volvuli affecting the junction of the ileum and colon. The exact cause of the extended cecum is unknown.

Studies of basal metabolism and blood circulation in germ-free animals show reductions in these measurements of function. Compared with conventional animals, the following reductions were measured in germ-free rats: basal metabolic rate, 20%; cardiac output, 32%; blood volume, 22%; arterial blood flow to liver, 50%.

Applications of germ-free animals

Surgical application

To avoid the risk of postoperative wound infection, surgical procedures are carried out in germ-free animals in germ-free isolators. Especially in the field of intestinal surgery, germ-free animals are frequently used to study aspects of wound healing.

Oncology

Some spontaneous murine hematopoietic neoplasms are affected by the absence of a conventional

microbial environment, whereas nonhematopoietic tumors are not. Conflicting data were obtained following the introduction of chemical carcinogens in germ-free and conventional mice. The effects of the natural microflora are unclear. Germ-free animals provide an important tool of research in the fight against cancer.

Dental research

The use of germ-free animals and the principles of gnotobiology have provided much information concerning the origin of caries and periodontal disease. The flora in these animal models can be modified, giving information about the cause of both diseases. Also, treatment and prevention can be studied with the help of gnotobiotic animal models.

Microbiology

Studies with germ-free animals are carried out on a large scale to investigate the complex microecological relationships between microbes themselves and between the microbes and their hosts. One of the most important functions of the intestinal microflora for the host is the phenomenon of colonization resistance, being the force withstanding oral infections. Only the presence of many hundreds of different biotypes of microorganisms in an intestinal tract provides a 'normal' colonization resistance.

Toxicology

In an intestinal tract of a 'normal' animal, the bacterial cells outnumber the cells of the whole body. This means that an enormous metabolic capacity is present in the intestinal tract. Detoxification of a broad variety of toxic products occurs by the normal microflora. With the help of gnotobiotic animals this phenomenon can be investigated in relation to the quality of the microflora.

Nutrition

From the outset of using germ-free animals, nutrition was of utmost importance, due to the sterilization of food necessary to rear germ-free animals. The microchemical environment in the intestine is determined by three factors: chemicals in the diet, chemicals of host origin and chemicals derived by the intestinal microbial flora. By means of gnotobiotic technology, it is possible to change the factors due to diet and microflora to assess the potential impact for the microchemical environment in the intestine.

Immunology

Germ-free animals have provided us with a unique model for studying a true primary response. A reaction to an antigen to which the animal has not previously been exposed is generally considered to be a primary response. During a normal lifespan, however, animals encounter a large number of antigens in the environment that might cross-react with antigens used in investigative work. Germ-free animals can be used as a model to study the question of whether *in vitro* immune responses were true primary or early secondary responses.

See also: Immune response.

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GERMINAL CENTER

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In secondary lymphoid organs mature B lymphocytes tend to aggregate in nodular structures, the lymphoid follicles, that are located in well-defined areas such as the outer cortex of lymph nodes, the marginal zone of the spleen and the Peyer's patches, and play a central role in the secondary immune response to T cell-dependent antigens (Ags). Two different types of follicles exist. Primary follicles are spherical collections of small resting B lymphocytes that cluster around specialized Ag-presenting cells (follicular dendritic cells, FDCs) and are found in germ-free animals as well as in human fetal lymph nodes from the 16th gestational week onward. After Ag challenge, i.e. soon after birth, primary follicles evolve into secondary follicles through the progressive generation of a mitotically hyperactive population of B cells which form an area called the germinal center (GC) that is surrounded by a mantle or corona of small resting B lymphocytes. The GC provides the unique microenvironment of cells, adhesion structures and cytokines that ensure the proper balance between activation/proliferation and apoptotic death of newly generated Ag-specific B lymphocytes and lead to an antibody (Ab) response of optimal affinity.

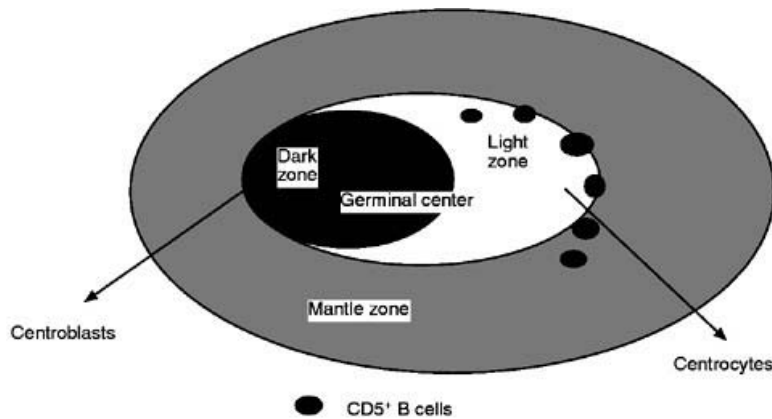
Organization and cellular composition

GCs are placed eccentrically within secondary follicles and have a zonal structure (Figure 1). The upper pole of a GC possesses a prominent crescent-like cap of B mantle lymphocytes: this area is defined as the light zone because of the weak basophilia of the cells. At the opposite (lower or basal) pole, the B mantle may be reduced to a tiny rim of a few cells

encompassing an area of highly basophilic cells: the dark zone. The cellular organization of a GC is very composite: B, T and accessory cells have a specific distribution in the different zones of the GC.

Different populations of B lymphocytes form the major cellular component of a GC. According to the level of activation and differentiation, GC B cells have specific morphological, phenotypical and molecular features and occupy different areas, but, in spite of these differences, have two common features. First, they are unable to bind to postcapillary high endothelial venules (HEVs) and lack the normal migratory mechanism that most small lymphocytes possess. Second, they strongly express the adhesion molecules responsible for homotypic adhesion of lymphoid cells, CD11a/CD18 and their natural ligand ICAM 1 (CD54). These properties suggest that GC B cells behave as sessile elements and that Ag-recruited B lymphocytes become transiently segregated into lymphoid follicles in order to properly respond to the stimuli.

The dark zone is made up by centroblasts, a highly proliferating pool of large to medium-sized immature-looking cells with nucleoli and a sparse, intensely basophilic cytoplasm. Centroblasts are surface (s) immunoglobulin (Ig)D⁺, sIgM weakly positive and express major histocompatibility complex (MHC) class II molecules, CD19 and low amounts of CD10 and CD38. The light zone is chiefly made up by centrocytes, B lymphocytes of a more mature appearance with indented (cleaved) nuclei and a pale cytoplasm. Centrocytes are frequently IgG⁺ or IgA⁺, CD10⁺, CD38⁺. Further, a few immunoblasts and rare plasma cells are also observed within the light



Centroblasts

CD10⁺
CD38⁺
CD39[−]
CD77⁺
CD44[−]
IgM⁺, IgD[−]

Centrocytes

CD10⁺
CD38⁺
CD39[−]
CD77[−]
CD44[−]
IgG⁺, IgA⁺

Mantle zone

CD10[−]
CD38[−]
CD21⁺
CD23⁺
CD39⁺
CD77[−]
CD44⁺
IgM⁺, IgD⁺

Figure. 1 Lymphoid follicles: main architectural features and phenotypic properties of the different B cell populations involved.

zone. Finally, at the edge between the GC and the follicular mantle, cells coexpressing sIgM and the CD5 antigen (CD5⁺ B cells) are seen.

FDCs are a unique population of accessory cells found exclusively in follicles where they represent a numerically minor cell population (about 2%) that, nevertheless, plays a crucial role in the development of the GC, as follicles devoid of FDCs are never observed. Also, the nodular dissemination of malignant lymphocytes in GC malignancies is characterized by the clustering of monoclonal B lymphocytes around FDCs. FDCs form a dense meshwork of cytoplasmic processes around lymphoid cells which is most prominent in the light zone but extends its branches also amidst B lymphocytes in the corona. FDCs are unable to phagocytose and use their Fc and C3b receptors to fix and localize Ag-Ab complexes on their surface. These complexes provide a long-standing stimulation of GC B lymphocytes as Ag can be held on the FDC surface for more than a year.

Tingible body macrophages are large macrophages that owe their name to their ability to actively phagocytose apoptotic lymphoid cells (the 'tingible bodies' observed in their cytoplasm are apoptotic bodies) and, scattered among proliferating centroblasts, give the typical starry sky appearance to developing GCs. T cells represent about 5% of the GC cellular composition. Although infrequent, they appear to play a crucial role in the generation of the GC, as GCs are

virtually absent in congenitally athymic mice; this deficiency can be reversed by the transfer of T cells. T cells are very rare in the dark zone; they are more abundant in the light zone and are Ag specific. Nearly all GC T cells have the CD4⁺ helper phenotype, but exhibit some unusual features, such as the expression of CD57 and the inability to produce interleukin 2 (IL-2).

Function

GCs have a fivefold function that is based upon a coordinated process of proliferation/maturation/selection. First, they ensure the Ag-specific oligoclonal proliferation of B cells in the T cell-dependent secondary immune response. Although unexplained, an Ag-unrelated B cell proliferation also takes place in the GC, which might contribute to the overall amplification of the B cell pool. Second, GCs are the site of somatic hypermutations of immunoglobulin (Ig) V-region sequences that are required for the affinity maturation of Abs. It is still debated whether the T cell receptor may also occasionally mutate in GCs. Third, GCs are the site at which the isotype switch from IgM to IgG or IgA occurs. It is widely held that the high degree of apoptotic death observed within the GC, and witnessed by the starry sky histology, reflects the mortality of the cells with inadequacies in the switch and somatic hypermutation processes of Ig genes. Fourth, the GC microenviron-

ment generates B memory lymphocytes which can be recruited for a further round of proliferation/differentiation/selection by a forthcoming Ag challenge, and plasma cell precursors (plasmablasts). The precursors of plasma cells undergo their terminal differentiation into Ab-secreting cells at sites distant from the GC (medullary cords in lymph nodes, marginal zone in the spleen, lamina propria in the gut, bone marrow). This may be a security mechanism to prevent the developing immune response being prematurely shut off by the local buffering activity of high concentrations of Ab in direct contact with the triggering Ag. Finally, the GC microenvironment selects mature B cells by censoring (i.e. leading to the apoptotic death of) autoreactive cells.

Development

The primary response to T cell-dependent Ag occurs in the T cell areas of secondary lymphoid organs and generates Ag-specific B cells which migrate into follicles and give rise to GCs. Newly formed GCs are oligoclonal, as, on average, each mature GC is derived from the colonization of no more than 1–3 B cell blasts. Ag-specific immigrant B cells gather around the Ag-holding cytoplasmic protrusions of FDCs and are interspersed with Ag-specific T lymphocytes. Cycling B cells in the dark areas of the GC (centroblasts) undergo somatic mutations of the V_H and V_L genes that generate Ab of optimal affinity. Point mutations accumulate in the V_H and V_L genes independently of the V region utilized or of self specificity and are preferentially localized in the complementarity-determining region (CDR)-encoding segments. Centroblasts transform into noncycling centrocytes that migrate towards the light zone of the GC, switch their Ig phenotype under the control of cytokines and are eliminated by apoptosis, unless further stimulated by additional signals. The specific Ag interaction with surface Ig is the main signal that prevents apoptosis as it ensures the specificity of the selection process. Additional signals delivered through the interaction of B cell CD40 with its ligand (CD40L) also prevent apoptosis as assessed by *in vitro* tests and *in vivo* observations on patients with CD40L deficiency (X-linked hyper-IgM syndrome) or on CD40L knockout mice. CD40L is expressed primarily by T cells, again reaffirming the T cell dependence of GC formation. Other less well defined antiapoptotic signals are delivered by other cells (FDCs, macrophages or B cells themselves) through CD40–CD40L interactions (FDCs express CD40L) and the stimulation of CD20, CD21 and CD38 molecules. All these stimuli prevent apoptosis by inducing the expression of Bcl-2 protein, a potent

antidote to apoptosis. The majority of cells within the GC fail to express Bcl-2: proliferating centroblasts are Bcl-2 negative and only the centrocytes that have survived the whole molecular screening process of the GC become Bcl-2 positive and acquire the ability to survive. The GC-based screening program includes the prevention of autoimmune reactivity by inducing the apoptosis of self reacting B cells. Some censoring mechanisms that prevent the development of antiself reactions during the process of B cell selection in the GC have been partially elucidated. First, the exclusion of self Ag-binding B cells from the normal migration route into lymphoid follicles. Second, the exposure of self Ag-specific B cells to excess soluble Ag. Also, centrocytes exposed to CD40L upregulate the expression of Fas/CD95, a member of the tumor necrosis factor (TNF)-receptor (R) superfamily that transduces apoptotic signals to B cells upon interaction with activated T cells expressing CD95L. Thus, additional pathways to eliminate unwanted B cells may be controlled by T cells by means of the CD95/CD95L system.

Cytokines and the GC

A number of cytokines are involved in the generation of GCs: interferon γ (IFN γ) and IL-2 impede while IL-4 and IL-6 favor GC formation. IL-10 prevents the spontaneous death of GC B cells by induction of Bcl-2 protein. Further, it has recently been shown that GCs fail to develop in lymphotoxin- α or type I TNF-R-deficient mice. Likewise, the stimuli delivered by cytokines that induce the isotype switches have been partially elucidated. Activated B cells switch from IgM to IgG1 or IgG3 in the presence of IL-10, and to IgA1 or IgA2 in the presence of transforming growth factor β (TGF β). IL-4, especially in conjunction with IL-13, induces IgE and IgG4 production.

The fate of the GC

GCs are fluctuating structures which acquire their fully fledged zonal aspect through different steps. In the experimental animal, the first step is the appearance, 3–4 days after Ag injection, of foci of actively proliferating centroblasts within primary follicles. After a further 24 hours, tingible body macrophages become apparent and give the typical starry sky histological picture. This phase continues over a period of 1–3 weeks and is characterized by the striking proliferative activity of centroblasts that divide every 6–7 hours. About 50% of centroblasts are in the S phase of the cell cycle 30 minutes after a single injection of [3 H]thymidine. Mutations are introduced in a stepwise manner throughout this proliferative period

and this phase is followed by the progressive transformation of centroblasts into centrocytes that are selected on the basis of their ability to interact with the Ag held on the surface of FDCs. Some centrocytes may re-enter the centroblast pool and undergo another round of proliferation (and mutation), thereby explaining the rapidity of the occurrence of a high-affinity secondary Ab response. Little or no trafficking exists between GCs: therefore, clonal evolution proceeds independently within each GC, which autonomously provides the optimal microenvironment to the selected clones. Centrocytes either give rise to B memory cells or to plasma cell precursors (plasmablasts), which then migrate to other areas. The choice between differentiation into plasma cell precursors or memory cells is a finely tuned process controlled by different genes and cytokines whose molecular details are still largely undefined.

With time, GCs may either be potentiated by the influx of new antigenic waves, or disappear or develop into the so-called 'regressively transformed GCs' or 'progressively transformed GCs'. The regressively transformed GCs are observed after corticosteroid therapy when no lymphoid cells, but only hyalinized PAS⁺ (periodic acid-Schiff reaction¹) material can be seen within GCs. The progressively transformed GCs are follicles observed in nonspecific lymphadenitis when the border between the GC and B mantle cells becomes first indeterminate and finally totally lost, with the formation of nodules containing abundant numbers of lymphocytes.

See also: Adhesion molecules; Antigen presentation via MHC class II molecules; Apoptosis; B lymphocyte activation; B lymphocyte differentiation; CD antigens; Cytokines; Immune complexes; Immunoglobulin class switching; Leukemia; Lymph nodes; Lymphoma; Somatic mutation.

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GLUCOCORTICOIDS

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The adrenal cortex produces two types of steroid hormones that are essential for life: glucocorticoids and mineralocorticoids. The outer zone of the cortex, the zona glomerulosa, produces the powerful mineralocorticoid aldosterone. The inner zones, the fasciculata and reticularis, produce the glucocorticoids and various androgens. Glucocorticoids act on almost all cells in the body, influencing a host of physiological processes including glucose metabolism, immune and inflammatory reactions, blood pressure regulation, and mental functions. They are essential at basal levels for maintenance of many homeostatic systems. Furthermore they are stress hormones, being secreted at higher than basal rates in response to stress and protecting the organism from stress.

Clinically, glucocorticoids are almost unique among hormones in that they are used for the treatment of a multitude of conditions apparently unrelated to hormone deficiency. Among these conditions are rheumatoid arthritis, asthma, allergy, autoimmune diseases, lymphocytic leukemias and lymphomas. Therapeutic doses used in such cases are often large enough to override normal physiological controls and functions of the glucocorticoids, producing unwanted side-effects.

Historical and physiological background

Thomas Addison reported in the mid-1800s on a series of patients who had succumbed to a disease – later named after him – associated with degeneration of the adrenal glands. Foreshadowing a relation between the adrenal cortex and the immune system, he noted that the blood of one patient ‘had a considerable excess of white corpuscles’. By the early 1900s patients with Addison’s disease, as well as adrenalectomized animals, were known to suffer from low blood glucose and salt loss. These effects were traced in the 1930s to two distinct hormones from the adrenal cortex, each essential for life: glucocorticoids, which influenced blood glucose; and mineralocorticoids, which regulated salt balance. The vital role of mineralocorticoids was well explained by their effects on salt balance, but that of glucocorticoids could not be accounted for by effects on glucose, and remained obscure for many years. By 1940 it was known that glucocorticoids exert numerous other actions, in particular on lymphoid

tissue: Addisonian patients and adrenalectomized rats had enlarged thymuses, and glucocorticoids caused atrophy of the thymus and other lymphoid organs.

Harvey Cushing in 1932 described a disease later ascribed to excessive secretion of glucocorticoids. Symptoms such as glucose intolerance, fat redistribution and heightened susceptibility to infection which accompany Cushing’s syndrome are nowadays most often encountered as undesirable side-effects of glucocorticoid therapy.

Around 1930 it became clear that there is an intimate relationship between glucocorticoids and stress. Stress from almost any source – cold, injury, infection, fright, and other challenges to homeostasis – stimulated glucocorticoid secretion. The resulting high levels of glucocorticoids protected against stress and sustained life, and for a long time were thought to somehow enhance the activity of normal defense mechanisms. A separate protective role for glucocorticoids emerged later from observations that adrenalectomized animals, which died if stressed, could survive moderate – though not severe – stress if maintained on basal levels of glucocorticoids. Such basal levels exert ‘permissive’ effects essential to maintain a wide range of homeostatic mechanisms, including blood pressure regulation and certain immune reactions.

The principal natural glucocorticoids are cortisol and corticosterone (**Figure 1**), the former predominating in humans and the latter in rodents. For many years cortisone, which was isolated in the 1940s, was thought to be the principal glucocorticoid, but it is now known to require conversion to cortisol for biological activity by reduction of the ketone group at carbon 11 to a hydroxyl group (**Figure 1**).

Until 1949 the main clinical use of glucocorticoids was for replacement therapy of patients with hormone deficiency. That year the biomedical world was electrified by the totally unexpected discovery that large doses of cortisone exerted powerful anti-inflammatory activity, dramatically alleviating the symptoms of rheumatoid arthritis. This development led to a spectacular rise in applications of glucocorticoids – the ‘miracle drugs’ of the 1950s – and set the stage for much of their current clinical use. Innumerable derivatives of the glucocorticoids were syntheses-

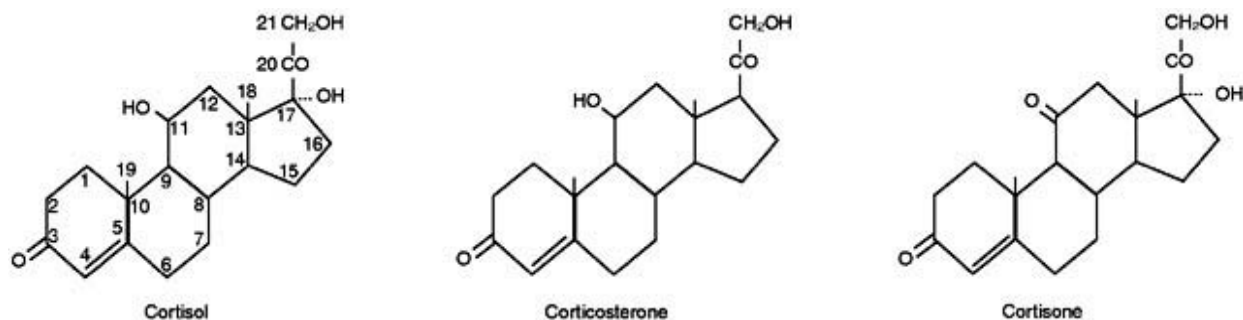


Figure 1 Structures of the natural glucocorticoids, cortisol and corticosterone, and of cortisone, an inactive metabolite derived from cortisol by oxidation of the 11-hydroxyl group.

ized in pursuit of compounds with reduced salt retention and other unwanted side-effects, a quest that yielded prednisolone, dexamethasone and other important steroids in current use (Figure 2).

The discovery of the anti-inflammatory effects also caused turmoil among adrenal physiologists, who were unable to reconcile the suppressive effects on inflammation with their assumption that stress-induced glucocorticoids stimulated defense mechanisms. Consequently they stigmatized the anti-inflammatory effects as pharmacological artifacts without physiological significance, leaving the major therapeutic applications of the glucocorticoids with no apparent physiological foundation. That paradoxical situation prevailed for over three decades. In recent years an alternative view of the protective role of glucocorticoids in stress that reconciles glucocorticoid physiology with therapy has gained strong experimental support. It proposes that in many circumstances high, stress-induced levels of glucocorticoids actually protect the organism from its own stress-activated defense mechanisms, suppressing those mechanisms to prevent them from overshooting and causing damage. This view places immunosuppressive, anti-inflammatory and other such actions at the center of glucocorticoid physiology.

Glucocorticoids can therefore be viewed as sustaining life through two different but related mechanisms: they 'permissively' activate some homeostatic defense mechanisms and, generally at higher levels, they prevent such mechanisms from overshooting. In the course of normal diurnal variation they probably exert both these influences to varying degrees; under stress, the second may predominate. Figure 3 illustrates qualitatively how activity of a defense mechanism might be influenced at various cortisol concentrations. Levels of free cortisol in humans vary diurnally over the lower range of the bell-shaped curve, rising in the morning to roughly the concentration for peak activity. Stress-induced levels can go well into the suppressive range.

Regulation of glucocorticoid secretion via the hypothalamo-pituitary-adrenal (HPA) axis began to be understood in the 1930s. ACTH (adrenocorticotrophic hormone) was identified as the anterior pituitary peptide that stimulates glucocorticoid secretion as well as growth of the inner zones of the adrenal cortex. Its secretion was eventually shown to be stimulated by the hypothalamic hormone CRH (corticotropin-releasing hormone) in conjunction with VP (vasopressin, also a hypothalamic hormone). Glucocorticoids in turn exert negative feedback on production of CRH, VP and ACTH, thus



Figure 2 Structures of the synthetic glucocorticoid analogs prednisolone and dexamethasone, and of the glucocorticoid (and progesterone) antagonist RU486.

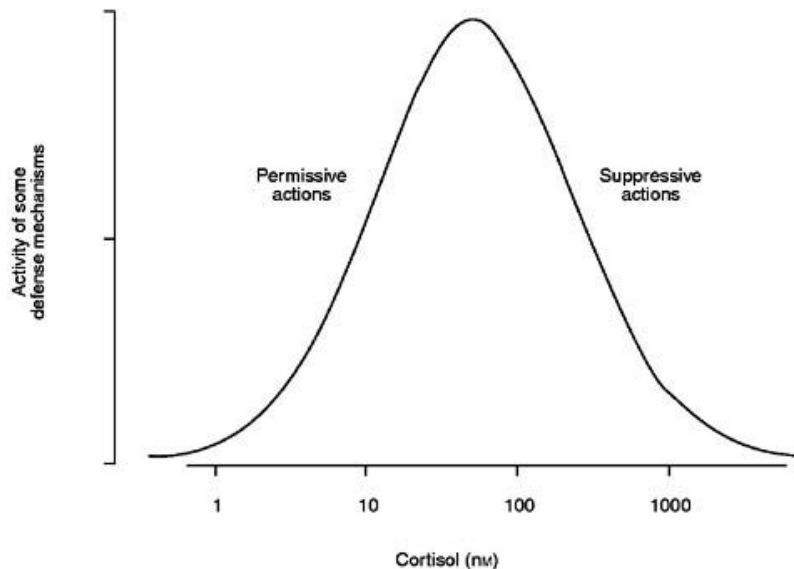


Figure 3 Bell-shaped curve that illustrates schematically how the capacity of certain homeostatic defense mechanisms to respond to challenges is permissively enhanced by basal levels of glucocorticoids and suppressed by stress-induced levels.

regulating their own secretion. Stress signals override negative feedback control, and via the hypothalamus stimulate the HPA axis to produce stress-induced levels of glucocorticoids. A consequence of negative feedback regulation is that glucocorticoid therapy can cause atrophy of the HPA axis, leaving a patient without the capacity to respond to stress. Some of these relationships are shown in Figure 4, where some glucocorticoid effects on peripheral tissues are depicted as primary, being exerted directly through glucocorticoid receptors in the cells that are affected, and others as secondary to modulation – usually inhibition – of production or activity of cytokines,

inflammatory agents and other mediators. Recent studies strongly suggest that certain cytokines, such as interleukin 1 (IL-1), which are produced during immune responses, act on the hypothalamus to stimulate secretion of glucocorticoids. Glucocorticoids, in turn inhibit cytokines, forming a negative feedback loop that can regulate immune reactions.

Glucocorticoid receptors and molecular mechanisms

Glucocorticoid receptors were first identified in the 1960s in thymus cells. They have since been found

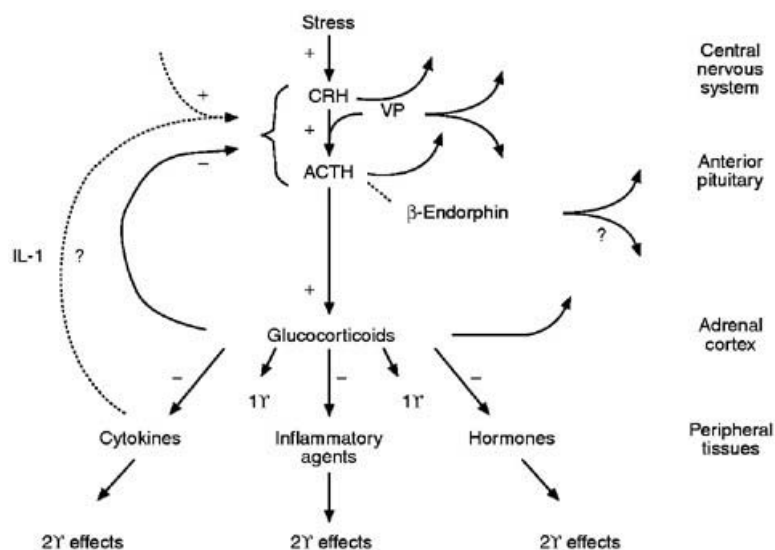


Figure 4 Feedback regulation of glucocorticoid secretion. See text for details.

in almost all cells, supporting the conclusion that almost all cells are targets for glucocorticoids. Like other members of the steroid and thyroid hormone receptor family, the type I (mineralocorticoid) and type II (glucocorticoid) adrenal steroid receptors are ligand-dependent transcription factors which act in the nucleus. Glucocorticoid receptors usually number from 1000 to 20 000 per cell. Before they are bound by hormone, the nonliganded glucocorticoid receptors (i.e. receptors without bound hormone) are seen mainly in the cytoplasm as large oligomers with HSP90 (90 kDa heat shock protein) and other heat shock proteins. When a glucocorticoid binds to its receptor, it forms a nonactivated hormone–receptor complex. Within seconds or minutes, the oligomer releases the heat shock proteins and is transformed to an activated hormone–receptor complex with high affinity for receptor-specific DNA-binding sites (hormone response elements or HREs) associated with hormone-regulated genes. Binding of glucocorticoid–receptor complexes as dimers to HREs alters transcription rates and levels of the mRNAs encoded by the glucocorticoid-responsive genes, eventually altering levels of the proteins that produce the hormone effects. This general scheme is illustrated in Figure 5.

A number of molecular mechanisms by which glucocorticoid receptors can modulate gene expression without necessarily binding to HREs have recently

been identified, and may underlie many immunosuppressive and anti-inflammatory actions. These include 1) blocking the transcription factor AP-1 from binding to its target genes, 2) binding to the transcription factor NF κ B, preventing it from binding to DNA, 3) increasing synthesis of I κ B α , an inhibitor which holds NF κ B in an inactive form in the cytoplasm, and 4) negatively regulating mRNA stability (e.g. for IL-1 and IL-6) by as yet unknown mechanisms.

In a surprising recent development, natural glucocorticoids have been shown to bind to type I receptors almost as strongly as aldosterone, the principal mineralocorticoid. Their affinity for mineralocorticoid receptors is, in fact, much higher than for glucocorticoid receptors. Since glucocorticoids are usually present in the body far in excess of aldosterone, the question arises as to how mineralocorticoid receptors can respond specifically to aldosterone. It turns out that mineralocorticoid target cells contain a unique enzyme, 11 β -hydroxysteroid dehydrogenase, which ‘protects’ mineralocorticoid receptors by rapidly inactivating glucocorticoids, converting cortisol, for example, to cortisone. The full physiologic implications of glucocorticoids binding to type I receptors is still being explored, but already it seems likely that certain actions of glucocorticoids in the brain are mediated through ‘unprotected’ mineralocorticoid receptors. What role

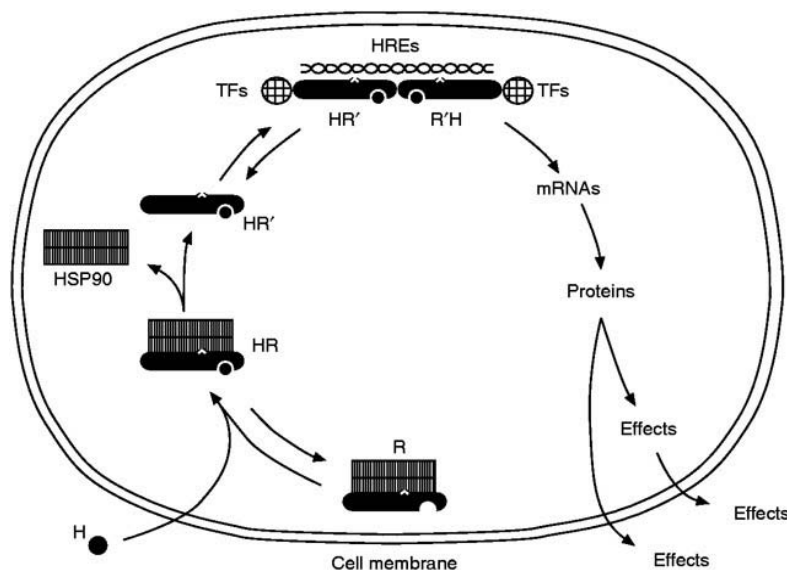


Figure 5 Receptor-mediated genomic mechanisms of action of steroid hormones. The hormone, H, is assumed to diffuse freely into the cell, where it binds reversibly to the nonliganded receptor R to form a nonactivated hormone–receptor complex HR. HR is rapidly activated, with dissociation of two HSP90 (90 kDa heat shock protein) subunits and other heat shock proteins, to give the activated complex HR' that can bind to nuclear structures to form nuclear complexes HR'n. Some of the HR'n associate with TFs (transcription factors such as AP-1 or NF κ B) and bind as dimers to hormone response elements (HREs) in the promoter regions of hormone-regulated genes. Stimulation or inhibition of the rate of transcription of those genes alters the levels of the mRNAs and proteins for which they encode. The proteins produce the hormone effects.

mineralocorticoid receptors may play in glucocorticoid actions on the immune system remains to be elucidated.

Anti-inflammatory and immunosuppressive effects

Suppression of inflammatory and immune reactions and treatment of cancers of the lymphoid system are among the major applications of glucocorticoids. As noted above, despite early assumptions that such 'pharmacological' effects had no physiological basis, it is now clear that they are as physiological as are the effects on glucose metabolism. For example, induction of adrenal insufficiency in experimental animals (by adrenalectomy or administration of the glucocorticoid antagonist RU486) enhances inflammatory and immune reactions, showing that these reactions are physiologically regulated by endogenous glucocorticoids. Studies by Sternberg and coworkers also strongly suggest that dysregulation of the HPA axis can result in autoimmunity.

Anti-inflammatory and immunosuppressive actions of glucocorticoids are now known to be closely related, affecting nearly all cells that participate in immunity and inflammation and sharing fundamental cellular and molecular mechanisms that are under glucocorticoid influence. Glucocorticoids dramatically alter the distribution of leukocytes, markedly decreasing blood counts of lymphocytes, monocytes, eosinophils and basophils within 1–3 h of glucocorticoid administration. Recent work has shown that changes in blood leukocyte distribution result from glucocorticoid interaction with the type II adrenal steroid receptor. While neutrophil counts rise in the blood, glucocorticoids decrease the accumulation of leukocytes at inflammatory sites. Glucocorticoids also kill lymphocytes, largely by the initiation of apoptosis, a striking effect that is applied in the treatment of lymphocytic leukemias and lymphomas.

Numerous reactions that are fundamental to immunity – antigen-induced T and B cell proliferation, early B cell differentiation, antigen presentation, NK activity, differentiation and activation of macrophages – are suppressed by glucocorticoids. Although some of these are probably direct effects on the target cell being measured, others certainly involve indirect effects resulting from glucocorticoid regulation of intermediary molecules. In the past 20 years, the ability of glucocorticoids to inhibit the production of various inflammatory enzymes and cytokines, by mechanisms such as those outlined above, began to be recognized. **Table 1** contains a partial list of mediators which are obligatory components of immune and inflammatory reactions

Table 1 Glucocorticoid regulation of mediators involved in immunity and inflammation

<i>Increased mRNA and/or protein</i>
Annexin I (lipocortin-1)
β_2 -Adrenergic receptor
Macrophage inhibitory factor
<i>Decreased mRNA and/or protein</i>
Interleukins 1, 2, 3, 4, 5, 6, 8, 11, 12, 13 (immune cytokines)
TNF α , GM-CSF, RANTES, MIP-1 α , SCF (inflammatory cytokines/chemokines)
iNOS (inducible nitric oxide synthetase)
COX-2 (inducible cyclooxygenase)
cPLA $_2$ (inducible phospholipase)
Endothelin-1 (bronchoconstrictor)
ICAM-1, VCAM-1 (adhesion molecules)
Collagenase, elastase, plasminogen activator (proteases)
Bradykinin, serotonin, histamine (inflammatory mediators)

and whose production is inhibited by glucocorticoids. Glucocorticoids also inhibit some effects of these mediators, such as responses of lymphocytes to IL-2 and of eosinophils to IL-3, IL-5, GM-CSF and IFN γ . By blocking communication via cytokines, glucocorticoids cut off signals required for clonal expansion and differentiation of antigen-specific T and B cells, as well as signals which activate effector functions that participate in inflammatory reactions. It seems likely, therefore, that a principal mechanism through which glucocorticoids control immune and inflammatory reactions is by suppressing cytokines and other mediators.

The complexity of the interplay between glucocorticoids and inflammatory mediators is evidenced by the increased expression of certain cytokine receptors in response to glucocorticoids, and probably by altered expression of glucocorticoid receptors induced by some cytokines. How might such effects fit with the models presented in **Figures 3** and **4**? Basal levels of glucocorticoid, and possibly the surge which occurs early in an immune response, may initially enhance the actions of cytokines by increasing expression of cytokine receptors. As cytokine concentrations rise, they act on the HPA to stimulate even higher levels of glucocorticoids which restrain further cytokine production unless very strong inflammatory signals persist. Normally, elimination of the antigen and resolution of the inflammation allows cytokine production to cease so that both cytokines and glucocorticoids return to basal levels. However, sustained inflammation with continued high levels of cytokines can persist in spite of high levels of glucocorticoid. This outcome might result in part from cytokine-induced reduction in glucocorticoid receptor expression, a mechanism postulated by some to contribute to steroid-resistant asthma.

See also: **Anti-inflammatory (nonsteroidal) drugs; Arachidonic acid and the leukotrienes; Immunosuppression; Annexins (lipocortins).**

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GLYCOSYLATION OF IMMUNE SYSTEM MOLECULES

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The secreted and membrane-associated polypeptides of higher organisms are subject to a variety of post-translational modifications involving carbohydrate moieties. Of these, the most common is *N*-glycosylation, in which an oligosaccharide is attached to the Asn residue of an Asn-X-Ser(Thr) by an *N*-glycosidic bond, and *O*-glycosylation in which an oligosaccharide is attached to a serine or threonine residue through an *O*-glycosidic bond. Both *N*- and *O*-linked oligosaccharides are branched, structurally complex molecules possessing secondary and tertiary structure. An individual *N*-glycosylation site generally does not contain a single oligosaccharide structure, but rather a *conserved population* of oligosaccharide structures. This phenomenon is usually referred to as microheterogeneity, and, since most glycoproteins have several *N*-glycosylation sites, it means that a polypeptide is diversified by glycosylation into a set of *glycoforms*, which differ with respect to the structure and location of oligosaccharides on a common amino acid sequence. That is, a

glycoprotein really exists as a set of discrete glycoforms, and it is this set of glycoforms which at any developmental stage constitutes the final expression of that polypeptide by the cellular population. This structural diversification in some cases at least (e.g. immunoglobulin G (IgG)) correlates with functional diversification, and the activity of a glycoprotein may really be a weighted average of the activity of each set of glycoforms. Further, glycosylation is cell type specific. A single polypeptide expressed in two different cell types (or tissues) of the same species is *N*-glycosylated differently. That is, cells have a unique *glycotype*. The final glycosylation of a polypeptide reflects numerous intracellular events, and changes in glycosylation are proving to be an increasingly valuable probe for cellular function and dysfunction.

The structural and functional consequences of polypeptide glycosylation in general are only slowly being elucidated, largely due to the difficulties in performing accurate structural analysis on the population of complex, branched oligosaccharides as-

sociated with a polypeptide. Nevertheless, it is emerging that oligosaccharides, being intrinsic and inseparable components of glycoproteins, modulate to a greater or lesser extent almost every aspect of glycoprotein function. For example, physicochemical properties, immunogenicity, and antigenicity, as well as individual biological functions, are all influenced by oligosaccharide.

Many of the molecules involved in the initiation, execution, and control of immunological processes are glycosylated, some very heavily. For example, most members of the immunoglobulin superfamily carry *N*-linked oligosaccharides as do many cytokines and most leukocyte cell surface proteins. IgG has been studied the most extensively with respect to the structure and function of oligosaccharides attached to molecules involved in immune responses and will therefore form the focus of this article.

Glycosylation of IgG

All normal serum immunoglobulins carry *N*-linked oligosaccharides. This is true for all classes and subclasses in every species studied to date. These *N*-linked oligosaccharides are located at conserved *N*-glycosylation sites on the Fc (the precise position depending on class), and at nonconserved sites on the Fab. In addition, immunoglobulins from certain species carry *O*-linked oligosaccharides. For example, rabbit IgG carries one or two (depending on allotype) *O*-linked oligosaccharides in the hinge region, but human serum IgG invariably lacks *O*-linked oligosaccharides. In the case of IgG, particularly detailed structural analysis of glycosylation has been performed.

Human serum IgG carries, on average, 2.8 *N*-linked oligosaccharides. Of these, 2.0 are invariably located in the C_H2 domain at the conserved *N*-glycosylation site of Asn297 (Figure 1). The additional oligosaccharides are found attached to the hypervariable regions of the Fab, and their frequency, location and incidence appears to depend predominantly on the generation of an Asn-X-Ser/Thr *N*-glycosylation sequon during formation of the immunoglobulin repertoire. Isolation, fractionation and structural analysis on the oligosaccharides of IgG, and its Fab and Fc fragments, has shown that 31 different complex-type oligosaccharides are associated with total human serum IgG. These structures are nonrandomly distributed between Fab and Fc. Those oligosaccharides on the Fab are characterized by a high incidence of sialylated structures, and of 'cores' carrying a 'bisecting' GlcNAc (Figure 1). The Fc oligosaccharides are characterized by a low incidence of sialylated oligosaccharides and of 'bisecting' GlcNAc

in the cores, and by the absence of galactose on the α 1-3 arm of at least one oligosaccharide chain in the Fc. The large number of different oligosaccharides associated with IgG is not principally due to the analysis of a polyclonal population, since monoclonal antibodies in general show a heterogeneity of glycosylation similar to that found in the total serum IgG of the corresponding species. Further, the pattern of *N*-glycosylation of serum IgG is broadly similar between different species. In general, the oligosaccharide structures on any IgG can be accommodated within the same set of 31 oligosaccharides found on human IgG. Only the relative incidence of individual structures varies between species. Crystallographic studies on IgG and Fc fragments suggest an important structural role for the Fc oligosaccharides (Figure 1), consistent with this degree of conservation. The two oligosaccharides in the Fc engage in both protein-oligosaccharide and oligosaccharide-oligosaccharide interactions. These interactions appear to fill the interstitial region between the C_H2 domains, keep the two domains apart and maintain the stable and relatively inflexible conformation characteristic of Fc. A particularly curious feature of IgG glycosylation is the variation in the structure of oligosaccharides with age. In a study of normal healthy individuals varying in age from 1 to 70 years, the relative incidence of oligosaccharides totally devoid of outer-arm galactose (i.e. agalactosyl structures) was found to vary continuously with age in a parabolic fashion, with the minimum incidence of agalactosyl structures occurring at about 25 years of age, irrespective of sex. This age-related molecular parameter is so far unique in varying continuously in the normal population, and raises the possibility that those age-related variations in immunological competence which are also parabolic, may reflect an underlying change in glycosylation.

Functional consequences of IgG glycosylation

The oligosaccharides of the Fc do not appear to be involved in binding of antigen, protein A, or recognition by rheumatoid factor. This has been shown by several authors who have compared aglycosyl IgG produced by culturing cells in the presence of tunicamycin, with native IgG. Proteolytic susceptibility and those interactions between IgG and C1q or cellular-bound receptors are, however, critically affected by Fc oligosaccharides. For example, binding to monocyte and macrophage Fc receptors, induction of cellular toxicity, rapid clearance of immune complexes from serum, and feedback immunosuppression are all severely reduced in agly-

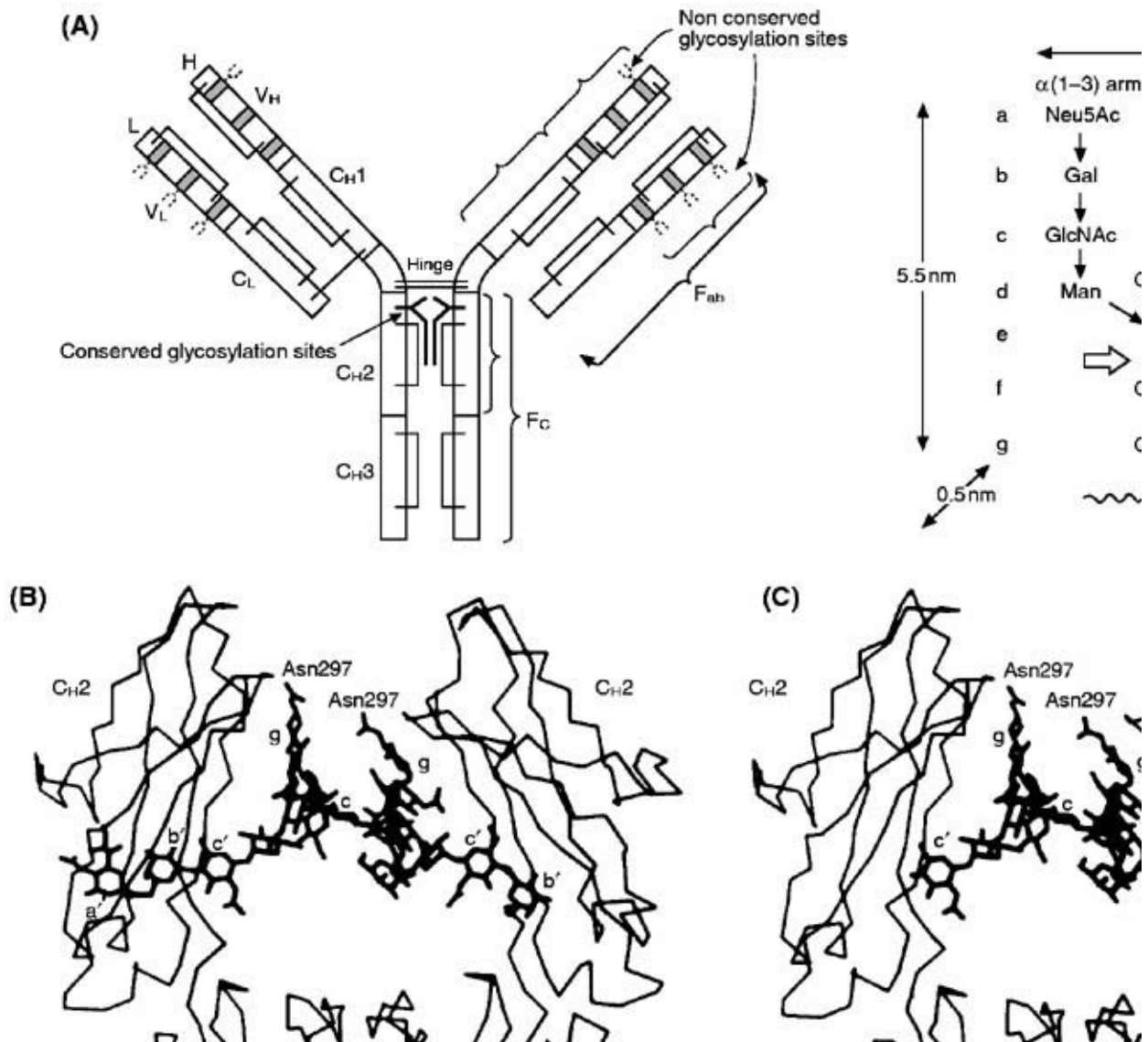


Figure 1 (A) A schematic representation of an IgG molecule indicating the positions of conserved *N*-glycosylation sites (at Asn297 in the C_H2 domains), and nonconserved *N*-glycosylation sites (in the hypervariable regions (dotted) of the Fab). The relative size of an immunoglobulin domain and a fully extended *N*-linked biantennary complex oligosaccharide are similar. Complex-type oligosaccharides present on IgG can be subdivided into an outer-arm region (a, a', b, b', c, c'), and the core, which is composed of a trimannosyl unit (d, d', e) and a *N,N'*-diacetylchitobiosyl unit (f,g). The 'bisect' GlcNAc (residue i) is linked $\beta(1-4)$ and the fucose (residue h) is linked $\alpha(1-6)$. The arrow between residues e and f indicates the site of interaction between the two oligosaccharides in B and C. (B) Refined structure at 2.80 Å of rabbit Fc fragment. The two carbohydrate chains, each attached at Asn297, differ in conformation, may also differ in sequence and bridge the C_H2 domains. The $\alpha(1-3)$ arm of the chain (left side) is always devoid of galactose and interacts through its $\beta(1-2)$ -linked GlcNAc residue (c) with the Man $\beta(1-4)$ GlcNAc segment of the opposing (right side) oligosaccharide chain (see A). The $\alpha(1-3)$ arm of the right chain extends outwards between the domains with no apparent steric constraints on its length. A Neu5Ac unit (a') is shown on one $\alpha(1-6)$ arm only (left). (C) Fragment containing oligosaccharides devoid of galactose and sialic acid on each of the $\alpha(1-6)$ arms. Since these residues in normal IgG are in contact with the surface of the protein (see B), their absence vacates oligosaccharide-binding sites in IgG from arthritic patients, and could make IgG 'sticky' by creating a lectin-like activity.

cosyl IgG. Further, serial lectin chromatography on total serum IgG has shown that there normally exists within serum IgG a *range of affinities* for monocyte Fc receptors, and this range is independent of subclass or antigenic specificity but correlates with Fc

N-glycosylation. That is, both the presence and nature of Fc glycosylation affect its interaction with the monocyte Fc receptor, and the naturally occurring glycoforms of IgG differ in this respect.

While interaction with cell surface receptors is

clearly sensitive to Fc N-glycosylation, Fab N-glycosylation appears to exert more influence on solubility and aggregation phenomena. Naturally occurring univalent (and therefore nonprecipitating) antibodies are formed by N-glycosylation of only one of the Fab arms (asymmetric glycosylation). Apparently, the presence of an N-linked oligosaccharide at certain positions of certain Fab moieties leads to a marked reduction in affinity for antigen at that Fab. Enzymatic removal of such an oligosaccharide restores the natural antigen affinity and renders the molecule bivalent. Interestingly, the oligosaccharide rendering some IgG molecules univalent almost invariably carries nonreducing terminal mannose residue(s), suggesting that such antibodies, though univalent, may activate the alternative complement pathway through interaction with serum mannan binding-lectin. The aggregation of IgG into IgG complexes is in some cases also Fab oligosaccharide-related. The cryoglobulin and cold agglutinin properties of some IgM and IgG monoclonal antibodies are dependent on the presence of certain Fab oligosaccharides.

A study of IgG N-glycosylation has provided new insights into the etiopathology of certain human diseases, particularly rheumatoid arthritis (RA), tuberculosis (TB), and Crohns disease. When the N-linked oligosaccharides of total serum IgG from normal individuals and patients suffering from active RA are compared, it is found that IgG from RA patients has a dramatically decreased incidence of structures terminating in outer-arm galactose, and therefore a corresponding increase in structures terminating with outer-arm N-acetylglucosamine ($P < 0.001$ by analysis of covariance). In effect, IgG totally lacking galactose [IgG(0)] is markedly raised. This change is also found in juvenile-onset RA (irrespective of the precise mode of onset), TB, Crohns disease, and systemic lupus erythematosus with Sjögrens. It is *not* found in myositis, scleroderma, psoriatic arthropathy, ankylosing spondylitis, osteoarthritis, reactive arthritis, sarcoidosis, multiple sclerosis, *Klebsiella* infection, or a variety of viral infections such as rubella and mumps. The change in galactosylation is therefore not a correlate of chronic inflammation, autoimmune rheumatic diseases, arthropathies, or infectious diseases, but is highly specific to adult and juvenile-onset RA, TB and Crohn's disease, and furthermore, the extent of the reduction in galactosylation closely parallels disease activity. Recent *in vivo* studies by Rademacher and

colleagues have demonstrated that IgG(0) is pathogenic in the collagen-induced arthritis model in DBA/1 strain mice.

See also: Adhesion molecules; Carbohydrate antigens; Lewis^x/sialyl-Lewis^x (CD15/CD15s); Cytokines; Immunoglobulin gene superfamily; Immunoglobulin structure; Lectins; Selectins (CD62-E/L/P).

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GOODPASTURE'S SYNDROME

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Since Ernest Goodpasture described the coexistence of hemoptysis and progressive renal failure in a young man after an episode of influenza in 1919, important advances have been made in our understanding of this unusual and, so far, exclusively human disease. Milestones have been the finding that the major pathogenic event is the linear deposition of circulating immunoglobulin G (IgG), the GP (Goodpasture) antibodies, along glomerular and alveolar basement membranes and the identification of the GP antigen as the C-terminal region of the $\alpha 3$ chain of basement membrane collagen IV.

Today the definitive diagnosis of GP syndrome is based on the detection of circulating antibodies against $\alpha 3(\text{IV})\text{NC1}$ in patients with renal-pulmonary involvement. Evidence suggests that the unique manner in which these high-affinity antibodies bind to the glomerular basement membrane efficiently interferes with plasma filtration. Restricted regions at both ends of the antigen are involved in antibody binding and in the formation of the GP epitope. Exclusively in humans, the N-terminal portion of this epitope can be phosphorylated and exists in multiple alternative antigen-related products that vary in their ability to be phosphorylated. In predisposed individuals, disturbance of these or other human-restricted processes may cause changes in the GP antigen peptide presentation and thereby initiate the autoimmune response.

The Goodpasture antigen and the pathogenic epitope

Basement membranes are specialized structures of the extracellular matrix found at the boundaries between cells and connective tissues. They play important roles in tissue and organ development and repair; in guiding cell migration and in signaling differentiation; and, in certain locations, such as in glomerulus, choroid plexus and alveolus, basement membranes control fluid exchange between the compartments they separate. In these three locations, the membrane appears to have arisen by the fusion of an endothelial and epithelial membrane. Interestingly these double-width basement membranes have been implicated in the autoimmune attack carried out by the GP antibodies (Figure 1).

A network of interacting collagen IV molecules

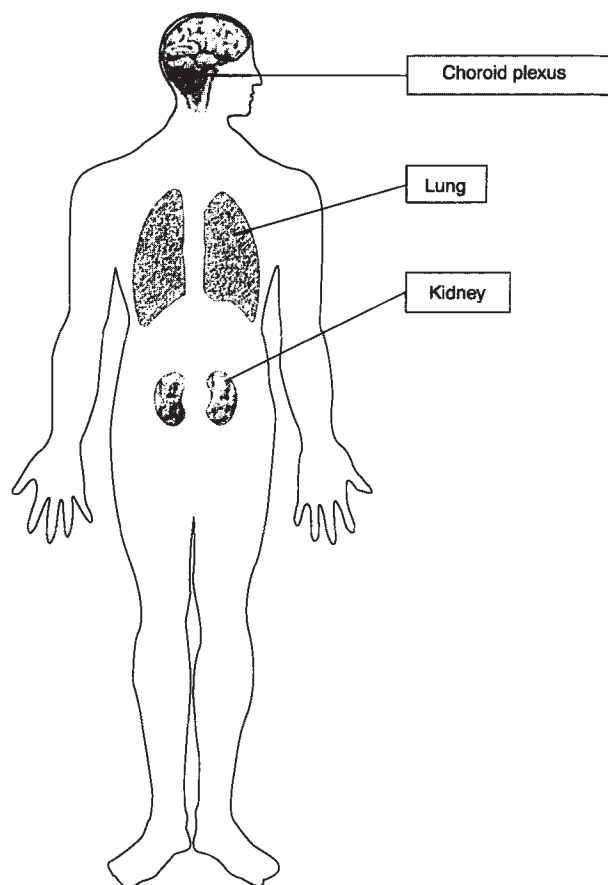


Figure 1 Principal organs affected in Goodpasture's syndrome.

(Figures 2 and 3) in close association with laminin (not shown) contribute respectively the structural strength and the adhesiveness required to build a typical basement membrane. The proportion and subunit composition of some of the constituents, including collagen IV, greatly varies among basement membranes.

Collagen IV is a nonfibrillar collagen exclusively found in basement membranes, where it is also the major structural component. As in other collagens, it is a triple helix composed of three intertwined α chains (Figures 2 and 3). So far, six different but related α chains, $\alpha 1(\text{IV})$ – $\alpha 6(\text{IV})$, have been characterized in collagen IV. The presence of noncollagenous sequences along each individual $\alpha(\text{IV})$ chain causes imperfections in the resulting triple helix that prevent its molecular assembly into fibrils. Among

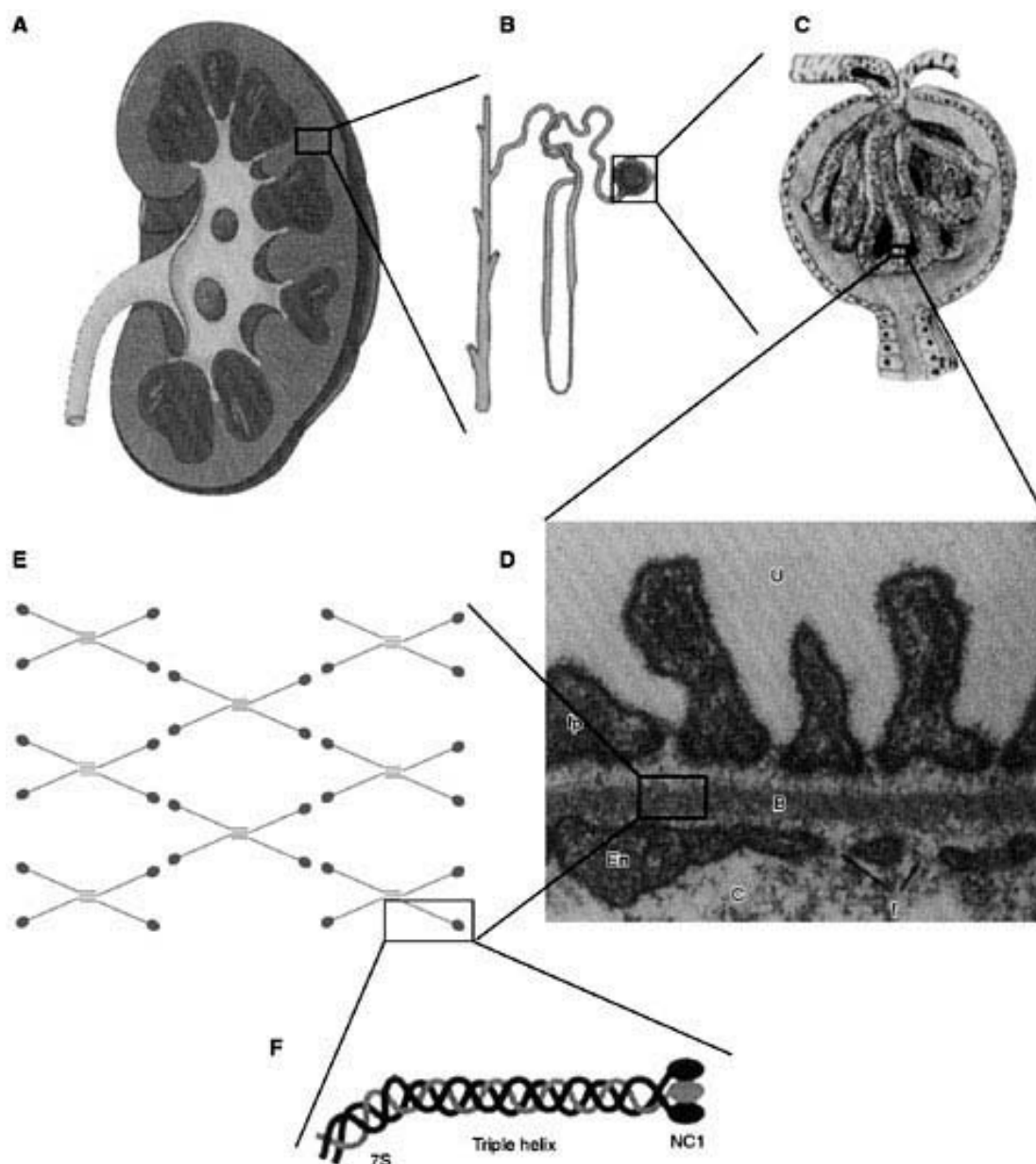


Figure 2 Diagram representing the localization and structural identification of the Goodpasture antigen from organ level to molecular level: (A) Cross-section of the kidney. (B) The functional unit of the kidney, the nephron. (C) The filtration unit of the nephron, the glomerulus. The blood enters the capillary tuft and the filtrate is forced out of the capillaries over the glomerular basement membrane into the surrounding Bowman's capsule. (D) Micrograph of the glomerular basement membrane (B) located between the fenestrated (f) endothelium (En) on the capillary (c) side and the epithelial foot processes (fp) on the urinary (U) side (Bowman's space). (E) Schematic drawing representing the collagen IV network of a basement membrane. Four collagen molecules are cross-linked through their N-terminal domains (7S) and two through their C-terminal domain (NC1). (F) Enlargement of a schematic collagen IV molecule in which the principal domains are indicated. (Figure courtesy of Charlotte Brunmark, originally presented in her doctoral dissertation 'Type IV collagen and renal disease'.)

the noncollagenous sequences, the C-terminal region or NC1 domain is the largest and conforms to a highly folded disulfide-rich region that guides, among other processes, the triple helix formation. In the basement membrane, two molecules of collagen IV interact through their NC1 domains to yield a

hexameric structure that can be released from the corresponding network by collagenase digestion (Figure 3). This hexamer is compacted by noncovalent forces and by disulfide bonds and yields monomer and disulfide-related dimers under denaturing conditions. The GP epitope is hidden within the iso-

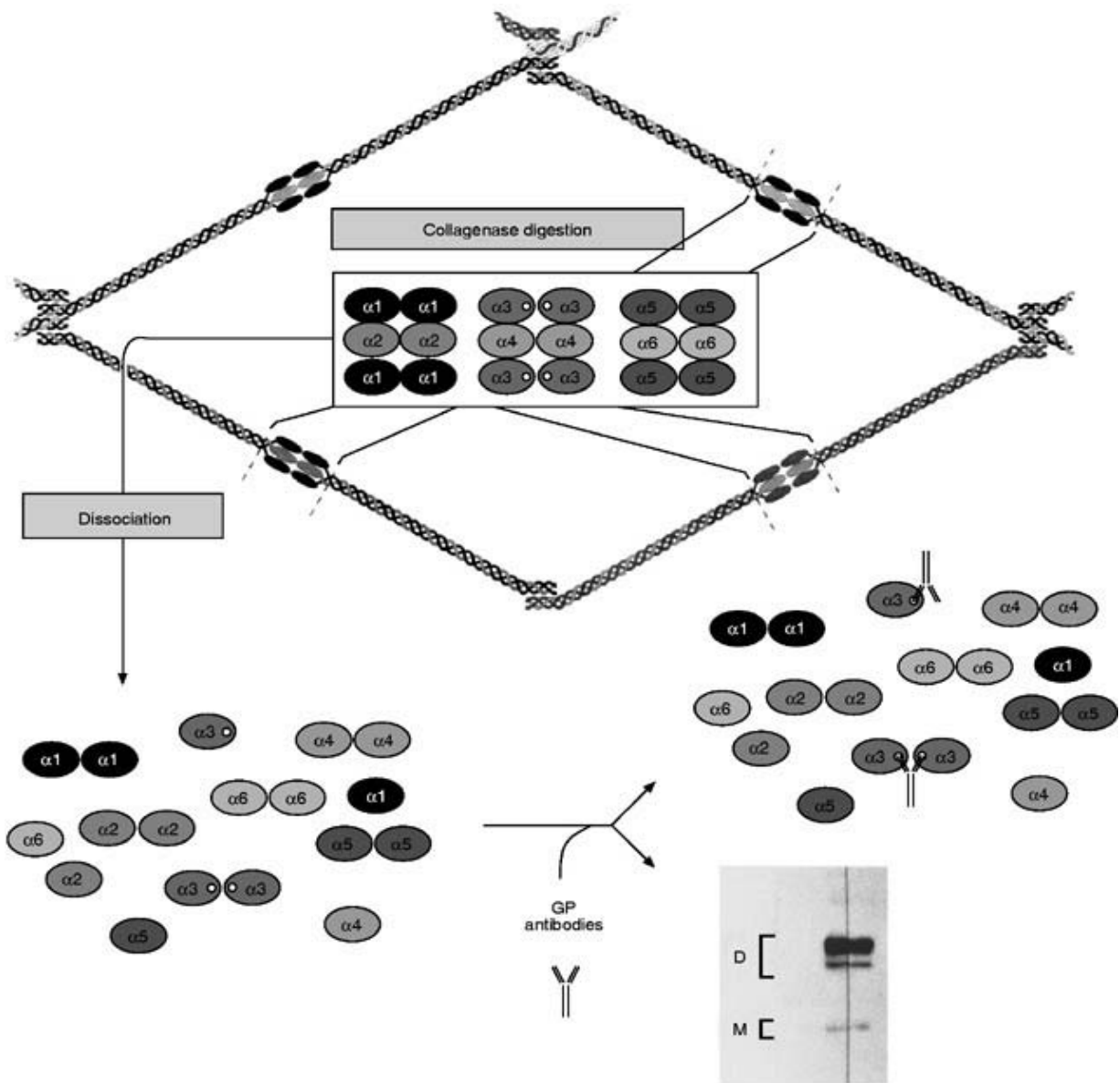


Figure 3 Representation of a hypothetical collagen IV network in which the six characterized $\alpha(\text{IV})$ chains are assembled into three types of collagen IV molecules ($(\alpha 1)_2\alpha 2$; $(\alpha 3)_2\alpha 4$; $(\alpha 5)_2\alpha 6$) and interactions through the NC1 domains are restricted to homologous molecules. There is evidence for the existence of $(\alpha 1)_2\alpha 2$ and $(\alpha 3)_2\alpha 4$ but not for $(\alpha 5)_2\alpha 6$. Other collagen IV compositions and interactions expected to occur are not represented in the drawing for simplicity. Bacterial collagenase digestion of the collagen IV network releases the hexameric NC1 domain that yields monomer and related dimers upon dissociation. The antigenic determinants recognized by the GP antibodies are hidden within the isolated hexamer and only exist in the $\alpha 3(\text{IV})\text{NC1}$ -containing material. At the lower right side, the hexamer was analyzed by denaturing electrophoresis and transferred to a nitrocellulose membrane. The membrane was sliced in two and the individual pieces blotted with Goodpasture serum (left) or with monoclonal antibodies recognizing the N-terminal region of the human $\alpha 3(\text{IV})\text{NC1}$ (right). The undistinguishable reactive patterns in both monomer (M) and dimer (D) regions localizes the Goodpasture antigen to the $\alpha 3(\text{IV})\text{NC1}$ domain.

lated hexamer since the unfolding of the hexamer is required for full GP antibody recognition (Figure 3).

The expression of each individual chain varies greatly among tissues with a more restricted distribution for the $\alpha 3(\text{IV})$ – $\alpha 6(\text{IV})$ chains. In the glomeruli, the $\alpha 3(\text{IV})$ – $\alpha 5(\text{IV})$ chains are coordinately

expressed and it appears that these three chains assemble into a collagen IV network different but connected to the more classic and ubiquitous one composed of the $\alpha 1(\text{IV})$ – $\alpha 2(\text{IV})$ chains. Typically in the Alport syndrome, a condition arising from mutations in the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$ or $\alpha 5(\text{IV})$ genes, the

glomerulus does not express the $\alpha 3(\text{IV})$ - $\alpha 5(\text{IV})$ chains. In Alport patients, the glomerular basement membrane displays a split structural appearance caused by the defective fusion of the endothelial and epithelial basement membranes, resulting in a severe impairment of the plasma filtration. This pathology reveals that the $\alpha 3(\text{IV})$ - $\alpha 5(\text{IV})$ network cements the double-width basement membrane and is a critical structural requirement for the glomerular filtration. This structural-functional relationship is further supported by the renal failure that results from the particular binding of antibodies to the C-terminal domain of $\alpha 3(\text{IV})$ in GP patients.

Collagenase digestion of the human $\alpha 3(\text{IV})$ chain releases the GP antigen, a 244-residue polypeptide consisting of a short nondigested collagenous sequence at the amino end followed by the NC1 domain (Figure 4). The molecular cloning of the different human $\alpha(\text{IV})$ chains and that of $\alpha 3(\text{IV})$ from other species has allowed comparative studies that have identified the pathogenic epitope and certain biological features unique to the human antigen. The GP epitope is discontinuous, disulfide-dependent and involves at least both ends of the antigen (Figure 4). In humans, the amino-side of the GP epitope is phosphorylated by type A protein kinases and antigen-

related products produced by alternative exon splicing regulate the process. These alternative products share the phosphorylatable amino-side of the epitope with the primary antigen and diverge from it at the C-terminal region. The shorter, divergent C-terminal regions do not contain the structural requirements to build a classic NC1 domain and therefore are not expected to guide triple helix formation. However, they do confer a distinct susceptibility to phosphorylation to the common N-terminal region.

The Goodpasture antibodies and the antigen-antibody reaction

The detailed characterization of the autoantibodies present in patients with renal-pulmonary syndromes is important since treatment and prognosis vary greatly depending upon antibody specificities. The poor prognosis associated with the presence of circulating GP antibodies justifies the 'toxic' quality by which they are known. The GP antigen is especially immunopathogenic and the specific way in which GP antibodies bind is critical in determining the severity of the resulting nephritis. This is supported by the fact that the development of experimental GP syndrome requires the administration of dimeric

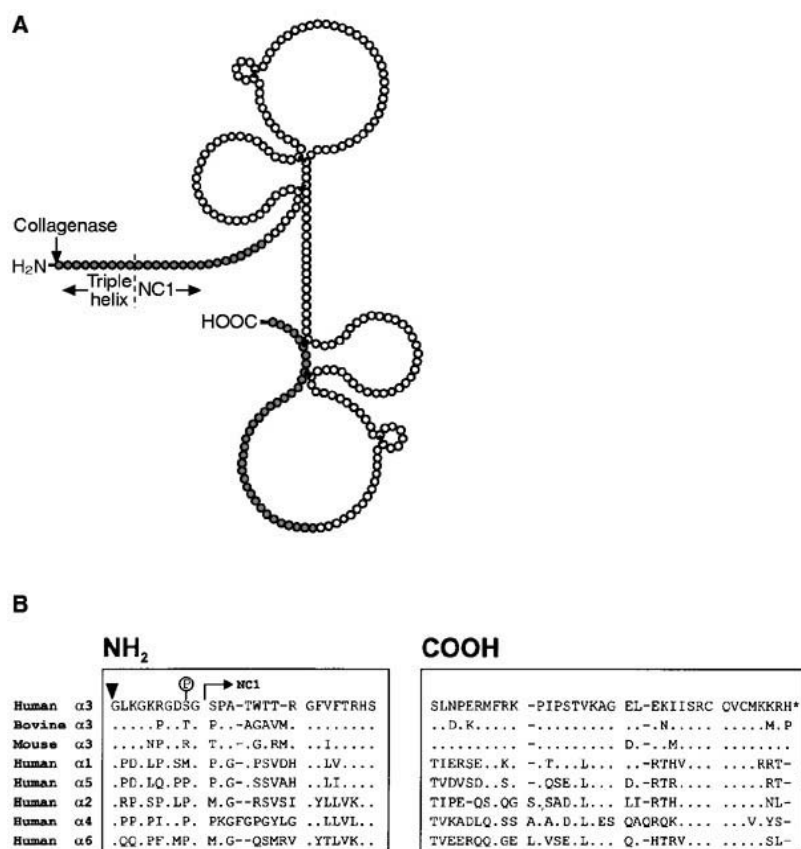


Figure 4 (A) Schematic representation of the 244 residues (circles) of the collagenase digestion product of the human $\alpha 3(\text{IV})$, the Goodpasture antigen, according to a previously reported model for the NC1 domain of a single $\alpha(\text{IV})$ chain (Siebold B. *et al* (1988) *European Journal of Biochemistry* **176**: 617-624; Kalluri *et al* (1991) *Journal of Biological Chemistry* **266**: 24018-24024). The disulfide bonds are represented by short black bars and the residues involved in the Goodpasture epitope are filled in dark. The collagenase cleavage site and the triple helix-NC1 junction are indicated. (B) Sequence comparison between the amino (NH₂) or carboxyl (COOH) sides of the Goodpasture epitope and the homologous regions of the indicated $\alpha(\text{IV})$ chains. The exclusive phosphorylation site in humans at the amino-side is indicated (P). The arrowhead and the bent arrow respectively indicate the beginning of the GP antigen (collagenase cleavage site) and the NC1 domain in the human $\alpha 3(\text{IV})$ sequence. Residues are represented in one-letter codes. The points and letters respectively indicate the identical and the divergent residues in the indicated collagen IV chains when compared to the human $\alpha 3(\text{IV})$ sequence.

$\alpha 3(\text{IV})\text{NC1}$ domain while immunization with other dimeric $\alpha(\text{IV})\text{NC1}$ domains also results in autoantibody production but not in evident disease. Furthermore, the administration of the $\alpha 3(\text{IV})\text{NC1}$ domain as part of the hexamer also fails to provoke the experimental syndrome although the animals display high titers of $\alpha 3(\text{IV})\text{NC1}$ autoantibodies.

Therefore, only the specific recognition of the GP antigen by GP antibodies appears to cause GP syndrome and neither the presence of other non-GP anti- $\alpha 3(\text{IV})\text{NC1}$ or antibodies against the other NC1 domains can cause the disease. Consistently, normal human sera contain high titers of IgM and IgG antibodies that bind the antigen in a nonpathogenic fashion; and individuals with high titers of antibodies directed against the more abundant NC1 domain of the $\alpha 1(\text{IV})$ chain do not develop a rapidly progressive glomerulonephritis.

It seems likely that the unique binding of GP antibodies to the $\alpha 3(\text{IV})\text{NC1}$ domain not only causes a general inflammatory response but also specifically interferes with critical molecular interactions required for plasma filtration.

Pathogenesis

Upper respiratory infection, flu-like illness and inhalation of hydrocarbon solvents, chlorine gas or hard metal dust have been implicated as triggering events of the GP syndrome. However the precise molecular mechanism by which these triggers initiate autoantibody production is unknown. Evidence concerning the participation of T cells in the immuno-

pathogenic response also is indirect and circumstantial. However, mononuclear interstitial infiltrate comprised mainly of CD4^+ helper T cells is consistently found in the affected tissues; and the major immunoglobulin subclass to which GP antibodies belong (IgG1) also suggests T cell-mediated autoantibody production.

As stated previously, normal human sera contain high levels of IgM and IgG that specifically recognize the GP antigen. Interestingly, these nonpathogenic autoantibodies are significantly reduced in some GP patients. The coincidence of this up- and downregulation of autoantibody production suggests that the bulk of the B cell clones involved in both natural and pathogenic autoreactivity may be the same. The repetitive presence of the GP antigen along the basement membrane collagen IV network and the existence of these 'natural antibodies' also suggest that these B cells are normally being stimulated in a T-independent fashion (Figure 5). During pathogenesis, a T-dependent stimulation of these same B cells may cause an isotype switch to IgG-producing cells. Subsequently, somatic mutations in the class-switched B cells along with persistent and efficient antigen stimulation could lead to the production of the high-affinity GP antibodies. Efficient antigen stimulation appears to be of pathogenic relevance since effective immunization of experimental animals requires the administration of an accessible dimeric form of the antigen.

It is accepted that self-tolerance is mainly achieved by downregulating autoreactive helper T cells. A consequence of bypassing this control would be the

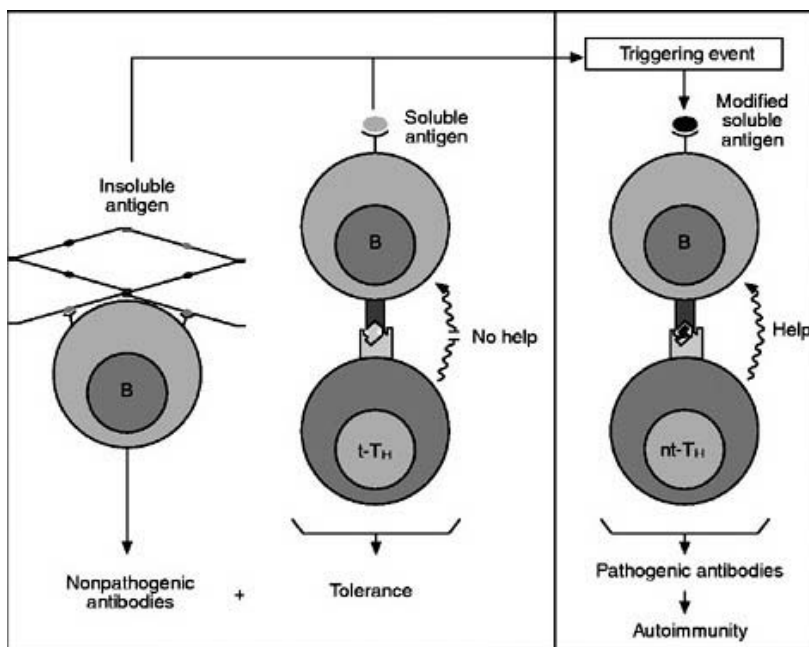


Figure 5 A hypothetical model for the autoimmune response in Goodpasture's syndrome. Soluble antigenic products are recognized by silent or T-independently induced B cells whose T-dependent induction cannot occur because the cognate T helper cells are tolerized ($t\text{-T}_{\text{H}}$). A triggering event may generate modified antigenic products that still are recognized by the B cells. In certain predisposed individuals, however, the modified products are distinctly processed and presented, and thereby bypass the tolerized T helper cells to initiate a T-dependent pathogenic response mediated by nontolerized T cells (nt-T_{H}).

induction of autoreactive cells leading to autoimmunity. In the present case, silent or T-independently stimulated autoreactive B clones could also be the target of a more solubilized form of antigenic products. Yet the corresponding T-dependent induction would not occur due to the presence of tolerized T helper cells. In certain genetically predisposed individuals, however, a triggering event causing antigen modifications may result in the presentation of 'new' peptides that bypass the tolerized T cells and thereby initiate a T-dependent autoimmune response (Figure 5).

In such an induction mechanism, the removal of altered antigen would terminate the immunopathological response and relapses would require a new effective trigger. This appears to be the case in the GP syndrome since kidney removal improves lung manifestations in some patients, and relapses are rare and related to re-exposure to a triggering agent. Furthermore, effective autoantibody removal from patient plasma commonly results in a virtual arrest of the autoimmune process indicating that the production of the pathogenic antibody is a temporally limited event.

Phosphorylation of certain serines by type A protein kinases and alternative splicing of the GP antigen are connected phenomena found exclusively in the human antigen but not in other species or in other human α (IV) chains. Both type A protein kinase and antigen phosphorylation have been independently implicated in the development of other autoimmune responses. Phosphorylation of specific serines modifies intracellular proteolytic processing of other proteins, suggesting that alterations in antigen phosphorylation can affect peptide presentation. The involvement of these or other unique biological processes yet to be discovered in the GP syndrome pathogenesis may explain why the natural disease is not described in other mammals, why other NC1 domains are not immunopathogenic and why this disease is so rare and with such a strong linkage to a particular presenting MHC haplotype (HLA-DR5).

See also: Antigen presentation via MHC class II molecules; MHC peptide-binding specificity; Antigens, T dependent and independent; Autoantigens; Autoimmune disease, induced experimental models; Autoimmune disease, pathogenesis; Autoimmune diseases; Autoimmunity; Extracellular matrix; Immunoglobulin class switching; MHC disease associations; Protein kinases; Systemic lupus erythematosus, experimental models; Tolerance, peripheral; Tolerance, models.

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GRAFT REJECTION

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Organ transplantation is currently the best clinical option for patients with end-stage renal failure, and indeed the only option for patients with failure of organs such as liver and heart. Although the survival rate for transplants (e.g. 1 year transplant survival for first cadaveric kidney transplants in the UK is ~80–85%) is now very high, one continuing problem with organ transplantation is that of graft rejection. In the 1940s, Medawar and his colleagues firmly established that graft rejection involved an immune response against the foreign antigens carried on tissues. A very large amount of work subsequently carried out in this area suggests that most facets of the immune system can be involved in the tissue destruction that results in graft rejection. Experimental models of transplantation continue to be of interest, not only to examine *in vivo* immune responses to alloantigens, but also to dissect the mechanisms of peripheral tolerance. The eventual goal of the latter work is to develop strategies to achieve graft-specific immunoregulation or tolerance in clinical transplantation in order that we may dispense with the nonspecific drugs that are currently used to prevent graft rejection.

Graft rejection may be divided into afferent and efferent phases – in the former the immune system becomes alerted to the presence of the foreign tissue, and in the latter the immune system destroys the graft.

Afferent phase

Antigens

It is the histocompatibility antigens present on cells of the transplant that activate the immune system of the host. Proteins encoded within the major histocompatibility complex (MHC) produce the most vigorous immune response, which, if left unchecked, will almost invariably lead to complete destruction of the graft. However, the minor histocompatibility antigens may also lead to graft rejection. Indeed,

multiple minor histocompatibility differences may lead to rejection of solid organ grafts as rapidly as that seen with MHC differences, and in bone marrow transplantation a single minor histocompatibility difference may lead to lethal graft-versus-host disease. Of the MHC antigens, class I antigens are found on probably all nucleated cells, but with a variable level. By contrast, class II antigens have a rather limited distribution on normal cells, their expression being restricted to B cells, some endothelial and epithelial cells, and cells of the dendritic cell lineage. The level of expression of both class I and class II antigens may be upregulated, and indeed class II-negative cells may be induced to express these antigens, *de novo*, in the presence of cytokines such as interferon γ (IFN γ) and tumor necrosis factor α (TNF α). Both class I and class II antigens become strongly upregulated on most cells of an organ transplant during graft rejection.

Differences of single class I or class II antigens may result in graft rejection, although the kinetics of rejection may vary with the incompatibility. The very great polymorphism of the MHC antigens means that it is usually only possible to transplant fully MHC-compatible grafts between siblings. Even then, minor antigen differences may occur. Indeed, it is only possible to graft tissue which is matched for both major and minor histocompatibility antigens between monozygotic twins. Thus, immunosuppression is always required in clinical transplantation and, even so, rejection episodes occur in many patients.

MHC antigens can be recognized either as intact proteins on the surface of the donor antigen-presenting cells (direct presentation) or as processed fragments in association with recipient MHC (indirect presentation). It is suggested that direct presentation is responsible for acute rejection, while indirect presentation may lead to the more insidious changes associated with chronic rejection.

Mice with disrupted expression of both β_2 -

microglobulin (class I^{-/-} mice) and class II genes (class II^{-/-} mice) have been generated and used as either donor or recipient of transplants. In many such experiments, a lack of class I or class II antigens alone on donor tissue has little effect on graft survival; however, in other experiments graft survival may be prolonged or permanent when donor tissue lacks either class I or class II or both class I and II antigens. Certainly, different results are obtained when different types of grafts are used, but the interpretation of apparently straightforward experiments is confounded not only by unusual features of the immune systems of these knockout mice but also by the suggestion that grafts from class I^{-/-} mice may be reconstituted in their expression of class I by serum β_2 -microglobulin in the recipient.

Recipients lacking class I or II antigens are severely depleted of CD8⁺ or CD4⁺ cells, respectively. Different workers obtain distinct results using such recipients, which is perhaps not surprising given the results of other experiments in which the role of these two subsets has been investigated. Further, despite an apparent decrease in CD8⁺ cells in class I^{-/-} mice, CD8⁺ cytotoxic T lymphocytes (CTLs) can be generated in large numbers following transplantation and may therefore be involved in the rejection process.

Cells involved in activation of the immune response

Mature splenic dendritic cells which express both class I and II MHC antigens are able to induce very strong primary immune responses to alloantigen. It is thought that the passenger leukocytes present within most or all tissues are members of the dendritic cell lineage. There is good evidence that such cells are able to migrate from a transplanted organ to the central lymphoid tissue, acquiring full T cell stimulatory activity where they can activate the immune system (direct antigen presentation). Depletion of passenger leukocytes from transplants has been shown under some circumstances to increase the survival of organ grafts in both experimental and clinical transplantation. The role of endothelial cells in provoking immunity to a graft is a subject of much discussion. Endothelial cells do not fall into the category of professional antigen-presenting cells, yet they do constitutively express MHC class II proteins (at least in humans) and therefore have at least part of the machinery required for CD4⁺ T cell activation. It is possible that their most important role in transplantation responses is to augment immunity and stimulate T cells which have already been exposed to antigen and thus are classified as memory or activated T cells.

Privileged sites

Allografts placed in certain 'privileged' sites may survive for prolonged times or even indefinitely. Probably the best known privileged site is the cheek pouch of the Syrian hamster, in which an allograft will survive indefinitely in an unsensitized animal. Other sites in which allografts or xenografts show prolonged survival are the anterior chamber of the eye, the cornea in humans, the testis and the brain. However, grafts placed at these sites will be rejected eventually in unsensitized recipients, or rapidly if the recipient is sensitized to the graft antigens. All of these sites have in common abnormal or reduced lymphatic drainage and it is possible that a delay in sensitization of the recipient affects the tempo of rejection. Recent experiments have shown that at least some of these privileged sites also express Fas ligand at very high levels. The suggestion from these data is that this Fas ligand induces apoptosis in Fas-expressing T cells, thus limiting their action in graft rejection. Indeed, insertion of cells expressing high levels of Fas ligand into a pool of syngeneic islet cells can protect the islets from rejection by an allogeneic recipient.

Efferent phase

T cell help and graft rejection

Because congenitally athymic (nude) and adult thymectomized, irradiated and bone marrow-restored (ATXBM) animals do not reject incompatible grafts, it is clear that T cells are required for graft rejection. It is also clear from adoptive transfer experiments that helper cells are essential for the initiation of the immune response to a graft. Thus, in most experimental models of transplantation, CD4⁺ but not CD8⁺ T cells are able to reconstitute ATXBM or nude animals with the ability to reject an allograft. This is slightly confused by the finding that only CD8⁺ cells are required to initiate graft rejection between certain mouse strains that differ in only a few amino acids of their class I molecules. The ability of CD8⁺ cells to cause rejection in this situation, however, seems to be a reflection of the high number of CD8⁺ helper cells activated in this strain combination, rather than a lack of requirement for T cell help. Although the adoptive transfer studies described above have shown that, perhaps not surprisingly, T cell help is required to initiate graft rejection, they give little indication of how graft rejection is effected, since both nude and ATXBM animals contain many leukocytes including B cells, macrophages, natural killer cells, and at least some cytotoxic T cell precursors. Any or all of these cells

may be recruited by T helper cells to cause tissue destruction.

Specificity of the effector phase

One key point about the effector phase of graft rejection is the exquisite specificity with which grafts can be rejected, a finding so elegantly demonstrated by Mintz and Silvers in a skin allograft model. In these experiments, allophenic or tetraparental mice derived from mice of different coat colors were used as skin donors. Skin was grafted to mice of either parental strain and the cells of nonidentical origin were rejected, leaving only the cells of recipient type intact and capable of hair growth. It is difficult to imagine that any mechanism of rejection involving nonspecific lysis of bystander cells (such as that involved in delayed-type hypersensitivity lesions) could be responsible for such specific tissue damage.

More recently, similar experiments have been performed by Rosenberg and colleagues. Interestingly they found that nonspecific inflammatory reactions could lead to total loss of grafts from allophenic animals if the area affected by the specific response was great enough. In other words, if the skin was predominantly of an MHC type different to that of the recipient, the entire graft could be lost. This suggests nonspecific inflammatory reactions may lead to tissue destruction if triggered by a massive and specific T cell response.

Antibody in graft rejection

Preformed antibodies reactive with MHC antigens are undoubtedly the cause of hyperacute rejection where grafts are destroyed within minutes of revascularization following binding of antibodies to the organ and complement fixation. The presence of such antibodies in potential donor-recipient pairs is now assessed before clinical transplants are performed using the cross-match test, and hyperacute rejection mediated by antibody is therefore avoided. Natural antibodies are responsible for the hyperacute rejection seen in discordant xenogeneic transplantation, although their target antigens are not completely defined. The role of antibody in acute and chronic rejection is less clearly understood and indeed there is usually a poor correlation of the presence of antibody with these types of rejection. However, work analyzing renal graft rejection across an MHC class I antigen disparity supports the concept that antibody may be involved in acute rejection.

Cytotoxic T cells

In tissue culture, cells from MHC mismatched individuals proliferate strongly in response to one

another in the mixed lymphocyte reaction (MLR). During this proliferation, cytotoxic effector cells (CTLs) are generated that are able to specifically lyse target cells carrying the same alloantigens to which they have been activated. The target cells are usually lymphocyte blasts or tumor cells that carry the correct MHC antigens. The majority of CTLs are CD8⁺ class I directed, but CD4⁺ class II directed CTLs may also be generated. In 1970, Hayry and Defendi suggested that the MLR was the complete *in vitro* correlate of graft rejection, the proliferation representing the afferent phase of the immune response to an allograft. This was a very attractive view and one which was widely held for many years. Indeed, CTLs may be recovered from grafts that are undergoing rejection. Further, cloned populations of CTLs have been shown to cause both tumor rejection and tissue destruction. However, more recently, several groups have recovered CTLs from grafts that will not be rejected and, further, grafts may be rejected in the apparent absence of CTLs. One problem with all these analyses is our questionable ability to assess the cytotoxic potential of cells using *in vitro* assays – it may be naive to assume that such assays accurately reflect the potential of cells to cause tissue damage or graft rejection *in vivo*. Indeed, Steinmuller and his colleagues have described CTL clones that have lost their ability to lyse ⁵¹Cr-labeled targets *in vitro* yet maintain their ability to cause tissue damage *in vivo*, and vice versa.

Some other evidence in favor of the involvement of CTLs in graft rejection comes from the work of several groups in which the tempo of graft rejection may only be completely restored to normal in T cell-deprived animals if both CD4⁺ and CD8⁺ cells are given at the time of transplantation. Other experiments implicating CTLs include those in which graft rejection is manipulated by injection of antibodies that react with CD4⁺ and CD8⁺ T cell subsets. In such experiments, greater prolongation of graft survival is often seen when both CD4⁺ and CD8⁺ subsets are depleted than when CD4⁺ cells alone are depleted. In summary, it seems likely that CTLs are capable of causing the tissue destruction associated with graft rejection but are unlikely to be the only cells involved and their presence within a graft may not always result in rejection.

Delayed-type hypersensitivity (DTH) and graft rejection

DTH reactions are specifically initiated via a CD4⁺, antigen-reactive cell. The DTH lesion itself is, however, nonspecifically effected and characterized by an infiltrate of lymphocytes and cells of the monocyte-macrophage lineage. It is noteworthy that the sever-

ity of DTH lesions varies greatly among different species, all of which reject allografts with comparable facility. Skin graft rejection across male-specific minor histocompatibility (H-Y) differences in mice correlates better with DTH responses than with CTL responses. Now that the nature of the H-Y antigen(s) is becoming clear it should be possible to assess more accurately the role of CTL and DTH reactions to H-Y in graft rejection. Activated macrophages are certainly found within rejecting grafts but this may simply be as a consequence of the tissue destruction that occurs in graft rejection. Indeed, large numbers of class II-positive macrophages may be seen in syngeneic skin grafts, presumably as a consequence of tissue damage rather than of an immune response.

Natural killer (NK) cells

NK cells do not require any activation in order to lyse a variety of tumor cell targets. It is unlikely that these cells are involved in the rejection of solid organ allografts for a number of reasons. First, they are found in high levels in nude animals which are unable to reject grafts. Second, they are present in grafts which are not rejected by, for instance, cyclosporine-treated or blood-transfused animals. Third, antibody treatments designed to deplete NK cells have no effect on graft survival.

Lymphokine-activated killer (LAK) cells

Lymphocytes cultured in high levels of interleukin 2 (IL-2) develop cytotoxic activity of an essentially nonspecific nature. Whether these cells are actually generated in *in vivo* immune responses remains to be clarified. Their role in solid organ graft rejection has not been studied, although they appear to be capable of causing tumor regression in both experimental and clinical settings.

The target cells of graft rejection

The precise target(s) cells of allograft rejection are not clearly defined, but it seems likely that damage to the vascular endothelium, which may express both class I and class II antigens, would result in rapid graft loss. The vascular changes that occur during graft rejection indeed suggest that this is the case. The parenchymal cells of the graft may also be targets for rejection, although it is likely that this will be secondary to the initial attack on the endothelium.

Cytokines and graft rejection

Cytokines clearly play an essential role in the regulation of all immune responses. The expression of cytokines in transplanted tissue has been studied

extensively, both in experimental models of transplantation and in the clinical setting. In allografts a predominance of the T_H1 -type cytokines (such as interferon γ) usually indicates the onset of rejection, although there are certainly exceptions to this rule. It has been suggested that, by contrast, a T_H2 -dominated response may be beneficial and indeed responsible for tolerance. While it is often the case that prolonged graft survival is associated with a maintained or even increased expression of T_H2 cytokines, there are very few examples in which tolerance can be explained on the basis of a T_H2 response. Indeed, it is possible to induce tolerance in animals that, through genetic manipulation lack IL-4. It seems probable that a T_H2 response may be less damaging to a graft than a T_H1 response and thus may provide a window in which the immune system can become tolerant of the graft.

Chronic rejection

Chronic graft rejection has now become the major reason for graft loss, yet remains poorly understood. It is suggested that both immunological (cellular and humoral) and nonimmunological mechanisms are involved in the damage that leads to eventual graft loss, but the immune effector mechanisms are ill defined. Experimental models of chronic rejection have begun to allow a description of the etiology of the obliterative arterial changes that are the hallmark of chronic rejection and may allow the development of therapeutic strategies aimed at preventing this problem.

Conclusions

The immune response that results in graft rejection is a complex phenomenon, with respect both to the manner in which the graft antigens are presented to, and recognized by, the host leukocytes, and in the effector phase of the response that generally results in graft damage. The CTL is still considered of primary importance in mediating tissue damage in graft rejection, but the DTH reaction may also be involved and is probably responsible for some of the later damage to a graft. Antibody is clearly involved in graft rejection in some special situations, but its role in acute and chronic rejection remains unclear. Specific regulation of these immune responses with resulting graft tolerance in the adult remains the goal of transplant biologists and, although achieved in many experimental models of transplantation, remains elusive in clinical transplantation.

See also: **Antibody-dependent cellular cytotoxicity;**

Antigen-presenting cells; Cytokines; Cytotoxic T lymphocytes; Delayed-type hypersensitivity; Effector lymphocytes; Graft-versus-host reaction; H-Y, the male-specific transplantation antigen; Immunosuppression; Lymphokine-activated killer (LAK) cells; Minor transplantation (histocompatibility) antigens; Natural killer (NK) cells; T lymphocytes; Tolerance, peripheral; Transplantation.

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GRAFT-VERSUS-HOST REACTION

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The graft-versus-host reaction was first described by Billingham, Medawar and their colleagues. In their original publications the prerequisites for the graft-versus-host reaction were the infusion of immunocompetent cells into a recipient incapable of rejecting the cells and the existence of a histocompatibility difference between donor and recipient. Thus, the graft-versus-host reaction could occur following the infusion of homozygous cells into heterozygote recipients, but not in the reverse setting. The graft-versus-host reaction occurred in totally allogeneic hosts only if the recipient was immunosuppressed prior to the infusion of immunocompetent cells and was not observed in the recipients of syngeneic cells.

Graft-versus-host disease

Confusion exists as to the difference between the graft-versus-host reaction and graft-versus-host disease. Graft-versus-host reaction identifies only the immunologic events that occur as a consequence of the infusion of allogeneic immunocompetent cells into a susceptible recipient. It has become apparent, especially from the studies in germ-free animals, that the clinical manifestations of the graft-versus-host reaction (graft-versus-host disease) is dependent upon many environmental factors, including the presence of endotoxin, the age of the donor and recipient, the sex of the donor, etc. Thus, strain combinations exist in which no graft-versus-host disease occurs when transplants are done between germ-free animals. However, the introduction of bacteria into

the stable post-transplant chimera results in the development of clinical graft-versus-host disease, presumably secondary to the effects of endotoxin and other bacterial products/antigens. An extensive literature exists documenting the role of endotoxin in increasing the severity of graft-versus-host disease. Graft-versus-host disease is more frequently seen in the recipients of female, as opposed to male, immunocompetent cells.

Clinically, graft-versus-host disease can appear as one of two distinct forms. Acute graft-versus-host disease has its onset early following the infusion of donor immunocompetent cells and is characterized by the cytolytic destruction of the recipient epidermal cells, hepatocytes and gastrointestinal cells, producing a characteristic cutaneous erythroderma, elevation in hepatocellular enzymes and diarrhea. A high rate of fatality is found in both animals and humans with severe acute graft-versus-host disease due to the denudation of the gastrointestinal tract, epidermal sloughing, sepsis and bleeding. Individuals, who do not die from acute graft-versus-host disease, are at risk of developing chronic graft-versus-host disease, which has many similarities to autoimmune diseases, including sclerodermatous skin changes, the production of autoantibodies, etc. In model animal systems, graft-versus-host disease is usually produced by the infusion of homozygous donor immunocompetent cells into F₁ recipients or of allogeneic bone marrow cells into irradiated recipients. Graft-versus-host disease can be seen following the infusion of

peripheral lymphoid cells, splenocytes and bone marrow or the transplantation of lymphoid-containing organs, such as the small intestine.

Histocompatibility differences

Graft-versus-host disease due to antigenic differences at the major histocompatibility complex (MHC) can be due to either CD8⁺ or CD4⁺ T lymphocytes. Acute graft-versus-host disease caused by class I histocompatibility differences is due primarily to CD8⁺ T lymphocytes, many of which are cytotoxic T lymphocytes, whereas acute graft-versus-host disease due to class II histocompatibility differences is due to CD4⁺ T lymphocytes, some of which may be cytotoxic T lymphocytes, while others are helper T lymphocytes. The specificity of each of these effector cell types can be seen in strain combinations in which isolated class I or class II histocompatibility differences exist. A role for cytokines, in addition to cytotoxic T lymphocytes, has been demonstrated in acute graft-versus-host disease. The administration of antibodies to murine tumor necrosis factor α (TNF α) resulted in a reduction in mortality and the abolition of the histopathologic changes associated with acute graft-versus-host disease. These results indicate that cytokines, in addition to cytotoxic T lymphocytes, may be important effector mechanisms in acute graft-versus-host disease.

Graft-versus-host disease due to non-MHC antigenic differences is more complicated, and debate still exists as to its effector mechanisms. Most investigators, however, agree that both CD4⁺ and CD8⁺ T lymphocytes are involved in the pathogenesis of acute graft-versus-host disease due to non-MHC differences, although the relative importance of the CD4⁺ and CD8⁺ T lymphocytes is strain dependent. The CD8⁺ T lymphocytes have specificity for recipient restricted minor histocompatibility antigens, while the CD4⁺ T lymphocytes have specificity for the shared class II antigens. Based upon *in vitro* depletion experiments, the CD4⁺ T lymphocytes may produce the cytokines (interleukin 2 (IL-2), IL-4) required for the *in vivo* differentiation and growth of the recipient-specific CD8⁺ T lymphocytes. When animals with chronic graft-versus-host disease have been analyzed, primarily CD4⁺ autoreactive T lymphocytes without cytotoxic capacity have been identified. Chronic graft-versus-host disease is characterized histopathologically by increased collagen deposition. The autoreactive CD4⁺ T lymphocytes are capable of producing cytokines (interferon γ , TNF α , IL-4) which can stimulate fibroblast collagen production, suggesting that chronic graft-versus-host disease is primarily a cytokine-mediated disease. IL-

4 could also be responsible for the observed increase in autoantibody production. Animal experiments, therefore, indicate that histocompatible acute and chronic graft-versus-host disease, besides differing in their clinical presentation, also differ in their cellular effector mechanisms and suggest that the pathogenesis of acute and chronic graft-versus-host disease differ.

Human graft-versus-host disease

Graft-versus-host disease has its major clinical significance in human bone marrow transplantation. Graft-versus-host disease is also seen following the infusion of nonirradiated allogeneic blood products into immunosuppressed recipients, including patients with primary cellular immunodeficiencies and cancer. Graft-versus-host disease due to infusion of histoincompatible immunocompetent cells usually results in the death of the recipient. Graft-versus-host disease that is usually seen following histocompatible bone marrow transplantation is much milder and usually does not result in the death of the patient if prophylactic anti-graft-versus-host disease drugs are given. When human bone marrow is transplanted, it usually contains 20–40% peripheral blood mononuclear cells, meaning that as many as 5–10% of the total transplanted cells are mature peripheral blood T lymphocytes. Following the transplantation of histocompatible human bone marrow, acute graft-versus-host disease is a frequent occurrence. The prophylactic administration of methotrexate has reduced the incidence of significant graft-versus-host disease ($\geq$ grade 2) to 40%. Combined immunosuppression with cyclosporine plus methotrexate or cyclosporine plus steroids has further reduced the overall incidence of significant acute graft-versus-host disease to 20%. The presence of significant acute graft-versus-host disease ($\geq$ grade 2) is the major prognostic factor that determines whether chronic graft-versus-host disease will occur. Acute graft-versus-host disease usually develops 10–30 days after bone marrow transplantation and is characterized by a measles-like rash, elevation of hepatocellular enzymes and diarrhea. Elevation of serum IgE levels can also be seen. Factors that are predictive of an increased incidence or severity of acute graft-versus-host disease include transplantation from alloimmunized female donors into male recipients, increased donor/recipient age, and the lack of prophylactic anti-graft-versus-host disease therapy. Patients with acute graft-versus-host disease, who do not respond to prophylactic graft-versus-host disease therapy, are treated with steroids and/or antithymocyte globulin (ATG).

Transplantation between family members that differ at only a single HLA allele has demonstrated that class II histocompatibility differences produce more graft-versus-host disease than class I differences. The recipients of matched unrelated donor bone marrow have an increased severity and frequency of acute graft-versus-host disease than the recipients of histocompatible sibling bone marrow. Again class II histocompatibility differences produce more severe graft-versus-host disease than class I differences. Transplantation with unrelated cord blood results in less graft-versus-host disease, presumably due to the relative immunologic immaturity of cord blood T lymphocytes. If the transplantation of unrelated cord blood results in less acute graft-versus-host disease, greater degrees of histoincompatibility between donors and recipients may be possible.

Chronic graft-versus-host disease has its usual onset between 90 and 150 days after transplantation. Patients are characterized by increased collagen deposition with sclerodermatous skin changes, pulmonary fibrosis, gastrointestinal malabsorption and sicca syndrome. Patients are also characterized by an immunodeficiency to environmental antigens in the context of autoantibody production. Patients have a particular inability to make antibodies to carbohydrate antigens, predisposing them to an increased incidence of infections with respiratory bacteria. Patients with chronic graft-versus-host disease are aggressively treated with cyclosporine and steroids. Combination therapy has resulted in significant clinical improvement and increased patient survival. Because of their immunosuppressed status, patients with chronic graft-versus-host disease are placed on prophylactic antibiotics (trimethoprim and sulfamethoxazole) and monthly replacement intravenous immunoglobulin therapy.

Autologous graft-versus-host disease

A special form of graft-versus-host disease, which was first observed in rodents and has now been seen clinically, is autologous graft-versus-host disease. Autologous graft-versus-host disease is found in recipients undergoing autologous or syngeneic bone marrow reconstitution and receiving cyclosporine in the post-transplant period. They develop an acute form of chronic graft-versus-host disease, in which autocytotoxic T lymphocytes with a broad specificity for autologous class II histocompatibility antigens can be identified. Such autologous graft-versus-host disease appears to be due to the effects of cyclosporine and irradiation on thymic differentiation. Although the mechanism of autologous graft-versus-host disease is not certain, the increased frequency of

autoreactive T lymphocytes seen in autologous graft-versus-host disease animals suggests that thymic damage, leading to the decreased negative selection of autoreactive T lymphocytes during normal T lymphocyte ontogeny, may be the basis for the autologous graft-versus-host disease.

A graft-versus-leukemia effect has been seen in the recipients of allogeneic bone marrow transplants with acute graft-versus-host disease. The graft-versus-leukemia effect is seen in patients with chronic and acute myelogenous leukemia and to a lesser degree in acute lymphoblastic leukemia. Clinical experiments are now underway to determine if the effector mechanisms involved in acute graft-versus-host disease can be separated from the graft-versus-leukemia effect.

Graft-versus-host disease is the clinical manifestation of the graft-versus-host reaction. Acute and chronic graft-versus-host disease are still major problems found in human bone marrow transplantation. Many basic immunologic questions still need to be resolved before a total understanding of both the graft-versus-host reaction and graft-versus-host disease is achieved.

See also: Antilymphocyte serum; Hematopoietic stem cell transplantation; Graft rejection; Immunosuppression; Transplantation.

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GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF)

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The colony-stimulating factors (CSFs) were characterized by their ability to facilitate survival and to promote clonal proliferation and development of hematopoietic progenitor cells *in vitro*. Granulocyte CSF (G-CSF) is one member of this family of glycoproteins, which also includes granulocyte-macrophage CSF (GM-CSF), macrophage CSF (M-CSF), and multi-CSF (also known as interleukin 3, IL-3). G-CSF was distinguished from the other CSFs by its ability to induce differentiation and clonal extinction in certain myeloid leukemia cell lines, and by its capacity to promote almost exclusively the development of neutrophils from normal hematopoietic progenitor cells. It shows a high degree of cross-specific activity, indicating strong evolutionary conservation.

Sources

Most tissues and freshly isolated monocytes, macrophages, fibroblasts and endothelial cells can produce G-CSF when cultured *in vitro*. Production *in vivo* is regulated, at least in part, by bacterial products: serum G-CSF levels are rapidly elevated in a variety of pathological conditions such as infection or following injection with bacterial endotoxin. Since this can occur in the absence of T lymphocytes or other leukocyte populations, cells of the nonhematopoietic tissues are presumably responsible for the production of the cytokine in response to the endotoxin. Indeed, *in vitro* data show that bacterial products can directly influence endothelial cells or fibroblasts to increase the production of G-CSF.

Molecular biology

Molecular cloning of human G-CSF has yielded purified material (rhG-CSF) that is biologically active in colony assays when used at a concentration of 5–10 pM. The mRNA encodes a 30 amino acid hydrophobic leader sequence that is characteristic of secreted proteins. A typical signal sequence cleavage site is followed by 174 amino acids, giving an *M_r* of 18 627: two disulfide bonds stabilize the molecule. Although native G-CSF is glycosylated, the sugar residues do not appear to be important for biological activity; recombinant material produced by *Escherichia coli* is apparently as active *in vitro* and *in vivo* as the native glycosylated material.

X-ray crystallography has shown that nonglycosylated G-CSF has four antiparallel bundles with crossing angles of 18°, a short 3–10 helix immediately after the first disulfide bond, and two long loops and one short loop connecting the helical core bundle. Although there is a structural relationship between G-CSF and other cytokines such as IL-2, IL-3, IL-6, erythropoietin and GM-CSF, it is most closely related to human growth hormone in terms of the four helices and folding and positioning of the helices within the core structure.

The gene for human G-CSF is single copy, does not share homology with other CSFs, and has been localized to the q21–22 region of chromosome 17 (unlike M-CSF, GM-CSF, IL-3, IL-4 and IL-5, which are found as a linked gene cluster on chromosome 5). This region of chromosome 17 is involved in the translocation between chromosomes 15 and 17 that is associated with acute promyelocytic leukemia.

Receptor

Single, high-affinity receptors, specific to G-CSF, are expressed on precursor and mature cells from the neutrophil lineage and some monocytes/macrophages. The receptor density varies from 30 to 3000 per cell and the K_d is about 100 pM. It is a 130–150 kDa glycoprotein, encoded by a single gene on chromosome 1 p35–p34.3, with a single transmembrane domain of 812 amino acids and an extracellular portion containing an immunoglobulin (Ig)-like domain, three fibronectin type-3 repeat units and the WSXWS motif common to the cytokine-receptor family. Ligand–receptor interaction is followed by rapid internalization of the complex and a slow degradation of the internalized G-CSF. Relatively little is known of the subsequent signal transduction events consequent upon G-CSF bonding to its receptor.

Hematopoietic target cells

G-CSF is a relatively poor stimulus for progenitor cell development – only a few colonies are found (in comparison with IL-3, GM-CSF or M-CSF) and these consist predominantly of mature neutrophils, although occasional monocytes and macrophages can be found. Generally, the colonies are small and contain 50–150 cells (cf. GM-CSF or IL-3 where colonies containing 10^4 – 10^5 cells can routinely be observed). However, G-CSF can work also in synergy with other cytokines: a combination of GM-CSF plus G-CSF or of M-CSF plus G-CSF can recruit proliferation and development of more primitive ‘target’ cells than either factor used alone.

In addition to its mitogenic effects on progenitor cells, G-CSF can prolong the survival time of mature neutrophils, and mature cells exposed to G-CSF demonstrate enhanced antibody-dependent cellular cytotoxicity (ADCC), and enhanced response to the chemotactic peptide *N*-formyl-Met-Leu-Phe in terms of the generation of the superoxide anion. These responses are presumably related to the role of G-CSF in the activation and recruitment of neutrophils in response to infectious agents.

Radioiodinated G-CSF has been used to determine its receptor-binding properties. It binds to (some) blast cells, promyelocytes, myelocytes, metamyelocytes and mature neutrophils, with the numbers of receptors increasing from around 300 (on blast cells) up to 3000 on mature blood neutrophils. A small proportion of promonocytes and monocytes also have G-CSF receptors, but cells of other maturing hematopoietic lineages are uniformly negative. Thus, the receptor data are in broad agreement with the

biological effects elicited by this cytokine. Analysis of leukemic cell populations has shown that receptors are present on most myeloid, but absent from lymphoid, malignancies. The receptor for G-CSF present on murine cells has recently been molecularly cloned and has some structural features shared by receptors for other CSFs and human interleukins.

Other target cells

It has been reported that G-CSF and GM-CSF can directly influence the proliferation and migration of endothelial cells *in vitro*. However, the physiological significance of this is unknown and, so far, no effect of G-CSF on neovascularization or angiogenesis *in vivo* has been reported. Also, certain solid tumor cell lines have demonstrated increased proliferative activity when exposed to G-CSF, but the significance of this effect is unclear and no growth stimulatory effect upon solid tumor cells has been reported in patients with malignant disease following treatment with G-CSF.

Preclinical and clinical studies

Existing data suggest that, in humans, G-CSF is the primary factor mediating neutrophil response to infection and neutropenia: in gram-negative and fungal infections, elevated blood G-CSF levels are found. In ‘knockout’ mice, lacking endogenous G-CSF, chronic neutropenia and impaired neutrophil function are found, suggesting that G-CSF is essential for maintaining a normal quantitative balance of neutrophil production during steady-state hematopoiesis in addition to its role in emergency granulopoiesis during infections.

In all mammalian species examined, the administration of G-CSF at a dose of about $10 \mu\text{g kg}^{-1} \text{ day}^{-1}$ leads to a dramatic increase in the numbers of neutrophils in the peripheral blood. A modest increase in the number of circulating monocytes and lymphocytes has also been reported, but no effect is seen on the levels of circulating eosinophils, basophils or platelets. In mice, G-CSF leads to a redistribution of hematopoiesis from the bone marrow to the spleen, presumably due to release and migration of early stem cells from the bone marrow to the spleen. Indeed, following treatment with G-CSF, circulating stem cell levels can increase up to 400-fold compared with controls. Such data have encouraged the use of cells harvested from the blood of patients treated with G-CSF as a source of stem cells for transplantation. Although long-term treatment of mice with G-CSF can lead to anemia as a consequence of suppression of erythropoiesis, neither

anemia nor thrombocytopenia have been consistently observed in patients following long-term treatment with this cytokine.

Phase I trials in patients (dose escalation and efficacy studies) have reproduced the effects seen in animals in terms of enhancement of circulating neutrophil levels (up to 15 times normal levels), a modest increase in monocytes and a dramatic increase in the number of circulating hematopoietic progenitor cells. The bone marrow of treated patients shows increased cellularity and a marked shift in the myeloid:erythroid ratio. Cell kinetic studies have shown that the increase in neutrophils arises via increased amplification in the maturation compartment in the bone marrow and that newly produced mature neutrophils are released into the circulation within 1 day, compared with a normal emergence time of 5 days. This effect of G-CSF is unlike that seen with GM-CSF or IL-3, where a normal emergence time of 5–6 days is seen.

Subsequent phase II and III clinical trials have shown that G-CSF can accelerate recovery of circulating neutrophil levels when given after chemotherapy in patients with malignant disease or following bone marrow transplantation. The clear advantages of this are reduced morbidity (i.e. decrease in infective episodes), shorter hospitalization, and dose intensification of chemotherapy. In

addition, G-CSF has been shown to be effective in recruiting myeloid leukemia cells into cell cycle and thus rendering them sensitive to chemotherapeutic agents, and in alleviating the neutropenia in patients with myelodysplasia, congenital neutropenia, aplastic anemia and acquired immune deficiency syndrome (AIDS).

See also: Bone marrow and hematopoiesis; Hematopoietic stem cell transplantation; Colony stimulating factors; Cytokines; Granulocyte-macrophage colony stimulating factor (GM-CSF); Interleukin 3; Macrophage colony stimulating factor (CSF-1).

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GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)

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Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the proliferation, differentiation and functional activation of hematopoietic cells. The effects of this cytokine observed *in vitro* have also been demonstrated *in vivo*, and GM-CSF now has an established role in clinical medicine.

Sources

Cellular sources of GM-CSF include T lymphocytes, macrophages, fibroblasts and endothelial cells. GM-CSF synthesis in these cells is induced by such stimuli as antigen, lectin, interleukin 1 (IL-1), lipopolysaccharide, tumor necrosis factor α and phorbol ester.

GM-CSF is undetectable in the circulation, suggesting that it is produced locally and becomes compartmentalized within the extracellular matrix.

Assays

GM-CSF is assayed biologically using clonal assays in which progenitor cells from bone marrow or peripheral blood are suspended in the presence of growth factor in a semisolid matrix. After 1–2 weeks of culture, the number of granulocyte, macrophage and mixed granulocyte-macrophage colonies that have developed are scored. GM-CSF can also be assayed using factor-dependent cell lines as target cells in

short-term assays which utilize the uptake of tritiated thymidine as a measurement of cell growth. Proliferation assays to quantitate GM-CSF have also utilized as targets primary leukemic cells isolated from patients with chronic myelogenous leukemia and leukemic cell lines. Enzyme-linked immunosorbent assays (ELISA) are also available to measure GM-CSF.

Structure

The structure of GM-CSF has been determined by both partial amino acid sequence analysis and by deduction from the nucleotide sequence of cDNA clones. Mature murine and human GM-CSFs contain 124 and 127 amino acids, respectively. In each species the mature protein is preceded by a 17 amino acid leader sequence. Although murine and human GM-CSF are 54% identical at the amino acid level, there is no cross-species biological activity. Both murine and human GM-CSF contain two potential N-linked glycosylation sites and two internal disulfide bridges. Native GM-CSF is glycosylated to approximately 23 kDa. Bacterial-derived, nonglycosylated GM-CSF has high biological activity, indicating that the attached carbohydrate is not absolutely required for the molecule to be functional. GM-CSF has been demonstrated by X-ray crystallography to combine a two-stranded antiparallel β sheet with an open bundle of four α helices. This structure is similar to that of other hematopoietic cytokines.

Molecular biology

The GM-CSF gene in both mouse and human is present as a single copy of approximately 2.5 kb that contains four exons and three introns. It has been mapped in mouse to chromosome 11 and in human to the long arm of chromosome 5 in the region 5q21-q32. In humans the IL-3 gene is located 9 kb upstream and a number of other growth factor and growth factor receptor genes also map to chromosome 5q. Interstitial deletions in this region are observed in the 5q-syndrome and in a number of therapy-related myelodysplastic syndromes and leukemias. A similar clustering of genes has been observed on mouse chromosome 11. Regulation of GM-CSF expression is both transcriptional and post-transcriptional (by alteration of mRNA stability). The human GM-CSF gene contains a proximal functional promoter region (−620 to +34) and a powerful enhancer located 3 kb upstream.

Biological effects *in vitro*

GM-CSF acts as a proliferation and differentiation factor for granulocyte, monocyte and eosinophil progenitors, and as an enhancer of the function of mature effector cells. In combination with erythropoietin, GM-CSF supports the development of erythroid burst-forming units (BFU-E) and, in combination with IL-2, promotes the growth of some T cell lines. It has also been shown to have activity on dendritic cells.

GM-CSF enhances the function of granulocytes and macrophages in immune responses. It prolongs the lifespan of neutrophils and eosinophils *in vitro* and increases their metabolic activity. Direct effects on neutrophils include degranulation, inhibition of migration, changes in receptor expression (e.g. formyl-Met-Leu-Phe receptors) and alterations in cell shape. GM-CSF also primes the neutrophil for enhanced responsiveness to a secondary stimulus by increasing leukotriene synthesis and superoxide production. The ability of neutrophils, eosinophils and macrophages to phagocytose and destroy a variety of microorganisms is enhanced by GM-CSF. This molecule is also important in increasing antibody-dependent cellular cytotoxicity towards tumor cells.

The biological effects of GM-CSF are mediated through binding to specific cell surface receptors. Receptor-binding studies identified low- and high-affinity binding sites for GM-CSF on hematopoietic cells, but also on some nonhematopoietic tumor cell lines. Because placental tissue exhibited GM-CSF binding sites, this was used as source of cDNA to clone a low-affinity receptor for GM-CSF. A second subunit was subsequently identified that, together with the first (α chain), reconstituted high-affinity binding. This β chain also participates in the high-affinity binding of IL-3 and IL-5, together with their respective α chains. Both chains of the GM-CSF receptor are members of the hematopoietic cytokine receptor family and contain characteristic structural features in the extracellular domain, including four positionally conserved cysteines and a WSXWS motif. The cytoplasmic domains contain no catalytic regions but the β chain has a membrane-proximal region with limited homology to some other receptors of the family (box 1 and box 2 motifs). It has been demonstrated that a subfamily of cytoplasmic tyrosine kinases termed the Janus kinases (JAKs) are involved in signaling by the hematopoietic cytokine receptor family. Following ligand-dependent receptor aggregation, JAKs associated with the membrane-proximal region of the receptor are cross-phosphorylated. This results in phosphorylation of the receptor itself, which provides docking sites for

SH2-containing signaling proteins including SHC and phosphatidylinositol-3' kinase. The signal transducer and activator of transcription (STAT) proteins are a substrate for JAK kinase activity and are important mediators of cytokine responses. GM-CSF signal transduction has been demonstrated to involve the JAK 1 and JAK 2 kinases and STAT 5.

Biological effects *in vivo*

Transgenic technology has provided additional information regarding the biology of GM-CSF. Overexpression of GM-CSF in transgenic mice led to the accumulation of macrophages in the eye, resulting in blindness, and infiltration of macrophages into striated muscle. This demonstrated that high concentrations of GM-CSF during development may have pathologic consequences and sounded some warnings for clinical trials.

Targeted disruption of the GM-CSF gene has resulted in homozygous mice with no demonstrable defect in steady-state hematopoiesis, but a progressive accumulation of surfactant lipids and proteins in the alveolar space. Extensive lymphoid hyperplasia was associated with lung airways and blood vessels. It would seem that GM-CSF is not essential for basal hematopoiesis but has an unexpected, critical role in pulmonary homeostasis. This role was underscored in studies where mice deficient in the GM-CSF-receptor β chain were generated and showed a similar pulmonary pathology.

The biosynthesis of recombinant GM-CSF protein has permitted a wide number of studies to be undertaken on the efficacy of GM-CSF as a therapeutic agent. As predicted from *in vitro* studies, GM-CSF has proved to be a potent stimulator of hematopoiesis when administered *in vivo*. Administration is generally well tolerated but may be associated with bone pain and influenza-like symptoms, including fever, flushing, malaise, myalgia, arthralgia, anorexia and headache. These effects are usually mild, are alleviated by antipyretics and resolve with continued administration. At higher doses, GM-CSF has been associated with a capillary leak syndrome.

GM-CSF has been used to support both standard and intensified doses of chemotherapy. The expense of hematopoietic cytokines has necessitated critical examination of cost-effectiveness, and routine clinical use has generally not been recommended simply to reduce the degree, incidence or duration of neutropenia. The therapeutic end-points of GM-CSF administration in the chemotherapy setting are improved quality of life and survival. Use at present is largely to maintain dose intensity where dose reduction is not a reasonable alternative, and to

support a regimen that has a proven beneficial impact on survival and a high (>40%) risk of febrile neutropenia.

GM-CSF has been used to manage cytopenias associated with human immunodeficiency virus (HIV) infection and is capable of increasing neutrophil counts at relatively low doses, even with the concomitant administration of zidovudine (AZT), interferon α or ganciclovir. Clinical studies have focused on the role of GM-CSF as a supportive agent in the management of secondary infection and following chemotherapy for acquired immune deficiency syndrome (AIDS)-related non-Hodgkin's lymphoma. Potential alternative uses in AIDS include augmentation of neutrophil and macrophage function and enhanced tumor cell killing. There is some concern regarding the effect of GM-CSF on HIV replication in monocyte/macrophages, although data regarding this are at present conflicting.

GM-CSF was the first hematopoietic growth factor approved for use after autologous bone marrow transplantation. In this setting, like granulocyte colony-stimulating factor (G-CSF), it has been shown to reduce the duration of neutropenia, decrease the incidence of serious infection and use of antibiotics, and shorten hospital stay. GM-CSF has been used, either alone or with chemotherapy, to mobilize peripheral blood progenitor cells (PBPCs) for collection by apheresis and subsequent transplantation. These mobilized PBPCs appear to result in more rapid hematologic recovery than nonmobilized PBPCs or bone marrow alone. In allogeneic bone marrow transplantation, GM-CSF has been used with apparent success in the management of graft failure.

Ongoing and future studies should not only more clearly define the role of GM-CSF alone in the management of a variety of clinical situations, but also examine how this cytokine might be used effectively in combination with other modulators of hematopoietic development.

See also: Hematopoietic stem cell transplantation; Colony stimulating factors; Granulocyte colony stimulating factor (G-CSF); Macrophage colony stimulating factor (CSF-1).

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GRANULOMA

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The germ granuloma was introduced by Virchow in 1865 to describe certain well-circumscribed swellings consisting of what he thought was granulation tissue, found in a number of chronic infectious diseases including tuberculosis, leprosy, syphilis and leishmaniasis. Later authors, including Cohnheim in 1877, distinguished large, swollen, epithelial-like cells. These they called epithelioid cells and differentiated them from the lymphoid elements in these lesions. In 1893, Metchnikoff formally recognized that these cells were related to the cells he had newly described as macrophages. The relation between granulomatous diseases and delayed-type hypersensitivity was recognized by Zinsser in 1925. Cells of the mononuclear phagocyte series are now considered to be the most important feature of granulomas. Although granuloma formation is generally a tissue response to particulate or fixed material in the tissues, they are not necessarily associated with infectious diseases or with delayed hypersensitivity. Hypersensitivity granulomas can be produced by the metals beryllium or zirconium and in sarcoidosis, a disease in which the role of an infectious agent has yet to be identified. Nonimmunological granulomas occur in silicosis and following the injection of metallic compounds such as aluminum hydroxide and kaolin. Nonimmunological granulomas can also occur in chronic infections where there is a specific failure of cell-mediated immunity, as in lepromatous

leprosy, systemic leishmaniasis (kala-azar) and the systemic mycoses. The granulomas of tuberculoid leprosy and localized forms of leishmaniasis are examples of hypersensitivity (immunological) granulomas and are associated with strong delayed hypersensitivity reactions, as are the granulomas produced by beryllium and zirconium (Table 1).

The predominant cell that features in nonimmunological granulomas is the typical phagocytosing macrophage. In lepromatous leprosy, this foamy cell, often referred to as the 'Virchow' cell, is packed full of globi of *Mycobacterium leprae*. In immunological granulomas, the cells of the mononuclear phagocyte system take on the appearance of epithelioid cells. These cells are poorly phagocytic and may have a rough endoplasmic reticulum and Golgi apparatus presenting a plasmacytoid appearance (type A); other epithelioid cells (type B) present a more vacuolar and lysosomal appearance. It is generally thought that epithelioid cells have a secretory function. However, no specific secretory agent has, as yet, been identified. As well as epithelioid cells, hypersensitivity granulomas contain giant cells; these are generally of the Langhans type with peripherally placed nuclei. Giant cells as a whole do not have a rough endoplasmic reticulum. Other cell types seen include lymphocytes and fibroblasts. Collagen synthesis leading to fibrosis is a particular feature of hypersensitivity granulomas. The relation between epithelioid cell

secretory function and fibroblast activation has yet to be fully defined. However, there is some evidence of increased prostaglandin and cytokine activity. Patients with immunological granulomatous diseases and silicosis are particularly associated with increased fibrosis in the area of the granulomas. Extreme examples of this are in sarcoidosis and berylliosis as well as tuberculosis. Silicosis is the only example of a nonimmunological granulomatous condition associated with epithelioid cell formation, fibroblast activation, increased collagen synthesis, and fibrosis generally in the lung.

There are two hypersensitive granulomatous states associated with a diagnostic skin test. The lepromin reaction is induced by the intradermal injection of killed *M. leprae*. The granuloma can be detected between 2 and 3 weeks as a nodular skin lesion which, on histological examination, shows all the features of an epithelioid cell granuloma. This reaction is positive in patients at the tuberculoid end of the leprosy spectrum, but negative at the lepromatous pole. In sarcoidosis the intradermal injection of an extract of spleen from a patient with sarcoidosis produces a Kveim reaction. This reaction is a nodular granuloma maximal between 4 and 6 weeks which, when examined histologically, shows all the features of an epithelioid cell granuloma.

Tissue destruction leading to caseation and cavitation is a particular feature of tuberculosis and is rarely, if at all, found in other immunological and nonimmunological granulomas. One of the agents that has been postulated as a cause of caseation is tumor necrosis factor α (TNF α), which is released from mononuclear phagocytes particularly by the action of endotoxin and can lead to vascular endothelial damage and fibrin deposition. It could be that there is increased release of TNF α by mononuclear phagocytes in patients with tuberculosis in the presence of certain components of *M. tuberculosis*, and this may account for the regular occurrence of caseation in this disease. Caseation may be a feature of the epithelioid cell granulomas or gummas of tertiary syphilis, and at the other end of the spectrum, absence of caseation is a diagnostic feature of sarcoidosis.

The association between epithelioid cell granuloma formation and delayed hypersensitivity in a number of diseases would explain the large number of T lymphocytes in the periphery of these lesions. Experimentally, it has been shown by transfer studies that granulomas in schistosomiasis are T lymphocyte mediated. They are also smaller in neonatally thymectomized animals. However, the presence of plasma cells and eosinophils in many granulomas of helminthic origin would indicate that B cells might

Table 1 Classification of granulomas

1. Immunological	
	Tuberculosis
	Tuberculoid leprosy
	Syphilis
	Schistosomiasis
	Sarcoidosis
	Zirconium
	Beryllium
2. Nonimmunological	
	Nontoxic, e.g. plastic beads, carbon particles, nontoxic metals (e.g. Fe)
	Toxic, e.g. silica, talc, asbestos
	Activation of C3, e.g. carageenan, kaolin, aluminum hydroxide

play an important part in their formation. Immunoglobulin and complement also may accumulate around eggs, particularly in *Schistosoma japonicum* infection. In immunological granulomas of T cell origin, it has been found that CD8⁺ T cells are found more peripherally in the granuloma, while CD4⁺ T cells are found more centrally.

The term 'foreign body' granuloma is used to cover the localized response to a wide range of substances that enter the body from the exterior. Granulomatous reactions may occur around thorns or other sharp objects that penetrate the skin. Many of these reactions are immunological responses against foreign antigens, either as part of the object or on microorganisms carried in by the object. Thus, the lesions are frequently epithelioid cell granulomas with giant cells and there is also fibroblast proliferation. Frequently, these lesions resolve by fibrosis. The lesions produced by talc are similar. Although talc is not antigenic, the reaction is probably related to its silica content. Silicosis does not fit easily into the simple classification of immunological/epithelioid cell granulomas leading to fibrosis and nonimmunological/phagocytosing macrophage granulomas without fibrosis. Silicosis is a highly fibrinogenic condition. It has been suggested that phagocytosis of silica leads to lysosomal damage. However, this is not the whole story as nonfibrinogenic granuloma-producing agents may have the same effect. It has been shown that asbestos, a silica-containing product, activates complement through the alternate pathway.

Mycobacterial diseases: the immunological spectrum of granuloma formation

The spectrum of granuloma formation, from the

Table 2 Ridley–Jopling scale for leprosy

	<i>Tuberculoid</i> (TT)	<i>Borderline</i> <i>tuberculoid</i> (BT)	<i>Borderline</i> (BB)	<i>Borderline</i> <i>lepromatous</i> (BL)	<i>Lepromatous</i> (LL)
Organisms (<i>M. leprae</i>)	0	0 or +	0 or ++	+++	+++
Lepromin test (Mitsuda)	++++	++	± or 0	0	0
Histology	Epithelioid cells	Epithelioid cells	Epithelioid cells	Phagocytosing macrophages	Phagocytosing macrophages

tuberculoid or high-resistance tuberculoid form to the low-resistance-phagocytic macrophage form, is known as the Ridley–Jopling scale (Table 2). Patients in the middle points of the spectrum can ‘upgrade’ and move to a higher immune position or ‘downgrade’ to a lower immune position, dependent upon the bacterial load in the tissues. However, polar tuberculoid and polar lepromatous patients usually remain in a fixed position. Patients on the TT or BT points on the scale are usually lepromin positive. The 14-day lepromin test is generally known as the Mitsuda reaction; a 48 h delayed hypersensitivity component is referred to as the Fernandez reaction. Spectra of granuloma formation based on immunological reactivity similar to that found in leprosy have been described in tuberculosis and leishmaniasis. High-resistance and low-resistance forms of granulomas may also be found in systemic mycoses such as histoplasmosis, coccidiomycosis and blastomycosis. The relation between the granuloma of the high-resistance form of disease, which is a hypersensitivity phenomenon, and resistance to infection has been a matter of controversy over many decades. Evidence for a dissociation between these two phenomena has been accumulating for some time.

See also: *Coccidioides*, infection and immunity; *Delayed-type hypersensitivity*; *Histoplasma*, infection and immunity; *Leishmaniasis*; *Macrophage activation*; *Mononuclear phagocyte system*; *Mycobacteria*, infection and immunity; *Sarcoidosis*; *Schistosomiasis*; *Treponema*, infection and immunity.

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GRANZYMES

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'Granzymes' (granule-associated enzymes) are a family of homologous serine proteases expressed specifically in the cytolytic granules of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). Individual granzymes appear to have distinct functions and while one, granzyme B (graB) is a key mediator of target cell death, others fulfil a variety of immunological functions to do with lymphocyte migration, amplification of cytokine networks, chemotaxis and may even directly reduce the infectivity of some viruses. Upon receptor-dependent conjugate formation between the effector lymphocyte and a target cell, exocytosis of the granule contents rapidly results in the delivery of a lethal signal to the target cell, resulting in apoptosis. Recent interest in granzymes has centered on the synergistic actions of graB and the pore-forming protein perforin (copackaged with granzymes in cytolytic granules) in the induction of a calcium-dependent apoptotic cell death pathway. The role of graB in apoptosis induction is dependent on its key ability to cleave substrates at specific aspartate residues, a property it shares with a family of cysteine proteases implicated in apoptosis in a range of diverse biological systems.

'Granule exocytosis' is one of two important contact-dependent lymphocytotoxic mechanisms, the other being granule- and calcium-independent and triggered by a classical receptor/ligand interaction involving the Fas ligand (FasL, CD95L) on the effector and Fas (CD95, Apo-1) on the target cell. Spontaneous (gld, lpr) and engineered (perforin or graB) gene knockout studies in mice have shown that granule- and Fas-mediated pathways are likely to have distinct functions *in vivo*. Defects in granule-mediated apoptosis result in a seriously deficient cellular immune response against some viruses (including reduced CTL, NK and delayed-type hypersensitivity (DTH) responses) and delays in rejection of some allogeneic tissue transplants, whereas inactivation or absence of Fas or its ligand cause persistence of autoreactive T cells in the peri-

phery, with consequent potentially fatal autoimmune sequelae such as nephritis. Despite this apparent dichotomy of function, it is likely that some Fas-sensitive target cells may be killed by the combined actions of both pathways.

Perforin and granzymes are the principal granule-bound CTL/NK effector molecules

The initial studies implicating protease activity in cytotoxicity were performed during the early 1980s, using generic serine protease inhibitors. Two groups headed by Pierre Henkart and Jurg Tschopp were later responsible for purifying cytolytic granules from mouse CTLs and demonstrating that they could reproduce the cytolytic activity of intact CTLs. The granules largely consisted of a battery of distinct but homologous granzymes and a potent 67 kDa membranolytic protein (perforin) which produced membrane pores structurally and functionally akin to those induced by the complement membrane attack complex. As many as eight different mouse granzymes have now been cloned, however only five (granzymes A, B, H, trypsin 2 and Met-ase) have been identified in humans. The granzymes have the 'charge relay triad' (histidine, aspartate, serine) and key active site serine residues of classical serine proteases like trypsin and can be purified on the basis of physicochemical properties such as charge and size, or by affinity purification with specific antibodies. The granzyme protein backbone is typically 26–28 kDa and is decorated to a variable extent with N-linked carbohydrate.

Each granzyme displays a particular proteolytic specificity, determined initially by cleavage of model synthetic tripeptide substrates (Table 1). GraA and trypsin 2 are trypsin-like and cleave adjacent to basic residues, GraB has a preference for acidic residues, Met-ase cleaves best at nonpolar nonaromatic amino acids (especially methionine and leucine) and the cleavage specificity of graH is yet to be defined.

Table 1 Molecules characterized from the cytotoxic granules of cytotoxic lymphocytes^a

<i>Molecule</i>	<i>Size (kDa)</i>	<i>Species^b</i>	<i>Function/specificity</i>
<i>Lytic molecules</i>			
Perforin	65–70	H,M,R	Ca ²⁺ -dependent pore-formation and synergy with granzyme B
<i>Granzymes</i>			
GraA	60 ^c	H,M	Multiple; tryptase protease
GraB	28–30	H,M,R	DNA fragmentation, Asp-ase protease
GraC	30	M	Unknown, protease
GraD,E,F	30	M	Unknown, tryptase proteases
GraG	30	M	Unknown, protease
GraH	30	H	Unknown, tryptase protease
Met-ase	30	H,M,R	Unknown, Met-ase protease
Tryptase-2	30	R	Unknown, tryptase protease
Leukophysin	30	H	Intracellular processing of cytotoxic granules
<i>Miscellaneous</i>			
Calreticulin	50	H,M	Chelates free Ca ²⁺ and keeps perforin inactive
Proteoglycans	>200	H,M,R	Bind activated granzymes electrostatically

^aThis is not a complete listing of all granule-associated proteins, only those directly relevant to this entry; other molecules described include lipase, casein, carboxypeptidase, arylsulfatase, β -glucuronidase and β -hexosaminidase; leukoloxins; TIA-15; TIAR.

^bH, human; M, mouse; R, rat.

^cDisulfide-linked homodimer of 30 kDa subunits.

Granzymes are synthesized as pre-propeptides. Following cleavage of classical leader peptides, they are transported through the Golgi, then targeted to the lysosome-like granules by binding to mannose-6-phosphate receptors. Immediately prior to packaging, cleavage of a remaining N-terminal dipeptide by dipeptidylpeptidase I results in refolding of the mature N-terminus into a cleft in the molecule, approximation of the 'charge-relay' residues and acquisition of protease activity. All granzymes are basic molecules (pI in the range 8–11) and are retained in cytolytic granules (pH ~5.5) electrostatically bound to chondroitin sulfate proteoglycans which are present in high concentration.

Perforin and graB synergize to cause apoptosis

In early studies, purified perforin was demonstrably a potent lysin (particularly for erythrocytes) but by itself was unable to reproduce apoptotic features such as blebbing of the cell membrane, DNA fragmentation and nuclear collapse in nucleated cells. Paradoxically, perforin-deficient mice first produced by Kagi and Hengartner exhibited normal T cell development and cytokine secretion but were immunodeficient due to a markedly reduced ability to induce target cell apoptosis. Kagi's observations were consistent with the hypothesis advanced by Greenberg and his colleagues that the combination of graB (fragmentin-2) and tiny (sublytic) doses of perforin could synergize to produce DNA fragmentation. Greenberg indeed demonstrated that cells

exposed to just these two purified molecules underwent apoptosis. Like perforin, graB applied alone to cells did not cause apoptosis. The requirement for an 'Asp-ase' specificity was demonstrated in that proteolytically inactive graB could not produce apoptosis, while other granzymes with non-Asp-ase activities (fragmentins 1 and 3, both of which possess trypsin-like activity) produced far slower apoptotic changes in combination with perforin.

Over the past several years, the mechanism of granzyme/perforin synergy has been the topic of intense investigation. Target cells loaded with protease inhibitors such as aprotinin could withstand apoptosis by CTLs, suggesting that graB acted intracellularly. This was confirmed by our own studies with purified fluorescein-conjugated graB which showed that cells co-exposed to perforin rapidly took up graB into the cytoplasm and ultimately also into the nucleus. These results were further supported by immunoelectron microscopy, which demonstrated graB within cytoplasmic vacuoles and free in the cytosol, while perforin staining was confined to the target cell membrane. Because it can form membrane pores, perforin had long been postulated to permit the diffusion of toxins such as granzymes into target cells. However this 'simple diffusion' model seems unlikely to be valid, as cells exposed to graB alone became vacuolated and contained cytoplasmic graB. Furthermore, graB/perforin-induced apoptosis could not be reproduced by substituting alternative pore-forming and membranolytic agents (including complement) for perforin. Overall, these findings suggested a highly specific requirement for graB

which cleaves intracellular substrates and the generation of an equally essential but yet undefined signal at the cell membrane by perforin.

As perforin and graB can kill a very broad variety of cells, it is likely that the signal generated by perforin utilizes a pathway present in virtually all cells. Whether the uptake of graB is receptor-mediated and/or due to endocytosis remains unclear. GraA has been shown to cleave the thrombin receptor and possibly other similar, unidentified cell surface receptors. Perforin, by contrast, undergoes a change in its tertiary structure from a globular to an extended configuration in the presence of calcium ions and binds to phosphorylcholine moieties in natural (and artificial) membranes to form oligomeric polyperforin clusters consisting of up to 18 monomers.

Generic cell death cysteine proteases are activated by graB cleavage

Studies in the helminth *Caenorhabditis elegans* have elucidated a key cysteine protease, CED-3 with Aspase specificity that is essential for the death of 131 somatic cells programed to undergo apoptosis during normal embryogenesis. The first mammalian homolog of CED-3 recognized was interleukin 1 β -converting enzyme (ICE), which was initially identified for its cleavage (at Asp residues) and activation of IL-1. All the caspases (ICE-like proteases) are synthesized as proenzymes which require cleavage at a specific Asp residue to produce an active heterodimeric molecule. Further autoprocessing then results in cleavage (also at Asp) of the propeptide, resulting in the fully active mature form. Expression of active ICE in a variety of cell lines resulted in apoptotic cell death. Subsequently, three subfamilies of related cell death proteases have been identified, whose individual members share closest structural similarity to either CED-3, ICE or ICH-1 (Nedd-2). It is the current view that some or all of these proteases are expressed in an inactive form in virtually every mammalian cell, and can be activated in response to a very diverse range of apoptotic stimuli including toxins, irradiation, growth factor withdrawal and intracellular pathogens.

That the Aspase activity of graB was indispensable for perforin/grab-mediated cell death strongly suggested it might activate ubiquitous cysteine protease cell death pathways. The lack of requirement for protein or RNA synthesis in CTL/NK-mediated apoptosis is consistent with latent cytoplasmic ligands/substrates 'ready' for cleavage by graB. Strong evidence now exists that graB can cleave multiple cell death proteases of the CPP32 (CED-3-like) subfamily that constitute a protease cascade in the

cytoplasm of target cells (Figure 1). CPP32 (also termed apopain and YAMA-1) activation by cleavage at Asp175 is followed by autoprocessing at Asp9, resulting in the fully active 19/12 kDa form that can cleave nuclear structures such as poly-(ADP ribose) polymerase (PARP). Cleavage (and inactivation) of PARP and other enzymes including DNA-dependent protein kinase (DNA-PK) has been identified in nuclei early in the apoptotic event. This can result in curtailed DNA repair and derepression of nucleases that then cleave exposed internucleosomal sites on DNA, contributing to DNA degradation and precipitating nuclear breakdown. In addition, graB can activate cytoplasmic proMch4 which in turn cleaves proCPP32 and proMch3, further amplifying the proteolytic cascade. Activation of Mch2 then results in the proteolysis of structural nuclear proteins such as lamins.

Evidence also exists that some graB substrates, such as proMch4 and proMch5 (FLICE), are high in the proteolytic cascade, and may also be able to couple Fas ligation to downstream proteolytic events. This is achieved in that proMch4 and proMch5 both contain two N-terminal FADD-like domains. These regions may associate with FADD bound to the cytoplasmic domain of Fas following FasL binding and receptor aggregation, and lead to activation of the cysteine protease domains of Mch4 and Mch5 to trigger downstream events in the cysteine protease cascade. Thus, in some instances CTLs may be able to activate proMch4/5 via Fas, and in other instances may use graB to activate them directly.

Remarkably, some viruses have developed ways of subverting generic cell death pathways by carrying protease inhibitors (serpins) whose expression can subvert 'altruistic' apoptosis of a cell, leading to a prolongation of the cell's life and greater time for viral replication and assembly. For example, the protein encoded by the cowpox crmA gene can block ICE activation and prevent apoptosis, while the baculovirus protein p35 can derail apoptotic mechanisms by a similar process. Serpins may also inhibit both cysteine and serine proteases of like-specificity (cross class inhibition). It is interesting that crmA is far more effective at blocking ICE than graB, suggesting that cells in which ICE activation has been blocked might still be susceptible to CTL/NK-mediated apoptosis due to graB.

Other functions of granzymes

Due to a multiplicity of protease specificities, granzymes have been suggested to contribute to many lymphocyte functions including extravasation

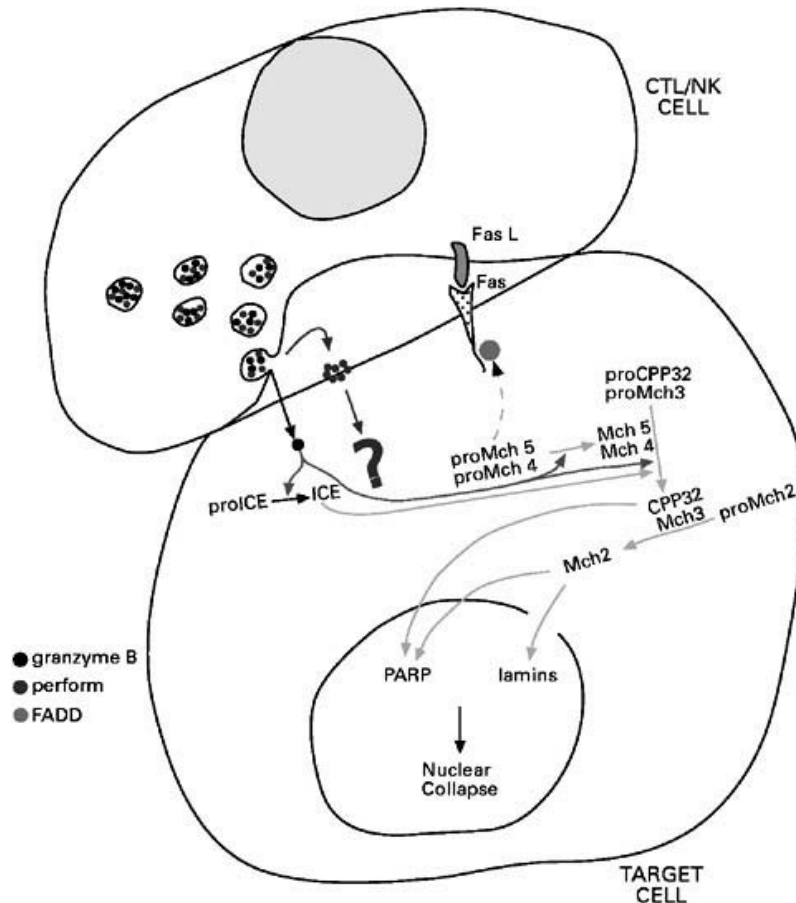


Figure 1 A hypothetical outline of the granule exocytosis mechanism of cellular cytotoxicity. Upon receptor-mediated conjugate formation between the effector lymphocyte and the target cell, cytolytic granules containing perforin and graB become oriented towards the site of cell–cell contact ('polarization'). Exocytosis of granule contents into the closed space between the two abutting cells permits perforin to intercalate the target cell membrane and polymerize in a Ca^{2+} -dependent manner. This generates a key signal permissive for apoptosis, the nature of which is poorly understood. Simultaneously, graB (and presumably other granzymes) enter the target cells, possibly by endocytosis, and are liberated into the cytosol. The Asp-ase activity of granzyme B is then instrumental in initiating a protease cascade within the target cell. Granzyme B cleaves (and activates) components of the apoptotic cascade at highly specific aspartate residues, resulting in activation of upstream proteases including Mch4 and Mch5 (FLICE), as well as CPP32 and ICE, which exert their effect further downstream. The result is amplification of the cascade and production of further activated proteases which can neutralize DNA repair enzymes such as PARP and DNA-PK and structural nuclear proteins such as lamins. This leads to DNA fragmentation and nuclear collapse, and the familiar apoptotic morphology. ProMch4 and ProMch5 (or highly analogous proteases) may also be recruited to the inner surface of the plasma membrane to subserve FasL-mediated signal transduction. These proteases contain conserved FADD-like domains which may interact with FADD molecules bound to the cytoplasmic tail of Fas upon Fas ligation. This interaction with FADD could result in activation of Mch4 and Mch5 with triggering of the proteolytic apoptotic cascade in the absence of granzyme B.

through capillary endothelium and penetration of intercellular connective tissue by mature T cells. GraB was also able to cleave molecular interactions between certain tumor cell lines and extracellular matrix proteins via the Arg-Gly-Asp (RGD)-binding motif, thereby inhibiting cell proliferation. To date, specific functions for Met-ase, graH and tryptase 2 have not been elucidated, however Met-ase is interesting in that its expression is restricted to NK cells, and therefore presumably plays a specific role in NK function.

The trypsin-like activity of graA has been shown

to cleave several connective tissue proteins including fibronectin and collagen *in vitro*. GraA can also cleave the thrombin receptor at the sequence Arg-Ser-Phe-Leu-Leu on various cells, stimulating such pleomorphic effects as contraction of processes on astrocytes and secretion of IL-6 and IL-8 from monocytes. GraA can also be trophic for fibroblasts and B lymphocytes, presumably by transducing a proliferative signal by cleaving analogous cell surface receptors.

Perhaps most remarkably, mice with a targeted disruption of the gene encoding graA generally dem-

onstrate normal immune-mediated apoptosis, but are extremely susceptible to infection with the poxvirus *Ectromelia*, possibly as the result of an inability to cleave envelope proteins that mediate adhesion to cell surface receptors. This is an important finding, as it indicates that some granzymes may have evolved highly specialized functions which are distinct from a direct role in cytolysis. The delineation of such specific granzyme functions will be a laborious task in a world populated by an almost limitless array of pathogens.

See also: Apoptosis; Cell-mediated immunity; Cytotoxic T lymphocytes; Cytotoxicity, mechanisms of; Degranulation; Effector lymphocytes; Exocytosis; Fas (CD95) and fas ligand; Cell-mediated lysis; Lymphokine-activated killer (LAK) cells; Natural killer (NK) cells; Perforin; T lymphocytes.

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GRAVITY, EFFECT OF SPACE FLIGHT ON IMMUNITY

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Soon after astronauts, cosmonauts and experimental animals began to travel in outer space, postflight studies of their immunological markers revealed differences from preflight controls. Differences included neutrophilia, slight T cell lymphopenia and a diminished blastogenic responsiveness of T cells in postflight blood samples. Eosinophilia was sometimes seen, even after flights of only several days duration. Neutrophilia (observed also in animals) was almost

always present, reaching values 2–3 times above normal, but there were no accompanying increases in band forms or other immature cells.

Lymphopenia, most frequent and consistent in cosmonauts who participated in prolonged missions, was typified by reductions in numbers of T cells and natural killer (NK) cells. Rats exposed to the microgravity of outer space for 20 days showed postflight hypoplasia of spleens and thymic glands, with

a markedly reduced number of lymphocytes in these tissues.

Changes were also noted in the postflight concentrations of immunologically important plasma proteins, including immunoglobulins, complement components, lysozyme, interferon and α_2 -macroglobulin.

Although postflight laboratory abnormalities reverted to normal within a week or two, they were not the only immunological concerns. Minor infections occurred with some consistency during space flights. Careful postflight dental examinations often revealed areas of newly acquired gingival inflammation. Also, the microbiological environment of space vehicles showed consistent changes (in both the US and USSR programs), with decreases in anaerobic bacteria and fungal isolates, and increases in potentially pathogenic organisms.

Despite recirculation of cabin air through microfilters, microgravity caused a suspension of food particles and droplets of liquids from all sources, including waste disposal systems. Thus, microorganism-containing aerosols tended to be the rule, rather than the exception. It was not surprising then, that the microflora of space travelers also showed extensive alterations. A broad redistribution of microbial species occurred among crew members, with increased numbers of body sites harboring microorganisms. Altered body microflora of space travelers included an increased number and variety of dermal organisms, and the presence of potentially virulent bacteria (including *Mycoplasma* and *Staphylococcus*) on the upper respiratory mucosa.

Because of concerns about the immunological effects of prolonged exposure to microgravity, NASA program managers sought the advice of senior immunologists. Ensuing research recommendations, provided by a FASEB Committee in 1985, were widely implemented during the next decade in both the US and USSR space programs. Inflight studies were conducted in humans, experimental animals and cultured cells, and ground-based surrogate model systems were developed. Immunological data on space flights in excess of several weeks were almost exclusively Russian.

Granulocyte and monocyte-macrophage findings

Granulocytosis was observed consistently in astronauts after short duration shuttle flights, but numbers of eosinophils and monocytes tended to decline after 6–8 days in space. Similarly, increased numbers of blood neutrophils were also seen in postflight rats, while eosinophil and monocyte counts were reduced. No reports have mentioned any effects of micro-

gravity on granulocyte diapedesis, chemotaxis, phagocytosis or superoxide formation.

Marrow cells obtained from rats flown in space showed decreased responses to granulocyte-monocyte colony-stimulating cytokines. Rat monocyte-macrophages also showed a reduced spreading, *in vitro*, and their differentiation was inhibited. There was an increase in a population of smaller, less granular blood monocytes which failed to express receptors for insulin or insulin-like growth factor I.

Cell-mediated immunity and T cell changes

The more recent studies of lymphoid cells confirmed a modest depression in cell-mediated immunity after both short and long (over 1 month) space flights. Inducer and cytotoxic T cell populations declined. Decreased numbers of both CD4⁺ and CD8⁺ lymphocytes were found in postflight blood samples of 30 shuttle astronauts. Humans were also found to have postflight reductions in phytohemagglutinin-triggered blast transformation of cultured blood lymphocytes. After prolonged stays in space, the proliferative index of cosmonaut T cells showed reductions in xenogenic graft-versus-host reactions.

Human NK cells showed reduced numbers and cytotoxic activities after both long and short space flights. NK cells from the marrows and spleens of rats flown in space showed significantly inhibited *in vitro* cytotoxicity for target cells, although their binding to these cells appeared to be unaffected. The surface expression of some cytokine receptors was reduced in rat NK cells.

Delayed-type dermal hypersensitivity (DTH) reactions showed reductions, or even disappeared, during protracted stays in space. When tested during space missions, DTH reactions were consistently reduced after the fourth day of weightlessness. Inflight measurements of DTH were facilitated by the use of a multitest skin puncture device that simultaneously injected predetermined doses of seven recall antigens and one control.

Cell-mediated immunity was also studied in rats. After short flights, rats showed decreased numbers of blood CD4⁺ and CD8⁺ lymphocytes. The proliferative response of rat spleen cells to mitogens or to interleukin 2 (IL-2) was inhibited after 1–2 week flights. After 10 days in space, the phenotype of rat lymph node or spleen lymphocytes showed several tissue-specific differences, including an increased percentage and fluorescent intensity of splenocytes which expressed CD4⁺ and CD8⁺ markers, as well as the integrins LFA-1 α and β . In contrast, these

markers remained unchanged, or showed decreases, in lymph node cells from the same rats.

The response to concanavalin A of cultured cells from lymph nodes (but not spleens) of space-flown rats was depressed, and could not be restored by supplementation with either IL-1 or IL-2. However, these cultured cells showed normal responses to other mitogens, normal production of IL-2, and normal expression of surface markers. Because some of the immunological effects of microgravity appeared to be lymphoid organ specific, it was postulated that the findings may have been influenced by variations in lymphocyte trafficking, brought about by body fluid compartment shifts associated with microgravity.

Humoral immunity and B cell observations

In contrast to the expanding database showing subtle dysfunctions of cell-mediated immunity, evidence has not shown the presence of concomitant defects in humoral immunity. After space flight, B cell numbers have decreased in rats, but their percentages, relative to total lymphocyte counts, remained unchanged. In a single study, B cell responses to lipopolysaccharide were increased in rats flown in space for 19 days.

The few attempts to study the antibody-mediated humoral immune system have been inconclusive. However, the productive phase of the specific immune response to T cell-dependent antigens was not inhibited by space flight. *In vivo* responses to antigenic challenge have yet to be reported. During long space flights, immunoglobulin A (IgA) and IgM concentrations were sometimes increased, but IgG values remained constant.

Cytokine responses

The cytokine network is influenced by space flight, with changes being observed in preliminary, exploratory studies of the production and activity of interferons, interleukins, colony stimulating factors and tumor necrosis factor. After long flights, lymphocytic production of IL-2 and interferon was significantly decreased in both humans and rodents. In contrast, human lymphocyte cultures produced interferon more intensely during shorter spaceflights, but responded poorly to concanavalin A stimulation. In addition, the ability of cultured human postflight lymphocytes to express surface IL-2 receptors was blunted, but this expression could be restored by a protein kinase C-activating phorbol ester. Lymphocytes of rats flown on a 10 day space mission showed a decrease in IL-2 production in response to a T cell

receptor-independent mitogen, but not to receptor-dependent mitogens. Lymphocyte-produced interferon, but not IL-3 production, was reduced in rats after 6 days in space.

Cell culture studies in microgravity

Single cells in culture change their behavior when studied in the microgravity of space. When a standardized lymphocyte-monocyte population was isolated from blood, resuspended in culture fluid, and flown in space, monocytes virtually failed to produce IL-1, and the activation of lymphocytes by concanavalin A was markedly inhibited. However, when these same cells were attached to microcarrier beads before a flight, T cell activation was doubled, and their production of interferon and IL-2 was greatly enhanced. It thus appeared that certain immunological effects of microgravity were caused by altered cell-to-cell interactions.

Conclusions

Although a myriad of questions remain, available evidence clearly defines a blunting of cellular immune mechanisms during space flight. But not all immunological functions are affected equally. The major variable during space flight is microgravity, with its primary impact on the distribution of body fluids and on cell-to-cell adhesions and interactions, and their secondary immunological consequences. But microgravity is not the only variable that must be considered. Short- and long-term effects of ever-varying solar and space-generated radiation can have troublesome, but yet unquantified effects on the immune system of space travelers. The altered and redistributed microbiological flora of space vehicles, and their crews, undoubtedly impact on body defenses and immunological functions, as do stress-induced responses of the endocrine and neuroendocrine systems.

Infectious diseases do occur with some frequency during space flights, but the clinical relevance of documented immune system changes has not been proven. Although the immunological dysfunctions observed in laboratory tests have not as yet been accompanied by serious communicable infections among crew members, immunological concerns remain highly relevant whenever longer and more distant space travels are planned.

See also: Neuroendocrine regulation of immunity; Radiation, effects on immune system; Stress and the immune system.

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GUINEA PIG, INBRED STRAINS OF

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The most widely used inbred strains of guinea pig in immunologic studies are the strain 2 and strain 13 animals which have been maintained for many years at the National Institutes of Health in Bethesda, Maryland, USA. These two strains originated from a colony maintained by the Department of Agriculture in Washington, DC. In 1896, this colony was moved to Bethesda, a distance of 11 miles. However, during the course of the move, the covered wagons containing the guinea pigs encountered a severe rainstorm. Following an outbreak of pneumonia, only 54 animals from the original colony of 800–990 animals survived. In 1906, a series of experiments to study the effects of inbreeding were started and 24 inbred families were established. Family 2 and family 13 originated from distinct males. In the 1920s, a crisis in funding led to the elimination of most of the inbred families, but families 2 and 13 were preserved because they were the most vigorous. These two strains of animals were used by Sewall Wright in studies of the population genetics of special traits including spotting, digit number, otocephaly, sizes and shapes of internal organs, and resistance to tuberculosis.

MHC class II molecules

Although strains 2 and 13 animals were also used by Wright for studies of transplantation antigens, very little information is available about the results of these studies. It was shown that the two strains had become sufficiently homozygous for pieces of organs transplanted between members of the same strain to

be accepted. In the late 1950s, it was shown that intrastrain skin grafts were successful, with no indication of Y chromosome incompatibility, as shown by the acceptance of male skin by females. All the interstrain grafts were rejected, with a mean survival of 8 days. An estimate of the number of histocompatibility gene differences was derived by challenging 27 F2 individuals with grafts from each parental strain. Fourteen grafts survived, giving a survival rate of 26%. This level of survival would correspond to five independent genes. The distribution of survival ranged from 8 to 52 days, with a suggestion of two modes at 8 and 14 days. Thus, it seemed possible that two major histocompatibility complex (MHC) loci could separate the two strains.

The guinea pig was used as an experimental animal for a large number of immunologic studies in the 1960s and 1970s, including the identification and characterization of MHC-linked immune response genes. Because inbred strains 2 and 13 animals differed in the expression of these genes, cytotoxic antisera were raised by cross-immunization of the strains. Comparative studies of the tissue distribution and the biochemical properties of the antigens identified by anti-2 and anti-13 sera demonstrated that they appeared to recognize cell surface antigens which were the guinea pig homologs of the products of the I region of the mouse H2 complex. These antigens were restricted in their expression to lymphocytes and monocytes/macrophages and could not be identified on nonlymphoid cells. In contrast to other species, guinea pig Ia antigens were readily identifiable on resting T cells, although

their expression is less than that seen on B lymphocytes.

Biochemical studies of the antigens identified by anti-2 and anti-13 sera confirmed their relationship with the murine I region products. At least three different types of Ia-bearing molecules, each with a characteristic molecular structure, could be identified. Sequential precipitation studies have shown that the Ia2 determinant expressed by strain 2 and Ia3 and Ia5 determinants expressed by strain 13 animals can be found on molecules composed of 25 and 33 kDa chains in noncovalent association. The Ia4 and Ia5 determinants of strain 2 and Ia7 determinants of strain 13 are borne on 58 kDa molecules in which the two chains are linked by disulfide bonds. The Ia1 and Ia6 determinants of strain 13 are found on a single chain structure of 26 kDa. These findings are most consistent with a model that the guinea pig I region is composed of three subregions, each of which encodes an Ia determinant borne on a molecule with a characteristic chemical structure.

MHC class I molecules

The identification of guinea pig MHC class I molecules was facilitated by analysis of cytotoxic antisera independently raised by cross-immunization of random bred guinea pigs maintained in a closed colony by de Weck at the Institute for Clinical Immunology, Bern, Switzerland. Three sera were obtained which identified antigens controlled by a single locus or region. The antigens identified by these sera were termed GPLA antigens B1, B2, and B3 (originally B,C,D). Strain 2 and strain 13 animals were both found to bear the B1 antigen and to lack B2 and B3.

A number of strains were established by de Weck and coworkers which were homozygous for the B1, B2 and B3 antigens. An additional antigen termed S was identified as was found to be present in inbred strain 2, strain 13 and some outbred animals. Antigen S was distinct from the antigen identified by anti-B1 sera, although some anti-B1 sera prepared in B3 animals also contained anti-S activity. Almost all B2-bearing outbred animals were S positive, whereas B3-bearing outbred animals were uniformly S negative.

Studies of the tissue distribution of the B series of antigens demonstrated that, in contrast to the antigens identified by anti-2 and anti-13 sera, they were readily identifiable on all cells, including nonlymphoid cells. The identity of these antigens to murine and human MHC class I antigens was confirmed by immunoprecipitation studies which revealed that the B antigens were composed of two chains of molecular weights of approximately 12 and 45 kDa. The 12 kDa peptide was shown to be the guinea pig equivalent of human β_2 -microglobulin. The biochemical characteristics of the S antigen were identical to those of the B antigens and it is likely that S represents the product of a second MHC class I locus.

Distribution of MHC antigens in various inbred strains

Table 1 summarizes the distribution of all the known specificities of the guinea pig MHC (GPLA complex) in the different strains that have been studied. For reference, we have included the known genetic polymorphisms of properdin factor B and the second and fourth components of the classical complement pathway. The genes coding for these three complement

Table 1 The specificities of the GPLA complex

Strain	B region			Cp region ^a			I region		
	B locus	S locus	Source ^b	BF locus	C4 locus	C2 locus ^c	Subregion I	Subregion II	Subregion III
2	B1	S1	NIH	SS	FF	A1A1	1a2	1a4,5(?6)	1a(?6)
13	B1	S1	NIH	SS	FF	A1A1	1a3,5	1a7	1a1,6
BI0B	B1	S1	WEK	FF	SS	BB	?	—	1a1,6
BI0C	B2	—	WEK	SS,FS	FF,FS	A1A1,A1B	1a8	1a7	1a1,6
BI0AC	B2	—	WEK	SS	FF	A1A1	1a3,5	1a7	1a1,6
BI0AD	B3	—	WEK	FF	SS	AA	1a3,5	1a7	1a1,6
R9	B3	—	LAC	FF,FS,SS	FF,FS1,S1S1	A1A1,A1B,BB	1a3,5	1a7	1a1,6
B/Lac	B3	—	LAC	FF	SS	NT	1a8	1a7	1a1,6
OM3	B3	—	LAC	FF	S1S1	BB	1a3,5	1a7	1a1,6
PCA	B3	—	NIH	NT	NT	NT	1a3,5	1a7	1a1,6

^aData supplied by D Bitter-Suermann (personal communication).

^bNIH, Division of Research Services, NIH, Bethesda, MD, USA; LAC, Laboratory Animals Centre, Carshalton, Surrey, UK; WEK, Institute for Clinical Immunology, Inselspital, Bern, Switzerland.

^cA, Acidic; B, basic; NT, not tested.

components are also linked to the GPLA complex. The order on the chromosome is arbitrary and the Ia antigens have been grouped on the basis of structural homology. In studies of outbred animals, there is a closer association between the B- and S-region antigens than between either the B- or S-region antigens and any of the I-region antigens. It is therefore likely that in the guinea pig, the I region lies outside the regions coding for the two MHC class I antigens. It has been observed in breeding studies that a cross-over occurred between the C4 allotype and the Ia antigens, which suggests that the region for complement proteins (Cp) maps either between the B and S regions and the I region or outside the rest of the GPLA complex.

Although the inbred guinea pig was widely used in the past as an experimental model for studies in immunology, very few studies have continued to use

inbred guinea pigs and large numbers of these animals are no longer available.

See also: Antigen presentation via MHC class I molecules; Contact hypersensitivity; Immune response (I_r) genes; Inbred strains.

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H

H2 CLASS I

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Mouse class I major histocompatibility complex (MHC) encompasses the classical class I genes (also known as class Ia genes) K, D and L as well as approximately 25 additional sequences (known as class Ib, formerly known as nonclassical class I) which include the TL and Qa region genes and the less closely related CD1 genes. Class Ia molecules survey the proteins produced inside cells and display a selection of peptides derived from those proteins on the cell surface for inspection by T cells. This allows the immune system to constantly monitor the array of proteins produced by cells and detect those which are infected by viruses or bacteria by the appearance of new peptides bound to class I proteins. This same function can also be of use in detecting tumors; especially those caused by viruses. The function of the class Ib proteins is largely

unknown, but some have been shown to bind antigens and present them to T cells.

History

Mouse MHC class I proteins are encoded by a multi-gene family of about 30 members. The classical antigens K, D and L, were first described by Peter Gorer who was attempting to define blood groups in mice in a manner similar to Rh blood groups in humans. Gorer found that if he immunized mice of one inbred strain with cells from another he could produce antibodies that would agglutinate the red blood cells of the donor and some other strains, but not the recipient strain. The agglutinating activity which would become H-2 was first defined as antigen II. The most

important breakthrough came when he collaborated with George Snell and showed that the genetic locus responsible for the blood groups he studied, was the same as the locus Snell studied which defined tumor graft rejection. Coupled with Snell's first production of congenic mice, this allowed the genetic definition of the complex H-2 locus. This complex is now referred to as H2. The congenic strains allowed Snell and many other investigators to define the function of the MHC by comparing the immune responses of strains that differed only in the H2 complex.

Structure of class I proteins

The K/D/L proteins are trimers consisting of β_2 -microglobulin, which is invariant, a polymorphic heavy chain and a highly variable peptide. The 45 kDa polymorphic K/D/L heavy chain is further divided into specific domains. The $\alpha 1$ and $\alpha 2$ domains each donate a helix and four strands of anti-parallel β sheet. These combine to form a cleft with two α helices running along the edges, a β sheet as the floor and the peptide bound in the middle, like a hot dog in a bun. This structure is quite robust. All the class I protein structures are very similar. The overall structure is independent of the allele or peptide bound. While the primary amino acid sequences of different class I molecules can differ by up to 30%, and they have completely unrelated peptides bound, the α carbon backbones are nearly identical. Surprisingly, the peptide, which provides much of the specificity of interaction with CD8 T cells, is largely buried in the structure with only a few atoms directly accessible to the T cell receptor.

Genetics of class I genes

Polymorphism

Many genes are polymorphic. Different individuals in a species have distinct sequence variants in the coding sequences of expressed genes. Class I genes are uniquely polymorphic in two regards. First, the sequences of alleles differ not by the common one or two amino acids, but by as much as 30% of their amino acids. Secondly, while most polymorphic loci have a few major alleles found at high frequency and variants found at low frequency, class I genes have a much larger number of alleles. Indeed, a new class I allele is found in almost every new mouse population which has been sampled. More than 100 distinct class I genes have been described in mice.

It has been suggested by many investigators that this polymorphism, which is a prominent feature of class I genes, is crucial to its function in resistance to

infectious disease. Thus the existence of many alleles confers on the species the ability to survive the introduction of new pathogens, since it is likely that at least one of the class I genes will be able to bind and present peptide from the new pathogen to T cells and that individual would be able to mount a protective immune response. Thus this polymorphism provides a great evolutionary advantage to species which possess it. **Figure 1** shows the structure of the K^b molecule with the vesicular stomatitis virus 8mer (RGYVYQGL) bound. The polymorphic residues in each domain are highlighted. As can be seen, they are not randomly distributed throughout the protein. They are clustered in the region that is necessary for peptide binding.

In contrast to the K, D and L genes, the remainder of the class I genes are much less polymorphic in sequence. Thus the sequence similarity among Tla and Qa genes from different mouse strains is limited to a small number of changes, which are found only rarely.

Gene structure

All class I genes share a similar organization in genomic DNA. They are composed of eight exons. The encode the cleaved leader sequence, two exons which comprise the peptide-binding domain (see Structure), a third extracellular domain which binds β_2 -microglobulin and five small exons which comprise the transmembrane and intracellular portions of the molecule.

Gene mapping

The majority of class I genes are located in a cluster on chromosome 17, at about 20 cM. This region has been extensively studied and physical maps have been derived from cosmid cloning of this region. The K and D genes flank the class I and class II regions. The Tl and Qa regions are located centromeric from the classical class I genes, with the Qa genes close to D. It is of interest that the sequences of the genes in the Qa and TL regions are conserved, but it is the organization and number of genes that varies among different inbred strains of mice rather than the sequences of the genes. One other class I-related gene deserves special mention: the CD1 genes map on chromosome 3, at 48 cM. This small gene family (four genes) is related in both sequence and function to the classical class I genes, and may be involved in antigen presentation of nonpeptide epitopes to T cells.



Figure 1 (A) Ribbon diagrams of the K^α molecule with the vesicular stomatitis virus 8mer (RGYVYQGL) bound. Polymorphic residues in the α1 domain (exon 2) are highlighted in blue, α2 (exon 3) in red, and α3 (exon 4) in purple. (A) T cell receptor view of the complex. (See also color **Plate 8A**.)

Function of class I proteins

The major function of the K, D and L class I proteins is to bind peptides produced inside the cell and to display them on the cell surface for inspection by the immune system. The result of this display of a new peptide/class I complex on the cell surface is the recognition of the complex by CD8⁺ cytotoxic T cells. This interaction results in the destruction of the cell expressing the complex. In a sense, expression of a virus-derived peptide by a cell is a 'KILL ME' sign. As a result, it is important that only the cells expressing the viral protein express the peptide on the cell surface. This has resulted in a mechanism in which the binding of the peptide to the class I protein is integral to its biosynthesis and assembly in the endoplasmic reticulum. The exchange between class I molecules on different cells is therefore minimized.

Synthesis and assembly of class I

MHC class I molecules are trimers with a single invariant light chain, β₂-microglobulin, which is complexed with the polymorphic K, D or L proteins and a peptide. On any given cell, many different peptides are bound to each kind of class I molecule, but only one peptide is found in each β₂-microglobulin K/D/L peptide trimer. Further, each cell will express a K, D and L molecule from each parent. Thus there is an array of different molecules on the cell surface. It is important to emphasize the relative lack of interchange of peptides on class I molecules. If there was rapid exchange of peptide between class I molecules on different cells, it could result in destruction of uninfected bystander cells which would be dangerous to the host during the immune response.

Class I heavy chains and β₂-microglobulin are syn-



Figure 1 (B) side view of the molecule. (See also color **Plate 8B**.)

thesized on ribosomes of the rough endoplasmic reticulum (ER) and are cotranslationally inserted into the lumen of the ER (**Figure 2**). There, they associate on the peptide-loading complex. The newly synthesized class I heavy chain first binds the chaperone, calreticulin. The complex then moves to the peptide-loading complex. This complex is composed of the peptide transporters TAP1, TAP2 and tapasin.

TAP1 and TAP2 are members of the ABC (ATP-binding cassette) transporter family. Association with this complex is required for efficient loading of peptides on to class I. Only when peptide is assembled into the K/D/L β_2 -microglobulin complex does the molecule achieve its final and stable conformation. It is then released, transferred to the Golgi complex and exported to the cell surface.

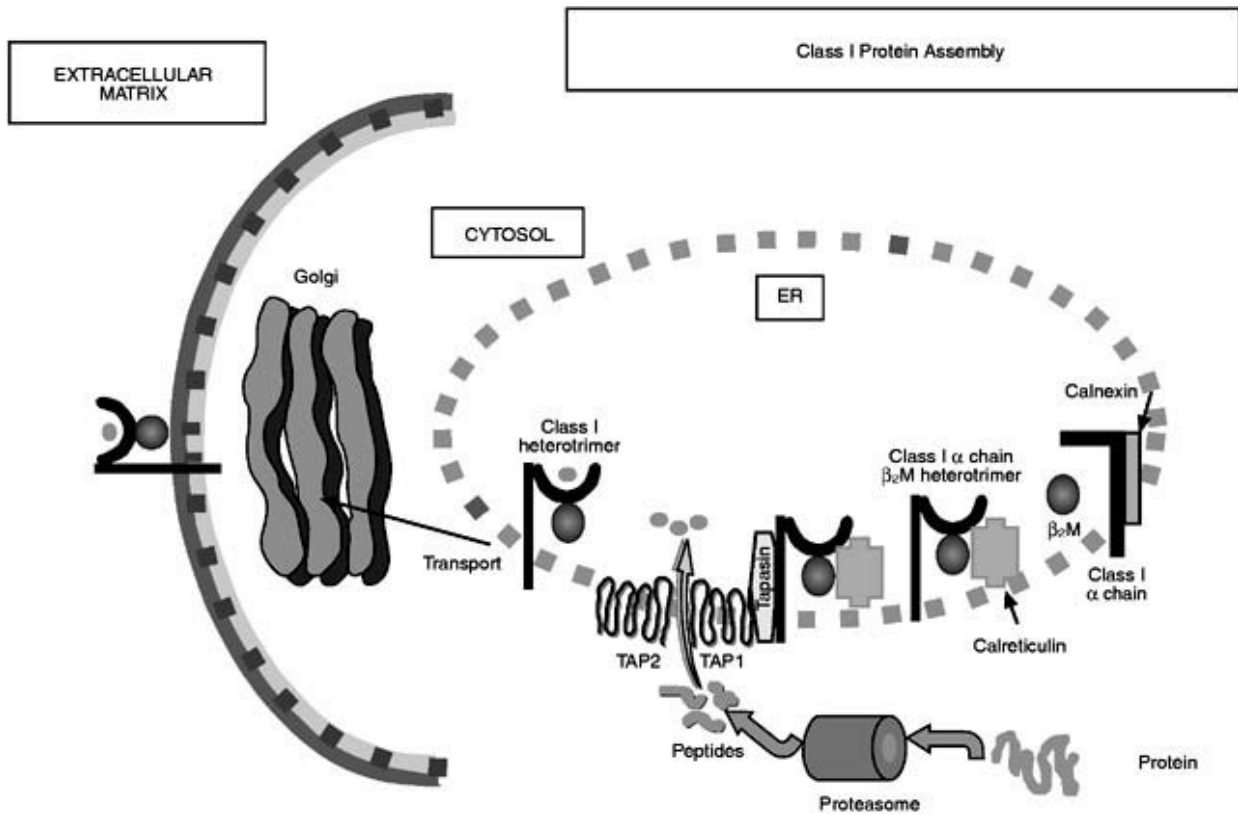


Figure 2 Pathway of class I assembly inside the cell. In the absence of the molecular interactions shown, minor pathways can be detected. The change in the shape of the heavy chain denotes a change in the molecule detectable by monoclonal antibody. (Drawn by Dr Amy Peace-Brewer, University of North Carolina.)

Specificity of peptide binding to class I

Class I molecules must be able to bind peptides with a variety of sequences. Thus, the binding must lack the exquisite specificity of antibodies. At the same time we know from biochemical binding experiments that binding is limited. These molecules utilize specific interactions with specific amino acids called anchor residues, as well as contacts with other atoms in the peptide. The anchor residues differ among the different K/D/L proteins, both in the position of interaction of the peptide and in the amino acid in the anchor position. For example K^k binds glutamic acid at position 2 and isoleucine at position 8, while K^d binds tyrosine at position 2 and leucine at position 9. Thus different K alleles will bind different sets of peptides, as will different D and L alleles. This maximizes the possibility that any virus will have at least one peptide that can be productively presented to the immune system.

The class Ib molecules appear to have distinct binding characteristics. For example, the M locus products bind peptides with N-formyl methionine as the N-terminus, suggesting a role in the presentation of peptides derived from intracellular bacteria. Some

CD1 proteins may present nonpeptide epitopes derived from cell membranes and cell walls of *Mycobacteria* to γδ T cells. The specificity of other class Ib molecules is unknown.

Transplantation

A consequence of recognition of class I proteins with virus peptides bound is that the immune system recognizes different alleles with considerable vigor. Class I proteins that differ in sequence bind different sets of peptides, but importantly are recognized by large numbers of T cells. The requirements for allorecognition are still a matter of debate, but clearly T cells which recognize allogeneic MHC bound to a specific peptide and those which are indifferent to the bound peptide both exist. The consequences of the recognition are profound. Mice that differ only in a single class I product, either by production of a transgenic line or by spontaneous mutation are able to mount rapid and effective graft rejection. The cellular mechanism of rejection involves both CD4 and CD8 T cells, and results in rapid organ graft rejection.

Mating preferences

Class I alleles also affect mating preferences. A female mouse will consistently choose to mate with males whose class I differs from her own. This has also been shown in large breeding experiments in experiments that mimic natural environments. While the exact mechanism for this is unknown, it appears to relate to odors learned in the nest. Mice can distinguish odors associated with urine whose donors differ only in the class I genes. It is also interesting that exposure of pregnant females to urine from MHC-different males results in resorption of the embryos. There is much speculation that these mechanisms may help to contribute to the maintenance of polymorphism at the population level at times when selection by pathogens is not occurring.

See also: Antigen presentation via MHC class I molecules; β_2 -microglobulin; *bm* Mutants; Cytotoxic T lymphocytes; Gene conversion; H2 class II; Immunoglobulin gene superfamily; Inbred strains; MHC, functions of; MHC restriction; Tumors, immune response to.

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H2 CLASS II

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The class II molecules encoded by the mouse major histocompatibility complex, H2, have as their predominant role, the binding and presentation of antigenic peptides for recognition by the antigen receptor complex of the T lymphocyte. They thus play a crucial role in self–nonself discrimination by the immune system. The development of recombinant inbred strains of mice by George Snell and others allowed the genetic mapping of class II genes, as well as many subsequent experiments performed by a large number of laboratories, which have revealed how class II molecules function to regulate immune responses. H2 class II molecules have now been shown to play critical roles in antigen presentation, which in turn affects both the activation of mature T lymphocytes as well as T cell development and selection. Studies of class II knockout mice have revealed a variety of defects in the immune response

which result from a lack of cell surface class II molecules. Although less well studied, class II molecules have also been suggested as playing a role in signal transduction to cells which express them.

Discovery of H2 class II molecules

In the mid- to late 1960s, a number of investigators reported the observation that when lymphocytes from genetically disparate individuals were mixed together, small ‘resting’ lymphocytes began to transform into large blast cells and divide. This phenomenon was termed the mixed lymphocyte reaction (MLR). Serologically defined determinants encoded by H2 genes, and shown previously to control allograft rejection in mice, were found to correlate with responsiveness in an MLR, although not all serologically detectable H2 antigens were found to stimulate

the MLR. It was subsequently shown, using recombinant inbred mouse strains, that MLR responses mapped to a specific region of the H2 complex, separate from those loci which encoded class I major histocompatibility complex (MHC) molecules.

Additional evidence for the existence of the genes now known as class II came from a variety of studies which showed that the effectiveness of the immune response to certain pathogens appeared to have a genetic component. The increasing availability of inbred, genetically defined strains of laboratory animals, such as mice and guinea pigs, coupled with the use of defined synthetic polypeptides as antigens allowed researchers to dissect the basis for these observations. During the course of these studies, the genes controlling these immune responses were dubbed 'immune response' or Ir genes. The use of recombinant and congenic inbred mice allowed the mapping of Ir genes to the middle of the H2 complex. It was concluded that responses to soluble protein antigens and the MLR are regulated by these Ir genes, which mapped to a distinct location from then known H2 loci controlling allograft responses. It was thus suggested by Jan Klein in the late 1970s that MHC molecules controlling the different responses be referred to as class II and class I, respectively – the designation which is now used.

Genetic organization of the H2 class II region

The region of the mouse H2 complex containing the class II genes was historically called the 'I' region, as it contains the aforementioned Ir genes, and the molecules encoded by these genes are called Ia antigens. The two classical class II antigens produced by the H2 complex are I-A and I-E. A current map of the class II region is shown in Figure 1. In addition to the genes which encode known functional class II molecules ($A\beta$, $A\alpha$, $E\beta$ and $E\alpha$), there are four additional class II genes (Pb, Oa, Ob and $E\beta 2$) whose function is not yet clear. Although Pb appears to be a pseudogene, transcripts from the Oa, Ob and $E\beta 2$ genes have been found. The Oa and Ob genes produce a nonpolymorphic, serologically recognized molecule with an expression pattern distinct from that of the classical H2 A and E molecules. That is, the H2 O molecules are expressed in spleen and

lymph node follicles, but only in B cell-rich areas, suggesting H2 O is not expressed by macrophages and dendritic cells. H2 O is also found in thymus, but restricted to a population of medullary epithelial cells which are virtually negative for H2 A and E expression. However, the functional role of the H2 O molecule remains elusive. The region between the Oa and Ob genes contains genes which encode proteasomal subunits (LMP1 and LMP2), the peptide transporters for class I molecules (TAP1 and TAP2), and the three H2M genes whose products are involved in proper association of class II with antigenic peptides (Ma, Mb1 and Mb2).

One of the great advantages which the mouse as an experimental animal has provided to the study of the structure and function of the MHC is the availability of a wide variety of H2 recombinant mouse strains. Over 60 such strains were made by several laboratories and quickly made available to the scientific community. Knowledge of the MHC complexes of other organisms has not been achieved as rapidly due to the paucity of such intra-MHC recombinants. The study of H2 recombinants in the I region has revealed a 'hot spot' for recombination events within part of a large intron between exons 2 and 3 of the $E\beta$ gene. Comparisons of intra-MHC recombinant strains show a much higher degree of polymorphism to the left of this hot spot, i.e., from the 5' end of $E\beta$ through the $A\alpha$ and $A\beta$ genes.

Gene structure and polymorphism of H2 class II molecules

Mouse class II genes contain 4–5 exons (Figure 2). The first exon encodes the leader peptide, and the second and third encode the external domains of each chain. In β chain genes, the fourth exon encodes the connecting peptide and the transmembrane



Figure 1 Genetic organization of the H2 class II region. Genes of known function are represented by filled rectangles.

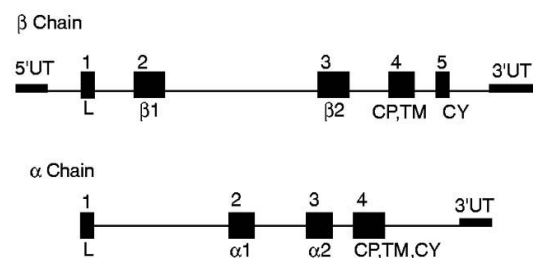


Figure 2 Gene structure of H2 class II I-A chains. Because of their chromosomal position, β and α genes are transcribed in opposite direction. Filled boxes represent exons; the exon number is above each. Portions of the mature class II protein which each exon encodes are indicated below the exon. L, leader peptide; $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ encode the external domains; CP, connecting peptide; TM, transmembrane domain; CY, cytoplasmic domain; UT, untranslated region.

domain, the fifth exon encodes the cytoplasmic domain, and the final exon encodes the 3' untranslated (UT) region. The α chain gene structure is similar, except that the fourth exon encodes the connecting peptide, transmembrane and cytoplasmic domains, as well as part of the 3' UT region.

H2 class II genes exist in a number of allelic forms, many of which have been cloned and sequenced. Sequence information reveals that there is extensive polymorphism at the nucleotide level, with the highest variability clustered in several short stretches of protein sequence in the first external protein domain of the A β , E β and A α genes, while there is little variability between alleles of E α . Most of the predicted amino acid substitutions encoded by these polymorphisms are nonconservative, and are in parts of the class II molecule predicted to form the binding pocket for antigenic peptides. Such variable amino acids can thus alter interactions with peptide, the T cell antigen receptor, or both.

The use of X-ray crystallography, together with chromatography techniques allowing the isolation and characterization of peptides bound in the class II binding pocket, has led to considerable current understanding of the structural constraints regulating class II-peptide interactions. In contrast to the peptide-binding groove of class I MHC molecules, the class II groove is open at both ends. This allows bound peptides to extend beyond the ends of the groove, and accounts for the greater average length and length variation of class II-binding peptides. However, although class II peptides can vary from 12 to 24 amino acids in length, class II molecules form many bonds with both the peptide main chain and side-chains, increasing binding affinity and potentially leading to similar conformation of peptides bound in the groove, despite differences in length and sequence. It is the side-chain contacts which show greatest variation among class II alleles, leading to allele-specific peptide sequence 'motifs'.

Expression of H2 class II genes and molecules

In contrast to class I molecules, class II molecules are expressed constitutively on only a limited number of cell types in the immune system. These include B lymphocytes, thymic epithelium, dendritic cells, and cells of the monocyte/macrophage lineage, including

Langerhans cells and hepatic Kupffer cells. While human HLA class II molecules are also found on activated T lymphocytes, this is not the case for mouse H2 class II molecules. A variety of stimuli can increase class II expression on these cell types, as well as induce class II expression on cell types such as fibroblasts, endothelial cells, and cells of the nervous system, which do not constitutively express detectable class II. B cell class II expression is enhanced by the lymphokines interleukin 4 (IL-4), IL-10 and interferon γ (IFN γ), as well as signals through the B cell antigen receptor complex and a number of microbial products. IFN γ also upregulates class II expression on macrophages and a number of cell types of nonhematopoietic origin. Additional cytokines and microbes and their products can also regulate class II expression in these cell types.

There is evidence for both transcriptional and post-transcriptional regulation of H2 class II gene expression, but it seems clear that the majority of regulation of amounts of class II protein occurs at the level of transcription. Thus, in a given cell population, the level of surface class II protein correlates closely with the level of class II mRNA. Most of the studies performed to date on detailed molecular mechanisms of control of class II gene transcription have focused upon human HLA class II genes. However, general principles appear also to apply to H2 class II gene expression in the mouse. Those studies which specifically examined control of mouse class II have focused primarily on control of α chain gene expression. The promoter region of the A α gene contains several *cis* regulatory elements located between 40 and 160 base pairs upstream of the transcriptional start site. These include the Y box (a 10 bp motif which contains a reverse CCAAT sequence), the X box (a 14 bp motif located adjacent to a palindromic regulatory element known as a cAMP response element or CRE), a 7 bp motif called the H box, and an element downstream of the Y box called the T box. The location of several of these elements is shown in Figure 3. Mutation or deletion of the H, X or Y boxes reduces both constitutive and IFN γ -induced class II expression in B cells. Additional experiments suggest that the X and Y boxes are also critical for expression of E α and E β .

It has been demonstrated that the X and Y boxes are recognition sites for DNA-binding proteins. A protein named NF-Y, which is ubiquitously



Figure 3 H2 A α promoter sequence. Positions of H, X1 and Y boxes are indicated by underlining. The X2 (CRE) element is indicated by boldface type.

expressed in all tissue types, recognizes the sequence CCAAT contained in the Y box of H2 class II genes, and consists of a heterodimer of two subunits, NF-YA and NF-YB. A protein called mXBP binds the A α X box sequence, and contains both a leucine zipper motif and a basic domain likely to bind DNA. mXBP binds as a homodimer to the A α CRE element, also called the X2 box, and can also form a heterodimer with the transcription factor c-Jun and induce its binding to CRE elements. As some of the stimuli which induce class II expression involve CRE motifs (e.g. engagement of the B cell antigen receptor), mXBP may play an important role in this induced expression.

Assembly, transport and peptide loading of class II molecules

The assembly of class II molecules occurs in the endoplasmic reticulum. The first event in this process is trimerization of the invariant chain (Ii) with which intracellular class II associates. Immediately after formation, this trimer associates with a pair of α and β chains, and two more heterodimers subsequently associate, to form a nonameric complex (Figure 4).

The chaperones immunoglobulin-binding protein (BiP) and calnexin enhance this process. The nonamers then travel across the Golgi and *trans*-Golgi to endosomal compartments. The nonameric complexes are unable to bind peptides, and there is strong experimental evidence that Ii plays an important role in preventing this association. Two different sequence motifs present in the Ii cytoplasmic domain have been shown to contribute to endosomal transport of class II, but Ii-independent transport has also been shown to occur. Sequences within the cytoplasmic domains of class II molecules themselves have been implicated in the regulation of Ii-independent transport.

Following endosomal localization, Ii is degraded by aspartic and thiol proteases into a nested set of peptides spanning Ii residues 81–104. These peptides are termed CLIPs. CLIPs associate with the peptide-binding groove of $\alpha\beta$ heterodimers, blocking their association with antigenic peptides. Removal of the CLIP peptides, which must precede normal class II peptide loading, is enhanced by the products of the H2M locus (see Figure 1). Recent evidence shows that these molecules directly promote CLIP release, as well as the release of peptides which bind class

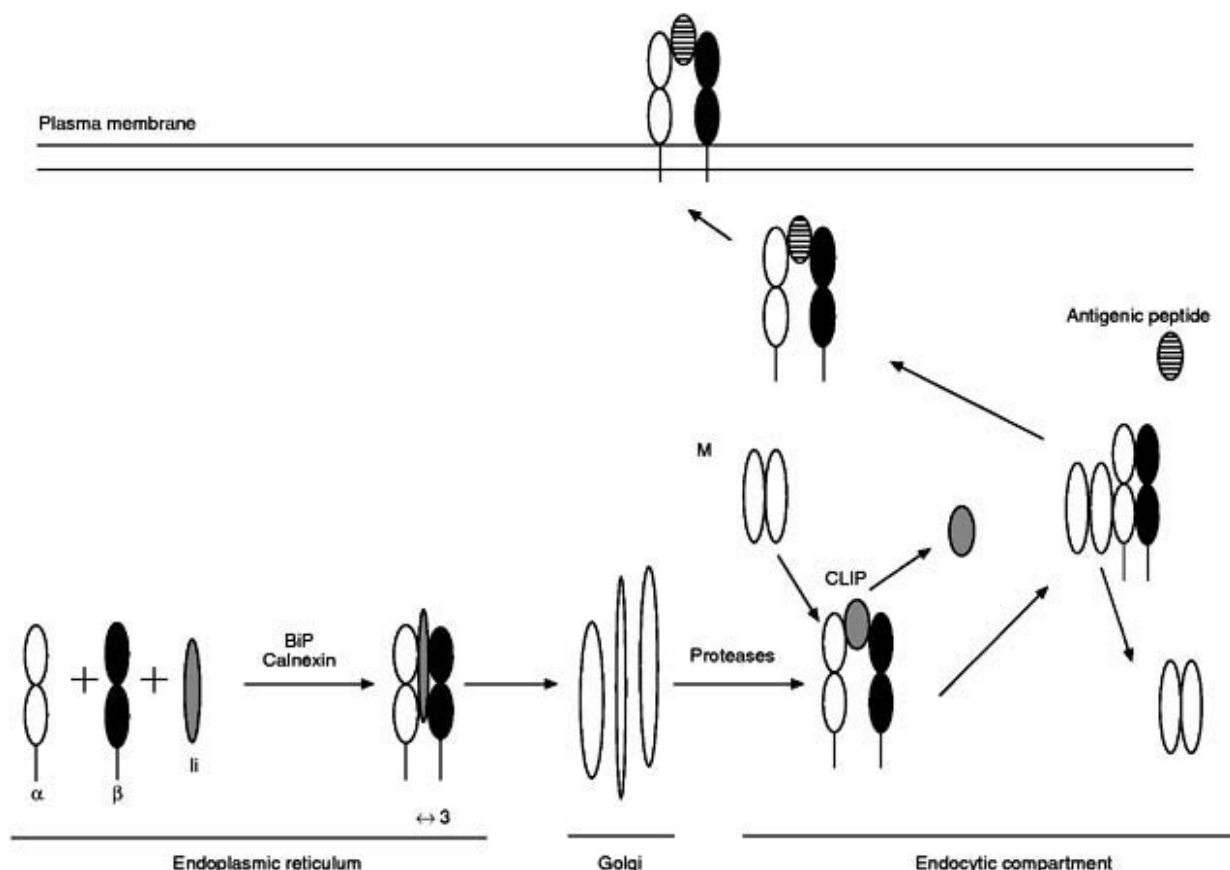


Figure 4 Class II assembly and transport to the cell membrane. Details of the process are described in the text.

II relatively weakly. Antigenic peptides which bind tightly are not susceptible to this enhanced release. The specific nature of the endosomal compartment where class II associates with exogenously derived antigenic peptides is not completely defined. A large body of evidence indicates that peptide association and CLIP dissociation is favored by an acidic environment, such as that found in late endosomes and lysosomes. However, data from a large number of studies suggest that class II molecules are found to some extent at many levels in the endocytic system. Several studies have also suggested the existence of a specialized class II-containing compartment, distinct from previously characterized endosomal/lysosomal compartments, called the MIIC. MIICs show a multilamellar structure and contain lysosomal enzymes; this compartment may have a specialized role in class II antigen presentation.

Functions of H2 class II molecules

Study of the class II molecules of the mouse MHC has provided a great deal of the current information on the functional roles played by class II molecules in the immune system. Genetic manipulations which are feasible in defined inbred mouse strains have allowed investigators to examine in great detail how class II molecules regulate responses to antigen. The advent of transgenic and knockout mouse technology in more recent years has created additional opportunities for studying the roles of class II molecules, in both immune system development and function. Following the development of inbred, genetically defined strains of rodents, it was observed that the allelic forms of class II molecules expressed by an animal determine its responsiveness to certain soluble antigens. In fact, it was this property of mouse class II genes which was the basis for their early designation as immune response (Ir) genes. However, it was not until decades later that details of how class II molecules exert this regulatory effect were understood.

A major advance in this understanding occurred in the early 1980s, with two major discoveries, each involving the contributions of many investigators. The isolation and characterization of the T cell antigen receptor (TCR) in 1983–1984 demonstrated that the same receptor recognizes both antigen and MHC molecules on an antigen-presenting cell (APC). This implied that antigen must physically associate with MHC molecules to be recognized by a T cell. The subsequent discovery (1985–1987) that purified MHC molecules bind tightly to antigenic peptides in an allele-specific manner revealed the nature of the critical role of MHC molecules in antigen presen-

tation. In subsequent years, the peptide-binding sites of both class I and class II MHC molecules have been crystallized, and physical characteristics of these binding sites determined.

The ability to bind antigenic peptides and present them to T cells endows class II molecules with their major functional role – to effect the selection and stimulation of T cells by delivering signals through the T cell antigen receptor complex. The study of transgenic and knockout mice has revealed that when and where class II molecules are expressed during development of the immune system profoundly affects the development of T cell populations and the T cell antigen receptor repertoire. Class II thus plays a pivotal role in the ability of T cells to distinguish self from nonself and to react appropriately to antigen stimulation. It is not surprising, therefore, that the expression and peptide-binding properties of particular allelic forms of class II molecules are also implicated in the development of some autoimmune diseases. In the mouse, H2 class II molecules have been shown to play important roles in the development of experimental models of insulin-dependent diabetes, autoimmune encephalitis, collagen-induced arthritis and a murine form of lupus nephritis, among others. Conversely, a lack of class II expression from the earliest stages of development results in profound defects in the development of CD4⁺ helper T cells and in both cellular and humoral responses.

Class II molecules have also been shown to deliver signals to cells which constitutively express them, such as B cells and monocytes. Ligation of class II molecules on mouse B cells *in vitro*, alone or in combination with ligation of the B cell antigen receptor, can induce or enhance cell proliferation, antibody secretion, homotypic adhesion and IL-6 production. The molecular events involved in this signaling pathway are not well defined, but evidence has been presented for the involvement of both tyrosine and serine/threonine kinases, and the elevation of intracellular cAMP. Class II signals have also been shown to enhance signals delivered via the B cell receptor, CD40. It is not yet clear what role such signals might play in the regulation of *in vivo* immune responses, but recent *in vivo* studies in tumor immunity and using class II knockout mice have suggested that class II signals may affect the efficiency of antigen presentation by APCs.

See also: Alloantigens; Antigen-presenting cells; Antigen presentation via MHC class II molecules; MHC peptide-binding specificity; H2 class I; Immune response (Ir) genes; MHC disease associations;

MHC, functions of; MHC restriction; Mixed lymphocyte reaction (MLR).

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H2 CLASS III REGION

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The H2 class III region is located between the class II H2-Ea and the class I H2-D genes in the mouse major histocompatibility complex (MHC) on mouse chromosome 17 (Figure 1). Based on pulsed-field gel electrophoretic analysis and partial genomic cloning, the distance between H2-Ea and H2-D has been estimated to be 900 kb. Over 60 genes have been identified in the human MHC class III region, but although the cDNA clones for several of these genes detect unique cross-hybridizing sequences in the mouse H2 class III region, only a few of these genes have been identified as independent transcriptional units in the mouse. The class III region genes in the mouse, the rat and the swine MHC are organized similarly to those in the human HLA, suggesting an interspecies conservatism in the MHC class III region. There are, however, some differences between the HLA and H2 class III region, because the distance between Bat1 and H2-D is only 6 kb, whereas the distance between the homologous genes in the human MHC is 200 kb.

It has not been established if the HLA genes identified within this 200 kb have mouse homologs mapping within the H2.

H2 class III genes

The proto-oncogene *Int3* is located approximately 200 kb telomeric from H2-Ea. *Int3* is a member of the mouse Notch receptor family which all transduce proliferative signals by directly interacting with the DNA-binding protein RBP-J kappa. The *Int3* locus is a target for integration of mouse mammary tumor viruses, which are frequently associated with primary mouse mammary tumors. Such tumors contain a truncated transcript containing the whole intracellular part of the *Int3* product. Analysis of transgenic mice containing the tumor-specific truncated forms of *Int3* suggest that deregulation of the *Int3* gene in the mammary epithelial cells limits their capacity to perform apoptotic decisions.

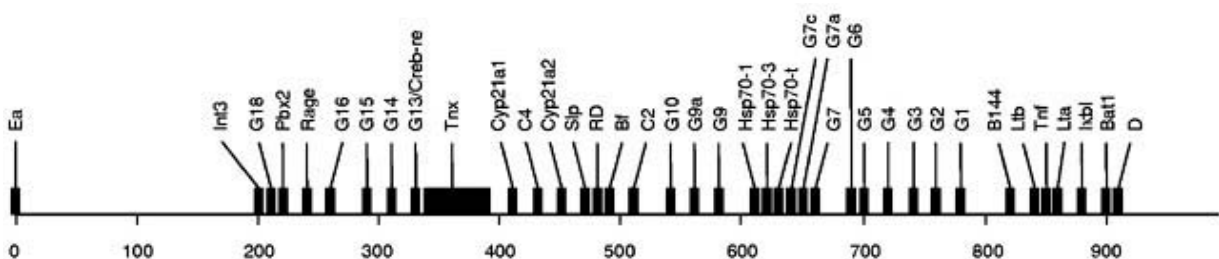


Figure 1 Mouse H2 class III region. The relative map location of the putative genes G9, G9a and G10, and of G13, G14 and G15, has yet to be confirmed.

Pbx2 encodes a protein which, together with PBX1 and PBX3, is a member of a new family of homeodomain proteins. In common with PBX1, PBX2 binds cooperatively to DNA with the Hox gene products HOXB-7 and HOXB-8. While it is known that PBX1 is involved in transcription activation in leukemia, the function of PBX2 is not clear. However, when fused with E2a all members of the PBX family can transform mouse fibroblasts, suggesting that PBX2 may have a latent oncogenic potential. The human genome contains three different Pbx2 genes located on chromosome 1, chromosome 3 and chromosome 6 respectively, however only the single Pbx2 gene mapping in the H2 class III region is found in the mouse genome.

Pbx2 is linked to Rage which encodes a 65 kDa transmembrane cell surface member of the immunoglobulin superfamily expressed on monocytes and endothelial cells. The Rage product associates with a lactoferrin-like protein to form a receptor that can interact with advanced glycation end-product (AGE)-modified proteins and induce the synthesis and release of a range of inflammatory cytokines. The advanced glycation end-product is a nonenzymatic modification of proteins, nucleic acids and lipids exposed extensively to reducing sugars. The AGE products are very resistant to proteolysis and accumulate as a consequence of aging. Since diabetes is associated with hyperglycemia, AGE-modified proteins accumulate more rapidly in the later stages of the disease, where they have been implicated in various pathological complications associated with diabetes. The Rage products have also recently been implicated in the pathogenesis of Alzheimer's disease.

Tnx encodes an extracellular matrix protein, tenascin X, which has been implicated in the formation of heart, muscles and limb. Tenascin X belongs to a family of matrix proteins, the other members of which are tenascin C (also called tenascin or cytactin) and tenascin R (also called restrictin). Tnx appears to have at least three promoter sequences, of which the most upstream overlaps with the promoter for a newly identified gene G13/Creb-re, which in humans is transcribed in the opposite orientation to the Tnx gene. The Tnx gene spans about 100 kb and overlaps in the 3' end with the Cyp21 gene. The 160 kb containing the human Creb-re/CYP-21 segment has at least 13 different transcriptional units, including several that overlap in the same orientation (Z within C4, P450c21 within Y, XB-S within XB) and in the opposite orientation (XA, XB-S and XB within P450c21). The segment appears to be conserved in rodents (and also in cattle) and thus may contain the same genes; how-

ever, the mRNA for the individual transcriptional units has not been identified in the mouse.

G13/Creb-re (cAMP-responsive element-binding protein-related protein) is a member of the Creb/ATF gene family and encodes a leucine zipper protein. The gene has a very broad expression pattern and is supposed to encode a general transcription factor.

The region between H2-Ea and Cyp21a1 contains, in addition to the genes listed above, the sequences cross-hybridizing with the cDNAs for the human genes encoding G18, located between Int3 and Pbx2, and G16, G15 and G14 located between Rage and Tnx.

C4 gene encodes the fourth component of the mouse complement system. Two different allelic forms of the C4 gene have been detected, characterized by high (C4^h) and low (C4^l) levels of expression. The Slp (sex-limited protein) gene shows structural similarities with the C4 gene, exhibits EDTA-independent complement activity and is expressed in the liver. In contrast to the C4 product, which is present in all mouse strains analyzed, Slp is only present in male mice with high C4 expression. Female expression has been noted in a few strains such as FM, PL/J and NZB mice, but is, in these strains, regulated by non-H2-encoded RIs (regulator of sex limitation) genes. In addition, the Slp gene in some wildtype haplotypes (H2^{w7}, H2^{w16}, and H2^{w19}) is constitutively expressed in both male and females, and the haplotypes contain several copies of C4 and Slp. Genetic analysis of these haplotypes have shown that the constitutive expression is caused by three copies of hybrid genes where the 5' part of the C4 gene is fused with the 3' part of the Slp gene, resulting in a hybrid gene regulated as the C4 gene.

Linked to the 3' end of both the C4 and Slp gene are the Cyp21a1 and Cyp21a2, respectively. Whilst Cyp21a1 is a pseudogene, Cyp21a2 encodes the steroid 21-hydroxylase which is expressed in the adrenal glands. This enzyme is involved in the biosynthesis of both cortisol and aldosterone. The linkage of the Cyp21 genes with C4 and Slp is conserved between the species and would suggest an early duplication of ancestral C4 and Cyp21 genes linked together.

The C2 and Bf genes are structurally and functionally related. Both genes encode soluble proteins involved in the classical complement activation pathway (C2) or alternative complement pathway (Bf). Both genes show very limited genetic polymorphism. Between Slp and Bf is the RD gene encoding a 44 kDa nuclear protein containing 24 tandem repeats of arginine and aspartic acid/glutamic acid dipeptides. The function of this gene has not yet been established.

The region between C2 and H2-D contains several

sequences related to the human HLA class III region genes; however, only a few of these have been analyzed at the transcriptional level. The MHC class III region contains three members of the heat shock 70 family, Hsp70-1/Hsp70-3/Hsc70-t, where Hsp70-1 and Hsp70-3 are two tandem heat-inducible genes and the expression of Hsc70-t is confined to the male germ cells. These genes are centromeric to the G7a gene that in humans encodes valyl-tRNA synthetase.

B144 (equivalent to HLA-gene LST-1) is preferentially expressed in B cells and macrophages. Although the function of the gene product has not been established, the expression pattern suggests a function for the B144 gene product in the immune response.

Centromeric to H2-D is the Tnf cluster containing genes for tumor necrosis factor α (Tnf), lymphotoxin α (Lta) and lymphotoxin β (Ltb). Both TNF α and LT α have multiple functions in the immune response and are expressed in a variety of tissues. Both cytokines can be expressed as a membrane-anchored and as a secreted protein. The membrane attached LT α forms a heterodimer with the structurally related LT β encoded by Ltb. The LT α -LT β complex is a ligand for the LT β receptor and is expressed early in the embryo, where it is involved in the development of the lymph node and splenic architecture. The LT α -LT β heterodimer is also important for the formation of germinal centers.

The region between Tnfb and H2-D contains two genes encoding nuclear proteins, I κ BL and BAT1. I κ BL encodes a protein structurally related to NF κ B, whereas the product of the BAT1 gene product is a member of the RNA hexicase superfamily with homology to the rat P47 nuclear protein and the WM6 gene of *Drosophila*.

Disease susceptibility

Several diseases have susceptibility genes mapping to the MHC. Although many of these involve specific alleles for the MHC class I and class II molecules, others have susceptibility genes mapped to the MHC class III region. In some instances, where there exist mouse models, inbred intra-MHC recombinant mouse strains with recombinant breakpoints in the MHC class III region have helped to map the genetic trait to the region between the C2 and Tnf gene clusters. In this region is the locus that controls the hematoopoietic histocompatibility antigen, Hh-1, the gene

that affects antibody responses to TNP-Ficoll, and the neuraminidase locus which has been identified as G9a in the human MHC. In addition the cortisone-induced cleft palate susceptibility gene, Cps-1, has been located between the Hsp70 cluster and G7a encoding valyl-tRNA synthetase. In this region is also the locus that determines susceptibility to experimental autoimmune orchitis, Orch-1.

See also: Complement, genetics; H2 class I; H2 class II; HLA class III region; Tumor necrosis factor α ; Lymphotoxin.

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HAEMOPHILUS, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Haemophilus influenzae is a gram-negative pleomorphic bacterium whose only ecological niche is the human host. While the primary residence of this human pathogen is the upper respiratory mucosal epithelium, it can invade these surfaces and cause serious systemic disease. An important virulence factor associated with *H. influenzae* is its capsular polysaccharide. The presence of this structure divides the species into encapsulated and nonencapsulated strains. Six different capsular serotypes have been described. *H. influenzae* serotype b (Hib), whose capsule is a repeating polymer of polyribosyl-ribitol phosphate (PRP), have accounted for the majority of invasive disease caused by *H. influenzae* until the recent widespread use of conjugate vaccines. Hib strains represent a clonal population. In contrast to Hib, nonencapsulated or nontypeable *H. influenzae* (NTHI) are a genetically and antigenically diverse group of organisms consisting of numerous clonotypes.

Encapsulated and nonencapsulated *H. influenzae* express other outer membrane surface structures, including fimbriae, lipooligosaccharides (LOS) and outer membrane proteins (OMPs), which are important in colonization of the human host and in pathogenesis of infection. Because of the clonal nature of the Hib strains, the surface antigens are relatively conserved among strains. By contrast, a characteristic feature of NTHI strains is the antigenic heterogeneity of surface antigens among strains. Two of the most abundant surface antigens are LOS and OMP P2, which comprises approximately half of the protein content of the outer membrane. These two molecules demonstrate striking heterogeneity among strains, particularly in the regions of the molecules, which are exposed on the bacterial cell surface.

H. influenzae colonizes the human upper respiratory tract. Almost all of the colonizing strains are NTHI. Colonization by NTHI begins early in childhood and continues through adult life. Analysis of prospectively recovered strains has shown that colonization by NTHI is a dynamic process; children eliminate strains and acquire new strains of NTHI frequently. Colonization with NTHI at an early age

is associated with an increased incidence of otitis media in children. Whether nasopharyngeal colonization is an immunizing process is not yet known.

The most serious systemic disease caused by *H. influenzae* is meningitis. The incidence of invasive Hib disease, particularly meningitis, has decreased dramatically since the mid-1980s. This decrease is due to the widespread use of vaccines which are composed of Hib polysaccharide capsule conjugated to protein carriers. While Hib causes invasive infections, NTHI causes predominantly mucosal infections. NTHI causes 25–30% of all cases of otitis media. Approximately 80% of all children will experience at least one episode of otitis media by the age of 3 years. NTHI is a common cause of lower respiratory tract infections in adults with chronic obstructive pulmonary disease. More recently, NTHI has been recognized as an important cause of pneumonia in adults with acquired immune deficiency syndrome (AIDS) and in children in developing countries. The incidence of invasive disease caused by NTHI strains is increasing. The conjugate vaccines are not effective in preventing disease caused by NTHI strains.

Immune responses of the host

In the 1930s, Fothergill and Wright demonstrated that serum bactericidal activity to Hib was associated with protection from infection. Subsequent work in animal models and human systems has established that much of this bactericidal activity is mediated by antibody to capsule and that antibody to the PRP polysaccharide capsule protects against invasive infection. These observations guided work on vaccine development in the 1970s and 1980s.

By the age of 3 years, most children have acquired natural antibodies to PRP. Children with invasive Hib infection have low levels or no detectable anticapsular antibody at the onset of infection. Even in the convalescent period, the titers of anticapsular antibodies in children remain low until approximately 2 years of age. These children are susceptible to reinfection and second and third episodes of Hib meningitis have occurred in the same child. The response to the PRP polysaccharide in children below

age 2 is typical of the response to a T-independent antigen.

Less is known about the host immune response to nontypeable strains of *H. influenzae*. A hallmark of infections caused by NTHI is the high rate of recurrences. Therefore, infection does not confer subsequent protection from infection by NTHI strains. Serum bactericidal antibody develops following otitis media due to NTHI and this antibody response is associated with protection. However, the immune response is specific for the infecting strain and the child remains susceptible to infection by other strains of NTHI. A similar scenario may occur with recurrent exacerbations of chronic obstructive pulmonary disease. The strain-specific immune response is directed at least in part to immunodominant surface epitopes on the P2 OMP. These observations regarding strain-specific immune responses may account for the observation that these apparently immunocompetent populations experience recurrent infections.

The LOS of *H. influenzae* is a major toxin associated with the species. There is a human humoral immune response to specific carbohydrate structures but little is known about the functional activity of this antibody. Recently, the *htrB* gene has been characterized in *H. influenzae*, *htrB* encodes for an acyltransferase which is responsible for the myristic acid substitution of *H. influenzae* lipid A. It has been shown that *htrB* mutants are less toxic than the parent strain from which they are derived, as they are less invasive in the rat nasal challenge model. In addition, *in situ* hybridization studies with primary human respiratory epithelial cells has shown that LOS from wild type *H. influenzae* generates a tumor necrosis factor α (TNF α) mRNA response that is ten-fold greater than LOS from *htrB* mutants.

Little is known about the mucosal immune response to NTHI. This is an important area of investigation because most infections caused by NTHI are mucosal infections.

Vaccines

The development of conjugate vaccines to prevent invasive infections by Hib represents a dramatic success in the prevention of an infectious disease. High concentrations of serum immunoglobulin G (IgG) to Hib capsular polysaccharide confer increased bactericidal and protective activity. PRP alone is a poor immunogen. Conjugating the PRP capsular polysaccharide to protein carriers has resulted in vaccine formulations which effectively induce protection from invasive Hib infections in infants. The antibody response to the vaccines is restricted. The predominant IgG subclass induced by vaccination is IgG1,

and the predominant IgG light chain expressed is V κ II α . Qualitative differences in antibody repertoires induced by natural exposure, purified PRP vaccine and each of the four conjugate vaccines have been observed. However, no major differences in protection afforded by the different vaccine formulations have been noted. Although antibody to PRP confers protection, an antibody concentration which correlates with protection cannot be precisely defined. Factors other than antibody concentration play a role in determining protection. These include avidity of antibodies and the priming effect of early vaccination with conjugates.

An unexpected but important effect of vaccination against Hib has been observed. Vaccinated children have a decreased incidence of nasopharyngeal colonization with Hib. This is probably one of the important mechanisms by which the vaccines prevent invasive infections.

The development of a vaccine to prevent infections caused by NTHI is the subject of intensive investigation. An approach has been to identify surface proteins which are relatively conserved among strains of NTHI and test these proteins for their ability to induce a protective immune response. Candidate antigens include P6, a 16 kDa OMP; P4, a 30 kDa OMP; fimbriae; high molecular weight adhesins; protein D; and others.

See also: Bacteria, immunity to; Bacterial cell walls; Neurological infections; Respiratory and cardiac infections; Vaccines.

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HAPTEN

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Hapten is usually defined as a substance that is not immunogenic as such but can bind to antibodies of the appropriate specificity (hapten-specific antibodies). A hapten can be made immunogenic by coupling it to an immunogenic protein (the carrier). The conjugate is then immunogenic not only with respect to carrier epitopes but also to the hapten. 'Antigenic' is sometimes incorrectly used as a synonym for 'immunogenic', but free hapten is 'antigenic' and not 'immunogenic'. Antibodies specific to a hapten can only be raised when the hapten is foreign to the individual to be immunized.

Small haptens are widely used as defined antigenic determinants (epitopes) in basic immunology. Unlike natural epitopes of proteins, haptens can be easily handled in the laboratory both in the coupled and in the free form. They have helped immunologists to evaluate the specificity, affinity and heterogeneity of antibodies, and to estimate the size of their antigen combining site. Haptens were used to obtain the first X-ray crystallography pictures of antibody–antigen complexes. The concept of T cells helping B cells to produce antibodies is heavily based on the demonstration of B cell hapten recognition, coupled to carrier molecule recognition by T cells. Use of haptens enabled the analysis of inherited and acquired elements of the antibody response. Another aspect is the development of immunoassays to quantify many different molecules in body fluids or other solutions.

Some compounds have a borderline immunogenicity but are considerably stronger immunogens when linked to proteins. For example, the type b capsular polysaccharide of *Haemophilus influenzae* (Hib) is very weakly immunogenic when given to children aged 1 year or less, but a significant immun-

ogen when given to adults. When Hib is coupled to immunogenic proteins such as diphtheria toxoid, young children also produce a fair amount of Hib-specific antibodies in response to vaccination. Hib is thus a hapten in young children but an immunogen in adults.

Many haptens are small molecules (≤ 1 kDa), and as such nonimmunogenic. When coupled to a protein a small hapten can become an antigenic determinant (epitope). An epitope can, however, include the hapten plus an adjacent region of the carrier as well. Thus, unless excluded by specificity controls, monoclonal antibodies to haptens may be suspected of having some affinity preference to the hapten–amino acid conjugate, as it was presented in the immunogen, over free hapten.

Other haptens are macromolecules (>100 kDa). It is not completely understood why they are haptens rather than immunogens, but one factor is that coupling to immunogenic proteins brings T cell help to the antihapten response.

A growing interest centers on the generation of artificial epitopes of proteins. A common approach has been to prepare conjugates of peptides and carrier proteins. This has, however, often failed to reproduce the orientation of the peptide in the original protein, to the extent that the antibodies obtained have been irrelevant to the aimed task. Newer approaches have included incorporation of a nucleotide sequence coding for the peptide epitope into the gene for another protein. The insert is placed at a position that is known to produce a protruding loop on the surface of the expressed protein. Carrier genes found suitable include those for influenza virus, hemagglutinin, penicillinase, etc.

Preparation of hapten conjugates

Suitable attachment points on the carrier protein molecule are presented in Table 1. Usually, the hapten is modified to make it 'reactive'. Exceptional haptens, e.g. penicillin and its relatives, spontaneously convert to the active form. Commonly used, artificially introduced reactive groups are shown in Table 1. Proteins usually function well as carriers without modification, but sometimes they have been improved by the addition of reactive groups like thiol or hydrazide groups. These groups can be set at a defined distance from the carrier molecule through a spacer.

When peptides are used as haptens, hydroxyl (OH) groups of tyrosines or amino (NH₂) groups of lysines are usually used as the groundwork of the reactive group. Failures have sometimes been observed due to destruction of the epitope by the coupling, through incorrect folding of the peptide, or through lack of appropriate postsynthetic modification like amidation, as has been observed for some polypeptide hormones.

Special expertise in chemistry is required for derivatization of steroid hormones toxins, or drugs to forms that can be bound to carrier molecules in the correct orientation. Because of the high demand for antibodies to these substances, several tailored derivatizations of these compounds have been published.

Monitoring of hapten binding

Monitoring is carried out in basic immunology, usually to determine affinity of antibodies, and in clinical chemistry, forensic science, etc., to quantify substances of low concentration.

The gold standard for measuring the affinity of a hapten-specific antibody is equilibrium dialysis. It requires that the concentration of specific antibody is known and that the concentration of the hapten can be determined at the concentration range where half saturation of antigen-binding sites occurs (usually 10⁻⁶ M or less). Therefore, the hapten must

carry a tracer, usually a radioactive atom – often considered too laborious to prepare.

An alternative reference method for affinity measurement is fluorescence quenching. A part of tryptophan fluorescence emitted by antibody molecules is quenched when the combining site is occupied by the epitope (hapten). The maximum degree of quenching varies but in some monoclonal antibodies it can be up to 80% of the total fluorescence. Exposed tryptophan residues must then be almost exclusively located around the antigen-binding site. Also, affinities can already be measured when only 20–40% of the fluorescence is quenched by the hapten binding. With suitable instrumentation the association and dissociation reactions can be evaluated separately. In practice, the operating range extends to lower affinities than can be measured with equilibrium dialysis. Both methods described above give actual binding constants but are laborious to carry out.

When it suffices to determine relative affinities in a set of antibodies, less laborious methods are available. Free hapten inhibition of binding of a labeled hapten to antibody may then suffice. Another means for practical affinity measurement is based on surface plasmon resonance (for example using the BIA core™ machine produced by Pharmacia), essentially a method for detecting the mass of substances collected to a surface through change in the refractive index for light. Both association and dissociation reactions may be followed in real-time mode. Some disturbances by surface effects may be possible. Therefore, the affinities obtained are to some extent relative but comparison of reagents and detection of interactions can easily be done.

Many practical immunoassays (measurement of drugs, hormones, toxins, etc.) depend on hapten binding to an antibody. The simplest monitoring of hapten binding is carried out without separating bound and free hapten (homogeneous methods). For example, in the fluorescence polarization method, the hapten is coupled to fluorescein and exposed to

Table 1 Structures on carriers and haptens that participate in coupling

Structure on carrier	Active group in the hapten
Tyrosine	Azo
ε-Amino group of lysine	Azide, <i>N</i> -hydroxysuccinimide, phenylisothiocyanate, aldehyde (coupling of saccharides by reductive amination, etc.)
Carboxyl group of aspartic or glutamic acid	Amino group via carbodiimide
Free sulfhydryl group of cysteine or of SPDP [3-(2-pyridyldithio)-propionic acid <i>N</i> -hydroxysuccinimide] modified lysine	Maleimide

polarized light. When an antibody binds this hapten, the rotation of the fluorescein is inhibited and the emitted light is polarized. The unbound hapten-fluorescein complex rotates freely and emits unpolarized light.

In most immunoassays, the bound hapten is separated from the free hapten. The oldest method is the Farr type competitive immunoassay. The hapten is labeled and, after incubation, bound and free hapten are separated, usually by precipitation and centrifugation. When the concentration of the hapten is very low ($<10^{-10}$ M in the case of growth hormone), a very high-affinity antibody is needed for such an assay. Other separating immunoassays (e.g. the immunometric assays) are less dependent on antibody affinity. Since they are also less laborious than the Farr assay, they have largely replaced it.

Drugs as haptens in clinical medicine

Adverse reactions to drugs are sometimes due to interaction of the drug and antibody to it. In order for a drug to be immunogenic, either the drug itself or its metabolite must be capable of binding to a large molecule of the body, most commonly a protein. β -lactam antibiotics (e.g. penicillin) are an example. They form covalent bonds to proteins due to spontaneous breakage of the β -lactam ring and a concomitant emergence of a chemically reactive group. When sufficiently high doses are used, some molecules of the body are apparently modified by several bound β -lactam molecules. An immunogenic molecule is then formed. Immunoglobulin M (IgM) and IgG anti-penicilloyl seem to cause cytolytic reactions; IgE class antibodies have been observed in anaphylactic reactions and in drug-induced fever reactions.

Some drug reactions are immunologically mediated but antibodies are not involved. Sulfonamides and several anticonvulsants yield metabolites that have an active prosthetic group able to bind to protein. The target for the binding is usually a nearby protein at the site of the metabolic event. The protein to be modified is thus mostly the enzyme itself that catalyzes metabolite production. Hence, the cytochrome P450 group enzymes are a preferential target. The altered protein is believed to yield an altered

peptide which binds to the major histocompatibility complex (MHC) of the cell, creating a target for T cell attack. Susceptibility to this kind of reaction is determined by the MHC phenotype of the patient and apparently also by allelic variation in detoxifying enzymes such as epoxide hydrolase.

Drug reactions have been described which seem to have an immunological basis, but the drug appears to be unable to couple covalently. α -Methyldopa is an example. A hypersensitivity reaction to this drug causes formation of autoantibodies and a lupus-like syndrome. The drug may or may not act as a hapten in such cases; for example, it may act as an epitope for the T cell antigen receptor, and covalent coupling may then be unnecessary for immunogenicity. The drug might also bind to protein noncovalently and change its conformation. Some cases previously thought to operate in this way have been recently explained by covalent protein binding activity of the (often ill-defined) metabolites of the drug concerned.

See also: Affinity; Anemia, drug induced immune hemolytic; Antigens; Carrier; Epitopes; Immunoassays.

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HEAVY CHAIN DISEASES

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Heavy chain (HC) diseases (HCD) are monoclonal immunoproliferative disorders characterized by the production of abnormally short immunoglobulin (Ig) HC without associated light chains (LCs). HCD are known for the three main Ig classes.

Clinical presentation and diagnosis

The clinical presentation usually varies according to the HC class. Apart from a very few cases exhibiting a respiratory disease, α -HCD, the most common HCD, usually affects young adults originating from areas with a high degree of infestation by intestinal microorganisms and having lived in conditions of poor hygiene. α -HCD is common in North Africa, the Middle East and southern Europe. It is also encountered in the Far East, southern Africa and Latin America. Patients usually present with a severe malabsorption syndrome due to a diffuse and massive infiltration of the small bowel and mesenteric lymph nodes, usually by mature plasma cells. More rarely, the proliferation is lymphoplasmacytic or lymphocytic. This first stage of the disease is possibly benign. It is susceptible to complete remission under oral antibiotics. The natural history is an evolution toward a malignant large cell lymphoma of the same clonal origin as the initial proliferation.

The clinicopathologic features of γ -HCD vary, with a lymphoplasmacytic proliferation similar to that of Waldenström's macroglobulinemia in 40% of cases and, in other cases, various forms of lymphoid proliferations, including lymphomas and chronic lymphocytic leukemia (CLL), or no detectable lymphoproliferative disorder. Association with autoimmune diseases is not uncommon. μ -HCD is rare. It presents with the same variety of immunoproliferative disorders as γ -HCD, typically CLL with vacuolated bone marrow plasma cells. Although truncated HCs (structurally different from HCD proteins) are able to deposit in tissues in amyloidosis and heavy chain deposition disease, there is no documented tissue deposition in HCD (a single case of amyloidosis of unknown composition was reported in γ -HCD).

Diagnosis is based upon the demonstration of monoclonal HCs without associated LCs by the immunochemical study of the serum (or of the intestinal fluid in certain cases of α -HCD without serum α -HCD protein). There is rarely a narrow band by

electrophoresis, HCD proteins being often heterogeneous in charge, especially α -HCD proteins which migrate from albumin to slow gammaglobulins in certain patients. μ -HCD proteins often migrate in the α -globulin region. Hence, HCD proteins may easily be missed. Immunoelectrophoresis can be either misleading (certain IgA λ fail to precipitate with anti-LC sera) or very useful, for example when using anti- α antisera containing antibodies to conformational epitopes of the Fab α region. The immunoselection method, a gel immunodiffusion technique in which anti-light chain antisera are included in the gel (thus preventing light chain-containing molecules migrating) can also be used as a diagnostic tool.

There is an HC subclass restriction, always α 1 in α -HCD and with γ 3 overrepresentation in γ -HCD. HCD proteins usually polymerize (except for certain γ -HCD proteins that are present in the serum as dimers) and are rarely present in significant amounts in urine. Free LCs that do not associate with HCs are produced in about 50% of μ -HCD cases, leading to urinary Bence Jones proteins. LC cast nephropathy was recently observed in such a case. Non-secreted cellular LCs are found in a few α - and γ -HCD cases. Nonsecretory forms occur in α -HCD, diagnosis being made at the intestinal plasma cell level. The clinicopathological picture of α -HCD is the same as that of the Mediterranean type or immunoproliferative small intestinal lymphoma (IPSID). Certain IPSID patients lack detectable α -HC in the serum and intestinal fluid. Nonsecretory α -HCD seems to account for only a minority of them.

Structural alterations of HCD proteins

Structural study of HCD proteins reveals normal carboxy-terminal regions and deletions involving the variable (V) region and most often the first constant (C_{H1}) domain (Figure 1). The normal sequence usually resumes at the beginning of the hinge region or of a C_{H1} domain. Comparison of the available sequence data shows four situations:

1. A few μ -HCD proteins lack the V domain only.
2. The absence of V and C_{H1} (and in one instance C_{H2}) domains is a feature of some α - and γ -HCD proteins. However, in such a case (Omm), the cellular HC precursor contained a normal leader (L) peptide and 15 residues from the V

Table 1 HCD genes

	<i>V region</i>			<i>Switch/C_H1 region</i>		<i>Remainder of the C region</i>
	<i>L-V intron</i>	<i>VDJ exon</i>	<i>VDJ 3' flank</i>	<i>5' to switch</i>	<i>3' to switch</i>	
μ -BW	Normal	1 bp deletion in V generating stop codons	A 138 bp deletion 3' to J4 with a 51 bp insertion (3' splice site of VDJ4 deleted)	NS	C _H 1 normal	Normal
γ_3 -Omm	Normal	A 320 bp internal deletion at V-J4 junction	Mutations, deletions, and a 37 bp duplication in the J4-J5 IVS	Normal?	A large deletion from switch to C _H 1 leaves its 3' end	Normal
γ_1 -Riv	A 80 bp deletion with 56 bp insertion (V 5' splice site deleted)	A 280 bp deletion with 88 bp insertion (containing inverted repeats) at V-J6 junction	V-J6 3' splice site mutated; mutations, deletions following J6	A 400 bp deletion preceding S μ	A large deletion from S γ to C _H 1 leaves its 3' end	Normal
α_1 -Yao	Normal	A 350 bp deletion at V-J5 junction	A 330 bp deletion with 62 bp insertion in the J5-J6 intron just 5' to J6	A short 10 bp deletion	A large deletion from S α to C _H 1 leaves its 3' end	Normal
α_1 -Mal	Complete deletion of the VDJ exon and its 5' and 3' flanks accompanied by a 360 bp insertion and followed with mutations and two short insertions			A 70 bp deletion with a 509 bp insertion and another 1.1 kb deletion preceding S μ	A large deletion from S μ to the C _H 1-C _H 2 intron	Normal
α_1 -Sec	A 17 bp deletion 5' to V (V 5' splice site altered)	A 330 bp deletion with 289 bp insertion (containing a direct repeat) at V-J5 junction	NS	1.3 kb deletion with a 57 bp insertion (containing a direct repeat) preceding S μ	A large deletion from switch to the C _H 1-C _H 2 intron	A 369 bp deletion 3' to C _H 3 involving the secreted form α poly (A) site

NS, not sequenced.

region, making this case actually belong to the following group.

- Most proteins contain an amino-terminal V region fragment of variable length followed by an internal deletion. Some such proteins also contain a sequence corresponding to part of J_H and hence have two deletions.
- A few proteins have an amino-terminal sequence with no homology with any known human sequence.

Most HCD proteins have a high carbohydrate content, with unusual moieties containing galactosamine.

Cellular and nucleic acid data

HCD proteins are synthesized as truncated HCs, without important postsynthetic degradation, although limited proteolysis is observed in a few cases, leading to heterogeneous amino termini in some. mRNA are short. Southern blotting shows clonal rearrangements of the relevant HC gene. The abnormalities of the productive gene are simple in the case of μ -HCD BW; alterations of splice sites of the VDJ exon by a deletion-insertion and mutations force a direct splice from the leader exon to C_H1. The other studied genes bear complex, different but similar abnormalities (Table 1). The VDJ exon is

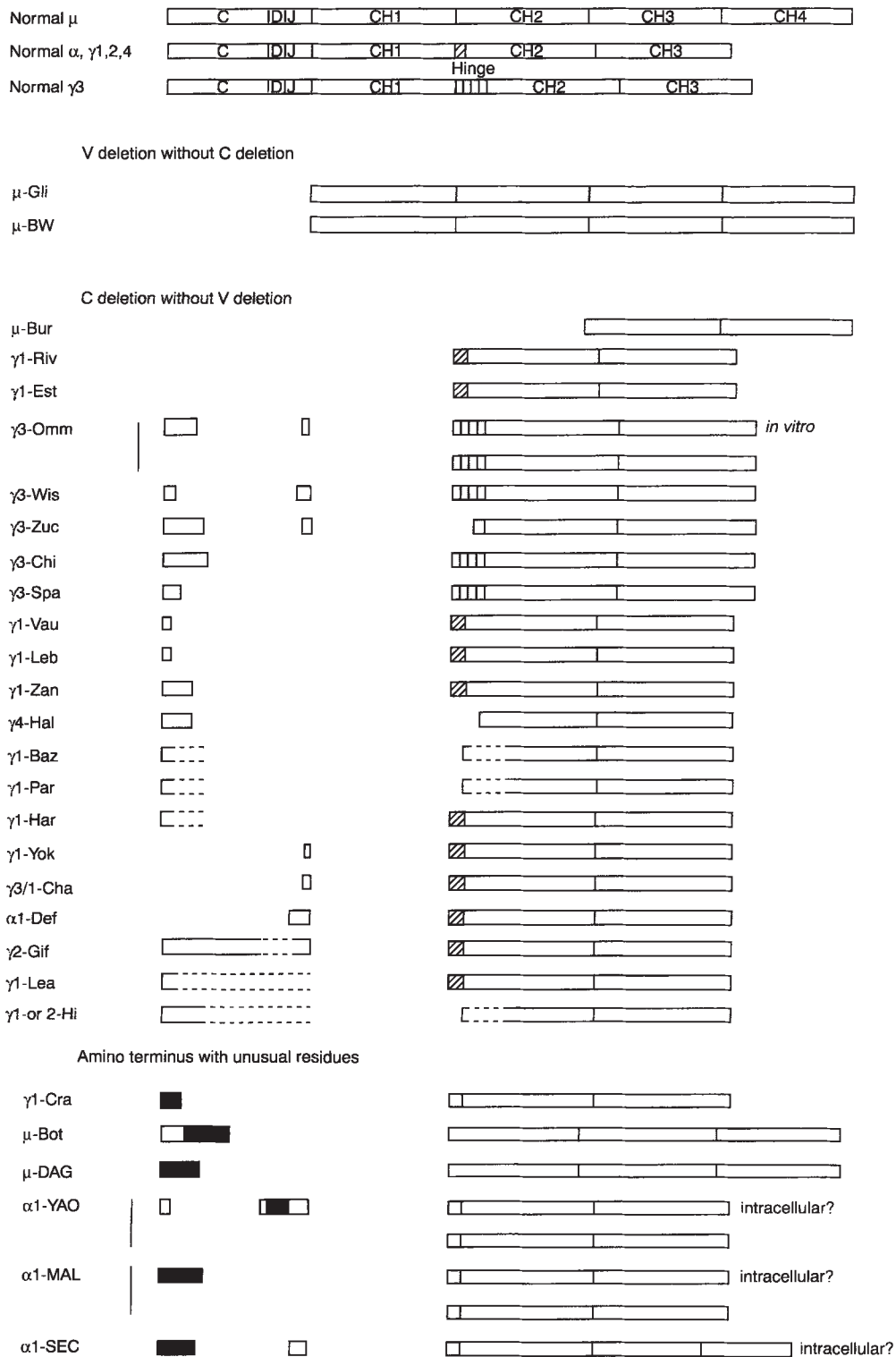


Figure 1 Structure of HCD proteins. Except when stated (*in vitro* for Omm in whom the native protein is known from biosynthesis experiments, and 'intracellular?' for protein sequences deduced from mRNA sequences), the structures shown are those of the serum proteins.

entirely or most often partly (leaving the 5' end of V and the 3' end of J_H) deleted, and its 3' flank bears insertions and deletions. Part of the switch region is removed by one or two deletions, one of which encompasses C_{H1} (entirely or partially). These abnormalities lead to various mRNA splicing patterns removing the possible residual 3' end of C_{H1}. Entire free HCs are not secreted. They are retained and degraded in the endoplasmic reticulum due to an interaction with chaperone proteins such as the immunoglobulin binding protein BiP which occurs through the CH1 domain of the HC. As most HCD proteins lack CH1, they escape the interaction with BiP. As for those rare cases of μ -HCD with a deletion of the V domain only, normal LCs are produced which are noncovalently associated with the HC intracellularly, preventing interaction with BiP. Insertion of DNA of unknown origin is constant, usually at the location of deletions. In three genes such insertions include an exon. The unknown sequences present in the mature transcripts are absent from the serum protein in the two cases studied, probably due to amino-terminal proteolysis. In the nonsecretory α -HCD SEC, a further genomic deletion removes the polyadenylation signal of secreted α chains, forcing plasma cells to produce membrane α chains only.

Although LC – mostly κ – gene rearrangement seems to be constant, there is usually no detectable LC synthesis in γ - and α -HCD. In the single such γ -

HCD case studied, a productive rearranged κ gene bore numerous mutations in the VJ region, some of which altered both splice sites of the VJ exon, with direct splicing from the leader exon to C κ . A deletion and two insertions were found in the 3' flank of VJ. These LC abnormalities are reminiscent of those of the HC genes.

See also: Immunoglobulin genes; Immunoglobulin structure; Lymphoma.

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HELICOBACTER PYLORI, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Helicobacter pylori is a gram-negative, S-shaped, motile, microaerophilic bacterium which is characterized by the production of significant quantities of urease and the presence of multiple (5–7), unipolar, sheathed flagella with terminal bulbs. *H. pylori* primarily colonizes the gastric epithelium of humans and some nonhuman primates. Infection is probably acquired in childhood and colonization, once established, is probably lifelong. This infection is almost always associated with histologic chronic (type B) gastritis and is a major risk factor for the develop-

ment of peptic ulceration, atrophic gastritis, gastric cancer and gastric lymphoma.

H. pylori isolates are biochemically and antigenically surprisingly homogeneous despite significant genomic variation. Recently the entire genome of one *H. pylori* strain has been sequenced. A number of antigens of *H. pylori* have now been identified, including the urease which is a 540 kDa multimeric, immunodominant protein comprising six subunits of two polypeptides (28 kDa and 61 kDa) encoded by the *ureA* and *ureB* genes respectively. Monoclonal antibodies directed against the 28 kDa polypeptide can inhibit the enzyme's activity. This urease is antigenically highly conserved among a wide variety of other spiral gastric colonizing organisms, indicating

that the enzyme has evolved with an essential role in gastric epithelial colonization. A third polypeptide (54 kDa), frequently associated with the urease, is an hsp60 homolog (GroEL-like) encoded by the HspB gene.

The flagella of *H. pylori* comprise two immunogenic flagellins (about 54 and 56 kDa) encoded by the *flaA* and *flaB* genes. The flagella sheath material is antigenic and at least some of its antigens are distinct from the outer membrane of the bacterium. Flagella-associated proteins are antigenically cross-reactive with the flagellins of the campylobacters.

About 60% of *H. pylori* strains express an 87 kDa antigen which is the polypeptide subunit of the 970 kDa multimeric VacA protein possessing vacuolating cytotoxic activity. The subunits are encoded by the *vacA* gene which is also present in nontoxigenic strains. There is also a cytotoxin-associated protein (120–140 kDa) expressed from the *cagA* gene and present in about 80% of strains. The CagA protein is not required for toxin activity but may have a role in its regulation. Both the CagA and VacA proteins are highly antigenic.

H. pylori produces a variety of hemagglutinins, including the *N*-acetylneuraminyl-lactose-binding fibrillar-like hemagglutinin encoded by the *HpaA* gene, but their role in specific adherence to gastric epithelial cells is still debatable.

Immune responses of the host

H. pylori colonization of the gastric mucosa induces detectable circulating and local humoral responses. The isotypes of the antibodies produced are consistent with a prolonged chronic mucosal infection. Circulating immunoglobulin G1 (IgG1) and IgG4, and frequently IgG2, antibodies are observed but IgG3 and IgM antibodies are rare. IgA antibodies are also usually observed. In a well-documented volunteer study, IgG seroconversion occurred between 22 and 33 days after infection. Subsequent studies showed an initial IgM response, followed much later by an IgA seroconversion. At the local mucosal level, IgA antibodies predominate and are secreted into the gastric juice. IgG and IgM antibodies are also produced locally, although IgG reactivity is rapidly lost in gastric juice. The systemic IgG response persists throughout infection and only falls slowly after successful treatment, attaining basal levels after 12 months or so. Recurrence of infection is accompanied by a rapid rise in titer.

The specificity of the circulating host antibody response to *H. pylori* is complex and extremely variable, which seems to be a reflection of differences in the immune responses rather than the antigenic

diversity of the bacterium, other than for variation in the VacA and CagA proteins. It seems likely that the epithelial cell damage, the massive infiltration of immunocompetent cells, the efficiency of the antigen-presenting cells and the chronic nature of the infection all contribute to this variation. Most infected individuals produce antibodies against a large number of antigens, including the urease subunits, the flagellins and the 54 kDa hsp60 homolog. When present in the infecting strain, there are usually circulating antibodies directed against the VacA and CagA polypeptides. The specificity of the host mucosal response is also complex but may differ from that of the systemic antibodies even when of the same isotype.

ELISA-based serological tests are used routinely for detection of infection in patients with dyspepsia and for seroepidemiological surveys. The antigens used vary in complexity but all include the urease. Most recently tests for salivary IgG antibodies have been introduced which are noninvasive and particularly useful in children. In general, tests to monitor the success of treatment have not been useful because of the persistence of the circulating antibodies.

H. pylori-associated gastritis comprises an infiltration of plasma cells, lymphocytes and monocytes into the superficial layers of the lamina propria, sometimes causing a loss of gastric mucosal architecture. This gastritis is usually active, involving a polymorphonuclear infiltrate adjacent to areas of bacterial colonization.

Immunohistology indicates that approximately half the lymphoid cells are mature B cells, with the production of primarily IgA, but also IgG and IgM antibodies. Evidence suggests that these cells secrete immunoglobulin directed against *H. pylori*. The T cells present are mostly of the CD8⁺ subset; however, there is a significant increase in CD4⁺ T cells in *H. pylori*-positive gastritis. There is also an increase in $\gamma\delta$ T cells, which may not be specific for *H. pylori* infection. During infection there is an aberrant expression of HLA-DR antigens on the gastric epithelial cells and a local production of lysozyme and lactoferrin. Additionally, *H. pylori* infection is associated with an increased production of interleukin 1 (IL-1), IL-6 and tumor necrosis factor α (TNF α) in the gastric mucosa. In particular *H. pylori* has been shown to induce the expression of IL-8 in gastric epithelium, which would stimulate neutrophil chemotaxis. This stimulation may be related to the cytotoxin/CagA phenotype of the strain. Many of these responses are related to the severity of disease. Evidence suggests that these cellular responses initiate long-term immunopathological events as a consequence of the infection. Such events could play

a key role in the pathogenesis of the diseases associated with *H. pylori* infection, including ulcerogenesis and atrophic gastritis leading to gastric cancer.

Non-Hodgkin lymphoma affecting the stomach (MALT lymphoma) is associated with *H. pylori* infection in about 92% of cases. A causative role for *H. pylori* in this disease is supported by evidence that *H. pylori* colonization can also induce the formation of lymphatic aggregates and lymphoid follicles in the gastric mucosa, and that antimicrobial therapy leading to eradication of the infection results in regression of disease.

Evasive strategies by the organism

H. pylori colonization persists in an apparent immunologically hostile microenvironment; however, no evidence for bactericidal activity of the local antibodies can be demonstrated. The gastric epithelium is not an immunoprivileged site. Immunoglobulin coating of bacteria, except those deep within the gastric pits, occurs *in situ* but these antibodies do not appear to be bactericidal. Bacterial mechanisms of immune avoidance may include a continuous loss of highly antigenic material, including the urease and flagella sheath, from the bacterial surface, thereby reducing the effectiveness of bound antibodies. Downregulation of the immune response during infection may also occur as antigen-specific responsiveness of local and circulating T cells is significantly reduced in *H. pylori*-positive compared with *H. pylori*-negative individuals, suggesting that these cells have been tolerized.

Vaccines

The ineffectiveness of the host immune response dur-

ing infection and frequent reinfection, or recrudescence of infection, in treated patients indicates that protective immune responses will be difficult to develop. Nevertheless, current results indicate that oral subunit vaccines, comprising bacterial sonicates, the UreB polypeptide (but not the UreA or jack bean urease) or the GroES and GroEL homologs, particularly when used with a mucosal adjuvant such as cholera toxin, can protect in animal models such as *H. felis* infection in mice. Interestingly, it also appears that oral immunization can eradicate a pre-existing experimental infection, suggesting that immune therapy may be an option. However, these animal models may not reflect human protective immune responses against *H. pylori* and more complex vaccines may be required. Moreover, the effects of vaccination on the immunopathological events associated with *H. pylori* infection need to be carefully considered before such an approach is adopted.

See also: Bacteria, immunity to; Bacterial cell walls: Gastrointestinal tract infections.

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HELPER T LYMPHOCYTES

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It was not until the late 1950s that lymphocytes were shown conclusively to be the essential specific cell of the acquired immune response. James Gowans showed that depletion of lymphocytes by chronic drainage of thoracic duct lymph, which contained only lymphocytes, abolished immune responses; the resulting immune deficiency could be restored completely by administration of thoracic duct lymphocytes. Within a few years Jacques Miller and others were able to define two separate lineages, initially on

the basis of function: T lymphocytes, which developed and matured in the thymus from precursor cells that originated first in the yolk sac and later in the bone marrow, carried out the functions of cell-mediated immunity; B lymphocytes, which were derived from cells that had their origin in the bursa of Fabricius in birds and the bone marrow in mammals, produced antibody. Although B lymphocytes were the cells which produced antibodies, it was quickly recognized that stimulation of B cells

not all T cell clones fit into these two categories, and additional subsets secreting other arrays of cytokines have been described. However, T cell clones secreting simultaneously the cytokines which have been attributed uniquely to T_H1 and T_H2 cells often are unstable. These clones may represent cells isolated before the process of differentiation has been completed.

In addition to having different functions, the various T cell subsets respond differently to several regulatory processes. Some of the factors that favor the selective growth of murine T_H1 or T_H2 clones have been defined. The patterns of immune responses observed in different situations suggest that the preferential growth and differentiation of one or another T cell subset can account for unusual features of some diseases. For example, infection with *Leishmania major* causes a progressive fatal disease in BALB/c mice, while limited disease and cure of infection are observed in C57BL/6 mice; the immune manifestations observed in these two mouse strains correspond to a predominantly T_H2 response to BALB/c mice and a predominantly T_H1 response in

C57BL/6 mice. The preferential involvement of subsets, mediated by particular immunoregulatory events, may also operate in autoimmune diseases.

See also: Antigen presentation via MHC class II molecules; B lymphocytes; Carrier; Cell-mediated immunity; Cooperation, mechanisms of cellular; Cytokines; Cytotoxic T lymphocytes; Effector lymphocytes; Hapten; Humoral immunity; Suppressor T lymphocytes; Thymus.

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HEMATOPOIETIC STEM CELL TRANSPLANTATION

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The last 10 years have seen a rapidly increasing use of hematopoietic stem cell transplantation as treatment for a variety of malignant and nonmalignant diseases. This has come about due to improvements in supportive clinical care which have reduced the morbidity and mortality of the procedure. In addition, discoveries in basic research have led to developments of new pharmaceutical and recombinant materials which are redefining the practice of stem cell transplantation. This entry will review the basic principles of stem cell transplantation, results of treatment, newer developments and likely future directions.

Background

Initially, most transplants were performed for younger patients with leukemia or aplastic anemia

and involved the use of marrow donors who were human leukocyte antigen (HLA)-identical siblings. Because of initial success and improving outcomes, the use of allogeneic stem cell transplantation has broadened to include a variety of severe hematologic diseases, including genetically-based diseases of the hematopoietic and immune systems (Table 1). Improvements in prevention of graft-versus-host disease (GVHD) have allowed the use of partially HLA-mismatched family donors, and increasingly, unrelated donors. An even greater increase has taken place in the use of autologous stem cell transplantation for hematologic malignancy and solid tumors, particularly as a treatment for breast cancer.

The conceptual basis for stem cell transplantation for malignancy is that it allows delivery for supralethal doses of chemotherapy and radiation in an attempt to overcome tumor resistance. In treatment

Table 1 Diseases currently treated with stem cell transplantation

Malignant	Nonmalignant
Acute leukemias (AML/ALL)	Aplastic anemia
Chronic leukemias (CML/CLL)	Immunodeficiency syndromes
Myelodysplastic syndromes	β -Thalassemia
Multiple myeloma	Sickle-cell anemia
Myeloproliferative disorders	Fanconi anemia
Breast cancer ^a	Inborn errors of metabolism
Ovarian cancer ^a	
Neuroblastoma	
Various sarcomas ^a	
Germ cell tumor ^a	

^aAutologous transplants almost invariably used.

of nonmalignant diseases such as aplastic anemia the goal is to replace a defective hematopoietic or immune system, and less intensive therapy may be sufficient to facilitate engraftment of donor cells. In both situations, long-term hematopoietic and immune functions are reconstituted from so-called stem cells transferred with the graft. These cells home to the marrow environment and give rise to long-term multilineage hematopoiesis.

Source of hematopoietic stem cells

Historically, most transplants were performed using marrow 'harvested' from the donor pelvis. Recently, there has been increasing use of hematopoietic stem cells collected from the blood by leukapheresis, and the term stem cell transplant now appears to be more appropriate than bone marrow transplant. Large numbers of hematopoietic progenitors circulate in the blood after treatment with chemotherapy and/or recombinant growth factors, such as granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), and transplantation of these cells, primarily to date for autografting, is associated with rapid hematopoietic recovery. Preliminary data using peripheral blood progenitors for allografting also indicate an engraftment benefit, but there may prove to be an increased risk of chronic GVHD due to the high doses of T cells given.

Donors used are usually HLA-identical siblings. Approximately 25% of patients have such a donor, and in some cases phenotypically HLA-matched or HLA-nonidentical family donors can be used. Rarely an identical twin is available, and from an immunologic standpoint these donors are ideal. Increasingly used are phenotypically HLA-matched unrelated

donors identified through national or international donor registries.

Autologous transplant requires the cryopreservation and storage of the patient's own stem cells. Since these transplants are almost always performed for malignancy, the graft may be treated *in vitro* in an attempt to remove residual tumor cells, e.g. with monoclonal antibodies or chemotherapeutic agents.

Conditioning regimens

The most frequently employed regimen used before allografting for the treatment of malignancy has been a combination of cyclophosphamide (CY) and total body irradiation (TBI). An antitumor dose-response relationship has been found for TBI as demonstrated by lower relapse rates in patients receiving high (15.75 Gy) as opposed to lower (12 Gy) doses of TBI (Figure 1A). Because of greater toxicity, there has been no survival gain from using the higher doses of radiation (Figure 1B). Fractionation of TBI reduces radiation-induced side-effects without significant loss of antileukemic activity. Attempts to increase the antileukemic efficacy of the regimens has involved

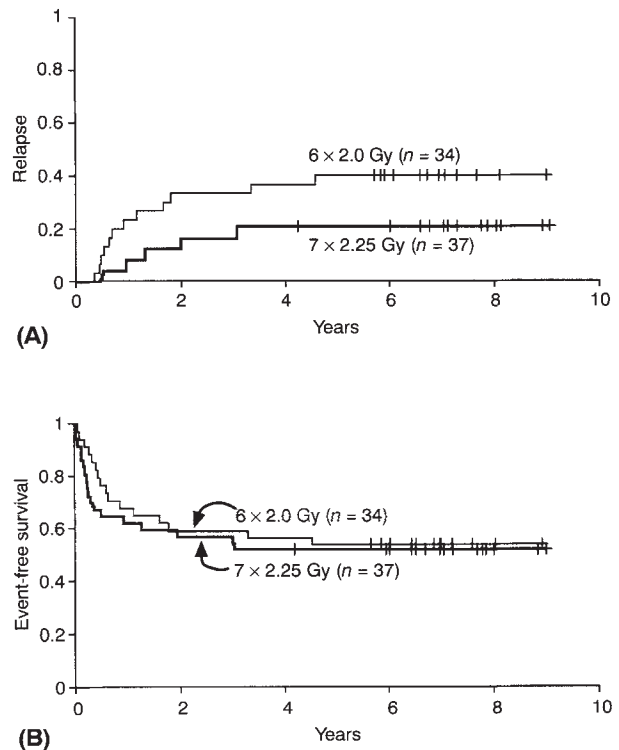


Figure 1 Outcome after allogeneic transplantation using HLA-matched sibling donors for AML in first remission illustrating effects of radiation dose on (A) relapse and (B) overall survival. Patients were treated in Seattle at the Fred Hutchinson Cancer Research Center and randomized to receive cyclophosphamide 120 mg kg⁻¹ and either TBI 12 Gy (2 Gy $\times$ 6) or TBI 15.75 Gy (2.25 Gy $\times$ 7). Seattle data as of 1996.

Table 2 Agents frequently employed in transplant conditioning regimens

Total body irradiation
Cyclophosphamide
Etoposide
Melphalan
Cytosine arabinoside
Thiotepa
Bischlorethyl nitrosurea (BCNU)
Carboplatin

replacing CY with other agents used in conjunction with TBI, or the use of regimens based on chemotherapy alone (Table 2). For autologous transplants where host immunosuppression is not required, multidrug regimens are often used. Despite a wide array of possible conditioning regimens that have been tested, no significant advances on the basic TBI-based regimens have been demonstrated to date.

Recombinant cytokines

Many cytokines have been identified that act on hematopoietic and immune cells (Table 3). Potential useful effects include stimulating hematopoietic progenitor proliferation, 'mobilization' of progenitors into the blood for collection, and stimulation of an immune recovery and antitumor activity. Cytokines currently in clinical use include G-CSF, GM-CSF, erythropoietin, interferon α and interleukin 2 (IL-2).

Table 3 Recombinant cytokines with a potential clinical role in stem cell transplantation

Hematopoietic growth factors ^a	Immunomodulatory factors ^a
GM-CSF ^b	Interferon α^b
G-CSF ^b	Interleukin 2 ^b
Erythropoietin ^b	M-CSF
Interleukin 1	Interferon γ
Interleukin 3	Interleukin 4
Interleukin 6	Interleukin 7
Interleukin 8	Interleukin 10
Interleukin 11	Interleukin 12
Stem cell factor	
Thrombopoietin	
FLT3/FLK2 ligand	
Leukemia inhibitory factor	
Oncostatin	

GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor.

^aMany of these factors are pleiotropic with effects on both hematopoietic and immune cells.

^bAvailable for clinical use.

Results of clinical stem cell transplantation

The vast majority of allogeneic stem cell transplants and almost all autologous stem cell transplants are undertaken for treatment of malignancy. The following section discusses the use of HLA-matched family donor transplants and autologous transplants. The choice of which type of transplant to perform is guided by a variety of factors, including disease type, patient age, likelihood of obtaining a useful autologous stem cell graft free of tumor, and physician preference.

Transplants for hematologic malignancy (Table 4)

Allogeneic stem cell transplantation is the only dem-

Table 4 Comparison of outcomes after allogeneic stem cell transplantation and conventional chemotherapy as treatment for hematological malignancies^a

Malignancy	Transplant		Chemotherapy ^b
	Relapse-free survival (%)	Relapse-free survival (%)	Relapse-free survival (%)
AML			
CR1 ^c	40	22	20-30
First relapse/CR2	25-34	45	<10
Refractory	15	80	Incurable
ALL			
CR1 ^c	54	35	40 (adults)
CR2	35	45	<5
Refractory	15	80	Incurable
CML			
Chronic phase	58	17	Incurable
Accelerated phase	30	45	Incurable
Blast crisis	15	70	Incurable
CLL			
Less advanced	60	10-20	Incurable
More advanced	20	Unknown	Incurable
Lymphoma (HD, aggressive NHL)			
CR2 ^c	40	20	<10
Refractory ^c	≤15	70-80	Incurable
Myelodysplasia			
No excess blasts	60	<10	Incurable
With excess blasts	32	50	Incurable

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; HD, Hodgkin's disease; NHL, non-Hodgkin's lymphoma; CR, complete remission.

^aNote that some variation in outcomes has been reported, both with transplantation and with conventional therapy, that may not be reflected in these comparisons.

^bPatients failing conventional chemotherapy almost always die as a result of malignancy.

^cSimilar disease-free survivals have been shown with autologous bone marrow transplantation, but relapse rates are higher.

onstrated curative therapy for diseases such as chronic myeloid leukemia (CML) and myelodysplastic syndromes in which the stem cell compartment of the marrow is abnormal. In CML, excellent results have been achieved for patients transplanted in chronic phase using conditioning regimens of TBI/CY or busulfan (BU)/CY. Results are best if transplants are performed early after diagnosis and within the chronic phase (Figure 2) with relapse-free 5-year survivals of 60–70%. For patients with myelodysplastic syndromes, approximately 40% of patients are cured, with the best results seen in patients with less advanced myelodysplasia, where survivals are approximately 60%.

For patients with acute myeloid leukemia (AML) in first remission, approximately 50% of patients are cured and 25% relapse (Figures 1 and 3). For patients in first relapse or second remission, 3-year disease-free survival is approximately 34% (Figure 3). Patients with advanced refractory AML have a cure rate of 10–15%. Similar results have also been seen after allografting for the same disease stages in acute lymphoblastic leukemia (ALL). Patients with

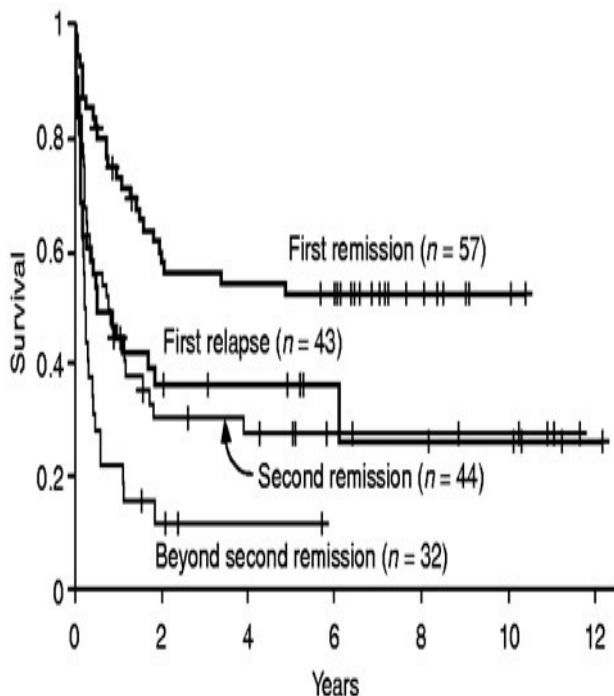


Figure 2 Survival after allogeneic transplantation from HLA-matched siblings for CML. Patients received regimens containing CY/TBI or BU/CY. (A) Survival according to phase of disease at transplant. (B) Survival after transplant for chronic-phase patients according to interval from diagnosis to transplant. Patients received CY 120 mg kg⁻¹ and TBI 12 Gy or BU 16 mg kg⁻¹ and CY 120 mg kg⁻¹. GVHD prophylaxis was a combination of short methotrexate (days 1, 3, 6 and 11) and cyclosporine. Seattle data as of 1996.

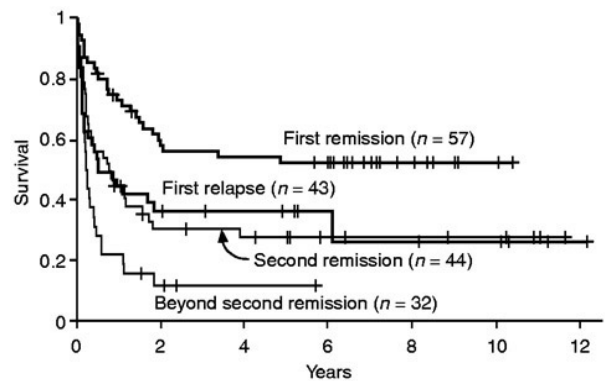


Figure 3 Survival after allogeneic transplantation for AML according to disease stage at diagnosis. Seattle data as of 1996.

ALL are not transplanted in first remission unless adverse prognostic features for relapse are present. The outcomes of allogeneic transplant and autologous transplants have been similar for acute leukemia in remission. However, there is more transplant-related mortality after allografting and a higher relapse rate after autografting.

Transplants have now been widely used in the treatment of non-Hodgkin's lymphoma, Hodgkin's disease and multiple myeloma and, to a lesser extent, chronic lymphocytic leukemia (CLL), almost always after failure of conventional therapy. As seen in acute leukemia, relapses are more frequent after autografting but toxicity is greater after allografting. Results of allografting and autografting are similar for lymphoma except for low-grade lymphomas, where allografting appears to have better curative potential. Allografting can cure patients of CLL and possibly multiple myeloma. Many autologous transplants have been performed for myeloma but cure using this approach has not been demonstrated.

Transplants for solid tumors

The use of high-dose chemotherapy followed by autologous transplantation has been tested in a variety of solid tumors. When patients have metastatic disease, results are poor. Best responses are seen in diseases that are often responsive to conventional dose chemotherapy, e.g. breast cancer. Because of the poor results in patients with metastatic disease, high-dose therapies are now being tested as adjuvant therapy for patients with chemosensitive solid tumors at high-risk of relapse, e.g. with breast or ovarian cancer. Preliminary results of autografting in patients with high-risk stage II and III breast cancer indicate that relapses are delayed, and possibly prevented with 3-year relapse-free survivals of 70% reported after transplant, as compared with 40–50%

for patients receiving conventional adjuvant chemotherapy.

Transplants for nonmalignant disorders

Stem cell transplantation for nonmalignant disorders has almost exclusively involved the use of allogeneic transplants from HLA-identical siblings. Initially, most experience involved transplants for aplastic anemia where stem cell transplantation could cure 50% of patients, but graft rejection and GVHD were significant problems. Improvements in conditioning regimens and GVHD prevention have led to impressive survival rates of 85–90% in allograft recipients (Figure 4). Equally impressive, if still somewhat con-

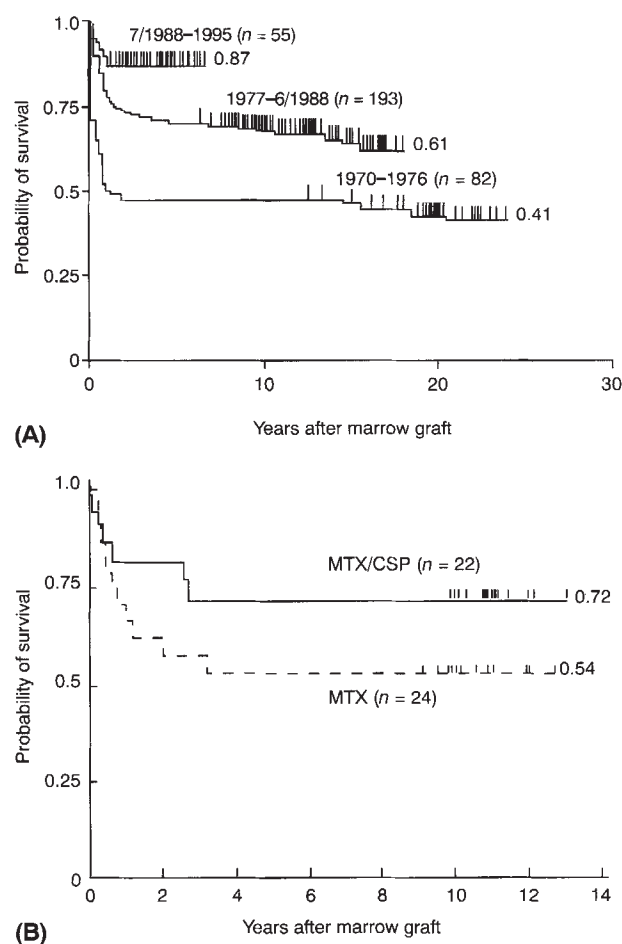


Figure 4 Survival after allogeneic transplantation from HLA-matched siblings for aplastic anemia. (A) Improved survivals of patients treated in the last decade were a result of improved conditioning regimens and GVHD prophylaxis. The addition of antithymocyte globulin to cyclophosphamide-based conditioning regimens has largely eliminated graft rejection as a problem (1988–1995). (B) Effect of GVHD prophylaxis on survival showing results of a randomized study comparing prophylaxis with MTX/CSP (methotrexate/cyclosporine) versus MTX alone. Previous studies had shown that MTX and CSP were equivalent as single agents. Patients received CY-only conditioning regimens. Seattle data as of 1996.

troversial, has been the use of allografting for treatment of β -thalassemia. Results of transplants, primarily performed by the Pesaro group, have shown a cure rate of more than 90% for patients transplanted early in the disease course (Figure 5). Preliminary data indicate that similar results may be possible in sickle-cell disease. Transplants for genetically-based immune deficiency syndromes can cure diseases such as severe combined immunodeficiency disease.

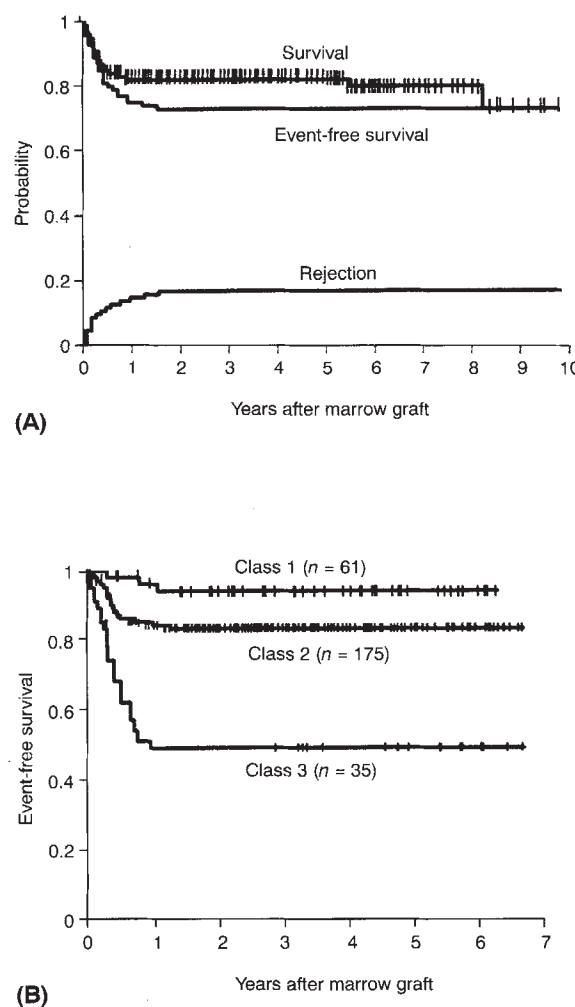


Figure 5 Survival after allogeneic transplant at Pesaro for β -thalassemia using HLA-identical sibling donors. (A) Probability of survival, event-free survival and graft rejection in all patients. (B) Event-free survival according to disease stage based on extent of liver damage (due to iron overload from multiple transfusions and ineffective erythropoiesis). Early stage (class 1) patients have >90% rejection-free survival. Patients received conditioning regimens containing BU/CY and GVHD prophylaxis with MTX/CSP. Class 1, no hepatomegaly, no portal fibrosis; class 2, hepatomegaly or portal fibrosis; class 3, + hepatomegaly, + portal fibrosis. (Reprinted by permission of Blackwell Science, Inc. from Lucarelli G (1994) Bone Marrow Transplantation in Thalassemia In: Forman SJ, Blume KG and Thomas ED (eds) *Bone Marrow Transplantation*, 833–835, Blackwell Scientific Publications, Boston, USA.)

HLA-nonidentical stem cell marrow grafts

To extend the available donor pool and thereby offer potentially life-saving treatment, studies using HLA-nonidentical family members and phenotypically HLA-matched unrelated donors have been performed. A high incidence of acute and chronic GVHD and increased risk of graft rejection have been observed. The use of one-HLA-antigen mismatched donors and HLA-matched unrelated donors have been associated with acceptable results. In patients with chronic phase CML, who underwent unrelated donor marrow transplantation in Seattle, an impressive 70% 3-year disease-free survival has been achieved.

Complications of transplant

Factors influencing outcomes of transplant include age, type of transplant, disease type and stage, and pre-existing medical problems. The principal complications that adversely affect outcome include toxicity of conditioning regimens, immunologic deficiency, poor graft function, GVHD and relapse of disease.

Regimen-related toxicities

To achieve maximum antitumor effects, regimens have been intensified such that significant nonhematopoietic toxicities are seen (Table 5). The main life-threatening toxicities are veno-occlusive disease (VOD) of the liver and interstitial pneumonitis. Serious VOD is seen in 15–28% of patients, and severe idiopathic pneumonia in approximately 3% of patients. Long-term side-effects include endocrine dysfunction, infertility, cataracts and, in chil-

dren, impaired growth, impaired sexual function and reduced intellectual function. Late secondary malignancy is also a significant problem. Unique to autologous transplant recipients is the development of myelodysplastic syndromes, occurring in up to 20% of patients. The only cure for this life-threatening side-effect is a second transplant.

Hematologic and immune recovery

Recovery of granulocytes and platelets is usually complete by 40–50 days after transplant. Recovery of natural killer (NK) cells, monocytes and macrophages occurs early. However, functional defects, including defective immune function and chemotaxis, may persist for many months. Lymphocyte dysfunction is often prolonged and characterized by diminished humoral and cell-mediated response to antigen. Peripheral blood B cell numbers and function are depressed, and the T cells show depressed CD4 counts, increased CD8 suppressor activity and inverted CD4:CD8 ratios. Immune reconstitution is often complete by 1 year, but in patients with chronic GVHD sustained clinical and laboratory evidence of immune deficiency persists.

Infectious complications

Early in the post-transplant period there is severe neutropenia of 2–3 weeks duration and, although bacterial infection is common, the availability of potent antibiotics means that death from bacterial sepsis is uncommon. More difficult problems are viral and fungal infections and, in particular, cytomegalovirus (CMV) infections. Reactivation of CMV from host or transfused cells in the early post-transplant months can cause life-threatening pneumonia, previously fatal in approximately 15% of those receiving allografts for malignancy. Effective preventative measures have included use of CMV-seronegative blood products in CMV-seronegative transplant recipients, and more recently prophylaxis with ganciclovir. Fungal infections due to candida or aspergillosis are increasingly seen and are often resistant to drug therapy. Herpes simplex is seen commonly after transplant and is prevented with acyclovir. Varicella zoster reactivation occurs in about 40% of cases and is treated effectively with acyclovir. *Pneumocystis carinii* infection, which previously caused about 10% of post-transplant pneumonias, is prevented with prophylactic trimethoprim-sulfamethoxazole.

Graft-versus-host disease

Acute GVHD is caused by alloreactive T cells directed at non-MHC (major histocompatibility

Table 5 Major toxicities caused by conditioning regimens

Early	Late
Mucositis ^a	Infertility ^a
Nausea/vomiting ^a	Endocrine insufficiencies
Enterocolitis	Secondary malignancy
Acute myocarditis	Cataracts
Veno-occlusive disease of the liver	Leukoencephalopathy
	Myelodysplastic syndrome ^b
Interstitial pneumonia	
Leukoencephalopathy	
Hemorrhagic cystitis	
Acute renal failure	
Erythroderma	
Alopecia ^a	

^aAlmost invariable side-effects of conditioning regimens currently used for treatment of malignancy.

^bAssociated with autologous transplants, may be due to pretransplant therapies.

Table 6 Major risk factors for the development of GVHD

<i>Acute GVHD</i>	<i>Chronic GVHD</i>
HLA disparity	HLA disparity
Unrelated donor	Unrelated donor
Advanced malignancy	Preceding acute GVHD
Sex-mismatched donor/recipient pair	Increasing patient age
Parous female donor	
Increasing patient/donor age	
Irradiation dose >1200 cGy	

complex) antigens recognized on host antigen-presenting cells, or against MHC antigens when MHC disparity exists. A complex series of cellular interactions that generates a cascade of cytokine release appears to be important in pathogenesis. Target organs for inflammation are the skin, liver and gastrointestinal tract. Principal risk factors for GVHD are summarized in Table 6. Between 20 and 50% of patients receiving HLA-identical sibling-donor transplants develop significant acute GVHD, and this is associated with significantly increased risk of transplant-related mortality, primarily as a result of infectious complications due to immune suppression.

Attempts to prevent GVHD are necessary and usually involve administration of immunosuppressive agents (Table 7), most commonly cyclosporine, methotrexate and corticosteroids. Indefinite im-

munosuppression is not required and transplant tolerance usually develops by 3–6 months. The combination of cyclosporine and methotrexate is better GVHD prophylaxis than either agent alone and leads to improved survival in patients with less advanced malignancy and nonmalignant disorders (Figure 4).

With aggressive malignancy, improved GVHD control may be associated with relapse rates, suggesting a loss of graft-derived antitumor effect. Newer, promising agents include FK506 and mycophenolate mofetil. A highly effective alternative approach to GVHD prevention involves removing T cells from the graft. However, this results in greater risks of graft rejection and relapse, with no survival gain. Treatment of established GVHD primarily involves the use of corticosteroids, antithymocyte globulin (ATG), cyclosporine and, increasingly, other agents listed in Table 7.

Chronic GVHD, defined as GVHD occurring after 100 days post transplant, clinically resembles autoimmune diseases such as scleroderma, Sjögren's syndrome and primary biliary cirrhosis. Severe immune deficiency persists and infection is the most frequent cause of death. Chronic GVHD of some degree occurs in 30–50% of patients receiving HLA-matched sibling transplants, usually within 2 years of transplant, and is associated with reduced probability of survival. The most effective therapy involves use of prednisone plus or minus cyclosporine. Other drugs with an increasing role include FK506 and thalidomide.

A relationship between relapse rates and previous GVHD has been established. Development of clinically significant acute or chronic GVHD provides some protection against relapse (graft-versus-leukemia effect), and for patients with advanced leukemia may lead to improved overall survival. In practice, it has not been possible to control GVHD with any precision to exploit this benefit during the transplant process. However, it has been possible to treat some cases of relapsed leukemia, most notably patients with CML, with infusions of donor buffy coat cells and induce second complete remissions, including possible cure.

Graft failure

Poor graft function is common after transplant but often resolves after contributing factors such as infection and drugs are dealt with. Persistent poor graft function is associated with a high mortality and the use of recombinant hematopoietic growth factors improves survival. True immunologic graft rejection is uncommon after the use of TBI-based conditioning regimens, except when HLA-mismatched donors are used and/or the graft is T cell depleted. The addition

Table 7 Agents currently used or under investigation for prophylaxis and treatment of GVHD^a

<i>Agent</i>	<i>Prophylaxis</i>	<i>Treatment</i>
Methotrexate	+	
Cyclosporine	+	+
Corticosteroids	+	+
T cell depletion	+	
FK506	+	+
Antithymocyte globulin	+	+
Anti-T-cell monoclonal antibodies	+	+
Mycophenolate mofetil ^b	+	+
Rapamycin ^b	+	+
CTLA4-Ig ^b	+	+
Azathioprine ^c		+
Thalidomide ^c		+
Psoralen ultraviolet A range (PUVA) therapy		+
Topical corticosteroids (skin, gastrointestinal tract)		+

^aCombination therapy is commonly given for prevention of acute GVHD.

^bPhase I or preclinical studies currently being performed.

^cMainly used for treatment of chronic GVHD.

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HEMOLYTIC DISEASE OF THE NEWBORN

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For this article, hemolytic disease of the newborn (HDN) is taken to be the condition resulting from blood group incompatibility between mother and fetus, and clinically 'rhesus' (Rh) disease is the most important example.

During pregnancy, and particularly during the trauma of delivery, some fetal red cells may cross the placenta and enter the maternal circulation. When the mother is Rh negative and the fetus Rh positive (paternally derived), the fetal cells may immunize the mother, and when this happens the important antibody which she produces is of the immunoglobulin G (IgG) (7S) class. This can cross the placenta and coat, thereby damaging the fetus's Rh positive red cells, causing anemia, jaundice and edema.

Immunization can now usually be prevented by injecting the mother after delivery with anti-Rh (D) gammaglobulin. This eliminates any fetal Rh-positive cells which have entered the mother's circulation and therefore protects the next Rh-positive baby. The first one in any case usually escapes, since the maternal IgG antibody is slow to form.

In 1938, Ruth Darrow, an American doctor who herself had had a succession of affected babies, was the first to suggest that an incompatibility was the cause, but she attributed this to differences between fetal and adult hemoglobin rather than between blood groups.

In 1939, Levine and Stetson described a group O woman who needed a transfusion after a delivery. Though she was given her husband's group O blood, she nevertheless developed a severe transfusion reaction and it was found that her serum agglutinated her husband's cells, suggesting the presence of a new antibody.

For some years, Landsteiner and Wiener had shown that rabbits injected with the blood of rhesus

monkeys formed antibodies which not only agglutinated the monkey's red cells but also those carrying the human M antigen. However, in 1940, they discovered that when the anti-M had been absorbed, the rabbit serum still agglutinated most human bloods independently of their MN types. The new blood group system was therefore designated Rh.

In 1941, Levine and colleagues put the Rh blood groups firmly on the map with regard to pregnancy, for they noted that transfusion accidents tended to be associated with pregnancy, the explanation being that the mother had previously been immunized by an Rh-positive fetus. They also discovered that it was necessary to test for Rh antibodies at body (not room) temperature, and this is why these antibodies had not been detected earlier.

It was obvious, therefore, that the first step in preventing RhHDN was to ensure that all Rh-negative women needing transfusion were given Rh-negative blood.

Naturally occurring protective mechanisms

Although 1 in 7 of all matings in the UK are between Rh-negative women and Rh-positive men, Rh immunization is much less frequent than expected for the following reasons:

1. The husband may be heterozygous.
2. Transplacental hemorrhage may never take place.
3. The mother may be a 'nonresponder' (about 30% of Rh-negative individuals do not make antibodies when injected with a small volume of Rh-positive cells).
4. The fetus may be incompatible with the mother on the ABO system. For example, in a group O

mother with a group A or B fetus, the mother's naturally occurring anti-A or anti-B (mostly IgM) will eliminate any fetal cells before they have time to stimulate the production of Rh antibodies.

Blocking (incomplete) antibodies

Sometimes the Rh antibody cannot be detected, even at body temperature, in mothers who have had RhHDN babies. This is because the antibody, though present, is 'hidden'. When Rh-positive cells are suspended in the suspected serum, and anti-D (IgM) is added, the cells are not agglutinated (as would be expected). This is because the antigen sites have already been blocked with the mother's IgG blocking, or incomplete, anti-D. ('Blocking' is the term used by Wiener – it is synonymous with 'incomplete', as used by Race.)

The antiglobulin test (Coombs' test)

When blocking antibody is present, the antiglobulin test, which is a test for any antibody on red cells, gives positive results.

The baby's red cells are washed and an equal volume of rabbit antihuman globulin (AHG) is then added. If there is IgG (i.e. blocking anti-Rh) on the red cells, the AHG will cause agglutination (this is the direct Coombs' (antiglobulin) test).

The antiglobulin test should be carried out regularly on the cord blood whenever HDN is a possibility.

Rh nomenclature

In the Fisher-Race nomenclature, an individual inherits a set of three Rh genes from each parent and the loci are all on chromosome 1. They are responsible for D or d, C or c, E or e, and the genotyping of an individual is carried out by testing his or her red cells against anti-D, anti-E, etc. (anti-d has never been found). D is clinically the most important of these antigens and there is a rare weak form called D^u. As the tests for detecting D^u become more sensitive, an individual's Rh blood group may very occasionally appear to change from Rh negative to Rh positive.

Clinical features

Hemolytic anemia is the result of the maternal antibody having coated the red cells of the baby, resulting in their removal to the spleen. Generalized bleeding, jaundice, edema, and sometimes damage to

the central nervous system occur. Hydrops (Figure 1), the result of low plasma protein, anemia and heart failure, is now increasingly rare because of modern therapy.

Serological aspects of HDN

The Rh D antigen is the most potent, and c next; then (outside the Rh system) the Kell antigen. Anti-Kell is usually the result of a blood transfusion, most people being Kell negative and therefore incompatible pregnancies rare. In theory, any IgG anti-blood group antibody present in the mother can cross the placenta and cause HDN.



Figure 1 Hydropic baby, the result of rhesus hemolytic disease. Anemia is often another feature.

Prediction of severity

There is a correlation between maternal anti-D concentration and the severity of HDN. Furthermore, there are subclasses of anti-D which are associated with different degrees of severity. The antibody-dependent cytotoxicity test (ADCC), using monocytes and lymphocytes, is also useful. Liley spectral absorption curves, whereby the severity of the disease is judged by the bilirubin content of the amniotic fluid, are now somewhat out of date. For the newborn baby, the best single criterion of severity is cord hemoglobin – normal 13.6–19.6 g dl⁻¹, moderately affected around 12.8 g dl⁻¹.

Treatment of the established disease

Transfusion with Rh-negative blood is the keynote. Exchange transfusion after birth is still used, because free maternal Rh antibody continues to act for several days after birth.

Before birth, intraperitoneal transfusion (blood is absorbed through the lymphatic vessels of the fetus) arrests the deterioration of the fetus and gains a few weeks gestation. More recently, direct intravascular fetal blood transfusion of Rh-negative blood, using ultrasound to guide the needle into the umbilical vein, is used. The quantity of blood to be transfused and the volume to be exchanged is calculated with the goal of achieving a hematocrit of 30–40%.

Plasmapheresis is popular but the beneficial effects are not proven. Phototherapy is of much more use in the jaundice associated with prematurity than in that due to Rh.

Prophylaxis

Fetal cells in the maternal circulation can be easily recognized using the Kleihauer technique (Figure 2), and the size of any transplacental hemorrhage thereby assessed. Anti-D gammaglobulin should be given to all non-immunized Rh-negative women after delivery of an Rh-positive baby, and it should be injected into the deltoid muscle of the mother within 48 h of delivery. The anti-D coats the D antigen sites on the fetal cells and prevents immunization. The standard dose is 500 iu but more may be needed, depending on the Kleihauer count. For an abortion up to 20 weeks 250 iu is the standard. Occasional failures of prophylaxis occur but if antenatal anti-D is given at 28 and 35 weeks as well as post delivery, the failure rate is very small. However, antenatal anti-D as a routine is wasteful (though monoclonal anti-D will eventually make supplies easier). An economical compromise is to restrict the antenatal

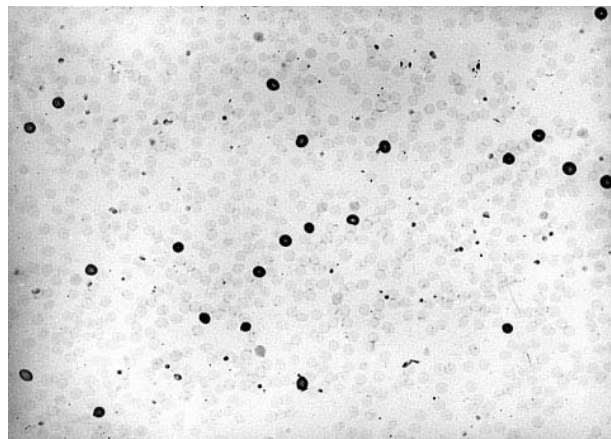


Figure 2 Fetal cells in the maternal circulation demonstrated by the Kleihauer technique. The adult hemoglobin in the mother's red cells is eluted by an acid phosphate buffer and they appear as 'ghosts', whereas the fetal hemoglobin is not eluted and the cells therefore stand out and can be readily counted.

regimen to primigravidae because with this a woman is highly likely to have at least two babies free of RhHDN.

A comparative aspect in monkeys

Interestingly in some species, the Rh mechanism is present but no disease results. Thus IgG antibodies are formed by the monkey mother, cross the placenta, and give a positive antiglobulin test on the fetal red cells, yet the offspring are never jaundiced. The probable reason is that the antibody concentration is not high enough to cause hemolysis.

ABO Hemolytic disease

ABO incompatibility has already been mentioned (see Naturally occurring protective mechanisms) and ABO hemolytic disease might be expected to be very common, but the anti-A and anti-B are mostly IgM and therefore do not cross the placenta. Mild cases are fairly frequent, but in some parts of the world there are areas where it is both much more common and more severe, e.g. Jamaica. Here ABO antibodies may arise from antigens derived from food and from the bacterial flora in the gut, and in Jamaica the antibody titers may be as high as 2000–4000. Interestingly, the infant may have overt evidence of hemolysis in the absence of a positive antiglobulin test, the converse of what often happens in RhHDN. The reason is not known, but the explanation may lie in differences between the Rh and ABO antigens.

The treatment of ABO HDN is on the same lines as for RhHDN – exchange transfusion is carried out

using group O red cells taken from a donor with very low anti-A or -B titers.

See also: ABO blood group system; Agglutination; Antibodies, detection of; Antibody-dependent cellular cytotoxicity; Antigens; Antiglobulin (Coombs') test; Antiserum; Blood transfusion reactions; Immunosuppression; Maternal antibodies; Plasmapheresis; Rh antigens.

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HEPATITIS A VIRUS, INFECTION AND IMMUNITY

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Hepatitis A is an acute, self-limiting infection of the liver, caused by an enterically transmitted picornavirus. As the virus is not cytolytic, liver cell damage is assumed to be immunologically mediated. While frequently asymptomatic, especially in the young, infection occasionally results in fulminant hepatitis and death. Recovery from the disease is accompanied by lifelong protection against reinfection. Protection is mediated by circulating antibodies and can be passively transferred.

Hepatitis A has a relatively short incubation period (15–49 days), is transmitted fecal orally and is followed by long-lasting immunity that can be passively transferred. In 1973, virus-like particles were identified in the feces of volunteers experimentally infected with the MS-1 strain of hepatitis A virus (HAV). These particles were specifically aggregated by convalescent, but not by preinfection, serum. The identification of the etiological agent – and the demonstration that infection could be trans-

mitted to marmosets – ushered in a new era of research leading to the development and licensing of a safe and effective vaccine.

Virology

Classification

Hepatitis A virus is a member of the Picornaviridae family and the sole member of the genus *Hepatitis A virus*. There are four recognized genotypes, but only one serotype.

HAV is spherical, nonenveloped and measures 27–28 nm diameter; it is more resistant to heat than other picornaviruses, remaining stable after incubation at 56°C for 30 min, several weeks at room temperature and years at –20°C, although it can be inactivated by heat (121°C for 30 min) or exposure to hypochlorite (1.5–2.5 mg l⁻¹ for 15 min). The virus is known to infect humans, juvenile chimpanzees and several *Saguinus* species of marmosets.

Epidemiology

Prevalence of infection

Although hepatitis A has a worldwide distribution, there are wide differences in its incidence. Under conditions of overcrowding, especially when there is limited access to clean water and inadequate disposal of human feces, HAV infection is common and occurs early in life. These infections are usually clinically inapparent. By contrast, where high standards of hygiene and sanitation apply, most children reach adult life without encountering the virus.

Epidemiologic patterns

Two patterns of age-specific prevalence of anti-HAV are commonly recognized: 1) Pattern A is typical in countries with poor standards of hygiene and sanitation: most children are infected by the age of 5 years and the prevalence of anti-HAV is virtually 100% by the age of 10 years. This pattern may also be seen in disadvantaged groups in affluent countries. 2) Pattern B is typical of most developed countries. The sigmoidal curve reflects a steady decline in the rate of infection over many years. Paradoxically, as living standards improve, the incidence of hepatitis A may rise, as most of the infections which occur are delayed until adult life, when they are more frequently associated with jaundice.

Hepatitis A has become a significant problem among travelers, as large numbers of people from low prevalence countries take holidays in regions with high rates of infection. Hepatitis A is now the most common vaccine-preventable disease of travelers, the risk of acquiring the disease being 100 times greater than typhoid fever and 1000 times greater than cholera.

Clinical features

Clinical course

The mean incubation period from infection to the onset of symptoms is 4 weeks. Patients with hepatitis A often describe a vague illness appearing between 1 and 7 days prior to the onset of dark urine.

The first objective sign of disease is the onset of dark urine, which is followed within a few days by pale or clay-colored feces and yellow discoloration of the sclera, skin and mucous membranes. The return of color to the stools, which usually occurs within 2 or 3 weeks after the onset of illness, is a good sign of resolution of the disease.

Laboratory diagnosis

Acute hepatitis A is not distinguishable from other forms of viral hepatitis on clinical grounds, although the diagnosis may be inferred in a patient with typical symptoms and the appropriate epidemiologic clues. Liver function tests, especially serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are sensitive measures of parenchymal liver damage, but are not specific for hepatitis A.

The diagnosis of acute hepatitis A is most commonly confirmed by detection of specific anti-HAV immunoglobulin M (IgM), using either ELISA or radioimmunoassay.

Prevention

General

Control of hepatitis A may require social, educational and engineering initiatives. Provision of clean water, proper disposal of feces and alleviation of crowded living conditions rapidly reduces the incidence of hepatitis A and other enteric infections.

Passive immunization

Until recently, the only means of preventing hepatitis A was administration of pooled human immunoglobulin (HNIG), which was first shown to be effective in large-scale studies among American soldiers during World War II. If administered prior to exposure, HNIG can reduce the incidence of hepatitis A by up to 90%.

Active immunization

Three parenterally administered hepatitis A vaccines have been developed and are currently licensed for use in humans. Each of these is produced from inactivated cell culture-derived virus and they obtain their effects by inducing circulating antibodies to antigens present on the surface of the virus.

Clinical trials indicate that inactivated hepatitis A vaccines are safe, highly immunogenic and provide excellent protection against infection. Several million doses of the vaccine have been administered without serious adverse events.

Therapy

As no specific therapy exists, management is largely supportive.

See also: Hepatitis B virus, infection and immunity; Gammaglobulin; Hepatitis C virus, infection and

immunity; Hepatitis E, F and G viruses, infection and immunity; Picornavirus, infection and immunity; Viruses, immunity to.

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HEPATITIS B VIRUS, INFECTION AND IMMUNITY

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Hepatitis B virus (HBV) infection is a major public health problem, infecting approximately 300 million people worldwide, and is a major cause of chronic liver disease and hepatocellular carcinoma (HCC). The majority of adults infected have a transient hepatitis from which they completely recover and clear the virus. Rarely the disease becomes fulminant and the patient may die. Between 5 and 10% of adults become persistently infected and develop chronic liver disease. In the majority of cases of vertical transmission the virus is not eliminated and the child becomes chronically infected, greatly increasing its risk of developing cirrhosis and HCC.

Virology

HBV is a member of the Hepadnaviridae, a related group of hepatotropic, enveloped, double-stranded DNA viruses which have small genomes. The HBV genome is 3.2 kb in size and contains four open reading frames (Figure 1). These encode the envelope (hepatitis B surface antigen, HBsAg), nucleocapsid

(hepatitis B core and 'e' antigens, HBcAg and HBeAg), DNA polymerase and X protein.

The envelope of the virus contains the large, middle and major surface proteins, which all contain the same carboxyl terminus of 226 amino acids. The middle protein has an additional 55 amino acids designated the pre-S2 region, and the large protein has a further 120 amino acids designated the pre-S1 region. All of these proteins also occur in a glycosylated form.

The core gene encodes two overlapping polypeptides. The longer of these polypeptides contains a signal peptide which directs it to the endoplasmic reticulum where the amino- and carboxy-terminal ends are cleaved and HBeAg is secreted into the circulation. The smaller polypeptide has a nucleophilic sequence at its carboxy-terminal end and associates with the RNA pregenome of the virus during the initial stage of the formation of nucleocapsids. The X open reading frame encodes a protein which has the ability to modify the transcription of both viral and host cell genes.

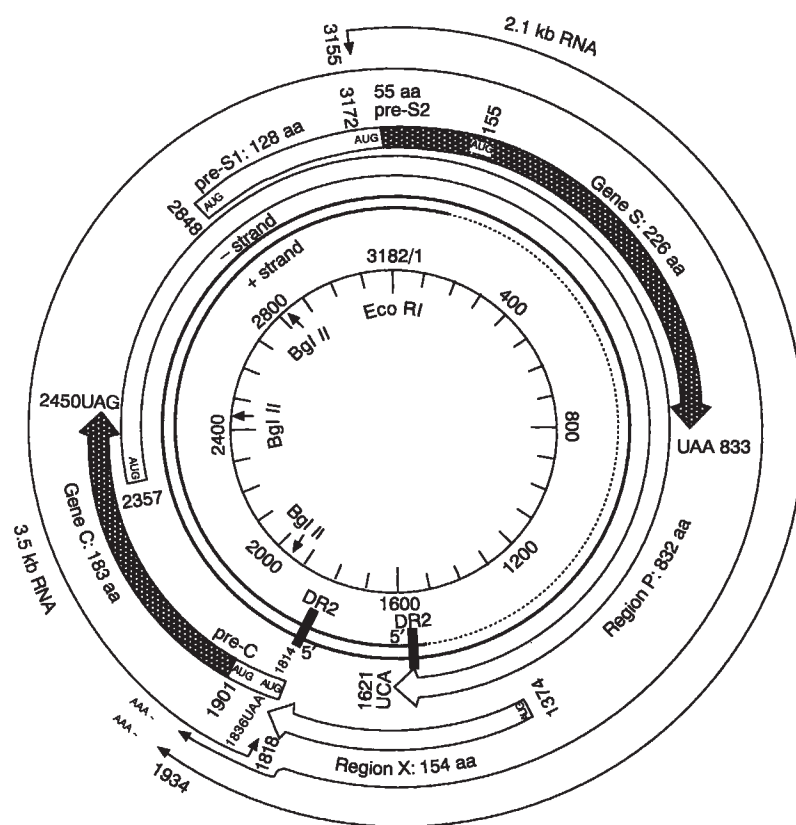


Figure 1 The genetic organization of hepatitis B virus. The HBV virion contains a circular, partially double-stranded DNA genome which contains four overlapping open reading frames. Gene C encodes the two nucleocapsid antigens, HBcAg and HBeAg, gene S encodes the small, middle and large surface antigen proteins, region P the viral polymerase and region X the X protein.

The largest open reading frame encodes for a bifunctional protein which has DNA polymerase and reverse transcriptase functions. This enzyme is central to the replication of the virus. The first stage of viral replication is the synthesis of a positive strand RNA molecule by host cell RNA polymerase. This positive RNA molecule acts as a pregenomic template for the viral polymerase/reverse transcriptase, allowing transcription of a DNA copy which is then itself transcribed to give the double-stranded DNA genome of the virus.

At some stage of chronic HBV infection, viral sequences become integrated into the genome of the liver cells. There is a preferred site within the cohesive end region of the viral genome, at which integration occurs. The integration into the host genome appears to be random. In some cases, this integration event has resulted in the activation of a proto-oncogene and malignant transformation has followed. In other HCCs, the site of integration of the virus is distant from the position of the activated oncogene. The role of the X protein in transactivation of endogenous oncogenes remains to be determined. In the UK, less than 50% of HCCs contain episomal or integrated HBV sequences and it has been suggested that in the majority of cases the association of the virus with HCC occurs because of the ability of the

virus to induce cirrhosis rather than because of a direct relationship to the activation of oncogenes. Rarely, when HCC occurs in a noncirrhotic liver, insertional mutagenesis is probably involved in carcinoma formation.

Immunobiology

HBV is not thought to be directly cytopathic; the liver damage in both the acute and the chronic disease are probably caused by the host immune response.

Acute hepatitis B infection

High titers of immunoglobulin M (IgM) anti-HBc, thought to be predominantly a thymus-independent response, appears early in the course of acute HBV infection, together with HBsAg and HBeAg (Figure 2). Anti-pre-S1 may also occur early in the course of infection. This is accompanied by a vigorous major histocompatibility complex (MHC) class I restricted cytolytic T lymphocyte (CTL) response against multiple epitopes throughout the structural and non-structural proteins of the virus. The CTLs, by direct lysis of infected hepatocytes, are thought to be important in clearance of the virus. There is also some evidence for a role in viral elimination of the

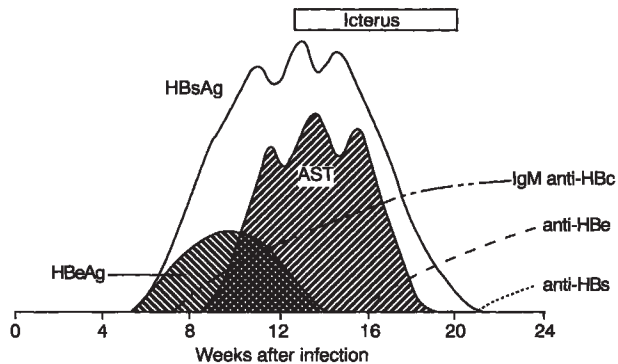


Figure 2 The natural history of acute HBV infection. HBsAg and HBeAg can be detected early in the serum, prior to the onset of clinical hepatitis. Antibody to pre-S1 can sometimes be detected during this early phase. AST (aspartate aminotransferase) is an indication of lysis of infected hepatocytes.

cytokines interferon γ (IFN γ) and tumor necrosis factor α (TNF α).

There is a strong MHC class II restricted T helper cell proliferative response to the nucleocapsid antigens, HBcAg and HBeAg, but not to the envelope proteins, during the early phase of acute hepatitis B. The association of recovery with the presence of the MHC class II locus DRB1*1302 supports an important role for the CD4 response in recovery.

Elimination of the virus from the liver results in the loss of HBeAg from the serum and the detection of anti-HBe. Loss of HBsAg and the appearance of anti-HBs occurs a little later.

Persistent hepatitis B infection

In some patients HBV is not successfully eliminated by the immune response and the patients become chronically infected. It is likely that there are many different reasons why patients develop the chronic carrier state, and the liver pathology is probably the result of a dynamic equilibrium between the replication of the virus and the host's immune response.

Mechanisms of viral persistence

Chronic HBV infection following exposure in neonatal life The majority of babies born to HBeAg-positive mothers become infected and over 90% of these develop a chronic carrier state. Although some cases have been documented of HBV infection during fetal life, it is thought that these infants probably receive a large inoculum of virus from maternal blood before or during birth, and by close contact with secretions soon after birth. HBeAg but not HBsAg has been detected in the cord blood of these neonates, suggesting that HBeAg crosses the placenta. The reason for the infants failing to clear the virus is unknown, but may relate to the induction of

immune tolerance to HBeAg. Children born to HBeAg-negative/HBsAg-positive carrier mothers who are asymptomatic rarely become chronically infected.

Chronic HBV infection following exposure after the neonatal period In marked contrast to the situation at birth, in young African children, more than half of whom have been infected by 7 years of age, only 15% fail to clear the virus. The majority of adults who become infected have an acute disease and eliminate the virus; in 5–10% of these cases, however, HBV persists. Persistent infection in adults is associated with poor T cell proliferative and cytotoxic responses to HBV antigens, although there is no evidence of a general immune suppression. This antigen-specific state of tolerance may be the result of the genetic background of the host: the MHC class II allele DRB1*1302 which is associated with clearance of the virus is virtually absent in adults and children who have persistent infection.

Natural history of persistent HBV infection There are four phases of the chronic infection (Figure 3).

HBeAg-positive phase During this phase there is little immune-mediated lysis of infected hepatocytes. The CD4 response to HBcAg is minimal and there is no response to HBsAg. The CTL response is largely undetectable.

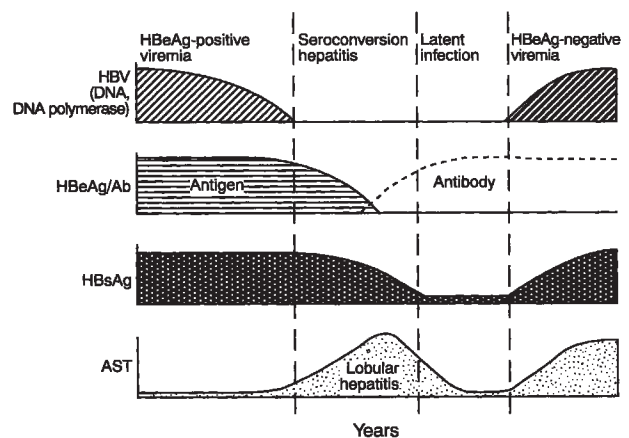


Figure 3 Natural history of persistent HBV infection after neonatal or adult exposure. During conversion from HBe antigenemia to the presence of anti-HBe, viremia ceases. The hepatitis associated with this seroconversion is the result of immune lysis of hepatocytes supporting HBV replication. This immune pressure may select an HBe-negative strain of HBV which causes viremia in the absence of HBeAg. AST, Aspartate aminotransferase (an indication of lysis of hepatocytes).

HBeAg/antibody seroconversion phase As the infection continues, the inflammatory liver disease becomes more active. Between 5 and 15% of persistently infected patients undergo spontaneous HBeAg/antibody seroconversion each year, as do approximately 40% of those treated with IFN α . This seroconversion event is usually accompanied by hepatocyte death, as indicated by raised liver enzymes. The mechanism by which seroconversion occurs is unknown. In the early stages of seroconversion an increased T cell proliferative response to the nucleocapsid antigens is seen, and is taken to indicate an increase in the numbers of nucleocapsid sensitized T cells. In addition, when serum transferases are at their highest a nucleocapsid antigen-specific MHC class I restricted CTL response can be demonstrated, although it is considerably lower than in acute disease.

Anti-HBe-positive patients with no evidence of HBV replication This phase follows the HBeAg/antibody seroconversion hepatitis which results in clearance of hepatocytes supporting HBV replication. The sera of these patients do not contain virus particles detectable by dot-blot hybridization. However, in some cases, low levels of viremia can be detected by the polymerase chain reaction. HBsAg in these patients is encoded by integrated HBV DNA sequences. This phase represents immune control of the virus.

Anti-HBe-positive patients with persistent HBV replication These patients are infected with a mutated HBV which is unable to encode HBeAg. The most common mutation is a G to A change at nucleotide 1896, creating a novel translational stop codon in the pre-core region of the genome. Chronic infection with this mutant virus may be associated with a severe and progressive liver disease and is more prevalent in Mediterranean and Far Eastern patients.

This mutation is found as a very minor species in some patients with an HBeAg-positive infection. The mutant is selected at the time of seroconversion to anti-HBe in some patients. These patients usually become HBV DNA negative by hybridization initially and chronic infection develops only after a long interval, presumably because the mutant virus escapes from immune control. The mutant virus can be shown to have a statistically higher rate of amino acid substitution in regions of the virus encoding known B cell epitopes, which may also contain CD4 T cell epitopes, presumably representing the emergence of variants that can replicate in the presence of these components of the immune response. The mutant virus is thought not to be more cytopathic

than the wild-type virus and the pathology is probably immune mediated.

Vaccines

HBsAg, derived from infected serum or produced by recombinant DNA technology, has been shown to induce protective immunity against HBV infection. These vaccines are now in widespread use throughout the world. When used to prevent neonatal transmission from infected mothers, the best protection is achieved when given with hyperimmune globulin.

Although the majority of patients vaccinated with HBsAg are protected from further infection, 2.5–5% of immunocompetent adults do not respond, probably for genetic reasons. This percentage is greater in some high-risk groups of patients, such as hemodialysis patients, in which the failure rate is approximately 40%; this probably represents a secondary immune deficiency due to the renal disease.

There has been considerable debate on whether the middle pre-S2-bearing and large pre-S1-bearing polypeptides should be added to existing vaccines. Experiments in mice suggested that both the pre-S1 and -S2 regions can recruit T cell help independently for the production of anti-HBsAg. It has also been suggested that pre-S1 is the region of the virus which binds to the hepatocyte during infection: antibodies to this region might be virus neutralizing and, therefore, inclusion of the pre-S1 region in a vaccine may be desirable.

The antibodies to HBsAg in convalescent serum (>80%) bind predominantly to the common 'a' determinant epitopes found in the hydrophilic region of the polypeptide between amino acids 124 and 147. The carboxy-terminal region of HBsAg has 14 cysteine residues and the antigenicity of this protein is highly dependent on its conformational structure. Using two cyclical peptides analogs of amino acids 124–137 and 139–147 and a panel of monoclonal antibodies binding to these regions, it has been possible to show in chimpanzees that antibody to the region aa124–137 of HBsAg prevents infection. Antibodies to this region, as well as to other epitopes on the surface antigen gene encoded polypeptide, are present in the serum of patients convalescent from HBV infection and in normal subjects immunized with plasma-derived and recombinant DNA-produced vaccine.

Genetic variation of HBsAg

Patients with chronic liver disease and no serum markers of current HBV infection except for HBV DNA have been described. The first indication that this

may be due to a mutation in the surface gene of the virus was described in a child born to an HBsAg-positive mother who became HBsAg positive despite passive and active immunization at birth and the development of high titer anti-HBsAg. A point mutation in the gene which resulted in an amino acid substitution from glycine to arginine at amino acid 145 of HBsAg was observed. This change was later shown to alter the common 'a' determinant so that it was not recognized by the known protective anti-HBsAg response.

This particular mutation has been found in a number of vaccine recipients around the world and in one patient not known to have been immunized. This virus was also shown to be transmissible in chimpanzees, and in mice the variant HBsAg, although eliciting a high titer antibody to the variant, produced only a low titer of antibody recognizing the native protein.

Other variants of HBV which have amino acid changes in the common 'a' determinant have now been described in patients and vaccinees with chronic liver disease. The effect of these mutations on the

immunogenicity and antigenicity of the HBsAg remains to be formally evaluated.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Cytotoxic T lymphocytes; Interferon α .

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HEPATITIS C VIRUS, INFECTION AND IMMUNITY

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Hepatitis C virus (HCV) was first characterized in 1989, following successful cloning from copy DNA extracted from infectious chimpanzee plasma. The natural route of infection is unknown, but HCV is blood borne, and therefore high-risk groups for infection include intravenous drug users and recipients of unscreened blood and blood products (e.g. hemophiliacs). Sexual transmission does not appear to be a major route of spread of infection. Vertical transmission from carrier mothers is around 2–10%. Estimates of the prevalence of infection vary between 0.1 and 1% of the population of Europe and the USA. There may be significant fluctuations from this in other countries – for example, rates of 15–20% anti-HCV positivity have been reported in Egypt. The majority (possibly 75%) of HCV infections result in chronic carriage of the virus. The percentage of chronic carriers who will progress to clinically significant liver disease (chronic hepatitis, cirrhosis, hepatocellular carcinoma) is unknown, but a conservative estimate is 20%. HCV infection is also

associated with autoimmune phenomena such as the anti-LKM-1 reactivity of autoimmune hepatitis type 2, and the development of mixed essential cryoglobulinemia type II. Viral and host factors which determine the variability in clinical outcome are poorly understood. Current therapy (interferon α , IFN α) is expensive, prolonged (6–12 months) and has a 'cure' rate of only 20%. More encouraging results have been reported in small-scale trials of combination therapy comprising IFN α plus ribavirin.

Characteristics of the organism and its antigens

HCV is an enveloped virus containing a single-stranded positive-sense RNA genome of 9.4–9.5 kb. It is classified as a separate genus within the family Flaviviridae. The genome includes a 5' nontranslated (noncoding) region (5'NCR) of 329–341 nucleotides, followed by a large single open reading frame

encoding a polyprotein precursor of 3010 amino acids (AA) in length, and a 3' NCR. The open reading frame is translated as a polyprotein containing (from N to C terminus) the core protein (p22), two envelope glycoproteins E1 (gp35) and E2 (gp70), and a series of nonstructural proteins (NS2-5) (Figure 1). The E2 gene contains a hypervariable region encoding a domain of 27 amino acids in length. The NS gene products are essential for virus replication, and possess a number of enzymatic properties: protease (NS2, NS3), helicase (NS3), and RNA-dependent RNA polymerase (NS5).

There is considerable sequence variability between isolates, which are classified into six (at present) major genotypes on the basis of 5'NCR and NS5 gene sequence analysis, each of which contains a number of subtypes. The most conserved part of the genome is the 5'NCR (>90%), followed by the core gene (>80%), the least conserved being the 3'NCR (26%), and the E1 and NS2 genes (55–60%). Genotype distribution around the world is uneven: types 1, 2 and 3 predominate in Europe and the USA, 4 is found in the Middle East, 5 in southern Africa and 6 in China. Studies of potential differences in pathogenicity between the genotypes have been inconclusive. All known genotypes may give rise to life-threatening liver disease. Genotype 1 viruses are less sensitive to IFN α therapy than are the other genotypes.

Diagnostic antibody tests utilize a series of recombinant or synthetic peptide antigens. First generation assays contained only an NS4-derived antigen, c100. These assays were of low sensitivity and specificity. Second generation tests added core (c22) and NS3 (c33) gene products to the c100, resulting in considerable improvement in diagnostic accuracy. Current, third-generation, assays also include an NS5 gene product. Commercially available screening assays are in standard sandwich ELISA format. Confirmatory assays, essential in the context of screening of low-risk sera, such as from blood donors, rely on the same antigens, but in immunoblot or 'line assay' format, where the antigens are presented on nitro-

cellulose strips. The presence of the relatively conserved core and NS3 gene products are essential to avoid false-negative reactivity arising through failure of antibodies raised against one viral genotype to recognize antigens derived from a different genotype. In contrast, the genetic diversity of the NS4 gene is reflected in sufficient antigenic differences between genotypes to allow synthesis of type-specific NS4-derived peptides for use in serotyping assays. Genome detection assays, such as the reverse transcriptase polymerase chain reaction (RT-PCR) using primers from the highly conserved 5'NCR, are useful for determination of carrier status in anti-HCV-positive individuals. Genotyping of the infecting virus may be determined by use of type-specific primers, by restriction fragment length polymorphism analysis of PCR products, or by hybridization of PCR products with type-specific probes.

Immune responses of the host

The sensitivity of the current serological assays is such that specific antibody responses are detectable in the vast majority of HCV-infected individuals. The only exceptions to this are patients who are immunosuppressed at the time of infection, where RT-PCR positivity may be the only indication of infection, and in the weeks following acute infection, as it may take up to 6 months for anti-HCV reactivity to develop. The clinical and biological significance of antibodies to HCV-derived polypeptides present in diagnostic assays is still unknown. They are clearly not protective, as the majority of anti-HCV positive sera are also RT-PCR positive. It is possible that antibodies against the envelope glycoproteins may have neutralizing activity, but assays for the detection of anti-E1 and -E2 are not widely available, and neutralization studies are severely hampered by the lack of a routine cell culture system for HCV.

Cellular immune responses to a variety of recombinant viral antigens have been studied in proliferation and cytotoxicity assays. CD4⁺ T cell proliferative responses to both structural (core, E1) and non-

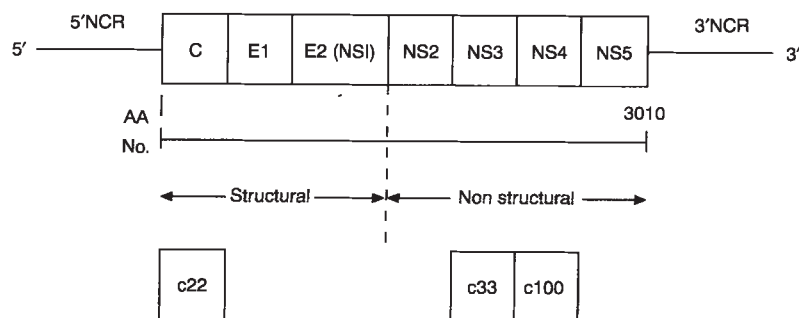


Figure 1 The hepatitis C virus genome. c22, c33 and c100 refer to the location of antigens used in diagnostic assays for the detection of anti-HCV.

structural (NS3, NS4) HCV-derived proteins have been identified in cells from both peripheral blood and liver tissue of anti-HCV positive individuals. Which (if any) of these responses correlate with clearance of virus, or at least with lack of progression of liver disease, is unclear. Preliminary data suggest that chronic progressive liver disease may be associated with T_H2 patterns of cytokine production from liver-derived T cells. HCV-specific cytotoxic CD8⁺ T lymphocytes (CTL) have also been identified in HCV-infected individuals, but again, variable results have been reported. Some groups suggest that these cells may be compartmentalized within liver tissue, but others have no apparent difficulty in finding such cells within peripheral blood. Lack of standardization of experimental assays may underlie these discrepancies. Target epitopes within both structural and nonstructural proteins have been mapped.

Evasive strategies by the organism

The majority of HCV-infected individuals become chronic carriers of the virus, even though they possess easily demonstrable, broad-ranging humoral and cellular immune responses. A number of hypotheses have been advanced to explain the failure of these responses to eliminate virus.

Both low- and high-density HCV virions may be found in human sera, the multiple densities reflecting association of virus with various lipoproteins and/or antibody. The ability of HCV to associate with very low-density lipoproteins, thereby rendering a major proportion of infectious virus nonneutralizable, may be of importance for virus persistence.

The existence of HCV as a quasispecies (i.e. a population of viruses each with slightly different sequences) may allow the virus to escape immune surveillance by changing its antigenic determinants. The hypervariable region of E2 shows considerably less genetic divergence in chronically infected patients who are agammaglobulinemic, as compared with immunocompetent hosts, while persistence of infection in chimpanzees has been associated with the emergence of cytotoxic T lymphocyte (CTL) escape mutants, with critical amino acid substitutions within T cell epitopes. Both of these lines of evidence support the concept that host immune responses, whether humoral or cellular, drive the evolution of variant viruses with an enhanced ability to persist in the host.

The existence of extrahepatic sites of replication

of HCV may be another mechanism of immune evasion. Most studies have relied on detection of negative-strand RNA, a replicative intermediate, as evidence of HCV replication. Strand-specific amplification assays are, however, technically fraught, and this topic remains controversial. Nevertheless, extrahepatic sites may exist, and may be important for virus persistence – for example, if replication within cells of the immune system is able to interfere with the host immune response, or if the site in question is inaccessible to the immune system.

Vaccines

Vaccine development is at a preliminary stage. Obstacles to be overcome include the diversity of HCV genotypes and subtypes and the hypervariability of HCV quasispecies within an individual host. An understanding of protective mechanisms of immunity would also greatly facilitate vaccine design. Experimental recombinant envelope glycoprotein vaccines induce anti-E2 responses in chimpanzees, associated with protection against low dose challenge with homologous virus. However, the duration and cross-reactivity of this protection appears to be very limited. Broadly-reactive CTL responses have been induced in mice using peptide-based vaccines derived from the core and NS5 genes.

See also: Adhesion molecules; Endothelium; Hepatitis E, F and G viruses, infection and immunity; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Lymph nodes; Lymphocyte function-associated antigen 1 (LFA-1); Lymphocyte trafficking; Mucosa-associated lymphoid tissue (MALT).

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HEPATITIS E, F AND G VIRUSES, INFECTION AND IMMUNITY

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In the 1970s the development of sensitive and specific serologic assays for markers of acute infection by hepatitis A virus (HAV) and hepatitis B virus (HBV) showed that enterically and parenterally transmitted viral hepatitis, not caused by HAV or HBV, occurred throughout the world. Two of these viruses, HCV and HEV, have been isolated, cloned and sequenced. In the 1980s the development of serologic assays for these viruses indicated that additional enterically and parenterally transmitted hepatitis-associated viruses exist. Research is ongoing to elucidate the immunology, clinical features and pathology associated with these viruses.

Hepatitis E virus

Hepatitis E virus (HEV) is a member of the Caliciviridae family. Hepatitis E (HE) is a self-limiting disease, similar to hepatitis A, with no known tendency for chronicity. Hepatitis E occurs as large scale epidemics in developing countries, and also as sporadic cases. Outbreaks have been reported and confirmed either serologically or with the reverse transcriptase polymerase chain reaction (RT-PCR) in over 30 countries throughout the world.

The primary source of infection is usually drinking water contaminated with water from a sewage system. Person-to-person contact has been noted as a very rare cause of transmission, and in a recent outbreak in India vertical transmission of HEV from infected mothers to infants during the third trimester of pregnancy was noted. Initial reports of HE epidemics failed to find cases of HE in pediatric populations; however, the development of diagnostic tests have shown HEV is a common cause of acute hepatitis in this age group. The attack rate is highest in young to middle-aged adults between 15 and 40 years of age. While the seroprevalence of anti-HAV may approach 70–90% in young children in endemic areas, seroprevalence of anti-HEV is less than 10% in young children and approaches 40% in middle-aged adults. In most reported outbreaks there is no significant difference in the attack rate of HE between genders in the general population (1–15%), but attack rates for pregnant women are significantly higher (17–40%), with case fatality rates approach-

ing 20%. The case fatality rate for the general population in endemic regions is about 0.5–4%.

Localization of HEV epitopes

HEV genomic RNA is composed of three open reading frames (ORFs) that code for the various proteins involved in the replication of nascent virions. The deduced amino acid sequence for the three ORFs has been used to synthesize overlapping peptides for enzyme immunoassays to detect potential epitopes in these ORFs. Although potential epitopes have been located in all three ORFs, epitopes near the carboxyl termini of the deduced ORF2 and 3 amino acid sequences have proven to be most useful in the development of immunoassays and prototype recombinant vaccines.

Clinical features

Typically the incubation period for HE is about 6 weeks, with a range of 2–9 weeks. Clinical HE occurs in two phases. First, the prodromal phase is usually characterized by fever and nausea. Second, the icteric phase, which lasts about 2 weeks, is characterized by jaundice and dark urine. General symptoms include abdominal pain, anorexia, hepatomegaly, splenomegaly and vomiting. Fulminant hepatic failure, particularly among pregnant women in the second and third trimester, has been associated with HE, although it is not a regular sequela of this disease. Diagnostic testing has shown that subclinical infection occurs in pediatric and adult populations.

The patterns of viremia and virus excretion are not well characterized in humans and only limited data exist from experimental animal models (cynomolgus macaques). In humans, viremia has been detected by RT-PCR within 2 weeks after the onset of illness and persists for 4–16 weeks in some individuals. Virus excretion has been detected by immunoelectron microscopy (IEM) in feces, predominantly in the first week following the onset of acute icteric hepatitis, and may persist for 2 weeks, with one report documenting excretion for up to 52 days. In macaques RT-PCR has been used to detect viremia within 4 days after experimental infection, which persists for up to 2 weeks after the onset of illness. Virus excretion in experimentally infected macaques has

been detected as early as 2 weeks before the onset of illness and may persist for up to 2 weeks after the onset of acute illness.

Pathology

Morphologic features of HE in liver tissue specimens include those of cholestatic and standard (noncholestatic) types of acute viral hepatitis. In cholestatic HE, bile stasis is observed in canaliculi and gland-like transformation is seen in parenchymal cells. Less frequently seen changes in liver cells include acidophilic bodies and focal necrosis. Portal ducts are enlarged with inflammatory infiltrate that may spill over into neighboring parenchyma. Infiltrates consist of lymphocytes, polymorphonuclear leukocytes and macrophages. In standard HE, ballooned hepatocytes, acidophilic degeneration of hepatocytes and acidophilic body formation are frequently seen. Focal intralobular necrosis of hepatocytes, with accumulations of lymphocytes, mononuclear macrophages and activated Kupffer cells, is also an important morphologic feature. In an IEM examination of liver biopsies taken from three patients, Dienes and colleagues found the predominant subpopulation of lymphocytes in the infiltrate were CD8⁺ T cells (51%), 16% were CD4⁺ T cells, 19% displayed Leu-7 (CD57) antigen, and 14% were positive for B1 (CD20). HLA-DR was found on all Kupffer cells, and HLA class 1 antigens were expressed on hepatocytes.

Antibody response in infected individuals

In humans an immunoglobulin M (IgM) response is mounted against HEV during the acute phase of the disease. The peak titer of HEV-specific IgM usually coincides with the peak of serum alanine aminotransferase activity and generally falls off to undetectable levels about 5 months after the immune response is initiated. HEV-specific IgG titers peak during the early convalescent phase of an HEV infection and decline over the next several months. Although HEV-specific IgG falls to undetectable levels in over 50% of postinfection individuals, studies have shown IgG is detectable in some individuals from 2 to about 14 years after infection. The presence of pre-existing HEV-specific IgG appears to prevent reinfection and clinical HE in young adults. In a study of HE patients in Somalia HEV-specific IgA titers appeared to rise and fall in conjunction with IgM titers, but the prevalence of specific IgA (52%) in these patients was lower than that of IgM (73%).

Prophylaxis

When travelers were passively immunized with

immune globulin prepared from plasma collected in nonendemic countries they were not protected from infection when they traveled to endemic regions. Studies using immune globulin prepared from donors in India, an HEV-endemic country, showed no difference in attack rates for HEV infection among passively immunized and nonimmunized individuals during an HE outbreak. However, passive immunization of macaques with convalescent phase plasma from a macaque previously infected with HEV prevented clinical disease when the immunized macaques were challenged with HEV, but failed to prevent viral replication or shedding. Active immunization of macaques with recombinant vaccine also led to prevention of clinical illness, but also failed to prevent viral replication or shedding.

Hepatitis F viruses

Serologic testing has identified five hepatropic viruses; HAV, HBV, HCV, HDV and HEV. These viruses account for almost all human viral hepatitis; however, a small percentage of patients exhibiting the signs and symptoms of acute viral hepatitis do not have serologic evidence of infection with any of these viruses. Researchers continue to search for the viruses associated with non-ABCDE hepatitis. Many of these enterically and parenterally transmitted viruses have been tentatively designated hepatitis F virus (HFV). However, at this time no single virus has been recognized as being HFV. Some researchers have suggested the non-ABCDE viruses are really one of the known hepatitis viruses and serologic testing has failed to detect the viral-specific antibodies present in infected individuals. Serologic testing of individuals with HCV and HEV infection has shown that the serologic tests available for these viruses do not detect every infected individual. It has also been suggested that a new serotype of HBV may be responsible for some so-called non-ABCDE hepatitis.

Hepatitis G virus

Recently, a new hepatitis-associated virus, hepatitis G virus (HGV), has been cloned and sequenced. HGV, an RNA virus, is a member of the Flaviviridae family, and is distantly related to HCV. Originally this virus was one of the viruses referred to as HFV. Although HGV has been identified as a hepatitis-associated virus and is associated with acute and chronic hepatitis, research has failed to prove that HGV is hepatotropic. At this time no serologic test exists for detection of HGV-infected individuals, as no HGV antibodies have been detected in infected individuals using either recombinant HGV proteins

or synthetic peptides. The only way to detect HGV is with RT-PCR.

See also: Calicivirus, infection and immunity; Hepatitis B virus, infection and immunity; Hepatitis A virus, infection and immunity; Hepatitis C virus, infection and immunity; Togavirus, infection and immunity; Viruses, immunity to.

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HERPES SIMPLEX VIRUS, INFECTION AND IMMUNITY

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Infection by herpes simplex virus (HSV)

There are two related subtypes of herpes simplex virus: types 1 (HSV-1) and 2 (HSV-2), now called human herpesviruses 1 and 2 respectively. Both are large, double-stranded DNA viruses (ca. 150 kb)

encoding 70 or more proteins (HSV-1 has now been completely sequenced). The virion has a large icosahedral nucleocapsid core containing the viral genome as a linear double-stranded DNA molecule. This is surrounded by an amorphous protein-filled tegument which is then enclosed in

a membrane envelope containing virion glycoproteins.

Infection starts with attachment of the virion to the cell surface. At present, a number of viral glycoproteins and cellular receptors have been implicated: glycoprotein B binds heparan sulfate on the cell surface whilst glycoprotein C binds complement receptor 1 (CR1 or CD35). Also involved are glycoproteins D and H as well as the cell's basic fibroblast growth factor receptor. Once attached, the virion envelope must fuse with the cytoplasmic membrane to release the viral core into the cell. The core is then transported to the nuclear pores where the viral genome is released into the nucleus to undergo replication and expression. Expression of viral genes may be divided into three broad categories: α (immediate early), β (early) and γ (late) genes with transcription and translation taking place in the nucleus and cytoplasm respectively. The progeny virion core is assembled in the nucleus and is thought to then bud out through the nuclear membrane to be transported to the endoplasmic reticulum and Golgi apparatus of the cell where it reacquires its envelope as it buds out from the plasma membrane.

This would be the normal lytic virus lifecycle seen in epithelial cells, however HSV can establish latent infections of neurons in the sensory nervous system. The actual mechanism for establishing, maintaining and reactivating latency is not understood although productive replication is not necessary for establishment. During a latent infection the viral genome is maintained as a multicopy circular episome in the nucleus. There is a family of spliced RNAs present called the latency-associated transcripts (LATs). These transcripts are not required for the establishment or maintenance of latency. Virus reactivates from neurons through an unknown stimulus although *in vitro* the presence of nerve growth factor stops reactivation.

HSV-1 is associated primarily with orolabial infections, whilst HSV-2 is usually associated with genital lesions; however, these sites are not mutually exclusive. Both viruses are transmitted by direct contact from a productively infected to an uninfected individual through skin abrasions or at mucosal surfaces. Vesicular skin lesions or shallow ulcers on mucosal surfaces result due to the lytic infection of epithelial cells by the virus. Large quantities of virus appear between the epidermal and dermal layer in fluid containing cell debris, inflammatory cells and giant cells. Progeny from this surface infection may pass into sensory nerve endings of the dermis and be transported up the nerve axon by retrograde transport to the cell body as a naked nucleocapsid. An acute infection of the peripheral nervous system may result

and usually a latent infection is established primarily in the sensory ganglia (see **Figure 1A**) but latently infected neurons have also been recovered from the central nervous system. There have been reports of latently infected tissues outside the nervous system but these have been difficult to confirm.

Latent infections in sensory ganglia are reactivated periodically by unknown means (emotional and physical stress, exposure to UV light, hormones and tissue damage have all been implicated) to produce viral progeny which are transported back down axons to the skin at or close to the original site of infection. Here the virus re-establishes a productive lytic infection which may result in asymptomatic virus shedding or a recurrent (recrudescent) lesion as is suffered by many in the population (**Figure 1B**). HSV-1 reactivates preferentially from cervical and trigeminal ganglia whilst HSV-2 reactivates more readily from sacral ganglia. Recurrences are more likely to occur after severe primary infections and their frequency decreases with time. Reactivated infections may well not reseed the original neuron, which survives infection as sensation is not lost from the periphery even after multiple reactivations.

The first problem for the host in defending itself against HSV is the site of infection. The skin and nervous system have been called 'immune privileged' sites and certainly the immune surveillance occurring in these tissues is not as great as in highly vascularized tissues such as the liver. To compound this problem, especially in recrudescent lesions, the virus is restricted to the epidermal layer until a well-established infection results in enough cell death to cause inflammation in the dermal layer. The second problem for the host is that once the virus has established a latent infection, no detectable viral products are expressed and so there is apparently no way for the host's immune system to attack latently infected cells. However, the immune system does have an important part to play in restricting the severity of HSV infection. Primary infections are usually confined to the skin and dorsal root ganglia but in unusual circumstances a disseminated systemic infection with brain damage and fatal consequences may result. This is seen in patients with T cell defects and neonates. Immunosuppression also increases the risk of recurrences whilst in normal hosts secondary infections are of shorter duration than primary infections.

Immune responses of the host

The immune response to HSV has mainly been studied in animal models of infection (e.g. mice, rabbits and guinea pigs). Although various routes of

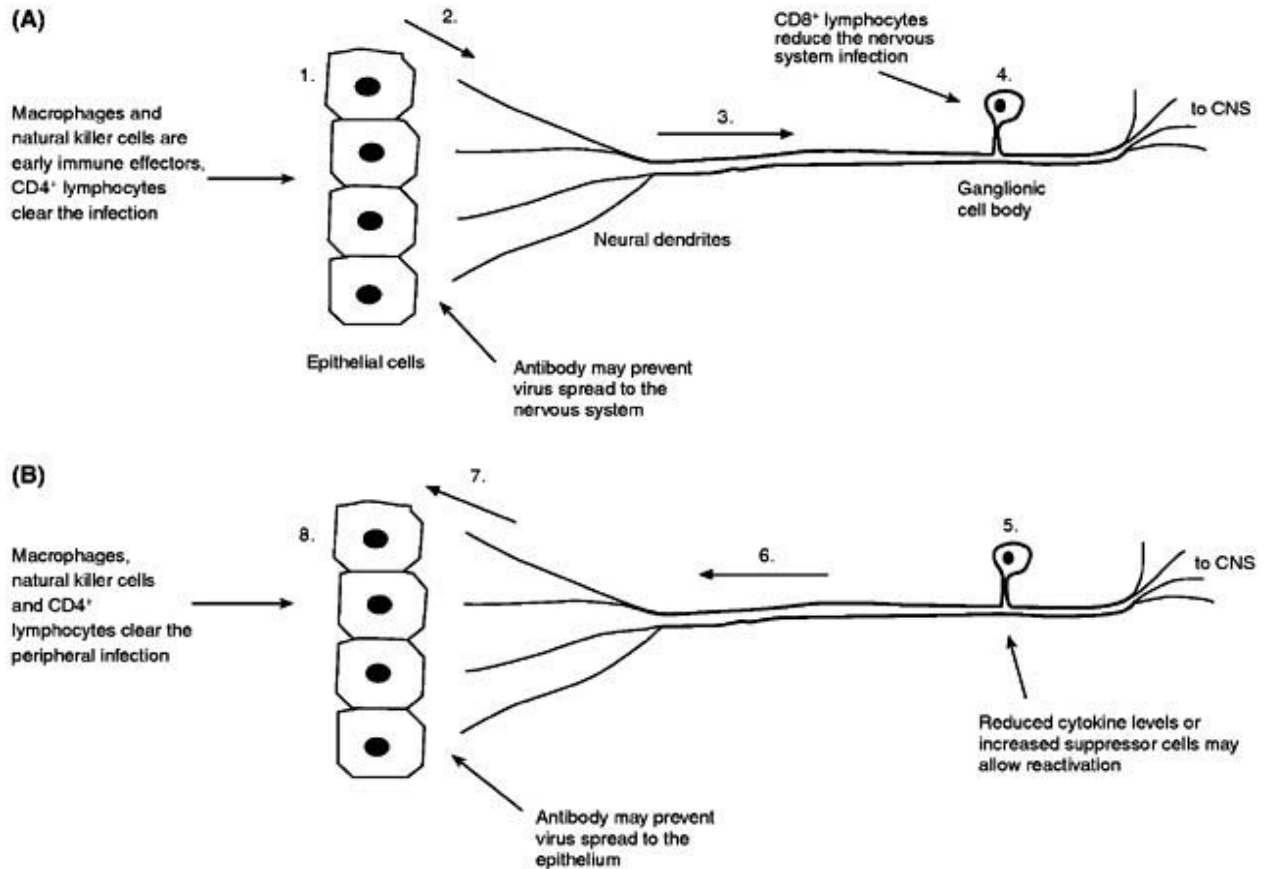


Figure 1 Map of HSV infection and immune responses. (A) Acute infection: 1, HSV productively infects cells of the epithelium; 2, viral progeny transfer to neural dendrites which they infect; 3, naked viral capsids are transported up the neuron axon to the cell body; 4, productive infection may occur or latency may be established in the nucleus. (B) Reactivation: 5, a latent infection is reactivated to produce progeny in the cell body; 6, progeny are transported down the nerve axon; 7, virions infect epithelial cells to cause a productive lytic infection; 8, virus may be transferred to an uninfected individual.

infection are used that are not always consistent with the natural route of infection in humans, some understanding of the host's response to these viruses has been achieved (Table 1).

The host animal has a natural genetic resistance to infection with HSV which may involve several mechanisms including macrophage and natural killer (NK) cell activity or interferon production. Many experiments in this field used unnatural routes of infection but it would seem that good NK cell activity and interferon production are important if the HSV infection occurs through mucosal surfaces

(Figure 1A). These responses would act immediately to restrict an infection but are not themselves sufficient to clear the virus; rather, it is the specific immune response of the host which will eventually remove an acute infection. Both antibody and T lymphocytes are induced a few days after a primary infection is established. These would be too late in preventing infection of neural tissue from this primary infection but they do restrict the peripheral and neural infection and eventually clear the virus.

Important target antigens for antibody are virion and infected cell glycoproteins, capsid proteins and

Table 1 Site of action of immune effectors

Site of infection	Immune effectors			
	CD4 ⁺ lymphocytes	CD8 ⁺ lymphocytes	Antibody	Innate immunity
Epithelium	+++	-	-	++
Epithelium to nervous system	-	-	+	-
Nervous system	-	++	-	-

some infected cell polypeptides. In normal naive mice, passively transferred antibodies to HSV will protect from lethal doses of HSV (mice would normally die from encephalitis) but antibody alone is not sufficient to clear virus from the periphery or the nervous system. Consequently it may have a role in retarding the spread of virus to and through the nervous system. However, to be effective it must be present in high titers within 24–48 h of infection and may act at several sites by restricting the area of epidermal infection, the number of progeny produced from this infection or interfering with the passage of virus from the epidermis to and through the nervous system (Figure 1). Several *in vitro* activities of HSV-specific antibody may be measured (e.g. virus neutralization, antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity) but the important activity *in vivo* is in dispute. However, it may prove to be the target antigen's function and/or concentration in the virion or infected cell which will determine the protective ability of an antibody. In humans, reactivation and recurrence or reinfection with HSV is often in the face of high neutralizing serum antibody levels, suggesting that antibody is not protective.

T lymphocytes are extremely important during HSV infections: T cell-depleted or nude mice die from encephalitis after HSV infection but are protected by the transfer of immune T cells. However, there are several T cell subsets involved and the role of each is gradually being clarified.

The cytotoxic T lymphocyte (CTL) response to HSV can be seen after lymphocytes are cultured *in vitro*. The response after stimulation with live virus antigen is major histocompatibility complex (MHC) class I-restricted and mediated by CD8⁺ T lymphocytes in both mice and humans. Glycoproteins have been the major protein group studied in mice with reports of recognition of glycoproteins B, C and D, although the MHC background of the host may affect the response. Nonstructural proteins of the virus also act as target antigens; the immediate early proteins of HSV account for up to 50% of the CTL response in some mouse strains. Humans have high CTL precursor levels in the blood probably because of constant stimulation by the virus and CTL clones derived from an HSV-2-infected individual were either HSV-2 specific or cross-reactive with HSV-1 and recognized immediate early and virion proteins and glycoprotein D-2.

The actual role of the MHC class I-restricted response during an HSV infection is best shown by the *in vivo* depletion of CD8⁺ T cells in mice. There is no effect on the clearance of a peripheral viral infection but there is a more florid ganglionic infec-

tion (Figure 1A). When high doses of virus are inoculated, depleted mice tend to die from the infection. This leads to the theory that CD8⁺ T cells may be involved in a reduction of the acute nervous system infection. CTLs do not kill neurons which lack expression of MHC class I however, and may act through cytokine release after recognition of a non-productive infection in capsular (microglial) cells. During immunopathological responses (e.g. herpes keratoconjunctivitis) CD8⁺ T lymphocytes may be involved in suppressing anti-HSV responses as depletion of CD8⁺ cells aggravates the condition.

The other major T cell subset, CD4⁺ (MHC class II-restricted) lymphocytes, is involved in several functions including the delayed-type hypersensitivity (DTH) response to HSV, CTL activity and T cell help (e.g. in antibody production). The predominant cells to infiltrate a cutaneous lesion are activated macrophages and CD4⁺ T cells, suggesting a role for both in virus clearance. Studies using gene knockout mice have shown that CD4⁺ cells are essential for virus clearance and it is the T_H1 subset which appears to be protective (Figure 1). It is likely that CD4⁺ cells mediate antiviral immunity directly by lysing MHC class II-bearing infected cells or through the release and activity of cytokines such as interferon γ (IFN γ) or tumor necrosis factor. Nitric oxide production may also play a role in virus clearance. Glycoproteins, virion (VP16) and immediate early (ICP27) proteins all induce CD4⁺ T cells but glycoprotein D has proven to be the most protective HSV protein studied to date in mice. $\gamma\delta$ TCR⁺HSV-specific T cell clones have also been isolated from humans, although their function is unknown.

Most experiments in mice use acute primary infections, but in the guinea pig vaginal model, recurrent infections may be studied. Here there is apparently an impaired cell-mediated response (decreased cytokine production and the presence of suppressor cells) to HSV in animals which suffer recurrent infections. The above dysfunctions in immune function have also been implicated in allowing recurrent infections in humans.

Evasive strategies by the organism

HSV has several mechanisms for evading the host immune response. Once a cell becomes infected with HSV the virus switches off host macromolecular synthesis. This also stops the production of new MHC molecules, therefore reducing the presentation of virus antigen to T cells. The virus has a more specific mechanism for the evasion of the CD8⁺ lymphocyte surveillance: an immediate early protein (ICP47) interferes with the peptide transporter (TAP) used to

move cytoplasmic peptides to the endoplasmic reticulum for complexing with MHC class I, thus reducing antigen presentation by infected cells. Additionally the virus produces both an immunoglobulin G Fc receptor (glycoprotein E $\pm$ glycoprotein I) and a complement C3b receptor (glycoprotein C) which are known to interfere with antibody and complement function protecting both virions and infected cells. HSV is known to encode genes which prevent apoptosis of differentiated cells, important in the infected neuron. Lastly and most importantly, HSV establishes a latent infection with no detectable protein expression which allows it to survive for many years in one host.

Vaccines

It is hoped that vaccination against HSV will protect from life-threatening infections and decrease recurrent episodes. Subunit glycoprotein (B-2 and D-2) vaccines have been studied in the guinea pig vaginal model and are known to reduce the frequency of recurrent infections. Unfortunately the avirulent or inactivated virus and subunit vaccines (glycoproteins B-2 and D-2) trialed in humans do not use placebo control groups and so any efficacy of the vaccines is hard to establish. Often antibody levels are unchanged after vaccination in the trial groups. The above vaccines are usually injected with alum but new adjuvants and ways of presenting antigen to the immune system are being investigated. Muramyl tripeptide appears to be a strong adjuvant. Delivery of antigen to the mucosal immune system is being studied using recombinant *Salmonella* or adenovirus. In animal trials, protection can be induced with glycoprotein D using recombinant *Salmonella* fed orally or intranasal administration of a glycoprotein B-expressing recombinant adenovirus. Direct DNA vaccination is also being studied with ICP27, glycoprotein B and D-2 genes. The type of immunity required for protection is not clear (see above) and it may prove that neutralizing antibody as well as T (CD4⁺ T_H1) lymphocytes will be important in

restricting the infection and perhaps in inhibiting the establishment of latent infections.

Finally, some of the most successful antiviral drugs are against HSV and these include acyclovir. Although these drugs cannot clear the latent infection once it is established, they can reduce the severity and length of productive infections.

See also: Cytotoxic T lymphocytes; Effector lymphocytes; Eye infections; Immune surveillance; Neurological infections; DNA vaccines; Privileged sites; Vaccines; Viruses, immunity to.

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Evasive strategies by the organism

HHV-6 infects cells of the immune system. A number of effects of such infection on cellular function have been described, although the *in vivo* significance of these phenomena are not clear. Virus-induced suppression of growth factor-induced maturation of marrow precursor cells and colony formation by marrow precursors may be important in inhibition of bone marrow engraftment following transplantation. Infection of peripheral blood lymphocyte cultures leads to interferon α , tumor necrosis factor α , and interleukin-1 β production, and suppression of T cell function as evidenced by reduced interleukin 2 synthesis and cellular proliferation. Infection of CD8⁺ T cells leads to expression of surface CD4, and resultant susceptibility to infection with HIV.

Vaccines

There are no reports as yet of attempts at development of protective vaccines.

See also: **Acquired immune deficiency syndrome (AIDS); Cytokines; Cytomegalovirus, infection and immunity; Human immunodeficiency viruses.**

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HERPESVIRUS-8, INFECTION AND IMMUNITY

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Human herpesvirus type 8 (HHV-8), or Kaposi's sarcoma-associated human herpesvirus (KSHV), is the first herpesvirus to be discovered by molecular biology techniques rather than by conventional tissue culture methods and probably represents the long-predicted infectious cause of Kaposi's sarcoma (KS).

Detection

Fragments of its genome were detected in 1994 by Chang *et al* using representational difference analysis (RDA), a polymerase chain reaction (PCR)-based technique involving subtractive hybridization and kinetic enrichment. HHV-8 genomes can be detected in all forms of KS, including human immunodeficiency virus (HIV)-associated KS, 'classic' KS in HIV-uninfected individuals. African 'endemic' KS (in HIV-uninfected individuals) and post-transplant KS. HHV-8 has also been found in a rare form of acquired immune deficiency syndrome (AIDS)-associated B-cell lymphoma, body cavity-based lymphoma (BCBL), or primary effusion lymphoma

(PEL), and in some cases of Castleman's disease. The detection of HHV-8 in other tumors from immunosuppressed individuals of Western countries has been reported by some, but not other groups. However, the significance of this finding remains to be established.

Relationship to other herpesviruses

HHV-8 is a γ_2 herpesvirus (rhadinovirus) and is the first human representative of this group. Several animal rhadinoviruses are known and some of them, herpesvirus saimiri (HVS), alcephaline herpesvirus 1 (AHV-1) and ovine herpesvirus 2 (OHV-2), cause lymphoid malignancies. Among human herpesviruses, HHV-8 is most closely related to Epstein-Barr virus (EBV), a γ_1 herpesvirus, which also causes lymphoid tumors in immunosuppressed individuals and may play a role in other malignant diseases (African Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma). While EBV-mediated cellular transformation has been studied in detail,

much less (with the exception of HVS) is known about the oncogenic mechanisms of rhadinoviruses.

Genome

HHV-8 genomes are present in KS spindle and endothelial cells, which are believed to represent the neoplastic component of this tumor. In KS tissue and in BCBL cells HHV-8 genomes are present as circular episomes and therefore probably persist in a latent form in these tissues. *In situ* hybridization studies using probes for latent HHV-8 genes have confirmed this conclusion. The HHV-8 genome contains several genes which could conceivably interfere with cell cycle control, cellular transcription or intracellular signaling; these include a bcl-2 homologue (orf 16), a cyclin D2-like protein (orf 72), an interleukin 6 (IL-6)-like protein (orf K2), chemokine homologs (orfs K4, K6), an interferon regulatory protein (orf K9), and a G protein-coupled receptor homolog (orf 74). The cyclin homolog is capable of interacting with cdk 6 to phosphorylate the retinoblastoma protein RB, and is expressed in KS tissue and in BCBL cells, but whether it, or any of the other viral proteins, plays a role in cellular transformation is not yet known and there is as yet no *in vivo* or *in vitro* evidence that HHV-8 is indeed a transforming rhadinovirus.

Does HHV-8 cause KS?

The epidemiological evidence for a causative link between HHV-8 and Kaposi's sarcoma is steadily accumulating. Detection of HHV-8 by PCR in peripheral blood correlates with, and predicts subsequent progression to, KS. Antibodies to a latent HHV-8 antigen and to a recombinant structural antigen are found in individuals with, or at risk for, KS but only rarely in healthy blood donors or members of those HIV risk groups (hemophiliacs, intravenous drug users) who are known to suffer from KS only exceptionally. Thus, the distribution of antibodies to these two HHV-8 antigens in Western countries mirrors that of KS, strongly suggesting a role for this virus in the pathogenesis of KS. Antibodies to the same HHV-8 antigens are more common in HIV-uninfected homosexual men and sexually transmitted disease clinic attenders than in the general population (blood donors). Among homosexual men in Northern Europe the likelihood of having antibodies to these defined HHV-8 antigens increases with the

number of partners and passive anal intercourse. As measured by these serological assays, HHV-8 was introduced into the homosexual community on a wider scale in the late 1970s/early 1980s. Infection with HHV-8 may, however, be more common in some Mediterranean countries and is very widespread in Africa. Thus, while HHV-8 may be a recently introduced sexually transmitted virus in some countries, it has existed for a long time in other populations, and 'classic' Kaposi's sarcoma was first described more than a century ago.

Prevalence in the general population

There is still considerable controversy about the prevalence rates of HHV-8. Antibodies to undefined structural HHV-8 antigens have been reported in up to 25% of North American blood donors, whereas antibodies to the recombinant capsid-related and latent HHV-8 proteins are found in 0–2% of North American and Northern European blood donors. Attempts to detect HHV-8 by PCR in peripheral blood and semen samples of healthy donors have yielded even more discrepant results. Some of these discrepancies may be the result of geographical variation in HHV-8 infection rates, but differences between assays probably also contribute.

See also: Acquired immune deficiency syndrome (AIDS); Epstein-Barr virus, infection and immunity; Viruses, immunity to.

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HETEROPHILE ANTIBODIES

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In 1911, John Forssman noted that immunization of rabbits with guinea pig kidney leads to the formation of antibodies that act upon sheep erythrocytes. Forssman referred to this rather unexpected cross-reaction using the existing term 'heterologous reaction'. Subsequently, however, the term heterologous has been reserved for cross-reactions to be expected, such as between human and chimpanzee serum albumin for example. The term 'heterophile reaction' has been coined to denote unexpected cross-reactions. Accordingly, heterophile antibody is an antibody, the production of which was stimulated by an antigen apparently unrelated to the one with which this antibody cross-reacts. Heterophile antigen is, then, an antigen that reacts with such a heterophile antibody. There are many known heterophile antigen-antibody systems. Of these, three systems that are of significant interest for biology and medicine will be discussed. The antibodies defining these three systems have one important common feature – they all agglutinate sheep red blood cells.

The Forssman (F) system

The F antigen is the antigen originally described by Forssman as an antigen shared by sheep erythrocytes and guinea pig kidney. All the animal kingdom may be divided into F-positive species containing this antigen (guinea pig, hamster, mouse, sheep, goat, horse, dog, cat, chicken, etc.) and F-negative species (rabbit, rat, humans, apes, monkeys, ox, goose, etc.) which do not have this antigen. Interestingly, the appearance of F antigen in various animals does not parallel their genetic relatedness. Sera of F-negative species, notably rabbit and humans, frequently have naturally occurring F antibodies. The antigen shared by all F-positive animals is a glycosphingolipid, with a pentasaccharide being the active epitope. There is evidence that F antigen may appear in tumors of F-negative animals, including humans. Increased titers of F antibodies were noted in some patients with malignancies. Serologically characteristic is the disappearance of agglutination of sheep erythrocytes after absorption of the F antiserum by guinea pig kidney suspension, but not by bovine erythrocytes.

The Hanganutziu-Deicher (H-D) system

H-D antibodies were first described under the name 'serum sickness antibodies' by Hanganutziu in 1924,

and then by Deicher in 1926 in patients who received injections of sera of foreign species origin administered for serum therapy. Subsequently, H-D antibodies were found in human sera from many pathological conditions, though usually in low frequencies. H-D antigen is found in the tissues of all mammals studied to date, but it is absent from normal human tissues. On the other hand, it has been demonstrated in some human pathologic tissues, notably lymphoma and melanoma. Chemically, H-D antigen is an *N*-glycolylneuraminic acid, but many H-D-positive human sera contain antibodies combining with other antigenic moieties found in the high molecular weight glycoprotein fraction of sheep and bovine erythrocytes. Serologically characteristic is the disappearance of agglutination of sheep erythrocytes after absorption of sera containing H-D antibodies with bovine red blood cells as well as with guinea pig kidney suspension.

The Paul-Bunnell (P-B) system

Antibodies characteristic for infectious mononucleosis were described by Paul and Bunnell in 1932. These antibodies are predominantly of IgM variety. They are conveniently detected in reactions with sheep or bovine erythrocytes. Two antigenic moieties participating in these reactions could be distinguished: B, present on bovine erythrocytes only; and BS, appearing on both bovine and sheep erythrocytes. Most sera from patients with infectious mononucleosis contains antibodies against both B and BS, but a small proportion of IM sera with anti-B only was described. P-B antibodies appear in over 90% of patients with infectious mononucleosis but, significantly, they were hardly ever noted in patients with other pathological conditions or in normal subjects. The mode of formation of these antibodies in infectious mononucleosis is still not definitely explained. An attractive hypothesis postulates that they are formed in response to an antigen that is created in the course of the pathologic process. P-B antigen appears to be a glycoprotein. In serological tests, antibodies agglutinating sheep erythrocytes are removed from infectious mononucleosis sera by absorption with bovine erythrocytes, but not with guinea pig kidney suspension.

See also: Epstein-Barr virus, infection and immunity; Forssman antigen; Serum sickness.

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HIGH ENDOTHELIAL VENULES

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High endothelial venules (HEVs) are specialized blood vessels which support the migration of naive lymphocytes from the bloodstream into secondary lymphoid organs. The majority of lymphocytes circulating in the blood are able to migrate into lymphoid organs via HEVs. This allows rare, antigen-specific lymphocytes to encounter small amounts of antigen on presenting cells within these organs and ensures effective surveillance of the body for antigen by the full repertoire of antigen-specific lymphocytes. The developmental origin of HEVs and their relationship to other types of blood vessel are not known. HEVs differentiate during neonatal life when the endothelial cells lining these blood vessels adopt a distinct, cuboidal morphology and express the peripheral and/or mucosal lymph node addressins. A precisely regulated sequence of adhesive interactions mediated by different families of adhesion molecules allows lymphocytes, but not other leukocytes, to adhere to the inner surface of HEVs which precedes their subsequent migration across the vessel wall and into the tissues. The peripheral and mucosal addressins, which are specific markers of HEVs are, in fact, cell adhesion molecules that mediate binding of lymphocytes to the surface of HEVs and, thus, they are critical for HEV function. Blood vessels with the morphology and adhesive properties of HEVs are found at sites of chronic immune-mediated inflammation and extranodal lymphomas. The development of these extranodal HEVs may exacerbate the disease process by facilitating the large-scale

migration of naive lymphocytes to nonlymphoid sites. Recent studies using transgenic techniques to direct the expression of inflammatory cytokines to extranodal sites have highlighted a role for cytokines in the development of HEVs at these sites.

What is an HEV?

Lymphocytes enter lymph nodes directly from the blood by migrating across the walls of specialized postcapillary venules which are located in the paracortex (T lymphocyte areas) of the node. These vessels have a characteristic morphology which distinguishes them from other types of blood vessel. The endothelial cells which line these vessels have a plump or cuboidal morphology, which contrasts to the flattened morphology of endothelial cells lining other types of vessel (**Figure 1**). It is the peculiar endothelial morphology that has engendered their common name – high endothelial venules (HEVs). The endothelial lining is surrounded by a thickened basement membrane, which is more pronounced than in other types of blood vessel. In immunocompetent adults, the localization of lymphocytes, but not other leukocytes, either attached to the inner (luminal) surface or within the vessel wall and the distinct morphological appearance are the most recognizable features of HEV. However, these vessels are also readily distinguishable from other blood vessels by histochemical, biochemical and immunological markers. The lining endothelial cells, which will be referred to as high endothelial cells (HECs),

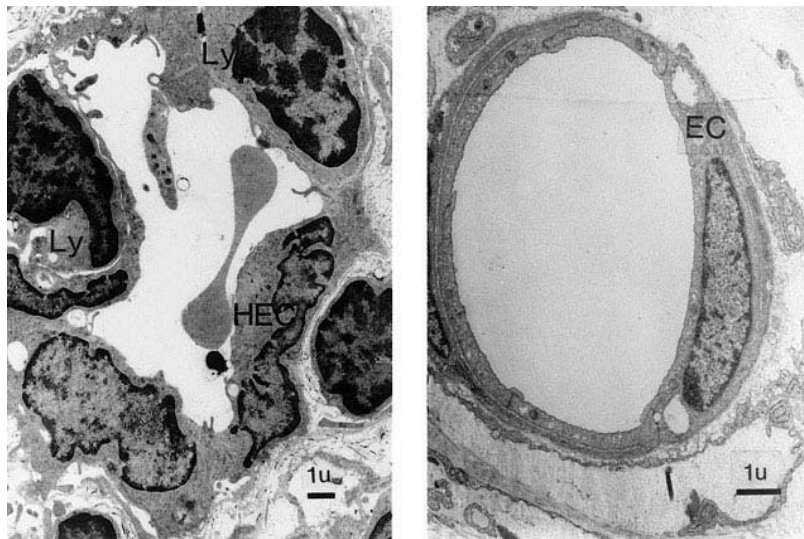


Figure 1 Distinct morphological appearance of HEVs. Transmission electron micrographs of an HEV in peripheral lymph node (left) and a capillary in the vasa vasorum of aorta (right) of the rat. Note numerous lymphocytes (Ly) trapped in the walls of the HEV and increased height of lymph node high endothelial cells (HEC) in comparison with capillary endothelial cells (EC).

stain more intensely with RNA-binding dyes such as pyronin and contain a prominent Golgi apparatus and more secretory vesicles than other types of endothelia. Early histochemical studies also distinguished HEC from 'flat' endothelial cells lining other types of vessel. HEC express higher levels of nonspecific esterase, lactate dehydrogenase and NADH reductase. Biochemical studies identified a unique biosynthetic pathway for inorganic sulfate in HECs which is crucial for the generation of the peripheral addressin, an important adhesion molecule which mediates L-selectin-dependent binding of lymphocytes to HECs (see below). Several monoclonal antibodies have been described that specifically stain HEVs and not other types of blood vessel. These include MECA 79 and MECA 367, which identify the peripheral and mucosal addressins respectively, HECA 452, which identifies part of the functional carbohydrate epitope in the peripheral addressin, the sialyl Lewis^x saccharide and MECA 325, for which the antigen has not yet been identified.

The actual height of the endothelial cells lining HEVs varies between species and according to the method of tissue collection; therefore these vessels cannot be defined solely by endothelial height. For example, endothelial cells (ECs) which line equivalent vessels in lymph nodes of sheep, rabbits and guinea pigs are not as 'high' as those in rodents. Additional markers such as the expression of the vascular addressins are therefore important to identify these specialized blood vessels. Collection of rodent lymph nodes under reduced arterial pressure results in a constriction of the vessel lumen and plumping up of the endothelium to 15–20 μm in height. If the patency of HEVs is preserved by perfusion during fixation, then the endothelium is only 5–7 μm in height, although this is still greater than that lining

non-HEV blood vessels, which is 1–2 μm thick (Figure 1). The relationship between vessel patency and endothelial height suggest that HEVs are contractile vessels. Both the endothelial cell and the underlying basement membrane could contribute to HEV contractility. Dynamic changes in HEV morphology *in vivo* may enhance the trapping and passage of lymphocytes from the blood. The term HEV will be used to describe those postcapillary venules within lymph nodes which support the migration of naive lymphocytes from the blood irrespective of exact endothelial height.

Distribution

In adult animals, HEVs are normally found in lymph nodes, gut-associated lymphoid tissues such as Peyer's patches, and aggregates of lymphoid tissues associated with other mucosal surfaces such as bronchus-associated lymphoid tissue in the lungs. HEVs are not found in the spleen although lymphocytes continually migrate into this organ. Lymph nodes are highly vascularized organs and HEVs are found at precise locations within the microvascular bed of the lymph node (Figure 2). The main hilar artery feeding the lymph node arborizes into a capillary bed in the outer cortex which leads directly (or indirectly via arteriovenous shunts) into the postcapillary venular network. This traverses the paracortex (T cell area) of the node and HEVs are located within this postcapillary venular network. Several major HEV trunks, each receiving 3–5 smaller side-branches as they traverse the paracortex, are found within each node. In the medulla, HEVs merge into segmental veins before joining the hilar vein. The basic structure of HEVs does not differ between lymphoid tissues; however, there is heterogeneity among indi-

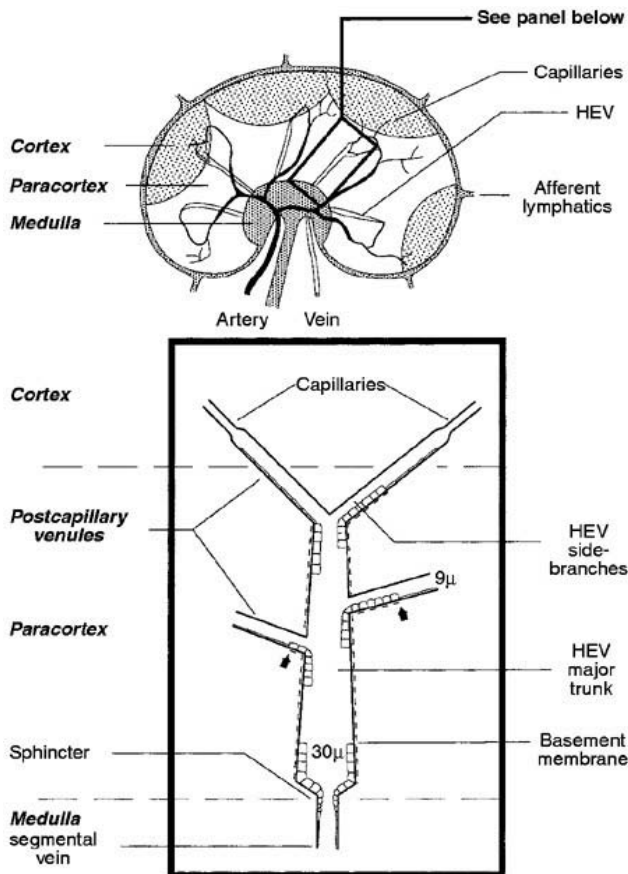


Figure 2 Location of HEVs within the lymph node. The main hilar artery into the lymph node arborizes into a capillary bed in the outer cortex which leads directly into the postcapillary venular network. This traverses the paracortex (T cell area) of the node and HEVs are located within this postcapillary venular network. Several major HEV trunks (30 μm in diameter) each receiving 3–5 smaller side-branches (9 μm in diameter) as they traverse the paracortex are found within a single node. In the medulla, HEVs merge into segmental veins before joining the hilar vein. Solid arrows denote transition from postcapillary venule lined with flat EC to HEV (i.e. postcapillary venule lined with high endothelial cells) and location of proliferating HECs in antigen-challenged node.

vidual HECs lining a single venule. The majority of HECs exhibit a basal level of activity but are interspersed with cells of increased cytoplasmic density due to abundant polyribosomes, dilated Golgi, and an invaginated luminal surface. This heterogeneity in metabolic activity is accompanied by varying levels of nonspecific esterase and 'hot spots' of enhanced lymphocyte extravasation. In contrast, expression of the vascular addressins is uniform on individual HECs within a single venule (see Figure 3).

HEV function in lymphocyte extravasation

To select sufficient antigen-specific lymphocytes for the initiation of immune responses, as many naive lymphocytes as possible should be sampled by antigen-presenting cells within lymphoid tissues. The clearly identifiable function of HEVs in this process is to recognize and sort lymphocytes from other leukocytes in the bloodstream and deliver them into lymph nodes. It is reasonable to assume that all of the HEV-associated properties described above may, in some way, regulate the selective migration of lymphocytes from the blood into the lymph node, but apart from the roles of individual adhesion molecules (see below) other properties have not been studied in relation to lymphocyte migration.

Structural considerations

The actual number of lymphocytes that migrate through a single node can be regulated in three ways: the extent of blood flow through HEVs, the size of the HEV network, and the rate of lymphocyte extraction per HEC. The level of lymphocyte migration through lymph nodes increases dramatically following antigen administration and a major contributor to this increased traffic is the rapid change in blood flow which can reach as much as three times control levels after 2 days. The rate of lymphocyte extraction is governed by the level of adhesion to and transmigration across the HEC layer. This can increase up to two-fold following antigen administration, which takes estimated extraction rates to a maximum of 60% of lymphocytes that flow through lymph nodes. The number of HECs, i.e. the size of the HEV network, also increases following antigenic stimulation and this will contribute to increased infiltration of lymphocytes. This increase occurs by endothelial proliferation but it is restricted to HECs at the boundary between 'flat' and high endothelium in the proximal region of the postcapillary venular network (see Figure 2).

Since the suggestion by Marchesi and Gowans that lymphocytes penetrated the HEC cytoplasm rather than taking a more conventional, intercellular pathway, the actual route of migration across the vessel wall has been a matter of debate. Ultrastructural studies have provided evidence in support of both intracellular and intercellular routes of lymphocyte migration. Scanning electron microscopic analysis of the luminal surface of HEV shows that HECs form a continuous lining and there are no obvious 'gaps' either between HECs or within HEC cytoplasm for lymphocytes to penetrate. Depending on the precise location in the HEV network, HECs are either

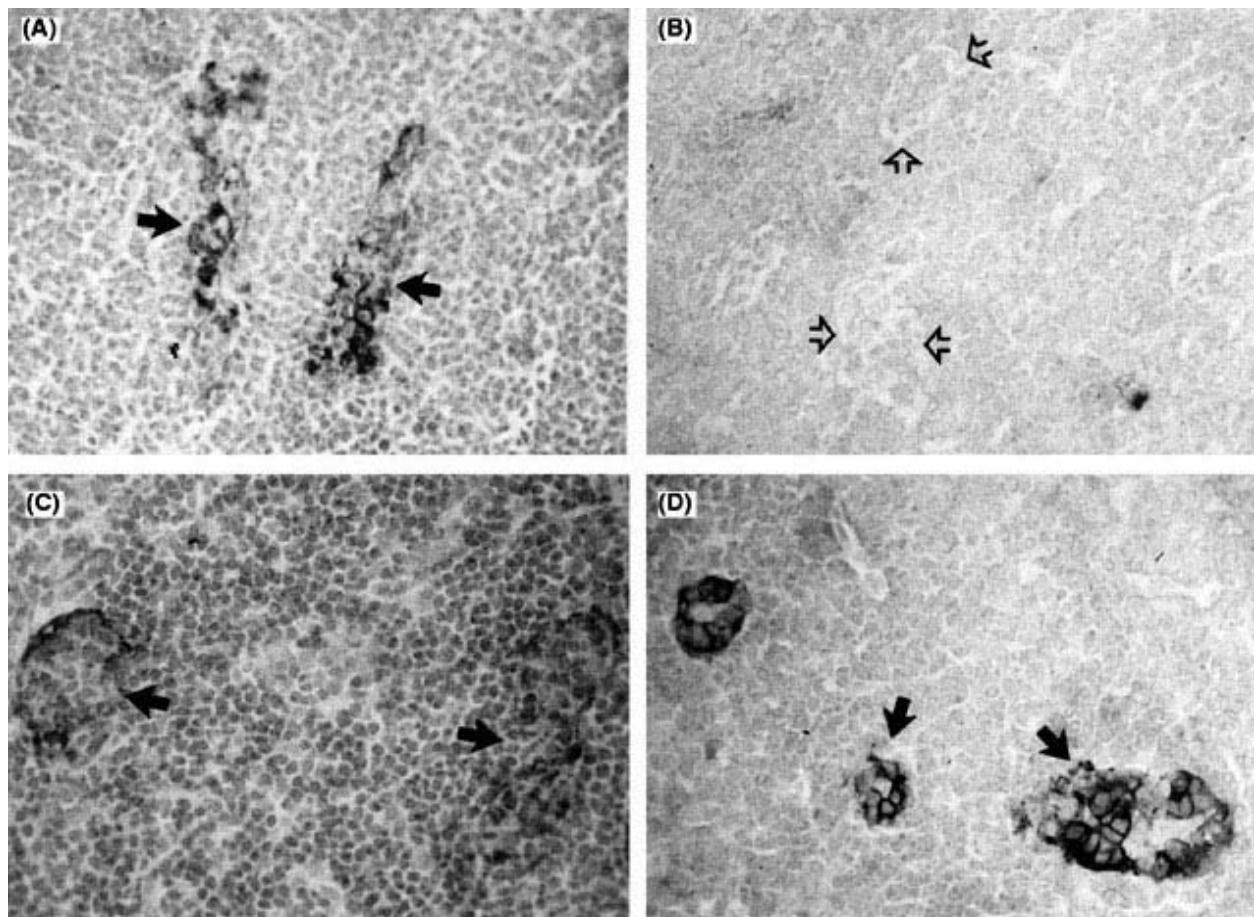


Figure 3 Organ-specific expression of the vascular addressins by HEVs. (A, C) The peripheral addressin (identified by monoclonal antibody MECA 79) is expressed by HEVs in (A) peripheral (cervical) and (C) mucosal (mesenteric) lymph nodes of adult BALB/c mice (solid arrows). (B, D) The mucosal addressin MAdCAM-1 (identified by monoclonal antibody MECA 367) is expressed by (D) mucosal lymph node (solid arrows) but not (B) peripheral lymph nodes HEVs (open arrows).

arranged in a cobblestone array (proximal segment) or as an interlacing network of cells (distal segment). However, all HECs have cytoplasmic extensions which overlap significantly with adjacent HECs. There are deep crevices in the luminal surface at these intercellular contacts and lymphocytes adhere preferentially at or near these junctions.

Lymphocytes undergo a dramatic change in morphology as they transmigrate the vessel wall. Circulating lymphocytes are spherical with numerous surface microvilli and they initially make contact with the surface of HECs via these microvillous projections. They then penetrate the HEC layer, during which the lymphocyte becomes polarized; this is accompanied by a loss of microvilli. During the migration of lymphocytes across the HEC layer very close intercellular, gap-like junctions of 2–4 nm are formed between lymphocytes and HECs, the significance of which is unclear. These are substantially closer than the 20–30 nm intercellular adhesive junc-

tions lymphocytes make with either the luminal surface of HEVs or the underlying basement membrane. Intercellular contacts between adjacent HECs are fewer in number than in other types of endothelia and they are limited to small areas of close apposition, referred to as 'spot welds'; this altered arrangement of inter-EC junctions may facilitate transendothelial migration of lymphocytes.

The potential role of the prominent basement membrane surrounding HECs in regulating lymphocyte extravasation warrants consideration. It comprises a layer of supporting cells (which are called pericytes in other types of blood vessel) embedded in a mixture of extracellular matrix molecules such as laminin, fibronectin and glycosaminoglycans. In addition a novel extracellular matrix glycoprotein has recently been demonstrated in HEVs. This protein, called HEVin, has homology to the antiadhesive extracellular matrix protein SPARC, and may play a role in regulating the morphology of HECs or the

permeability of the basement membrane. It is not clear whether the lymphocyte needs to degrade the basement membrane to cross it, but it does not appear to present a substantial barrier to lymphocytes. The basement membrane, but not the endothelial lining, is however, a barrier to small molecules in the blood stream. Following intravenous administration, soluble molecules accumulate on the endothelial side of the membrane. Conversely, molecules entering the node via the afferent lymphatics, somewhat surprisingly, have access to the intravascular compartment. There is evidence that the basement membrane underlying HEC is, in fact, an extension of the reticular network that extends throughout the paracortex of the node and that this network of reticular fibers, fibroblast reticular cells and extracellular matrix components functions as a conduit for bulk flow of soluble molecules from the afferent lymphatics to HEVs. This one-way traffic of soluble molecules from the node to the vascular compartment has been implicated in the transfer of chemoattractants that play a crucial role in regulating the adhesion of lymphocytes from flowing blood and subsequent transmigration across the vessel wall (see below).

Nonrandom migration of lymphocytes

The traffic of recirculating lymphocytes through lymph nodes in different regions of the body is not entirely random, which adds a further order of complexity to lymphocyte-HEV recognition. The most marked asymmetry is found between peripheral lymph nodes draining skin sites and lymphoid tissues associated with mucosal surfaces, such as Peyer's patches in the gut wall. Lymphocytes (T and B cells) collected from nodes draining either of these two sites in sheep preferentially migrate or 'home' back to their site of collection. In rodents, the discovery of lymphomas which adhere to either peripheral or Peyer's patch HEVs has identified a molecular basis for homing. Lymphocytes express 'homing receptors' that recognize ligands (termed 'vascular addressins') expressed by HECs in an organ-specific manner (Figure 3). The peripheral and mucosal homing receptors have been identified in mice as L-selectin and $\alpha_4\beta_7$ integrin respectively. Naive lymphocytes, which are randomly distributed between peripheral and mucosal lymph nodes, express both types of homing receptor. Following activation by antigen, L-selectin is downregulated from the lymphocyte surface and thus these cells lose the ability to migrate to peripheral lymph nodes. Selective expression of a mucosal homing receptor is presumed to form the cellular basis of the mucosal immune immunoglobulin A (IgA) response which occurs independently

of systemic immunity. Recent studies have demonstrated clearly that homing receptor/vascular addressin recognition represents just the first step in a sequence of adhesive interactions required for successful lymphocyte extravasation, that of binding to the inner surface of the HEVs from flowing blood.

The multistep adhesion cascade: roles of adhesion molecules and chemoattractants

A sequence of adhesive interactions, the multistep adhesion cascade, is required to arrest the lymphocyte from flowing blood on the inner surface of the HEV wall, prior to its directed migration across the vessel wall and into the lymph node. The adhesion cascade can be dissected into two distinct stages: a reversible, adhesive interaction that manifests as binding from flow and rolling on the inner surface of the vessel wall followed by a stable, adhesive interaction that completely arrests the leukocyte from blood flow (Figure 4: steps 1 and 2). Rolling is thought to allow the lymphocyte time to integrate and respond to stimuli presented on the endothelial surface which activate integrins and completely arrest the leukocyte on the vessel wall. Migration across the vessel wall (diapedesis) can also be dissected into distinct steps including transendothelial migration and migration across the underlying basement membrane (Figure 4: steps 3 and 4). Studies of lymphocyte migration have not distinguished between these steps and thus they are poorly understood. It is assumed that they are regulated by chemoattractants derived from the surrounding tissues which have access to the vascular compartment as described above.

The adhesion molecules which mediate lymphocyte rolling and stable adhesion in HEVs in Peyer's patches and peripheral (subcutaneous) lymph nodes of mice have been identified. L-selectin (the peripheral lymph node homing receptor) on the lymphocyte mediates binding from flow and rolling via recognition of sulfated, fucosylated, sialylated oligosaccharides (commonly called the peripheral addressin and identified in mice and humans by monoclonal antibody MECA 79) on proteins such as CD34, Sgp200 and GlyCAM-1 as well as MAdCAM-1 in Peyer's patches. L-selectin is less important in Peyer's patches where rolling is also mediated by $\alpha_4\beta_7$ integrin (the mucosal homing receptor) binding to MAdCAM-1 (the mucosal addressin). The subsequent stable adhesion of lymphocytes in all HEVs is mediated by the CD18 integrin, LFA-1, which requires a pertussis toxin (PTX)-sensitive activation step for its function. The stimuli that activate LFA-1 and lead to stable adhesion of lymphocytes, but not other types of leukocyte, on the

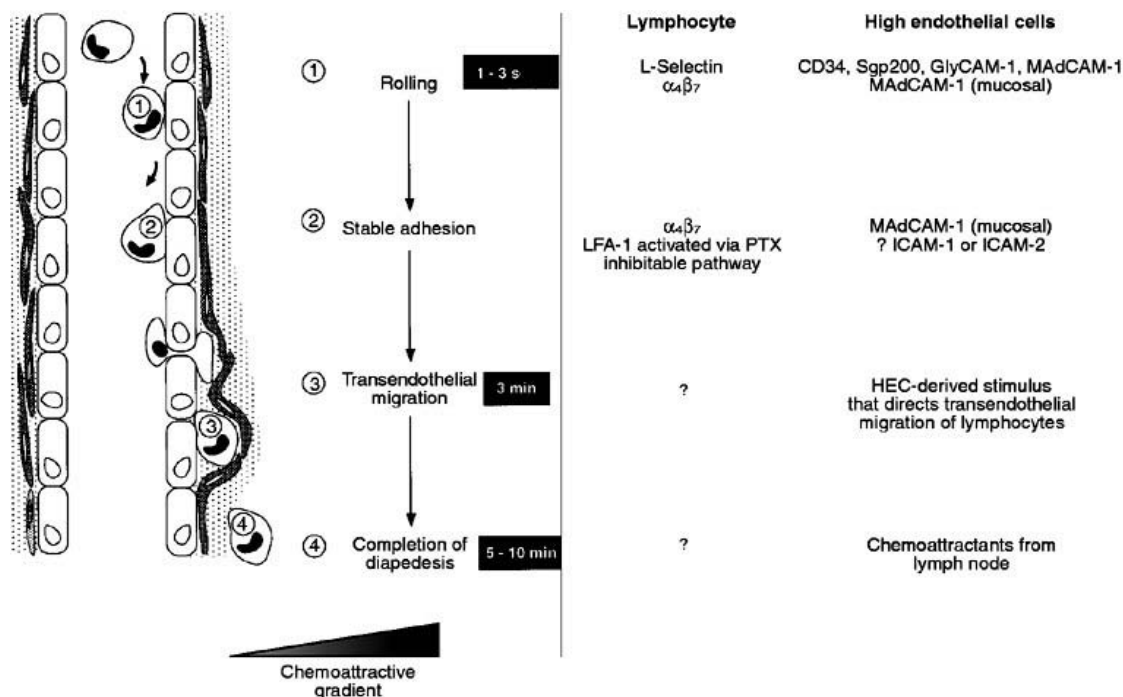


Figure 4 The sequence of events that regulate lymphocyte migration from the bloodstream across the walls of HEVs into lymphoid organs. Lymphocytes use L-selectin (and/or $\alpha_4\beta_7$ integrin in mucosal tissues) to roll on the HEC surface (step 1). Rolling is rapidly converted to integrin-dependent stable adhesion within 1–3 s (step 2). This is mainly mediated by LFA-1 following its activation via a pertussis toxin (PTX)-sensitive pathway. The ligands and activating stimulus for LFA-1 on the HEC surface are not worked out. Lymphocytes migrating to mucosal lymph nodes also bind via $\alpha_4\beta_7$ integrin recognition of MAdCAM-1. Transendothelial migration of bound lymphocytes (step 3) is regulated by a putative 'lymphocyte migration stimulus' expressed by HECs. The molecular basis of the final stage of migration across the basement membrane (step 4) is not understood but it is thought to be regulated by lymph node-derived chemoattractants.

inner surface of lymph node HEVs are undetermined. By analogy with the neutrophil, chemoattractant molecules operating on PTX-inhibitable G protein-linked receptors are likely candidates for this stage. Lymph node-derived chemoattractants have been proposed to direct the migration of lymphocytes across the HEV wall, although candidate molecules have not yet been identified. Since the majority of lymphocytes migrating into lymphoid organs are unactivated T and B lymphocytes, it is predicted that there are chemoattractants for these lymphocyte populations. Studies using cultured HECs suggest that transendothelial migration, the first step in diapedesis, can be initiated by the endothelial cells, suggesting that lymph node-derived chemoattractants may not be required for this step.

The crucial roles of L-selectin and β_7 integrins in directing lymphocyte migration to peripheral and mucosal lymph nodes respectively have been confirmed in 'knockout' mice; the requirement for fucosylation of the functional ligand for L-selectin on HEVs is also confirmed in mice in which the enzyme fucosyltransferase VII, which generates the sialyl Lewis^x modification has been deleted. This is, how-

ever, one of several post-translational modifications required to generate the peripheral addressin, the other being sulfation of sialyl Lewis^x and, possibly, other sugars. The endothelial selectins, E- and P-selectin, do not regulate lymphocyte migration via HEVs under basal conditions because they are not normally expressed by HEVs and the functional ligands for these two selectins are not expressed by naive T and B lymphocytes, which comprise the bulk of the migrating population.

HEV development

Morphologically distinct HEVs are not visible until birth in mice, rats and humans. Blood vessels expressing sialyl Lewis^x are detectable in lymphoid organs during embryonic life in humans; it is not clear whether these represent a precursor to HEVs which are not yet fully differentiated. Expression of the mucosal addressin is detectable at birth in mice, however its expression is widespread in both peripheral and mucosal lymph nodes. Expression of the peripheral addressin commences after birth and is accompanied by loss of MAdCAM-1 expression in

peripheral lymph nodes during the first few days of life. The peripheral addressin is, however, not strictly 'peripheral' since its expression is detectable in Peyer's patch HEVs in some mouse strains and migration to Peyer's patches is partially dependent on L-selectin.

The origin of HEVs is not clear. An obvious suggestion is that they are normal postcapillary venules in which the endothelial cells have been activated by their immediate microenvironment. Alternatively they be of a distinct developmental lineage arising from a different endothelial stem cell (Figure 5). Recent studies showing expression of a newly identified member of the endothelial cell-specific growth factor receptor family, FLT4, on HEVs and lymphatic endothelium, rather than on blood vessel endothelium, suggests the latter hypothesis. The developmental origin of cells in the basement mem-

brane of HEVs and their potential role in regulating the activity of the lining endothelial cells are also unknown.

In adult mice the expression of vascular addressins and the function of HEVs are actively maintained by, as yet, unidentified stimuli. Ligation of afferent lymphatics which drain into a lymph node from the surrounding tissues (Figure 2) has a dramatic effect on the phenotype and function of HEVs. Lymphocyte adhesion to HEV *in vitro* decreases dramatically and the migration of lymphocytes into lymph nodes *in vivo* is undetectable 3 weeks after deafferentization. Blood flow through the deafferentized lymph nodes is severely reduced, which limits lymphocyte entry *in vivo*. There is a gradual flattening of endothelial cells lining HEVs and a reduction in peripheral addressin expression. The components in afferent lymph which maintain fully functional HEVs and whether they have direct or indirect effects (e.g. via the underlying basement membrane) on HEVs are unknown. HEV morphology and function are normal in T and B lymphocyte-depleted mice (SCIDs, nudes and irradiated mice), which suggests that factors from accessory cells or the direct delivery of cytokines from surrounding tissues will be important. In congenitally athymic rodents, the endothelial cells lining peripheral lymph node HEVs are considerably flatter than in their euthymic litter mates. However, these blood vessels express the peripheral addressin and are able to support the migration of recirculating B and T cells. Thus the morphology and function of HEVs are independently regulated. HEVs in T cell-deprived mice acquire plump morphology on reconstitution with T cells. If the height of endothelial cells lining HEVs is related to vessel contractility (see above), this suggests that HEV contractility may be regulated by T cells or their products.

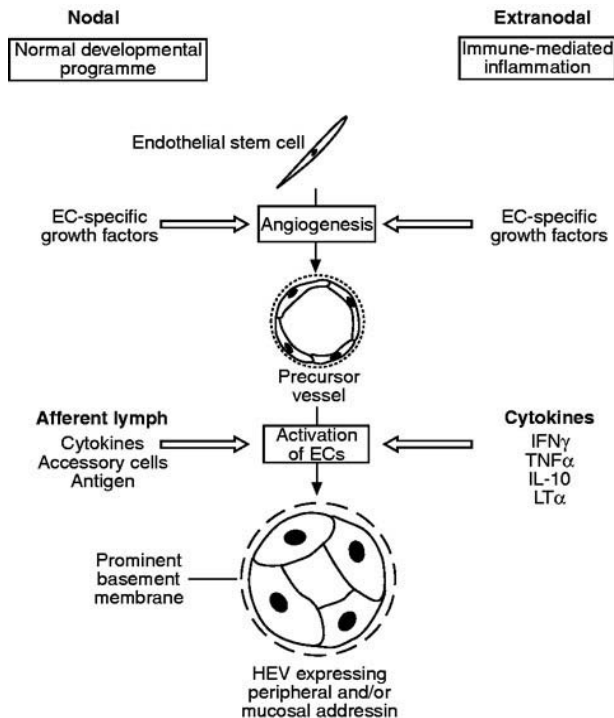


Figure 5 Developmental origin of HEVs. *Nodal*: In lymph nodes HEVs may arise from uncommitted postcapillary venules which have undergone a normal developmental program in response to EC-specific growth factors or from a distinct high endothelial stem cell. The morphology, expression of vascular addressins and ability of HEVs to support the migration of lymphocytes is maintained by components gaining access to the node via afferent lymph. *Extranodal*: HEV-like vessels expressing vascular addressins may represent uncommitted blood vessels in which the endothelial cells have been activated by locally produced cytokines or may arise from a distinct high endothelial stem cell which proliferates during the angiogenic response associated with chronic inflammation. The developmental origin of cells in the basement membrane of HEVs is unknown.

Extranodal development of HEVs: relationship between high endothelium and other types of vascular endothelium

Blood vessels with the distinct morphology, biochemical properties, and antigenic profile of HEVs have been reported at sites of chronic immune-mediated inflammation, such as in rheumatoid synovia, the pancreas of NOD mice and in rejecting allografts. Blood vessels in the lamina propria of mice express the mucosal addressin MADCAM-1 and the HEC-specific antigen MECA 325, although they do not have the morphology of HEVs. As in lymph nodes, it is not clear whether these extranodal HEVs are formed from existing vessels via a response of the endothelial cells to local inflammatory mediators, or

whether they are formed *de novo* from a distinct progenitor cell during the angiogenic response associated with chronic inflammation (Figure 5). HEV-like properties have been reported in peritubular capillaries of the rat within 3 days of transplantation of fully allogeneic kidneys, indicating that microvascular changes can be rapid and are not restricted to postcapillary venules.

Studies using transgenic techniques to direct the expression of cytokines to extranodal sites demonstrate a role for cytokines in the formation of HEV-like vessels at these sites. Expression of interferon γ (IFN γ), tumor necrosis factor α (TNF α), lymphotoxin α (LT α) and interleukin 10 (IL-10) in islets of Langerhans in the pancreas of mice induces HEV-like blood vessels expressing the peripheral and mucosal addressins. The formation of HEV-like vessels correlates with the accumulation of unactivated T and B lymphocytes within these organs. It is not clear whether these cytokines have direct or indirect effects on blood vessels, however, some HEV-associated properties can be induced in nonlymphoid vascular endothelium in response to cytokine stimulation *in vitro*. IFN γ induces expression of the MECA 325 antigen and TNF α induces expression of MAdCAM-1 on murine endothelial cell lines. Induction of the peripheral addressin on cultured endothelial cells has not, however, been demonstrated. Studies in cytokine gene 'knockout' mice do not support roles for IFN γ or TNF α in the normal developmental program of HEVs. However, lymph nodes fail to develop in the absence of LT α or following blockade of the LT β R, which binds a cell surface LT α /LT β complex. It is possible that LT β R interactions regulate the development of lymph nodes via a direct effect on the vasculature inducing HEVs in embryonic life or by promoting the formation of the lymph node anlage, the precursor to lymph node formation, which may itself direct HEV development. The effects of IFN γ and TNF α at extranodal sites may therefore be exerted via indirect effects on the expression of either LT α / β complex or LT β R. The capacity of afferent lymph to affect HEV function is not dependent on this signaling pathway since once lymph nodes have formed they are unaffected by LT β R blockade.

Summary

What then is the function of HEV? The answer will depend on which HEV-associated property is under consideration. During the development of the immune system, HEVs are crucial for its formation and function in that they localize recently matured

T and B lymphocytes to secondary lymphoid organs. The unique structure of HEV provides an efficient collector of lymphocytes from the blood in that rapid changes in blood flow, trapping and migration of lymphocytes are facilitated. The migration of lymphocyte subsets to different regions of the immune system via organ-specific vascular addressins suggests that HEVs can directly affect the outcome of a local immune response. Finally, HEV-like vessels at sites of chronic inflammation will promote the immigration of naive lymphocytes and thus may amplify and maintain ongoing immune responses. Identification of the stimuli that induce HEV formation at these sites may, therefore, provide a means of controlling chronic immune-mediated inflammation.

See also: Adhesion molecules; Lewis^x/sialyl-Lewis^x (CD15/CD15s); Cytokines; Diapedesis; Endothelium; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Lymph nodes; Lymphocyte function-associated antigen 1 (LFA-1); Lymphocyte trafficking; Mucosa-associated lymphoid tissue (MALT); Selectins (CD62-E/L/P).

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HISTAMINE

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The biogenic amine histamine has long been incriminated as an essential mediator in immediate type I hypersensitivity and anaphylactic reactions. However, the amine also regulates the activity of other immune cells and has a fundamental role in the control of gastric acid secretion, the central nervous system and cell proliferation and cancer.

Chemistry, metabolism and distribution

Histamine (1*H*-imidazole-4-ethanamine) is formed by the decarboxylation of the amino acid histidine. There are two major pathways for the catabolism of the amine in humans (Figure 1). The most important route involves ring methylation and is catalyzed by the enzyme histamine-*N*-methyltransferase. The product, *N*^τ-methylhistamine, is then largely converted by monoamine oxidase to *N*^τ-methylimidazole acetic acid. In the second pathway, histamine is oxidatively deaminated to imidazole acetic acid by the enzyme diamine oxidase. The latter is also known as histaminase. The various metabolites have little or no pharmacologic activity and are excreted in free or in conjugated form in the urine.

Histamine is widely distributed throughout the various tissues of the human body; the name derives from the Greek word for tissue, *histos*. Every mammalian tissue that contains the amine is capable of synthesizing it from histidine by virtue of L-histidine decarboxylase. In most tissues the bulk of the amine is stored in the secretory granules of mast cells. In the blood, histamine is contained in the circulating counterpart of the mast cell, the basophil leukocyte. Histamine and other inflammatory mediators are released by the immunological activation of these cells in the course of immediate hypersensitivity reactions and thereby contribute to the symptoms of allergy and anaphylaxis. Histaminergic neurons of the central nervous system constitute an important non-mast cell source of the amine in humans (see below).

Histamine receptors

Histamine acts on a large variety of different cell types, including smooth muscle cells, neurons, endocrine and exocrine cells, blood cells and cells of the immune system. The biological effects produced are mediated through distinct receptors of which three subtypes, designated H₁, H₂ and H₃ respectively, have now been identified. Activation of H₁-receptors appears to lead to the breakdown of membrane inositol phospholipids and the consequent generation of diacylglycerol and the mobilization of calcium ions. The latter then act as second messengers for transduction of the biological response. In contrast, H₂-receptors are coupled to adenylate cyclase and actions of histamine at these sites are mediated through a rise in the intracellular level of cyclic AMP. The genes for the H₁ and H₂ receptors have been cloned and show limited homology, suggesting that they are not derived from a common ancestral precursor.

H₃ receptors are located on presynaptic nerve endings of histaminergic and nonhistaminergic neurons in the brain. The second-messenger system associated with these receptors remains to be fully elucidated but they function as autoreceptors and heteroreceptors to regulate the release of histamine and other central neurotransmitters.

Responses mediated through H₁ receptors are antagonized by classical 'antihistamines' such as mepyramine and chlorpheniramine, those through H₂ receptors are antagonized by cimetidine and ranitidine, and those through H₃ receptors by thioperamide.

Pharmacologic effects

Localized allergic reactions to food or aeroallergens are generally confined to particular target organs, such as the airways, eye, gut and skin. However, more generalized, systemic responses may occur, typically in anaphylactic or anaphylactoid reactions to various drugs, agents used in surgical or proper-

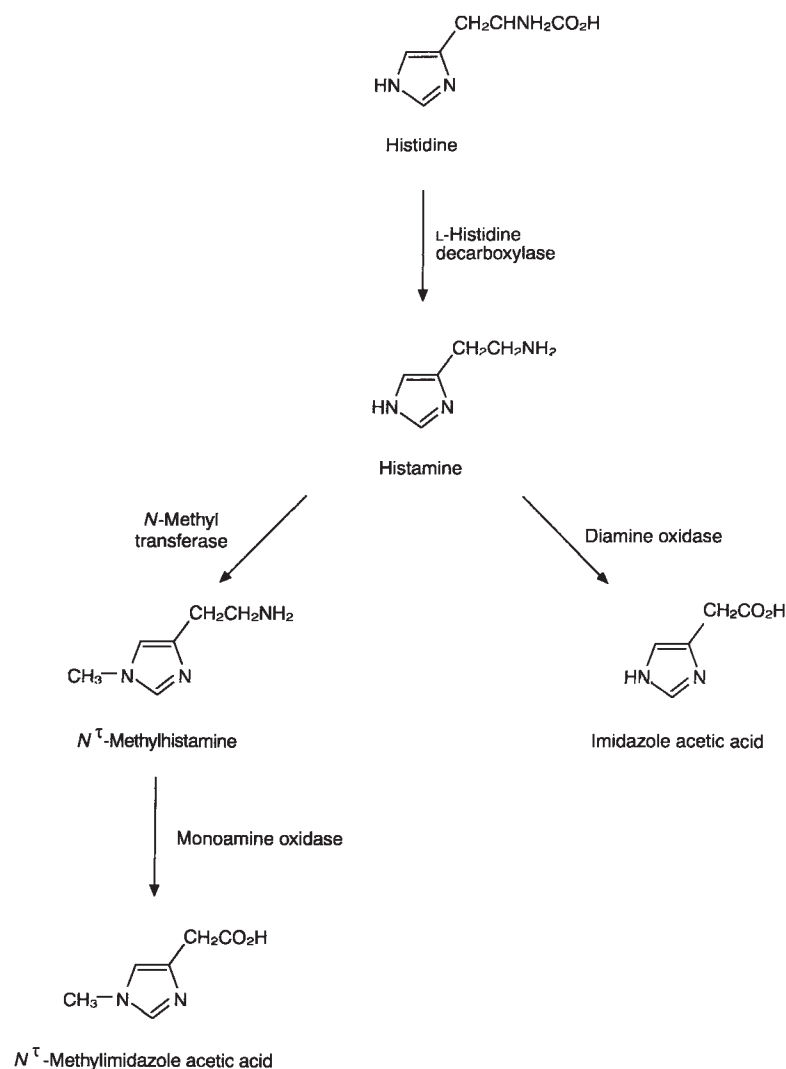


Figure 1 Synthesis and catabolism of histamine.

ative procedures such as anesthetics and their vehicles, plasma substitutes and radiographic contrast media, and to certain insect stings, particularly those of the bee.

Anaphylactic reactions in humans are accompanied by a sharp rise in the plasma concentration of histamine and the classical signs of intoxication by the amine. The clinical manifestations may vary considerably in severity. Mild cutaneous reactions involving erythema, urticaria and pruritus, often accompanied by metallic taste, headache and nasal congestion, are associated with plasma histamine levels below 1 ng ml^{-1} . Systemic responses with generalized skin reactions, discomfort, gastrointestinal disturbance, tachycardia, cardiac arrhythmias and medium hypotension involve elevations in plasma histamine above this basal level. Life-threatening responses comprising severe hypotension, ventricular fibrillation, bronchospasm and cardiac and respiratory arrest are typically associated with

plasma histamine levels in excess of 12 ng ml^{-1} (Table 1). These responses may be most conveniently discussed in terms of the various systems involved.

Cardiovascular system

The hemodynamic response to histamine involves an effect of the amine on the blood vessels and a direct action on the heart. In humans, and most but not all other animals, histamine has a predominantly dilator effect on the peripheral blood vessels. This dilatation results in a characteristic flushing, a decrease in total peripheral vascular resistance and a fall in blood pressure. Both H_1 and H_2 receptors appear to be involved in these responses, with H_1 receptors largely mediating the initial vasodilator effect and H_2 receptors becoming progressively involved during sustained exposure to the amine.

The above, and an important additional effect, are involved in the so-called 'triple response' produced by intradermal injection of histamine. This response

Table 1 Classification of histamine-mediated systemic responses by severity

Severity grade	Clinical symptoms	Plasma histamine (ng ml ⁻¹)
I: Cutaneous	Erythema, urticaria and/or dermal pruritus <i>only</i>	≤ 1
II: Systemic	Generalized skin reactions, discomfort, tachycardia, arrhythmias, medium hypotension, respiratory distress	> 1
III: Life-threatening	Severe hypotension, ventricular fibrillations, cardiac and respiratory arrest	> 12

Adapted from Ennis M and Lorenz W (1985) Hypersensitivity reactions induced by anaesthetics and plasma substitutes. In: Dean J (ed) *Immunotoxicology and Immunopharmacology*, pp 457–474. New York: Raven Press.

comprises a localized red spot, which is confined to a few millimeters around the site of injection and which develops within a few seconds, a flare of irregular outline that extends several centimeters beyond the original point of injection and develops more slowly, and a wheal that is discernable within 1–2 min and occupies the same area as the original red spot. The red spot results from the direct vasodilatory effect of histamine, while the wheal is due to an increase in capillary permeability and edema formation. The flare response is more complex and involves an axon-reflex mechanism whereby histamine stimulates afferent C-fiber nerve endings. The resulting impulses may spread antidromically into arborizations of the C fiber, leading to the release of neuropeptides, such as substance P, which act directly on the vasculature to propagate the inflammatory response.

In the human heart, histamine increases both the frequency and force of myocardial contraction as well as cardiac output. The amine also has a variety of arrhythmogenic effects, including enhancement of normal automaticity, induction of abnormal automaticity, induction of triggered tachyarrhythmias, depression of atrioventricular conduction and an increase in the vulnerability of the ventricles to fibrillation. A combination of H₁- and H₂-directed antihistamines is needed to block this diversity of effects, indicating the involvement of both receptor types. Although somewhat controversial, both the positive inotropic and chronotropic effects of histamine appear to be largely mediated through H₂ receptors, although H₁ receptors may additionally be involved in the former response.

Nonvascular smooth muscle

The effect of histamine on various types of smooth muscle varies according to the source and the species; however, the amine typically contracts nonvascular smooth muscle through activation of H₁ receptors, although occasional relaxant responses, largely mediated through H₂ receptors, are observed.

Inhalation of histamine in humans leads to bron-

choconstriction. This effect is most pronounced in asthmatic subjects in which the dose of histamine (PC₂₀ histamine) required to produce a 20% reduction in the forced expiratory volume in 1 second (FEV₁) is widely used as a measure of the degree of bronchial hyperreactivity. The initial bronchoconstrictor response to inhaled allergen in humans appears to be largely due to mast cell activation, with at least half of the effect being produced by the release of histamine and the remainder by the liberation of arachidonic acid metabolites. Among experimental animals, bronchial smooth muscle of the guinea pig is exquisitely sensitive to histamine and a profound, fatal bronchoconstriction is the characteristic feature of anaphylaxis in this species. Unusually, histamine causes relaxation in the sheep bronchus and cat trachea.

The response of gastrointestinal smooth muscle also varies with the species; however, the classical effect is again contraction and this response in the terminal ileum of the guinea pig forms the basis of the original bioassay for histamine.

Gastric acid secretion

Histamine plays an essential role in the regulation of acid secretion by oxyntic cells in the stomach. The source of this histamine varies according to the species but in humans the amine is located largely in mast cells in the gastric mucosa. The control of acid output is complex, with neurocrine, endocrine and paracrine factors acting together in interdependent fashion. In particular, acetylcholine, gastrin and histamine modulate the release process.

Two theories have evolved to account for the control of acid secretion. In the 'permission hypothesis', the oxyntic cells express acetylcholine, gastrin and histamine (H₂) receptors on their surface, with the effects of acetylcholine and gastrin only being fully expressed if the histamine receptors are occupied. That is, the former agents act by 'permission' of histamine. In the 'transmission hypothesis', histamine is the final common chemostimulant for secretion, and bloodborne gastrin and neurally released acetylcho-

line act on mast cells located adjacent to the oxyntic cells to induce release of the amine. The secreted histamine then acts through H_2 receptors on the oxyntic cells to cause the release of acid. Histamine thus 'transmits' the actions of acetylcholine and gastrin. Of these two models, the permission hypothesis has probably received general acceptance although the transmission hypothesis has recently been vigorously revived. In any event, histamine clearly plays a key role in the process and H_2 antihistamines are potent inhibitors of acid secretion induced by all physiologic mechanisms. Clinically, these agents have revolutionized the treatment of peptic ulcer disease.

The central nervous system

There are two major cellular sources of histamine in the human brain, namely mast cells and histaminergic neurons. The mast cells occur in the largest numbers in those brain structures with a high degree of vascularization, such as median eminence, the pineal gland and the meninges. They are found in close association with blood vessels and may play a role in the control of brain blood flow and vascular permeability. Histaminergic neurons are confined exclusively to the tuberomammillary nucleus in the posterior hypothalamic region. Individual neurons project axon collaterals to many different areas and essentially innervate the whole brain. These morphologic features suggest that the central histaminergic system provides a regulatory centre for whole-brain activity. Indeed pharmacologic studies using histamine agonists, antagonists and inhibitors of the synthesis of the amine have suggested that histaminergic neurons may control the sleep-wakefulness cycle, feeding and drinking behaviours, locomotor activity, the neuroendocrine system, body temperature and circulation. Brain histamine may also be involved in aging, neurodegeneration and dementia.

Cell proliferation and cancer

It has been known for many years that histamine may promote wound healing and that proliferating tissues synthesize the amine at a very high rate. Various treatments capable of stimulating cell proliferation in the skin, such as mechanical trauma or topical application of phorbol esters and detergents, also induce local histamine synthesis. The time of maximal epidermal hyperplasia coincides with a significant increase in the tissue level of the amine and a sharp fall in the activity of the histamine-catabolizing enzyme diamine oxidase. The proliferative effect of histamine may be mediated through a novel intracellular receptor (H_{IC}).

Newly formed, nascent histamine may also be involved in tumor growth and development. High

levels of the amine and of its synthesizing enzyme histidine decarboxylase are found in growing tumors in experimental animals, and an increased activity of the enzyme has been reported in both human mammary and colorectal carcinoma tissues. In contrast, diamine oxidase activity is diminished in the latter tissue compared with the surrounding colonic mucosa. Often, the histamine concentration in the tumors does not parallel the histidine decarboxylase activity, probably indicating the rapid release or metabolism of the amine. Significantly, inhibitors of histidine decarboxylase produce a marked inhibition of tumor growth in different animal models.

The above data would suggest a positive role for histamine in tumor induction. Consistently, studies in laboratory animals have indicated that administration of H_2 -receptor antagonists may lead to a decreased tumor growth and metastatic development, and an increased survival time. However, H_1 antihistamines promote the growth of some tumors, and abolish the antitumoral effect of the H_2 blockers, while selective H_1 -receptor agonists can again inhibit tumor development.

These apparently confusing findings may be rationalized into a convenient working model in which histamine plays a dual role in carcinogenesis. During tumor growth, newly synthesized histamine exerts an immunosuppressive effect by activating host suppressor T lymphocytes through H_2 receptors on the cell surface (see below). H_2 antagonists may block this activation and facilitate the natural immune response to, and destruction of, the tumor cells. In parallel, histamine, or appropriate experimental agonists, may exert an immunostimulatory effect by activating T contrasuppressor or effector cells through H_1 receptors. Blockage of this potentially beneficial effect by H_1 antihistamines would promote tumor development. In addition, H_1 and H_2 receptors have recently been described on different tumor cells and histamine could then directly regulate proliferation by acting on these receptors.

Cells of the immune system

The role of histamine in allergy and atopy is well established. As discussed, the amine is released from tissue mast cells and circulating basophil leukocytes by immunoglobulin E (IgE)-dependent mechanisms. This release may be modulated by histamine H_2 autoreceptors which appear to be present on the surface of the human basophil but not the lung mast cell.

In addition, histamine may have a more general role in the regulation of both humoral and cell-mediated immune responses. The amine suppresses lymphocyte proliferation, T cell-mediated cytotoxic-

ity of allogeneic target cells, and lymphokine production. All of these effects appear to be mediated through H₂ receptors. Histamine also suppresses the production of antibodies by B lymphocytes, natural killer cell cytotoxicity, complement production by monocytes, lysosomal enzyme release by neutrophils and neutrophil chemotaxis. Thymocyte maturation, eosinophil chemotaxis and expression of eosinophil complement receptors are also affected.

See also: Atopic allergy; Basophils; Mast cells; Hypersensitivity reactions; IgE.

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HISTOPLASMA, INFECTION AND IMMUNITY

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Characteristics of *Histoplasma capsulatum* and its antigens

The pathogenic fungus, *Histoplasma capsulatum*, exists in nature as a filamentous organism that produces two asexual spores, microconidia and tuberculate macroconidia. The natural habitat of the organism is soil enriched by bird droppings. Chromosomal mapping studies of this fungus have revealed substantial variability in the genomic organization of *H. capsulatum* strains. The Downs strain, which is thermointolerant, possesses seven chromosomes, whereas two thermotolerant strains, G217B and G186B, contain three and four chromosomes, respectively. Thus, a small number of chromosomes regulate a considerable number of biological events.

Infection is acquired via inhalation of microconidia or small mycelial fragments that settle in terminal bronchioles and alveoli of lungs. Within a period of hours to days, these fungal elements convert into yeasts which represent the tissue-specific phase. The transformation of mycelia to yeasts is

accompanied by an orderly sequence of biochemical events that can be divided into three stages. Initially, there is a reduction in the intracellular levels of adenosine triphosphate and a decrease in the respiratory rate of mycelia. In the second stage, the cells enter a dormant phase for 4–6 days. Subsequently, the rate of respiration returns to normal, and there is an induction of a yeast phase-specific cysteine oxidase. At this time, the morphological switch to yeasts is completed.

Phase transition is also associated with several alterations in gene expression. One to three hours after a temperature shift from 25 to 37°C, there is a marked increase in the expression of the genes encoding heat shock protein 70 and *cdc2*, which is a regulator of cell division. On the other hand, conversion of mycelia to yeasts produces a 2–3-fold reduction in expression of α -tubulin and β -tubulin mRNAs. A yeast phase-specific gene, *yps3*, is expressed only by strains that are thermotolerant and virulent. The avirulent Downs strain contains a 287 bp insertion that

disrupts the open reading frame of *yps3*. A precise function for this gene and its protein product has not been defined. It is likely that it regulates, at least partially, the morphological conversion of *H. capsulatum*.

Infection of humans with *H. capsulatum* is usually asymptomatic or it produces an influenza-like illness. A small percentage of individuals will develop a cavitary pulmonary disease or a disseminated infection that extensively involves the reticuloendothelial system. Although cases of histoplasmosis have been reported worldwide, the endemic areas are the Ohio and Mississippi river valleys in North America, and Central and South America. It is estimated that 200 000–500 000 new infections occur annually. Disseminated histoplasmosis has become a prominent cause of morbidity and mortality in patients with acquired immune deficiency syndrome or in those receiving potent immunosuppressive agents.

The originally described *H. capsulatum* antigens consisted of the culture filtrate of aged mycelia, otherwise known as histoplasmin, and yeasts treated with formalin. Histoplasmin is currently the principal antigenic mixture that is employed for analysis of both humoral and cell-mediated immune responses in humans and experimental animals infected with *H. capsulatum*. This preparation contains two glycoprotein antigens, M and H, which elicit both cell-mediated and antibody responses and a carbohydrate moiety, termed C antigen, which is recognized by circulating antibody from infected humans. The gene encoding H antigen has been cloned, and the deduced amino acid sequence indicates that the protein is homologous to extracellular β -glucosidase. M antigen appears to be a member of the catalase family. The carbohydrate portions of M and H antigens and the C antigen are rich in mannose and galactose. *In vitro* studies have shown that deglycosylation of M and H sharply reduces the capacity of these antigens to stimulate *Histoplasma*-reactive T cell clones. Antibody to C antigen can cross-react with antigens from heterologous fungi including *Blastomyces dermatitidis* and *Coccidioides immitis*.

Numerous other preparations of antigens have been isolated from disrupted mycelia and yeasts or from supernatants of actively growing organisms. Because much of this effort has been applied to improving serologic diagnosis of histoplasmosis, biochemical characterization of antigens has centered on identifying determinants that are recognized by circulating antibody. Polysaccharide determinants that contain galactomannan or glucomannan often react with sera from patients infected with *H. capsulatum*. More recently, several antigens that stimulate a cell-

mediated immune response have been identified. Heat shock protein 60, an 80 kDa protein with homology to heat shock protein 70, and *yps3* stimulate proliferation of lymphocytes by *H. capsulatum*-exposed mice or humans.

The organism does not secrete any known virulence factors. However, the α -(1,3)-glucan content in the cell wall of yeasts appears to correlate directly with virulence. Thus, avirulent variants contain up to 1000-fold less α -glucan than virulent parental strains.

Immune responses of the host

It is generally acknowledged that cell-mediated immunity is the critical host defense mechanism triggered in response to infection with this fungus. In humans, the specific cellular immune responses are believed to be activated in lymphoid organs and other tissues 7–18 days after exposure to conidia. Concomitant with stimulation of cellular immunity, the lesions in organs begin to heal. Granulomas are formed, with necrosis developing in the central portion. Lymph nodes draining the sites of infection enlarge, resolve by encapsulation, and then may calcify. *In vivo* or *in vitro* delayed-type hypersensitivity responses to histoplasmin can be detected approximately 4 weeks after infection.

H. capsulatum is a facultative intracellular pathogen that parasitizes macrophages and neutrophils. Resting mononuclear phagocytes provide an intracellular milieu that allows the yeast form not only to survive but also to replicate. Unopsonized yeasts and conidia bind to epitopes of the lymphocyte function-associated antigen 1 (CD11a/CD18), complement receptor type 3 (CD11b/CD18), and the p150–95 complex (CD11c/CD18) of adhesion-

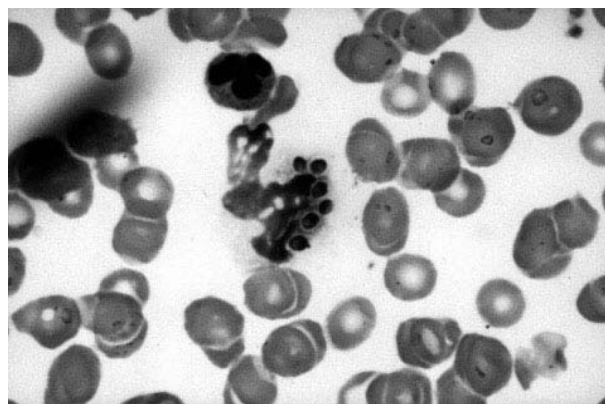


Figure 1 Circulating polymorphonuclear leukocyte infected with *Histoplasma capsulatum* yeast cells from a patient with human immunodeficiency virus infection. (With permission from Judith Rhodes.)

promoting receptors on human monocyte-derived macrophages and alveolar macrophages. Binding of yeasts to phagocytes is rapid and requires divalent cations. Once ingested, unopsonized yeasts trigger production of hydrogen peroxide by human macrophages. By contrast, *Histoplasma* yeasts fail to stimulate a respiratory burst as measured by ferricytochrome *c* reduction when phagocytosed by murine peritoneal macrophages. Ingestion of yeasts prompts phagosome-lysosome fusion. Within the phagolysosome, the yeast raises the intracellular pH to approximately 6.0–6.5. This elevation presumably enhances survival of the fungus intracellularly because it blunts the activity of acid proteases. The pH must be tightly regulated because the organism must scavenge iron from the intracellular contents of macrophages.

Both protective immunity and delayed-type hypersensitivity are transferred by antigen-specific CD4⁺ T cells. *In vitro*, antigen-reactive murine T cells undergo blastogenesis in response to *Histoplasma* antigens and secrete several lymphokines including interferon γ (IFN γ). CD8⁺ T cells also contribute to protective immunity. Depletion of these cells by monoclonal antibody to CD8 from mice impairs clearance of the fungus. However, the mice recover. In contrast, mice whose CD4⁺ T cells are eliminated by administration of monoclonal antibody to CD4 die after challenge with a sublethal inoculum. Thus, both T cell subpopulations participate in host defence, but CD4⁺ T cells appear to play a more pivotal role.

In mice, several cytokines appear to influence the outcome of infection in naive animals. The monokines, interleukin 12 (IL-12) and tumor necrosis factor α , are crucial determinants of survival of infected mice. IL-12 appears to act through induction of IFN γ . Depletion of IFN γ renders mice incapable of controlling infection. In contrast, anti-IL-4 treatment of mice inoculated with a lethal challenge of yeasts improves survival. The action of IFN γ is thought to be key in stimulating the anti-*Histoplasma* activity of macrophages. In this regard, recombinant murine IFN γ activates peritoneal macrophages from mice to inhibit but not kill intracellular yeasts. The finding that macrophages exert fungistasis rather than a fungicidal effect is of major clinical significance as yeasts are believed to remain viable, albeit in a dormant state, in tissues for many years.

However, human IFN γ does not arm human macrophages to express an anti-*Histoplasma* effect. The cytokines IL-3, granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor activate human macrophages to inhibit the

growth of yeast cells. Thus, there is an apparent disparity between human and murine macrophages.

Evasive strategies by the organism

Transformation of conidia to yeasts is one mechanism by which the fungus adapts for survival. Once inside mononuclear phagocytes, yeasts may decrease the release of toxic oxygen products. Additionally, yeasts are markedly more resistant to the microbicidal effect of hydrogen peroxide than are conidia. How the yeast escapes killing is not known.

Infection with this fungus can perturb cellular immune responses. In experimental animals or humans with disseminated histoplasmosis there is a considerable impairment of delayed-type hypersensitivity responses and *in vitro* proliferative responses to mitogens and antigens. In mice with disseminated disease, diminished reactivity is associated with the presence of CD8⁺ suppressor cells and a suppressor macrophage-like cell in spleens and lymph nodes of infected animals. These CD8⁺ T cells release a suppressor factor that inhibits cellular immune responses *in vitro*. Poor responses also may be secondary to defective production of IL-1 and IL-2, two cytokines that are critical in the amplification of T cell-mediated immunity.

Vaccines

No licensed vaccine exists yet. However, recent work with *H. capsulatum* heat shock protein 60 indicates that this molecule can function as a possible vaccine candidate. The native as well as the recombinant antigen can confer protection to mice with pulmonary histoplasmosis. There is evidence an oligopeptide from this protein confers protection when used as a vaccine. Additional studies are seeking to determine if a peptide from heat shock protein 60 can mediate protection.

See also: Cell-mediated immunity; Delayed-type hypersensitivity; Fungi, immunity to; Granuloma; Opportunistic infections; Respiratory and cardiac infections; Suppressor T lymphocytes.

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HLA CLASS I

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A multigene family

The human major histocompatibility complex (MHC) is designated the human leukocyte antigen (HLA). This genetic region is located on the short arm of chromosome 6, and consists of genes that encode three classes of molecules, HLA class I, class II and class III.

To date, the most extensively characterized members of the HLA class I gene family are the genes encoding the major transplantation antigens, HLA-A, -B and -C, also termed the classical class I genes. A genetic map of the short arm of chromosome 6 based on recombination fractions places HLA-B 0.2 cM (centiMorgan) proximal of HLA-C, and HLA-A 0.8 cM distal to HLA-C. These genes encode highly polymorphic 44 kDa antigens that are non-covalently associated on the cell surface with an invariant 12 kDa subunit, β_2 -microglobulin (β_2 m). The gene encoding β_2 m is located on chromosome 15. Class I heavy chain genes consist of eight exons. Exon 1 encodes the hydrophobic leader peptide. The second, third, and fourth exons encode the extracellular $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains respectively. The transmembrane domain and the first five amino acids of the cytoplasmic region are encoded by exon 5. The remainder of the cytoplasmic segment is encoded by exons 6–8.

Molecular genetic studies indicate that the HLA-A, -B and -C genes represent only a small subset of an extensive gene family. There are approximately 17 HLA class I genes per haploid genome. Most of these are HLA class I pseudogenes that do not seem to be capable of encoding an expressed HLA molecule. Three non-HLA-A, -B -C class I genes which are expressed have been isolated. These are designated HLA-E, -F and -G. Sequence analysis of these three class I genes shows that each is intact and encodes a class I heavy chain that can associate with β_2 m.

Each gene has intact exons 1–5, i.e. the leader peptide through the transmembrane domain.

The HLA-E gene is located between the HLA-A and HLA-C genes. The HLA-F and HLA-G genes are located distal to HLA-A. In the HLA-E gene, there is a deletion of five nucleotides from exon 7, resulting in an in-frame termination codon, and a class I protein one amino acid shorter than most HLA-B molecules and four amino acids shorter than most HLA-A chains. The HLA-F gene differs from other class I genes in that the 3' splice recognition site of intron 6 has a variant dinucleotide sequence: AA instead of the conserved AG sequence. This alteration in the 3' splice site of intron 6 in HLA-F results in the removal of nucleotides within exon 7 of HLA-F during mRNA processing. Thus, the HLA-F protein has a cytoplasmic segment shorter than that of the HLA-A, -B and -C class I genes. The HLA-G gene also differs from other class I genes in that an in-frame termination codon is located at the second codon of exon 6. Thus, most of exon 6 and all of exons 7 and 8 are not translated into the HLA-G protein.

Patterns of HLA class I gene expression

HLA-A, -B and -C genes are expressed on virtually all nucleated cells. The amount of expressed antigen varies considerably, depending on the cell type and the HLA class I locus, with lymphocytes expressing the highest levels of HLA-A, -B and -C antigens. Among lymphocyte subsets, B lymphocytes express HLA-A, -B and -C antigens at a higher level than T lymphocytes, and immature T lymphocytes express a relatively lower level of HLA-A, -B and -C antigens than mature T lymphocytes. Northern blot analysis indicates that the difference in HLA-A, -B and -C gene expression of B and T lymphocytes is reflected by the steady-state level of HLA class I mRNA. There is a considerable variation in the level of

expression between different class I loci within the same cell type. HLA-C products are found at the cell surface at about one-tenth the amount of HLA-A and HLA-B molecules.

Some factors that modulate the immune response have been shown to affect the expression of the HLA class I antigens in various cell types. Interferons α , β and γ increase the expression of HLA-A, -B and -C antigens and β_2m in fibroblasts and lymphoblastoid cells. This increase in antigen expression is preceded by an increase in class I and β_2m mRNA. Tumor necrosis factor (TNF) is another immune modulator that enhances HLA class I expression. *In vitro*, human TNF has been shown to increase the mRNA level and cell surface expression of HLA-A, -B and -C antigens in normal human vascular endothelial cells and dermal fibroblasts. Both interferon and TNF have a role in the enhancement of cytotoxic T lymphocyte lysis of virus-infected cells by their ability to increase the transcription and the antigen expression of HLA class I genes.

Each of the HLA-E, -F and -G genes have been shown to encode an HLA class I heavy chain after DNA-mediated gene transfer studies. HLA-E, -F and -G genes have been studied following their transfer into a HLA-A, -B, -C-null B lymphoblastoid cell line (B-LCL 721,221). The B-LCL transfected with cloned HLA-G DNA became positive at the cell surface for binding of a monoclonal antibody (mAb) to monomorphic determinants of class I antigen, w6/32, and binding of an anti- β_2m mAb, BBm.1. Binding of these mAbs was not detected on the B-LCL after transfection with either the HLA-E or the HLA-F DNA. However, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of immunoprecipitates with w6/32 and BBm.1 showed that lysates from the B-LCL cell line transfected with HLA-E and HLA-F DNA contained a class I heavy chain that coprecipitated with β_2m . Thus, each of the HLA-E, -F and -G genes encodes an HLA class I heavy chain that associates with β_2m ; however, only the HLA-G gene encodes a class I protein that gets to the cell surface of the B-LCL.

RNAase protection has been used to examine the expression of HLA-E, -F and -G transcripts of different human cell lines and tissues. The pattern of expression of HLA-E and HLA-F transcripts is, for the most part, very similar to that of HLA-A, -B and -C genes. HLA-E and HLA-F transcripts were detectable in RNA from almost all cell lines and tissues examined. The amount of HLA-E and HLA-F transcripts detected varied between the different cell lines and tissue types. Higher levels of HLA-E and HLA-F transcripts were found in lymphoid cells as compared to nonlymphoid cells. Moreover, within

lymphoid tissues, mature T cell lines expressed higher levels than immature T cell lines. The highest level of HLA-E transcript was detected in resting T lymphocytes, but this was decreased dramatically upon activation by PHA. HLA-E and HLA-F transcripts were also detectable in RNA from nonlymphoid tissues, and liver had a very low amount of these mRNAs. There are two HLA-E transcripts, 1.95 kb and 3.3 kb, differing only in the extent of the 3' untranslated region. Whereas the 1.95 kb transcript uses one of the first two polyadenylation signals, the 3.3 kb mRNA terminates after a distant polyadenylation signal. The ratio of the two mRNA species is dependent on cell type and the differentially regulated two HLA-E mRNA species might be important in the control of its function.

The HLA-G gene is unique among HLA class I genes identified so far in that its expression is highly restricted. The HLA-G transcript has been found only in extraembryonic tissues during gestation, eye tissue and at a very low level in fetal thymus. Furthermore, the level of HLA-G transcript in placenta and extravillous membrane changes with time of gestation. At the 10th week, HLA-G transcript was found in RNA from placenta and not in RNA from extravillous membrane. At the 21st week of gestation, both placental and extravillous membrane RNA contained HLA-G transcript. At term, the HLA-G transcript was detectable only in RNA from extravillous membrane.

An interesting point concerns the cellular sites of HLA-G expression with the placenta and extravillous membrane. HLA-G transcript has been detected in chorionic cytotrophoblasts at term. Both HLA-G transcript and protein were detected in the first trimester villous cytotrophoblasts, and were greatly reduced in the third trimester villous cytotrophoblasts. These data suggest that the fetal cytotrophoblasts are the cellular sites of HLA-G expression.

HLA class I gene polymorphism and functions

An important aspect of the HLA-A -B, and -C genes is their high level of polymorphism. Currently, over 80 alleles at the HLA-A locus, 185 at the HLA-B locus, and 42 at the HLA-C locus have been defined. Comparison of sequences from a single locus shows the class I heavy chains are divided into a variable part comprising the $\alpha 1$ and $\alpha 2$ domains and a relatively conserved part consisting of $\alpha 3$, the transmembrane, and the cytoplasmic domains. The $\alpha 1$ and $\alpha 2$ domains are known to bind diverse antigen peptides and form the site of interaction with variable T cell receptors. There are 20 amino acid positions with

high variability, all in the $\alpha 1$ and $\alpha 2$ domains, which have been defined from a comparison of 39 HLA-A, -B and -C sequences. From the HLA-A2 structure, 47 residues were hypothesized to make contact with bound peptide, or to interact with the T cell receptor. Of the 20 residues of high variability, 19 residues are included in this group. In contrast, HLA-E, -F and -G genes show relatively little polymorphism.

The HLA-A, -B and -C antigens are the major antigens recognized by the host during tissue graft rejection. Cytotoxic T lymphocytes recognize viral antigens only in the context of a class I antigen. T lymphocytes elicited by such an exposure are restricted in their killing to the cells which bear the same class I antigens as were present on the sensitizing cells.

It has been postulated that HLA-C is a declining class I gene, since HLA-C has fewer alleles than HLA-A and HLA-B, differing by smaller numbers of substitutions which are frequently at positions not directly involved in contacting the bound peptide and the T cell receptor. In addition, there is much less HLA-C antigen than HLA-A and HLA-B antigen on the cell surface. However, HLA-C molecules are capable of antigen presentation, as demonstrated by experiments with transgenic mice. Whether there are sufficient HLA-C restricted T cells for this to happen with any frequency in the human immune response is a matter for question. All this evidence supports the idea that HLA-C contributes little to human immunity.

So far, no function has been assigned to HLA-E, -F and -G class I products, although it has been demonstrated that HLA-G is able to present peptides in a similar fashion to the classical class I molecules. Restriction of HLA-G expression to fetal extraembryonic tissue implies that HLA-G subserves a function critical to this tissue. Within the extraembryonic tissues, fetal cytotrophoblasts are exposed to the maternal decidua. It has been suggested that fetal trophoblasts function to shield the fetus from recognition by the maternal immune system. Clearly, the lack of HLA-A, -B and -C expression by these cells is consistent with such a function. Expression of the nonpolymorphic class I gene HLA-G should not result in fetal rejection by the maternal immune system, assuming the maternal immune system does not recognize HLA-G as foreign. It has been proposed that HLA-G may function to directly protect fetal cytotrophoblasts by activation of maternal suppressor cells or blockage of maternal cytotoxic cells.

HLA-G expression by fetal cytotrophoblasts may also function to enable the maternal immune system to survey these fetal cells, much as it does other maternal somatic cells. An immune surveillance

capability seems warranted since fetal trophoblast cells play a crucial role in the development of the embryo. HLA-G on the surface of fetal cytotrophoblasts could afford a mechanism by which an antigen is presented to the maternal immune system, for example, in case of a viral infection, while keeping the fetal cells from being seen as foreign by the maternal immune system. HLA-G may have non-immunologic roles in modulating receptivity to soluble growth and differentiation molecules or it may be required for interplay with decidual cells.

Conclusions

Human histocompatibility class I molecules consist of a MHC-encoded heavy chain and an invariant β_2m . In the HLA gene complex, there are 17–20 class I heavy chain genes including various pseudogenes and gene fragments. Only six genes (HLA-A, -B, -C, -E, -F and -G) are known to encode β_2m -associated molecules. HLA-A, -B and -C molecules are present on virtually all nucleated cells. HLA-E and -F transcripts have been detected on almost all cell lines and tissues examined. In contrast, HLA-G expression is restricted to extraembryonic tissues during gestation, eye tissue and at a very low level in fetal thymus. HLA-A, -B and -C genes are highly polymorphic and encode antigen-presenting molecules. On the other hand, HLA-E, -F and -G genes show very little polymorphism and their functions are unknown.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; β_2 -microglobulin; Cytotoxic T lymphocytes; H2 class I; Linkage disequilibrium; MHC, evolution of; MHC, functions of; MHC restriction; Minor transplantation (histocompatibility) antigens; Tissue typing.

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HLA CLASS II

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HLA class II molecules are absolutely essential in the generation of a proper immune response. They function to display peptides at the surface of antigen-presenting cells for recognition by CD4⁺ T cells. HLA class II molecules consist of polymorphic α/β chain heterodimers encoded in genes of the human major histocompatibility complex (MHC). The first indication for the existence of a genetic region, distinct from HLA class I, that was important for the immune response came in 1967 when Bach and Amos observed the proliferation of mixed lymphocytes derived from HLA class I-matched donors. Genetic studies, reported in 1971 by Yunis and Amos, established what is now called HLA-D as the region controlling mixed lymphocyte responses. Thus, it is because of the high degree of allelic polymorphism in HLA class II genes that they were discovered, long before their structure or function were known. This polymorphism also led to a serological characterization of these molecules with alloantisera, first for HLA-DR, and later for HLA-DQ. The class II allelic specificities, defined either by cellular typing (Dw specificities) or serologically (DR and DQ specificities), correlated well with graft rejection between unmatched individuals. A new allelic series, designated HLA-DP, was defined by primed lymphocyte typing. Biochemical studies demonstrated that DR, DQ, and DP class II molecules consisted of distinct α/β heterodimers. The molecular cloning of class II genes in 1982 opened a new era. Recombinant DNA technology has made it possible to determine the sequence of most class II alleles. Molecular typing procedures are now in use to define allelic specificities with greater precision than cellular or serological techniques. Furthermore, expression of class II molecules after DNA-mediated gene transfer into cell lines or mice has become a powerful tool to study their function.

Structure and diversity

HLA class II genes map to position 6p21 on the short arm of chromosome 6, in the D region of the human MHC. The molecular description of the D region was derived from a combination of genetic studies, of *in vitro*-derived deletion mutants, and of mapping with cosmid DNA clones and pulsed-field gel electrophoresis of large DNA fragments (Figure 1). The D region is divided in three major functional subregions encoding the DR, DQ and DP molecules. A functional pair of α and β genes is present in each subregion.

In addition, the DP and DQ subregions contain a pair of α and β nonfunctional genes. The DR subregion is more complex. It contains a single α gene and several β genes, the number of which varies among different HLA haplotypes. DR1 and DR8 haplotypes express a single functional β gene, whereas most

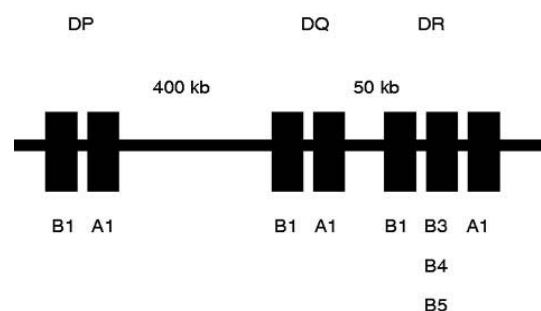


Figure 1 Molecular map and gene products of the HLA-D region. The orientation is: centromere to the left, class I genes and telomere to the right. The genes, grouped within subregions DP, DQ and DR, are represented as boxes and are not drawn to scale. Genes encoding α chains and β chains are designated A and B respectively. The DR subregion usually only contains two expressed B genes: B1 and one of either B3, B4 or B5. The nucleotide sequences of A and B genes within a subregion are more related than they are to those of genes in other subregions.

other DR haplotypes express two isotypic β genes, the product of which pairs with the same α chain. One DR molecule shows a higher degree of allelic polymorphism, and is encoded by the B1 gene (or B5 for the DR2 haplotype). A second DR molecule, with less allelic polymorphism and strong linkage disequilibrium with the first, is encoded by gene B3 or B4 (or B1 for DR2 haplotype). Gene B2 is nonfunctional.

Two additional expressed genes for which no product or function has been assigned yet are DNA and DOB. They are located between the DP and DQ subregions. The fact that they encode a translatable class II α or β chain, respectively, and their evolutionary conservation suggest that they serve some function.

The α and β chains are transmembrane glycoproteins with relative molecular masses of 35 000 and 29 000 respectively. After their cotranslational translocation into the endoplasmic reticulum, they assemble into a very stable noncovalent trimer with the invariant chain, a nonpolymorphic protein with a relative mass of 33 000. Prior to surface expression of class II molecules, the invariant chain is removed by proteolytic cleavages in an acidic intracellular compartment.

The allelic diversity of class II molecules is much greater than their isotypic diversity. Nucleotide sequencing of class II genes from different individuals revealed a greater heterogeneity than had been detected by serological or cellular typing. According to the 1996 WHO Nomenclature Committee for Factors of the HLA System, there are 215 recognized sequences for the DRB genes, 18 for DQA, 31 for DQB, 10 for DPA and 77 for DPB genes. The coding region for the extracellular portion of the DR α chain is nonpolymorphic.

The DR specificities defined by serology describe an allelic series that has been rather stable through evolution. Certain existing alleles have clearly preceded speciation, and closely related sequences can be found in nonhuman primates. A practical result of this genetic stability in DR alleles is that most DR specificities can be determined based on restriction fragment length polymorphism (RFLP) of DNA. However, many DR specificities can be subdivided by cellular typing (the Dw series). These 'splits' of DR specificities are due to small sequence alterations, affecting mostly the third variable region of the β chain, which is critical in specific T cell contacts. This variability must have evolved more recently than the DR allelic series; it has not been detected by RFLP.

A large number of diseases with an autoimmune component occur at elevated frequencies in individuals with certain class II specificities. The precise definition of class II specificities and of their underlying amino acid sequences has led to improved disease

associations. An important goal in understanding the basis for autoimmune disease is to identify the particular class II molecule, and the particular self peptide, that may be involved in triggering autoimmune T cells.

Function

The main function of HLA class II molecules is to present peptides to CD4⁺ T lymphocytes. By analogy with the mouse immune system, it is likely that this presentation occurs already in the thymus, where maturing T cells undergo both negative and positive selection. Mature peripheral CD4⁺ T cells are stimulated by antigen-presenting cells (APCs) when the proper combination of antigenic peptide and class II molecule is displayed at the surface of the APC. Several types of cells express class II molecules and can serve as APCs: dendritic cells, monocytes, macrophages and B cells. Class II expression on APCs is regulated by cytokines; it is enhanced in monocytes by interferon γ (IFN γ) and in B cells by interleukin 4 (IL-4). Cytokines may also induce class II expression in a variety of cells that are otherwise class II negative, such as fibroblasts. These cells can then acquire the ability to present antigen to CD4⁺ T cells.

Antigenic proteins are generally not recognized as such by T cells, but have to be processed into a form that can bind to MHC molecules. In general, antigen processing for presentation by class II molecules involves antigen uptake into an endocytic compartment where processed peptides encounter either newly synthesized class II molecules on their way to the cell surface, or surface class II molecules that are internalized and recycled. The biosynthetic route of class II molecules is distinct from that of class I molecules, in that class II molecules are targeted to a post-Golgi compartment, accessible to endocytosed material, where they are retained for a few hours. This specialized transport to the cell surface is presumably what makes class II molecules so efficient in the presentation of exogenous antigen. However, class II molecules are not limited to the presentation of exogenous antigens, because, at least in some cases, class II molecules are able to present endogenously processed antigen.

A failure to mount an immune response to a particular antigen can be caused by 1) an inability of the host's APCs to process the antigen, 2) the lack of a class II molecule to which the foreign peptides can bind, or 3) the absence in the T cell repertoire of a T cell receptor (TCR) with the proper specificity. To avoid the second possibility, and because the isotypic diversity in class II molecules is small, each class II

molecule must be able to bind many different peptide sequences. Although this is the case, some specificity in peptide binding can be defined. Only one or two crucial amino acids within a peptide account for the binding specificity. This explains why many different peptides, apparently unrelated in sequence, bind to the same class II molecule.

The structure of the peptide-binding site on class II molecules consists of a binding groove or cleft which, unlike the class I binding groove, is open at both ends, permitting peptides of 12–24 residues to be bound. Polymorphic amino acid residues that define the specificity in peptide binding lie on the floor of the binding groove, formed by the parallel β strands at the amino-terminal ends of the $\alpha 1$ and the $\beta 1$ domains, and on the inner face of the α helices that span the top of class II molecules. Residues on the α helices facing up have been identified that are critical for T cell recognition, in particular in the third variable region of the DR $\beta 1$ domain.

A particular class of antigens, termed 'superantigens', bind to class II molecules and elicit the stimulation of a large proportion of T cells, according to the V β chain of their TCR. Superantigens include a number of bacterial toxins produced by staphylococci, streptococci and mycoplasma. They do not require processing for T cell stimulation and display a hierarchy of affinities for different class II molecules. They apparently make external contacts with class II molecules and with the V β chain of TCRs, away from the sites involved in peptide binding and recognition.

Class II molecules may well carry out other functions besides presentation of antigens to T cells. Signaling via class II molecules occurs and could play a role in the control of cell differentiation during hematopoiesis. Surface expression of class II molecules is induced on activated human T cells; no role has been attributed yet to this induction. The expression of DR, DQ and DP molecules is not always coordinated in hematopoietic cells. The possibility that the multiplicity in class II isotypes serves specific functions, other than the expansion of potential immune responses to diverse antigens, must be considered.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Graft rejection; H2 class II; Immune response (I r) genes; MHC disease associations; MHC, func-

tions of; MHC restriction; Mixed lymphocyte reaction (MLR); Polymerase chain reaction (PCR) amplification; Superantigens; Tissue typing.

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HLA CLASS III REGION

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The human major histocompatibility complex (MHC) spans approximately 4 Mbp on the chromosome band 6p21.3 and is divided into three regions. The class I and II regions contain the classical MHC genes, which encode cell surface glycoproteins involved in the presentation of antigenic peptides to T cells. These are interspersed with a large number of other genes, some of which encode proteins involved in antigen processing. The 1100 kb segment of DNA that separates the class I and II regions is generally termed the class III region and contains at least 63 genes which encode proteins of diverse function (Figure 1).

The class I and II regions of the MHC are typified by a high level of gene duplication and extensive polymorphism. These features are less apparent in the class III region, but this does contain several clusters of related genes, for example, those encoding:

four components of complement; three members of the 70 kDa heat shock protein (hsp 70) family; and the cytokines tumor necrosis factor (TNF), lymphotoxin α (LT α) and LT β . The MHC class I chain-related (MIC) gene family has two members (MICA and MICB) between HLA-B and BAT1 and a further three related gene fragments within the class I region. The PERB (Perth MHC β -block) sequences also include class I-like pseudogenes and gene fragments. Most of the other genes in the class III region are single copy, but some have related genes elsewhere in the genome.

Encoded proteins

Genomic or cDNA fragments corresponding to most of the class III region genes have been cloned and

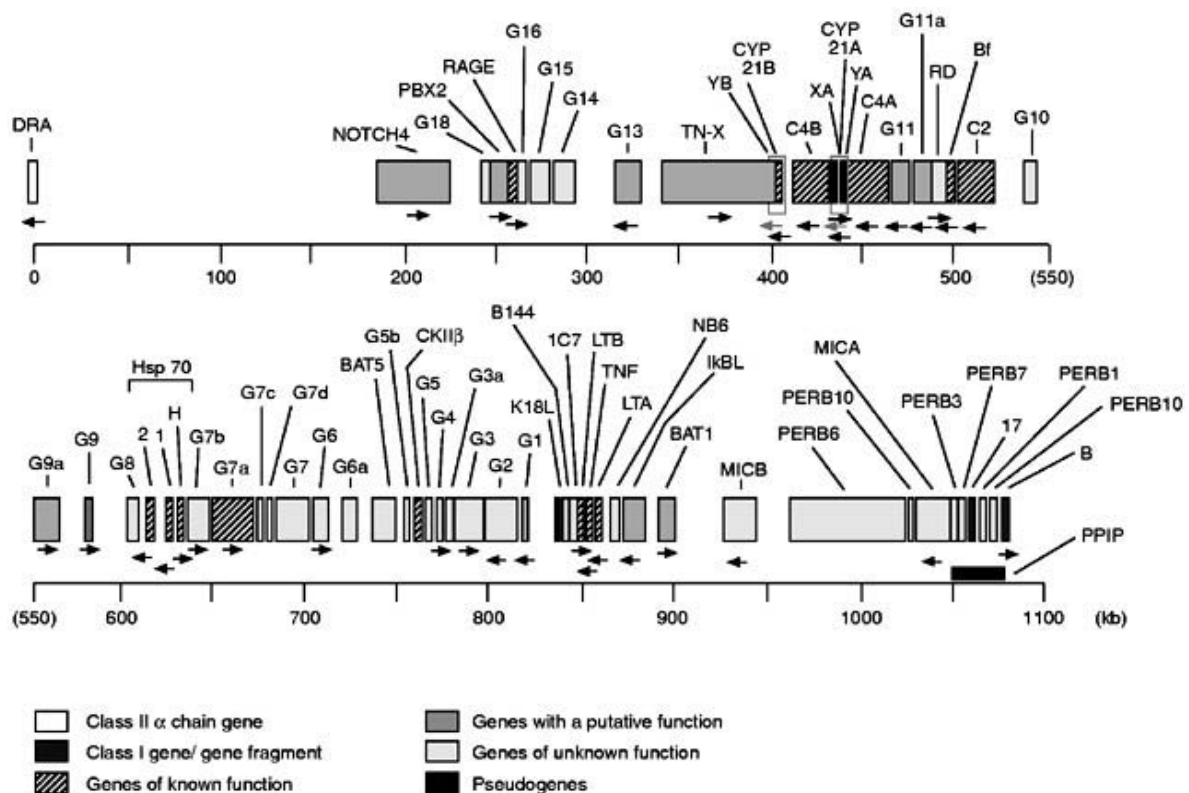


Figure 1 Molecular map of the human MHC class III region. The positions of genes, pseudogenes and gene fragments are indicated by filled boxes, as indicated in the key, except for the YA and YB genes, which are indicated by open boxes. The approximate region within which the cyclophilin pseudogene (PIIP) is known to lie is marked by a horizontal black box. The directions of transcription of genes, where known, are shown by horizontal arrows.

sequenced. Those that encode proteins with known or putative functions are discussed here and, together with the others, are listed in Table 1.

NOTCH4, the human counterpart of mouse mammary tumor gene, *int-3*, encodes a member of the Notch family of transmembrane proteins. Notch was first identified as a *Drosophila* neurogenic locus required for cell lineage determination in embryogenesis. However, Notch and its homologs are widely expressed during embryonic and adult development and participate in cell-cell interactions, mediating a variety of regulatory events.

The G17 gene encodes PBX2, a homeodomain-containing protein related to PBX1. The latter is involved in acute pre-B cell leukemias. While most of the *Drosophila* homeodomain-containing proteins have been implicated in developmental processes, the functions of the majority of the non-*Drosophila* proteins remain unknown. However, it has been suggested that these proteins function as sequence-specific DNA-binding proteins that play an important role in transcriptional regulation.

RAGE (a receptor for advanced glycosylation end-products of proteins) is a member of the immunoglobulin superfamily of cell surface molecules. AGEs are nonenzymatically glycosylated proteins that accumulate in vascular tissue as a result of aging, and at an accelerated rate in diabetes, due to the prolonged exposure of proteins to aldoses such as glucose and ribose. RAGE mediates monocyte migration and activation in response to AGEs, but other binding sites may also contribute to this. Binding of AGE to endothelial RAGE induces cellular oxidative stress, resulting in increased vascular permeability and activation of NF- κ B. RAGE may have other functions in addition to the binding and subsequent uptake of AGEs.

The G13 protein contains a leucine zipper consensus sequence preceded by two basic clusters, separated by an alanine spacer (bZip domain). This domain is conserved in many transcription factors including the cyclic AMP response element-binding protein (CREB) family, which comprises proteins that show sequence-specific binding to DNA (via a region rich in basic amino acids) and use dimerization to control function (via a leucine zipper domain).

The XB (TN-X) and XA genes overlap the 3' ends of the P450c21B/A genes by 481 bp, respectively, and are transcribed in the opposite orientation to the C4, P450c21 and Y genes. The protein encoded by XB has been termed tenascin-X (TN-X), due to its structural similarity to tenascin (TN-C). The tenascins are a family of extracellular matrix proteins, the functions of which are unclear, but TN-C can facilitate cell movement by counteracting the cell adhesion

and spreading activity of fibronectin *in vitro*, and may also be important in embryogenesis. A short form of XB (XB-S) is transcribed from a promoter within intron 26 of TN-X. The nucleotide sequence of the XA gene is more than 99% identical to XB-S, but XA contains premature stop codons, so it is unlikely to encode a protein.

The P450c21B and P450c21A genes share 97% overall nucleotide sequence identity. P450c21B encodes the adrenal enzyme steroid 21-hydroxylase, which is involved in the conversion of progesterone and 17-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, the intermediate steps in mineralocorticoid and glucocorticoid biosynthesis, respectively. P450c21A contains deleterious mutations and so cannot encode a functional protein.

The YB and YA genes utilize the p450c21B/A gene promoters, and are transcribed in the same orientation as the latter, but use a different array of exons and introns. It has not been established whether the YA/YB transcripts are translated into protein.

The complement system is the principal effector mechanism of humoral immunity and consists of at least 24 serum and 11 membrane-bound proteins. Its activation via the classical or alternative pathway leads to cell lysis, opsonization, clearance of immune complexes, regulation of B cell responses, and the generation of peptides that have potent anaphylactic and chemotactic activities. C2 and C4 are major effector proteins of the classical pathway, which is activated by the binding of C1 to immunoglobulin G (IgG) or IgM, complexed with antigen, or following the interaction of mannan-binding protein with carbohydrate ligands on the surfaces of bacteria or viruses. Factor B (Bf) is a component of the alternative pathway, which is activated by a diverse set of substances including components of yeast and bacterial cell walls and is usually antibody-independent. C4A and C4B differ by only four amino acids, but this results in a profound difference in their covalent binding activities. Both isotypes of C4 are highly polymorphic, whilst C2 and Factor B show more limited polymorphism.

Genomic sequence analysis of G11 has shown that its 3' end was involved in the duplication event giving rise to the two C4/P450c21/X/Y gene clusters. The predicted protein products of G11 contain bipartite nuclear localization signals and partial sequence similarity with the tyrosine kinase-transforming protein from the fujinami sarcoma virus. This, and the presence of potential sites for phosphorylation at Ser/Thr and Tyr residues, may suggest that G11 has a novel kinase-like activity.

The G11a (SK12W) protein contains an RNA helicase domain and a leucine zipper motif, and shows

Table 1 MHC class III region genes and their protein products

<i>Gene</i>	<i>Approx. Gene size (kb)</i>	<i>No. Exons</i>	<i>mRNA size (kb)/tissue distribution^a</i>	<i>Protein</i>	<i>Protein size (aa)</i>	<i>Function</i>
NOTCH4	30	—	— —	NOTCH4	—	Cellular interactions?
G18	4.5	—	1.5/1.4 L, M	—	—	—
G17	6	9	3.2/2.4 L, M	PBX2	430	Transcription factor?
RAGE	3	11	1.5 Endothelium, lung	RAGE	404	Receptor for advanced glycosylation end-products of proteins (AGEs)
G16	4	—	1.2 L, M	—	—	—
G15	7.5	—	2.1 L, M	—	—	—
G14	11	—	2 L, M	—	—	—
G13	17	—	2.8 E, H, L, M	G13	703	Transcription factor of CREB family
XB (TN-X)	65	39	12 Fetal tissue	TN-X	3626	Extracellular matrix protein. Member of tenascin family
XB-S	4.5	13	2.6 Adrenal gland*	XB-S	673	Truncated TN-X (C-terminal 673 amino acids)
YB	13	11	7.5/3 Adrenal gland*	—	—	—
P450c21B	3.3	10	2.4 Adrenal cortex*	21-OH	495	Mineralocorticoid and glucocorticoid biosynthesis
C4B	22/16	41	5.5 Liver*	C4	1725	Complement classical pathway thioester-containing protein
XA	4.5	13	2.6 Adrenal gland*	TN-X (Ps)	xx	Pseudogene
P450c21A	3.3	10	— —	21-OH (Ps)	xx	Pseudogene
YA	13	11	7.5/3 Adrenal gland*	—	—	—
C4A	22	41	5.5 Liver*	C4	1725	Complement classical pathway thioester-containing protein
G11	9.1	7	1.4–1.6 E, H, L, M	G11	368/258	Protein kinase?
G11a	9	—	3.9 E, L, M	SK12W	1241	Helicase?
RD	6	10	1.6 H, L, M	RD	371	—
Factor B	6	18	2.6 Liver, Mφ*	Factor B	739	Complement alternative pathway serine protease
C2	18	18	2.9 Liver, Mφ*	C2	732	Complement classical pathway serine protease
G10	4	—	2.6 E, H, L, M	—	—	—
G9a	17	—	3.3 E, H, L, M	G9a	1001	Regulation of transcription?
G9	3.5	6	1.9 E, H, L, M	G9	415	Sialidase enzyme, acidic pH optimum
G8	6	3	1 H, L, M	G8	126	—

Table 1 Continued

<i>Gene</i>	<i>Approx. Gene size (kb)</i>	<i>No. Exons</i>	<i>mRNA size (kb)/tissue distribution^a</i>	<i>Protein</i>	<i>Protein size (aa)</i>	<i>Function</i>
HSP70-1	2.5	1	2.4 Ubiquitous after heat shock	HSP70 (HSP72)	641	Heat-inducible hsp70. Chaperone in recovery of cells from stress
HSP70-2	2.3	1	2.4 Ubiquitous after heat shock	HSP70 (HSP72)	641	Heat-inducible hsp70. Chaperone in recovery of cells from stress
HSP70-HOM	4	2	3 E, testis (mouse)	HSP70-HOM	641	Constitutive hsp70
G7b	10	—	0.9 E, L, M	—	—	—
G7a	12	—	4.3 E, H, L, M	Val ^{Trs}	1265	Valyl tRNA synthetase
G7c	—	—	— F, H, L, M	—	—	—
G7d	4	—	— F, H, L, M	—	—	—
G7	22	—	3 E, H, L, M	—	—	—
G6	7	—	1.5/1.4 H, L, M	—	—	—
G6a	—	—	1.6 H, T*	—	—	—
BAT5	—	—	2 F, L, M	—	—	—
G5b	3.6	—	— F, L, M	—	—	—
CKIIβ (G5a)	5.2	7	1-1.1 E, L, M	Casein kinase II β	215	Cell growth?
G5	—	—	1.6 E, F, H, L, M	—	—	—
G4	3	—	2.5 H, L, M	—	—	—
G3a	6	—	1.1 H*	—	—	—
G3 (BAT3)	14	—	3.8 E, F, H, L, M	BAT3	1132	—
G2 (BAT2)	15	—	6 E, F, H, L, M	BAT2	2142	—
G1	1	3	0.6 M, T*	G1	93	Intracellular activation pathway in macrophages/T cells?
K18L	—	—	— Fetal tissue	Cytokeratin (Ps)	xx	Pseudogene
B144 (Lst-1)	3	2	0.8 E, M, L, spleen	Lst-1	85	—
1C7	—	—	— Spleen	—	—	—
LTB	2	4	1 Activated lymphocytes*	LTβ	244	Cytokine. Anchors LTα to cell membrane
TNF	2.5	4	1.5 Activated Mφ and lymphocytes, NK cells, fibroblasts	TNF	233	Cytokine. Antitumor activity. Roles in inflammation/immunomodulation

Table 1 Continued

Gene	Approx. Gene size (kb)	No. Exons	mRNA size (kb)/tissue distribution ^a	Protein	Protein size (aa)	Function
LTA	2.5	4	1.3 Activated lymphocytes*	LT α	205	Cytokine. Role in lymphoid organ development and germinal centre formation
NB6	—	—	— H, L, M	—	—	—
IkBL	13.5	—	1.6 E, L, M	Ikbl	381	Inhibitor of Rel family transcription factor(s)?
BAT1	10	10	1.7 F, L, M	BAT1	428	RNA helicase?
MICB	20	6	2.4 E, F, M*	MICB	383	—
PERB6	80	—	5.8/4.2 Lung 9.5/7.5/5/4/3.4 Liver	—	—	TNF α /receptor-associated protein-like?
PERB10	—	—	0.5 Ubiquitous	—	—	—
MICA	20	6	1.4 E, F	MICA	383	Indicator of cell stress in gastrointestinal epithelium
PERB3	—	—	—	—	—	MHC class I-like pseudogene or gene fragment?
PERB4	—	—	—	—	—	MHC, class I gene fragment?
PERB7 (P5-6)	—	—	—	—	—	—
HLA17	1	3	—	—	—	MHC class I gene fragment
PERB1	—	—	5.5/3/0.5 Testis (mouse)	—	—	Tyrosine kinase receptor?
PPIP	—	—	— Fetal tissue	Cyclophilin (peptidylpropyl isomerase) (Ps)	xx	Pseudogene

Information not available (—); pseudogene (Ps); no expressed protein (xx).

^aTissue distribution is based on published data. The names of tissues and primary cells are given in full, except for macrophages (M ϕ). Single letter codes indicate tissue culture cell lines corresponding to: epithelial cells (E); fibroblasts (F); hepatocytes (H); lymphoid cells (i.e. T and B cells) (L); myeloid cells (including some or all of erythrocytes, granulocytes, monocytes, macrophages and promyelocytes) (M); and T cells (T). *Indicates that an mRNA is expressed specifically in the cell line(s) or tissue(s) shown. Where an mRNA is expressed at particularly high levels in a cell line or tissue this is shown in bold text.

extensive similarity to the yeast antiviral protein Ski2p and the human protein KIAA0052. Helicases are essential enzymes, being required to unwind DNA during replication, repair, recombination and transcription, and RNA during splicing and translation. Many RNA viruses do not contain a poly(A) tail and/or this is not capped. Ski2p exerts its antiviral action by translational inhibition of poly(A)⁺ RNA.

The C-terminal region of G9a (which binds zinc *in vitro*) shows ~33% amino acid sequence identity with the C-terminal regions of *Drosophila* trithorax and its human homolog HRX, both of which are involved in the regulation of transcription during development. G9a also contains six contiguous cop-

ies of the 33 amino acid ANK repeat. This repeat is thought to mediate protein-protein interactions and is found in proteins of diverse function, including *Drosophila* Notch and NF- κ B.

The G9 gene encodes a protein with 28% sequence identity to the *Salmonella typhimurium* sialidase and recombinant G9 protein has sialidase activity with an acidic pH optimum, characteristic of a lysosomal enzyme. Sialidases are important in modulating cellular functions by regulating the sialic acid content of glycoproteins and glycolipids. The *Neu1* locus, which encodes liver sialidase activity and regulates the sialic acid content of several lysosomal enzymes, has been mapped to the murine class III region. Neu1

activity is upregulated in activated T cells with a concomitant reduction in cell surface sialic acid content. The latter is probably due to hyposialylation of cell surface proteins and is a requirement for T cell responsiveness to alloreactive B cells.

The hsp70s are a highly conserved family of proteins, found in all species, and are expressed both constitutively and in response to a variety of stress factors. HSP70-1 and -2 both encode the major heat-inducible HSP70 and their expression is upregulated in response to heat shock. However, sequence variation in their 5'- and 3'-untranslated regions may result in differential expression in response to other stress factors, such as oxidative injury and inflammation. HSP70 is normally expressed at low levels in the cytosol, but following heat-shock it is found predominantly in the nucleus and nucleoli where it aids cell recovery by catalyzing the refolding and disaggregation of damaged and denatured proteins. HSP70-HOM encodes a protein 90% identical to HSP70-1/-2, but its expression is not heat-inducible. All hsp70s can bind unfolded proteins and peptides and act as chaperones in the synthesis, folding, assembly, translocation and degradation of proteins during normal cellular processes and following stress. Sequence variation between the ~18 kDa peptide-binding domains of the hsp70s may be reflected in functional diversity. There is evidence that HSP70 can bind to immunomodulatory peptides and act as an immunophilin. Cytosolic hsp70s have also been implicated in the transport of peptides during antigen processing in tumor cells.

The G7a protein shows 48.3% identity with the valyl-tRNA synthetase (Trs^{Val}) of *Saccharomyces cerevisiae*, and functional studies on recombinant G7a have confirmed that it is the human Trs^{Val}. G7a has a unique N-terminal region which may be involved in the interaction of human Trs^{Val} with elongation factor 1H.

The G5a gene encodes the casein kinase II (cII) β subunit. CkII is a highly conserved serine protein kinase, consisting of a heterotetramer of α and α' catalytic subunits and two regulatory β subunits. The β subunit is required for high activity phosphorylation of the α subunit. CkII has been linked to the regulation of cell growth and metabolism as many of its substrates (such as insulin, epidermal growth factor and a variety of cellular and viral oncoproteins) play key roles in cell growth.

The G1 protein contains two potential 'EF-hand'-type calcium-binding domains, similar to those found in calmodulin. The restricted expression pattern of G1 suggests a possible role in an intracellular signaling pathway during T cell activation or macrophage stimulation when intracellular calcium levels rise.

The cytokines TNF, LT α and LT β belong to the TNF ligand superfamily. TNF occurs as membrane bound and secreted homotrimers, both of which are biologically active. LT α also forms secreted homotrimers, but can be retained at the cell surface as heterotrimers with LT β . TNF has antitumor activity and exhibits numerous inflammatory and immunomodulatory activities. LT α shows similar activity *in vitro*, but is often less potent than TNF. Recent studies suggest a unique role for secreted LT α in the generation of germinal centers, and membrane-bound LT α has been implicated in the development of the spleen, lymph nodes and Peyer's patches.

The ikbl protein contains two copies of the 33 amino acid ANK repeat followed by a third poorly conserved ANK repeat and a short region rich in acidic amino acids. A similar pattern of sequence elements is found in the I κ B family of transcription factor inhibitors. The Rel family of transcription factors, which includes NF- κ B, regulates the expression of a wide variety of genes including cytokines, cytokine receptors, and stress proteins. In unstimulated cells these transcription factors are maintained in the cytosol due to their association with inhibitors belonging to the I κ B family. Stimulation of cells by agents such as phorbol myristate acetate (PMA), IL-1 β , TNF and lipopolysaccharide (LPS) results in the phosphorylation and subsequent degradation of the I κ B protein, allowing translocation of the free Rel proteins to the nucleus.

The BAT1 protein, which is localized in the nucleus, shows significant sequence similarity to the DEAD-box protein family of ATP-dependent RNA helicases. RNA helicases participate in diverse cellular functions such as initiation of translation, RNA splicing, ribosome assembly, spermatogenesis, oogenesis and cell growth and division.

The MICA and MICB proteins share 83% identity and have the same domain structure as the class I heavy chains. However, they are highly divergent from the classical and nonclassical class I genes and differ in their patterns of expression. The MIC proteins lack the residues of the class I heavy chains involved in CD8 binding. MICA does not associate with β_2 microglobulin and is stable in the absence of class I peptide ligands. MICA is mainly expressed in the gastrointestinal epithelium and is upregulated in response to cell stress, suggesting a possible role in the gut mucosal immune system.

Disease association

The MHC is associated with a large number of diseases, many of which are autoimmune in nature. Allelic variants of the class II, and to a lesser extent

class I, molecules account for some of these associations, but there is evidence to suggest that the class III region may also contain disease susceptibility genes. For instance: the central segment of the class III region, containing the RD, G11 and G11a genes, has been implicated in IgA deficiency and common variable immunodeficiency (CVID); Orch-1, a susceptibility gene for orchitis, an autoimmune disease of the testes, has been mapped to the region between G7 and HSP70-HOM in the mouse; and the region between the TNF/LT gene cluster and HLA-B is thought to be important in susceptibility to type I diabetes and myasthenia gravis.

In addition, the functions of some proteins suggest possible roles in disease. For example, RAGE could contribute to diabetic complications by modulating cellular function following interaction with AGEs and expression of RAGE is upregulated in the vasculature in a range of vasculopathies, in atherosclerotic vascular lesions and in immune inflammation vasculitides. Casein kinase II activity appears to be upregulated in the neurofibrillary tangles in the brains of Alzheimer's Disease (AD) patients and associations of HLA-A2 and -DR3 with AD have been reported.

Increased expression of hsp70s has been observed in autoimmune diseases such as scleroderma and systemic lupus erythematosus (SLE), but it is not clear whether this is a contributory factor or a consequence of disease. However, exposure of keratinocytes to ultraviolet light results in accumulation of HSP70 in the nucleoli and a concomitant increase in binding sites for autoantibodies to nuclear antigens which are a feature of erythematosus lesions. Some allelic variation has been found in the MHC-linked hsp70 genes, but numerous studies have failed to show a direct contribution of any of the variants to a particular autoimmune disease.

Complete deficiency of C4 is rare and is correlated with severe immune complex disease, whereas partial deficiency of C4 is more common and is thought to be associated with an increased susceptibility to SLE, scleroderma and primary biliary cirrhosis. Deficiency of P450c21B is the most frequent cause (95%) of congenital adrenal hyperplasia (CAH), which is characterized by accumulation of 17-hydroxyprogesterone and production of excess androgen. Classical CAH occurs as the milder simple virilizing form and the more severe, and life-threatening, salt-wasting form. In addition there is a nonclassical late-onset form where individuals remain asymptomatic or develop the symptom of an excess of androgens in childhood or at puberty. Deficiency in sialidase activity can result in sialidosis, a lysosomal storage disorder characterised by various developmental and neurological abnormalities. Mice carrying a *Neu1*

allele that confers only some 17% of normal liver sialidase activity are small at birth, and show a high incidence of amyloidosis and low lymphocyte response to the mitogen phytohemagglutinin.

Conclusion

Detailed characterization of the MHC class III region has so far led to the localization of over 60 transcriptional units, with sufficient intergenic gaps for this number to increase significantly. Many of the genes are very closely packed: for example G11 and C4 are separated by 611bp; RAGE and PBX2 by approximately 500 bp; and C2 and factor B by just 421 bp. The 120 kb segment of DNA containing the duplicated C4/P450c21/X/Y gene cluster is one of the most complex in the human genome and contains overlapping genes and genes within genes. An understanding of the functions of all the proteins encoded in the class III region will be very valuable in identifying those genes that may be involved in disease susceptibility.

See also: Autoimmune diseases; Complement, alternative pathway; Complement, classical pathway; Complement deficiencies; Complement, genetics; H2 class III; HLA class I; HLA class II; Linkage disequilibrium; MHC disease associations; MHC, evolution of; MHC, functions of; Stress proteins; Tumor necrosis factor α ; Lymphotoxin.

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HOOKWORM DISEASE

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In its widest context, the term 'hookworm disease' may be applied to any of the pathologic consequences resulting from infection by hookworms, i.e. nematode parasites from the family Ancylostomidae. More specifically, the term is used in a restricted sense in reference to hypochromic, microcytic, iron-deficiency anemia, the principal manifestation of pathology in humans. Since the disease is entirely dependent on the presence of adult hookworms in the small intestine and the blood loss resulting from their feeding activities, resistance to hookworm disease is reliant on host-protective immune responses against the parasites.

Characteristics of the organisms and their antigens

Three species of hookworms have the capacity to mature in humans and to cause disease; others parasitize domestic animals and cause similar diseases. Each has its own characteristic features, with differences in important aspects of their biology, including the possession of species-specific antigens and the induction of species-specific pathology. Immunity in hookworm disease must therefore be considered against this background of heterogeneity in the causative organisms and associated immune responses by the host.

Two species, *Ancylostoma duodenale* and *Necator americanus*, are essentially anthrophilic and in nature transmission is maintained only by the reser-

voir of mature egg-laying parasites within infected people. The third species, *A. ceylanicum*, is primarily a parasite of carnivores but develops to maturity in humans, as evidenced among Dutch soldiers returning from active service in West New Guinea and by volunteer experiments among laboratory personnel. There are recent reports from Australia of *A. caninum* causing patent infections in humans, but this species is not generally regarded as being capable of developing fully in the human host. The pertinent details of these species and others are summarized in Table 1.

All hookworms have a characteristic life cycle which begins with the liberation of parasite eggs in host feces. These take a few days to hatch and the larvae then develop through three free-living larval stages, each separated by a molt, into the infective third stage (L3). The species differ in the routes used for entering a potential host. *N. americanus* is an obligatory skin penetrator and can only mature after a larval migration phase; L3 larvae stay at the skin site for about 2 days before migrating through the blood stream to the lungs, where further development lasting about 1 week takes place. The advanced L3 larvae then migrate up the trachea and are swallowed, passing through the esophagus to the stomach and intestine. During this migration the worms molt to the fourth stage. It is now generally accepted that *N. americanus* cannot establish directly in the intestine upon ingestion of third-stage infective larvae, however, during oral infection some larvae may pen-

Table 1 Hookworm species affecting humans and domestic animals

<i>Necator americanus</i>	Both virtually restricted to humans
<i>Ancylostoma duodenale</i>	
<i>Ancylostoma ceylanicum</i>	Closely related, both are parasites of carnivores, particularly dogs. However, <i>A. ceylanicum</i> can cause patent infections in humans whereas <i>A. braziliense</i> is commonly associated with cutaneous larva migrans
<i>Ancylostoma braziliense</i>	
<i>Ancylostoma caninum</i>	These species are also closely related to each other. <i>A. caninum</i> can cause cutaneous larva migrans but does not generally mature in humans, although some reports from Australia have identified adult worms in human subjects
<i>Ancylostoma tubaeforme</i>	

In addition to the parasites listed above, a variety of other members of the family Ancylostomatidae affect domestic animals. Among the best known are *Gaigeria pachyscelis* (sheep), *Bunostomum trigonocephalu* (sheep), *B. phlebotomum* (cattle) and *Uncinaria stenocephala* (dogs). To some degree all have the capacity to aggravate human skin during attempts at penetration.

erate the buccal mucosa and travel to the lungs to complete development in the normal way.

In contrast, members of the genus *Ancylostoma* can readily establish in the intestine following oral infection, without the need for a tissue migration. In addition *Ancylostoma* spp. have the capacity to migrate to the intestine after skin penetration, but unlike *Necator*, *Ancylostoma* do not develop until they arrive in the small intestine. In comparative trials *A. duodenale* established more successfully after oral compared with percutaneous infection, the reverse being true for *Necator*.

The L4 and adult stages of all hookworms live in the small intestine, although in heavier infections parasites may be encountered in the large intestine. *A. duodenale* is considered to be more pathogenic than *N. americanus* and this is probably related to its deeper penetration of the mucosa during feeding.

In recent years it has been recognized that *A. duodenale*, in common with most other *Ancylostoma* spp. has the capacity to undergo arrested development. This has important implications for the epidemiology of the disease and also for consideration of immunological intervention in controlling hookworm disease. Essentially, most of the larvae acquired in the transmission season in hot climates (invariably the rainy and postmonsoon season), instead of immediately completing development, follow a protracted course of development and begin to produce eggs only a few weeks before the anticipated onset of the following rainy season. The parasites thus conserve resources (which would otherwise be spent in unsuccessful reproduction) for the period when transmission is most likely to succeed. *Ancylostoma* spp. probably evolved initially as parasites of carnivores and only secondarily adapted to primates. Indeed it has been suggested that *A. duodenale* may have evolved from *A. caninum*, as a consequence of the long association between humans and dogs. Carnivore parasites profit from arrested development in being able to utilize paratenic hosts such as rodents. The larvae migrate to muscle sites in abnormal hosts

and can survive in an arrested form for over 12 months, generating an opportunity for transmission to the definitive host through the food chain. *A. duodenale* appears to exploit this capacity to maximize transmission efficiency during the limited period when conditions are appropriate. In fact oral infection with *A. duodenale* via meat-borne larvae has been demonstrated experimentally. As far as is known, *Necator* cannot undergo arrested development.

One consequence of the differences in the biology of hookworms outlined above is that the quality, intensity and duration of antigenic stimulation for the host is dependent on the species of hookworm involved. In common with other nematodes, hookworms produce a variety of excretory-secretory (ES) molecules, some of which have important functional roles for the parasites and the majority of which are immunogenic to some extent. Comparisons between the species have again identified key differences but to date only a few defined ES antigens have been studied in detail.

Acetylcholinesterase is secreted by *Necator americanus* and other species of intestinal nematodes and is believed to interfere locally with the muscular activity of the intestinal walls, conceivably providing a better environment for the worms. The enzyme may also play a role in downregulating local mucus secretion and leukocyte activation. *A. duodenale* secretes only 12% of the quantity released by *N. americanus* and *A. tubaeformae* and *A. ceylanicum* produce minimal quantities. *N. americanus* secretes antioxidant enzymes which reduce the concentration of free radicals in the vicinity of the parasite. High concentrations of superoxide dismutase, glutathione-S-transferase and catalase activity have been detected.

Adult hookworms secrete proteases, among which hyaluronidase and anticoagulase properties have been identified. The anticlotting activities include prevention of platelet aggregation and blockage of factor Xa, the latter caused by *A. caninum* anti-

coagulant peptide (AcAP). This species also secretes NIF (neutrophil inhibitory factor, a 41 kDa glycoprotein) which blocks CD11b/CD18 (Mac-1) neutrophil-dependent function, including release of oxidative mediators and adherence to the capillary endothelium. A metalloprotease (36–37 kDa), with anticoagulant and elastolytic properties, which is partially protective when used in an experimental vaccine has also been isolated from *A. caninum*. *N. americanus* secretes several proteases which have a range of pH optima, varying molecular weights and which show a number of biochemical properties, distinguishing them from the enzymes produced by *Ancylostoma* spp. Among the properties of these enzymes is the capacity to digest IgA, the main immunoglobulin isotype associated with the mucosa of the intestine. In general, the proteases secreted by *Ancylostoma* spp. are more similar to each other than to those produced by *Necator*, but some degree of serological cross-reactivity implies that common epitopes are expressed by the proteases of the two genera. In view of their important functional roles in parasite biology, inactivation of ES proteases through vaccination may offer a potential route to future immunoprophylaxis.

The molecular components of the hookworm cuticle have also been studied. The surface molecules are only loosely attached and overlie more important structural elements which are hidden from the host's immune system. It has been proposed that a sustained antibody response to the surface components may result in their removal. Subsequent exposure of the now unprotected deeper elements to a second wave attack of specific antibody, may result in lethal damage to the worms.

Hookworms are highly complex organisms and there is still much to be learned about the possible targets of immune attack. Sensory organs of hookworms have been relatively neglected and yet impairment of the parasite's sensory systems could have severe consequences for parasite viability and may prove a fruitful path to the development of effective vaccines.

Immune responses of the host

Epidemiology

People living in endemic environments become exposed to hookworm larvae from early childhood and consequently worm burdens build up throughout life or, in some communities, until a point of relative stability is achieved in late teenage years. The parasites are extremely long-lived. *Necator* has been shown to live up to 18 years in an experimentally infected volunteer. In the field, parasite longevity is

probably shorter and a turnover of worms must occur sooner or later, particularly in those communities which show stability of worm burdens in the 20–50 year age group. However, there are significant differences in epidemiology between the major species affecting humans, which are not always apparent in the field because mixed species infections are common. Where single-species infections have been studied, *Necator* causes stable infection intensities throughout the year whereas *A. duodenale* oscillates in a clear annual cycle with peak parasite burdens just after the transmission season.

The principal feature of hookworm infections in endemic regions is the absence of declining intensities of parasite burdens in older age groups which have experienced continuous exposure to infective larvae throughout life. If immune mechanisms are involved in regulating worm burdens they must operate at a very high threshold or alternatively immunity may be expressed selectively in a small resistant subset of the host population. Evidence in support of this latter hypothesis comes from observations that hookworm burdens are highly aggregated in affected populations, suggesting that some people may be more predisposed to infection than others. Several studies have confirmed that predisposition is marked. The inhabitants of villages in endemic environments treated for hookworms and re-examined several months later showed significant positive correlation between pretreatment worm burdens and those acquired after treatment. In other words, people with initial high worm burdens became more intensively infected after treatment. However, the results may also be explained through mechanisms other than immunity: behavioral predisposition to infection resulting from differences in standards of hygiene is one possibility. Wearing/not wearing shoes is another.

Volunteer infections

Several reports of infective larvae being administered to human volunteers have been published. Single-pulse primary infections with all three species of hookworm are well documented, but repeated infections have only been reported for *N. americanus*. In all the recorded trials secondary, tertiary and even quaternary were as successful as primary infections in respect of parasite establishment and survival.

Animal experiments

There is little doubt that both *A. caninum* and *A. ceylanicum* elicit strong protective immunity in dogs. Evidence is available for acquired immunity to *N. americanus* in a mouse model: in this case immune animals resist infection both at the skin site

of challenge and during the pulmonary phase of development.

Antibody responses in humans

Human hookworms elicit a variety of antibody responses in their hosts. These have been detected with a range of different assays including ELISA and radioimmunoassay. Responses to ES products and to surface cuticular antigens are particularly prominent and infection is often accompanied by a significant rise in particular immunoglobulin isotopes. Marked increases in total serum IgE are commonly observed in human cases of hookworm disease, often exceeding the quantities typical of patients with IgE myeloma disease. Most of the additional immunoglobulin in the circulation is not specific to parasite antigens and results from potentiation of unrelated IgE response, such as those to environmental antigens. An interesting study in India has reported a reduction in total IgA in the intestinal aspirates of hospitalized hookworm patients, followed by rapid restoration of normal levels when the worms were cleared by anthelmintic treatment, supporting the idea that hookworm proteases are able to digest host IgA and implying that this class of immunoglobulin poses a particular threat to the worms.

Analysis of age-dependent changes in antibody levels to stage-specific antigens of *N. americanus* and parasite burdens have produced some interesting results. In general, the greater the worm burdens the more intense the antibody responses to adult antigens. However, whilst in young children the relationship between antilarval IgG and parasite burden was consistently positive, in older age groups negative correlations were detected, implying that despite overall heavier worm burdens among older sectors of the community those who had fewer worms also had higher concentrations of antilarval antibodies and hence may have been resistant to further larval challenge. Data suggest that hookworms elicit T_H2-type responses with involvement of specific IgE responses to ES products and eosinophilia, but apart from an indication that the more intense responses are associated with smaller and less fecund adult worms there is little to suggest that the T_H2-dependent immunity has a major role to play in providing significant host-protective immunity to hookworms.

Cellular responses in humans

Few studies have investigated cellular responses to hookworms. Blast cell activity and reactivity to mitogenic as well as antigenic stimulation have been measured using human peripheral blood and bronchoalveolar lavage leukocytes. Increased reactivity was recorded but was confounded by considerable

variation between volunteers infected with *N. americanus*. However, significance differences in lymphocyte reactivity were observed during experimental human exposure to *A. ceylanicum* as well as in dogs during primary and secondary infections. Despite these studies, no clear picture has emerged from the use of techniques measuring lymphocyte activity, and the significance of changes in lymphocyte responsiveness are not understood in the context of host-protective immunity to hookworms.

Eosinophilia has been recognized for some time as a common manifestation of hookworm infection and impressive increases in the percentage of hypodense eosinophils have been described in volunteers with *N. americanus*. The same study revealed increased production of superoxide and enhanced chemotaxis of eosinophils from infected donors. Eosinophils have the capacity to adhere to L3 of *N. americanus* in *in vitro* systems. However, as above, a link between elevated eosinophil activity, and host-protective immunity to hookworms has yet to be established. Research on the cellular side of immunity to hookworm infection is a neglected aspect of this disease.

Evasive strategies by the organisms

It is clear from hookworm epidemiology and from experimental infections, that the parasites must employ evasive strategies to survive but information on possible strategies for evading the host's immune response is still scarce.

Several ideas have been put forward as to how immunity may be avoided but none of these has been established as a principal method of avoidance. *A. caninum*, for example, changes feeding sites within the host intestine at regular intervals, staying in place for no longer than 6 h at a time. Continuous relocation may be one way to minimize the effects of local inflammatory reactions as long as the gut does not respond to infection at a gross organ level. The identification of functional proteases in hookworms offers the intriguing possibility that some of these digest antibody on the parasite surface, a strategy utilized by many species of mucosal bacteria. The antioxidant enzymes of hookworms probably protect the worms from effector molecules released from local inflammatory cells. Hookworms may also cause general immunosuppression and probably utilize immunomodulatory molecules to downregulate immunological activity locally in the intestine as other gut nematodes are known to do. In *Ancylostoma* species, NIF may reduce local inflammation and undoubtedly other molecules with related or other immunosuppressive functions will be described

in future studies. However, such aspects of hookworm biology have only recently begun to be explored and it is still too early to present a realistic appraisal of their significance in overall avoidance of host immunity.

Vaccines

There are no vaccines for use against hookworm diseases in humans. However, a highly effective vaccine, based on the use of irradiated larvae, developed against *A. caninum* in dogs induced good protective immunity and it was considered that the tissue stages (possibly the lung phase) of the parasite provided the principal immunogenic stimulus as well as the main target of immunity following subsequent naturally acquired larval challenge. Vaccinated dogs were protected against anemia, but still harbored residual worms and the fact that sterile immunity was seldom induced contributed significantly to the commercial failure of the vaccine which was eventually withdrawn from the market.

Irradiated larval vaccines are never likely to be accepted for human use and the specificity of hookworms for their definitive hosts precludes the large-scale use of laboratory animals in supplying worms for antigenic preparation. Recent attention has therefore been focused on the scope for recombinant DNA technology as a solution to the provision of large quantities of relevant parasite antigens for research. Rapid progress has already been made and several genes encoding functional antigenic molecules have been cloned from *N. americanus* and *A. caninum*.

An alternative approach, which is being currently evaluated, is the development of cryptic epitope (also known as concealed or hidden antigen) vaccines. This approach has been shown to induce high levels

of protection in sheep against the blood-sucking nematode *Haemonchus contortus*. The basis of this vaccination strategy is to elicit in the host high levels of antibody against epitopes which the host normally does not have an opportunity to see, such as epitopes on intestinal cells of the parasite. When the worms then take a blood meal containing the antibodies their intestines are severely damaged and the worms may be expelled.

The next decade should see major strides in our understanding of hookworms and their host-parasite relationships as a consequence of the implementation of these new technologies.

See also: Eosinophils; IgE; Parasites, immunity to.

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hu-SCID MICE

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The severe combined immune-deficient (SCID) mouse

The SCID mutation spontaneously occurs in BALB/c mice, where it impairs double-strand DNA break repair. V(D)J rearrangement is primarily affected, resulting in a defective coding joint formation which

prevents the development of mature B and T cells. The molecular consequence of the SCID mutation is a genetically determined deficiency of the DNA-dependent protein kinase (DNA-PK) which induces DNA break repair by forming an activated complex with the DNA end-binding Ku proteins (p80 and p70) upon association with the damaged DNA.

there is improved human lymphocyte trafficking from the SCID mouse peritoneal cavity to peripheral tissues.

Conclusions

Despite its drawbacks, the hu-SCID mouse chimera (mainly obtained by intraperitoneal transplantation of human lymphocytes) represents one of the most accessible models for studying some of the aspects of human autoimmune diseases and, in general, of the human immune system. Although long-term studies are probably hampered by the fact that the mouse thymic environment operates a T cell selection restricted to murine antigens (Figure 1), the continuing improvements in achieving a longer persistence of non-antimurine-reacting human cells in the SCID mouse after transplantation are encouraging and a better and more complete engraftment of human lymphoid cells into mouse organs able to sustain a correct lymphopoiesis is conceivable in the near future.

See also: B lymphocyte differentiation; Chemokines; Immunodeficiency, animal models; Severe combined immunodeficiency; T lymphocyte differentiation; Xenotransplantation.

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HUMAN IMMUNODEFICIENCY VIRUSES

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Within a decade, the virus causing the acquired immune deficiency syndrome (AIDS) emerged from obscurity to become the best characterized of all retroviruses, the first of such viruses to have its cellular receptor defined and the one which has proved

to have the most complex genomic organization. The many facets of current AIDS virology are such that only a 'thumbnail' sketch of the virus structure and its behavior can be outlined here.

Classification and structure

The etiological agent of AIDS, human immunodeficiency virus type 1 (HIV-1), now endemic worldwide, was discovered in 1983. Two years later, a second virus (HIV-2) that caused the same disease but was significantly different from HIV-1 serologically and molecularly to be considered a separate, but related, virus was discovered in West Africa. Between 1985 and 1989, several nonhuman primates were shown to harbor similar viruses, some of which cause disease in the natural host and some of which appear to be quite harmless. All of these viruses have an RNA genome and replicate via a DNA intermediate (a 'provirus') by means of viral RNA-directed DNA polymerase, more commonly called reverse transcriptase. It is this 'backward' transfer of genetic information from RNA to DNA which classifies these viruses as retroviruses.

Each virion measures approximately 120 nm in diameter and has condensed cylindrical core surrounded by a lipid membrane. The envelope 'spike' glycoprotein is embedded in and protrudes from this membrane. The interrelationship of genomic RNA, core proteins and surrounding envelopes is more clearly demonstrated schematically (Figure 1) and more readily understood by considering the genomic organization.

Structural and functional organization of the HIV-1 genome

The HIV virion contains two identical copies of single-stranded RNA, 9.2 kb in length, of positive polarity with respect to translation. The proviral DNA resulting from reverse transcriptase has three genes which code for the virion structural proteins: gag, pol and env encoding the core proteins, enzymes for viral replications, and the envelope glycoproteins, respectively. Each gene product is initially synthesized as a high molecular weight polypeptide precursor which is subsequently proteolytically cleaved to yield the final, smaller structural components of the virus.

The genome (Figure 2) contains at least nine genes, flanked by long terminal repeat sequences (LTR) containing promoters of gene expression and specific enhancer elements which control viral gene expression and which are themselves influenced by cellular transcriptional proteins.

HIV structural genes

The primary product of the gag gene is a 55 kDa precursor polypeptide (p55) which upon proteolysis yields p17, the myristylated matrix protein (MA), and antigens of the viral core, the capsid (CA) p24,

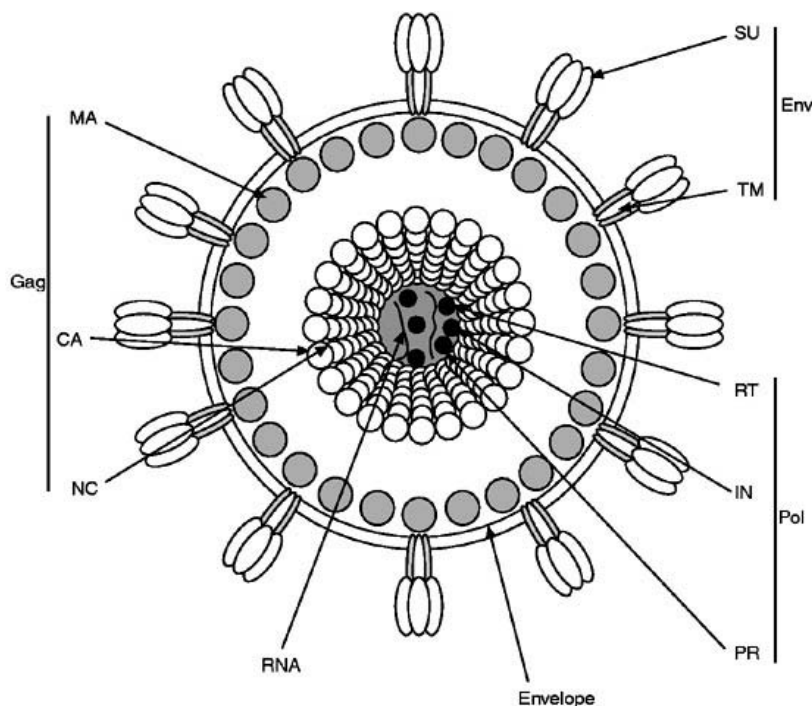


Figure 1 Schematic representation of an HIV virion. The relative positions of HIV structural and other virion proteins are indicated. NC, nucleocapsid; CA, capsid; RT, reverse transcriptase; IN, integrase (endonuclease); MA, matrix; TM, transmembrane; PR, protease; SU, surface.

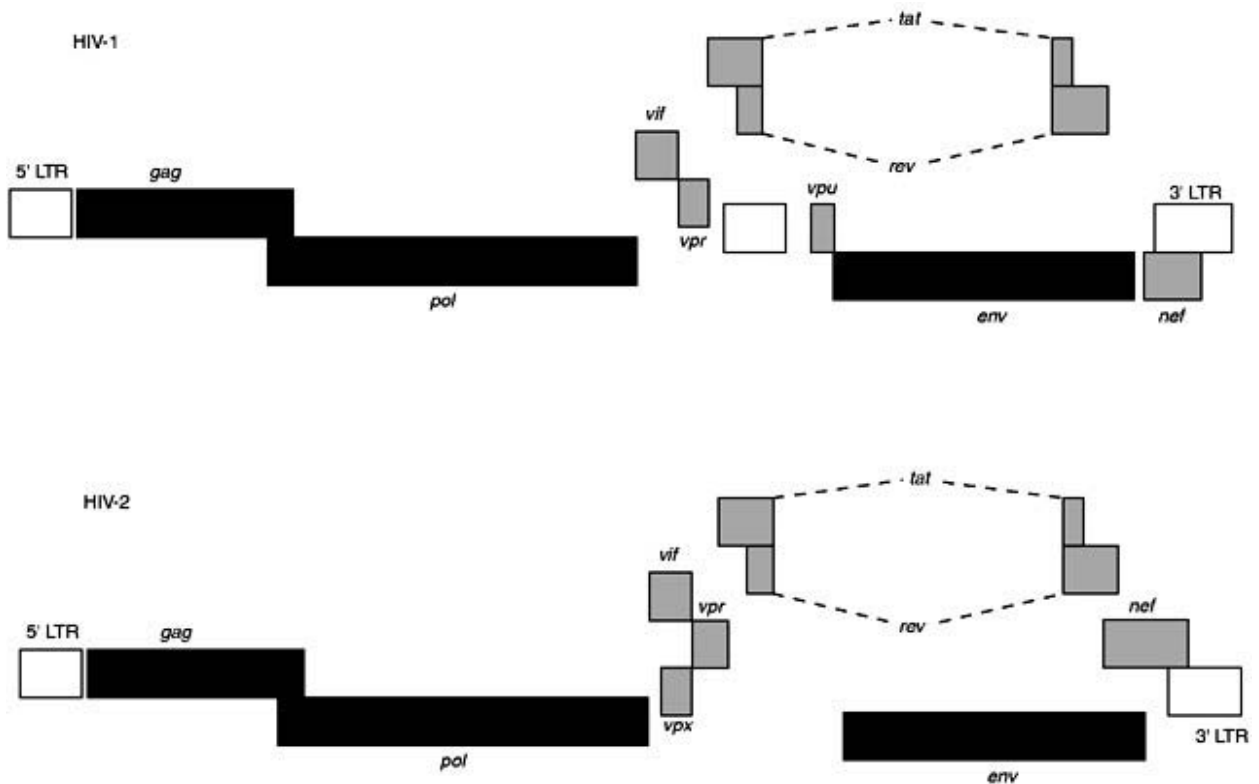


Figure 2 Genomic organization of HIV-1 and HIV-2. The genomes, which are between 9 and 10 kb long, are flanked by long terminal repeat (LTR) sequences. The *vpu* gene is only found in HIV-1 and the closely related simian virus from the chimpanzee, *SIV_{cpz}*. The *vpx* gene is common only to HIV-2 and its related simian counterparts isolated from the macaque and the sooty mangabey monkey, *SIV_{mac}* and *SIV_{smm}* respectively.

the nucleocapsid (NC) p7 and p6 which facilitates virion budding from the cell.

Since the *gag* and *pol* genes overlap in different reading frames (Figure 2), translational frameshifting is necessary to produce the Gag-Pol polyprotein precursor (p106) which also undergoes proteolytic cleavage by the virus-encoded protease to produce 1) the protease (p10) responsible for the breakdown of p55 *gag*, 2) the enzyme reverse transcriptase, p66 and p51, and 3) an endonuclease or integrase (p32) to catalyze the integration of the provirus into the chromosomal DNA of the host cell. Thus, both the Gag and Gag-Pol precursor proteins are broken down to their final products by the same protease, p10, during or after budding from the cell surface.

The initial 160 kDa glycoprotein product of the *env* gene undergoes cleavage to leave a newly glycosylated surface (SU) protein of 120 kDa (gp120) at the N-terminus, and the C-terminal gp41 transmembrane envelope glycoprotein. The viral gp41 serves to anchor gp120 to which it remains noncovalently attached and to effect membrane fusion via a hydrophobic domain at its N-terminus.

HIV regulatory and accessory genes

In addition to the usual three retroviral structural genes, the HIV-1 genome includes six further genes encoding regulatory and accessory proteins translated from various multiply-spliced HIV RNAs. The three genes regulating virus replication are *tat*, *rev* and *nef*. Two of these genes, *tat* and *rev*, are nuclear proteins which are absolutely essential for virus replication. Proviruses defective in these genes fail to replicate. The two exons of the *tat* gene code for a 14 kDa diffusable effector protein which binds directly to specific responsive sequences (the transactivating responsive region, TAR) within the LTR. In so doing, the synthesis of both structural and regulatory viral proteins is either directly or indirectly increased at the level of transcription initiation, elongation or both.

Viral structural protein synthesis is additionally controlled by the action of the *rev* gene, the 19 kDa phosphorylated product of which is located within the nucleolus and binds to Rev response elements (RRE) located within the *env* gene. The functional

expression of the Rev nuclear protein acts to facilitate the transport from the nucleus to the cytoplasm of multiply-spliced transcripts encoding regulatory proteins (Tat, Rev, Nef, Vpr) early in infection, or unspliced or partially spliced mRNA from which the Gag and Env proteins are translated.

The *nef* gene encodes a 27 kDa myristylated protein which is highly conserved in all immunodeficiency virus genomes (HIV-1, HIV-2 and the SIVs), yet is not essential for replication of the virus *in vitro*. Its importance would appear to be connected to its role in pathogenesis *in vivo*, since macaques infected with *nef*-deleted mutants fail to develop simian AIDS.

The remaining accessory genes, *vif*, *vpr*, *vpu* and *vpx*, which are dispensable for *in vitro* replication of the virus are, nevertheless, highly conserved in HIV and SIV strains, suggesting that, like *nef*, these genes have an important role *in vivo*, either in contributing to the efficiency of virus replication or to the pathogenic effect of the virus in the infected host. The *vif* and *vpu* genes contribute to virus replication by increasing the efficiency of infection of cell-free virus (*vif*) and in facilitating the assembly, binding and release of mature virus particles (*vpu*). In the absence of *Vif*, the 23 kDa cytoplasmic gene product which associates strongly with the cell membrane, virions have an abnormal morphology and a reduced capacity to synthesize proviral DNA when they infect new cells. Only the HIV-1 genome and that of the closely related chimpanzee immunodeficiency virus, SIV_{CPZ}, contain the *vpu* gene, the 16 kDa phosphorylated gene product of which is located outside the nucleus, close to the endoplasmic reticulum (ER) and Golgi apparatus. It appears to direct the transport of envelope proteins to the cell surface by mediating degradation of CD4 molecules in the ER. Mutant virus deficient in *vpu* replicates *in vitro* at reduced titer due to the accumulation of virus particles in intracytoplasmic vesicles.

The *vpr* gene induces subtle increases in the kinetics of virus infection. It appears to enhance virus production in primary macrophages and, to a lesser extent, in some T lymphocyte cell lines. The *vpx* gene is present in HIV-2 and the SIVs, but not in HIV-1 and, like *vpr* is packaged within the virion. Its function is ill-defined.

The lifecycle of HIV

Infection by HIV is initiated when either cell-free or cell-associated virus binds to a receptor in the target cell by means of the virus envelope. The major receptor for HIV, CD4 (see below), is a membrane protein

found on the surface of certain hematopoietic cells, such as T helper lymphocytes and monocytes.

After fusion of the virus envelope protein with the cell membrane, the virus core enters the cell and single-stranded RNA, still associated with capsid protein, is converted to double-stranded proviral DNA through the dual polymerase and ribonuclease activities of the viral reverse transcriptase.

In T cells, the newly made provirus then enters the nucleus, integrates with host DNA by the action of the viral integrase and establishes an infection which is lifelong. The proviral DNA acts as a template for the production of viral RNA. This, in turn, serves both as messenger for synthesis of new viral proteins and as genomic RNA which is subsequently packaged into viral particles. Virus assembly occurs at the surface of the membrane. First, the capsid protein condenses beneath the cell membrane to form the budding particle. The viral RNA attached to the capsid precursor is folded into the particle while the envelope protein aggregates on the outer surface. The virus particle is then released from the cell surface and the capsid protein is processed into mature proteins by the viral protease. This completes the life cycle.

Replication of HIV is determined at least in part by the level of activation of the target cell. It is well established that HIV will not replicate in resting T cells, one level of restriction is at the level of provirus synthesis. Apparently, only a partial provirus is formed in the resting cell and upon activation provirus goes on to completion. Another restriction is at the level of RNA transcription. The HIV LTR contains binding sites for at least two transcription factors which are activated in stimulated T cells. These are Sp1 and NFκB. Interaction of one of these two factors with the LTR sequences is sufficient to confer the virus with responsiveness to mitogenic stimulation. A number of DNA viruses (CMV, HSV), as well as the human T cell leukemia virus (HTLV-1), have been shown to activate the NFκB site. Many other cellular factors bind to specific DNA sequences in the HIV LTR and conceivably regulate HIV expression. The abundance and variety of these factors will vary according to cell type and cell cycle.

The HIV receptor

The CD4 molecule is a T cell surface glycoprotein of 55 kDa that characterizes the T helper subset of the human lymphocyte population. It associates with major histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells to effect a humoral and cellular immune response.

Classically, three main lines of evidence clearly

established CD4 as an essential component of the HIV receptor. First, monoclonal antibodies directed against the CD4-binding site completely inhibited infection of susceptible cell lines. Second, it was shown that the envelope glycoprotein of HIV-1, gp120, binds specifically and with high affinity (K_d 10^{-9} M) to the CD4 molecule on T helper lymphocytes. Third, transfection of the CD4 gene into non-susceptible (i.e. nonlymphoid) cell lines of human origin rendered them infectible by HIV. Cells of non-human origin, however, remained refractory to infection, leading to the idea of a species-specific cell surface cofactor in addition to CD4 being required for viral entry. Subsequent studies showed that strains of HIV-1 that had been adapted for growth in transformed T cell lines (T tropic strains) could infect primary T cells, but not primary monocytes or macrophages. In contrast, many primary viral strains were found to infect monocytes, macrophages and primary T cells, but not transformed T cell lines. This difference in tropism which results from sequence differences within the variable domains of the outer envelope glycoprotein, gp120, suggested that multiple cell type-specific cofactors might be necessary for entry, in addition to CD4. The molecules in question turned out to belong to the family of seven transmembrane signal transduction receptors for chemoattractant cytokines (chemokines). Interestingly, while many unsuccessful attempts had been made to molecularly clone the HIV entry coreceptor, it was the clinical observation that individuals who were homozygous for a 32 bp deletion in the chemokine receptor CCR5 were resistant to HIV-1 infection which led to the recognition of CCR5 as a coreceptor for macrophage-tropic (M-tropic) HIV-1 strains. Other strains adapted for growth and syncytium induction in transformed T cell lines (T tropic strains) utilize the CXCR4 receptor.

All HIV-1 strains tested *in vitro* use either CCR5 or CXCR4 coreceptors or both, suggesting that these coreceptors are likely to be determinants of infection and pathogenesis *in vivo*. However, receptor usage by HIV-1 is not restricted to either of these molecules. Many strains can use other members of this receptor signaling family, such as CCR3, CCR2b, and some even utilize the recently identified SIV coreceptors currently called 'Bonzo' and 'Bob' (brother of Bonzo!). The rather flippant nomenclature reflects both the freshness of their discovery and the anonymity of their natural ligands, although since each appears to be identical to known receptors, Bob may be known as GPR15 and Bonzo as STRL33.

In all, six coreceptors which are exploitable by HIV-1 for host cell entry are now known. These have

all been identified from experiments carried out *in vitro*. Currently, the extent to which they are used by the virus *in vivo*, or the selective pressures existing to influence their use are not understood. Since this active area of investigation has only recently opened up, much remains to be learned about the contribution of each coreceptor to the determination of infection, the pathogenic sequelae and about how a knowledge of coreceptor usage can be exploited for drug development.

Antigenic variation in HIV

With the availability of extensive HIV nucleotide sequence data, genetic diversity among AIDS viruses can be compared. The envelope gene of HIV-1 contains five hypervariable regions which are flanked by conserved regions. Sequence diversity is a characteristic of many RNA viruses. They are genetically error-prone by virtue of their DNA-dependent RNA polymerase, a step in their replication cycle which escapes correction by host cell enzymes ('sub-editing'). It has been estimated that the HIV-1 reverse transcriptase transcribes on average one error per genome in each replication cycle. These errors are mainly point mutations producing amino acid substitutions, stop codons, etc., although insertions and deletions have also been observed.

Genetic variability leading to antigenic diversity are means by which viruses can evade immune surveillance. For example, as well as retroviruses, influenza virus does this very successfully by generating an ever-changing repertoire of antigens. For many lentiviruses, genetic variation offers a selective advantage by allowing adaptation to different biological environments. If such variation is subjected to immune selection resulting in temporary evasion of host defense mechanisms, variant viruses result. Genotypic variation has been noted within a 'single isolate' of HIV-1 (i.e. taken from one individual), among isolates taken over time for the same individual, and among very different isolates within populations. Such variation is rapid *in vivo*, producing a great many related but distinct genotypes which can evolve in parallel and coexist during chronic infection, raising the possibility of the emergence of viruses with recombinant genomes. Thus, in addition to rapid mutation, recombination can contribute to the genetic diversity which characterizes the AIDS virus.

Since the discovery of the virus in 1983, the HIV-1 genome has been extensively studied, partly to determine the range of strains contributing to the global epidemic (and this information has obvious implications for vaccine design and evaluation) and

partly in an attempt to understand the evolution of HIV-1 over time. On the basis of sequence analysis and alignment of the gag, env and tat genes chosen from diverse geographical isolates of HIV-1, two major groups have emerged: group M, the major group comprising most isolates, and the distant (about 50% within env) group O or 'outlier group'. Even within the 'O' group, the HIV isolates are not closely related, being approximately 30% different from one another in their env gene sequences. Within the major group, the HIV-1 isolates can be subdivided into at least 10 genetic subtypes called clades (A–J). All subtypes are approximately equidistant from one another, the distance separating them being about 30% in the env and 14% in the gag gene. For comparison, HIV-1 and HIV-2 differ by at least 50% in both genes. On a similar basis, HIV-2 isolates can be classified into five clades.

Recombination between isolates of different clades makes its own contribution to the range of strains found in the global pandemic of AIDS. It is calculated that at least 10% of reported HIV-1 strains represents mosaic (recombinant) forms of the viral genome, i.e. bearing sequence characteristics of two different genetic subtypes. This can occur at any point along the genome and recombination break-points have been mapped in the LTR, structural and regulatory genes. For example, subtype E viruses mainly found in South-East Asia are really recombinant viruses composed of genetic material from subtype A and from an undefined parental subtype. The existence of recombinant viruses suggests that HIV-1 strains of different clades can coexist in the one individual, resulting in viable infectious progeny viruses of mixed genetic background, all of which adds to the dynamic and unstable nature of the HIV-1 epidemic worldwide and to the difficulties of designing an all-purpose vaccine.

The immunology of HIV infection

The immunology of HIV infection at first sight appears to be simple: HIV targets CD4⁺ cells, resulting in a declining CD4 cell count and loss of CD4 helper T cell function. As the CD4 lymphocytes are so pivotal to the immune system, providing help for other functions directly or through cytokines, it is not surprising that other immunological functions also decline as disease progresses. However, as more HIV⁺ people are studied in more detail for longer, it is clear that the pathogenesis and the nature of the protective immune response are considerably more complicated than originally perceived. The most antigenically dominant domain on the envelope of HIV, the V3 loop of gp120, induces neutralizing

antibodies and this factor has driven vaccine development. Because this region is highly variable, the virus is able to escape a strong antibody response. Moreover, it is not clear that disease progression is greatly influenced by neutralizing antibodies on their own as the immune response broadens to different V3 loops as disease progresses. Therefore, the nature of the protective immune response that clearly exists in long-term partners of AIDS patients must be defined in detail and the mechanism of how the virus induces disease needs to be carefully elucidated. Unfortunately, after over a decade of research the answers to these questions are only just beginning to be more than just theoretical.

The immune response to HIV and the correlates of protection

HIV induces neutralizing antibodies, antibody-dependent cellular cytotoxicity (ADCC), and cytotoxic T lymphocyte (CTL) activity in infected hosts. Unfortunately, it remains unclear as to whether any one, or all, of these activities can prevent infection and disease progression in the majority of individuals. It is important to appreciate that the quality of the immune response that protects against infection and the type of response required to contain infection may be different. This, of course, will have important implications for the development of preventative and therapeutic vaccines. This problem is further compounded by the fact that HIV-infected chimpanzees do not appear to develop an AIDS-like disease, and hence they may have a vital immunological difference that allows a vaccine to prevent HIV infection that will not extrapolate to protection of humans against the same infection.

Over the last few years it has become evident that a number of people have been consistently exposed to HIV but have remained seronegative, and that others have seroconverted but remained disease free. Studies on these patients show that they are more likely to have a strong cell-mediated or T_H1 cytokine profile (IL-2, IFN γ and IL-12) than those who develop disease. Moreover, they have good mucosal immunity and higher IgA levels as well as antibody responses to the C-terminus of gp120. The CTL response is confusing, as although CTL responses would expect to contain the virus, the activity is often low (and indeed hard to detect at all in HIV-1-infected chimpanzees).

The HIV vaccine challenge

The principle of vaccination is straightforward: the inoculation of a safe antigen to induce an immune

response against the etiological agent of an infectious disease and in doing so confer protection to subsequent infection. Successful vaccines currently available against virus-induced diseases include live attenuated strains that produce infection without inducing disease, such as the Sabin polio vaccine. Such strategies are not practically applicable to HIV-1 because the safety of attenuated strains cannot be evaluated before use. Even extensive genetic modification (e.g. deletion of regulatory genes critical for virus replication) would not necessarily ensure against reversion of the virus to virulence. While this strategy of vaccination with mutants deleted of key regulatory genes protected adult macaques from experimental SIV challenge, it proved fatal to immunocompromised neonatal animals.

An alternative strategy is the subunit vaccine which has already proved successful for viruses such as hepatitis B. In this respect the advent of recombinant DNA technology has allowed vaccine development to enter a new era. Individual virus genes can now be cloned and expressed in prokaryotic and eukaryotic cells to yield enough antigen for subsequent purification and use in vaccine studies. The viral proteins most likely to be exploited as compound candidate vaccines are those of Env and Gag. Antibodies directed against gp120 can neutralize HIV *in vitro*, while Gag protein has been shown to have epitopes recognized by both helper and cytotoxic T lymphocytes.

Neutralization epitopes of HIV-1 fall within the five hypervariable regions (V1-V5) of the envelope glycoprotein, gp120 and gp41, with the major neutralization epitope lying within the V3 domain. Serum and monoclonal antibodies directed against this region neutralize free virus infectivity and syncytium formation resulting from the coculture of HIV-infected cells with uninfected CD4⁺ cell lines. The amino acid sequence of the V3 neutralizing domain can elicit group-specific antibodies, i.e. antibodies which are effective in neutralizing a narrow range of HIV-1 isolates *in vitro*. In addition to being the principal neutralization epitope, V3 is an MHC class I restricted CTL epitope and a helper T cell epitope. *In vitro* studies have demonstrated that a minor mutation within the env gene can significantly alter the sensitivity of HIV-1 to neutralization. This implies that viral heterogeneity and immune selection *in vivo* allows HIV-1 to escape immune surveillance, leading to disease progression in the presence of neutralizing antibodies.

A major difficulty facing vaccinologists is that primary or field isolates of HIV-1 (i.e. those that have not adapted to growth in cell culture) are relatively resistant to neutralization by antibody. At the

same time, certain monoclonal antibodies and sera collected from infected individuals have the ability to neutralize some field isolates. The principal determinant on primary viruses against which neutralizing antibodies are raised has not yet been identified. Consequently, nothing is known of the breadth of these effective reagents or the extent to which this putative principal neutralizing domain would be present in primary isolates from different clades. Nevertheless, in the absence of defined correlates of protective immunity to field isolates of HIV-1, it must be assumed that high titers of specific neutralizing antibody and a significant cell-mediated response should ideally be induced in the immunized host. Since gp120 carries the major neutralizing determinant and the major CTL epitopes are located in the Gag, Pol and Nef proteins, it would suggest that a 'cocktail' of antigens may be more effective than a single component. A number of candidate vaccines have already been shown to be capable of inducing CTL response to HIV-1, among them live viral vectors delivering envelope genes, e.g. vaccinia-gp160 or avipox gp160, both used with a recombinant gp120 booster.

Whatever the adopted strategy for the development of an HIV vaccine, many complicating factors must be taken into account:

1. It is not yet known what combination of viral proteins in a subunit vaccine could best mimic the response induced by whole virus particles. Moreover, monomeric gp120 subunits, a likely vaccine component, may not reflect the more complex native envelope structure in the virus particle.
2. HIV can be spread by cell-free virus in the bloodstream or by the fusion between infected and uninfected cells. Both are important routes of infection and any potential vaccine candidate would have to have the capacity of preventing infection by both routes.
3. Testing the safety and efficacy of a new HIV vaccine before its general use may well present a considerable problem. There is no ideal animal model of HIV infection. Chimpanzees have been experimentally infected with HIV-1, but they do not manifest signs of clinical disease. Putative vaccines could, of course, be tested for their ability to inhibit infection, but this does not provide the ideal *in vivo* experimental system. An alternative approach would be to run parallel studies using the SIV-macaque system. Indeed, the most convincing evidence yet that a vaccine against HIV will prove possible has been the protection of macaques from simian AIDS fol-

lowing immunization with formalin-inactivated SIV particles.

4. Any useful vaccine must have a broad specificity of action. A strain-specific response which would protect only one strain of a mutation-prone virus would be futile. However, recent work investigating the neutralization properties of three human monoclonal antibodies against 10 viruses from a number of different clades suggests that an immunogen able to elicit significant neutralizing activity against a representative set of primary isolates from one clade, may also be able to induce a cross-clade response. This remains to be further investigated.
5. As candidate vaccines become available for evaluation, moral, ethical and safety considerations will simultaneously raise difficult issues concerning the use of primates and of human subjects in such studies. For example, HIV diagnosis is carried out serologically by ELISA and confirmatory western blot. In the event of a multicomponent vaccine entering a clinical trial, the question of distinguishing those subjects who are seropositive from those who have been immunized becomes problematic.
6. The generation of 'enhancing' antibodies which could increase viral infectivity needs to be avoided.
7. CTL activity could also be more harmful than helpful depending on the other factors operating within the immune system at the time.

In order to understand some of these issues, the pathogenesis of HIV will be reviewed.

How HIV causes disease

The correlates of disease are easier to identify than the correlates of protection and have been well documented by many investigators. Studies on the early stages of disease at the time of infection and seroconversion show that the viral load and the nonspecific activation markers of the immune system such as soluble TNF receptor levels are both highest in those that progress to disease rapidly. However, even in those who progress rapidly towards disease the high initial virus load is quickly reduced, presumably by the immune response, although the intrinsic dynamics of viral replication could also explain at least part of the decline. It may be that the initial interaction between the virus and the immune response sets the future pattern of disease. The issues affecting the establishment of infection at this stage are numerous and include properties of the virus as well as the host. Nonspecific sera and complement factors may play an initial role and the responsive-

ness of the immune system is probably also critical. A number of studies have reported a strong link between HIV disease progression and nonspecific activation of the immune system. Not only is there a close association with the rate of progression to disease, but also the baseline activation associated with immunogenetic markers such as the HLA system itself determines the rate of disease progression.

Immune activation and disease

Retroviruses are not particularly virulent viruses, at least in the short term. In order to infect cells and increase their chances of establishing a high viral load they prefer activated cells and employ sophisticated mechanisms to activate resting cells. Some retroviruses encode a superantigen which can activate approximately 10% of the T cell population. In spite of some publications to the contrary, there is no good evidence that HIV encodes a classic superantigen. Some authors maintain there is no need to encode a superantigen as the virus turns over fast and varies its dominant antigens, thus multiply stimulating the immune response. A disease which causes immunodeficiency by pan-activation and concomitant immunosuppression is chronic graft-versus-host disease (CGVHD). This is of interest with regard to HIV, as prior to the identification of HIV, the clinical similarities between AIDS and CGVHD had been recognized and the role of alloantigens in semen and blood proposed as an activation stimulus. Although this is extremely unlikely, the fact that HIV encodes several HLA-like regions makes the possibility that the virus itself induces allostimulation intriguing. The concept has recently been supported by the ability to induce murine AIDS with chronic, repeated stimulation with alloantigens. It is of further interest that several of the specific immune defects are similar, such as the early loss of antigen-specific responses.

HIV: virus turnover

The ability of HIV to kill CD4 cells directly and indirectly (such as by the signaling of apoptosis and the induction of fusion by envelope contact) should in itself be enough to gradually reduce the CD4 count. However, the rate at which it can do so has been questioned, and the need to invoke other mechanisms in addition to direct cytopathic effects has been raised by a number of authors. The ability to perform accurate quantification of viral load has allowed the new antiretroviral therapies to be assessed in detail. Such studies have allowed the overall rate of virus turnover and the half-life of the

virus, a virally infected cell, and the time from budding from one cell to virus release from a newly infected cell to be calculated. There were some surprises: the amount of virus turning over in the peripheral circulation per day was in the region of 10^9 to 10^{10} , the half-life of the virus less than 6 h, the half-life of productive cells was 1.6 days, and the time from budding to productive infection, 2.6 days and the half-life of long-lived cells was 1–4 weeks. Based on these calculations and the response to therapy, it has been calculated that effective treatment would need to be given for about 3 years.

The finding that the virus has such a high turnover rate suggested an immediate explanation for the CD4 cell decline *in vivo*. However, it may be that the decline is very slow, even with this turnover rate which would need to influence the precursor compartment before any effect on the CD4 cell numbers would normally be seen. In this regard a reservoir of HIV exists within follicular dendritic cells in the lymph nodes, as well as in mononuclear cells in the periphery and the spleen. It is the former that probably contributes most to the circulating virus load.

A major source of controversy has been whether or not HIV kills cells directly or indirectly. Although HIV can kill cells readily *in vitro* by inducing fusion and cytopathic effects, the indirect effects whereby apoptosis is induced without productive infection may also play a major role in the disease. The ability to infect antigen-presenting cells would greatly enhance this ability as well as interfering with antigen-presenting functions directly. The presence of gp120 alone during antigen presentation can inhibit antigen-specific responses, and this coupled with the ability to infect the most potent antigen-presenting cells would allow for a major perturbation of normal immunological function.

Immunogenetics and the cytokine system

The association with different HLA types and disease progression has been reported in most cohorts studied, although only a few of the associations stand up to multicenter analysis. One of the most striking associations is the association with HLA-B27 and nonprogressors and in Caucasian populations the association of HLA-A1, B8, DR3 with more rapid progression to disease. The latter haplotype is also associated with enhanced baseline immune activation that in turn is associated with other diseases of chronic activation such as systemic lupus erythematosus (SLE). It is of some interest that chimpanzees, which are 98% genetically similar to humans, do not have these haplotypes associated

with disease progression. The haplotypes determine a number of immune responses apart from peptide selection, including cytokine and complement usage. The fact that nonprogressors and chimpanzees have a quiet T_H1 -type immune response may be important with regard to the conditions that determine the initial interaction between the virus and the immune system. The loss of T_H1 responses is associated with progression and several authors report a T_H2 -dominant response as disease progresses although this also appears to become deficient with time in that IL-4 *per se* also disappears, leaving IL-10 and TNF more dominant.

A major issue is whether or not the immunological perturbation of HIV infection can be permanently correctable with antiviral treatment and whether or not an effective immune response can be induced, allowing this therapy to be withdrawn. Many patients have a dramatic 2-log decrease in viral load with therapy accompanied by a rise in the CD4 cell count. Unfortunately, attempts to stop therapy leads to a bounce back in viral load within 2–4 weeks. Even patients who are benefiting from therapy may have progressive disease of some associated condition, such as Kaposi's sarcoma, suggesting that immune competence may require 'reprogramming'; for example by inducing a T_H1 -dominant response.

The fact that much of the immune response is activated as disease progresses would suggest that if this is causal, then immunosuppression should be beneficial. There are suggestions that this may be so: studies with steroids and cyclosporine have suggested a delay in disease progression and lymph node radiotherapy, an effective form of immunosuppression, has prevented disease progression in SIV-infected macaques.

The immunology of HIV is clearly complicated and no single issue would appear to be of overriding therapeutic importance, other than the complete elimination of all virions. However, even this may not reverse immunodeficiency if irreversible changes in the immune system have been induced. Nevertheless, the following major points are worth summarizing:

- HIV only causes disease in susceptible hosts who bear the CD4 receptor and the appropriate secondary receptors, such as CCR5.
- The immune response appears to be genetically controlled and can determine outcome in that a cell-mediated T_H1 response is associated with a better prognosis than a T_H2 -dominant response.
- An activated immune system and a vigorous immune response to the virus are often associated with progressive disease.

See also: **Acquired immune deficiency syndrome (AIDS); Apoptosis; Chemokines; Dendritic cells; ISCOM (immuno-stimulating complex); Rhabdovirus, infection and immunity; Vaccines.**

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HUMANIZED ANTIBODIES

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The techniques for humanization of antibodies originated in 1986 when Jones and his colleagues demonstrated that antigen binding specificity could be transferred from a mouse monoclonal antibody into a human antibody by replacing the complementary determining regions (CDRs) of the human antibody with those of the murine – called CDR grafting. Subsequently it was found that in most cases additional subtle alterations to the structure of the human antibody were necessary to completely restore antigen binding affinity. The processes of introducing changes in addition to CDR grafting is called reshaping human monoclonal antibodies or humanization of antibodies.

Application of such techniques became possible with the development of methods for rapid cloning, sequencing and expression of antibody genes in mammalian cell lines and major advances in understanding the structure and computer graphic modeling of antibodies. In particular, the domains of the antibody which interact to provide the antigen binding surface were elucidated.

The rapid widespread use of the technologies in recent years has been driven by the disappointing behavior of rodent monoclonal antibodies as *in vivo* imaging and therapeutic agents and the continual failure to develop methodologies for routine provision of high-affinity human monoclonal antibodies.

Antibody-based therapies are attractive clinical and commercial targets. Over the last 20 years, many thousands of rodent monoclonals have been produced by hybridoma technology, many against unique targets of clinical significance. Humanization is perceived as the way to develop these as successful human therapeutics.

General background

Serum therapy using human immunoglobulins as intravenous immune globulin (IVIG) has been used successfully in a number of disease states. However, such polyclonal sera is expensive to collect, particularly high titer sera against specific diseases, and problems of contamination with viruses such as hepatitis B and human immunodeficiency virus limits the use of human materials.

In the last 15 years, over 50 different rodent monoclonals have been evaluated in studies of imaging (tumors, blood clots) and therapy (tumors, blood clots, immunosuppression). While the data show that these monoclonals can effectively target *in vivo*, they

have not proved clinically successful except in a few specialized cases.

Clinical use is limited by three factors. First, short serum half-lives of only 15–50 hours mean that regular repeat dosing regimens would be required, making the cost of treatment excessive. Human antibodies by contrast have serum half-lives of several weeks. Second, the effector function recognition signals for complement-dependent lysis of targets or Fc receptor driven cytotoxicity which reside in the constant region of rodent antibodies are not efficiently recognized by the human immune system. Thus other killing moieties such as radioactive isotopes or toxins have been attached to rodent antibodies, thereby increasing their toxicity. Third, the human immune system recognizes the rodent antibody as foreign and mounts a human antimouse antibody (HAMA) response, resulting in patient antibodies against the rodent monoclonal. HAMA responses develop in about 50% of patients after a single dose and in about 90% after a second treatment. Subsequent doses of antibody are then removed before they reach the disease target.

The aim of humanization is to provide antibodies essentially free of the three limitations on clinical use described above. Over 100 antibodies have now been humanized as a means of exploiting the existing vast array of rodent monoclonals as human therapeutics.

General structure of antibodies

The antigen binding site is in the Fab fragment and the effector functions in the Fc fragment. Six segments (CDRs) within the variable regions (V_H and V_L) of the heavy and light chains loop out from the framework (FR regions) globular structure of the rest of the antibody and interact to form an exposed surface at one end of the molecule (Figure 1). This is the antigen binding domain. Generally 4–6 of the CDRs will be directly involved in binding antigen, although fewer than this will provide the main binding motifs.

CDR grafting

The first step in humanization is CDR grafting (Figure 2). This involves five steps.

1. Amplification of cDNA for heavy and light variable chain genes of any murine hybridoma is possible using the polymerase chain reaction and a small subset of synthetic oligonucleotide primers with a small level of degeneracy which will amplify all families of murine V genes.
2. Amplified genes are sequenced and synthetic oli-

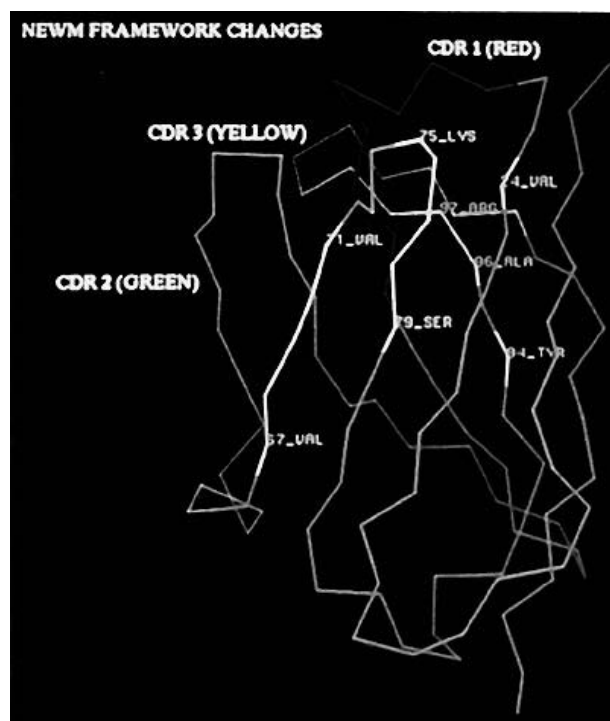


Figure 1 Computer graphic model of the structure of the V_H component of NEWM human antibody. The CDRs loop out from the scaffold structure of the framework regions (blue) to provide the antigen binding surface at one end of the molecule. The framework residues marked are those most commonly involved in maintaining the correct conformation of the CDR loops. (Reproduced with permission from Harris and Cunningham (1995).) (See also color Plate 9.)

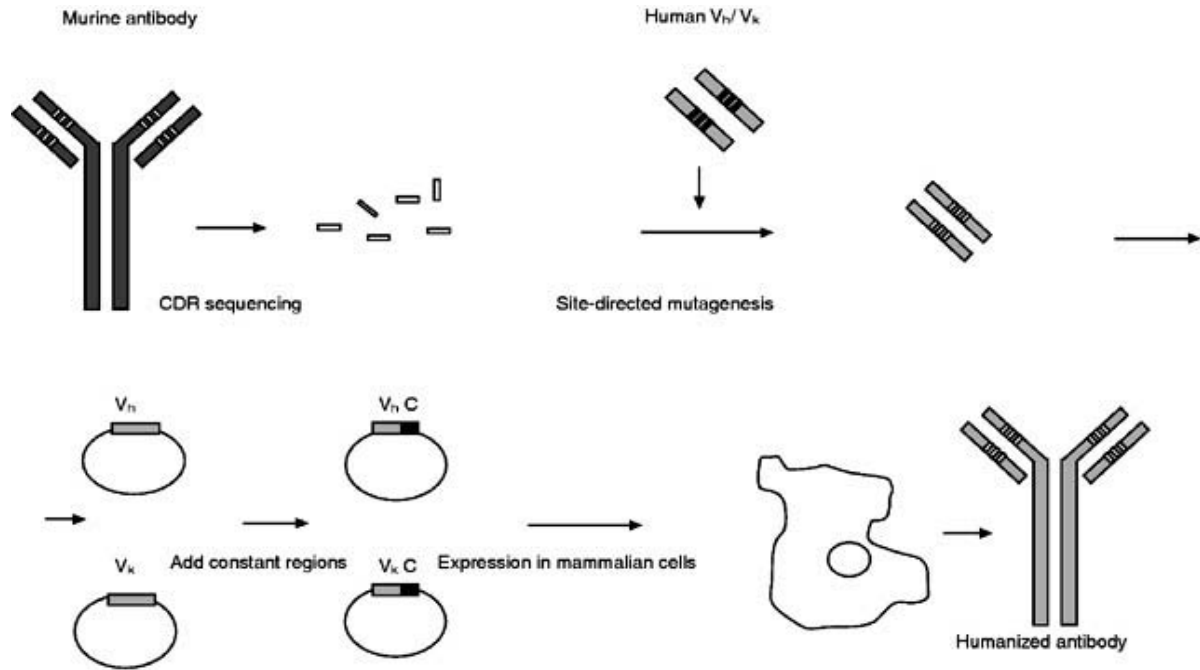


Figure 2 The method of CDR grafting of antibodies. Genes encoding CDR sequences from a donor rodent monoclonal antibody are sequenced. Synthetic oligonucleotides representing these sequences are then chemically synthesized and used to replace the corresponding CDRs in selected previously cloned human acceptor V_H/V_K genes. CDR grafted V_H/V_K are introduced into expression vectors, human constant genes of choice attached and the intact antibody expressed in mammalian cells.

gonucleotides which span the six CDR regions are synthesized. Earlier work selected CDR sequences as defined by Kabat on the basis of linear amino acid sequence homology across a range of antibodies. However, there are discrepancies between these sequences and loop segments and it is better to select those regions which encompass the loop structures.

3. The six synthetic oligonucleotides are synthesized with approximately 15 additional nucleotides at each end which are complementary to the corresponding nucleotides in the human immunoglobulin V genes chosen as acceptor molecules. These regions of homology then provide annealing sites for replacing CDRs in the human antibody with murine CDRs by oligonucleotide-directed mutagenesis.
4. Humanized V_H and V_K genes are cloned into separate expression vectors which include effective promoter and enhancer elements such as immunoglobulin (Ig) signals and human constant domain genes of choice (IgG1, IgG2, IgG3, IgG4), usually as genomic constructs. V_H and V_K vectors are cotransfected for permanent expression into cell lines such as mouse NS0, rat YB20, or CHO cells. Expression levels are of the order of 5–15 µg ml⁻¹.
5. It is usual for an initial CDR-grafted antibody

to have reduced antigen binding affinity compared to the parental rodent monoclonal. A number of alternate constructs are usually made with additional changes, particularly in the V_H framework regions (see below). These modifications are commonly screened by transient expression in COS or CHO-K1 cells.

Methods of humanization

The high-affinity binding of antigen shown by a monoclonal antibody is achieved by the loop structures in the antigen binding site being in the 'correct' conformation. This conformation is determined to some extent by interaction with certain residues in the underlying 'framework' region of the antibody (Figure 1). These loop-framework interactions may not be retained in an initial CDR-grafted antibody and therefore they do not generally have the same binding affinity for antigen as the original murine antibody.

Although the structure of loops can be assigned within a limited number of canonical forms, computer graphic modeling is not sufficiently developed to permit prediction of the 'correct' loop conformation and it is not known which loops provide the main binding energies to antigen. For these reasons

Table 1 Representative examples of humanized antibodies in preclinical and clinical studies

<i>Antibody</i>	<i>Antigen</i>	<i>Company/Institution</i>
<i>Cell surface markers</i>		
OKT3	CD3	Celltech/RW Johnson PRI
YTH12.5	CD3	Cambridge Pathology
UCHT1	CD3	Genentech
OKT4a	CD4	Celltech/RW Johnson PRI
CAMPATH-9	CD4	Cambridge Pathology
LL2	CD22	Immunomedics
Anti-Tac (Zenapax)	CD25	ProteinDesignLabs/Roche
CAMPATH-6	CD25	Glaxo-Wellcome
CDP771	CD33	Celltech/American Cyanamid
M195	CD33	Protein Design Labs
C4G1	CD41a	Protein Design/Yamanouchi
Hp1/2	CD49a	Scotgen/Biogen
YTH34.5	CD52	Glaxo-Wellcome
022	CD64	Scotgen/Medarex
Hu23F2G	CD11/CD18	Icos Corp.
<i>Tumor associated antigens</i>		
425	EGFR	MRC/E Merck
4D5	p185 ^{HER2}	Genentech
A5B7	CEA	Celltech
BW431/26	CEA	Behringwerke
COL-1	CEA	Scotgen/Dow
MN-14	CEA	Scotgen/Immunomedics
CC49	TAG-72	Scotgen/Dow
CDP671	HMFG	Celltech/American Cyanamid
ABL364	Lewis Y antigen	Protein Design Labs/Sandoz
CDP833	Colorectal antigen	Celltech/American Cyanamid
7E11.C5.3	Prostate antigen	Scotgen/Cytogen
<i>Cytokines</i>		
CDP571	TNF α	Celltech/Bayer
AF2	IFN γ	Protein Design Labs
<i>Viral proteins</i>		
RSHZ19	RSV F-protein	Scotgen/SmithKline Beecham
Fd79	HSV-1 gB	Protein Design Labs
Fd138-80	HSV-1 gD	Protein Design Labs
0.5b	HIV-1 _{IIIB} gp120	MRC Collaborative Centre
NM-01	HIV-1 _{MN} gp120	Scotgen/Nissin

EGFR, epidermal growth factor receptor; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IFN, interferon; RSV, respiratory syncytial virus.

Adapted from Adair and Bright (1995).

indirect methods have been developed to restore antigen binding affinity to a humanized antibody.

A major aspect is the selection of human V_H and V_K genes as acceptor molecules. A wide range of adult human immunoglobulin genes have been cloned which represent all seven families of V_H and V_K in the human genome and the entire germ line repertoire of human V_H and V_K has been sequenced. Two general strategies for humanization have been developed to exploit these resources.

1. CDR grafting using human V_H and V_K frameworks with the highest linear amino acid sequence homology to the donor rodent frameworks (homology matching). With this approach, the likelihood of the human framework having the same amino acid residue as the rodent in a 'crucial' position is maximized. Different human V_H and V_K combinations are used with each antibody and may include combinations not used in nature.

Table 2 Representative examples of humanized antibodies in cynomolgous monkeys and humans

Antibody	Doses (mg kg ⁻¹)	Monkeys		Man	
		t _{1/2} (hours)	M anti-mouse response (%)	t _{1/2} (hours)	HAMA
Anti-Tac	0.05–5.0	214	10–20	138	0/12
Anti-TNF α	0.05–1.0	66	10–20	150	0/20
RSHZ19	0.05–5.0	500	10–20	550	0/12
Murine antibody	1.0–10.0	50	100	50	8/10

Adapted from Harris and Cunningham (1995).

- CDR grafting using a limited subset of human framework V_H and V_K combinations selected on the basis of common usage *in vivo* (fixed frameworks). Those most commonly used are human NEWM V_H and REI V_K. These frameworks are used irrespective of linear amino acid sequence homology with the donor rodent antibody.

In both cases a few additional murine framework residues may have to be introduced. These are usually drawn from a small subset of residues 'critical' for framework–CDR interactions. Many have been defined by comparative analysis of X-ray crystallographic studies of antibodies or predicted from computer modeling (Figure 1).

Nearly 100 antibodies have been humanized by these two methods and, if care is taken, antigen binding affinity can be restored with the inclusion of only a few (1–5) additional murine framework residues. *In vitro* antigen binding affinity as measured by ELISA is usually equivalent or 2–5-fold reduced compared with the parental rodent antibody, although higher affinity is occasionally achieved. Evidence is accumulating that *in vitro* ELISA assays are not necessarily an accurate predictor of *in vivo* activity and humanized antibodies with apparent 3–5-fold reduced *in vitro* affinity are equally effective *in vivo*. It is likely that restoration of 'off rate' is a more meaningful predictor and this can now be measured with biosensor technology.

Veneered antibodies

An alternative method of humanization is described as veneering or resurfacing and aims to replace the surface exposed amino acid residues on the rodent monoclonal with corresponding residues from a closely matched human antibody. Computer graphic modeling of the rodent monoclonal is used to identify these surface residues which are unlikely to be involved in maintaining the correct conformation of loops. These are then substituted. The method has

not been widely used. A few examples have been described, although no clinical data is available as yet.

Humanized products

Over 100 humanized antibodies have been described in the scientific and patent literature. They represent a broad range of target antigens including anti-infectives, anticancer, immunosuppressor and immunomodulators for treatment of autoimmune disease. The majority have been humanized as IgG1 molecules. This has been out of choice to maximize the facility for complement-dependent killing and Fc-mediated cytotoxicity. In cases where blocking of target antigen alone is preferred and to reduce binding to Fc receptors on non-target cells, as with CD4 or tumor necrosis factor α (TNF α) targets, IgG4 or IgG1 mutants defective in FcR binding have been produced.

Preclinical and clinical studies with humanized antibodies

Table 1 is a representative list of humanized antibodies advanced in preclinical and clinical studies and Table 2 describes their general properties *in vivo*. Preclinical studies in monkeys have shown no toxicity, extended half-lives and little host immune response. Clinical studies also suggest that humanization has overcome the major clinical problems of rodent antibodies. The inclusion of human antibody constant regions has entirely restored effector function activity. Humanized antibodies have serum half-lives of the order one would expect for human antibodies against the same target antigens. Greatly reduced HAMA response has been observed, although anti-idiotypic responses are sometimes elicited.

As with any drug, therapeutic success requires the target to be carefully chosen. In particular, where the target is a normal body component, the therapeutic

threshold over toxicity due to blockage of normal biological function must be carefully defined. Two of the early humanized antibodies CAMPATH-1H and Anti-Tac did not show efficacy in phase II trials for bone marrow transplantation and graft-versus-host disease, respectively, but are being evaluated for other applications. The clinical effectiveness of humanized antibodies will undoubtedly vary widely but one can be optimistic that humanized antibodies will provide a new class of therapeutics in the coming years.

See also: **Antibodies, antigenicity of; Antibodies, bispecific; Domains, immunoglobulin-type; Gamma-globulin; Immunoglobulin genes; Immunotherapy of tumors.**

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HUMORAL IMMUNITY

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When a foreign substance enters the body, an immunological reaction may occur. Traditionally, immune responses are divided into two different types of reaction: a cell-mediated and a humoral immune response. However, for both types of responses, immunocompetent cells called *lymphocytes* are of major importance. The term humoral response derives from the fact that the antibodies which mediate it are dissolved in body fluids (humor) such as blood, tears, sweat, etc. These antibodies – characteristic of the humoral immune response – are secreted by a subset of lymphocytes called B cells. The other major subset of lymphocytes, T cells, contribute to the humoral response and are largely responsible for cell-mediated immunity.

The first antibodies made by a B cell are not secreted. They are inserted into the plasma membrane, where they serve as receptors for foreign substances. As predicted by the clonal selection theory,

all of the antibody molecules made by an individual B cell have the same antigen-binding site. Once a B cell has bound antigen, it is stimulated to proliferate. Activated B cells will develop into terminal effector cells, called plasma cells and, through a complicated and poorly understood series of events, into memory cells. Plasma cells secrete large amounts of antibody molecules into the blood plasma and the tissue fluid. Memory cells provide long-term protection. They enable an individual who has been exposed to an antigen once to respond more rapidly and efficiently to a second encounter.

Immunoglobulins (Igs) are present throughout the vertebrate kingdom but have not been identified in invertebrates. A basic feature, maintained during evolution, is the four-chain structure, comprising two light (L) and two heavy (H) chains (**Figure 1**). The amino-terminal portion of the H and L chains is the variable, and the carboxy portion the constant

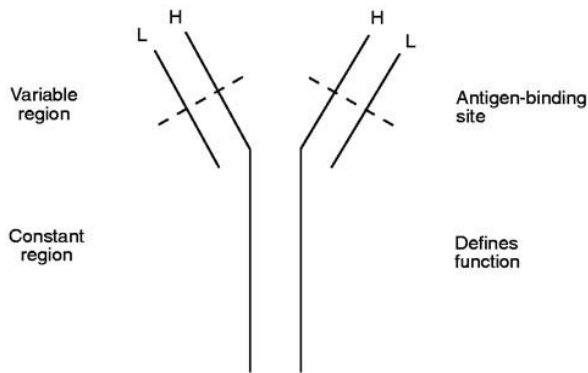


Figure 1 Basic four-chain structure of immunoglobulin.

part of the molecule. The variable regions of H and L chains together form the antigen-binding site.

During the evolution of vertebrates, various mechanisms have evolved to allow the individual to produce an enormous number of antibody molecules with different variable regions and hence of different specificity. The potential repertoire of the mouse could be as high as 10^{10} different molecules.

The L chain constant regions exist in two distinct forms, giving rise to two distinct types of L chains known as κ and λ . The constant region of the H chains define the antibody class and is responsible for a variety of characteristic effector functions. In higher vertebrates there are five different types of H chain constant regions and hence five different classes of antibodies: IgM, IgG, IgA, IgD and IgE. In addition, there are a number of subclasses of IgG. The function of the different H chain classes is shown in Table 1.

During the development of B cells, one particular combination of H and L chain variable regions will be found associated with antibodies of different immunoglobulin classes. Therefore, the same specificity can be attached to functionally different constant regions.

Table 1 Effector function of different immunoglobulin classes

IgM	Early immune response; first class of antibody to appear in serum; complement fixation; stimulation of ingestion by phagocytes
IgG	Produced later in the immune response; major antibody in serum; complement fixation; stimulation of ingestion by phagocytes; placental transfer
IgA	Major class of antibody in external secretions; protects epithelial surfaces from invasion by microorganisms
IgE	Involved in reactions to allergens; triggers inflammation through stimulation of mast cells; induced by parasitic infections
IgD	Very little secreted; function not clear
-	-

Cellular and molecular basis of the humoral immune response

An antigen, e.g. a bacterium, virus or toxin, may stimulate the differentiation of B cells into plasma cells. There are two different pathways by which B cells can be activated to differentiate into plasma cells:

1. *T-independent response.* In this case, B cells will be activated even in the absence of T cells. There are two types of T-independent antigens: T1-1 antigens activate a proportion of B cells polyclonally, irrespective of the specificity of their antigen receptor. T1-2 antigens are normally large polymeric molecules with repeating antigenic determinants (e.g. polysaccharides with identical subunits), such as the capsular antigens of microbial organisms, which directly cross-link the B-cell antigen receptor.
2. *T-dependent response.* In this case, the response depends on both T and B cells recognizing the antigen. In such a response, the differentiation of the B cells depends on the interaction with T cells.

In an immune response which is independent of T cells, the antibodies produced are mainly of the IgM class. Even in a T-dependent response, B cells first develop into plasma cells which secrete IgM antibodies. However, in a T-dependent response, factors secreted by the T cells (cytokines) will direct B cells to switch to other Ig classes: IgG, IgA or IgE. Consequently the B cell product (antibody) will acquire the biological properties characteristic of each Ig class.

Maturation of the immune response

In a T-dependent response, the affinity of the secreted antibody for the antigen increases. This is referred to as affinity maturation of the immune response. Analysis of the primary structure of antibody molecules has shown that the increase in antibody affinity correlates with an accumulation of somatic mutations in the variable region of the antibody molecule. Antigen-activated B cells migrate into the primary follicles of the lymphoid organs. There, proliferation of B cells leads to the formation of germinal centers. During clonal expansion in the germinal centers the hypermutation mechanism which is activated diversifies the B cell repertoire. Intracloal diversity is generated by nucleotide substitutions specifically introduced into the H and L chain variable region genes. Antigen presented on Follicular Dendritic Cells (FDC) permits selection of the few high-affinity variants which may then differentiate into memory and plasma cells. In this way through hypermutation and selection an affinity maturation

of the immune response is ensured and the affinity of the secondary response antibodies is generally higher than that of the primary response antibodies. The immune system 'learns' from the first contact with an antigen, so that at a second encounter the humoral immune response will not only be of greater quantity but also of greater quality.

Function of the humoral immune response

Among the major functions of a humoral immune response are:

1. Neutralization of either toxin or virus.
2. Agglutination of bacteria and other pathogens followed by phagocytosis.
3. Activation of the complement system via antigen-antibody complexes, which leads to cell death through lysis or phagocytosis or to acute inflammatory processes.
4. Antibody-dependent cell mediated cytotoxicity (ADCC).
5. Protection from microorganisms by preventing their attachment to epithelial cells.

Antigen-antibody complexes activate complement

When IgM and most subclasses of IgG (human: IgG3 $\geq$ IgG1 $\gg$ IgG2, mouse: all IgG subclasses except IgG1) bind to foreign antigens, they may trigger the classical complement pathway. Three major effector mechanisms are activated by complement:

1. *Lytic pathway of complement.* Following complement activation by an antigen-antibody complex, a cascade of events is initiated; this culminates in the lysis of the attacked cells.
2. *Immune adherence and phagocytosis.* Phagocytic cells have specific receptors for the complement cleavage product C3b on their surface, which promotes the process of immune adherence and phagocytosis of microorganisms.
3. *Acute inflammatory reaction.* Along the reaction sequence of the complement system, cleavage products of complement proteins are generated and released. These cleavage products have chemotactic activity and attract large numbers of phagocytic polymorphonuclear leukocytes, which lead to the development of an acute local inflammatory reaction.

Phagocytic response

The principal phagocytes of the blood – the granulocytes – can bind and ingest foreign particles,

especially those which have been 'opsonized' by binding antibodies and/or complement. Human antibodies of the subclasses IgG1 or IgG3 directly act as opsonins, whereas antibodies of the IgM class only enhance phagocytosis in the presence of complement. In the first step, the variable region of the antibody molecule binds specifically to the surface of the pathogen or parasite. The constant region of the IgG H chains will then attach to specific receptors (Fc receptor, Fc γ R) on the surface of the phagocyte. In the case of antibodies of the IgM class, immune adherence takes place via an antibody-complement complex. A cleavage product of complement (C3b) binds to specific receptors on the phagocytic cell (CR1, CR2), thus completing a bridge between bacteria and phagocytic cells. The microorganisms can then be phagocytosed. Similar mechanisms apply to tissue phagocytes (e.g. macrophages, Kupffer cells, microglia, etc.).

Antibody-dependent cellular cytotoxicity

Various cell types, which express Fc receptors, including natural killer cells, are capable of lysing target cells. However, lysis will occur only when the target cell is precoated with specific antibody. Natural killer cells express a low affinity receptor (Fc receptor, Fc γ RIII or CD16) with specificity for determinants in the constant part of the IgG H chain. Recognition of the IgG bound to the target cell through the Fc receptor will signal the cytolytic pathway. Eosinophils express Fc receptors with specificity for determinants in the constant part of the IgE H chain. In this way parasites, such as helminths, which induce an IgE response, can be killed by eosinophils.

Protective function of IgA antibodies

Immunoglobulins are found not only in serum but also in various body secretions such as saliva, sweat, milk, tears, and respiratory and intestinal secretions. Binding of an IgA to the Poly Ig receptor enables IgA antibodies to be transported across mucosal tissues into secretions. Hence IgA is the predominant immunoglobulin class in the external secretions of most species. By combining with microorganisms, it prevents their attachment to mucosal or epithelial surfaces and thus inhibits invasion of the body.

Aberrant humoral responses

Self-directed humoral immunity

Immunological tolerance is the process that normally prevents lymphocytes from reacting with self antigens. Under certain circumstances, immunological tolerance breaks down and autoimmune disease

results. Humoral antibodies to self antigens on cell surfaces can lead to cell death by direct complement-mediated cell lysis, by ADCC or by opsonization. Antibodies directed against hormones or against cell surface receptors may interfere with cell function. One such example is the autoimmune disease myasthenia gravis, where antibodies react with the receptor for acetylcholine at the neuromuscular junction.

In addition in autoimmune diseases such as systemic lupus erythematosus, the continued production of autoantibody to self antigens leads to the formation of large amounts of immune complexes which are deposited in various tissues. These complexes activate the complement cascade, which leads to acute local inflammation at the site of deposition. Various serious diseases such as vasculitis, nephritis, endocarditis or rheumatoid arthritis may be the result.

Immediate-type hypersensitivity reaction

The immediate-type reaction, in contrast to delayed-type hypersensitivity, is always a humoral immune response. In an organism sensitized by a given allergen, a new contact with the allergen will lead to an immediate reaction. The localization of the allergen by specific antibodies of the IgE class (reagin) on to mast cells leads to the activation of the mast cells. The resulting degranulation of the mast cells releases mediators such as histamine, serotonin, heparin and proteolytic enzymes. The result is an immediate inflammation reaction which may range from a local skin reaction to a potentially lethal anaphylactic shock.

Manipulated responses

Passive immunization

Protection can be transferred passively by injection of specific antibodies. This effect of humoral immune protection was first realized by Kitasato and Behring who treated diphtheria in children by using sera of animals immunized with the toxins. The duration of protection depends on the amount of antibody injected and on the half-life of the class of immunoglobulins introduced.

One important application for passive immunization is the rhesus (Rh) prophylaxis, whereby Rh-negative mothers are injected with anti-Rh antibodies *post partum*. This prevents the activation of the mother's immune system by Rh-positive blood cells of the newborn child.

A natural passive immunization occurs during pregnancy when IgG antibodies are transferred through the placenta into the fetus, thus providing humoral immune protection. A passive immunization is also given by antibodies of the IgA class in milk and colostrum, which may protect the gastrointestinal tract of the newborn.

Active immunization

The immune system can be directly stimulated by injection of an antigen into the organism. Such an active immunization is referred to as vaccination. In a humoral response, B cells will be activated and differentiate into plasma cells and memory cells. Thus long-term protection will be achieved. The enormous power of vaccination can be seen in the fact that by this means diseases such as polio or diphtheria can be controlled or even, in favorable circumstances, eradicated (smallpox).

See also: **Acute inflammatory reaction; Affinity; Affinity maturation; Agglutination; Antibody-dependent cellular cytotoxicity; Antigen-binding site; Autoimmunity; Mast cells; B lymphocyte differentiation; Cell-mediated immunity; Helper T lymphocytes; Complement receptors; Cytokines; Diversity, generation of; Fc receptors; Germinal center; Hypersensitivity reactions; Immune adherence; Immunoglobulin class switching; Immunoglobulin, functions; Immunoglobulin structure; Lymphocytes; Memory, immunological; Neutralization of biological reactions by antibodies; Opsonization; Phagocytosis; Vaccination, methods of administration.**

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HYBRIDOMAS, B CELL

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Historical background

Hybridoma production has as its background the joint application of two independently derived biological research tools. The first of these was the availability of induced monoclonal plasmacytomas in the mouse, for which Potter is primarily responsible. The availability of these lines and their ability to be transplanted *in vivo* and their subsequent adaptation to *in vitro* culture and fusion with normal B cells are described below. The second earlier-derived technology was that of cell fusion in which two somatic cells could be fused, resulting in the production of hybrid cells. The frequency of isolation of hybrid lines was then greatly facilitated by the use of selective media described by Littlefield, coupled with advances in the use of new fusion-inducing agents such as Sendai virus, lysolecithin and polyethylene glycol.

The origins of monoclonal antibody-producing hybridomas sprang from attempts to study immunoglobulin (Ig) gene expression and regulation in plasmacytoma cells. First, when two immunoglobulin-producing plasmacytomas were fused, both sets of immunoglobulin light and heavy chains continued to be expressed by the hybrid cells, resulting in 1) formation of hybrid molecules, 2) the failure of new immunoglobulin chains to be produced, and 3) that when a variant plasmacytoma that no longer expressed either of the wild-type light or heavy immunoglobulin chains was fused with an immunoglobulin-producing line, the nonproducer did not switch off immunoglobulin production by the other parental cell but continued to support the production of immunoglobulin from the other parent.

General aspects of hybridoma production

The immune system is capable of mounting an immune response to an almost limitless number of different antigens. Within the antibody response to each simple or complex antigen, many clones with differing specificities, affinity and avidity can be activated to antibody production by the multiple antigenic determinants (epitopes) of an immunogen. Few, if any, true monoclonal antibody responses occur in responses to antigens, although in some

responses a very restricted number of B cells clones may be engaged in antibody production.

In 1976 Köhler and Milstein described details of a powerful method in which B cells secreting antibody of one specificity could be fused with a continuously growing plasmacytoma. Clones of hybrid cells derived in this way, which express the immunoglobulin genes from both the normal antibody-forming B cell and the plasmacytoma, inherit the potential for unlimited growth from the continuously growing plasmacytoma parent and the genetic material determining a given antibody specificity from both antibody-secreting parents. The term hybridoma was coined to describe the fusion product of a normal antibody-forming cell with a plasmacytoma; for this important scientific advance Köhler and Milstein received the Nobel Prize for Medicine or Physiology in 1984.

As illustrated in **Figure 1**, when an animal is immunized with a given antigen, the antibody products secreted by the array of B cell clones triggered by the various epitopes on the immunizing antigen subsequently appear as a heterogeneous mixture in the immune serum. However, by constructing hybridomas from a member of each epitope-specific clone and then cloning these hybrids, monoclonal antibodies can be separated from other monoclonals with specificities toward the same or other epitopes on the same antigen. It is then possible to isolate a number of monoclonal antibodies specific for different epitopes on the same molecule or immunogen. Such highly specific antibodies can be used to probe the structure and function of different parts of molecules and various cell types without need for immunoadsorptions, and at the same time a virtually unlimited supply of antibody can be obtained that is constant in its specificity and affinity over time.

Hypoxanthine, aminopterin, thymidine (HAT) metabolism and selection procedures

In fusion procedures between two cell populations the frequency of fusion is low (1 per 10^4 of the lymphoid cells). Therefore, in order to select against the neoplastic plasmacytoma or lymphoma lines used in B cell hybridoma formation, a strategy must be employed to kill the nonfused cells because they have the potential for continuous *in vitro* growth and would eventually overgrow the minority hybrid cells.

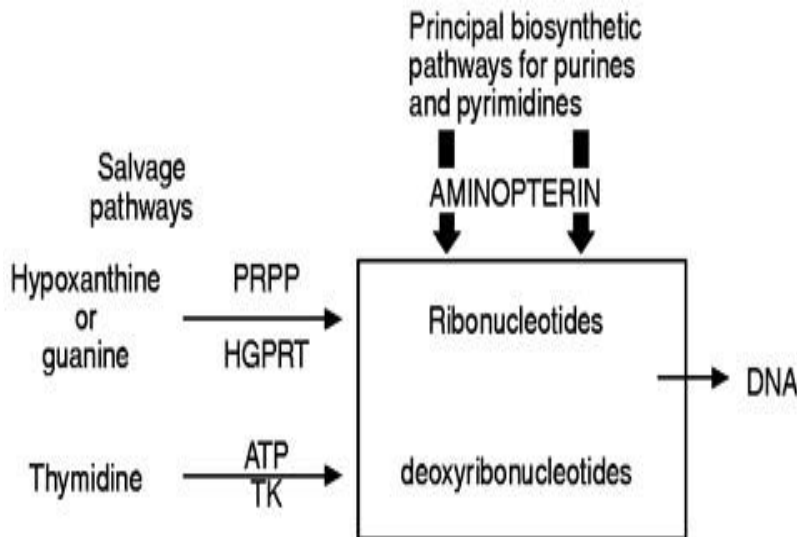


Figure 1 Comparison of conventional anti-sera production *in vitro*. By cloning the B cell-derived hybridoma lines, the antibody (Ab) products of each individual clone can be separated and propagated indefinitely. In this way individual epitopes (Ep) of an antigen (Ag) can be analyzed because of the monospecificity of monoclonal antibodies.

The method most commonly in use was developed by Littlefield and relies on the initial isolation of mutant plasmacytomas (Figure 2) lacking the enzymes responsible for the reincorporation of purines and pyrimidines into DNA and RNA (Table 1). Cells that have mutations resulting in the functional absence of these enzymes will be the only cells to survive when these toxic base analogs are incorporated into the growth media. Thymidine kinase-negative (TK⁻) or hypoxanthine guanine phosphoribosyl transferase-negative (HGPRT⁻) plasmacytoma cells can be selected, cloned and isolated in this way.

Once such mutant lines have been isolated it is

Table 1 Toxic base analogs and associated enzyme deficiency

Analog	Enzyme deficiency
6-Thioguanine (TG)	Hypoxanthine guanine phosphoribosyl transferase
8-Azaguanine (AG)	Hypoxanthine guanine phosphoribosyl transferase
Bromodeoxyuridine	Thymidine kinase

possible to select against the growth of these enzyme-deficient plasmacytoma lines by forcing them to use the salvage pathway for DNA synthesis. This is achieved by using the folic acid analog, aminopterin, which blocks the normal pathway for purine and pyrimidine synthesis, as shown in Figure 2. The plasmacytoma is then forced to use the salvage pathway but because the mutants lack one of the necessary enzymes they do not survive in the HAT medium.

The genetic defect in such mutant plasmacytoma lines that results in the absence of a functional salvage pathway is of little importance during growth of these cells in normal medium because these enzymes are involved *only* in salvage pathways synthesis, and under normal conditions this alternative route of nucleotide synthesis is unnecessary. Spleen cells, on the other hand, can synthesize a complete set of salvage pathway enzymes and, when fused to the HGPRT⁻ or TK⁻ plasmacytoma cells during hybrid formation, enable the hybrid cells to produce sufficient HGPRT or TK for the fused cells to survive in the HT medium which contains hypoxanthine and thymidine. In this way, nonfused plasmacytoma cells are killed, while the hybrids resulting from fusions with normal cells continue to grow. In contrast,

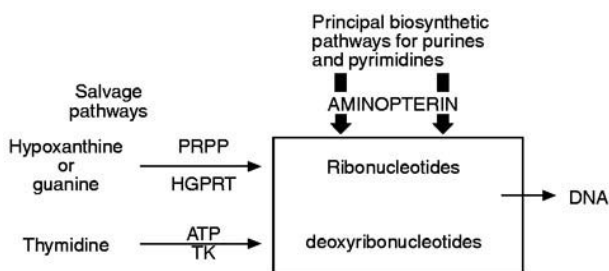


Figure 2 HAT medium metabolism: principal and salvage pathways involved in the synthesis of DNA. The toxic base analog described in Table 1 can be incorporated by the salvage pathways. In mutant lines lacking either TK or HGPRT such bases cannot be incorporated and are therefore nontoxic for such mutant lines and these cells live. Growth of these mutants will not occur when aminopterin is used to block the principal pathway of synthesis of purines and pyrimidines because both enzymes are necessary. Only those hybrid cells that contain normal levels of HGPRT from the nonmutant parental B cell partner will be able to live in HAT medium and the remaining unfused myeloma cells die. HGPRT, hypoxanthine guanine phosphoribosyl transferase; PRPP, 5-phosphoribosyl-1-pyrophosphate; TK, thymidine kinase.

normal unfused B cells have only a limited lifespan *in vitro* because they are not transformed, do not proliferate and eventually die.

Choice of fusion partner

Since the hybridoma cells resulting from cell fusion have the capacity to continue synthesis and secretion of both sets of immunoglobulin light and heavy chains from the fusing partners, scrambled hybrid molecules can be synthesized, which may lead to a corresponding decrease in specific antibody titer and/or creation of new specificities by the combination of inappropriate light and heavy chains. This problem can be avoided by using a plasmacytoma for fusion that has lost the ability to synthesize its own immunoglobulin chains.

Practical immunization procedures

Lymphoid cells from a variety of sources including spleen and lymph nodes have been used in the formation of useful antibody-secreting hybridomas. Most foreign proteins, polysaccharides or whole cells can be administered with or without adjuvants by a variety of routes. Priming immunization is generally followed by several subsequent immunizations at various intervals. Between 1 and 2 days after the last immunization, the spleen cells are removed and fused with the chosen plasmacytoma line.

Protocols for immunizations vary considerably between investigators and depend to a large extent on the quantities and the type of antigen(s) used. The use of lymph node cells has one advantage in that a high proportion of IgG-producing hybrids is obtained. These antibodies are preferable because of the greater ease in preparation and isolation of IgG antibodies over IgM monoclonals. The preparation of hybridomas, subsequent screening, testing for specificities and production of monoclonal antibodies are illustrated in Figure 3.

Heterohybridomas

Interest in producing monoclonal antibodies from species other than mice has been high and there have been reports of the use of murine plasmacytoma cells to construct interspecies hybridomas secreting non-mouse antibodies of human, rat and rabbit origin. However, the utility of these hybridomas is limited by the rapid chromosome segregation that characteristically occurs when cells from differing species are fused. With interspecies antibody-secreting hybridomas made with mouse plasmacytomas, the human, rat and rabbit chromosomes are lost preferentially. These chromosome losses appear to be related to the degree of phylogenetic disparity between the fusing species. Factors causing this chromosome loss are not well understood but may involve preferential loss of human chromosomes derived from the slower-growing human parental cells. Other factors that may lead to chromosome loss include faulty interaction between chromosomes and spindle fibers of disparate origin resulting in preferential loss of nonmouse chromosomes.

In contrast to the difficulties involved in the use of these interspecies hybridomas for actual antibody production and isolation, this preferential segregation of one set of chromosomes has been extremely useful in determining the chromosomal location for certain structural genes. By monitoring the loss of human chromosomes by cytochemical techniques, accompanied by chromosome banding and identification, it has been possible to associate certain chromosomes with appropriate phenotypic expressions. Location of genes to particular chromosomes can then be made and in this way the human heavy chain cluster of genes has been mapped to human chromosome 14 and, by a modified method using cDNA probes on these interspecies hybrids, the assignment of the light chain and loci was located to specific mouse chromosomes.

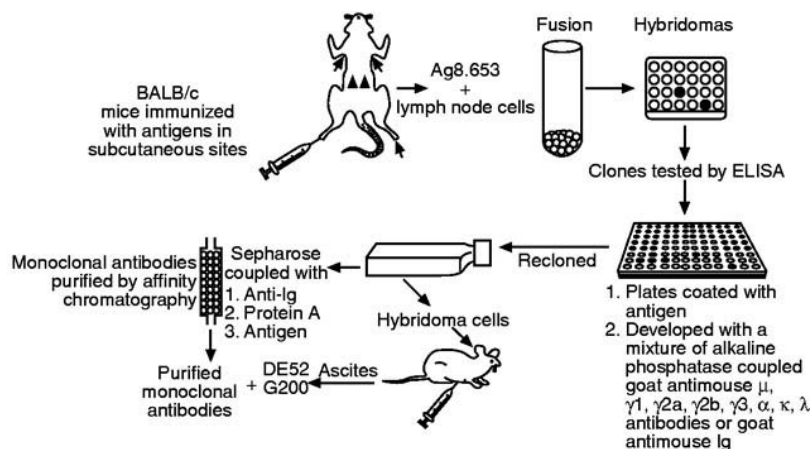


Figure 3 Outline of steps involved in production of monoclonal antibodies.

Although attempts have been made to produce cell lines from other species that would be useful in the isolation of stable hybridomas from these same species, these attempts have been met with little practical success. Recently however plasmacytomas have been derived from rabbits transgenic for *c-myc* and *v-abl* and used to make fusion partners for B cells derived from immunized rabbits. Rabbit-rabbit antibody-producing hybridomas that are stable and appear to secrete specific antibodies have been made using this cell line.

Examples of hybridoma use

B cell diversity

Although related to the production of specific monoclonal antibodies, the ability to construct B cell hybridomas has also led to a greater understanding of the genetic mechanisms of the generation of antibody diversity. An extremely informative use of hybridomas has been in the analysis of the diversity of antibodies in immune responses.

Epitope recognition by monoclonal antibodies

As already discussed under general aspects of hybridoma production, the unique attribute of a monoclonal antibody is its ability to recognize one epitope on a complex antigenic structure. This provides a powerful tool for biological analysis of complex structures. However, monoclonal antibodies may sometimes cross-react with unrelated molecules due to sharing of three-dimensional molecular structure between different epitopes. Thus it is important that molecular identification be made not solely on reactivity with one monoclonal antibody. Other means of characterization should also be used. On the other hand, cross-reactivities may lead to the identification of important structural and biological relationships between macromolecules.

See also: Antibodies, secretion; Cryopreservation of immune cells; Epitopes; Hybridomas, T cell; Limiting dilution analysis; Monoclonal antibodies (mAbs); Specificity.

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HYBRIDOMAS, T CELL

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The experimental problems in T lymphocyte biology include the low frequency of specific T cells and the minute amount of active T cell products. These have been circumvented by the isolation and propagation of T cell lines and clones of defined specificity. Two basic strategies have been devised. The first is to clonally expand normal immune T lymphocytes by repetitive stimulations with appropriate antigens and antigen-presenting cells. The other, involving T cell hybridoma technology, is to immortalize normal T lymphocytes by fusion with continuously replicating tumor cells. Since the fusion can randomly immortalize T cells independently of their antigen specificity and genetic restriction, the resultant T cell hybridomas may represent the initial T cell repertoire with less bias than T cell clones, although there is no direct evidence for this. The advantage of the hybridoma technique over T cell cloning is the relative ease in obtaining large numbers of T cells of interest (without contamination with other types of cells) and their biologically active products. Thus, T cell hybridomas have provided us with a source of lymphokines and other regulatory molecules, together with their mRNA and DNA. They have also proved useful in evaluating T cell receptors and their antigen recognition mechanism. Furthermore, some murine T cell hybridomas specific for certain autoantigens can induce autoimmune diseases after adoptive transfer.

Generation of T cell hybridomas

The methods used to generate T cell hybridomas are essentially the same as for B cell hybridomas. Antigen-specific normal T cells are fused to a T cell tumor line by means of a fusion-inducing agent. Hybrids between normal T cells and tumor cells are screened for hybridomas of interest and then cloned.

Choice of T lymphoma line

Normal T cells are fused to homologous tumor partners because of the instability in interspecies hybrids. Table 1 summarizes the murine and human T lymphoma lines that have served as the tumor partner in successful fusion experiments. The most common is the murine AKR thymoma BW5147, which has yielded hybridomas with different functions, despite its Lyt-2 phenotype, which is characteristic of a cytotoxic/suppressor T cell. Besides the ability to yield functional and stable hybridomas efficiently, the tumor lines are required to possess a drug marker that makes it easy to select hybrids of normal and tumor cells. A commonly used selective marker is sensitivity to medium containing hypoxanthine, aminopterin and thymidine (HAT). Other selection methods have also been devised (see below).

Table 1 Tumor cell lines used for fusion with T cells

Cell line	Origin	Marker	Selection
BW5147	AKR/J thymoma	HGPRT	HAT
BW1100.129.237*	[BW5147]	HGPRT	HAT
AKR-A	AKR/J thymoma	HGPRT ⁻	HAT
L5178	DBA/2 leukemia	HGPRT ⁻	HAT
EL-4.BU	C57BL/6 T lymphoma	TK	HAT
CEM-T15	Human leukemia	HGPRT	HAT
CEM-AG ^R	Human leukemia	HGPRT	HAT
CEM-T3	Human leukemia	HGPRT ⁻	HAT
CEM-6	Human leukemia	HGPRT	HAT
Jurkat-6TG-3	Human leukemia	HGPRT	HAT
Hut102-B2	Human leukemia	HGPRT	HAT
DIR11(KE37)	Human leukemia	TK ⁻	HAT
CEM	Human leukemia	—	EA
MOLT-4 or Jurkat	Human leukemia	—	Soft agar

HGPRT⁻, Deficiency of hypoxanthine-guanine-phosphoribosyl transferase (HGPRT); TK⁻, deficiency of thymidine kinase (TK); HAT, hypoxanthine, aminopterin and thymidine; EA, emetine and actinomycin D.

*This line, derived from BW5147, lacks the genes of both T cell receptor α and β chains.

Enrichment of specific T lymphocytes

Since there are very few cells specific for any one given antigen in a population of immune-lymphocytes, it is crucial to enrich those T cells prior to fusion. Antigen-specific helper T cells recognize a processed antigen in the context of class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs), and proliferate. Therefore, they are enriched by restimulating immune T lymphocytes with the antigen and appropriate APC. Cytotoxic T lymphocytes (CTLs) are enriched by restimulation with the specific target cells in the presence of interleukin 2. Although the nature of the cell type that actually fuses is not known, activation of the immune lymphocytes appears to select cells that are favored for the fusion. Thus, some of the procedures described above can serve to enrich cells that are not only specific for antigens but also amenable to fusion.

Cell fusion

Cells are fused by means of biological, chemical or physical methods. Among them, the chemical method, which uses polyethylene glycol (PEG) as a fusogen, is the most common.

PEG method All procedures must be done at 37°C, and the medium must contain no serum because PEG precipitates protein. First, the mixture of normal and tumor T cells is washed in serum-free medium and pelleted. Next, 50% PEG solution is added dropwise, and the mixture is gently stirred. This maximizes cell contact with PEG, resulting in many fusion products. Then, warm serum-free medium is added slowly to dilute the PEG gradually without lysing the cells. Finally, the cells are centrifuged, resuspended in medium with 10% fetal calf serum (FCS), and dispersed in 0.2 ml aliquots into 96-well plates. Successful fusion is influenced by lot-to-lot variation in PEG and FCS, requiring tedious batch testing.

Electrofusion Cell fusion can also be induced by a physical method, which uses an electric pulse. This method, compared with PEG, is highly efficient, but needs a special device. Cells are suspended in fusion buffer (0.2 mM Tris-HCl, pH 7.2, containing mannitol, CaCl₂ and MgCl₂) and transferred into a fusion chamber. The cells are brought together in a low-amplitude alternating electric field (300 V cm⁻¹ for 5 s), and then subjected twice to a high-amplitude short pulse (3000 V cm⁻¹ for 10 μs), which causes an electrical breakdown of cell membranes. Cell fusion results when the membrane breakdown occurs in areas of cell-cell contact. The cells are cultured

overnight in a 60 mm dish, and then dispersed in 0.2 ml aliquots into 96-well plates.

Selection of hybrid cells

Fusion is a random process. Fusion of a population of normal and tumor T cells results in a mixture of normal-normal, normal-tumor and tumor-tumor hybrid cells together with unfused cells. Since normal T cells have a limited growth potential, most normal-normal hybrids (and normal unfused cells) die before 2 weeks of culture. Therefore the selection methods have only to kill tumor-tumor hybrids (and unfused tumor cells) and allow normal-tumor hybrids to keep growing. The most common method is HAT selection.

HAT selection When the parental tumor cells lack an enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) or thymidine kinase (TK), selection is accomplished by culturing the fusion mixture in HAT medium. Aminopterin blocks the *de novo* synthesis of DNA. To survive in HAT medium, cells must make DNA via the so-called salvage pathway, in which hypoxanthine and thymidine are incorporated into DNA by means of HGPRT and TK, respectively. Therefore, tumor cells lacking either of these enzymes cannot grow in HAT medium, unless they fuse with normal cells provided with the required enzymes. Thus, the only cells able to grow in HAT medium are the normal-tumor hybrids. Preparation of mutants lacking these enzymes involves first mutagenizing T cell tumor lines and then exposing them to toxic base analogs that are incorporated into DNA via the salvage pathway. The toxic agents used are 8-azaguanine (or 6-thioguanine) and bromodeoxyuridine for the mutants lacking HGPRT and TK, respectively. The drug-marked variants listed in Table 1 have been selected and isolated in this way. These cell lines, however, can possibly regain enzyme activity, resulting in revertant cells that are no longer susceptible to HAT medium. Therefore it is recommended to maintain periodically the tumor cells in the presence of the appropriate toxic base analog.

Emetine and actinomycin D selection Tumor cells are treated with emetine and actinomycin D before fusion. Emetine inhibits protein synthesis in mammalian cells at the trans-location that involves movement of mRNA along a ribosome, and actinomycin D inhibits RNA synthesis irreversibly. The tumor cells treated with these drugs therefore die of ribosomal depletion, unless they fuse with normal untreated cells and are provided with the devices required for replication. Thus, only normal-tumor

hybrids are permitted to grow. Although this method is applicable to any tumor cell line, the optimal conditions of the treatment must be carefully determined for each line. This method seems to yield hybrid cells more frequently than HAT selection (10^{-4} versus 10^{-6}).

Soft agar selection An alternative method is culturing cells in soft agar after fusion. This strategy is based on the ability of T cell hybridomas to form colonies in the soft agar, whereas neither fusion partner forms colonies. By using this selection, T cell hybridomas have been successfully established from the fusion between antigen-specific normal human T cells and HAT-resistant human T lymphoblastoid cell lines such as MOLT-4 or Jurkat.

Screening of specific hybridomas

The method of selecting a few specific hybridomas out of a great number of hybrid cultures must be fast and simple enough to allow screening of many samples, and it must be unequivocal. In principle, the methods that serve to enrich specific T cells mentioned above can also be used for selecting the antigen-specific hybridomas. Thus, antigen-specific helper hybridomas are screened by examining their lymphokine-producing capability in an antigen-specific and MHC-restricted activation. Cytotoxic hybridomas are selected by a standard CTL assay using an appropriate target.

Cloning

Cloning, the initiation of a cell line from a single progenitor, can be achieved either in soft agar, by limiting dilution, or by using a cell sorter. Cloning by limiting dilution is performed by culturing hybridoma cells in 96-well plates at <1 cell per well together with feeder cells. Even on this condition, a significant proportion of wells receive two or more cells and hence cannot initiate a true clone; therefore cloning must be repeated to ensure the homogeneity of the hybrid lines. To verify the clonality it should be demonstrated that their T cell receptors are homogeneous at the level of proteins, mRNA or genes.

Problems of T cell hybridomas

Despite the advantages mentioned above, several

experimental problems with T cell hybridomas have made them less useful than continuous T cell clones. First, T cell hybridomas, particularly human hybrids, show a greater tendency to be unstable in their function during culture. This may be due to a rapid loss of chromosomes or somatic mutations. Therefore it is essential to cryopreserve large numbers of vials of cloned hybridomas as well as to repeat recloning. Another problem to be borne in mind is that T cell hybridomas are not necessarily the faithful models of normal antigen-specific T cells. Even pairs of cloned normal T cells and T hybridoma cells derived from them (i.e. fusion products of an established T cell clone and tumor cells) show a number of differences in activation requirements, affinity, lymphokine secretion and effector functions. These differences may result from the use of T cell receptor genes of the tumor fusion partner, from different gene regulation in the hybridomas, or from differences in the screening methods for preparing them. Thus, despite the validity of T cell hybridomas as models of the corresponding T cell clones, caution must be used in such an extrapolation. To minimize such differences, parental tumor cell lines that lack T cell receptor genes have been devised (Table 1).

Cell lines are available from various sources, including the American Type Culture Collection (ATCC) [Phone 301 881 2600; Fax 301 816 4367; Internet <http://atcc.org>] and the European Collection of Animal Cell Cultures (ECACC).

See also: Antigen-specific cells, enrichment and isolation; Helper T lymphocytes; Cryopreservation of immune cells; Cytotoxic T lymphocytes; Hybridomas, B cell; Limiting dilution analysis; MHC restriction; T lymphocyte activation.

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HYDROSTATIC PRESSURE, EFFECT ON IMMUNE SYSTEM

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In most eukaryotic systems, thermodynamic parameters such as temperature, volume or pressure are kept constant or allowed to modulate in a well-controlled narrow range. Temperature or pressure can be used for induction of preconceived irreversible changes in constitution and structure of biological tissues, like the cell plasma membrane, which could be then applied in an *in vivo* system (e.g. vaccination). In this respect, pressure is the method of choice, as it does not involve chemical modification and is generally applied under isothermal conditions.

Pressure can operate either as a transient vector (e.g. pressure difference across a membrane) or as an intrinsic scalar (i.e. hydrostatic pressure). Biological processes related to vectorial pressure include gas equilibrium across biological membranes (e.g. oxygen-carbon dioxide exchange in the lung alveoli or cell rupture upon sudden change in pressure) and osmotic regulation of membrane transport. Hydrostatic pressure is an internal thermodynamic factor which is in equilibrium with the ambient atmospheric pressure. A change in the latter by a magnitude of ΔP is reflected in a change in the internal energy of the system by $\Delta P \cdot \Delta V$, where ΔV is the overall change in volume. When a heterogeneous system, such as a biological tissue, is subjected to external hydrostatic pressure, only the compressible elements, i.e. those where ΔV is significant, will comply with pressure. Compartments with low compressibility ($\Delta V \sim 0$) will remain practically unaffected.

Compressible compartments

Water, solutes and most soluble macromolecules are of low compressibility under hydrostatic pressures below 1000 atm. Compression of biological membranes at this range originates predominantly from intermolecular free space, which is characteristic of lipid and protein assemblies. The lipid layer in biological membranes is readily condensed by pressure. As a result, the free space between the phospholipid molecules is reduced and the lipid structure becomes more ordered and viscous with respect to the magnitude of the applied pressure. In the extreme case, the lipid can undergo a phase transition to an ordered

gel phase, which is highly dense and therefore of low compressibility. Upon decompression, the lipid layer returns practically to its initial state.

Protein assemblies and multimers also include appreciable free space which can be released under pressure. In this case, the overt effect of pressure is not gradual, as in the case of lipids, but is quite abrupt in correspondence with the pressure threshold which induces dissociation to monomers. Reduction of pressure below this threshold level will then allow the proteins to reassemble.

When hydrostatic pressure in the range of 1000–1500 atm is imposed on cells, the increase in order and microviscosity of the membrane lipids is similar in magnitude to that induced by doubling the level of membrane cholesterol. The cytoskeletal network, which is composed of microtubules and microfilaments, is almost completely dissociated to form soluble monomers. As a result, some of the membrane proteins are displaced vertically, and a part of them is shed off into the aqueous domain, due to excessive displacement and dissociation from the cytoskeletal anchorage. Most other membrane proteins aggregate nonspecifically to form segregated domains (Figure 1). When a slow decompression is allowed, the affected cellular systems readjust with respect to the ambient pressure through a series of equilibria until atmospheric pressure is attained. Along with decompression, the protein aggregates will dissociate in part, while proteins of some affinity will remain associated as the cytoskeletal boundaries are reassembled.

Another, somewhat unexpected, effect of hydrostatic pressure is on protein synthesis. Similarly to protein assemblies, complexes between various suppressors and genetic matrices can dissociate under pressure and immediately evoke protein synthesis. This process can be classified as stress induced (e.g. analogously to heat shock protein (hsp) synthesis). Some of the pressure-induced proteins presumably take part in the surface presentation of antigens, as shown in Figure 2, and thus contribute to the apparent immunogenicity. The modulation in surface antigens which follow such a process, in particular the overt augmentation of major histocompatibility complex (MHC) presentation, is a combination of lateral and vertical displacements, presented in Figure 1,

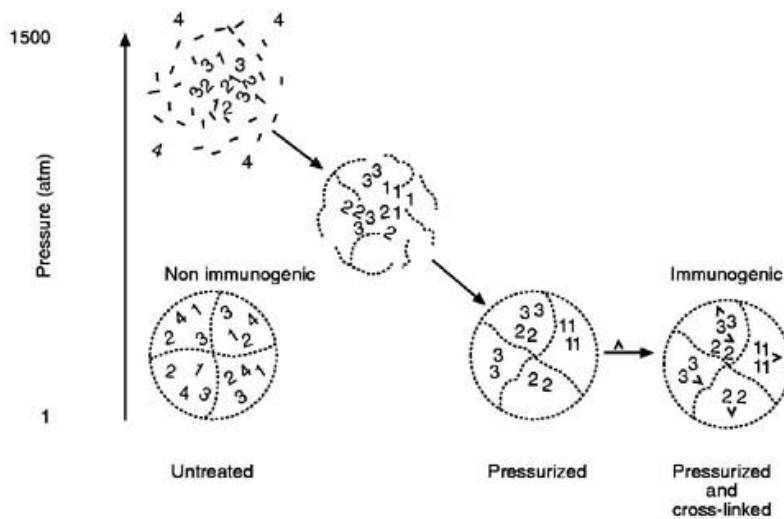


Figure 1 Structural changes on the cell surface induced by high hydrostatic pressure. Four schematic membrane domains ('unit membranes'), separated by cytoskeletal boundaries, are displayed. Under high pressure (as exemplified by 1500 atm), the boundaries are fully disintegrated, and the lipid regions become highly ordered and dense (not shown). Some proteins are then shed off to the outer space (4), while most other proteins are aggregating into segregated domains. Gradual release of pressure will allow the lipids to regain their fluid nature and protein aggregates to dissociate, except for proteins with some affinity (presented in the figure as proteins of the same kind) which will remain associated. The cytoskeletal boundaries which reassemble with release of pressure will maintain the new topology as long as membrane biogenesis has not been effective (up to about 24 h). Chemical cross-linking can fix the new protein topology in pressurized cells. This is the basis of the technology of combining hydrostatic pressure and cross-linking (PCL) for the preparation of tumor vaccines.

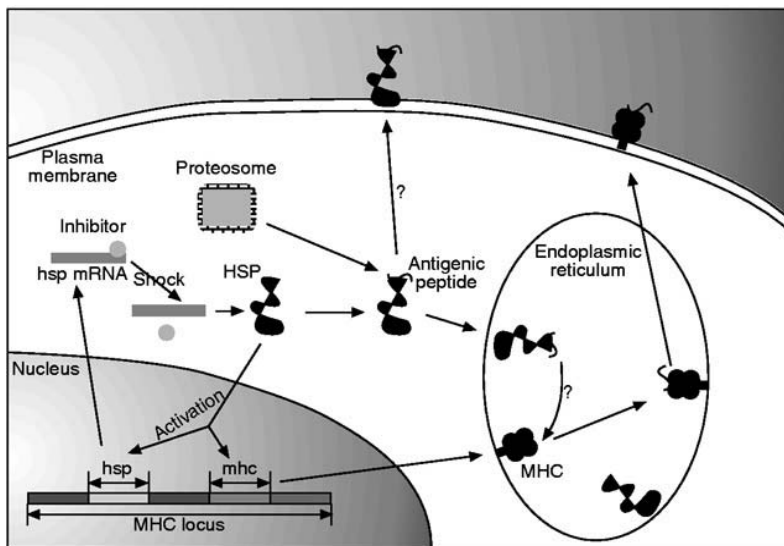


Figure 2 Pressure induction of protein synthesis leading to increased immunogenicity. Synthesis of numerous proteins is enhanced under hydrostatic pressure. Among them is HSP96, which on its own can carry processed antigenic peptides and can presumably dock on the cell surface. In parallel, the processed antigens carried by HSP can be loaded on the MHC molecules in the endoplasmic reticulum to be further transported to the cell surface.

and pressure-induced protein synthesis, described in Figure 2.

Application of hydrostatic pressure of the above range in a biological system *in vitro* (e.g. cells in suspension) can be carried out by placing the sample in a cylindrical metal chamber equipped with a sliding metal shaft. After filling the space with water or buffer, the chamber is closed, excluding air space or bubbles. Pressure is then applied gradually and maintained at the level where the expected modifications in cell structure take place. Upon slow release

of pressure back to the atmospheric level, the treated sample is collected and applied in further experiments.

Only limited information on the effect of hydrostatic pressure on immune function is currently available. It is discussed in the following sections under two distinct conditions – while under pressure and after return to atmospheric pressure.

Immune function under pressure

Physiologically relevant hyperbaric conditions, in general, refer to deep water or fast motion, as in an aircraft or spacecraft. In both, the ambient pressure does not exceed 10 atm. Rotational motion such as in an ultracentrifuge can build hydrostatic pressure of about 100 atm. However, under such pressures, the changes in structure and density in the compressible compartments remain by and large insignificant and the observed effects in function can only be attributed to a dynamic factor.

Formally, this could be related to ΔV in a transition state of a molecular process, which in principle can become significant even upon small changes in pressure. Technically, however, the resolution of such specific pressure-sensitive dynamic events in the framework of physiologic processes is complicated. The majority of the observed physiologic changes induced by pressure at this range can, as a first approximation, be attributed to indirect effects caused by changes in blood flow or oxygen level ('hyperbaric oxygenation').

It should be stressed that when a viable biological tissue, or even a whole animal, is kept under hyperbaric conditions for sufficient time (e.g. several days), processes of homeostasis will rectify the affected functions with respect to the ambient pressure (i.e. adaptation). In such cases, upon release of pressure, these functional aberrations will appear again and eventually be rectified by the same homeostatic mechanisms (readaptation).

The scarce experimental results on immune function under hyperbaric conditions of about 10 atm indicate an overt immune suppression, most of which might be attributed to hyperbaric oxygenation. Practically, a full recovery in immune function has been observed shortly after return to the ambient pressure.

As shown in **Figure 1**, cells under high hydrostatic pressure of about 1000 atm undergo massive topological changes. Despite the fact that such high pressure is far beyond any physiologically relevant scale, cells can apparently tolerate pressures of such magnitudes for a few hours, and occasionally even longer. Immune or other physiologic functions under such high pressures have not been reported, but it is very likely that, owing to the massive alterations in structure and dynamics, virtually all biological functions will be markedly suppressed, if not completely abolished. Merely the considerable decrease in the membrane lipid fluidity under such pressures is expected to reduce the overt function of immune cells severalfold, as can be extrapolated from the analogous

effect of decrease in membrane fluidity by cholesterol, which markedly suppresses immune function.

Pressure-augmented immunogenicity

Intact cells subjected to high hydrostatic pressure (above 1000 atm) for up to 1 h and then returned to atmospheric pressure, in general retain viability. For fixation of the transient surface topology in the pressure-treated cells, chemical cross-linking of the pressurized cells with a special impermeant reagent like 2'3'-adenosine dialdehyde (AdA) is effective, especially when plasma membranes are to be isolated and used as immunogens.

Pressurized tumor cells, as well as pressurized autoimmune cells, were both demonstrated to possess a high and specific immunogenicity. Such pressure-treated cells were successfully used in pre-vaccination trials in murine models of cancer and autoimmune diseases. These observations open novel approaches for both the diagnosis and treatment of cancer and autoimmune disease, which are currently under investigation.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Gravity, effect of space flight on immunity; Membrane-associated cytoskeleton: role in regulating immune cell function; Stress proteins; T cell vaccination.

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H-Y, THE MALE-SPECIFIC TRANSPLANTATION ANTIGEN

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The male-specific transplantation antigen, H-Y, was first discovered in the mid-1950s when Eichwald and Silmsker exchanged skin grafts between males and females within the same inbred mouse strain. They discovered that females of some, but not all, strains could reject skin grafts from males of the same (syngeneic) strain. In contrast, male-to-male, female-to-female and female-to-male grafts within the same strain were never rejected. Snell, because of his interest at the time in other minor transplantation antigens, interpreted these data as implying the presence of a minor H antigen gene on the Y chromosome, and it was Snell who named it H-Y, for histocompatibility (= transplantation) antigen on the Y chromosome. H-Y was a weak transplantation antigen in comparison with those of the major histocompatibility complex (MHC), H-2 in mice: it was therefore used by Billingham and his colleagues to explore tolerance induction during the neonatal period, following their classic work on neonatal tolerance induction across H-2 barriers. It proved much easier to induce tolerance to H-Y than H-2: fewer cells were needed in the tolerance-inducing inoculum, which could be given up to 10 days after birth.

***In vivo* responses to H-Y**

The fact that females of some but not all strains of mice rejected syngeneic male grafts suggested that there were genetic factors controlling the ability to mount an *in vivo* immune response to H-Y. These were explored in the early 1970s by Bailey, who found that genes of the MHC were the most important of the immune response (Ir) genes and, in particular, mice of the H-2^b haplotype, regardless of other genetic differences, were able to reject syngeneic male grafts, whereas very few non-H-2^b could do so. The H-2 genes controlling H-Y graft rejection were mapped toward the K end of the H-2 region by Bailey and subsequently, in 1978, into the class II region by Hurme and colleagues. The nature of non-responsiveness was explored from a different angle by Silvers in the early 1970s. He established that failure to respond was not due to the absence of the antigen in non-responder strains, that responsiveness was dominant in F1 hybrids between a responder and non-responder strains, and he found no evidence for allelism in H-Y. These findings have subsequently

been confirmed and extended by others, including those investigating *in vitro* T cell responses to H-Y.

***In vitro* responses to H-Y**

The skin graft rejection responses to H-Y implied cell-mediated immunity, as defined by Medawar for graft rejection responses by recipients of grafts from donors with much broader genetic disparities. However, the concomitant humoral immune responses, with the production of antibodies at least to MHC antigens, made the search for antibodies to H-Y a sensible one; had they been successful, the molecular characterization of the H-Y antigen would have been straightforward. However, despite a promising start by Goldberg and her colleagues, who in 1971 found in the serum of female mice immunized with male tissues factors which preferentially bound to sperm cells and epidermal cells from male mice, these proved to be of very low titer and difficult to work with. No satisfactory monoclonal antibodies (mAbs) specific for H-Y were produced from the time when mAbs were shown to be so useful for molecules which would otherwise never have been characterized. In retrospect, the logic which equated the specificity of the low titer, sperm-binding antibodies with the previously described male-specific minor transplantation antigen, H-Y, could be faulted but, at that time, with no other characteristics attributed to the H-Y chromosome (but see below) it was understandable.

Shortly after her work with putatively H-Y specific antibodies, Goldberg published the first paper showing that H-Y-specific cytotoxic T cells could be detected *in vitro* in the draining lymph nodes of female H-2^b mice that had recently rejected a syngeneic male graft. Subsequently, the level of cytotoxicity found in such populations was shown by Simpson and colleagues to be raised following a secondary mixed lymphocyte culture (MLC) step after the *in vivo* immunization, and they were the first to demonstrate that such H-Y-specific T cells were MHC restricted, in the same way that Bevan showed for cytotoxic T cells specific for other minor transplantation antigens. The ability to generate H-Y-specific T cells restricted by some but not all MHC molecules proved to be a very powerful way of analyzing the manner in which MHC genes acted as Ir genes and

establishing that the Ir gene products were in fact the MHC molecules. The work with H-Y established that not only were MHC class II molecules the Ir gene products, as was clear from the pioneering Ir gene work of McDevitt and Benaceraff with T helper cell and antibody responses to synthetic polymers, but the MHC class I molecules were the products of Ir genes controlling cytotoxic class I-restricted (CD8) T cells. In humans, CD8⁺ cytotoxic T cell clones specific for H-Y and restricted by HLA class I molecules have been isolated by Goulmy from female patients rejecting tissues and organs from HLA-matched males.

MHC and non-MHC Ir genes controlling T cell responses to H-Y

The identification of those alleles of MHC class I and II molecules that were permissive for T cell recognition of H-Y established the basis for understanding why some but not all strains could respond to H-Y by skin graft rejection and/or *in vitro* cytotoxic and proliferative responses. However, it was clear that genetic factors other than those of the MHC were also involved. Skin graft rejection appeared to require only a permissive H-2^b class II gene and, with few exceptions, only female mice with this allele could reject primary syngeneic male skin grafts. On the other hand, the generation of cytotoxic T cells *in vitro* following *in vivo* immunization, required a combination of a permissive class II and a permissive class I allele – the first to excite a CD4⁺ cell specific for H-Y, which would then provide help for the clonal expansion of H-Y specific, class I-restricted, CD8⁺ cytotoxic cells. The relative efficiency of different class II alleles in providing this help also depended on the route of *in vivo* immunization. For female mice with the H-2-A^b allele, any route would serve, i.e. following skin grafting or intraperitoneal or footpad injection of syngeneic male cells; for mice of the other commonly encountered H-2 haplotypes, H-2^{k,d,s,q}, the only way to generate cytotoxic T cell responses was by footpad priming. However, among mice of each of these latter haplotypes, there was variation in the ability to respond; some H-2^k strains did, others did not, and examples of responders and non-responder strains were found for each haplotype. This result implied that other, non-MHC Ir genes were also able to influence the response and a number of these have been chromosomally located, although not yet characterized at the molecular level. Similarly acting non-MHC Ir genes have been reported in controlling immune responses to a number of other antigens – some extrinsic, like hen egg lysozyme (HEL) and some, like H-Y, intrinsic, such

as autoantigens. For example, in type I insulin-dependent diabetes mellitus (IDDM) in humans as in the genetically predisposed inbred mouse strain NOD (non-obese-diabetic), in addition to the key MHC-associated Ir genes, there are non-MHC Ir genes. The manner in which they operate is at the moment unclear but, by analogy with the T cell receptor (TCR) repertoire selection function of self-ligands associated with self-MHC molecules, it seems possible that some might interact with MHC molecules, so that the self-non-MHC ligand/MHC molecule complex could positively or negatively select the TCR repertoire and hence affect the ability to respond to antigens.

The putative role of H-Y in sex determination

In the mid-1970s, following the reports of MHC-restricted T cell recognition of H-Y, and the possibility of there being antibodies which might detect the same antigen (see section entitled *in vitro* responses to H-Y), Ohno put forward the very attractive hypothesis that the sex-determining role of H-Y was mediated by H-Y that was viewed as a molecule associated with MHC molecules in the cell membrane and therefore well placed to provide cell-cell signaling during embryogenesis. Since few other genes had been mapped to the Y chromosome, the presence of which was clearly crucial for male sex determination in mammals, a hypothesis which equated the transplantation antigen H-Y, the serological entity then called by the same name and testis-determining function, was one which caught the imagination and inspired a number of experiments, some better controlled than others, to prove or disprove it. The denouement has been that both in mouse and man, using H-Y-specific MHC-restricted T cell clones to H-Y type cells from individuals carrying various deletions and mutations of the Y chromosome, that part of the Y chromosome which carries the H-Y gene is separable from that which carries the testis-determining gene, so that they cannot be one and the same. In man, the testis-determining gene (SRY) is on Yp, the short arm, while the H-Y antigen gene (HYA) is on the long arm, Yq. In mouse, the testis-determining gene (Sry) and the H-Y antigen gene (Hya) are closely linked on the short arm but have been separated in the mutant Sxr^b (Hya absent, Sry present) and by the several Y chromosome mutations which have lost Sry function but retained Hya. The serological reagents are not sufficiently discriminatory for them to have been used in the HYA/Hya chromosomal mapping analyses, for while they can distinguish a group of positive

individuals (males) from a negative one (females) in terms of an average measurement for each group, individuals cannot be confidently assigned to either.

Molecular identification of H-Y genes and peptide epitopes

The work on deleted and mutant Y chromosomes which separated Hya/HYA from other Y chromosome genes provided detailed mapping information which has led to the positional cloning of the genes and the identification of several H-Y epitopes in mouse and one in humans. New Y chromosome genes have also been discovered during the course of these studies and it has transpired that different H-Y peptide epitopes are derived from several linked genes, making Hya, like the loci encoding other minor transplantation antigens (see section on Minor transplantation antigens), a gene complex rather than a single gene. The first H-Y epitope to be characterized molecularly was discovered following expression cloning using H-Y-specific T cells to detect the presence of the epitope on cells transfected with DNA containing the relevant gene. The H-Y epitope associated with H-2K^k (H-Y/K^k) was identified as TENS^gKDI, the product of one of the 3' exons of the Smcy gene. The human H-Y epitope (H-Y/HLA-B7) is the peptide SPSVDKARAEL, a product of the homologous human gene, SMCY. This was deduced following the alternative approach of peptide elution coupled with microcapillary liquid chromatography and electrospray ionisation mass spectroscopy, and comparing peptide sequence with the known DNA sequence of the SMCY gene. The mouse H-YD^k epitope is also a product of the Smcy gene, being derived from one of the 5' exons, but an H-Y/D^b epitope, WMHHNMDLI, is encoded by a separate but adjacent gene, Uty. Smcy and Uty have X chromosome homologs, Smcx and Utx, which escape X inactivation; X and Y copies of both genes are ubiquitously transcribed, suggesting that two copies may be needed for function, which is currently unknown, but from their sequence the products could be DNA- or protein-binding proteins, and be involved in transcription. Perhaps unsurprisingly the H-Y epitopes, which are the basis for male-specific transplantation rejection responses, are derived from sequences at which the X and the Y homologs diverge.

Summary

Forty years ago, H-Y was defined as a minor transplantation antigen. Immune responses to it were

studied first *in vivo* and then in great detail *in vitro*, thereby contributing an understanding of the relationship between MHC restriction and MHC Ir gene function. The non-MHC Ir gene control of cytotoxic T cell responses to H-Y has provided a model for exploring non-MHC Ir gene control of T cell responses to other antigens, including autoantigens. Immune responses to H-Y have also allowed the exploration of an intriguing hypothesis about mammalian sex determination and have contributed to the chromosomal localization of genes on the sparsely populated Y chromosome. This in turn has led to the identification of genes encoding H-Y peptide epitopes, following expression cloning and peptide elution approaches. Reverse genetics has thus provided a route to characterizing molecules, or fragments thereof, recognized well by T cells but poorly if at all by antibodies.

See also: Cell-mediated immunity; Graft rejection; Immune response (Ir) genes; Minor transplantation (histocompatibility) antigens; Tolerance, peripheral; Transplantation.

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HYPERGAMMAGLOBULINEMIA

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Hypergammaglobulinemia results from an overproduction of immunoglobulin by plasma cells, most commonly including an increase in plasma cells in the bone marrow. The plasma cell increase and the associated increase in immunoglobulin may be polyclonal or monoclonal. In polyclonal hypergammaglobulinemia, nonneoplastic plasma cells increase in number in response to an antigenic stimulus such as infection, inflammation or a neoplasm. Monoclonal increases are not dependent on such a stimulus, but rather reflect a neoplastic increase in a single clone of plasma cells. Hypergammaglobulinemic disorders are recognized clinically when a physician orders a serum protein electrophoresis during a general medical examination, or when looking for a specific disorder.

Recognition

Suspicion of an underlying infectious, inflammatory or neoplastic disorder triggers a physician to order a serum protein electrophoresis. This is a simple screening technique commonly used in clinical medicine that displays serum proteins by their electric charge (Figure 1). Media include cellulose acetate and agarose. Separate bands are seen for albumin, alpha-1 globulin, alpha-2 globulin, beta and gamma globulin. Polyclonal or monoclonal increases may be detected when the electrophoretic bands are scanned. Capillary electrophoresis gives better resolution of separate bands. When the increase is polyclonal it appears as a broad band and the electrophoretic tracing shows a broad-based increase reflecting heterogeneous immunoglobulins being produced by polyclonal plasma cells. This increase is usually in the gamma region but may extend to the beta region. When the increase is monoclonal, the electrophoretic band is narrow and the electrophoretic tracing shows

a narrow-based band reflecting the homogeneous electric charge of the monoclonal immunoglobulin.

Polyclonal hypergammaglobulinemia (polyclonal gammopathy)

Pathophysiology of polyclonal gammopathy

Polyclonal hypergammaglobulinemia is usually due to an underlying infectious, inflammatory or neoplastic disorder such as a carcinoma. Antigen is processed by antigen-presenting cells which activate T cells to induce proliferation of antigen-specific B cells. Under the influence of cytokines such as interleukin 6 (IL-6), these B cells differentiate into antibody-producing plasma cells. These polyclonal plasma cells increase in number in the bone marrow, gut, lymph nodes, spleen and liver. They produce electrophoretically heterogeneous immunoglobulins which are detected in the serum as a broad increase, chiefly in the gamma globulin band. The polyclonal increase in immunoglobulin reflects expansion and differentiation of plasma cells, collectively producing more than one isotype of immunoglobulin (Ig) heavy chain. Both κ and λ light chains are represented. Rarely, no underlying cause is found, and the increase is attributed to an unknown antigen exposure or defective regulation of B cell proliferation and differentiation. The increase in plasma cells and gamma globulin almost never causes symptoms. The presence of symptoms reflects the specific underlying disease.

Rarely, the polyclonal hypergammaglobulinemia may be restricted to one or more subclasses of immunoglobulin (Ig). Hyper-IgM syndrome is described in another article in the encyclopedia. Hyper-IgE, or Job's syndrome, is characterized by a polyclonal increase in IgE alone. In these patients the catabolic destruction of IgE is slowed. The primary

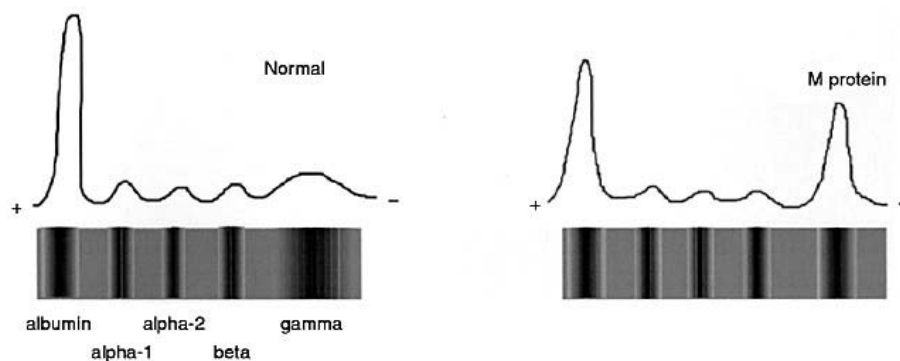


Figure 1 Serum protein electrophoresis. On the left is a normal pattern. Note the gamma globulin band is wide and the densitometric tracing is broad-based. On the right is a monoclonal gammopathy pattern. The gamma region contains a narrow band and the densitometric tracing shows a tall narrow 'spike'.

cause of the IgE increase is not known. Hyper-IgD syndrome has also been described. These patients may present with constitutional symptoms including fever and headaches, and they may have lymphadenopathy. Still another group of patients may have hyper-IgG syndrome. This is a familial disorder. There is no underlying infectious or inflammatory condition, and there is no evidence of an autoimmune disease or lymphatic neoplasm. The class restriction has been linked to class-specific regulatory T cell clones.

Underlying diseases causing polyclonal gammopathy

The most profound polyclonal hypergammaglobulinemia is found in chronic liver disease, rheumatoid arthritis and Sjögrens syndrome. The gamma globulin increase in Sjögrens syndrome is characteristically associated with very high levels of rheumatoid factor (IgM anti-IgG immunoglobulin). Uncommonly, serum hyperviscosity occurs, producing signs and symptoms of impairment of blood clotting and blood flow.

Known infectious causes of polyclonal hypergammaglobulinemia include parasitic infections such as malaria, schistosomiasis, strongyloidiasis and leishmaniasis, and fungal infections such as paracoccidioidomycosis and histoplasmosis (Table 1). Other causes include bacterial infections such as brucellosis, spirochete infections such as borrelia (Lyme disease), and rickettsial infections. Various viral infections may also cause polyclonal hypergammaglobulinemia. These include hepatitis, vaccinia, and varicella zoster infections.

Autoimmune disorders commonly manifest polyclonal hypergammaglobulinemia. Among these,

rheumatoid arthritis and Sjögrens syndrome cause the most profound polyclonal increase in gamma globulin. In systemic lupus erythematosus, the increase in polyclonal immunoglobulin is usually modest but many different autoantibodies may be present, such as antinuclear antibody, rheumatoid factor, cardiolipin antibodies and antibodies against red blood cells, neutrophils and platelets. The deregulated expression of IL-6 has been implicated in the polyclonal B cell activation and hypergammaglobulinemia seen in autoimmune diseases. Continuous IL-6 stimulation in mice produces hypergammaglobulinemia and mimics the chronic B cell hyperplasia of Castleman's disease, or giant lymph node hyperplasia. Increase in serum β_2 -microglobulin (the light chain of the class I major histocompatibility complex) accompanies the polyclonal hypergammaglobulinemia seen in lupus and other autoimmune disorders such as Sjögren's syndrome. A monoclonal gammopathy or B cell lymphoma may develop in patients with Castleman's disease or Sjögrens syndrome.

In common with other neoplasms, some malignant lymphomas and lymphoproliferative disorders may be associated with polyclonal hypergammaglobulinemia. Among these are non-Hodgkins and Hodgkins lymphoma and acute lymphocytic and myelogenous leukemia. While polyclonal hypergammaglobulinemia is uncommon in these conditions, it occurs in up to one-half the cases of peripheral T cell leukemia/lymphoma and chronic myelomonocytic leukemia. Polyclonal hypergammaglobulinemia is common in patients with angioimmunoblastic lymphadenopathy with dysproteinemia (AILD). The polyclonal hypergammaglobulinemia and other disease manifestations may disappear when a causative

Table 1 Causes of polyclonal gammopathy

<i>Infectious and inflammatory</i>	<i>Autoimmune</i>	<i>Neoplastic</i>	<i>Other</i>
Chronic liver disease	Rheumatoid arthritis	Metastatic carcinoma	Angioimmunoblastic
Parasitic infections	Sjögrens syndrome	Malignant lymphoma	lymphadenopathy with
Malaria	Systemic lupus	Hodgkins disease	dysproteinemia (AILD)
Schistosomiasis	erythematosus	Acute lymphocytic leukemia	Natural killer cell/large granular
Strongyloidiasis	Polymyositis	Acute myelogenous leukemia	lymphocyte syndrome
Leishmaniasis	Mixed connective tissue	Peripheral T cell	Castlemans disease
Fungal infection	disease	leukemia/lymphoma	Acquired immunodeficiency
Paracoccidioidomycosis		Chronic myelomonocytic leukemia	syndrome (AIDS)
Histoplasmosis			Benign hypergammaglobulinemic
Bacterial infection			purpura of Waldenström
Brucellosis			
Tuberculosis			
Spirochetes			
Lyme disease			
Viral infections			
Hepatitis			
Varicella zoster			
Vaccinia			
Rickettsial disease			

medication is discontinued. In most patients with AILD however, a latent T cell lymphoma is present. Patients with natural killer (NK) cell/large granular lymphocyte (LGL) syndrome have a polyclonal hypergammaglobulinemia. Another idiopathic acquired and uncommon polyclonal hypergammaglobulinemia is benign hypergammaglobulinemia purpura of Waldenström (BHPW). Patients have characteristic vascular purpura of the lower extremities. Malignant transformation in BHPW is uncommon.

Polyclonal hypergammaglobulinemia is frequently seen in disorders characterized by immune deficits. One example is the acquired immune deficiency syndrome (AIDS). In AIDS, there is a decreased CD4/CD8 T cell ratio, yet the level of polyclonal Ig is paradoxically increased. B and T lymphocytes have a decreased response to mitogen. Some AIDS patients develop monoclonal gammopathy and eventually a neoplastic process of B cells such as lymphoma or multiple myeloma.

Monoclonal hypergammaglobulinemia (monoclonal gammopathy)

Recognition of monoclonal gammopathy

Monoclonal gammopathy is recognized when the physician notes a monoclonal peak on the serum protein electrophoresis. Immunoelectrophoresis and immunofixation to identify the type of monoclonal peak reveals an IgG, IgA, IgM or rarely an IgD or IgE monoclonal protein with either κ or λ light chains. Urine electrophoresis may reveal free monoclonal light chain.

Pathophysiology of monoclonal gammopathy

In contrast to polyclonal processes where IL-6 acts as a B cell differentiating factor, in monoclonal gammopathy and myeloma IL-6 is important for myeloma cell growth. Increased serum levels of IL-6 may be found in myeloma. Serum levels of C-reactive protein, which is synthesized by the liver in response to IL-6, are also elevated. Evidence has been presented for both autocrine and paracrine IL-6-induced growth of myeloma cells. IL-6 levels are elevated in the serum of patients with active, high tumor burden disease. Soluble receptor for IL-6 acts as a cytokine agonist, enhancing the response to IL-6. Other interleukins such as IL-3 probably also play a role in sustaining myeloma cell growth. IL-6 also inhibits glucocorticoid and Fas-induced apoptosis, further contributing to myeloma cell growth.

Myeloma cells grow selectively in the marrow. Compared to B cells, myeloma cells have lost surface CD45 and CD19, and gain CD38. Normal plasma cells do not lose CD19. Myeloma cells probably home to the marrow using surface adhesion molecules which can trigger stromal cells in the marrow to release IL-6. CD56, a neural cell adhesion molecule (NCAM) not present on normal plasma cells but present on the surface of most myeloma cells, probably aids in this process. Myeloma cells produce IL-1 β which may increase the expression of surface adhesion molecules and induce stromal cells, osteoblasts and osteoclasts to produce IL-6.

Cytokines produced by myeloma cells such as IL-1 β or tumor necrosis factor α (TNF α) are osteoclast activating factors which can cause bone damage.

Table 2 Most common causes of monoclonal gammopathy

<i>Malignant</i>	<i>Premalignant</i>	<i>Other</i>
Multiple myeloma (MM)	Monoclonal gammopathy of undetermined significance (MGUS)	Amyloidosis
Solitary plasmacytoma		Heavy chain diseases
Malignant lymphoma	Smoldering multiple myeloma (SMM)	
Waldenström's macroglobulinemia		

Bone lysis is stimulated and new bone formation is inhibited. Widespread bone destruction and bone pain is commonly seen in multiple myeloma. Free light chain produced by myeloma cells (Bence Jones protein) may be toxic to the kidney and cause renal insufficiency. Patients may be predisposed to infections from *Diplococcus pneumoniae*, *Hemophilus influenzae* or other encapsulated bacteria because of the lack of a primary antibody response and hypogammaglobulinemia.

T cells may regulate the growth of myeloma cells. T suppressor cells are elevated in the stable phase of the disease. Activated suppressor T cell subsets are increased especially in poor prognosis patients. Clonal idiotype reactive T cells have been described in myeloma. Diminished CD4 and T_H1 cells in myeloma are associated with increased infection and decreased response to vaccines. Myeloma patients have increased susceptibility to herpes zoster reactivation.

B cells are decreased in patients with myeloma. Decreased CD19⁺ B cells are associated with an increased risk of infection, and shorter survival. Although B cells are decreased, pre-B cells may be increased. Whether these are malignant plasma cell precursors or abnormally regulated normal B cells requires further investigation. *Ras* oncogene is rearranged and *ras* oncogene product may be measurably elevated in myeloma cells of some patients by flow cytometry. *C-myc* oncogene is rarely rearranged but may be overexpressed.

Serum β_2 -microglobulin is often elevated in patients with myeloma. The source may be the myeloma cells themselves. Elevated serum β_2 -microglobulin is a major prognostic factor in myeloma. Other prognostic factors include elevated plasma cell labeling index (the percent of plasma cells in S phase of the cell cycle), plasmablastic morphology, elevated serum levels of IL-6, soluble IL-6 receptor, C-reactive protein, lactic dehydrogenase and thymidine kinase, or decrease in normal CD19 B cells or CD4 T cells. *Ras* mutations in myeloma cells are also associated with a poor prognosis.

Monoclonal gammopathy-associated diseases

Among the monoclonal gammopathies, the most important disease is multiple myeloma, a malignant neoplasm of plasma cells causing bone lesions, anemia, renal failure and death, often from infection (Table 2). Occurring in just 3:100 000 people per year, it increases to 40–60:100 000 per year at age 60 and causes 2% of cancer deaths. Clonal plasma cells proliferate and replace normal bone marrow. A monoclonal protein is detectable in the serum or urine in 98% of cases. The heavy chain is either IgG, IgA, IgD or IgE; IgM myeloma is rare. Light chains are often secreted in excess of heavy chains, so free light chains may be detected in the urine, so-called Bence Jones proteinuria. A total of 75% of patients will have a monoclonal peak in serum electrophoresis and 65% will have a monoclonal peak in the urine due to free light chain; in total, 98% will have a monoclonal protein detected by either immunoelectrophoresis or immunofixation. Of the remaining 2%, most have monoclonal protein detected in the marrow plasma cells.

While multiple myeloma is uncommon, its 'benign' counterpart, monoclonal gammopathy of undetermined significance (MGUS), is about fifty times more frequent. It increases with age, reaching a frequency of 3% by age 70. At 10 years after diagnosis of MGUS about 18% of patients will have developed myeloma or another serious monoclonal gammopathy-associated disease. MGUS is distinguished from myeloma by a lower amount of M protein in the serum and urine and fewer plasma cells in the marrow, usually less than 5%. Patients do not have bone lesions, renal failure or anemia. Any class of Ig may be represented. All patients with MGUS should be followed closely for the development of myeloma. Some patients with multiple myeloma do not have progressive disease. These patients are termed smoldering multiple myeloma (SMM). Distinguishing patients with SMM from those with overt myeloma may be difficult. Correct diagnosis is essential, as SMM may not require treatment for years. The plasma cell labeling index using a bromodeoxyuridine monoclonal antibody to detect plasma cells in S phase of the cell cycle is helpful in diagnosis. In SMM the labeling index is almost always <0.4%, while in overt myeloma it is often >1%.

Amyloidosis (AL) occurs when a monoclonal light chain cannot be degraded completely and the fragments of the variable region of the light chain reassemble in the form of a β -pleated sheet that stains with Congo red. They assume a characteristic 100 Å periodicity and nonbranching fibrillar appearance under the electron microscope. Organ damage

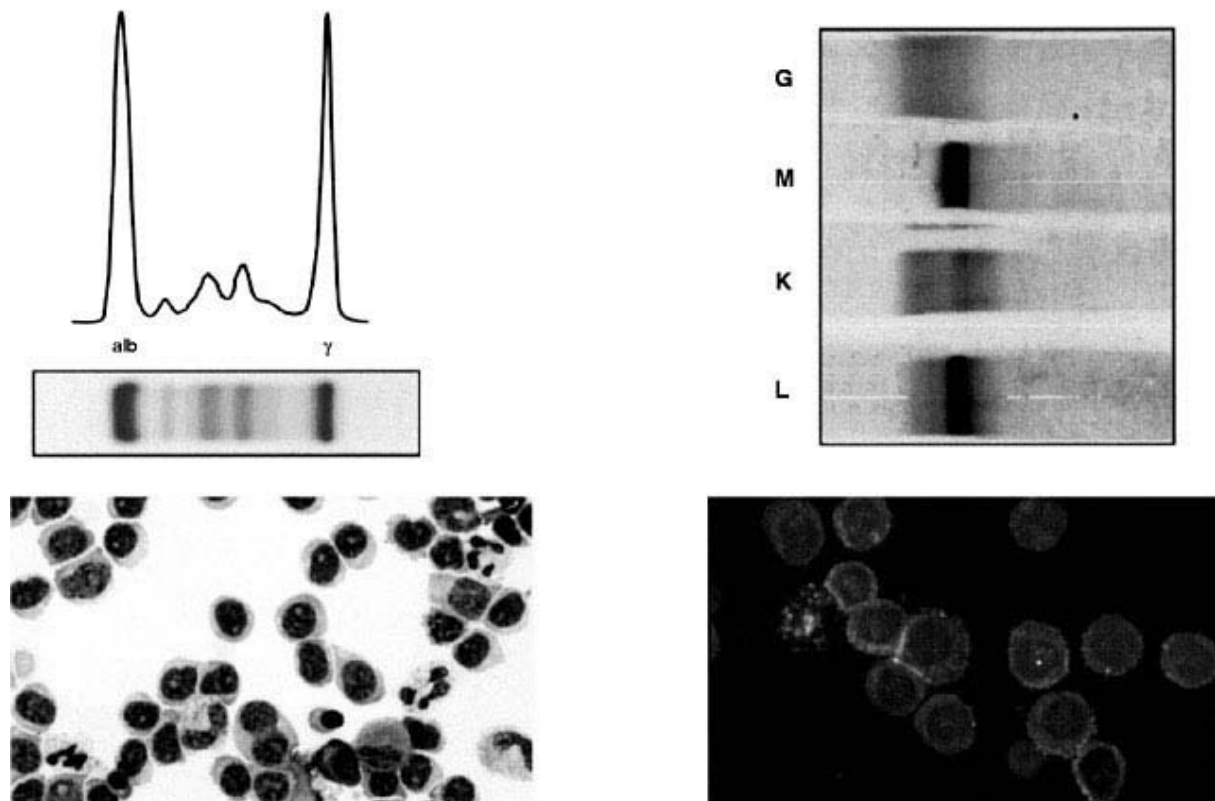


Figure 2 Macroglobulinemia is a disease in which monoclonal plasma cells in the marrow, depicted in the lower two panels, secrete a monoclonal IgM protein, shown in the upper two panels. The plasma cells are monoclonal as shown with fluoresceinated antibody to kappa light chain (lower right panel). The secreted IgM monoclonal protein is shown to be kappa light chain restricted by immunofixation (upper right panel).

Table 3 Miscellaneous monoclonal gammopathies

Lichen myxedematosus (also called papular mucinosis or scleromyxedema)
Light chain deposition disease
Cryoglobulinemia (type I and type II)
Adult acquired Fanconi syndrome
Systemic capillary leak syndrome
Acquired C1 esterase inhibitor deficiency
Cold agglutinin syndrome

reflects deposition of amyloid. The kidneys and heart, nerves, liver, gastrointestinal tract, tongue and blood vessels are prone to develop amyloid deposits. The recognition of AL and being able to distinguish it from the nonimmunoglobulin forms of secondary, familial and senile amyloid depends on the recognition of monoclonal plasma cell proliferation by immunoelectrophoresis and immunofixation of the serum and urine, and by the detection of a monoclonal increase in plasma cells in the marrow using immunofluorescence or immunohistochemical stains

for κ and λ light chain. If a monoclonal plasma cell process is not established it is necessary to specifically stain amyloid tissue using immunohistochemical techniques.

A monoclonal IgM in the serum often indicates the presence of a malignant lymphoproliferative disorder. The outcome reflects the histology of the underlying lymphoproliferation rather than the type or amount of IgM produced. Waldenström's macroglobulinemia is the syndrome that develops when the IgM levels are high, usually greater than 3 g dl^{-1} . Serum hyperviscosity, anemia, and bleeding can result (Figure 2).

Miscellaneous monoclonal gammopathies (Table 3) include lichen myxedematosus, primarily affecting the skin; light chain deposition disease; types I and II cryoglobulinemia; adult acquired Fanconi syndrome, primarily affecting the kidney; systemic capillary leak syndrome, causing episodic hypotension and hemoconcentration; osteosclerotic myeloma causing blastic bone lesions, associated with neuropathy and endocrinopathy; acquired C1 esterase inhibitor

deficiency, causing episodic hives and angioedema and loss of C3 and C4; and cold agglutinin syndrome, causing hemolytic anemia. Heavy chain diseases are covered in another article in this work.

See also: Adhesion molecules; Amyloid; Autoimmune diseases; B lymphocyte differentiation; β_2 -microglobulin; Bone marrow and hematopoiesis; CD45 (the leukocyte common antigen); Cold agglutinins; Complement deficiencies; C-reactive protein; Cryoglobulin; Cytokine receptors, soluble; Cytokines; Fas (CD95) and fas ligand; Gammaglobulin; Heavy chain diseases; Immunoelectrophoresis; Immunoglobulin, functions; Immunoglobulin structure; Interleukin 6; Interleukin 6 receptor; Sjögrens syndrome; Stromal cells; Varicella-Zoster virus, infection and immunity.

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HYPER-IgM SYNDROME

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The hyper-IgM syndrome is a rare immune deficiency disorder which was first described in 1961 in two independent reports by Rosen and colleagues and by Burtin. It is characterized by a defect in immunoglobulin isotype switching resulting in low or undetectable serum levels of IgG, IgA and IgE but normal or elevated IgM levels. Most patients exhibit an X-linked mode of inheritance; some families with affected females suggest autosomal recessive or dominant modes of inheritance. The hyper-IgM phenotype has also been associated with ataxia telangiectasia and can present as an acquired disorder that is associated with congenital rubella, administration of anti-epileptic drugs, chronic lung disease, autoimmune hemolytic anemia or tumors. In 1993, five different groups discovered that the X-linked hyper-IgM syndrome results from defects in the gene encoding CD40 ligand (CD40L), a glycoprotein expressed on activated T cells that drives immunoglobulin iso-

type switching in B cells through interaction with CD40.

Diagnosis and clinical characteristics

Most patients with X-linked hyper-IgM present in the first 2 years of life with recurrent sinopulmonary infections, while some patients have presented with *Pneumocystis carinii* pneumonia. Low or undetectable serum IgG, IgA and IgE levels with a normal or elevated IgM level in a boy suggests the diagnosis of X-linked hyper-IgM. The presence of a normal number of mature B cells in X-linked hyper-IgM differentiates it from X-linked agammaglobulinemia. Normal numbers of T cells and T cell subsets with normal T cell proliferation to mitogens differentiates X-linked hyper-IgM presenting with *P. carinii* infection from severe combined immunodeficiency or HIV infection. Identification of a defect in the CD40L

gene confirms the diagnosis of X-linked hyper-IgM. Patients with X-linked hyper-IgM have been found to produce specific antibodies in response to vaccination, but the antibodies are restricted to the IgM isotype. Patients lack germinal centers in their lymph nodes and do not exhibit memory responses to repeated immunizations. Because the genetic defect is not lethal to T cells, carriers have random inactivation of the X chromosome in their T cells. As few as 30% of the carriers' CD4⁺ T cells may express normal CD40L with no apparent effect on immune function (Figure 1).

Patients with X-linked hyper-IgM are also susceptible to infections with *Histoplasma capsulatum* and cryptosporidium; the latter is associated with chronic diarrhea. Other manifestations of the disease include recurrent episodes of stomatitis that may be associated with neutropenia. The cause of the neutropenia is not known; it may be secondary to the development of antineutrophil antibodies (although such antibodies are usually not detected) or to a defect in myeloid differentiation. The presence of autoantibodies has been reported in X-linked hyper-IgM and may lead to the development of anemia, thrombocytopenia or nephritis. Arthritis may result from autoantibodies or be secondary to viral or mycoplasma infections. Patients have an increased risk of developing B cell lymphoproliferative disease that is usually polyclonal but nevertheless can be fatal.

Molecular pathology

Most patients with X-linked hyper-IgM have a defect in the gene encoding CD40L. The gene for CD40L consists of five exons and is located on the X chromosome at Xq26.3–Xq27.1. CD40L is a 39 kDa glycoprotein member of the tumor necrosis factor α family and is expressed transiently on the surface of CD4⁺ T cells. The various reported defective forms of CD40L share an inability to bind to CD40 and consist of single amino acid substitutions, truncations, or in-frame or out-of-frame deletions. To date there has been no correlation between specific mutations and clinical phenotype.

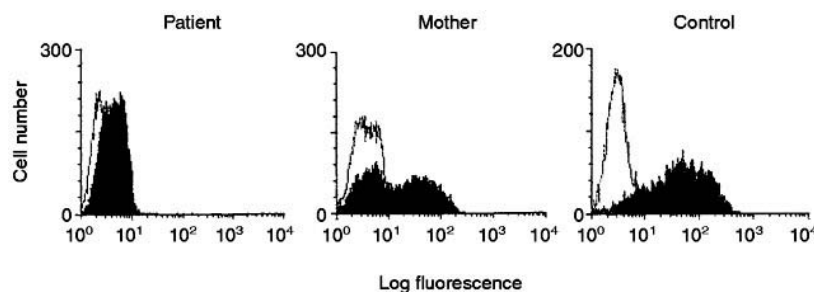


Figure 1 CD4⁺ T cells from a patient with X-linked hyper-IgM, his mother and a normal control were stained 6 h after stimulation with phorbol ester and ionophore. None of the patient's CD4⁺ T cells expressed any detectable CD40L in contrast to >90% of the control's CD4⁺ T cells. Fifty per cent of the mother's CD4⁺ T cells expressed CD40L consistent with her carrier status. ○, IgG1 control antibody; ■, CD40L antibody.

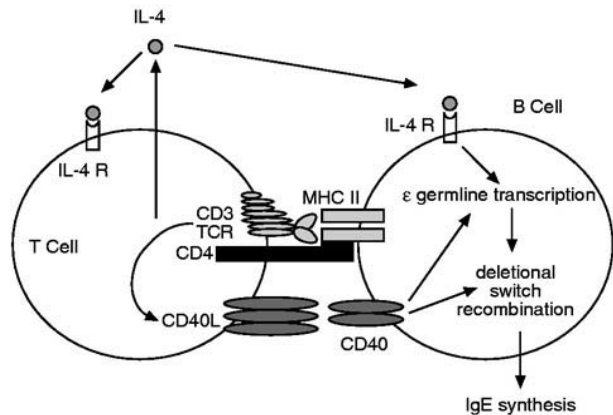


Figure 2 Mechanism of immunoglobulin isotype switching using IgE as an example. When the T cell receptor/CD3/CD4 complex is engaged by MHC and peptide antigen/allergen, interleukin 4 (IL-4) is secreted and CD40L expressed on the cell surface. IL-4 stimulates B cells to initiate ϵ germline transcription. Engagement of CD40 by CD40L enhances ϵ germline transcription and induces deletional switch recombination allowing synthesis of ϵ heavy chain and subsequent IgE production.

A few patients with a phenotype similar to the X-linked hyper-IgM syndrome and apparent X-linked inheritance have been found to have normal CD40L with an unidentified defect in signal transduction through CD40. These patients demonstrate that the cause of hyper-IgM is the absence of a stimulatory signal resulting from the interaction between CD40 and its ligand. Defects in CD40 or its signaling pathway may be responsible for autosomally inherited cases of primary hyper-IgM. In mice, gene targeting of either CD40 or its ligand results in a phenotype that resembles the human disease. Gene-targeted mice lack T cell-dependent immunoglobulin isotype switching, B cell memory responses and germinal center formation.

Pathogenesis of the clinical picture

Failure of interaction between CD40 and its ligand results in a defect in B cell differentiation which is reflected in the patients' serum immunoglobulin levels and their response to immunization. In X-

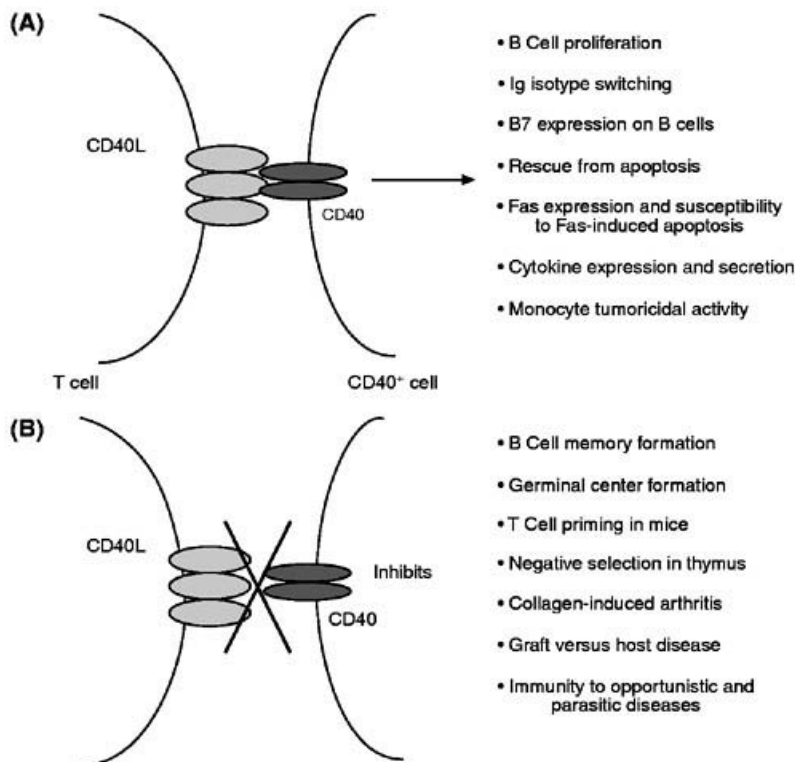


Figure 3 Consequences of interaction between CD40 and its ligand. (A) Effects of engagement of CD40 by mAb or by CD40L. (B) Functional consequences of inhibition of CD40/CD40L interaction by CD40L mAb, by soluble CD40, or by gene targeting.

linked hyper-IgM, mature B cells do not undergo immunoglobulin isotype switching (Figure 2) nor do they exhibit memory responses. Elevation of IgM levels in some patients but not in others remains unexplained but may be secondary to recurrent infections or to lymphoproliferative disease. Susceptibility to opportunistic infections in X-linked hyper-IgM, in contrast to X-linked agammaglobulinemia, suggests that CD40L plays a direct role in clearing opportunistic infections. Indeed, protection of severe combined immunodeficient mice from *Pneumocystis carinii* infection by reconstitution with human T cells was abrogated by antibody to CD40L. The mechanisms by which CD40L deficiency leads to the development of neutropenia, stomatitis and lymphoproliferative disease have not been determined. In B cells, engagement of CD40 results in the expression of Fas (CD95) and in a susceptibility to Fas-mediated apoptosis. This may be an important mechanism for killing proliferating B cells as well as autoreactive B cells and may explain the development of lymphoproliferative disease and autoimmune disease, two hallmarks of Fas deficiency, in patients with hyper-IgM syndrome. Successful interaction between CD40 and its ligand has further profound effects on normal immune function that are inhibited when the interaction is disrupted (Figure 3).

Therapy

Therapy of X-linked hyper-IgM is currently limited to provision of passive immunity by administration of intravenous immunoglobulin, which reduces the frequency and severity of infections but does not prevent lymphoproliferative disease. Infections are treated aggressively with specific antimicrobial therapy. There are conflicting reports on the benefit of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the therapy of neutropenia. Bone marrow transplantation can cure this disease and may be feasible when an HLA-matched sibling is available. Identification of the genetic defect has allowed carrier detection and prenatal diagnosis and may open the door to gene therapy in the future.

See also: B lymphocyte differentiation; Hematopoietic stem cell transplantation; CD40 and its ligand; Cryptosporidiosis; Fas (CD95) and fas ligand; Flow cytometry; Gene therapy; Germinal center; Immunodeficiency, animal models; Immunodeficiency, primary; Immunodeficiency, secondary; Immunoglobulin class switching; Memory, immunological; *Pneumocystis carinii*, infection and immunity.

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HYPERSENSITIVITY REACTIONS

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Hypersensitivity reactions occur as a result of an immune response that is inappropriate or excessive and causes various types of tissue injuries.

Following Coombs' and Gell's classification in 1963, there are at least four categories of hypersensitivity reaction. The first three, type I (immunoglobulin E (IgE)-mediated), type II (cytotoxic or antibody-dependent cytotoxicity) and type III (immune-complex mediated), are all immunoglobulin-dependent reactions involving a humoral, B lymphocyte-mediated response which operates mainly as a protection against extracellular infections. Conversely, type IV (delayed-type hypersensitivity) is a cell-mediated immune response that is T rather than B lymphocyte dependent. This type of reaction is more effective against intracellular infections (Table 1).

More recently, a type V hypersensitivity reaction has been added, named stimulatory or antireceptor hypersensitivity. This is caused by circulating auto-antibodies stimulating cell receptors of the affected tissues.

The *in vivo* pathologic picture is generally the consequence of overlapping immunologic mechanisms rather than the outcome of a single immune response. Thus, the interaction of cell-mediated and antibody-dependent immune mechanisms determine the type and effectiveness of the immune response.

T_H1 and T_H2 subsets: differential roles

This traditional classification has, however, to be seen in the context of the now recognized paradigm establishing the existence of two fundamentally

Table 1 Classification of immunologic diseases

Type of hypersensitivity	Pathologic immune mechanisms	Mechanisms of tissue injury and disease
Type I Immediate	IgE antibody	● Mast cells and their mediators (vasoactive amines, lipid mediators, cytokines)
Type II Antibody-mediated	IgM, IgG antibodies against tissue or cell surface antigen	● Complement activation ● Recruitment and activation of leukocytes (neutrophils, macrophages) ● Abnormalities in receptor functions
Type III Immune complex-mediated	Immune complexes of circulating antigens and IgM or IgG antibodies	● Complement activation ● Recruitment and activation of leukocytes
Type IV T cell-mediated	CD4 ⁺ T cells (delayed-type hypersensitivity)	● Activated macrophages, cytokines

Reproduced with permission from Abbas *et al* (1995).

polarized forms of specific immune responses which depend on the profile of cytokine production. Recent experimental evidence has shown that CD4⁺ T helper clones produce two distinct (T_H1 and T_H2), mutually exclusive patterns of cytokine secretion whose role is critical for the differential selection of the type of effector mechanism to be activated during an immune response. T_H1 clones mainly produce interleukin 2 (IL-2), interferon γ (IFN γ) and tumor necrosis factor β (TNF β) and are responsible for IgG2a production (in mice) and for cell-mediated immune responses, whereas T_H2 clones mainly secrete IL-4, IL-5 and IL-10 and direct IgG1 and IgE humoral immune responses. T_H0 clones have also been identified as a common precursor subset producing both T_H1 and T_H2 cytokines. Some cytokines, such as IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF α are secreted by the two T cell subsets. T_H1 cells dominate protective responses against intracellular microbial infections and are involved in delayed-type hypersensitivity (DTH). Conversely, T_H2 cells are more efficient towards extracellular host defenses through antibody production, particularly of the IgE isotype. This distinct T_H1 and T_H2 cytokine profile does not exist among naive T cells. The transition toward effect of T_H1 and T_H2 functions occur through an obligate, intermediate state expressing multiple cytokines, but the decision as to what effector function is required is predetermined by a series of factors. Similarly to CD4⁺ T cells, CD8⁺ cells appear to secrete a polarized pattern of cytokines, although they tend to make T_H1 rather than T_H2 cytokines.

Type I hypersensitivity

The first description of an allergic response was made by Prausnitz and Kustner, who succeeded in provoking an allergic reaction by transferring the serum of Kustner, who was reacting to fish, to Praus-

nitz, who was allergic to pollen. Reinjection of fish antigen immediately triggered a wheal and a flare at the site of reinjection. This observation goes back to 1921. It is surprising that the term allergy or atopy (altered reactivity) has become synonymous with a type I hypersensitivity only in more recent times when the term 'allergen' was introduced for proteins and chemicals responsible for this reaction, and the term 'anaphylaxis' was established for the resulting generalized immune reaction. The localized form of anaphylaxis is exemplified by hay fever, asthma, eczema and urticaria (skin wheals). The systemic anaphylaxis characterized by sudden shock and dyspnea, frequently leading to death, occurs in extremely sensitive individuals.

Since the early transfer experiment, one of the main achievements in this field was the discovery by Ishizaka of a novel class of immunoglobulin, the IgE responsible for the type I response. IgE exists in levels between 10 000 and 100 000 fold less than IgA and IgG, respectively.

Characteristics of IgE

1. Like the other immunoglobulins, IgE possesses two light and two heavy chains but, as in IgM, displays five instead of four domains in the heavy chains.
2. It is unable to fix complement or to cross the placenta.
3. It differs from the other immunoglobulin classes in its:
 - (a) activity, which is destroyed by heat inactivation at 56°C for 30 min;
 - (b) shorter half-life (2 versus 21 days for most IgG subclasses), although the sensitized mast cells retain the ability to respond for up to 3 months;
 - (c) ability to bind selectively to specific receptors on mast cells and basophils through its Fc portion specific for ϵ heavy chains.

The IgE receptors enable the IgE to initiate both the afferent and the efferent immune reactivity in the presence of low antigen concentration. Three types of receptors have been identified: FcεRI, FcεRII and εBP.

High-affinity receptor for IgE (FcεRI)

This is the classical IgE receptor present on mast cells and basophils. Recently it has been found also on Langerhans cells of the skin. Its structure consists of one α, one β and two γ chains with the γ chains linked by a disulfide bond. Only the α unit mediates IgE binding, whereas β and γ chains may be involved in signal transduction and all three chains are important for FcεRI function.

Low-affinity receptor for IgE (FcεRII)

This receptor (also named CD23) belongs to the lectin superfamily, although it binds to noncarbohydrate structures on the IgE. In humans, its expression is tightly regulated, being induced by IL-4 and suppressed by IFNγ. FcεRII is generally overexpressed in highly atopic patients and in those affected by parasitic infestations. Its structure consists of a 45 kDa sialoglycoprotein which exists in an α form and a β form. The two isoforms have differing tissue distribution. The β form is expressed on macrophages, B cells, monocytes, platelets and eosinophils only after stimulation, whereas the α isoform is expressed constitutively on B cells. FcεRII on a variety of cells plays a role both in antigen 'capture' and presentation and in effector cell functions.

IgE binding protein (εBP)

εBP, a member of the thiol-dependent lectin superfamily, is attached to the cell membrane by binding to glycolipids and glycoproteins. This receptor is present in various hematopoietic cell types, such as eosinophils and neutrophils, and can be disrupted easily. εBP binds the ε chain through carbohydrate structures. Its role is not fully elucidated.

IgE regulation and function

Atopic individuals produce high levels of IgE in response to particular antigens. This depends on: 1) the nature and dose of the antigen; 2) genetic factors; and 3) the type of cytokine production.

1. Antigens able to evoke a strong immediate type I response are called allergens. These are relatively small and highly soluble proteins which, by entering across mucosal surfaces in very low doses, favor the activation of T_H2 cells and IgE production. Additional factors, i.e. specific

mechanisms of antigen processing, the presence of proteases and for some airborne protein material, the combination with environmental adjuvants (i.e. pollutants, such as smoke, diesel exhaust) might all contribute to the enhancement of IgE synthesis and the clinical manifestation of allergy.

2. If both parents are allergic there is more than a 50% chance that the children will develop allergies; with only one parent being allergic, there will be approximately a 30% chance. A gene associated with atopy has been linked to chromosome 5q31.1, a region where both the gene for IL-4 and IL-13 have also been mapped. This would suggest that polymorphism in the coding region of a differential transcriptional regulation of IL-4 and IL-13 gene expression in atopic individuals could contribute towards T_H2 cell development.
3. T_H2 development and B cell isotype switching to IgE result from an environment rich in IL-4. The IL-4 requirement for T_H2 priming has been demonstrated in IL-4 knockout mice which have an impaired T_H2 response and an absent IgE production. Recently, IL-3, IL-5, IL-6 and TNFα have been claimed to contribute to the IgE isotype switching, whereas IL-12, IL-8, IL-10, IFNγ and transforming growth factor β (TGFβ) have inhibitory effects. The IgE production is initiated by allergic presentation by local APC to T_H2 cells. This subset releases sets of soluble cytokines important in the differentiation and activation of eosinophils (IL-3, IL-5, GM-CSF), in the survival of mast cells (IL-3, IL-4, IL-10) and in IgE production (IL-4, IL-13). In addition to cytokine production, T and B cell physical interaction occurring between CD40 ligand (CD40L) expressed on activated T cell subsets and CD40 expressed on B cells is required for the expression of productive mRNA and for the synthesis of IgE.

Type I hypersensitivity: role in the afferent limb of the immune response

There is evidence that IgE can play a critical role in antigen presentation at the mucosal sites. Cells expressing FcεR on their surface, such as Langerhans cells, follicular dendritic cells, activated B cells and macrophages, are able to 'capture' and 'focus' antigen-bound IgE on their surface, thus enhancing antigen presentation to CD4⁺ T cells. FcεRII is involved in this process.

Type I hypersensitivity: principal role in the effert limb of the immune response

Following antigen stimulation, monomeric antigen-specific IgE binds to Fcε receptors on circulating basophils and mast cells present in the lymphoid areas of the skin and respiratory and gastrointestinal tracts. The result of this interaction is mast cell sensitization. At the second exposure to the same antigen, cross-linking by the multivalent antigen of the surface-bound IgE will occur, causing degranulation of these cells (Figure 1).

Mass cell activation and immune modulator release

In addition to antigen-IgE complex cross-linking, mast cells can also be stimulated by complement components (the anaphylatoxins C3a and C5a). The result of stimulation is a cascade of reactions leading to intracellular influx of Ca^{2+} from the endoplasmic reticulum. Ca^{2+} in the cytoplasm triggers an intracellular series of events which induce the release of two types of mediators: preformed mediators, such as histamine (the most abundant and fastest acting), proteoglycans, proteases and heparin, which are released by exocytosis, and newly synthesized lipid mediators derived from arachidonic acid. Particularly important are three types of metabolites, those derived from the cyclo-oxygenase, such as prostaglandin D_2 (PGD_2) and thromboxane A_2 , those derived from the lipoxygenase, such as leukotrienes (CTB_4 , LTC_4 , LTD_4 and LTE_4), and platelet activat-

ing factor (PAF) (Figure 2). Following activation, mast cells can also release cytokines ($\text{TNF}\alpha$, IL-5, IL-6, IL-8). All of these mediators have essentially three important functions which directly affect target organs:

1. Vasodilatory and increased vascular permeability activities.
2. Chemotactic action on polymorphs, lymphocytes and platelets through neutrophil chemotactic and platelet-activating factors.
3. Spasmogenic activity with a direct effect on bronchial smooth muscle and an indirect increase in mucus secretion.

Different mediators are released from different tissue mast cell and blood basophil subsets, thus in turn triggering different clinical manifestations.

Clinical tests for allergy

Skin-prick tests

These are the most common tests in atopy and they are characterized by an itchy wheal and erythema at the introduction of an antigen into the skin in an allergen-sensitized individual. The reaction site is small, allowing a great number of tests to be done at once. The skin prick test consists of a biphasic reaction. The immediate phase lasts about 1 h, and is caused by the release of histamine from degranulatory mast cells. This is often followed by a late reaction, lasting several hours, which is generally

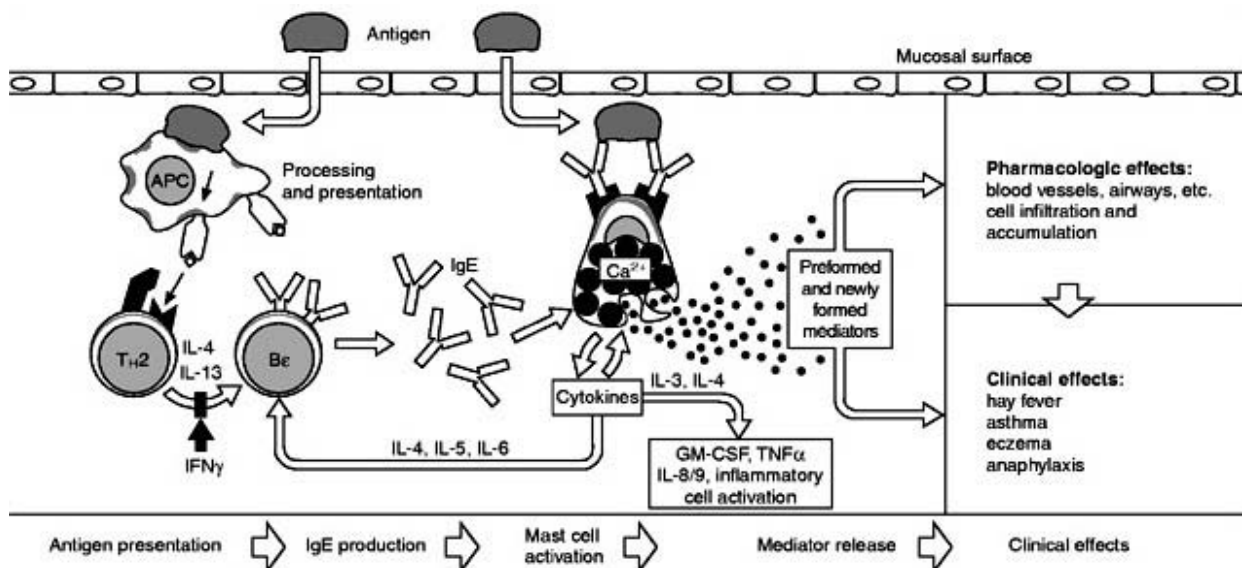


Figure 1 Induction and effector mechanisms in type hypersensitivity. Antigen presentation is performed at the mucosal level. Production of allergen-specific IgE results from the release of $\text{T}_\text{H}2$ cytokines from activated T cells. IgE binds to mast cells through FcεR and sensitizes these cells. Allergen re-exposure cross-links surface-bound IgE and triggers Ca^{2+} release. This leads to the production of mediators causing allergic symptoms. (Reproduced with permission from Roitt *et al* (1996).)

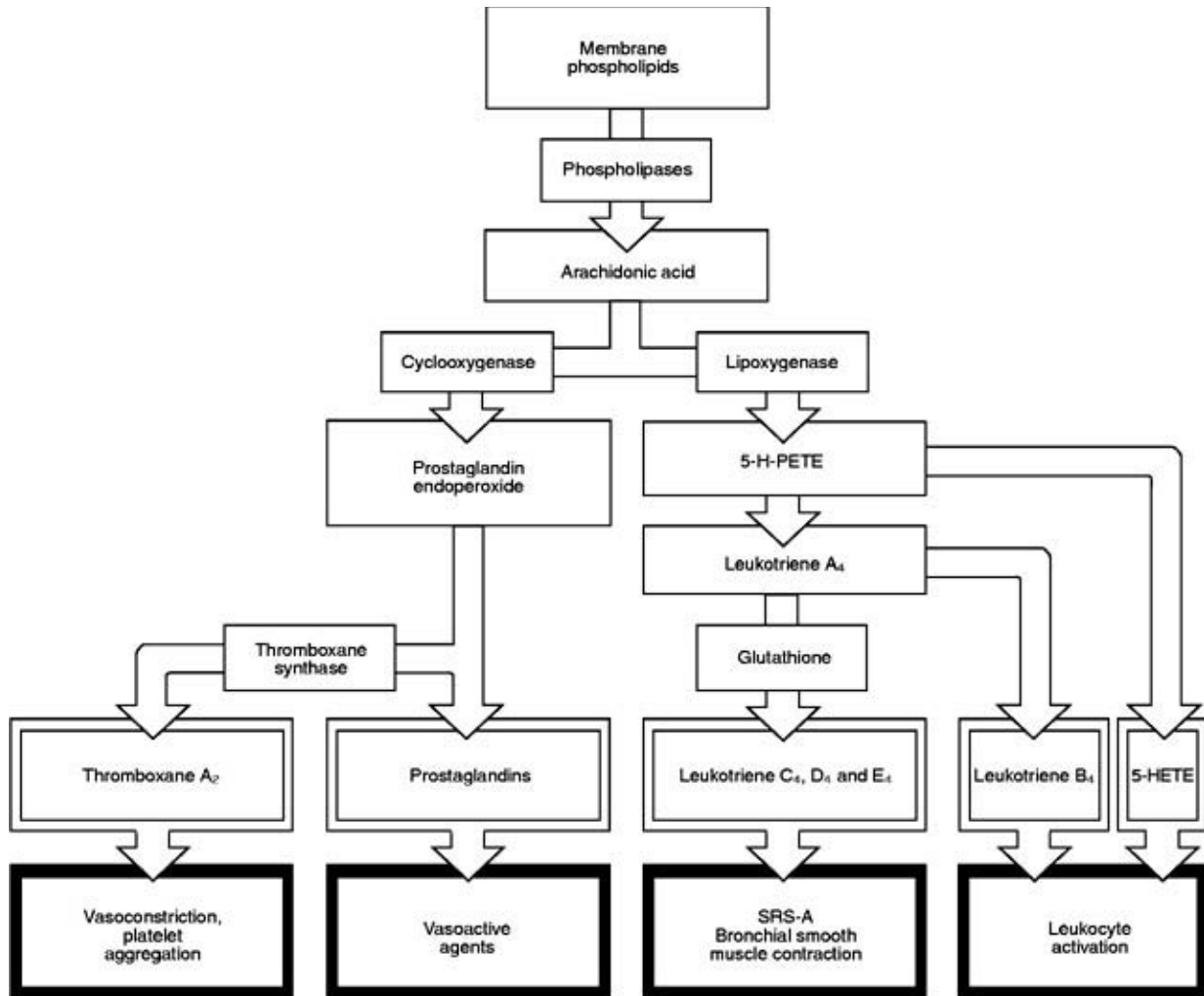


Figure 2 Lipid mediators of inflammation synthesized by mast cells upon stimulation by antigen binding to IgE. (Reproduced with permission from Janeway and Travers (1995).)

larger, harder and moderately painful. This reaction is caused by the release of PGD₂ and leukotrienes by T cells and eosinophils.

RAST (radioallergosorbent test)

RAST evaluates antigen-specific IgE in the serum. This usually correlates with skin-prick tests. There is also a group of allergic patients who are both skin test- and RAST-negative. These individuals make a local antibody response and this is shown by a positive nasal or bronchial provocation test with specific antigen.

Beneficial role of IgE

If the entry of a parasite is not blocked at the level of the gut by a defensive, neutralizing IgA mechanism, the contact of this agent with IgE-sensitized mast cells and basophils will release immune mediators. The response to helminth and nematode

infections is due to the induction of T_H2 cells to secrete IL-3, IL-4 and IL-5, which promote B cell and mucosal mast cell proliferation and eosinophil activation. The result is an increased IgE production and eosinophilia, which are the hallmarks of this type of immune response. Eosinophils have evolved specifically as a defense against parasites that are too large to be phagocytosed; the mast cells have evolved primarily to localize eosinophils near the parasites and to enhance their antiparasitic function. In contrast to allergy, the IgE response to parasites operates against a broad range of parasitic antigens. This response saturates the high-affinity receptors on mast cells, rendering them refractory to further triggering by parasites.

The effector role in host defenses is mainly performed by the eosinophils and IgE-sensitized mast cell interactions with the parasites via IgE FcεRII and eBP. The consequences are: 1) release of granule con-

tents, including the eosinophil major basic protein which lyses the worms; 2) secretion of mucus, which creates a barrier to the attachment and invasion; and 3) increased vascular permeability and chemotaxis, which allows the penetration of complement, IgG and effector cells back into the gut, thereby enhancing the expulsion of the worms. Furthermore, production of proinflammatory cytokines by activated macrophages ultimately increases mucus secretion and facilitates the expulsion of the worms.

Type II hypersensitivity

Type II hypersensitivity results from antibodies of either the IgG or IgM class binding to specific antigens on a cell surface or extracellular matrix, with subsequent activation of the classical complement cascade. This leads either to direct lytic damage of the target cells or facilitates opsonization by attracting polymorphs and macrophages/monocytes bearing C3bR. C3 components can also bind C3bR on mast cells and basophils and trigger the production of molecules which attract and activate other effector cells. In addition, IgG is able to directly cross-link effector cells. In this case, the antibody acts as a bridge between the target cells and the effector cells through the engagement of Fc receptors on effector cells (Figure 3). It is known that only antigen bound IgG (but not free IgG) can bind Fc γ R with high avidity. Fc γ receptors are present on a variety of effector cells: phagocytic (macrophages, neutrophils), non-phagocytic (platelets, eosinophils), and large granular lymphocytes (killer (K) and natural killer (NK) cells). Different IgG subclasses vary in their ability to bind Fc γ R-bearing effector cells, human IgG1 and IgG3 being able to bind more efficiently than IgG2 or IgG4. The destruction of antibody-coated cells by NK cells is referred to as antibody-dependent cellular cytotoxicity (ADCC) and represents a mechanism by which an antibody can direct an antigen-specific attack by an effector cell lacking specificity for the antigen. Thus, target specificity of NK cells is broader than that of cytotoxic T cells, but it does not occur at random.

Type II reactions can involve the activation of a membrane oxidase complex responsible for the secretion of oxygen radicals. Cytotoxicity also follows the activation of phospholipase A₂, which produces arachidonic acid secretion and consequently leukotriene/prostaglandin release. These vasoactive amines contribute to the target cell damage.

Reactions to red cells represent the most clear examples of type II hypersensitivity. The observation made by Landsteiner, at the beginning of the twentieth century, that the serum of some human blood

donors agglutinates the red cells of other normal blood donors, underlies the basis of the present understanding of two life-threatening reactions: hemolytic anemia of the newborn (erythroblastosis fetalis) and blood transfusion reactions. In both cases, the blood group of the 'donor' does not match with that of the 'recipient'.

Erythroblastosis fetalis

In this condition it is the rhesus (Rh) antigen system on the red cell surface which is responsible for the reaction causing fetal-maternal incompatibility. The name has derived from a similar antigen recognized by the serum of rabbits immunized with rhesus monkey red cells. The human Rh(D) antigen is encoded by a dominant gene which is present in approximately 80% of the population. There are therefore Rh⁺ and Rh⁻ individuals. The disease follows the gradual sensitization of a Rh⁻ mother to the blood group Rh⁺ antigen of fetal red cells bearing the paternal Rh⁺ antigen. This happens at the time of first delivery when the mixing of maternal newborn blood occurs. As a consequence, Rh⁻-specific IgG is made in the postpartum period. With subsequent pregnancies, these antibodies cross the placenta into the fetal circulation and react with fetal red cells, destroying them. Therefore the disease mainly occurs with the second or subsequent Rh⁺-incompatible children. The symptoms are hepatosplenomegaly, elevated bilirubin with jaundice, hemorrhage and kidney failure.

Transfusion reactions

Among the 19 different human blood group systems, the group ABO is the most important. Nearly all individuals make IgM antibodies to the antigens of the ABO system that they lack. These antibodies do not cross the placenta; they occur naturally, without prior immunization, possibly through cross-reactions of similar antigens on a variety of environmental agents. If an individual is blood group A, he or she will be tolerant to A and agglutinate B red cells, and vice versa for a blood group B individual. Conversely, a blood group O person will make anti-A and anti-B as natural antibodies and will only tolerate blood transfusions from group O individuals. Thus, if mismatched blood is transfused, it will cause blood agglutination, complement activation and intravascular hemolysis in the recipient. The hemolytic damage is characterized by jaundice, fever, kidney failure and rapid death of the patient.

A less common example of type II hypersensitivity is the autoimmune hemolytic anemia (AHA), in which patients react to their own red blood cells. The diagnosis is generally made by a positive Coombs

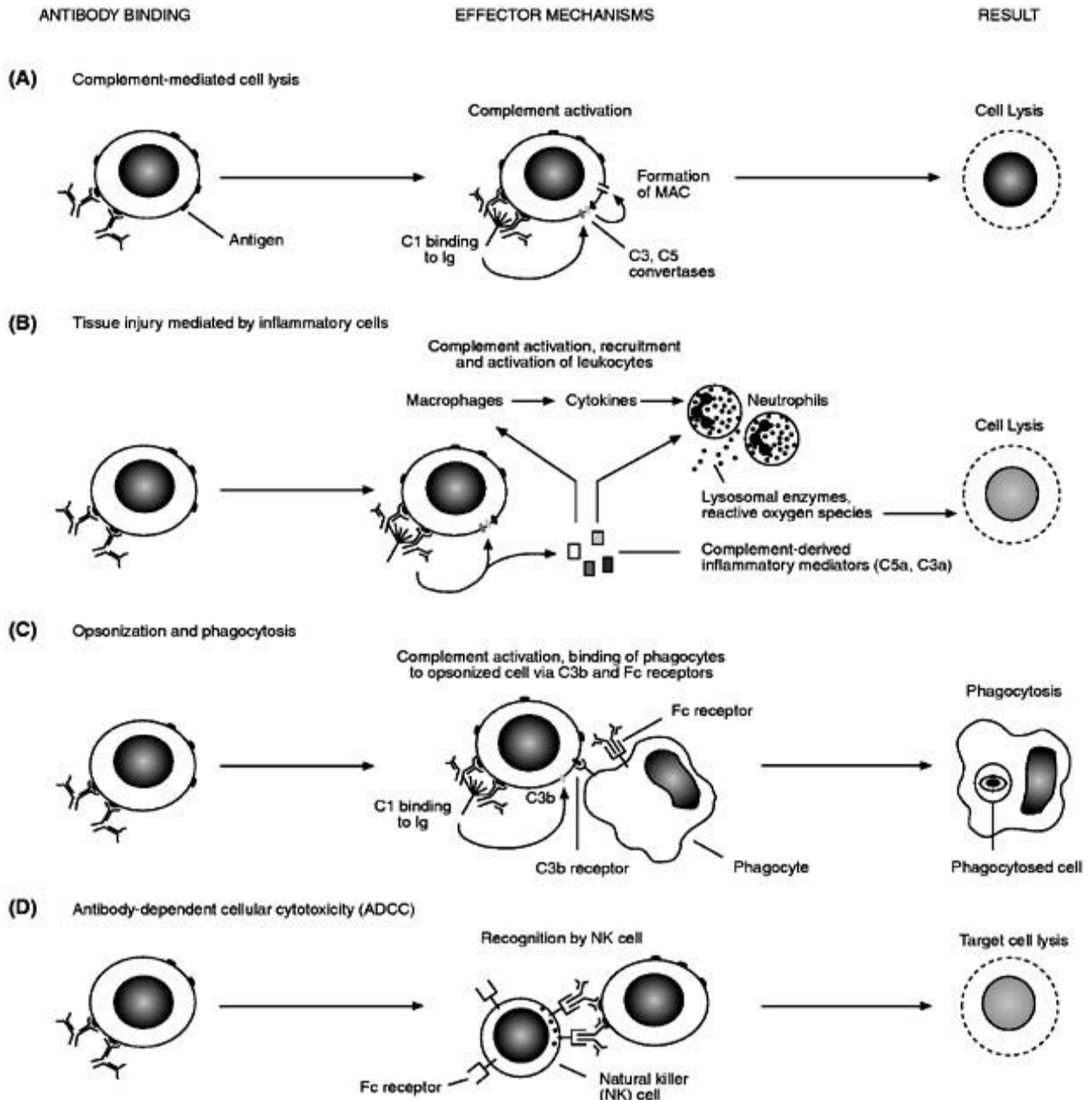


Figure 3 Effector mechanisms in antibody-mediated cell injury. (A) Activation of complement and cell lysis. (B) Activation of complement and recruitment/activation of leukocytes leading to cell lysis. (C) Phagocytosis of opsonized cells. (D) Cell lysis by natural killer cells. (Reproduced with permission from Abbas *et al* (1994).)

test in which the autoimmune reaction is detected by an agglutination obtained with the addition of human-specific immunoglobulin which agglutinates the autoantibody-coated cells.

Finally, in certain autoimmune diseases, type II reactions can cause immunopathologic damage as the autoantibodies are directed to cell surface or to cytoplasmic antigens. Goodpastures syndrome, pemphigus and myasthenia gravis are all caused by auto-

antibodies directed to cell surfaces antigens. In Goodpastures syndrome the autoantibodies are directed to the glomerular basement membrane, causing glomerulonephritis, and sometimes also to the alveolar basal membrane, causing lung hemorrhage. In pemphigus the pathogenetic antibodies are directed to desmosome antigens and cause the breakdown of the epidermis. In myasthenia gravis the autoimmune destruction is directed against acetyl-

coline receptors and is responsible for extreme muscular weakness. Conversely, in other organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM) and Hashimoto's thyroiditis, the autoantibodies are mainly directed against cytoplasmic antigens (islet cells and thyroid peroxidase, respectively). Their pathogenic relevance is still uncertain. There are indications that IDDM and Hashimoto's disease might be caused by a T rather than B cell mediated mechanism.

Type III hypersensitivity

Antibodies binding to equivalent amounts of soluble antigens give rise to immune complex formation. The antigens involved in inducing the pathogenic immune response can be either foreign or self, and the antibodies either IgG or IgM. When complexes are formed, they usually activate complement and then bind to C3b receptors on erythrocytes. The erythrocytes are then responsible for removing complexes from the plasma and transporting them to the sinusoids of the liver and spleen, where they are removed by macrophages. However, if immune complexes are formed in great excess of antigen, or if the complex is very small and soluble, the reticuloendothelial system will not be able to degrade them efficiently. These complexes will circulate in the blood and subsequently deposit in various tissues of the body, inducing local inflammation. The resulting pathogenic features depend on the sites of immune complex deposition.

Factors that influence immune complex deposition

1. Size: larger immune complexes fix complement more efficiently and are removed more quickly than those of medium size.
2. Isotype: IgA complexes do not bind efficiently to erythrocytes, thus they tend to deposit to a greater extent.
3. Defective phagocytosis and/or complement binding systems which allow immune complexes to bind to the endothelial wall.
4. Biochemical properties of the antigen and antibody: altered glycosylation of IgG has been found in rheumatoid arthritis and this may hamper phagocytosis.
5. Hemodynamic factors: the plasma goes through the capillary walls of the glomeruli and synovia at high hydrostatic pressure, thus facilitating immune complex deposition at these sites.

Immune complexes mediate the inflammatory reaction through several mechanisms:

1. Activation of complement components, with production of C3a and C5a fragments (anaphylatoxins) which bind to complement receptors on:
 - (a) basophils and mast cells, causing the release of preformed and newly formed mediators, such as histamine, PGD₂, PAF, leukotrienes and cytokines. This results in the series of events already described for mast cell degranulation following cross-linking by antigen of mast cell-bound IgE, i.e. increase in vascular permeability, which facilitates the deposition of immune complexes in the blood vessel wall and further production of complement, recruitment of inflammatory cells and smooth muscle contraction;
 - (b) polymorphonuclear leukocytes, causing increase in their adherence to the vessel walls, in their ability to ingest particles and in their attraction to the affected areas.
2. Binding to Fc receptors on basophils and platelets, triggering, on the one hand, the release of vasoactive amines, which leads to increase in vascular permeability and hemorrhage, and, on the other, platelet aggregation and microthrombosis.
3. Stimulating macrophages to produce pro-inflammatory cytokines.

Diseases caused by immune complexes generally follows:

1. *Persistent bacterial or viral (low-grade) infections: streptococcus, leprosy, syphilis, viral hepatitis.* Acute post-streptococcal glomerulonephritis is due to immune complex deposition on the basement membrane of the glomeruli. In lepromatous leprosy and secondary syphilis, the mycobacterium and the treponema antigens, respectively, can cause deposition of complexes in the joints, skin and kidney, with consequent local damage.
2. *Persistent exposure to and inhalation of environmental antigens, i.e. moulds and animal danders.* Examples are the extrinsic alveolitis found in farmers' lung and pigeon fanciers' lung. The antibody involved is IgG, not IgE.
3. *Persistent autoantibody formation in autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).* SLE is characterized by autoantibodies against nuclear constituents, such as DNA and nucleoprotein antigens. Large numbers of immune complexes are produced, tending to deposit in the small vessel walls of the glomeruli, joints and brain and causing injury. In RA, IgG

Table 1 Continued

<i>Gene</i>	<i>Approx. Gene size (kb)</i>	<i>No. Exons</i>	<i>mRNA size (kb)/tissue distribution^a</i>	<i>Protein</i>	<i>Protein size (aa)</i>	<i>Function</i>
HSP70-1	2.5	1	2.4 Ubiquitous after heat shock	HSP70 (HSP72)	641	Heat-inducible hsp70. Chaperone in recovery of cells from stress
HSP70-2	2.3	1	2.4 Ubiquitous after heat shock	HSP70 (HSP72)	641	Heat-inducible hsp70. Chaperone in recovery of cells from stress
HSP70-HOM	4	2	3 E, testis (mouse)	HSP70-HOM	641	Constitutive hsp70
G7b	10	—	0.9 E, L, M	—	—	—
G7a	12	—	4.3 E, H, L, M	Val ^{Tra}	1265	Valyl tRNA synthetase
G7c	—	—	— F, H, L, M	—	—	—
G7d	4	—	— F, H, L, M	—	—	—
G7	22	—	3 E, H, L, M	—	—	—
G6	7	—	1.5/1.4 H, L, M	—	—	—
G6a	—	—	1.6 H, T*	—	—	—
BAT5	—	—	2 F, L, M	—	—	—
G5b	3.6	—	— F, L, M	—	—	—
CKIIβ (G5a)	5.2	7	1-1.1 E, L, M	Casein kinase II β	215	Cell growth?
G5	—	—	1.6 E, F, H, L, M	—	—	—
G4	3	—	2.5 H, L, M	—	—	—
G3a	6	—	1.1 H*	—	—	—
G3 (BAT3)	14	—	3.8 E, F, H, L, M	BAT3	1132	—
G2 (BAT2)	15	—	6 E, F, H, L, M	BAT2	2142	—
G1	1	3	0.6 M, T*	G1	93	Intracellular activation pathway in macrophages/T cells?
K18L	—	—	— Fetal tissue	Cytokeratin (Ps)	xx	Pseudogene
B144 (Lst-1)	3	2	0.8 E, M, L, spleen	Lst-1	85	—
1C7	—	—	— Spleen	—	—	—
LTB	2	4	1 Activated lymphocytes*	LTβ	244	Cytokine. Anchors LTα to cell membrane
TNF	2.5	4	1.5 Activated Mφ and lymphocytes, NK cells, fibroblasts	TNF	233	Cytokine. Antitumor activity. Roles in inflammation/immunomodulation

Table 2 Delayed hypersensitivity reactions

Type	Reaction time	Clinical appearance	Histology	Antigen
Contact	48–72 h	Eczema	Lymphocytes, later macrophages, edema of epidermis	Epidermal, e.g. nickel, rubber, poison ivy
Tuberculin	48–72 h	Local induration	Lymphocytes, monocytes, macrophages	Intradermal, e.g. tuberculin
Granuloma	21–28 days	Hardening, e.g. skin or lung	Macrophages, epithelioid cells, giant cells, fibrosis	Persistent antigen–antibody complexes or non-immunoglobulin stimuli, e.g. talc

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hypersensitivity, foreign antigen presentation is performed by specialized Langerhans cells (HLA class II⁺, CD1⁺, FcγRII⁺) of the skin. These cells can process the antigen and transport it to the paracortical areas of the draining lymph nodes, where contact with naive T cells occurs and memory CD4⁺ T cells are generated. In addition, epidermal keratinocytes may be able to present antigen directly to class I HLA-restricted T cytotoxic cells. The elicitation step is triggered by the re-exposure of the sensitized skin to the same antigen. Activated endothelial cells lining postcapillary venules operate as antigen-presenting cells and present the foreign antigen to primed CD4⁺ T cells. IFNγ produced by CD4⁺ T cells is responsible for upregulation of a range of adhesion molecules and of HLA molecule expression on dermal endothelial cells and on keratinocytes, which further increases the efficiency of local antigen presentation. Adhesion molecules on endothelial cells bind to the corresponding ligands on leukocytes, thereby attracting them into the inflammatory site. Moreover, the activation of keratinocytes and macrophages by IFNγ produces proinflammatory cytokines, which enhance T cell and other leukocyte recruitment until the source of foreign antigen is eliminated by macrophages. Once macrophages are attracted to the skin, downregulation begins. The skin inflammation peaks within 72 h, when the epidermis becomes edematous. CD8⁺ cytotoxic T cells are also involved in the destruction of the hapten-conjugated epidermal cells. Suppression of the inflammatory reaction is mediated by the release of PGE produced by macrophages and keratinocytes and by TGFβ from mast cells and keratinocytes.

Tuberculin hypersensitivity

Tuberculin hypersensitivity was originally observed by Koch in patients with tuberculosis, and subsequently found in patients sensitized by *Mycobacterium leprae*, *Leishmania*, fungi, protozoa, and also by nonmicrobial agents. The reinjection of the spe-

cific sensitizing agent produces a generalized reaction of fever and vomiting and a local reaction of induration and swelling, reaching a peak at 48 h. The skin reaction is used to test for sensitivity to various organisms following previous exposure. After tuberculin challenge, cytokines, such as TNFα, are produced by antigen-specific T cells and these mediators are able to upregulate adhesion molecule expression on endothelial cells of dermal vessels. Adhesion molecules bind to corresponding receptors on leukocytes, thus recruiting inflammatory cells to the site of the reaction. Consequently, the migration of these inflammatory cells from the vessels into the dermis leads to the disruption of collagen structure. The tuberculin lesion usually resolves within a week but if the lesion progresses then it becomes granulomatous.

Granulomatous hypersensitivity

This reaction occurs as a result of the intracellular persistence of an antigen, usually microbial, particularly if it cannot be destroyed by macrophages. The reaction represents a defensive mechanism which is not always successful. Relevant diseases are leprosy, tuberculosis, schistosomiasis, sarcoidosis, Crohn's disease, berylliosis and Wegener's granulomatosis. Clinically, this is the most important form of delayed hypersensitivity. Antigen is presented to activated T cells which secrete proinflammatory cytokines (IFNγ, TNFβ). Cytokines are responsible for the activation of macrophages, whose role is to limit the dissemination of the intracellular microorganisms. When macrophages fail, chronic inflammation occurs. Following cytokine stimulation macrophages can fuse together and form giant cells which function to isolate the pathogens. Characteristically, granulomas consist of a central area of macrophages surrounded by lymphocytes. In tuberculosis the central zone can become totally isolated and the tissue undergoes necrosis. Macrophages and giant cells surround this area. Lymphocytes and

fibroblasts constitute the outer mantle. Foreign body reactions, such as talc and silica, can also present with granulomatous formation but they are distinct because of the absence of lymphocytes.

Measurement of cell-mediated immunity

Tests are developed to assess T cell memory, not protective immunity.

1. *Delayed hypersensitivity skin tests.* The antigen is injected into the skin and the positive inflammatory lesion is documented at the site of injection 48 h later (Mantoux test).
2. *Lymphocyte transformation tests.* This is a test to assess T cell memory. Sensitized lymphocytes are cultured in medium containing specific suspected antigen. A radioactively labeled DNA precursor (thymidine) is added, the mixture incubated, and the incorporation of radioactivity measured. The blastogenesis falls in subjects who are immunodepressed.
3. *Ability to produce cytokines following re-exposure to specific antigens.* Lymphocytes and macrophages are incubated with antigen; memory T cells will produce T_H1 and T_H2 cytokines, which can be measured in the supernatants of the cultured cells and within the cell lysates.

Type V hypersensitivity

Type V hypersensitivity occurs when autoantibodies are produced against cell surface receptors, with subsequent stimulation of the receptor. The most common clinical example is found in Graves' thyrotoxicosis, which, in up to 80% of cases, is characterized by the presence of circulating autoantibodies to the thyrotropin (TSH) receptor. There is a correlation between the autoantibody titer and the severity of the clinical condition, as the antibody stimulates via the same mechanism of adenylyl cyclase activation used by TSH itself. More recently, other clinical examples of type V reactions are gradually emerging, such as autoantibody against the β_1 -adrenergic receptor in myocarditis.

See also: Acute inflammatory reaction; Allergens; Anaphylatoxins; Antiglobulin (Coombs') test; Arthus reaction; Atopic allergy; Autoimmune diseases; Blood transfusion reactions; Cell-mediated immunity; Contact hypersensitivity; Delayed-type hypersensitivity; Eczema; Food allergy; Granuloma; Hemolytic disease of the newborn; Immune complexes; Rhinitis, allergic; Sarcoidosis.

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IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Idiopathic thrombocytopenic purpura (ITP) manifests into two relatively distinct clinical syndromes: acute ITP and chronic ITP. Both are characterized by immunologic destruction of the blood platelets. Because these syndromes differ significantly, they will be discussed separately.

Acute ITP

Acute ITP is a self-limited disorder occurring almost exclusively in children, most commonly between the ages of 2 and 5; there is no sex predilection. A similar syndrome occurs occasionally in adults. The disorder frequently appears 1–6 weeks after a viral illness and usually presents with the abrupt onset of petechiae, bruising and often epistaxis. More serious bleeding from the gastrointestinal tract occurs less commonly and central nervous system bleeding (the most feared complication) is rare (0.5–1.0%). Physical examination shows bleeding manifestations consistent with the level of the platelet count. Enlargement of the spleen is infrequent.

The initial platelet counts are often very low ($<10\,000\ \mu\text{l}^{-1}$); the leukocyte count and hemoglobin are normal unless significant bleeding has occurred. Bone marrow examination shows normal or increased numbers of megakaryocytes. Elevated levels of platelet-associated immunoglobulin G (IgG) are seen in essentially all patients with acute ITP, although recent studies show that this assay is relatively nonspecific and reflects primarily the presence of young platelets with increased α granule IgG. Antigen-specific autoantibodies are rarely demonstrable and, if present, are borderline positive.

Pathogenesis

Little experimental data are available. The fact that acute ITP is frequently seen after a viral illness suggests that the child may be responding to viral antigens by antibody production, immune complex formation and subsequent platelet destruction. Plate-

let removal may be due either to the absorption of viral antigens on to the platelet surface followed by antibody binding and destruction, or to the binding of preformed immune complexes to the platelet Fc receptor, with subsequent destruction. The clinical observation that these patients frequently respond to intravenous gammaglobulin or to anti-D antibody, both of which block the Fc receptors on phagocytic cells, suggests that platelets are destroyed primarily by phagocytosis. Since patients with acute ITP may respond to splenectomy (although this is rarely employed), the spleen is probably involved in platelet destruction.

Therapy

About 80–90% of patients with acute ITP recover spontaneously within the first 6 months; another 5–10% of patients recover over the next several months. Recovery occurs regardless of the therapy which is administered. Three potential therapeutic approaches have been championed by different investigators: 1) intravenous gammaglobulin; 2) high doses of corticosteroids; and 3) no therapy. In view of the self-limited course of acute ITP, some physicians advise no therapy because the mortality rate is extremely low ($<1\%$). However, the fear of severe mucosal bleeding or central nervous system bleeding pushes most physicians to treat their patients. In general, patients without mucosal bleeding, platelet counts $>20\,000\ \mu\text{l}^{-1}$ and a normal bleeding time can be safely observed; others should be treated. Randomized studies show that treatment of patients with either intravenous gammaglobulin or high-dose corticosteroids results in a more rapid recovery of the platelet count than is seen in untreated patients. Due to the low mortality rate of acute ITP, it is not possible to show a survival advantage of either treatment or no treatment. Platelet transfusions are indicated only under emergency circumstances for control of significant bleeding.

A certain proportion of children with acute ITP

fail to improve spontaneously (5–10%) and this group is felt to have an autoimmune condition similar to chronic ITP, which will be described next.

Chronic ITP

This is an autoimmune disorder which occurs most commonly in adults between the ages of 20 and 50 and is more common in women than in men (approximately 2:1). Unlike acute ITP, this disorder appears more insidiously, and extremely low platelet counts and severe mucosal bleeding are uncommon, although they may occur. Physical findings, as with acute ITP, reflect the degree of thrombocytopenia. An enlarged spleen or the presence of enlarged lymph nodes suggests another cause. A similar disorder, often associated with autoantibodies, is seen with a variety of illnesses, including collagen vascular disorders (e.g. systemic lupus erythematosus), lymphoproliferative disorders and certain infections, particularly human immunodeficiency virus (HIV). Blood counts are normal, except for the reduced platelet count, and bone marrow examination shows normal or increased numbers of megakaryocytes. Somewhat surprisingly platelet production studies show that, in the majority of chronic ITP patients, platelet production is either normal or reduced, rather than increased as would be expected. This suggests that the autoantibody may be binding to similar antigens on megakaryocytes and effecting thrombopoiesis.

Elevated levels of platelet-associated IgG are seen in about 90% of patients, but, as noted above, these findings are nonspecific. However, unlike acute ITP, antigen-specific platelet-associated and plasma autoantibodies can be demonstrated in about 80% and 50% of patients, respectively. Autoantibodies are most commonly directed to either the platelet glycoprotein IIb/IIIa complex (the platelet fibrinogen receptor) or to the glycoprotein Ib/IX complex (the platelet von Willebrand factor receptor), although autoantibodies rarely bind to epitopes which interfere with platelet function. As shown in Table 1, antibodies to the former complex occur more com-

monly. Other glycoproteins and glycolipids have been implicated as autoantigens in ITP.

Pathogenesis

Chronic ITP is due to the formation of an autoantibody against a platelet-associated antigen which results in subsequent phagocytosis of platelets by the reticuloendothelial system. The studies of Harrington and Shulman in the 1950s and 1960s showed that infusion of blood or plasma from ITP patients into normal recipients resulted in thrombocytopenia. This antiplatelet factor could be adsorbed with platelets and was present in the IgG-rich fraction of human plasma. Subsequent studies have shown that the autoantibody is produced *in vitro* by both splenic and bone marrow cells from patients with chronic ITP and that the autoantibody binds to both platelets and megakaryocytes. The importance of the spleen in disease pathogenesis, as both a source of autoantibody production and a platelet destruction site in ITP, is supported by the clinical observation that approximately two-thirds of patients are cured of their disease by removal of this organ. Autoantibody studies done before and after surgery show that levels of both platelet-associated and plasma autoantibodies fall precipitously and approach normal levels in patients responding to splenectomy. Conversely, autoantibody levels remain high in those who do not.

The role of complement activation in chronic ITP is unclear. Platelet-associated C3, C4 and C9 levels are elevated on ITP platelets, and *in vitro* incubation of platelets with selected autoantibodies results in binding of C3, C4 and C9 to the platelets and subsequent platelet lysis. How important complement activation is under *in vivo* circumstances is not known.

Although there have been numerous studies on cellular immunity in chronic ITP, the role of cellular immunity in this disorder is unclear. The present evidence suggests that the humoral immune system is the more important.

Platelet destruction in ITP is due to autoantibody binding to antigen, with or without complement activation, followed by phagocytosis of platelets by the reticuloendothelial system. Although complement-mediated platelet lysis, initiated by selected antiplatelet antibodies, has been demonstrated *in vitro*, phagocytosis is thought more important *in vivo*. Phagocytosis can be triggered by either the Fc portion of the IgG molecule or by platelet-bound C3b. The Fc mechanism is known to be important because therapy with either intravenous gammaglobulin or anti-D antibody results in an increase in the patient's platelet count in many chronic ITP patients and the mechanism of action of both agents is thought due

Table 1 Autoantibody results in patients with chronic ITP^a

Patients	Platelet-associated antibody	Plasma antibody
Total studied	142	128
Total positive	112	54
Anti-GPIIb/IIIa	81	43
Anti-GPIb/IX	24	7
Both autoantibodies	7	4

^aAutoantibody was measured using the immunobead assay (McMillan, 1990).

to Fc blockade. In addition, a few patients have been treated with an anti-Fc monoclonal antibody, with temporary improvement of their platelet count.

Therapy

Patients with chronic ITP are treated initially with high doses of corticosteroids ($1\text{--}2\text{ mg kg}^{-1}\text{ day}^{-1}$). Approximately 75–80% of patients will respond by increasing their platelet counts; however, upon tapering this treatment the majority relapse. It is estimated that about 10% of ITP patients are cured with corticosteroids. However, the mainstay of therapy in chronic ITP is splenectomy, which results in a complete and lasting normalization of the platelet count in approximately 60% of patients; an additional 10–15% show persistent improvement of their platelet counts to safe levels ($>30\,000\ \mu\text{l}^{-1}$).

Treatment of the 25–30% of patients who do not respond to corticosteroids and splenectomy is much more difficult. Multiple therapies have been tried with varying degrees of success, including: danazol, colchicine, cyclophosphamide, azathioprine, IgG and immune complex adsorption from plasma by insolubilized staphylococcal protein A, high-dose intravenous cyclophosphamide, and combination chemotherapy. Patients who are refractory to corticosteroids and splenectomy have significant morbidity and mortality from both the therapy and their disease. The overall mortality rate in chronic ITP is approximately 4%; however, refractory patients have a mortality rate closer to 15–20%.

See also: Autoimmune diseases; Autoimmunity; Platelets.

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IDIOTYPE

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About 50 years ago, it was demonstrated that antibody molecules bear antigenic determinants similar to foreign antigens and they can be defined by immunoglobulin (Ig)-specific sera prepared in xenogeneic or allogeneic animals. These studies led to the definition of two types of antigenic determinants called isotypic and allotypic specificities.

In 1955, Slater and coworkers studied the specificity of antisera prepared in rabbits against human myeloma proteins. They observed that such antisera still reacted with the initial immunogen after extensive absorptions on unrelated myeloma proteins or

normal immunoglobulins. This new category of antigenic determinants was called 'individual' antigenic specificities. Several years later, Oudin and Kunkel described several antigen specificities on rabbit and human antibodies. Oudin coined the term 'idiotype' from the Greek – which means 'identical form' – for this new antigen borne by immunoglobulins.

Further immunochemical studies localized the idiotypic determinants initially on Fab fragments and later on Fv fragments. These studies led to the conclusion that the idiotypes are markers of variable genes encoding the specificity of antibodies or of the

immunoglobulin receptor of clones exhibiting the same antigen-recognizing specificities. Studies conducted in the early 1980s showed that idiotypic determinants can also be serologically defined on the antigen receptor of T cells.

It is now generally accepted that the idiotype represents the antigenic determinants of variable regions or phenotypic markers of variable region genes encoding the specificity of a lymphocyte antigen receptor. Each individual antigenic determinant which makes up the *idiotype* of a given receptor is termed an *idiotope*.

Immunochemical requirements for the expression of idiotypes

Initial studies carried out with human myeloma proteins and rabbit antibodies have shown that both variable regions of heavy and light chains contribute to idiotypic determinants. These results strongly indicated that the idiotypes are conformational antigenic determinants. The development of new techniques, such as western blotting assay, preparation of hybrid heavy-light chain molecules by immunochemical methods or transfectoma technology, and the utilization of synthetic peptides corresponding to protein sequences of variable regions, showed that there are situations in which a given idiotype is localized on only heavy or light chain or in very rare cases on both. The idiotype located on a single chain or identified by anti-peptide antibodies are more likely sequence dependent and therefore are linear antigenic determinants.

Combined utilization of serologic properties of anti-idiotype antibodies with electron microscopic analysis allowed the mapping of idiotopes on V-regions of streptococcus group A-specific antibodies. These studies showed that the idiotopes could individually be located over much of the surface of the V region extending from the combining site to the constant region.

Classification of idiotypes

Idiotypes are generally defined by serologic reagents collectively designated as anti-idiotype antibodies. Several criteria were used to classify the idiotypes based on the type of interaction between idiotypes and anti-idiotype antibodies.

The immunochemical criterion used to classify the idiotype consists of the effect of antigen on the interaction between idiotype and anti-idiotype antibodies. In the case of some idiotypes, this reaction is inhibited by the antigen, whereas in other cases it is not. Because it is generally accepted that hyper-

variable regions make up the combining site of antibodies and amino acid residues are responsible for binding to antigens, antigen-inhibitable idiotypes are considered 'idiotypes associated with the combining site'.

The second category of idiotypes that interact with anti-idiotype antibodies are non-antigen inhibitable and are considered to be located outside the combining site, and are usually called 'framework associated idiotypes'.

Genetic criteria were used to classify the idiotypes at a clonal level. Individual idiotypes result from a unique somatic recombination event of immunoglobulin genes or as a consequence of somatic mutation. Individual idiotypes are expressed on the antigenic receptor of a single lymphocytic clone in a single individual of a given species and are not inherited.

Cross-reactive idiotypes (CRI), (IdX) are expressed on antibodies or T cell receptors (TCRs) with the same specificity from various individuals. This category of idiotypes is inherited in a mendelian manner and probably represents phenotypic markers of germline genes. Cross-reactive idiotypes were identified on antibodies with the same antigen specificities produced in various species: amphibians, mice, rats, guinea pigs, pigs, rabbits, monkey and humans. They have been identified on antibodies against foreign antigens (haptens, polysaccharides or proteins), cellular antigens (HLA-DR, H-2, erythrocyte antigens such as Rh, I or P), self antigens (DNA, Sm, thyroglobulin, basal membrane, collagen II), and cellular receptors (such as AChR, TSHR, insulin receptor, and β receptor).

In 1981, we classified the cross-reactive idiotypes into three major groups:

1. *IdX linked to allotype*. These idiotypes are expressed in all inbred or recombinant inbred strains which share the same allotypes of heavy or light chains. The restriction of the expression of the V region genes of immunoglobulin molecules by C genes is not fully understood.
2. *Interstrain idiotypes*. This category of idiotypes is expressed on various inbred strains or on a major fraction of individuals of an outbred species independent of genetic allelism of the constant region.
3. *Interspecies idiotypes*. These are cross-reactive idiotypes shared by antibodies displaying the same antigen specificity produced by individuals of various species. These IdX are markers of germline genes that are conserved during evolution.

The same idiotypic determinants of antibodies with the same antigen specificity can be shared

independently on various immunoglobulin classes or isotypes. This phenomenon is due to the ability of a given V_H region gene to recombine with various constant region genes during clonal development, differentiation and switching of B cells subsequent to antigen stimulation.

The idiotypes were also classified based on their expression in the immunoglobulin repertoire, variation during the immune response, or their role in the immune response. With respect to their expression, the idiotypes can be classified into:

1. Dominant idiotypes expressed on the majority of antibodies exhibiting the same antigen specificity. As an example, one may cite T15 IdX expressed on antiphosphocholine antibodies, A5A IdX expression on ACHO-streptococcus A polysaccharide-specific or CRI of arsonate-specific antibodies. The dominance of certain idiotypes can be related to the phylogenetic selection of clones involved in the immune response against microbes.
2. Minor idiotypes expressed on a small fraction of antibodies produced in a response against a particular antigen.
3. Silent idiotypes, which are not expressed during a conventional immune response but which can be detected or even can become dominant subsequent to activation of clones by anti-idiotypic antibodies or certain regimens of immunization.

From a functional point of view, the idiotypes can be classified into two major categories:

1. Conventional idiotypes, which can be defined by anti-idiotypic antibodies obtained in various animals and which do not show variations during the immune response.
2. Regulatory idiotypes were described on antibodies specific for both foreign and self antigens. They are involved in the regulation of responses to antigen and may show variations during the immune response.

Polymorphism of the idiotypic system

The localization of idiotype on Fv fragments of antibodies and the subsequent concept that the idiotypes are phenotypic markers of variable region genes encoding the antigen receptor of lymphocytic clones, led to another concept; namely, that there is a relationship between the combining site, hypervariable regions and idiotypes. However, this concept was challenged by the experiments of Oudin and Cazenave, who showed for the first time that the same idiotype can be shared by antibodies with various specificities. This observation clearly indicated

that the idiotypic system is polymorphic. The polymorphism of the idiotypic system has important implications for the physiology of the immune system, indicating the existence of a certain degree of connectivity between lymphocyte clones and a possible cross-regulation by anti-idiotypic antibodies produced by B cells or by anti-idiotypic T cells. There are numerous experimental data demonstrating that the idiotypic determinants expressed on the lymphocytic receptor are the target of regulatory processes involved in clonal expansion or suppression.

The idea of polymorphism of the idiotypic system is supported by several observations demonstrating that the cross-reactive idiotypes can be shared by antibodies with various specificities, antibodies specific for various epitopes of multivalent antigens, or antibodies with unknown specificities.

The idiotypes shared by antibodies specific for various antigens were initially described by Oudin and Cazenave, who found that rabbit antibodies specific for chicken, duck, and turkey ovalbumin share a serologically defined cross-reactive idiotype. This discovery was quite surprising, as the idiotypes were considered clonal markers. This observation was confirmed in various systems. Thus, cross-reactive idiotypes were found on antibodies specific for the immunodominant sugar of lipopolysaccharide of gram-negative bacteria, on antibodies specific for various type A influenza virus subtypes, etc.

It should be mentioned that idiotype-like structures can be shared by antibodies and other substances which are not immunoglobulins. Volkanis and Kearney actually showed that C-reactive protein which binds to phosphocholine and phosphocholine-specific antibody both interact with T15 anti-idiotypic antibodies. T15 idiotype is a dominant idiotype shared by murine phosphocholine-specific antibodies.

Idiotypes shared by antibodies specific for various epitopes of an antigen molecule were also demonstrated in several systems. Such shared idiotypes have been identified on guinea pig antibodies specific for different epitopes of hen egg white lysozyme, allo-type-specific antibodies or antibodies specific for PR8 virus laboratory-selected variants that differ from the parental virus by a single point mutation.

Shared idiotypes were also identified on antibodies with unknown specificities. These types of antibody are called 'parallel sets'. It is unknown whether these antibodies represent genuine anti-anti-Id antibodies interacting with the idiotypes of anti-Id antibodies or they exhibit an unidentified antigen specificity. In practice, it is very difficult to exhaust by screening all possible antigen specificities.

The antibodies sharing cross-reactive idiotypes can

be encoded by V genes from various V_H or V_K families. This indicates that contacting residues able to interact with the same anti-idiotypic antibody which serologically defines a given idiotypic can be produced by various combinations of V_H or V_K families.

Recognition of idiotypes by T cells

It is well documented that T cells can recognize the antigenic determinants of immunoglobulins as they are able to recognize foreign, allo or syngeneic antigenic determinants. This was shown for various subsets of T cells, such as suppressor or helper regulatory T cells or effector cells mediating cytotoxic, delayed-type hypersensitivity or contact sensitivity reactions. Anti-idiotypic T cells can recognize serologically defined idiotypes as well as idiotypes that are not recognized by anti-idiotypic antibodies. A regulatory idiotypic shared by PC-binding antibodies recognized by T cells but not by anti-idiotypic antibodies was identified by Gleason and Kohler. Recent data suggest that the recognition of idiotypes in association with major histocompatibility complex (MHC) antigens requires the processing of native immunoglobulins by antigen-presenting cells in the case of both CD4 and CD8 T cells.

Idiotypes of TCR

TCR, like Ig of B cells, bears idiotypic antigenic determinants that can be located on α or β chain or both. These determinants should be distinguished from antigenic determinants borne by $V\alpha$ or $V\beta$ chains encoded solely by $V\alpha$ or $V\beta$ gene segments. These latter can be considered as the equivalent of V_H or V_K subgroup or allotypic determinants.

Idiotypic determinants of TCR were defined by anti-idiotypic antibodies or T cells. In early studies, Eichmann and Rajewsky showed that the T cells and B cells exhibiting the same antigen specificity, i.e. ACHO carbohydrate of streptococcus A, share the same idiotypic. These studies were not confirmed later. Actually, idiotypic determinants of TCR are generally different from those of Ig because of the low degree of homology between CDRs of V genes of immunoglobulins and of TCR. Idiotypic determinants defined by antibodies or by T cells were identified on various subsets of T cells such as suppressor and helper T cells, and T cells mediating delayed-type sensitivity, contact sensitivity, or resistance against graft-versus-host reaction. It is important to mention that antibodies specific for idiotypes of TCR can function as antigen surrogates; namely, to activate or suppress T cell function.

Structural correlates of idiotypes

Two major approaches were used to determine the structural correlates of idiotypes. The first method consisted of mutational analysis of antibody molecules followed by screening with anti-Id antibodies, and the second method employed X-ray crystallographic analysis of the Fab fragments of Id-anti-Id complexes.

Comparison of the sequences of the V_H regions of anti- α 1-3 dextran monoclonal antibodies reacting with anti-Id specific for J558 or MOPC106 myeloma proteins established that one amino acid difference located in the CDR3 of V_H expressed in an anti- α 1-3 dextran monoclonal antibody (i.e. MOPC104) accounted for the expression of an individual idiotypic specificity.

Similar studies of the sequences of V genes encoding antibodies specific for haptens such as 4-hydroxy-3-nitrophenyl-acetyl (NP), *p*-azo phenyl arsonate (Ars) or phosphocholine (PC) demonstrated that one to a few amino acids correlate with idiotypes. These observations strengthened the concept that individual idiotypes are the result of somatic mutations which took place in an individual clone.

Site-directed mutagenesis contributed efficiently to the definition of the amino acid residues of idiotypes involved in the interaction with anti-idiotypic antibodies. The information provided from these studies established that:

1. Single amino acid substitutions may have dramatic effects on the expression of individual or regulatory idiotypes.
2. Amino acid residues in both CDRs and frameworks may contribute to the expression of idiotypes but that CDR3 appears to have a prominent importance.
3. Both V_H and V_L chains can contribute to the expression of a given idiotypic.

These conclusions are strongly supported by X-ray crystallographic analysis of Id-anti-Id complexes which represent the definitive and most subtle approach to determining the nature of idiotypic-anti-idiotypic interactions.

Study of Fab complexes of an anti-lysozyme and anti-Id monoclonal antibody showed that 13 amino acid residues of the idiotypic interact with the anti-idiotypic antibody. These 13 contact residues are associated with five CDRs and one framework. In this case the idiotypic is a conformational epitope representing a solvent accessible area of approximately 800 Å determined by the participation of various CDR loops of both the V_H and V_L region.

See also: Antibodies, antigenicity of; Antigen-binding site; Idiotypic, internal image; Idiotypic network.

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IDIOTYPE, INTERNAL IMAGE

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The idiotype network theory proposed by Jerne suggests that, in the steady state, the immune system is in equilibrium due to the existence of complementary clones that are interconnected by idiotypic links. This concept implies that the receptor of a lymphocytic clone is able to recognize a particular antigen by its paratope, and to be recognized by other clones through idiotypes which are expressed on the antigen receptor of all lymphocytic clones. Each receptor has several idiotypes which are collectively designated as its idiotype. Operationally, the clones of the immune system can be divided into two major categories: those which recognize the antigen (Ab1) and those which recognize the idiotypes (Ab2). This classification is strictly operational, since one of the essential features of the network theory is that it introduces the concept of internal image of the antigen as a statistical necessity. This means that among the multitude of three-dimensional, steric structures of the variable domains of lymphocytic receptors, there are structures which are similar, if not identical, to the epitopes of conventional foreign or self antigens.

Lindeman suggested that it is likely that such structures are expressed on anti-idiotype (anti-Id) antibodies, because if the idiotype of an Ab1 associated with the combining site is the negative imprint of the antigen, the idiotype of an Ab2 could be the

positive imprint of the antigen, i.e. its internal image. The obvious conclusion to this concept is that the internal antigens play a role equally as important in the network as external antigens because they represent links between the outside world of the antigens and the immune system of a given individual.

Two major functions can be thought of for the internal antigens. First, the idiotypes as internal antigens can prime the Ab1 precursors for self or foreign antigens. From a physiological standpoint, this is important because the host can respond promptly to an invader. There are data to indicate that a mother's idiotypes transferred via the placenta can actually expand clones which become dominant during a conventional immune response elicited by a bacterial polysaccharide and which are otherwise silent.

The second function of internal image can be related to the stimulation of memory cells and particularly their long survival after extinction of the immune response related to clearance of the antigen. Minute amounts of residual internal image antibodies could have a stimulatory effect on memory cells, as it is well established that the lymphocytic receptor of such cells have high affinity for antigen and therefore a surrogate stimulus may suffice to stimulate their proliferation and survival. The first demonstration of an internal image idiotype came

from the work of Sege and Peterson. These investigators showed that anti-idiotypic antibodies prepared against bovine anti-insulin antibodies were able to mimic some of the properties of insulin. Such antibodies bound receptor and stimulated some physiological properties of insulin itself.

Anti-idiotypic antibodies carrying the internal image

The idiotypes of a lymphocytic receptor can be located on various segments of the variable regions and may require the contribution of both the heavy and light chain. Some idiotopes are associated with the combining site, since their interaction with anti-idiotypic antibodies can be inhibited by antigen. Other idiotypes are located on variable region segments which are not involved in the combining site; the interaction of these idiotypes, collectively designated the 'framework associated idiotypes', with anti-Id antibodies is not antigen inhibitable. Therefore, it appears that the clones recognizing idiotypes (Ab2) represent a heterogeneous population displaying various specificities.

These clones express surface immunoglobulin and secrete antibody which can be classified into four major categories (Figure 1):

1. Ab2 α , which is specific for idiotypes associated with the framework segments of the variable region. The binding of Ab2 α to Ab1 does not alter the binding of Ab1 to antigen. These antibodies can exhibit a regulatory effect, priming

or suppressing Ab1 clones, but they cannot replace an antigenic stimulus. It was shown that allogeneic A/J anti-T15Id antibodies, which are not antigen inhibitable, can induce a long-lasting suppression of T15⁺ anti-phosphocholine antibodies. Similarly, a monoclonal antibody specific for a cross-reactive idiotypic shared by antibodies specific for influenza virus neuraminidase was able to prime animals for a specific response but not elicit by itself anti-neuraminidase antibodies.

2. Ab2 γ are antibodies that recognize combining site-associated idiotypes. Such antibodies can inhibit the binding of Ab1 to antigen but cannot elicit an Ab1 response when injected into an animal. Such antibodies can have a regulatory effect, despite the fact that they are devoid of internal antigen properties.
3. Ab2 β are anti-idiotypic antibodies mimicking the antigen. Such anti-Id antibodies bind to the combining site of the lymphocytic receptor and are able to stimulate the expansion of clones and their maturation in antibody-forming cells. The interaction of Ab2 β with the combining site of Ab1 is obligatorily antigen inhibitable, since Ab2 idiotypic is an antigen surrogate.
4. Ab2 ϵ (epibody) are antibodies which bind both to idiotypic and to an epitope of self or foreign antigen.

The major questions concerning the stimulatory effect of anti-Id antibodies are related to:

- The ability to distinguish Ab2 α or Ab2 γ from Ab2 β .
- The determination of the immunogenic and tolerogenic doses. If the idiotypes are surrogates of the antigens, then they may obey the same rules of immunogenicity as the antigens.
- The requirements of MHC-restricted recognition of internal image antigen.

Criteria that define Ab2

Several criteria have been proposed to distinguish Ab2 α from Ab2 β . Immunochemical criteria are based on the molecular mimicry concept, which are predicted that all Ab2 β resemble the antigen and therefore Ab1–Ab2 β interactions are antigen inhibitable. If this feature can distinguish Ab2 α from Ab2 β , it is not sufficient on its own to distinguish Ab2 β from Ab2 γ . This latter category is also antigen inhibitable but cannot elicit an antibody response to the antigen by itself.

The best criterion is a structural one. Ab2 β may share some amino acid sequence with the antigen. An

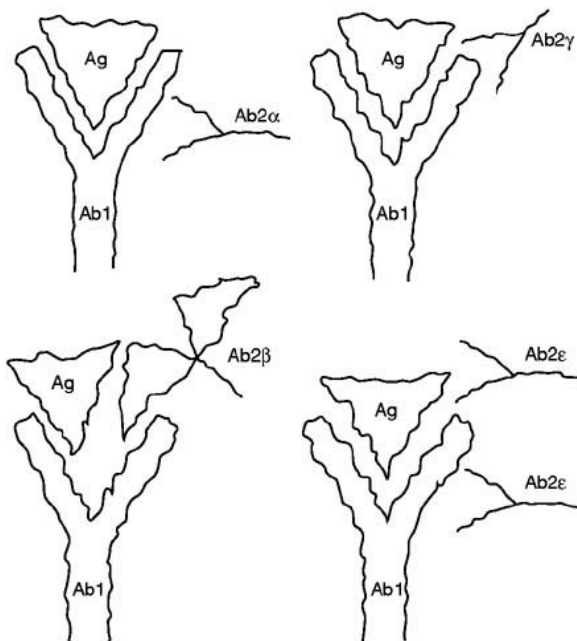


Figure 1 Various types of anti-idiotypic antibodies.

Ab2 β specific for anti-glutamic acid-alanine-tyrosine synthetic terpolymer (GAT), in which GT is the major immunodominant epitope, has a GTT surface-exposed tripeptide in the D region. The injection of this Ab2 β into animals elicits an anti-GAT or GT response. Similarly, an Ab2 β specific for anti-hemagglutinin reovirus antibody shares a tetrapeptide located in the CDR2 of the variable region of the light chain with the protective epitope of viral hemagglutinin. This antibody, as well as the synthetic peptide that corresponds to its shared sequence, can elicit an anti-viral immune response and can be recognized by cytotoxic T cells specific for viral hemagglutinin. It should be noted that Ab2 β , which mimic nonprotein antigens such as polysaccharides and glycolipids, and steroidal hormones such as aldosterone, have also been described.

Perhaps the most practical criterion for defining Ab2 β is the functional one; namely, their ability to elicit the production of Ab1 antibodies specific for the nominal antigen.

Immunization with anti-Id antibodies can, in principle, lead to the production of several types of antibody molecules: the Ag⁺/Id⁺/Ab1 type, which binds to nominal antigen and expresses the idiotype of the antibody used to prepare Ab2 β ; the Ag⁺/Id⁻/Ab1 type, which binds to nominal antigen but fails to express the idiotype of the Ab1 used as immunogen; and Ag⁻/Id⁺, which are Ab3 antibodies able to bind to the idiotype of Ab2.

In principle, the outcome of the immunization with Ab2 α or Ab2 γ antibody will be the activation of clones producing Ag⁺/Id⁺ and Ag⁻/Id⁺ antibodies, since these antibodies can interact only with Ab1 clones bearing the corresponding idiotypes or with Ab3 clones producing only anti-anti-id antibodies. In contrast, the outcome of the immunization with Ab2 β , which by virtue of its idiotypes could mimic the antigen will activate Ag⁺/Id⁺ as well as Ag⁻/Id⁻ clones, both of which are able to recognize the nominal antigen or its idiotypic surrogate. Obviously, Ab2 can also activate true Ab3 clones.

Therefore, the functional criterion which define an Ab2 β is the ability to initiate the production of antibodies specific for nominal antigens able to express a variety of idiotypes in the absence of the immunization with antigen itself.

Structural basis of internal antigen mimicry

Only the study of the three-dimensional structure of antigen-Ab1 (idiotype) and of Ab1 and Ab2 β was able to provide exhaustive information on the molecular basis of internal image immunoglobulin.

Fields *et al* determined the crystal structure of an idiotype and anti-idiotype to a resolution of 1.9 Å. Fv fragments were obtained from D1.3, a monoclonal antibody (mAb) specific for lysozyme, and from the E5.2 anti-idiotypic mAb. E5.2 exhibits the properties of an Ab2 β because when injected into BALB/c or C57BL/6 mice it elicits the production of anti-lysozyme antibodies.

Of the 18 residues of D1.3 that contact the anti-Id antibody and the 17 that interact with lysozyme, 13 were in contact with both lysozyme and the anti-Id antibody. The position of the atoms by which the anti-idiotype contacts the anti-lysozyme antibody are close to those by which D1.3 contacts lysozyme. The structural mimicry observed involves van der Waals forces, hydrogen bonds and solvent interactions.

Ban and colleagues determined the crystal structure of the Fab complex of a mAb specific for the E2 peplomer of feline infectious peritonitis virus and of an anti-Id antibody. This anti-Id also exhibited Ab2 β properties since when it was injected into animals it elicited the production of virus-neutralizing antibodies. The two Fab fragments interact by direct juxtaposition of their complementary CDRs. The CDR loops of the two Fabs were in contact through van der Waals forces and hydrogen bonds between 58 atoms of the virus-specific antibody and 59 atoms of the anti-Id. In total 19 residues of idiotype and 17 residues of anti-idiotype participate in the interaction. The CDR1 loops of the anti-Id light and heavy chains have a near identity with a hexapeptide of the antigen. These two regions of homology of the CDR1 of the light and heavy chain of Ab2 provide important contacts with the idiotype of the antibody specific for viral antigen.

These results indicate that the structural correlates of Ab2 β mimicry can be related to discontinuous determinants with contact residues located in various CDR loops. However, in other cases such as the feline peritonitis virus system some linear sequences located in CDRs can play a major role in antigen mimicry.

While there is a wealth of evidence that the surface Ig of B cells can recognize either discontinuous or linear epitopes on the native antigen, T cells recognize only peptides generated from the fragmentation of antigens which are then bound to self MHC antigens. This implicates that internal image-bearing peptides recognized by T cells are derived from immunoglobulin processed by antigen-presenting cells and selected by binding to either class I or class II major histocompatibility complex (MHC) antigens. The peptides associated with class I recognized by CD8 T cells are generally nonamers, whereas those associated with class II antigens recognized by

CD4 T cells are larger. Thus, an internal image immunoglobulin able to stimulate T cells should generate peptides identical to those generated by the processing of foreign antigens.

Practical applications of internal image idiotypes

In the early studies on idiotypic regulation, Eichmann and Rajewsky showed that polyclonal xenogeneic anti-Id antibodies of a particular isotype injected into mice can stimulate the production of antibodies against a polysaccharide antigen of streptococcus group A. Similar results have been obtained with monoclonal anti-Id antibodies in a hapten system, namely the activation of clones producing anti-TNP antibodies expressing 460Id.

Important information regarding the stimulatory aspect of anti-Id antibodies was provided by Hiernaux and colleagues, who showed that the injection of minute amounts of anti-Id antibodies after birth can expand silent clones. Knowledge of the expansion of silent clones was important for an understanding of the effect of anti-Id in tumor therapy.

However, Nisonoff and Roitt predicted that the idiotypic mimicry of foreign antigens can serve as a basis for the development of new vaccines based on the preparation of anti-Id antibodies. This hypothesis stimulated studies of the utilization of anti-Id antibodies as vaccines against bacterial, viral and parasitic antigens and was largely extended to other areas. An area in which anti-Id antibodies have seen practical application is tumor therapy both for lymphocytic tumors, in which the idiotypic functions as tumor-associated antigen, as well as solid tumors. In this case, the injection of antibodies against tumor-associated antigens can elicit the production of internal image, which expands the clones involved in anti-tumor immunity.

Other applications of internal image consist of the utilization of Ab2 β to identify or purify cell receptors for biologically active substances such as enzymes, hormones, or drugs. Anti-Id antibodies raised against antibodies specific for biologically active ligands will bind to the cell receptor and therefore permit its identification or eventual isolation.

The internal image concept has provided a theoretical basis for the practical application of anti-idiotypic antibodies in biology and medicine.

See also: **Antibodies, antigenicity of; Idiotypic; Idiotypic network; Molecular mimicry; Vaccines.**

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IDIOTYPE NETWORK

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The idiotypic network theory formulated by Jerne views the immune system as being interconnected by idiotypes which are the antigenic determinants of variable regions of a lymphocytic receptor. Each receptor has several idiotypes which are collectively designated as its idiotypic.

The immune system is composed of lymphocytic clones that are able to recognize through their combining site (paratope) the foreign as well as the self antigens. The immune receptor of each clone expresses several idiotypes that are a special category of self antigens which can be recognized by other T or B cells bearing complementary paratopes. Operationally, the receptors recognizing the idiotypes are called anti-idiotypes or Ab2, whereas those expressing the idiotypes are called Ab1.

The idiotypes can be considered a special category of self epitopes which are autoimmunogenic without the need of breaking of self tolerance.

Background to the idiotypic network theory

The autoimmunogenicity of idiotypes was one of the first observations that led to the development of the network theory. This was first shown experimentally by the induction of an autoantibody response specific for idiotypic determinants borne by antibodies isolated from a rabbit and reinjected with its own antibodies several months later. Such rabbits produced antibodies able to bind to idiotypes of its own antibodies. The occurrence of autoanti-idiotypic antibodies was also demonstrated in physiological conditions during conventional immune responses elicited by an antigen.

A reciprocal fluctuation between Ab1 and Ab2 clones was observed in the immune response elicited by phosphocholine or inulin as well as in the florid and remission phases of certain autoimmune diseases such as systemic lupus or myasthenia gravis.

It should be pointed out that not all idiotypic (Id) determinants seem to be equally immunogenic. The dominant idiotypes that are expressed in high amounts in the circulation rarely cause anti-Id responses compared to individual or minor idiotypes. However, experiments carried out with animals in which the expression of cross-reactive idiotypes were suppressed after birth showed that such animals are able to produce anti-Id antibodies subsequent to injection with cross-reactive idiotypes.

These observations clearly show that all idiotypes are immunogenic but that the stimulation and/or proliferation of anti-Id clones depends on the concentration of idiotypes in the circulation.

Also contributing to the development of the idiotypic network theory were data that demonstrated that idiotypes are expressed on the immune receptor of lymphocytic clones; namely, the surface immunoglobulin of B cells or the TCR of T cells. These observations were of great importance for understanding clonal connectivity through idiotypes; namely, that the clones can speak one to another using an idiotypic dictionary that overlaps with the V gene repertoire of lymphocytes.

There is much data to demonstrate that there are lymphocytes in both B and T cell compartments able to recognize the idiotypes of B or T cell receptors. There is strong evidence that B cells are able to produce antibodies specific for the idiotypes of surface immunoglobulin of other B cells or for TCR of T cells. Similarly, in the T cell compartment it was shown that there are T clones able to recognize the idiotypic determinants of B cells (anti-idiotypic T cells) or of other T cell clones (Figure 1). Both CD4⁺ and CD8⁺ cells can recognize idiopeptides derived from the processing of V regions within the cytoplasm via the endogenous pathway. The idiopeptides resulting from the V region polypeptides are translocated to the membrane via the secretory pathway with either class I or class II major histocompatibility complex (MHC) antigens, where they are recognized by anti-idiotypic T cells. These two observations – namely, the autoimmunogenicity of idiotypes and the presence of idiotypes on the immune receptor of lymphocytic clones – represent the major contributions to the idiotypic network theory, which views the immune system as a web of V domains.

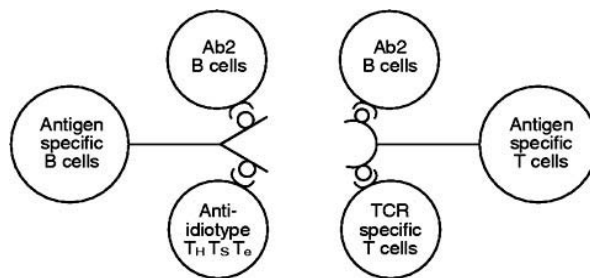


Figure 1 Symmetry of idiotypic recognition in B and T lymphocytic subpopulations.

An important question arising from this concept is whether or not all lymphocytes are idiotypically interconnected. There are two major categories of lymphocytes – short-lived and long-lived lymphocytes. Memory cells belong to the second category. Certain immunologists consider that the idiotypic network is predominant among the short-lived lymphocytes because these cells mainly produce multi-specific antibodies that can bind to both self and foreign antigen. However, there are data to suggest that autoanti-Id antibodies can only be detected against the idiotypes of antibodies produced during a secondary immune response, which are mainly the product of memory cells. Such secondary idiotypes are not detected during the primary response, which has been documented in the hen egg lysozyme system. Actually, the idiotypic network concept views the immune system as a dynamic process, which implies many types of interactions in the various stages of the development of an immune response as well as among the subpopulations of lymphocytes.

In the steady state, there is an equilibrium between Ab1 and Ab2. The major pattern of this equilibrium is the suppression of expansion of Ab1 clones by Ab2 lymphocytes. This probably represents an important physiological mechanism for the control of clonal homeostasis preventing the overwhelming of the immune system by certain clones, as can happen in the case of lymphocytic tumors.

When an organism encounters the antigen, the immune system decides which pathway should be taken – the suppressive or immune response. This is a transitional stage in which memory cells are generated, indicating that the immune system has the capacity not only of learning and deciding but also remembering the choice for a long time. In the transitional stage, this equilibrium is shifted by the antigen, which causes the expansion of Ab1 clones. In general, a reciprocal fluctuation between Ab1 and Ab2 clones is observed during this transitional state.

It is known that the antibody response, particularly against T dependent antigens, is associated with somatic mutations, a process believed to be crucial in the production of antibodies with high affinity. Somatic mutations can be important in establishing new idiotypic interactions and new regulatory patterns in the idiotypic network. Therefore, the transitional state that represents the immune response is determined by the clonal elements of the steady state as well as by variants which have been generated.

The system evolves toward a new steady state after the diminution of the immune response. However, this new steady state is qualitatively different, since it is associated with the generation of memory cells. The anti-Id antibodies that are produced during the

immune response can play an important role in the establishment of this new steady state. It is possible that an important fraction of anti-Id antibodies generated during the immune response carry the internal image of the antigen. These antibodies (termed Ab2 β) can persist in the body after the disappearance of the antigen and can contribute to the stimulation and survival of memory cells (Figure 2).

Postulates of idiotypic network theory

Idiotypic network theory is based on three major postulates:

1. The idiotypes of the immune receptor of lymphocytes are recognized within the immune system and they function both as links between clones and the target of regulatory processes.

Self-non/self discrimination is a central element of the clonal selection theory which views the immune system as a collection of clones programmed to recognize foreign antigens. While foreign antigens selectively induce the proliferation of clones bearing complementary immune receptors, self reactive clones are deleted or suppressed during ontogeny. In contrast, with the idiotypic network concept, the self/non-self discrimination ceased to be a central problem as the idiotypes that are self antigens are recognized and can be immunogenic. The major question of whether the immune system is directed outward (i.e. the recognition of foreign antigens) or inward (i.e. the recognition of self antigens) becomes meaningless.

Furthermore, there are a legion of experimental findings indicating that idiotypes are the target of regulatory processes. Both anti-Id antibodies or anti-idiotypic T cells can activate or suppress the expansion of clones bearing corresponding idiotypes. There are data to show that the maternal transfer of anti-Id antibodies via the placenta in the newborn, and of the injection of anti-Id antibodies in neonates and adults causes longlasting suppression of clones bearing the corresponding idiotypes. This suppression can be due either to a direct effect of anti-Id antibodies on the precursors or the generation of Id-specific suppressor cells. Maternally induced, neo-

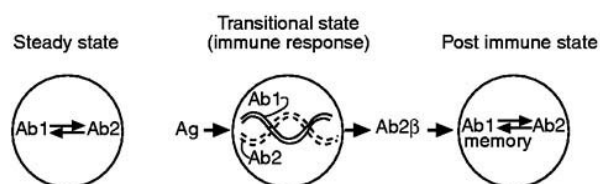


Figure 2 Idiotypic interactions in steady-state and immune response.

natal or adult idiotype suppression generally requires a high concentration of anti-Id antibodies. In contrast, minute amounts of anti-Id antibodies exhibit a stimulatory effect. They can expand the dominant clones in place of the antigen or stimulate the expansion of minor and even silent clones. Antibodies specific for the idiotypic determinants of TCR can replace antigen in the stimulation or suppression of T cell clones.

It should be mentioned that not all serologically defined idiotypes are targets of regulatory processes. We defined a category of idiotopes – designated regulatory idiotopes – which are mainly involved in idiotype-determined regulatory processes. The regulatory idiotopes can be distinguished from conventional idiotopes by the following criteria:

- (a) They are autoimmunogenic.
- (b) They can be expressed on antibodies with various specificities.
- (c) They are recognized by regulatory T cells and could become dominant idiotopes.

The expression of regulatory idiotopes on antibodies with various specificities represents the structural basis of cross-regulation of clonal expansion by anti-Id antibodies. There are data which clearly show that the administration of anti-Id antibodies in minute amounts causes the activation of a clonally heterogeneous population with various antigen specificities and that the clones specific for nominal antigen (Ab1) represent only a fraction of the activated clones.

2. Idiotypes as internal image of the antigens.

This postulate is based on the idea that the idiotypes can mimic the foreign and self antigens. The idiotypes as markers of the variable region of immunoglobulin molecules or TCR can be envisioned as the negative imprint of the antigen. In contrast, the idiotype of anti-idiotype antibody can be seen as the positive imprint of the antigen. This postulate is not a simple consequence of the 'lock and key' principle of the interaction between an antigen and an antibody, but rather is based on molecular mimicry; namely, that the idiotype of a given antibody molecule can mimic the antigen. The concept of idiotypes as internal image of antigen indicates that the idiotypes represent links between the universe of foreign antigens and the immune system and that actually the immune repertoire is already primed by internal stimuli and therefore can promptly react to foreign antigen.

3. Through the self-recognition process, idiotypes can activate a cascade of complementary clones.

The concept is based on various experimental data which demonstrate that idiotypes can induce a chain of anti-idiotypic antibodies; namely, Ab1→Ab2 (anti-Id antibody)→Ab3 (anti anti-Id antibodies)→Ab4. Initially, the idiotype cascade was demonstrated experimentally in allotypically matched outbred rabbits, but later it was shown that it can happen in a single animal. The demonstration that the cascade can occur in a single animal strengthens the conclusion that the idiotype network is not a pure accident and has a precise functional role. Studies of the immunoreactivity of complementary anti-Id antibodies showed that Ab1 resembles Ab3 and Ab2 resembles Ab4. These data clearly show that the idiotype network is not open ended, as was initially thought and predicted by certain mathematical models.

The degeneracy of idiotype-anti-idiotype interactions within the cascade introduces a high degree of connectivity between lymphocytic clones and builds a complex web of reciprocal interactions. In fact, so-called Ab3 – which are produced subsequent to stimulation by Ab2 (anti-idiotypes) – are heterogeneous. They constitute a population of antibodies that cannot be distinguished from Ab1, a population that reacts with the idiotype of Ab2 (true Ab3) and, finally, a population that can exhibit a wide array of specificities (parallel sets).

Ontogenetic and phylogenetic significance of the idiotype network

The rearrangement of DNA segments encoding the specificity of the variable region of the immune receptor of T and B lymphocytes occurs during the development of these lineages. The V region genes are clustered in families. The members of the same family show a high degree of DNA homology. The DNA homology can be observed among certain V gene families of various species. Because the rearrangement of some V genes occurs during ontogeny before the expression of antigen receptor, it appears that the idiotype network does not play an important role in the establishment of the pre-immune repertoire. However, there is strong evidence to indicate that it plays an important role in the establishment of the primary repertoire in neonates and adults.

Maternal or neonatal idiotype suppression leading to ablation from the repertoire of the clones bearing the corresponding idiotypes is one of the best and most studied examples. Similarly, maternal anti-Id antibodies or idiotypes transferred via the placenta to neonates can play an important role in shaping the primary immune repertoire. For example, it was

shown that the injection of idiotype into mice during pregnancy stimulates the generation of idiotype-specific helper cells in neonates, which can expand silent clones to become dominant. Similar results can be obtained with anti-Id antibodies administered after birth. In physiological terms, it is advantageous for progeny to inherit the immune experience of the mother with an expansion of clones that are specific for common pathogens of a given species. This is particularly important in the period of postnatal life when the concentration of antibodies provided from the mother starts to decline.

The absence of an idiotype-induced expansion could explain the widespread epidemics and pandemics caused by pathogens that have not been encountered by previous generations, such as the pandemics caused by influenza viruses resulting from a major antigenic shift or the current human immunodeficiency virus (HIV) pandemics.

In certain cases, the maternal idiotypes can have deleterious effects. Thus, it is well known that an important fraction of newborns born to myasthenic mothers can develop transient symptoms of myasthenia due to the mothers' autoantibodies specific for acetylcholine receptors. However, in certain cases, the infants develop severe and chronic disease. It has been found that these patients produced anti-Id antibodies, which were probably responsible for the activation of autoreactive clones producing anti-acetylcholine receptor antibodies and which are involved in the pathogenesis of the disease. It is important to note that recent studies have shown that a relatively high fraction of the clones (about 7%) of neonates produce anti-Id antibodies and such antibodies can have either a stimulatory or suppressive effect. These observations clearly indicate that the idiotype network plays an important role in the establishment of the primary immune repertoire and that idiotypes or anti-idiotypes can act in lieu of the antigen in the fixation of the repertoire.

The immune response continuously generates clonal variants because of somatic mutations as well as the recombination of various segments of DNA during the rearrangement of immunoglobulin and TCR genes. The sequencing of V genes of antibodies specific for haptens and viral proteins has shown that each antibody has a different sequence from the germline V gene from which the rearranged V gene

was derived. This would imply that idiotype regulatory networks should exist for each clone. However, it has been shown that in each species there are regulatory patterns that are inherited. Despite the fact that each of the anti-arsonate antibodies in A/J mice are encoded by V_H genes that differ from the VH36-65 germline gene from which their rearranged genes derive, anti-Id antibodies specific for CRI of anti-arsonate antibodies will cause the suppression of the arsonate response in all A/J mice. This suggests that the idiotype network can play an important role during phylogeny in the elimination of variants that are not the subject of idiotype-determined regulatory processes and in the maintenance of the stability of the repertoire in a given species.

That the idiotype network is important both in selecting the initial available repertoire and in idiotype-determined regulation during immune responses elicited by both foreign and self antigens is a broadly held view based on a large number of experimental findings and clinical observations.

See also: Antigen-binding site; Autoimmunity; B lymphocyte repertoire; Clonal selection; Idiotype; Idiotype, internal image.

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Ig α /Ig β (CD79a/CD79b)

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In the pro-B cell stage, the rearrangement of immunoglobulin heavy chain gene leads to the expression of surface membrane immunoglobulin (mIg μ). mIg μ assembles with Ig α -Ig β and surrogate light chains in pre-B cells, or conventional light chains in B cells, to form the precursor B cell receptor (pre-BCR) and B cell receptor (BCR), respectively. The pre-BCR is sufficient for the pre-B cell transition, and imposition of allelic exclusion. In the more mature B cell stages, specific recognition of antigen by the BCR triggers B cells for subsequent differentiation and proliferation into terminally differentiated plasma cells. Interaction of the Ig α /Ig β heterodimer with immunoglobulin heavy chains is essential for the signal transduction.

Protein tyrosine kinases are associated with BCR through the cytoplasmic tails of Ig α and Ig β

Ig α (CD79a) and Ig β (CD79b) are expressed prior to immunoglobulin heavy chain gene rearrangement and are the products of the mb-1 and B29 genes, respectively. The B29 gene contains six exons. In murine B cells, Ig α is about 34 kDa and Ig β is about 38 kDa. Both are extensively glycosylated and occur as disulfide-linked heterodimers, which are noncovalently associated with mIg. Both proteins are members of the immunoglobulin supergene family, each contains one Ig-like extracellular domain, a transmembrane spanning segment and a cytoplasmic tail. The cytoplasmic tails of Ig α and Ig β proteins contain a conserved sequence motif, which is called the immunoreceptor tyrosine-based activation motif (ITAM). Signaling through BCR induces a rapid sequence of phosphorylation steps. Upon receptor crosslinking, four members of the Src family of protein tyrosine kinases, including Blk, Lyn, Fyn and Lck, are activated to phosphorylate the Ig α and Ig β tails, causing the association of another tyrosine kinase, Syk, to the receptor. Syk contains two SH₂ domains, which associate with the conserved tyrosine residues in the ITAM motifs only when they are phosphorylated. This association results in the activation of Syk and augments transmission of the signal. Evidence that Ig α and Ig β couple the BCR to

intracellular protein tyrosine kinases comes from the following observations: 1) Ig α and Ig β exhibit low levels of tyrosine phosphorylation in resting B cells and are hyperphosphorylated in response to anti-Ig; 2) analysis of Lyn-deficient (Lyn^{-/-}) mice showed that the number of B cells decreased by half in the peripheral tissue due to the impaired proliferation; in addition, these B cells do not respond normally to a number of stimuli, including BCR crosslinking and CD40 ligand; 3) analysis of lymphoid cells from Syk-deficient (Syk^{-/-}) mice showed the total ablation of BCR signaling and blockage of the differentiation of B-lineage cells. The reconstitution of irradiated mice with Syk-deficient fetal liver revealed the importance of Syk kinase in pro-B to pre-B cell transition. Thus, Ig α and Ig β can couple B cell receptors to intracellular protein tyrosine kinases.

Ig α and Ig β mediate signals

At the immunoglobulin heavy chain locus, the rearrangement begins by the joining of diversity genes (D_H) with the joining elements (J_{H1}) and is followed by variable region (V_{H1}) gene rearrangement (V_{H1} → DJ_{H1}). Heavy chain gene assembly precedes light chain gene rearrangement. The ordered rearrangements of immunoglobulin heavy chain and light chain genes lead to the expression of surface immunoglobulin which is essential for B cell development. However, it has been shown that mIg μ molecules in which transmembrane polar residues have been mutated (Tyr-Ser:Val-Val, YS:VV) are unable to activate signal transduction. This disruption may be due to blockage of the association with Ig α and Ig β which comprise the signal transducer unit in each tail. In order to examine whether the pre-B cell transition and the inhibition of V-DJ joining is the result of signaling through the ITAM motifs of Ig α and Ig β tails, we generated an Ig μ transgene with the YS:VV mutation. In contrast to the wild type Ig μ transgene, B cells carrying the mutant transgene could not mediate allelic exclusion of endogenous loci. However, this mutant can be restored to wild type competence by addition of the cytoplasmic tail of Ig α and Ig β to the carboxyl terminus of Ig μ . Replacement of ITAM tyrosines in the Ig α and Ig β tail with phenyl-

alanine cannot restore this activity. These findings imply that allelic exclusion is mediated through signal transduction via Ig α and Ig β and is dependent upon intact tyrosine residues. To test whether Ig α and Ig β are required for the pre-B cell transition, strains of RAG $^{-/-}$ transgenic mice were created which carried a YS:V μ with or without the cytoplasmic domain of Ig α and Ig β . Mice carrying the mutant transgene could not produce pre-B cells. However, mice carrying the YS:V μ Ig α and Ig β chimera transgene developed pre-B cells. Thus, a single ITAM from either Ig α and Ig β is essential for the pre-B cell transition.

Since Ig α and Ig β are expressed at the pro-B cell stage, it has been postulated that an earlier checkpoint may be regulated by this signal transducer. In order to investigate the role of Ig α and Ig β at the pro-B cell stage, Ig β $^{-/-}$ mice were created by targeted gene disruption. These mice had normal levels of D $_H$ to J $_H$ rearrangement but V $_H$ to D $_H$ and V $_K$ to J $_K$ rearrangements were severely diminished. Since the B cells of these mice were arrested at the pro-B cell stage and did not express Ig μ , these mice failed to produce pre-B, mature B and plasma cells. These results revealed an independent role of Ig β outside the BCR in the pro-B cell stage.

D $_H$ to J $_H$ rearrangement has three reading frames (RFs). However, it has been shown that RF1 joints are overrepresented in functional murine heavy chains. This RF bias was considered to be due to the counterselection of RF3 on the bases of stop codons and of RF2 through the expression of the membrane-bound D $_{\mu}$ protein, since RF2 joining activates heavy-chain transcription from a previously inactive promoter 5' to D $_H$ and the resulting mRNA encodes a truncated μ protein, D $_{\mu}$. It has been proposed that D $_{\mu}$ may signal through Ig α and Ig β and activate the allelic exclusion of machinery which inhibits further VDJ recombination and suppresses the expression of functional Ig μ . Thus, cells containing D $_{\mu}$ will be arrested from further development. Results from the Ig β $^{-/-}$ mice showed that D $_{\mu}$ signaling is totally blocked and there was no bias against RF2 in D $_H$ joining. This revealed that the Ig α /Ig β signal transducer also plays a critical role in the counterselection against D $_{\mu}$. This finding is in accordance with the other observations that there is no counterselection in the absence of an Ig μ transmembrane exon, Syk, and λ 5. Taken together, these results suggest that the conserved ITAM motif in Ig α and Ig β tails couples the BCR to cytoplasmic tyrosine kinases which mediates signaling to the nucleus.

ferentiation; Diversity, generation of; Immunoglobulin, cell surface; Immunoglobulin genes.

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IgA

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Immunoglobulin A (IgA) is of special interest because of its predominance in exocrine secretions where it is thought to provide the host with a first line of immune defense against foreign microorganisms. The relatively high concentration of IgA in the exocrine secretions is due to two principal factors: 1) an enrichment in mucosal tissues of cells committed to IgA synthesis; and 2) the presence of a specific receptor on epithelial cells (poly-Ig receptor or pIgR) which transports newly synthesized polymeric IgA (pIgA) across the epithelium and discharges it into the secretory lumen.

Synthesis and distribution

IgA is found in species which emerged after the appearance of amphibia, although evidence has been presented that IgA-like molecules may exist in fish. In most higher vertebrates, the majority of IgA is synthesized by gastrointestinal lymphoid tissue, with smaller amounts synthesized at other mucosal sites such as the respiratory tract, salivary glands and reproductive tract. During lactation, mammary tissue contains substantial numbers of IgA-producing cells. The majority of these are thought to migrate from the gut, thus protective IgA antibodies generated by the mother against gastrointestinal organisms in the environment may be transferred to the infant via breast feeding. An important species variation should be noted: in addition to IgA, ungulates have even greater concentrations of IgG1 antibodies in milk, due to the presence in mammary epithelium of a specific receptor which transports IgG1 from blood to milk.

While much of the IgA produced by mucosal tissue is transported into the secretory lumen, some inevi-

tably enters the lymphatic circulation and is transferred to blood via the thoracic duct. In some species (e.g. rabbit, rat, chicken and mouse), polymeric IgA entering the blood is returned to the intestinal secretion via the liver, which, in these species, expresses the pIgR on hepatocytes.

The total quantity of IgA synthesized in humans has been estimated to be 66 mg kg⁻¹ day⁻¹, of which approximately two-thirds is derived from mucosal lymphoid tissue. The relatively large amounts of IgA synthesized daily may reflect the fact that the mucosal surfaces are constantly infected with organisms that are either commensal to the host or pathogenic. The bone marrow also provides an additional source of IgA, especially in humans.

IgA production is regulated by T cells. T cell-derived transforming growth factor β (TGF β) has been identified as the primary cytokine that promotes B cells to switch from IgM-bearing cells to IgA-bearing cells. Interleukin 5 (IL-5) has been identified as the major cytokine that drives the differentiation of IgA-bearing B cells to IgA antibody-secreting plasma cells.

Catabolism of IgA is thought to occur primarily in the liver. In humans, this appears to be mediated by the receptor for asialoglycoproteins, which targets by endocytosis, asialoglycoproteins to lysosomes.

Structure

The three-dimensional structure of IgA determined by crystallographic studies has not yet been reported. Consequently, there is still some uncertainty in the conclusions drawn from available data. However, computer models of IgA structure have been put forward based on analogy with the known three-dimensional structure of IgG.

The numbers of IgA isotypes vary between species. In humans, there are two isotypes (IgA1 and IgA2), whereas in rabbits as many as 13 have been reported. The principal difference between human IgA1 and IgA2 is the absence in IgA2 molecules of a 13 amino acid sequence in the hinge region, which is rich in proline, threonine and serine-containing O-linked glycosyl side-chains. This region is also the target of proteolysis by IgA1 proteases secreted by many bacteria which inhabit mucosal surfaces. In humans, the IgA2 isotype exists in two allotypic forms: IgA2m(1) and IgA2m(2). IgA2m(1) molecules also lack the cysteine in the heavy chain which links, through disulfide bonds, the α heavy chain to the light chain. In mice, the α chain is expressed as a single isotype and has been reported to exist in two allelic forms. BALB/c mice have IgA molecules that are similar to human IgA2m(1), in that their light chains are disulfide-linked to each other, rather than to the corresponding α heavy chain. IgA in the NZB strain contains the α chain cysteine which links α and the light chain by a disulfide bond. Allotypic variation also exists in the rabbit α chains. A diagram of the principal features of the human α_1 heavy chain of IgA is shown in Figure 1.

The advantage of the extended hinge region in IgA1 molecules has not yet been assessed but it could provide IgA1 molecules with greater segmental flexibility, compared with IgA2 antibodies, which in turn might enhance antigen binding of this isotype. This advantage might be offset by increased susceptibility of IgA1 molecules to attack by IgA1-specific proteases secreted by bacteria inhabiting the mucosal surface.

All α chains terminate in an octadecapeptide 'tail' sequence not found in the prototype immunoglobulin IgG. IgM also possesses a homologous extended tail at its C-terminus. IgA is secreted from lymphocytes as a four-chain 'monomer' subunit, as well as in polymeric forms consisting of dimers, trimers and tetramers. Polymer formation is accompanied by disulfide linkages between J chain (a 15 kDa protein synthesized by antibody-producing cells) and a cysteine present in the extended 'tail' as the penultimate residue of the α chain, at amino acid position 471.

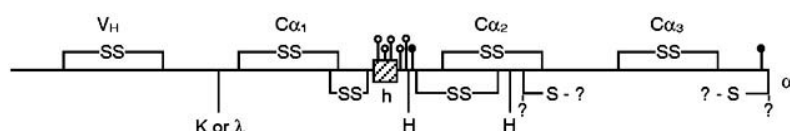


Figure 1 The human α_1 heavy chain. h, Hinge region which is absent in α_2 chains; $\dagger$, O-linked carbohydrate; $\ddagger$, N-linked carbohydrate. The intrachain disulfide bonds within domains are shown above the line [SS]; other intrachain disulfide bonds and the interchain disulfide bonds are shown below the line. The cysteines at residues 311 and 471 are hypothesized to form an intrachain disulfide bond in IgA monomers and an intersubunit disulfide bond in dimeric IgA.

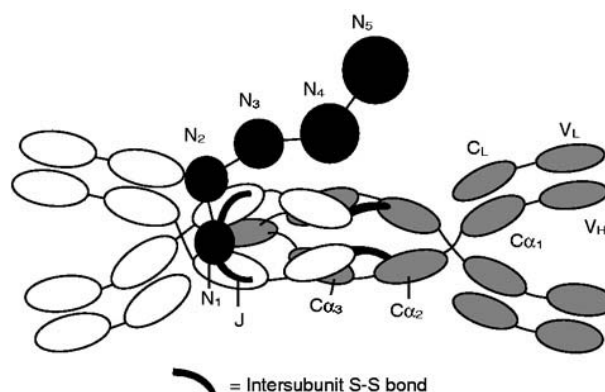


Figure 2 Secretory IgA dimer. The asymmetry of the disulfide bonds that form the dimer is emphasized. Disulfide bonding of the first domain of secretory component (N1) is shown linked to the $C\alpha_2$ domain, hypothesized to be at residue 311. (Reprinted with permission from Underdown BJ (1990) Transcytosis by the receptor for polymeric immunoglobulin. In: Metzger H (ed) *Fc Receptors and the Action of Antibodies*. Washington DC: American Society for Microbiology.)

Polymer formation confers on IgA enhanced avidity for binding antigen and is required for interaction of pIgA with the pIgR.

The arrangement of disulfide bonds joining the various subunits together in pIgA appears to be asymmetric. One model proposes that J chain is disulfide-linked through cysteine 471 to the two α chains of one monomer subunit. The two monomer subunits are then envisioned to be linked by the cysteines at position 471 of the monomer lacking J chain to cysteines in the $C\alpha_2$ domain (possibly residue 311) of the monomer which contains J chain. Formation of secretory IgA occurs with the addition of secretory component which, in humans, is known to be disulfide-linked to only one of the monomer subunits, presumably at residue 311. Dimeric secretory IgA is illustrated in Figure 2.

Function

The unique structural characteristics of secretory IgA provide some clues to its function. For example, the ability of polymeric IgA to bind to the pIgR and be transported to the mucosal secretions, coupled with

the relatively high proportion of plasma cells committed to IgA production at mucosal sites, provides the mucosal surface with substantial amounts of antibody. Being polymeric, secretory IgA has relatively high avidity for antigen, which should promote its antigen-binding function. The presence of secretory component appears to protect secretory IgA against proteolytic enzymes in the gut. Binding of secretory IgA antibodies to microorganisms would be expected to reduce the ability of the microbes to attach and enter the epithelium of the mucosae, two processes which seem crucial for the initiation of infection. Indeed, good experimental evidence exists to support this function of IgA. In addition to preventing attachment of microorganisms, secretory IgA is thought to perform an 'immune exclusion' function by combining with the myriad of soluble antigens in the diet, as well as those released from commensal and pathogenic flora, preventing them from entering the body. It has also been demonstrated that during its transcytosis through epithelial cells, IgA antibody comes into contact with, and neutralizes virus in infected epithelia.

Elimination of infectious agents from the mucosae may also involve cooperative interaction of IgA antibodies with other effector elements of the immune and inflammatory systems. For example, cells such as macrophages, neutrophils and eosinophils have been reported to have receptors which bind to various forms of IgA. Activation of the alternative pathway of the complement system by IgA has been demonstrated *in vitro*. The importance of these properties in combating infections at the mucosal surface is less evident, as it is not clear whether the relatively small number of inflammatory-type cells observed in the mucosal lumen are functional, nor is there evidence that an intact complement system exists at the mucosal surface or in external secretions.

The functional role of systemic IgA is not clear. Clearance of antigen in the form of antigen-IgA antibody complexes has been demonstrated and it is possible that this is particularly important in the case of antigens which have crossed the epithelial barrier into the circulation. This function would be expected to be carried out best by IgA antibodies induced by mucosal antigen.

Deficiency

In Caucasians, IgA deficiency is the most common form of selective Ig isotype deficiency. Estimates vary between 1:300 and 1:800 in a normal population. Among the Japanese, IgA deficiency is reported to be much less frequent. Individuals with IgA deficiency have normal α chain structural genes, but may

express a higher frequency of particular major histocompatibility complex (MHC) haplotypes. This suggests that IgA deficiency is associated with defects in immune regulation (e.g. secondary to cytokine deficiency) and evidence has been presented which indicates that IgA deficiency may be linked to the presence or absence of particular HLA haplotypes. In addition to generalized deficiency of IgA, selective deficiency in one or the other IgA isotype or in systemic versus mucosal IgA have been reported.

There appears to be general consensus that individuals with IgA deficiency are at risk for mucosal infection, although many such individuals are healthy. Individuals who develop a compensatory increase in IgM antibody synthesis appear to be indistinguishable from normal individuals with respect to frequency of mucosal infection. Infants who are transiently deficient in IgA in the first year of life are prone to develop atopic disease. An increased frequency of autoimmune diseases has also been reported in IgA-deficient individuals, which may reflect the importance of a putative defect in immune regulation, although increased exposure of the host to foreign antigens as a result of the loss of 'immune exclusion' may play a role. In contrast to humans, IgA deficiency has not been reported in many species. A group of IgA-deficient dogs has been reported.

Associated immunopathology

There are several diseases in which IgA is deposited in tissue in a pattern which suggests it may play a role in inducing pathology. For example, IgA nephropathy is one of the most common Ig-associated nephropathies. Typically, deposits which consist primarily of IgA1, often together with components of the complement alternative pathway, are found in the renal mesangium. While there have been a large number of studies *in vitro* indicating the potential of IgA to activate the alternative pathway of complement, only a few have used antigen-antibody complexes in which the antigen did not have the potential to activate the complement system in an aggregated form. However, some studies have avoided this pitfall and it appears that IgA has the capacity to activate the alternative pathway with less efficiency than IgG. The association of mucosal infection with either the onset or exacerbation of IgA nephropathy gives further credence for the pathogenic role of IgA in this disease. It should be noted that IgA nephropathy is a disease with a broad clinical spectrum and, in approximately half the patients, IgG and IgM are also observed associated with IgA deposited in the renal mesangium. These data raise the possibility that these latter isotypes are required

to produce a more severe clinical course. Dermatitis herpetiformis is a disease characterized by deposition of IgA at the dermal–epidermal junction. Here again, components of the alternative pathway of complement are deposited along with IgA. Dermatitis herpetiformis is often associated with elevated levels of gliadin-specific antibodies and the clinical course of some patients may be reduced by a gluten-free diet. A bullous disease of childhood has been described in which linear deposits of IgA are detected along the basement of affected subepidermal areas, suggestive of the deposition of specific anti-basement membrane IgA antibodies.

Induction of IgA responses

Immunization at mucosal surfaces is known to be the most effective method for inducing IgA antibody responses. Migration of IgA-committed cells to other mucosal sites is known to occur, but sufficient protection of these sites probably requires expansion of seeded cells by direct application of antigen to the secondary sites. Parenteral immunization also results in a brief synthetic boost of pIgA in blood, the function of which is not known; however, there is evidence that parenteral priming will seed mucosal sites for IgA production to a secondary mucosal challenge with antigen.

To date, immunization at mucosal surfaces is accomplished best by living organisms, followed by mucosal adjuvants such as cholera toxin. Nonliving antigens are, in general, not effective unless targeted to Peyer's patches.

See also: Cytokines; Gastrointestinal tract infections; Immunoglobulin structure; Joining J chain; Mucosa-associated lymphoid tissue (MALT); Oral immunity; Secretory component (the polymeric Ig receptor).

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IgD

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The IgD immunoglobulin isotype was discovered in 1965 by Rowe and Fahey, who found a human myeloma protein with unusual properties. They also found small amounts of immunoglobulin of the same type in normal human serum. Subsequently, immunoglobulins that could be generally defined as IgD were found in all mammalian and avian species. This evolutionary conservation points to a defined functional role of this immunoglobulin, yet, after a third of a century of intensive investigation the specific function of IgD, as distinct from the other immunoglobulins, remains to be defined.

The protein

Human IgD is a monomer of 175 kDa with 9% carbohydrate. The human heavy chain (δ) has three domains and an unusually long hinge region that is highly sensitive to proteolysis. The murine δ chain is shorter and is missing a domain corresponding to the human C_δ2. The membrane form of the δ chain has in addition a transmembrane domain and a short intracytoplasmic extension with the sequence Lys-Val-Lys, analogous to that of the membrane form of the μ chain.

The gene

The gene that codes for the constant portion of the δ chain is located downstream of the μ gene. In the mouse it consists of three exons ($C_{\delta}1$, $C_{\delta}2$ and $C_{\delta}3$) that code for the body of the δ chain, plus three small exons that code for the C-terminus of the secreted (δs) or the membrane form of the δ chain ($\delta M1$ and $\delta M2$); all these are separated from $C_{\delta}3$. It is noteworthy that there is no switch region upstream of $C_{\delta}1$. The simultaneous synthesis of μ and δ chains, with the same variable region, by one given B cell occurs by the production of a transcript that includes both the μ and the δ genes, followed by alternate splicing to produce separate μ and δ mRNAs. When B cells mature to an IgM-secreting plasma cell, transcription usually stops after the μ gene and therefore the δ message is not included in the transcript. Alternatively, when the B cell matures to an IgD-secreting plasma cell (a relatively rare event), the μ gene is deleted.

Serum IgD

In humans, serum IgD levels vary from 3 to 40 $\mu\text{g ml}^{-1}$, with some relationship to the Gm type of the individual. These low IgD levels are the product of IgD plasma cells that are found in low numbers in the spleen and, more frequently, in the tonsils. The low numbers of IgD plasma cells in the lymphoid tissues, together with the short half-life of the immunoglobulin (2.8 days), explain the low levels of IgD in the serum. The relative paucity of IgD plasma cells also explains the rarity of IgD myelomas. Similar conditions are also found in mice, in which there are two IgD allotypes, termed Ig5^a and Ig5^b.

It is curious that no specific antibodies have been found so far in serum IgD, even after immunization, even though humans exposed to chronic antigen stimulation tend to express relatively higher serum IgD levels. Conversely, no specific effector function has been ascribed to the IgD isotype. Another curious finding with human serum IgD, but not membrane IgD, is that it is more commonly associated with λ than with κ light chains; similarly, human IgD myelomas with λ chains are about five times more frequent than those with κ chains.

Membrane IgD

In sharp contrast to the low levels of serum IgD, both in humans and in mice, IgD is expressed on the membrane of the majority of B lymphocytes. In mice, membrane IgD appears around the end of the first week postpartum, and the percentage of B cells with

membrane IgD then rapidly increases, more or less in parallel with the maturation of the T cell system of the animal. However, δ chains that are not expressed on the membrane are apparently synthesized quite early in developing B cells. A completely rearranged δ gene causes allelic exclusion in transgenic mice.

Membrane IgD and IgM molecules, with the same V_H and with the same light chain, and therefore with the same antigen-binding site, are expressed together on the majority of B lymphocytes. Since μ and δ chains do not pair, there are no hybrid μ/δ molecules. The ratio between the two isotypes may vary between high δ /low μ and the reverse; it may be rather stable in some cells, like the Ly1-B cells, that are low δ as a rule, or it may be variable even in single clones of B cells, like some human Epstein-Barr virus (EBV)-transformed cells where it has been found to vary between the two extremes in the same clone. The ratio of IgM to IgD on a given B lymphocyte must have functional consequences that are not as yet totally clear; it is established, however, that membrane IgD is reduced in memory B cells and that it disappears with the process of maturation to plasma cells.

After cross-linking with anti- δ antibodies, membrane IgD is internalized by the cells, much in the same way as the other membrane immunoglobulin isotypes; however, in some B cell lines, differences in the intracytoplasmic location of the IgD/anti-IgD complexes versus the IgM/anti-IgM complexes have been observed; this difference may have relevance to the role of the two isotypes in the processing of antigens by B lymphoid cells.

Membrane IgD molecules are associated with a heterodimer consisting of two proteins of 35 kDa (IgD- α) and 39 kDa (IgD- β); the presence of the IgD- α chain is necessary for the membrane expression of IgD. Membrane IgM is also associated with a heterodimer formed by the same IgD- β chain discussed above and by an IgM-specific IgM- α chain of 34 kDa. Thus the two membrane immunoglobulin isotypes are associated with companion molecules which are related but different; these biochemical observations may go a long way toward the understanding of the function of the two classes of antigen receptors that coexist on the membrane of B lymphocytes.

So far, however, no gross differences have been detected between the biochemical events that take place in B cells after cross-linking IgM and those seen after cross-linking IgD; therefore, the immediate signals given to the B cell by the two classes of antigen receptors seem to be equivalent.

However, a difference in the kinetics of phos-

phorylation of the protein tyrosine kinases after cross-linking of IgM and IgD with the same antigen specificity has been detected by Kim and Reth. In IgM-expressing cells phosphorylation reaches a maximum in 1 min and decreases after 60 min, whereas in IgD cells the phosphorylation of the protein kinases reaches a maximum in 60 min and persists longer than 240 min after exposure to antigen. This may be relevant in conditions where the B cells are competing for antigen, as in the germinal centers.

The function of IgD

Notwithstanding a long series of experiments and hypotheses, the immunological function of IgD is still far from being completely understood. Many of these experiments were performed with heterologous or allogeneic anti- δ antibodies administered *in vivo*. The early ones were performed on monkeys using rabbit antihuman IgD antisera that cross-react with monkey IgD. The result was a marked polyclonal stimulation of immunoglobulin production, mostly of the IgG class, that included several antibodies to the different serum proteins of the heterologous anti-IgD antiserum. The mechanism of this marked reaction, that developed in a couple of weeks after the injection of the antiserum, was not clarified. With the isolation of murine IgD from a myeloma, and with the availability of purified hetero- or alloantimouse IgD antibodies, the study of the effect of administration of anti-IgD *in vivo* could make considerable progress.

Finkelman and coworkers have clearly shown that the polyclonal stimulation of B cells induced by heterologous anti-IgD, that eventually results in the production of IgG1, takes place in two stages, of which the first is T independent; the second involves T helper cells, activated by the heterologous immunoglobulins, that help the IgD⁺ cells to mature, switch to IgG1, and secrete the latter. The simplest interpretation is that B cells bind anti-IgD, process this foreign molecule, and present its fragments to T helper cells specific for it. However, this interpretation is not the only one. In fact, experiments with transferred congenic B cells of different allotype also point to the possibility that B cells activated by anti-IgD can use in a nonspecific fashion the large amount of cytokines produced by the activated T helper cells. This last possibility would be in agreement with the production of specific antibodies observed in the early experiments conducted with monkeys, that, as already noted, suggested an 'adjuvant' effect of the injection of heterologous anti-IgD antibodies.

It is clear, however, that protein ligands bound to membrane IgD can be processed very efficiently for

presentation to T cells. Another possible interaction between IgD and T cells is through a receptor for IgD that is present on the membrane of a subpopulation of T cells; through this receptor IgD can stimulate T cells in a nonspecific fashion.

Double transgenic mice, which express both the gene for hen egg lysozyme (HEL) and the genes for a high-affinity anti-HEL antibody; are tolerant to HEL but contain high numbers of B cells with anti-HEL membrane Ig that is IgD and not IgM. Since tolerance to the antigen cannot be explained by suppressor T cells, it appears that this is somehow an intrinsic property of the IgD⁺ B cell population. IgD may therefore have a role in B cell tolerance. Curiously, such a role may favor tolerance, a view opposite to that entertained previously, when the observation that membrane IgD in murine B cells appeared after expression of IgM seemed to favor the interpretation that IgD could prevent B cell tolerance.

Interesting observations that shed some light on the function of membrane IgD have been made by Roes and Rajewsky in mice in which the δ gene had been knocked out. In heterozygous $\delta^{+/-}$ mice the wild-type IgH locus was considerably overrepresented amongst the peripheral B cells and in particular in cells producing IgG1 in response to a T-dependent antigen. In homozygous IgD-deficient mice affinity maturation of antibodies in a primary T cell-dependent response is delayed. This is again consistent with a role of the IgD receptors where the B cells are competing and are being selected for receptor affinity, such as in the germinal centers.

See also: B lymphocyte activation; B lymphocyte differentiation; Fc receptors; IgM; Immunoglobulin, cell surface; Immunoglobulin class switching; Immunoglobulin structure; Tolerance, peripheral.

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IgE

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IgE (immunoglobulin E) is the least abundant, but in many respects the most potent, of the various antibody classes found in mammals. In normal individuals the concentration of IgE in serum (100 ng ml^{-1}) is 10 000 times less than that of IgG (15 mg ml^{-1}), but it is elevated in allergic individuals, in certain parasitic diseases (e.g. schistosomiasis) and in the hyper-IgE syndrome. The designation IgE stems from the observation that injection of this antibody into skin, followed by antigen at the same site, causes passive cutaneous anaphylaxis, characterized by the appearance of a wheal (edema) and flare (erythema) reaction at the site of the injections. Erythema ensues when antigen crosslinks IgE antibody bound to the high-affinity IgE receptor on skin mast cells, which leads to the release of agents, such as histamine, that cause vasodilatation and pooling of blood in the tissue. This is part of the typical inflammatory reaction caused by immune processes involving IgE antibodies and mast cells (see below). IgE is best known for its association with allergy, and individuals with abnormally high serum concentrations of IgE are said to be 'atopic'; however, epidemiological and experimental evidence suggests that IgE antibodies may have a beneficial function in the rejection of parasites.

Structure of IgE

Like antibodies of any other class, IgE is composed of four polypeptide chains, two heavy chains and two λ or κ light chains; its main features are described in Table 1. IgE is characterized by its ϵ heavy chains, which have four invariant or constant (C) region domains, C ϵ 1–C ϵ 4 (Figure 1), corresponding to those in the μ chain of IgM (C μ 1–C μ 4), but differing from the three that occur in the γ - δ - and α -heavy chains of IgG, IgD and IgA, respectively. The extra domain is C ϵ 2 (or C μ 2 in IgM), which occupies the equivalent position to the hinge found in IgG, IgD and IgA. IgE can be proteolytically cleaved into Fab and Fc fragments in the same way as IgG. The cleavage site lies between C ϵ 1 and C ϵ 2, and thus the Fc fragment has three (C ϵ 2–C ϵ 4) rather than two (C γ 2 and C γ 3) immunoglobulin domains per chain. The homology between the C ϵ 3–C ϵ 4 region of IgE and the IgG Fc, and the existence of a high-resolution crystal structure of the IgG1 Fc, has provided the basis for a model of this region of the IgE structure (Figure 1).

IgE is heavily glycosylated, the carbohydrate contributing 13% of the total mass. There are seven putative glycosylation sites, three in the C ϵ 1 domain of the Fab and four in the Fc region. The latter include the conserved site in C ϵ 3 (Asn397; homolo-

Table 1 Characteristics of IgE

Cell distribution	Committed and selected B cells
Molecular weight	≈ 190 000
Glycosylation sites (human)	Asn145, 173, 219, 265, 371, 383, 394
Receptors	FcεRI, FcεRII, galectin 3 (ε-BP or Mac-1)
Domain structure – heavy chain	V _H – C _ε 1 – C _ε 2 – C _ε 3 – C _ε 4
– light chain (κ or λ)	V _L – C _L
Isoforms	Secreted, and long and short membrane forms

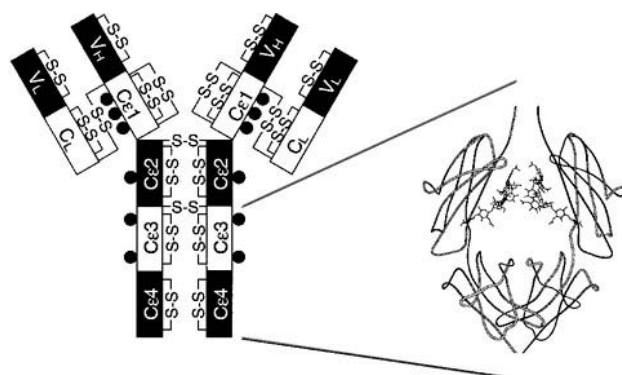


Figure 1 Structure of IgE. Each of the immunoglobulin domains is represented by rectangles. Intrachain and interchain disulfide bridges are represented by S-S, and external glycosylation sites are shown as black circles. Also shown is the α -carbon backbone trace of the C_ε3 and C_ε4 domains, modeled on the homologous IgG1 structure, with the conserved internal (buried) carbohydrate between the two C_ε3 domains.

gous to Asn297 in C_γ2), which is buried between the two ϵ chains in the model of IgE (Figure 1), and three additional sites (Asn265 in C_ε2 and Asn383 and Asn371 in C_ε3), which should be exposed on the surface of IgE. Two of these, Asn371 and Asn383, however, do not appear to be fully occupied.

The crystal structure of IgG does not allow prediction of the structure of C_ε2 or of its disposition with respect to C_ε3, but X-ray scattering data on the IgE-Fc suggest that there is a sharp bend between these domains. This is consistent with evidence from fluorescence energy transfer experiments, which indicates that IgE is bent in solution and remains so when bound to its high-affinity receptor (Figure 2).

Receptors for IgE

There are two cell surface receptors for IgE: FcεRI and FcεRII (also known as CD23). Their properties are set out in Table 2.

FcεRI comprises four polypeptide chains: one α , one β and two γ chains (Table 2). Only the first possesses an appreciable length of extracellular sequence; this contains two immunoglobulin-like domains and can be modelled on the nuclear mag-

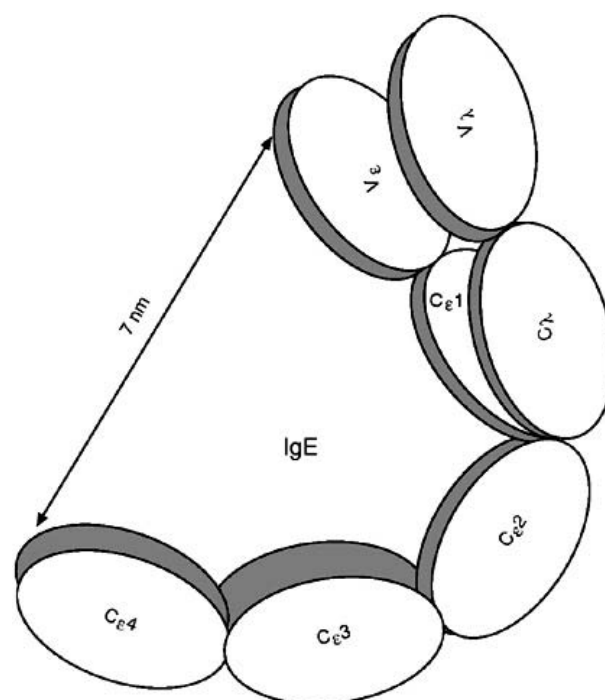
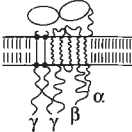


Figure 2 IgE adopts a bent conformation in solution. The immunoglobulin domains of IgE are represented by ellipses, and are arranged in a conformation compatible with available structural data. Also indicated is the measurement between the ends of the molecules as determined by fluorescence energy transfer methods; the diagram is to scale.

netic resonance and crystal structures of two homologous proteins (CD4 and CD2). It is known to form the binding site for IgE. The other three chains have more extensive intracellular sequences and are responsible for signal transduction. The affinity of FcεRI for IgE ($K_A \sim 10^{10} \text{ M}^{-1}$) is at least 1–2 orders of magnitude higher than that of any other Fc receptor–antibody interaction. FcεRI forms a 1:1 complex with IgE, attaching to one or both of the C_ε3 domains. C_ε4 sequences, however, are required for binding with full high affinity, probably because of their influence on the structure or disposition of the C_ε3 domains. The activities of FcεRI are a function of the cells in which it is expressed, and include IgE antibody-dependent antigen presentation in Langer-

Table 2 Characteristics of IgE receptors FcεRI and FcεRII

Feature	FcεRI	FcεRII
Structure		
Alternative names	High-affinity IgE receptor	Low-affinity IgE receptor, CD23
Cellular distribution	Mast cells, basophils, eosinophils, Langerhans cells, activated monocytes	B Cells, T cells, platelets, macrophages, natural killer (NK) cells, follicular dendritic cells, eosinophils, epithelial cells
Subunit composition	One α, one β and two γ chains	Homotrimer, cleaved to soluble fragments
Transmembrane regions	α = 1, β = 4, γ = 1 (×2), total = 7	1 per monomer, total = 3
Orientation	Intracellular C-termini	Intracellular N-terminus
Structural motifs	α = two immunoglobulin domains	C-type animal lectin, α-helical coiled coil, reverse RGD (Arg Gly Asp) adhesion sequence
Post-translational modification	Seven N-glycosylation sites in α, Ser, Thr, Tyr phosphorylation in α	Single N-glycosylation site
Affinity for monomeric IgE	$K_a \sim 10^{10} \text{ M}^{-1}$	$K_a \sim 10^7 \text{ M}^{-1}$
Binding site on IgE	C _ε 3	C _ε 3
Binding site on receptor	α Chain, membrane proximal Ig domain	Lectin domain; all soluble fragments bind
Function	Degranulation of mast cells	Growth and differentiation factor for hematopoietic cells; rescues germinal center B cells from apoptosis; IgE antibody dependent antigen presentation; regulation of IgE expression; IgE antibody dependent cellular cytotoxicity (ADCC)

hans cells and a wide variety of effector functions in mast cells, basophils, macrophages, eosinophils and platelets.

FcεRII/CD23 is a type II integral membrane protein (cytoplasmic N-terminus), which occurs in two forms, the a-form and the b-form, differing by 6–7 amino acids at their N-termini. The former is constitutively expressed in activated B cells and the latter is upregulated by interleukin 4 (IL-4) on a variety of cell types. FcεRII/CD23a mediates IgE antibody-dependent presentation of antigen to T cells and FcεRII/CD23b the phagocytic activity of macrophages. The IgE-binding portion of FcεRII/CD23 is a C-type (Ca²⁺-dependent) lectin domain, differing from other known Fc receptors expressed on cell membranes, all of which utilize immunoglobulin domains. The lectin domain is near the C-terminus of the protein, which lies at the distal end of the extracellular sequence. Separating this domain from the transmembrane sequence is a region that forms an α-helical coiled-coil stalk, responsible for the association of FcεRII/CD23 into trimers in the cell

membrane. FcεRII/CD23 is cleaved by endogenous proteases into fragments with important cytokine activities, not least of which is in upregulation of IgE synthesis via complement receptor 2, also known as CD21. Binding of IgE protects FcεRII/CD23 from such fragmentation. The membrane-bound form of FcεRII/CD23 is involved in feedback regulation of IgE synthesis.

A third protein, galectin 3 (also known as ε-binding protein or ε-BP), an S-type lectin, binds to IgE (and indeed to FcεRI), recognizing carbohydrate chains, and may be another modifier of the IgE response.

Control of IgE synthesis

IgE is distinguished from other antibody classes (IgM, IgD, IgG and IgA) by its ε heavy chains. The heavy chain immunoglobulin gene cluster including the gene segments encoding the constant region of the ε chain (C_ε) are found on chromosome 12 in mouse and chromosome 14 in humans. The antigen

specificity of a B cell clone is determined by the formation of a functional μ heavy chain gene, following the productive recombination of variable (VDJ) and C_μ gene segments. Association with light chains then results in the expression of IgM (and IgD) in the cell. Upon appropriate stimulation of the B cell, the rearranged gene may then undergo further rearrangement within the heavy chain gene cluster, resulting in apposition of the same VDJ sequence to the C region encoding an antibody of a class other than IgM. Heavy chain switching may be directed to C_γ (for IgG), to C_ϵ (for IgE) or to C_α (for IgA), which all lie downstream of C_μ , and is a necessary step in the expression of the new antibody class, with its characteristic effector functions. IgG, IgE and IgA are recognized by class-specific Fc receptors on different types of cells, which have different tissue distributions and distinct functions in immunity.

The mechanism of heavy chain gene switching is partially understood. Upstream of each of the C cassettes are two important sequences, the so-called I exon and the switch or S region. The selection of C_γ , C_ϵ or C_α is determined by the exposure of the B cell to particular cytokines: interferon γ for three of the four IgG subclasses, IL-4 or IL-13 for IgE and the fourth IgG subclass (IgG1 in mouse, IgG4 in humans), and transforming growth factor β for IgA. These cytokines act by inducing the expression of germline gene transcripts, initiated upstream of S and terminating beyond the C cassette. The germline gene transcript is spliced to form a transcript with a germline exon, I, and the exons in the C cassette. This transcript cannot be translated because it contains multiple stop codons in the I exon. This exon is looped out and lost when VDJ recombines with C to form the switched gene.

The direction of heavy chain switching is determined by the microenvironment of the B cell. IL-4 is produced by activated T_H2 cells and mast cells. When B cells present antigen to T_H2 cells, they induce IL-4 secretion and expression of CD40-ligand (CD40L), which provides the second signal required for heavy chain switching. This process occurs in lymphoid tissue, and also elsewhere (even *in vitro*) if B cells, IL-4 and a CD40L, such as an antibody to CD40, are present in the medium. Figure 3 shows how the release of IL-4 upon the degranulation of mast cells might stimulate a T_H2 response. The differentiation of T_H0 cells to T_H2 cells is stimulated by IL-4, first from an exogenous source, in this case mast cells, and later in an autocrine fashion. Antigen presentation to T cells via MHC II (major histocompatibility complex class II), following internalization of antigen-IgE complexes, bound to Fc ϵ R/CD23, activates the T cells to secrete IL-4 and express

CD40L, thus stimulating heavy chain switching to IgE in the B cell. Antigen stimulation of B cells, soluble fragments of Fc ϵ R/CD23 and IL-6 are perhaps further required for differentiation of the B cells into plasma cells which secrete IgE.

Several factors are known to stimulate the growth and differentiation of B cells in an IgE class-specific manner. Three of these (EBV, soluble fragments of Fc ϵ R/CD23, and anti-CD21) are ligands for CD21, expressed on the surface of B cells. The original name of this protein, complement receptor 2, reflects the function of CD21 as the receptor for the activated products of the third component of complement, C3. These have the effect of lowering the threshold antigen concentration for clonal selection of B cells. Fc ϵ R/CD23 apparently stimulates IgE synthesis by a mechanism analogous to that by which C3 stimulates the synthesis of specific antibodies; this involves upregulation of the 'survival gene' Bcl-2 in the cell nucleus. Accordingly, both C3 and Fc ϵ R/CD23 may effect clonal selection by preventing apoptosis (cell death) and so permit cell proliferation. Interestingly, while C3 promotes specific antibody synthesis, Fc ϵ R/CD23 stimulates IgE synthesis, regardless of the specificity of the antigen receptor.

Figure 3 illustrates how IgE is switched on, but it does not reveal how this positive feedback mechanism is controlled to set an upper limit to the concentration of IgE. The properties of the Fc ϵ R/CD23 knockout mouse demonstrate that Fc ϵ R/CD23 is important in the downregulation of IgE synthesis, though it does not exclude an additional upregulatory function, as explained below. *In vivo* inhibitory mechanisms should operate at IgE concentrations above the K_D for the IgE-Fc ϵ R interaction; the relatively low affinity of Fc ϵ R/CD23 for IgE (compared with Fc ϵ R) may thus be inseparable from this function. IgE synthesis is inhibited by the binding of IgE, or, better, antigen-IgE complexes, to Fc ϵ R/CD23 in the B cell membrane. Presumably, the greater efficacy of the antigen-IgE complexes results from their ability to crosslink Fc ϵ R/CD23 molecules in the B cell membrane, and thereby deliver a negative signal to the cell. An analogous mechanism for feedback inhibition by IgG, involving a low-affinity IgG receptor, has been studied in greater detail with regard to the signal transduction pathway.

The activity of Fc ϵ R/CD23 in both upregulating and downregulating IgE synthesis may be explained by the fact that it is cleaved at the cell membrane by endogenous proteases into fragments that stimulate IgE synthesis by binding to CD21, as described above. But IgE binding to Fc ϵ R/CD23 inhibits proteolysis, and thereby prevents the release of the stimulatory fragments. Moreover, crosslinking of

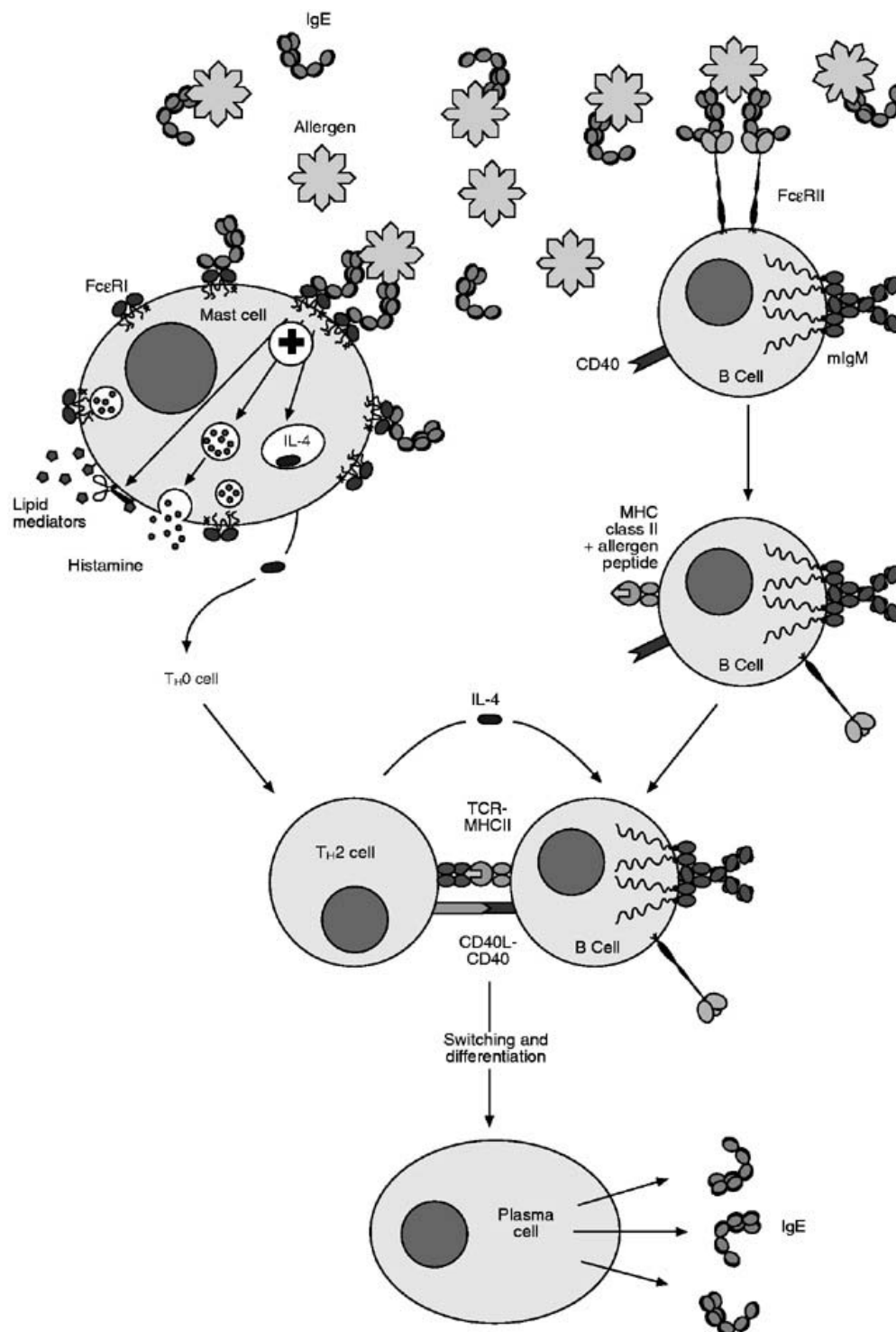


Figure 3 Synthesis of IgE. Simplified diagram of the mechanisms and key molecules involved in B cells switching to IgE after exposure to allergen. On binding to allergen, IgE interacts with its high-affinity receptor on mast cells, causing the cells to degranulate and release histamine, lipid mediators and IL-4. The IL-4 stimulates T_H0 cells to become T_H2 cells expressing IL-4. In addition, IgE–allergen complexes bind and crosslink CD23 (FcεRII) on B cells expressing surface IgM. The allergen is then internalized, processed and presented on the cell surface by class II MHC. The MHC–allergen complex is then recognized by the TCR on the T_H2 cells, and this cell–cell contact induces the expression of CD40L on the T_H2 cells, which then binds to CD40 on the B cell. This signal, in conjunction with the IL-4 produced by the T_H2 cells, induces switching and differentiation of the B cell, ultimately into either a memory B cell or a plasma cell secreting IgE.

FcεRII/CD23 by antigen-IgE complexes delivers a negative signal to the cell. Therefore, FcεRII/CD23 stimulates IgE synthesis at low IgE concentrations and inhibits IgE synthesis at high IgE concentration: it is pivotal in the regulation of IgE.

Biological functions of IgE

The biological function(s) of IgE in cellular immunity can be related to its partnership with the high-affinity receptor, FcεRI, on mast cells and Langerhans cells. Its interactions with the same receptor on eosinophils and monocytes are undoubtedly important also, but their significance is still somewhat obscure. Langerhans cells are bound in the epithelium and are thus favorably placed for the capture and processing of antigens recognized by IgE antibodies bound to FcεRI. Following antigen capture the cells travel through the circulation to lymphoid tissues, where they stimulate T cells with specific receptors. Mast cells are distributed throughout the mucosa of tissues lining the skin, lungs and gut (major organs affected by allergic disease). When multivalent antigens bind to IgE antibody-sensitized mast cells and crosslink the receptors, several processes ensue: 1) the cell degranulates, causing release within minutes of histamine and other stored mediators of inflammation, including the potent cytokines, IL-4 and tumor necrosis factor α (TNF α); 2) lipid mediators are synthesized and released (also within minutes); and 3) several genes encoding inflammatory cytokines, including IL-4, IL-5, IL-6, IL-13, granulocyte-macrophage colony-stimulating factor and TNF α are activated (over a period of many hours). The local high concentrations of the mediators and cytokines leads to: 1) vasodilatation; 2) increased capillary permeability, causing the leakage of macromolecules and cells into the tissues (which results in the edema, referred to above); 3) airway smooth muscle contraction (which impedes entry of further airborne antigens to the lungs) and bowel movement (to expel parasites); and 4) upregulation of adhesion molecules (e.g. ELAM, VCAM-1 and ICAM-1) on endothelial cells lining the tissues, which results in the recruitment of inflammatory cells (notably, T cells, eosinophils and macrophages). These conditions initiate a 'leukocyte cascade' or inflammatory response, centering on the sites of antigen provocation. The allergic response is divided into two phases, an 'immediate' response, termed 'type I hypersensitivity' in the medical literature, and the 'late phase response', or subsequent inflammatory reaction.

It has long been thought that localised anaphylaxis evolved in mammals to enable them to eliminate parasites, but that it has become largely deleterious

in humans, since, at least in allergic individuals, it is triggered by innocuous substances and is then manifested as allergy. It must be kept in mind, however, that parasites such as *Schistosoma mansoni* are endemic in many parts of the world and epidemiological (if not experimental) evidence suggests that IgE is protective. It is also possible that this response, essentially unique to the IgE antibody class, has as yet undiscovered functions.

On the whole, allergy is a concern in the more highly industrialized parts of the world, where immunology is mainly practised and where intensive efforts are being made to understand the 'pathogenesis' of allergy. Allergic conditions include hayfever or rhinitis (the most common), asthma, atopic dermatitis, food allergy and allergic uveitis (affecting the eyes). Anaphylactic shock is the only manifestation of the response that is systemic in nature, and thus differs from the others, which predominantly affect single organs. There has been an alarming increase, corresponding to a 10-year doubling rate, in the incidence of allergic disease in the industrialized parts of the world over the last few decades. Now one in five adults suffers from some form of allergy and a similar proportion of children suffer from asthma. IgE is implicated in the pathogenesis of allergy, as a key component in both the afferent (by way of dendritic cells and B cells) and the efferent (by way of mast cells and basophils) limbs of the immune response. The reasons why some individuals, but not others, are predisposed to this outcome are not well understood.

See also: Allergens; Atopic allergy; Basophils; Mast cells; B lymphocytes; CD40 and its ligand; Fc receptors; Immunoglobulin structure; Interleukin 4.

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IgG

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Immunoglobulin G (IgG) is the simplest, most abundant antibody class in vertebrates constituting some 75% of total immunoglobulin in humans. These molecules are glycoproteins formed of four polypeptide chains, consisting of two identical copies of each of two kinds of polypeptide chain: light (L) (which may be of either the κ or λ class), and heavy, gamma (γ) (which may be of one of four subclasses), linked together by disulfide bonds and noncovalent forces in the form L–H–H–L. Individual immunoglobulin G molecules will differ in their amino acid sequence but within an IgG molecule the two L chains are identical and the two H chains are identical to each other, a consequence of the fact that only one L chain gene and one H chain gene is expressed in any antibody-producing cell. The function of the molecule is to effect a liaison between antigen and the effector systems of the body. It is a multifunctional molecule, bivalent with respect to antigen, structurally differentiated with the antigen-binding sites at one end, one in each Fab, and a variety of sites for engaging non-specific effector functions in the Fc region of the molecule.

Structure

Internal sequence homologies provided the first indication that the L and H polypeptide chains fold into discrete and independent domains, each consisting of about 110 amino acids and containing a 60 residue disulfide loop. This has been confirmed by electron microscopy and X-ray diffraction analysis. The domains are numbered from the N-terminus: V_L and C_L (κ or λ) in the case of the light chain, and V_H and C_H1 , C_H2 and C_H3 in the case of the heavy chain. The V or variable domains show extensive sequence variation associated with their antigen-binding specificity, while the constant domains exhibit only subclass (isotypic) and allotypic variation in amino acid sequence. There are four subclasses of the heavy (γ) chains and there are allotypic variations within these subclasses (Table 1).

The mid-section of the γ chain is characterized by a region of extended polypeptide chain known as the hinge region. Its purpose seems to be to extend the molecule and to facilitate segmental flexibility of the parts of the molecule. Such flexibility must be of critical importance in enabling the two antigen-binding sites which are formed by the N-terminal variable

Table 1 Physical, chemical and biological properties of the four human immunoglobulin G subclasses

Property	IgG1	IgG2	IgG3	IgG4
H chain type	γ_1	γ_2	γ_3	γ_4
Mol. wt	146 000	146 000	155 000	146 000
Hinge amino acid number	15	12	62	12
Number of interchain disulfide bonds	2	4	11	2
Plasma half-life (days)	21–23	20–23	7–8	21–23
Serum concentration (mg ml ⁻¹)	8	4	0.8	0.4
Complement fixation	+++	++	++++	+/-
Placental transfer	+	+/-	+	+
Fc receptor Fc γ RI	+++	-	+++	+
Fc receptor Fc γ RII	+++	+	+++	+
Fc receptor Fc γ RIII	++	-	++	
<i>Hinge sequences</i>				
Upper: starting at residue 216	EPKSCDKTHT	ERK	ELKTPLGDTTHT	ESKYGPP
Middle:	CPPCP	CCVECPCPCP	CPRCP(EPKSC DTPPPCPRCP) ₃	CPSCP
Lower: (N-terminus of C γ 2) 231	APELLGGP	APPVA-GP	APELLGGP	APEFLGGP
<i>Allotypic markers</i>				
	Gm1	Gm2	Gm3	Gm4
	a(1)	n(23)	b1(5)	4a(1)
	x(2)		c3(6)	4b(1)
	f(3)		b5(10)	
	z(17)		bo(11)	
			b3(13)	
			b4(14)	
			s(15)	
			t(16)	
			gf(21)	
			c5(24)	
			u(26)	
			v(27)	
			g5(28)	

domains of the light and γ chains to engage bivalently with randomly spaced epitopes. Bivalent binding greatly enhances the functional affinity (avidity) of the antibody. The function of the hinge region, which is the site of most of the sequence differences between the γ chains of different subclasses is yet to be fully understood. Interest is focused on the variation in length of the hinge and its possible importance in spacing the efferent and afferent functional parts of the molecule.

The heavy chains are linked together at their mid-section, i.e. in the hinge region, by disulfide bonds and also through the pairing of the C γ 3 domains by strong noncovalent interactions. Each light chain is linked through its C-terminal cysteine to a heavy chain by a single disulfide bond and adheres also as a result of extensive noncovalent interaction through the pairing of the V_H and V_L domains, and of the C γ , and C_L domains. The strength of these noncovalent interactions is such that the polypeptide chains do not normally dissociate when the interchain bonds are broken unless denaturants are present.

The domains may be regarded as compact cylinders and the interdomain regions as short sections of

extended chain permitting a degree of flexibility in the interdomain regions, especially in the hinge region. The cylinders are resistant to proteases but proteolytic enzymes such as papain, trypsin and fibrinolysin can cleave IgG molecules at the hinge region, dividing the molecule into two basic functional units: a pair of fragments (Fab), which continue to express the antigen-binding function, and a single fragment (Fc) which readily crystallizes at low ionic strength and which interacts with complement (C1q) and continues to engage most of the effector functions of IgG. Pepsin cleaves C-terminal to the hinge disulfide leaving the two Fab fragments still disulfide linked as the F(ab')₂ bivalent antigen-binding fragment, but degrades the N-terminal domain of the Fc fragment leaving only the intact C-terminal domain, C γ 3, as the pFc' fragment. Fragments similar to the pFc' fragment (Fc') are also obtained from Fc by prolonged exposure to trypsin, papain and fibrinolysin. Specific proteolytic cleavage in the interdomain regions can be achieved by carefully controlled proteolysis of Fab or Fc following brief exposure to denaturing conditions such as low pH (~4.0). Fragments corresponding to individual intact

domains appear to retain their native conformation and continue to exhibit interdomain association as well as some of the biological properties associated with that domain.

The immunoglobulin fold

Each domain folds in a similar and characteristic way typical of all heavy and light chain domains as well as other polypeptides which form part of the immunoglobulin 'supergene' family. The polypeptide chain forms two layers of antiparallel β -pleated sheet to create a cylinder, one layer (X) consists of four β strands, the other (Y) of three. These layers form a hydrophobic envelope containing the intrachain disulfide bond which links them. The two cysteines that form this bond are conserved in almost all molecules that form the immunoglobulin fold. The β -strands are connected by loops which are evidently flexible, being rich in glycine residues. All constant region domains fold according to this basic structure.

The domains of the variable region have a slightly different structure in that there are two extra strands of β -pleated sheet associated with the three-strand (Y) face. This extension allows more scope for variety in the antigen-binding site. When the constant region domains pair, as in $C_L:C_{\gamma 1}$, $C_{\gamma 2}:C_{\gamma 2}$, $C_{\gamma 3}:C_{\gamma 3}$ the four-strand faces of the constant region domains face each other and, with the exception of $C_{\gamma 2}$, adhere on the basis of noncovalent interactions between conserved residues. The $C_{\gamma 2}$ domains are separated by a solvent channel lined on the X face with a carbohydrate moiety attached by *N*-glycosidic linkage through Asn297. The spatial relationship between the $C_{\gamma 2}$ domains is maintained by the hinge disulfide bonds and the interactions of the $C_{\gamma 3}$ domains. The biological significance of this feature is not understood, but loss of the disulfide or removal of the paired $C_{\gamma 3}$ domains disturbs this spatial relationship and may interfere with biological effector function. Additionally, the carbohydrate may play a biological role in securing this structure. Elimination or modification of the carbohydrate often has damaging consequences for the biological activity of IgG. Carbohydrate may also occur in the variable region if the acceptor sequence Asn-X-Ser/Thr occurs, that is in approximately 15% of V regions.

γ chain subclasses

Four subclasses are known in human ($\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$) and also in mouse IgG ($\gamma 1$, $\gamma 2a$, $\gamma 2b$ and $\gamma 3$) originally defined on the basis of specific antisera. There is little correspondence between the subclasses of the two species and it is thought that the subclasses evolved from a common ancestor indepen-

dently and subsequent to the divergence of the two species. While there are major differences in the biological properties of the subclasses in each species the sequence differences are small and confined mainly to the hinge region. The human subclass constant domains are approximately 95% identical but there are major structural differences in the hinge regions in terms of the number of disulfide bonds and the length of the region. The $\gamma 3$ hinge contains four tandem replications of the 15 residue segment (216–230) of the $\gamma 1$ hinge sequence. Electron micrographs reveal that this results in a considerably extended structure for the $\gamma 3$ protein. There are differences too in the point of attachment of the light chain disulfide bond to the heavy chain. In the IgG2, 3 and 4 subclasses the cysteine lies between the V_H domain and the $C_{\gamma 1}$ domain (residue 131) while in IgG1 the cysteine lies at the N-terminal end of the hinge (residue 220). The consequences of this are minimal since through the folding of the H chain these two points of attachment are close to one another.

Biological properties

Antigen binding is the primary function of the Fab region of IgG. The apposition of the three-strand faces of the V regions of the L and H chains leaves a cavity between them into which small molecules may fit, and the surface display of the hypervariable regions of the combined V regions forms a surface at the end of the Fab fragment which may bind in a noncovalent fashion to a protein or other macromolecular antigen over an area as large as $750 \text{ \AA}^2$. The $C_{\gamma 1}$ and C_L domains secure the interchain bonding between the H and L chains to provide a base for the antigen-binding V regions. The complement C4b fragment can bind to residues in the Fab heavy chain (Fd fragment) following activation of the classical complement pathway.

The Fc region is the locus for most of the other interactions of the molecule. Some of these have to do with the regulation of catabolism and excretion. The Fc fragment has a half-life in the circulation equivalent to that of the IgG from which it is derived. This is also true of the isolated intact (monomeric) $C_{\gamma 2}$ domain fragment of $\gamma 1$. Fab and $F(ab')_2$ fragments are rapidly excreted in the urine, usually unchanged. When desialylated, the carbohydrate on the Fc is recognized by the asialoglycoprotein receptor in the liver. Transfer across the placenta or by other means to the young is effected by a site in the Fc region. Binding to an Fc-specific receptor on the human placental syncytial trophoblast has been demonstrated with an affinity constant of the order of 10^7 M^{-1} . Subfragments of Fc are not able to bind to

this receptor, since the site extends over both Fc domains and requires an appropriate spatial relationship between the C γ 2 domains.

Complement activation by the classical pathway through C1q binding is effected through the C γ 2 domain. The intrinsic affinity for C1 is weak ($K_a = 10^4 \text{ M}^{-1}$). C1 activation takes place when multiple binding of the C1q occurs to several adjacently bound IgG molecules, thereby increasing the functional affinity to a value of the order of 10^8 M^{-1} . There is a marked difference in the ability of the subclasses of IgG to activate C1. The presence of glutamate at position 318 and lysine at both 320 and 322 has been shown to be essential for complement activation. This sequence, however, is present in all human IgG subclasses including those which activate complement only poorly (γ 2 and γ 4). Other residues are evidently important in the interaction or by facilitating it. Notable among the relatively few sequence differences is proline at position 331 (serine in γ 4). Replacement of these residues has been shown to affect the complement-activating activity of the modified immunoglobulin. Hypotheses based on differences in the hinge length or its relative flexibility appear now to have been disproven.

IgG binds to cell surface receptors on many types of cells to bring about an assortment of effects: the enabling of phagocytosis (monocytes, macrophages, neutrophils) or antibody-dependent cellular cytotoxicity (monocytes, macrophages and lymphocytes) or to effect feedback control on antibody synthesis (B and T lymphocytes). These properties vary among the IgG subclasses and in most cases the Fc fragments have the same property as the intact IgG, i.e. it does not appear to be modulated by the hinge or the Fab. The cellular Fc receptors have been classified into three categories according to their structure and affinity. All the sites on IgG which interact with these separate receptors appear to be located in the Fc region and, in the case of the Fc γ RI (which is the highest affinity receptor class), the site involves residues 233–237, at the N-terminal end of the C γ 2 domain close to the hinge region but coded in the C γ 2 exon. Site-directed mutagenesis experiments have indicated the specific requirement for a leucine residue at position 235 in Fc γ RI-binding molecules such as human IgG1, IgG3 and mouse IgG2a. Mouse IgG2b has glutamate in this position and does not bind Fc γ RI. The need for proline in position 331 has also been demonstrated. Fc γ RII binds to the same or a closely overlapping region since leucine 234 has been shown to be a necessary residue. It is postulated that Fc γ RIII may require sites on both Fc domains.

The implication that the FcR-binding region of IgG involves the near-hinge region of the molecule

presents conceptual problems for the topography of this interaction and runs counter to the simple view of the Fc receptor-bound antibody molecule sitting in the receptor on the cell surface like a letter Y sitting on its stem.

Binding of IgG also occurs to bacterial cell wall proteins such as protein A of *Staphylococcus aureus*. The site for this interaction has been shown by X-ray crystallography to involve residues in the inter-domain cleft between the C γ 2 and the C γ 3 domains, notably histidines 310 and 435. Histidine 435 is replaced by arginine in two allotypes of IgG3 and accounts for their not binding protein A. There is also evidence for secondary interaction between protein A and the Fab region of IgG.

See also: Allotypes, immunoglobulin; Antibodies, bispecific; Antibodies, detection of; Antibodies, secretion; Antibodies, specificity; Antibodies, synthesis; Antigen-binding site; Complement, classical pathway; Electron microscopy, immunological applications; Fc receptors; Glycosylation of immune system molecules; Immunoglobulin structure; Bacterial immunoglobulin-binding proteins.

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IgM

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Antibodies of the IgM class are the first immunoglobulins (Ig) appearing in the phylogeny and ontogeny of the immune system. Due to their predominance in large animals immunized with certain pathogenic bacteria for the purpose of generating protective antibodies, IgM were the first Ig separated to near absolute homogeneity. Although their concentration in the circulating blood is almost ten times lower (approximately 1.5 mg ml^{-1}) than that of the most represented Ig class, IgG, IgM play a major role in both the afferent and effector branches of the immune response. For instance, they serve as specific receptors for antigens on the surface of B lymphocytes, they constitute the major component of the primary antibody response to a specific antigen, and they efficiently activate the complement cascade, thereby enhancing opsonization of particulate antigens, such as bacteria and viruses.

Structure of IgM

IgM, previously known as γ -macroglobulin, exists as a variable size polymer of identical subunits. Each subunit (monomer) displays a molecular weight of 180 kDa and a sedimentation rate of 7.8 S. The IgM molecule possesses a double heterodimeric structure, consisting of two identical μ heavy (H) (μ_2) and two identical κ or λ light (L_2) chains. In healthy humans, the predominant polymer is the pentamer ($(\mu_2, L_2)_5$), with a molecular weight of 970 kDa and a sedimentation rate of approximately 19 S. The IgM pentamer contains one joining (J) chain of about 15 kDa molecular weight. The J chain is produced by the same B cell that makes the IgM. It contains at least six cysteins (Cys), but it does not share any amino acid sequence, nor does it display any antigenic cross-reactivity with any Ig H or L chains. Other human IgM polymers include the tetramer ($(\mu_2, L_2)_4$) and the hexamer ($(\mu_2, L_2)_6$), with the hexamer being the most frequent polymer after the pentamer. IgM are present as pentamers throughout the vertebrate evolutionary ladder, from the less evolved bony fishes (Osteichthyes) to mammals. Remarkable exceptions are most present-day bony fishes (Actinopterygii) and the amphibian *Xenopus laevis* which display IgM only as tetramers and hexamers, respectively. At the low end of the evolutionary ladder, cartilaginous fishes (Chondrichthyes) display IgM in the monomeric

form only. Monomeric IgM (μ_2, L_2) occur at a low concentration in the circulation of healthy humans, and are synthesized as such, rather than resulting from the degradation of polymers. Monomeric IgM are common in patients with Waldenström's macroglobulinemia, systemic lupus erythematosus, rheumatoid arthritis and ataxia telangiectasia. Many of these patients also display relatively high levels of circulating tetrameric and hexameric IgM.

Polymeric IgM can be split into (μ_2, L_2) monomers by mild reduction at neutral pH. Each of the two identical H chains of a monomer consists of five domains, i.e. one variable (V) and four constant, $C_{\mu}1$, $C_{\mu}2$, $C_{\mu}3$ and $C_{\mu}4$, H chain domains or regions, encoded by five different exons. The C_{μ} domains range in length from 105 ($C_{\mu}1$) to 111 ($C_{\mu}4$) amino acids (Figure 1). In contrast to IgG, IgA and IgD, IgM lacks a hinge region, which is substituted by a full $C_{\mu}2$ domain. The lack of a hinge region confers on IgM a more rigid structure than that of other Ig. Comparison of the IgM H chain primary structure to that of the other Ig classes entails the alignment of $C_{\mu}1$, $C_{\mu}3$ and $C_{\mu}4$ with the other Ig C_{H1} , C_{H2} and C_{H3} domains, except for IgE that also lacks a hinge region and possesses a four-domain C_{ϵ} H chain. Overall comparison of the amino acid sequence of human C_{μ} to that of the human $C_{\gamma}1$, $C_{\gamma}2$, $C_{\gamma}3$, $C_{\gamma}4$, $C_{\alpha}1$ and C_{ϵ} H chains yields identity values of 31, 31, 31, 34 and 28%, respectively. An extra 'tail' of 19 amino acids, including one Cys, is attached to the C-terminal end of the $C_{\mu}4$ domain (amino acid residue 557). This is not considered a domain and its primary structure is unrelated to that of any of the four C_{μ} domains (Figure 1). Studies of the mouse IgM have shown that the entire tail plus the amino acid at position 557, a glycine (Gly), are missing in the mature transcript of the membrane-anchored form of IgM (mIgM), and are substituted by a sequence of 41 amino acid residues that contains a highly hydrophobic (cell membrane anchoring) C-terminal stretch. Elements critical to the structure of the human IgM monomer are the Cys140 ($C_{\mu}1$) and the Cys337 ($C_{\mu}2$), which allow for the formation of the intrasubunit disulfide bonds to the L and H chain, respectively. Elements critical to IgM polymerization are the Cys414 ($C_{\mu}3$) and the Cys575 (tail). These Cys provide anchoring points

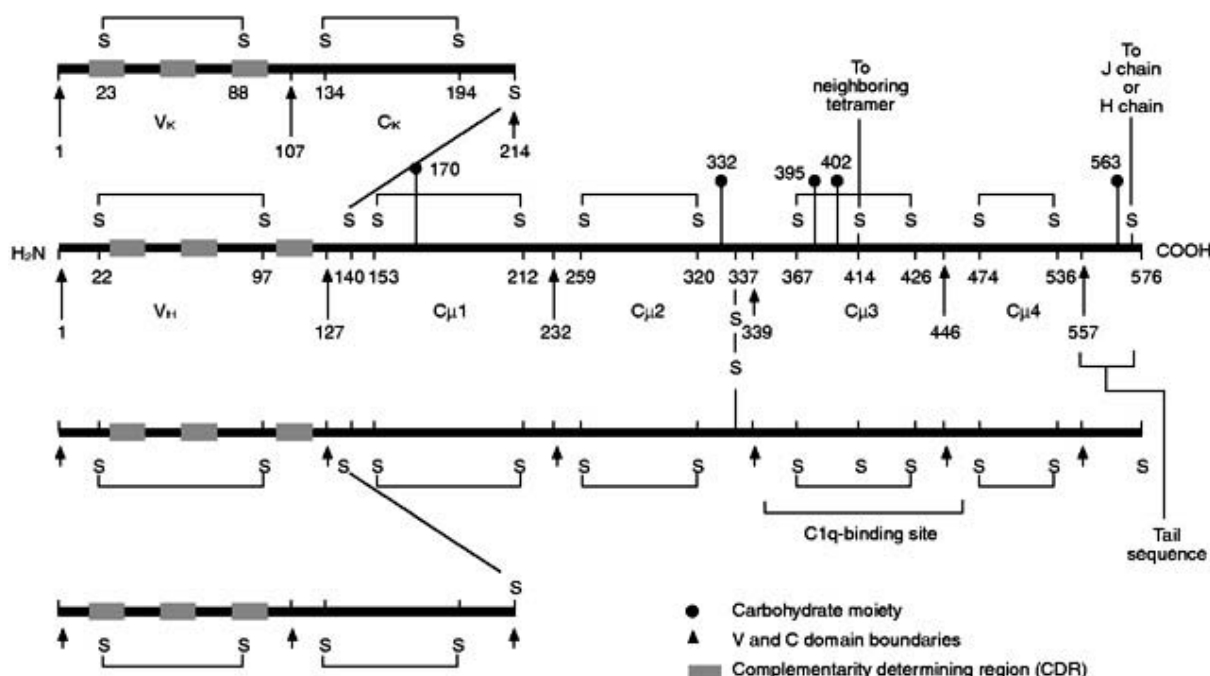


Figure 1 Human IgM subunit. V_H, V_κ and C_μ region domain boundaries, carbohydrate moieties, and complementarity determining regions are depicted. The C1q-binding site encompasses the C_μ3 domain only or the C_μ3 and part of the C_μ4 domains.

for the intersubunit disulfide bridges. Site-directed mutagenesis experiments involving serine (Ser) for Cys substitutions suggest that the availability of Cys575 in a Ser414 mutant monomeric molecule readily leads to assembly of a pentamer, that comprises one J chain bridging two subunits (dimer 'clasp'). Conversely, the availability of the Cys414 in a Ser575 mutant subunit readily leads to assembly of hexamers that do not include the J chain. The importance of the J chain for the assembly of the pentamer but not the hexamer is further emphasized by the observation that IgM hexamers readily assemble when monomeric subunits are allowed to spontaneously associate *in vitro* in the absence of the J chain. In the presence of the J chain, two predominant configurations of pentamers occur, in which Cys337-Cys337 is in series with both Cys414-Cys414 and Cys575-Cys575 (Figure 2). These configurations likely reflect the polymeric IgM structures most commonly occurring *in vivo*. Polymer IgM assembly can occur in the absence of the J chain and the Cys575, but it cannot be brought about by subunits lacking the 20 amino acid C_μ tail. Finally, IgM polymer assembly cannot rely merely on noncovalent subunit interaction, but requires disulfide bridging, as suggested in experiments involving Ser for Cys substitutions, by the ability of double Ser414, Ser575 mutants to generate IgM monomers, but the failure of these double mutants to generate polymers.

IgM, along with IgD and IgE, have a high carbo-

hydrate content of 10–12%. Carbohydrate groups are found in IgM H chains wherever the carbohydrate group acceptors are present, that is, one in C_μ1 (shared also by the corresponding domain in C_α1 and C_ε1), one in C_μ2, two in C_μ3 (one of which is also shared by the corresponding domain in C_γ1, C_δ and C_ε), and one in the tail (**Figure 1**). The carbohydrate group acceptor consists of the short sequence asparagine (Asn)-X-Ser/threonine (Thr), where X stands for any amino acid. It is suited for N-glycosidic linkages, in which N-acetylglucosamine attaches to the amino (-NH₂) group on the side chain of Asn. Both high mannose and 'complex' oligosaccharides with a molecular weight of 2500–3000 Da are present in IgM. 'Complex' oligosaccharides contain mannose, N-acetylglucosamine, galactose and sialic acid. The carbohydrate groups enhance the solubility of IgM in aqueous solution, and may stabilize their tertiary structure.

Initial electron microscopy examination of free IgM molecules revealed a typical planar 'star'-like shape for the pentameric form, with five pairs of Fab arms protruding from a central (Fc)₅ disc. More recently, it has been suggested that the planar axis of the F(ab')₂ arms may be at a right angle to the (Fc)₅ disc. After binding to a multivalent antigen on solid-phase matrix, e.g. flagella of *Salmonella*, a distortional conformation change takes place in the IgM polymer resulting in a dislocation of the central (Fc)₅ disc, possibly due to a rigidity of the F(ab')₂ arms

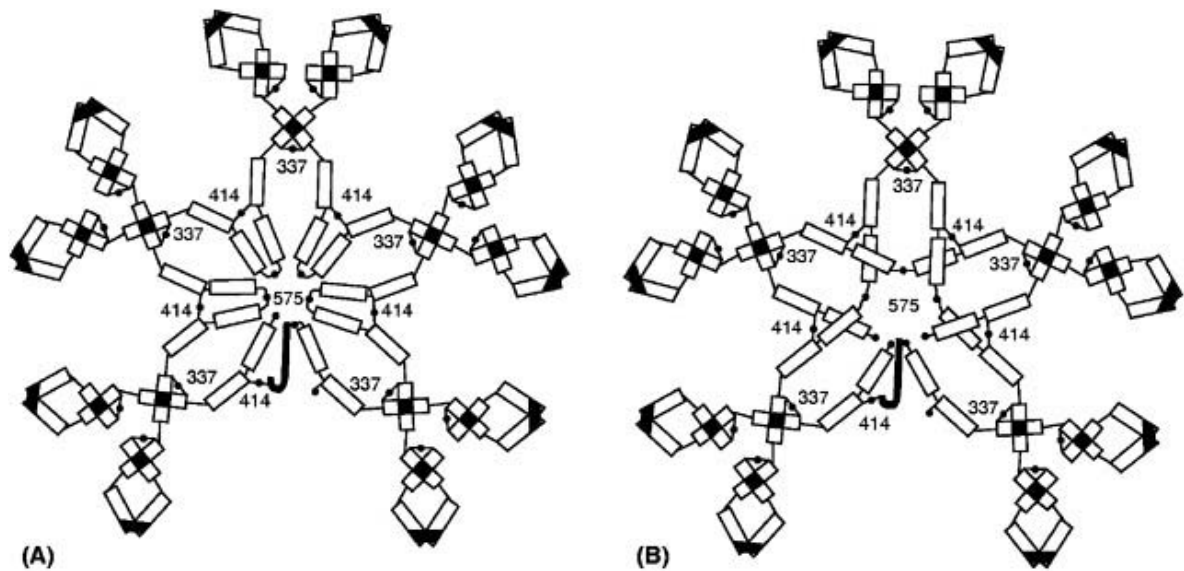


Figure 2 Structures of pentameric human IgM. (A) and (B) depict configurations of pentameric IgM based upon the two most likely disulfide bonding patterns. Other disulphide bonding patterns are possible, but are not considered for the purpose of this article. Open boxes depict V_H , V_L and C_μ domains. Black areas depict overlapping domains held together by noncovalent interactions. Full and half circles depict disulfide bonds and unpaired Cys residues, respectively. (A) depicts C_μ chains joined by homo pairs Cys414-Cys414 in parallel with Cys575-Cys575, and both in series with Cys337-Cys337; (B) depicts C_μ chains joined by homo pairs Cys337-Cys337 in series with both Cys414-Cys414 and Cys575-Cys575. In both (A) and (B), J chain is shown as bonded to Cys414 as well as to Cys575. (Courtesy of Ann C. Davis and Marc J. Shulman, modified from Davis and Shulman, 1989.)

which are not inherently altered. As a result, IgM engaged in antigen-binding takes the shape of a 'staple' or a 'multi-legged stool' with the $(Fc)_5$ disc on a plane different from that of the $F(ab')_2$ 'legs'.

IgM valency and complement activation

Reaction of pentameric IgM with haptens or small antigens results in the occupancy of all Fab antigen-binding sites, making IgM decavalent. Due to steric hindrance, reaction of IgM with larger ligands may result in occupancy of only five, or fewer, antigen-binding sites, suggesting that the valency of IgM is a function of the nature of the actual ligand. Owing to its high valency, the overall avidity of a whole IgM molecule for a given antigen, displaying repetitive epitopes, greatly exceeds the value of the intrinsic affinity of each Fab for the single epitope. Thus, the antigen size and nature have an important impact not only on the valency of an IgM molecule, but also on its binding strength. This is an important feature when one considers that naturally occurring proteinic and/or polysaccharidic antigens on bacteria, viruses and other microorganisms do often display repetitive epitopes.

The first step in the activation of the classical complement pathway is the binding of the C1q moiety of the latent C1 esterase to IgG or IgM. While at least two antigen-bound IgG are required for

complement fixation, a single IgM molecule can efficiently activate complement after binding Ag. In IgM, the C1q binding sites are located on the $C_{\mu}3$ and/or $C_{\mu}4$ domains (Figure 1). In its planar conformation, IgM bears a single C1q-binding site, allowing the binding of only one of the six globular heads present on each C1q molecule. This monovalent binding is characterized by a high dissociation constant (K_d), in the order of magnitude of 10^4 – 10^5 M^{-1} (low affinity). Dislocation of the IgM $(Fc)_5$ disc brought about by binding of the Fab arms to a multivalent particulate antigen leads to exposure of at least one more C1q-binding site on the $(Fc)_5$ disc. This allows multivalent binding of C1q, resulting in a 2–3 orders of magnitude increase in binding strength. Multivalent binding of C1q is enhanced in the IgM hexamer which activates complement up to 20-fold more efficiently than the canonical pentamer. Consistent with the notion that enhanced C1q binding by IgM results from a distortional process rather than an allosteric change, is the observation that antigen–IgM complexes efficiently activate C1 when prepared in antibody, but not antigen, excess. Thus, the engagement of IgM in a lattice is an absolute prerequisite for complement fixation by IgM following the binding of soluble antigens. It is conceivable that such an engagement entails a distortion of the Fab arms that results in a better exposure of the $(Fc)_5$ disc, in a fashion similar to that following the bind-

ing of IgM to particulate antigens. Activation of the C1 esterase by IgM leads to the assembly of the C3-convertase, C4b2a, and subsequent deposition of C3b, and its inactivated forms, iC3b and C3d, on the antibody molecules engaged in the lattice. C3b, iC3b and C3d can greatly enhance the engulfment of antigen by readily mediating attachment of the antigen-IgM complexes to the complement receptors CR1, CR3, and CR2 or CR4, respectively, that are present on the surface of different blood cells, including erythrocytes, granulocytes, monocytes, macrophages, natural killer (NK) cells, B lymphocytes and T lymphocytes.

IgM in the antigen-induced antibody response

Monomeric membrane (m)IgM, along with mIgD, constitute the receptors for antigen on all virgin B cells. mIgM⁺ lymphocytes, whether mIgD⁺ or mIgD⁻, make up the whole B cell repertoire in neonates and in axenic animals, and account for the majority of the B cell repertoire clonotypes in adults. Cross-linking by antigen of mIgM and mIgD on virgin IgM⁺IgD⁺ B cells triggers the mechanisms that lead to lymphocyte activation, proliferation, and, eventually, substitution of mIgM by mIgG, mIgA or mIgE in differentiated antibody-producing B cells and memory B cells. It has been speculated that the rigidity of IgM (due to the lack of a hinge region) plays a major role in signal transduction into the B cell. Polyclonal recruitment of surface IgM⁺IgD⁺ B cells during the early phases of a primary antibody response results in production of IgM antibodies displaying, in general, a low to moderate affinity for antigen. These IgM constitute the bulk of a primary response, and are encoded, in general, by unmutated V(D)J gene segments. They are heterogeneous in their degree of polymerization with a relatively high representation of the hexameric form. In contrast to these early IgM, the IgM of the 'late' primary response can display a significant level of somatic point-mutations. Selection of some of these mutations by antigen can occur prior to Ig class switching. Full-blown antibody somatic diversification and selection, however, and the resulting increase in antibody affinity are associated, in general, only with IgG, IgA or IgE class-switched B clonotypes. Ig class switching is dependent on T cell help that includes engagement of CD40 on the surface of B cells by CD40L on T cells. A defective CD40L molecule, as occurring in patients with inherited mutations, does not allow for effective CD40 engagement and Ig switching, and it is associa-

ted with absolute lack of IgG, IgA or IgE, and compensatory levels of IgM, 'hyper-IgM syndrome'.

IgM as natural antibodies

Most of the IgM antibodies that are produced in the early stages of an antigen-induced primary response or as a result of an antigen-nonspecific polyclonal B cell activation, e.g. by Epstein-Barr virus in humans or bacterial lipopolysaccharides (LPS) in mice, display the functional features of the Ig that are referred to as 'natural antibodies or autoantibodies'. Natural antibodies bind a variety of microorganisms, including viruses, bacteria, protozoa, metazoa parasites and fungi, as well as heterologous plasma and tissue components. The presence in the normal sera of a population of molecules reactive with a broad array of different antigenic determinants and displaying physicochemical properties similar to those of the antibodies that appear, usually at much higher titer, following immunization has been recognized for almost a century. The terms 'natural antibodies' or 'spontaneously produced antibodies' had been used to distinguish these Ig from the antibodies that have been elicited by intentional or known immunization. The vast majority of the natural antibodies are IgM. Their spectrum of reactivity is not limited to foreign antigens, but also includes self antigens, such as hormones, e.g. insulin or thyroglobulin, cell constituents, e.g. DNA, myosin, actin, tubulin, glycoproteins or phospholipids, and antibodies, particularly, the Fc fragment of autologous IgG (rheumatoid factor, RF). Most natural IgM antibodies are polyreactive, i.e. they bind, in a dose-saturable fashion, two or more antigens, even very different in nature, such as proteins, polysaccharides and nucleic acids, in general with low to moderate affinity (K_d , 10^5 – 10^7 M⁻¹). In most polyreactive IgM, the binding to a given antigen can be efficiently cross-inhibited by another antigen, suggesting that structures responsible for binding different antigens vastly overlap. Experiments involving shuffling of H and L chain complementarity-determining regions (CDR) and framework regions (FR) of human natural antibodies suggest that the somatically generated H chain CDR3 provides the main structural correlate for binding multiple antigens.

Most polyreactive IgM are produced by a discrete subset of the B cell repertoire expressing, at low intensity the surface CD5 marker (B-1 cells). B-1 cells account for a significant proportion of the normal human B cell repertoire. They constitute the vast majority of B lymphocytes in the late stages of fetal development and in the newborn. Expression by B-1 cells of unmutated polyreactive IgM would provide

the structural correlate for the high degree of 'connectivity', i.e. interclonal idiotypic cross-reactivity, characteristic of the fetal and neonatal B cell repertoires. As a consequence, B-1 cells and natural IgM antibodies may play a major role in shaping the overall diversity and specificities of the developing, and, eventually, the adult, B cell repertoire. These cellular elements and IgM antibodies play a major role in the first line of defense against invading microorganisms, as well as in blood group incompatibility, and in hyperacute rejection of xenografts. Their importance in the defense against infections is further suggested by their high degree of representation in primordial phylogenetic stages: polyreactive IgM are the only antibodies found in sharks and torpedo fish. Owing to their polymeric nature and multivalency, polyreactive IgM efficiently aggregate bacteria and viruses, mediate complement-dependent lysis, promote and enhance opsonization and phagocytosis. The high levels of circulating IgM binding to the IgG Fc fragment and other self antigens found in patients with bacterial, viral and parasitic infections suggest that most pathogens do readily activate B-1 cells to secrete polyreactive antibodies. Not only do these IgM directly bind antigens on invading microorganisms, but they also amplify, through their effective RF activity, the biological activity of specific IgG aggregated on the surface of bacteria, viruses or parasites. Antigen-aggregated IgG, present in the circulation from the beginning of a secondary response, would, in turn, efficiently boost RF production. In contrast to the RF IgM appearing early in the primary response, some of the RF IgM arising during the secondary response possess a much higher affinity for the IgG Fc fragment, as exemplified by the ability of RF IgM arising in rats during the specific response to *Trypanosoma lewisi* to terminate the *T. lewisi* infection when transferred into animals displaying only trace amounts of circulating specific IgG.

IgM, autoimmunity and neoplasia

Owing to their broad antiself antigen reactivity, natural IgM antibodies could provide the templates for affinity matured class-switched autoantibodies, and B-1 cells can, under certain circumstances, play a role in the establishment of autoimmune phenomena. B-1 cells are greatly increased in a number in patients with rheumatoid arthritis and Sjögren's syndrome. In rheumatoid arthritis patients, these cells spontaneously proliferate and secrete large amounts of IgM with RF activity. Some of these IgM RF are polyreactive, display a low affinity for the IgG Fc fragment, and are encoded by unmutated V genes. Others are monoreactive, display a higher affinity for

IgG Fc fragment (K_d , 10^{-7} M $^{-1}$), and are encoded by extensively mutated V genes. The inherent proliferative potential of IgM-producing B-1 cells and their stimulation by antigen may be synergistic and may contribute to premalignant or malignant B lymphoid transformation. A relatively high incidence of B lymphoid malignancies occurs in patients with Sjögren's syndrome and rheumatoid arthritis. Further evidence pointing to a link between IgM-producing B-1 cells, autoimmunity and malignant transformation stems from the features of the monoclonal antibodies associated with a variety of human B cell neoplasms, including chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma, Waldenström's macroglobulinemia and other paraproteinemias. The vast majority of CLL B cells are B-1 and produce, in general, IgM. Like monoclonal Waldenström's IgM paraproteins, many of these monoclonal IgM possess RF and other antiself antigen reactivities. Other autoantibodies of the IgM class that are characteristically associated with malignant B cell neoplasia or benign monoclonal gammopathy are cold agglutinins that react with various polysaccharidic red cell determinants. Transient cold agglutinins of the same IgM class and similar antigen-reactivity occur commonly in patients with infections, particularly those by *Mycoplasma pneumoniae* or by Epstein-Barr virus (infectious mononucleosis). Thus, the neoplastic transformation of natural IgM antibody-producing B clones is associated with fidelity of the function defining the physiological B cell clonotypic progenitor.

See also: Affinity; Antigen-binding site; B lymphocyte activation; Complement, classical pathway; Idiotypic network; Immunoglobulin genes; Joining J chain; Leukemia; Natural antibodies; Rheumatoid arthritis, animal models; Rheumatoid arthritis, human; Sjögren's syndrome; Systemic lupus erythematosus, experimental models; Systemic lupus erythematosus (SLE), human.

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I-J

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The background to this controversial molecule lies in studies reported in the 1970s by both Murphy and Tada and their colleagues, suggesting that suppressor T cells bear determinants which mapped to a novel locus of the I region of the murine major histocompatibility complex (H-2 complex). This site was termed the Ia-4 locus, and this locus was found to mark a new I-region gene which was termed I-J. Through the use of certain recombinant mouse strains, the I-J gene locus was found to map to a region between what are now referred to as the I-A and I-E loci of the H-2 complex. The recombinant mouse strains utilized for the mapping of the I-J locus included the B10.A(5R)[I-J^k], B10.A(3R)[I-J^b], B10.A(9R)[I-J^k] and B10.HTT[I-J^s] strains. The recombination points of the B10.A(5R) and B10.A(9R) strains define the centromeric (left) boundary, and the recombination points of the B10.A(3R) and B10.HTT strains define the right boundary of the I-J locus. It has been assumed that B10.A(3R) are congenic with respect to the I-J locus with B10.A(5R) mice, and the same is true of the B10.A(9R) and B10.HTT strains.

alleles have been generated successfully. A number of monoclonal antibodies against I-J have been generated as well. It has been the experience of numerous investigators that these antibodies react with antigen-specific suppressor T cells. Many of these cells are capable of secreting factors which are themselves suppressive. The I-J-specific antibodies also appear to react with an epitope present as a part of certain of these suppressor cell factors. The relationship of the membrane and secreted forms of the I-J-bearing molecules remains unclear.

The actual function of the I-J molecule is uncertain but may be related to the interaction of the individual suppressor cell populations. It has been found, in certain cases, that the functional activity of suppressor cells (and suppressor factors) is restricted at the I-J locus. This is additional evidence of the significance of the I-J gene product in the function of these regulatory cells. In addition, this suggests the possibility that the target cell population for the I-J-bearing suppressor factor must bear an I-J allele-specific binding structure for the factor.

Relationship of I-J to suppressor T cells

By utilizing a number of recombinant mouse strains, anti-I-J alloantisera which recognize many of the I-J

Cellular source of the I-J molecule

The initial studies which defined the I-J locus showed rather convincing evidence that I-J is produced by

suppressor but not helper T cells. More recent studies have shown that I-J is not expressed in B cells. On the other hand, a subpopulation of helper cells, and some (but not all) helper T cell clones, appear to express the I-J molecule. It has been proposed that the I-J gene product expressed on T helper cells may represent an alternative 'form' of the I-J molecule, as certain monoclonal I-J-specific antibodies react only with suppressor or helper T cell I-J epitopes. Other monoclonal I-J-specific antibodies appear to react with both 'forms'.

Evidence has been reported by certain laboratories suggesting that I-J may be produced by macrophages. The nature of the expression of I-J on accessory cells remains unsettled. There is some evidence to suggest that macrophages bear a binding structure for I-J-positive suppressor factors. Anti-idiotypic antisera (generated in response to monoclonal I-J-specific antibodies) react in an I-J haplotype-specific manner with accessory cells. It has been suggested that the I-J restriction in the interaction between suppressor cell populations actually represents an I-J restriction between the suppressor cell factor and an accessory cell.

Biochemical characteristics of the I-J molecule

The actual I-J structure has not been identified with any certainty. However, the suppressor factors which are recognized by polyclonal and monoclonal I-J-specific antibodies are protein in nature. The I-J-bearing polypeptide may be found either alone or linked (by a disulfide bridge) to a second (non-I-J-bearing) polypeptide. The two-chain factors are usually composed of an I-J-bearing chain linked to an antigen-binding chain. The I-J-bearing chain appears in most cases to be a polypeptide of 25–30 kDa. The antigen-binding chain may represent some part of the T cell receptor α chain.

The I-J structure expressed on the surface of both helper and suppressor T cells has also been examined. Using NP-40 lysates and immunoprecipitation with I-J-specific antibodies, the I-J molecule is detected as an 81–90 kDa dimer composed of 42–46 kDa glycoprotein subunits. In these studies, the I-J dimer was found to be distinct from the T cell receptor α/β heterodimer.

Molecular biology of the I-J gene

The region of the H-2 complex which spans the interval between I-A and I-E from the congenic strains B10.A(3R) and B10.A(5R) has been cloned and sequenced. This interval is less than 3.4 kb, a size that may be too small to encode for a polypep-

tide larger than 20 kDa. Moreover, T cells which express the I-J determinant do not appear to transcribe this region. These cells do not possess a rearrangement in the I-A or I-E genes either. Clearly, the structural gene for I-J must be elsewhere.

In view of these results, it is difficult to explain the genetic mapping initially reported for the I-J locus. It has been suggested that I-E or I-A genes contribute to the expression of the I-J gene product. Both I-A and I-E transcripts are undetectable in T cells which express I-J determinants. However, studies with both I-E chimeric and I-E $_{\alpha}$ transgenic mice show convincing evidence that suggests that the I-E locus contributes to the I-J phenotype. This notion is consistent with related evidence that shows that I-E has a significant impact on the T cell receptor V $_{\beta}$ allele repertoire in various mouse strains.

Conclusions

The I-J locus and gene product remain matters of great controversy at this time. Much of the skepticism concerning I-J will be alleviated once the I-J gene(s) has been cloned. More recent studies have suggested that the I-J determinant is associated with a 13 kDa protein termed the glycosylation-inhibiting factor. The glycosylation-inhibiting factor isolated from suppressor cells is recognized by I-J-specific antibodies and possesses suppressive activity. Additional questions remain, however, regarding the basis for the genetic restriction in the function of this factor, and the apparent haplotype-specificity in the recognition by I-J-specific antibodies.

See also: H2 class II; MHC restriction; Suppressor T lymphocytes.

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IMMUNE ADHERENCE

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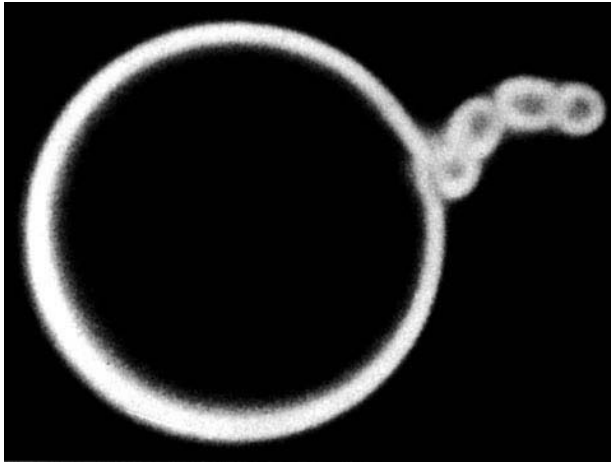


Figure 1 Immune adherence of *Streptococcus pneumoniae* to human erythrocytes. Dark ground illumination. (Photograph courtesy Dr RA Nelson.)

The term immune adherence (I-A) was first used by RA Nelson to describe a phenomenon that had already been known for 50 years and had been referred to as the red-cell adhesion test. I-A is part of one of the most important biological activities of the complement system, being involved in the phagocytosis of complement-coated particles by cells of the neutrophil and mononuclear phagocyte system. Nelson described the adherence of particulate antigens such as bacteria to human and other primate erythrocytes (Figure 1) or rodent platelets (Figure 2) in the presence of antibody and complement. It is

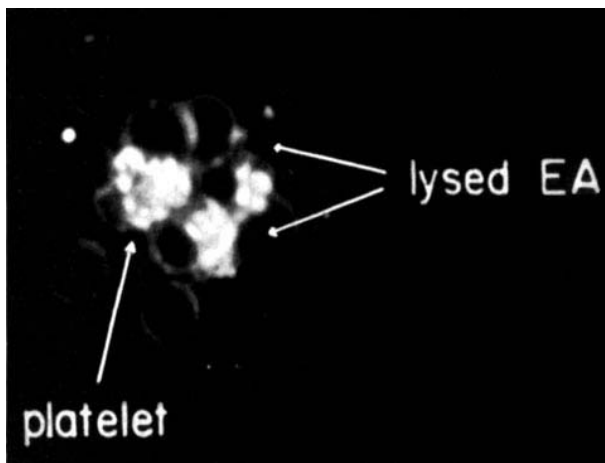


Figure 2 Immune adherence of sheep erythrocytes to guinea pig platelets. (Photograph courtesy Dr RA Nelson.)

now known that adherence is through the CR1 receptor through C3b, iC3b, and C4b. Complement activation in I-A may be through the classical or alternate pathway. Thus, I-A of zymosan by the alternate pathway of complement can occur in the absence of antibody. I-A to erythrocytes and platelets is part of a wider phenomenon including immune adherence to the CR1 receptor also present on neutrophils and macrophages. I-A to red cells and platelets is also involved in the clearance of complement-coated particles by neutrophils and cells of the reticuloendothelial system, acting as a means of presentation of antigenic particles or immune complexes to phagocytic cells in these tissues.

Originally described with bacteria and protozoa, I-A also involves the surface of helminths and viruses, as well as soluble antigens such as ovalbumin. It has been used as a diagnostic test in the clinical laboratory, particularly in syphilis serology, where the TPI-A (*Treponema pallidum* I-A) test has had varying popularity. I-A serology may be used by observing the adherence of particulate antigens such as bacteria to the red-cell surface under the microscope or by observing the hemagglutination patterns at the bottom of the wells of a titration dish (Figure 3). An I-A hemagglutination assay has been applied to the study of herpes simplex and varicella-virus infections, and also used for the quantitative estimation of infantile gastroenteritis antigens in the stools.

Other uses of I-A have been to demonstrate activation of C3 on the surface of macrophages which have ingested asbestos *in vivo*, detected by I-A of human, but not sheep, red cells. Moreover, in studying the complement reactivity of soluble immune complexes, I-A has been a useful marker of the ability of the complexes to activate C3 and to bind to the CR1 receptor. The I-A phenomenon has been used to demonstrate the CR1 receptor sites in solid tissues, using the adherence of EAC (erythrocyte antibody complement) complexes to tissue sections. The demonstration of CR1 sites in glomeruli using this technique has suggested an alternative arrangement for immune complex deposition in the kidney. The use of EAC complexes to quantitate B cells in the blood or exudates is also an example of the I-A phenomenon, as B cells are known carriers of the CR1 receptor.

See also: **Agglutination; Complement, alternative**

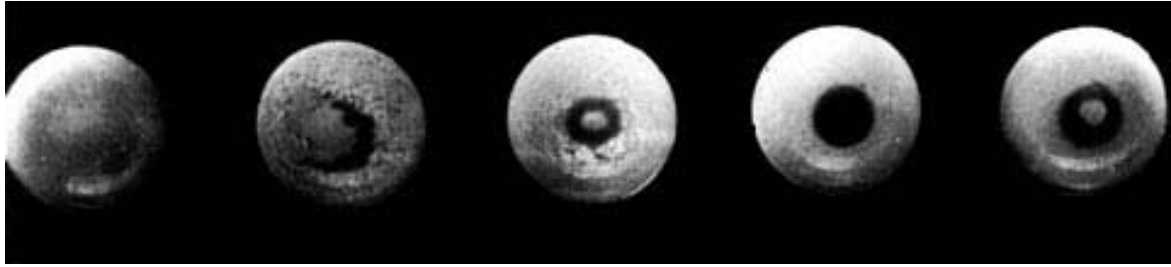


Figure 3 Hemagglutination patterns caused by immune adherence. The samples on the left show the greatest degree of agglutination.

pathway; Complement, classical pathway; Complement receptors; Erythrocytes; Immune complexes; Opsonization; Phagocytosis; Platelets; Rosetting techniques.

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IMMUNE COMPLEXES

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Binding between antigen and antigen-specific antibody results in a reaction product which is an antigen–antibody complex or simply ‘immune complex’. Immune complexes interact with many cells of the immune system, causing activation or inactivation, and they might trigger the cytokine network. In the presence of plasma proteins, especially those belonging to the complement system, immune complexes will grow to more than strictly complexes between antigen and antibody. In fact, a number of different proteins have been identified as becoming fixed to immune complexes, thereby building up large protein constructions of which antigen and antibody are only a portion. The proteins found on immune complexes include the complement proteins C1q, C4b, C3b and factor H as well as anti-antibodies (rheumatoid factors, anti-idiotypes) and fibronectin. Both exogenous (foreign) and endogenous (self, autologous) antigens may trigger immune responses that result in the formation of immune complexes.

Immune complexes are three-dimensional structures forming a lattice-type of sequence between antigens and antibodies with a ratio that may vary essentially according to the size of the antigen, the density of antigenic epitopes, and the isotype, subclass, poly/monoclonality and affinity of the antibody. The interaction between antigen and antibody does not result from chemical bonding but rather is due to electrostatic forces. Therefore, the physicochemical properties of a given immune complex preparation is the result of a dynamic process between build-up and breakdown, contributing to constant variability of the characteristics of immune complexes *in vivo*. The immune complex structure is favoured by the Y-shaped configuration of the immunoglobulin molecules and by their abundance of water-repelling nonpolar groups known to favor autoaggregation of immunoglobulin M (IgM) or IgG in solution.

Detecting immune complexes in bodily fluids and tissues

Detection of immune complexes in clinical samples is of interest in a number of human and animal diseases. Furthermore, in countless test systems, immune complexes are the *in vitro* reaction product of laboratory diagnostic tests for detection of either specific antibodies or antigens. For demonstration of *in vivo* formed immune complexes in serum or other body fluids, where the antigen involved is unknown, two approaches may be pursued: 1) quantitative estimation of immune complexes with characterization of the complexed immunoglobulin isotype and bound complement component using conventional screening tests; 2) analysis of the antibody specificity and possibly of complexed antigen using more elaborate technology (Figures 1 and 2).

Rapid screening tests for the detection of immune complexes in a large number of samples are mostly based on the ability of immune complexes to interact with naturally occurring humoral and cell receptors. A classical technique is the C1q-binding assay that is based on the ability of immune complexes to interact with the globular head domains of the first component of human complement, C1q: purified, radiolabeled C1q binds to the immune complexes

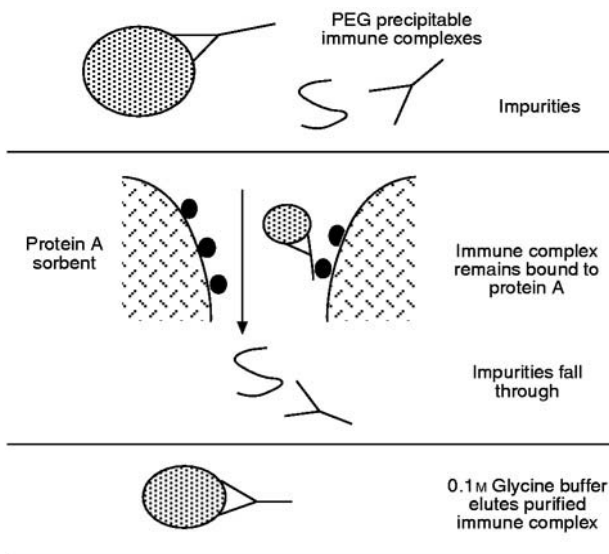


Figure 1 Isolation of immune complexes from the remainder of serum proteins. The figure depicts a possible protocol starting with coprecipitation of macromolecular proteins using 2–3% polyethylene glycol which precipitates proteins larger than approx 300 kDa. The precipitate is enriched in immune complexes but is still contaminated with other macromolecular proteins. In order to remove the latter proteins, the precipitate is passed over solid phase staphylococcal protein A which retains immune complexes; after thorough washing, the complexes are eluted from the column with glycine buffer.

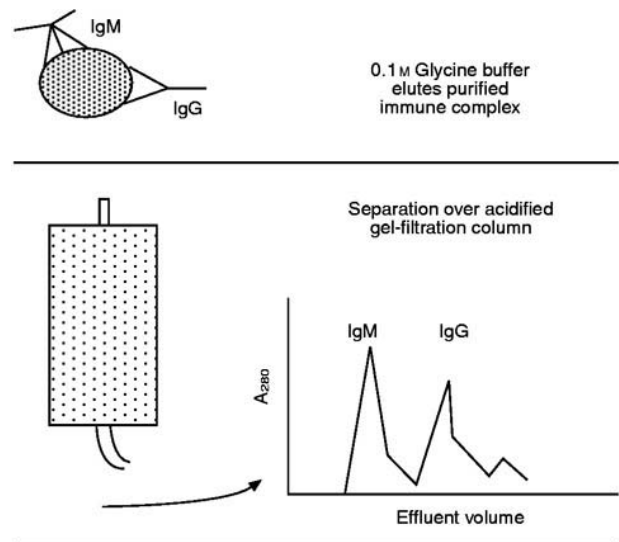


Figure 2 Analysis of immune complex composition and specificity. The purified immune complexes are now ready for further analysis, e.g. for separation and examination of antigen and/or antibody. An easier procedure for purifying immune complexes is by cryoprecipitation but only a few immune complexes (e.g. a portion of complexes with hepatitis C infection) have the property of precipitating in the cold.

and the resulting C1q-immune complex assemblage is detected using size exclusion with polyethylene glycol (PEG). An even simpler technique mixes serum with low concentrations of PEG known to precipitate oversized macromolecules with subsequent analysis of the pellet. Monoclonal antibodies to C1q insolubilized on microtiter plates may be used to capture C1q in the analytical sample already bound to immune complexes *in vivo*, with subsequent demonstration of immunoglobulins in the captured material. Other solid-phase immune complex detection assays are possible using conglutinin (K) or rheumatoid factors as capture for immune complex bound complement. It is said that K preferentially binds small complexes and those having a high antigen:antibody ratio. On the other hand, C1q-binding assays identify immune complexes with a lower antigen:antibody ratio and a greater size range. In addition, K binds complexes that contain the small residual degradation fragment of C3, C3d, whereas C1q only binds those activating complement by the classical pathway of complement activation.

For detection of immune complexes in more elaborate assays, cellular receptors may be used, such as the Fc receptor (FcR) present on macrophages or platelets and, in the most successful of this type of assay, the Raji cell test, iC3b receptors (complement receptor CR3) present on *in vitro* transformed lymphoblastoid cell lines. The erythrocyte-antibody-complement (EAC) rosette inhibition test measures

the inhibition by immune complexes of rosette formation between a subpopulation of peripheral B lymphocytes and sensitized sheep erythrocytes carrying C3. The sensitivity of these techniques is generally good and is in the microgram per milliliter range, but the disadvantage is that cell preparations must be used, which makes them tedious and cumbersome for routine use. Standardization is difficult, but a fair comparison of results among different laboratories is noted with use of international reference preparations. Similar technology using cellular receptors may provide insights into the immune complex constituent that actually ligates to cell surface receptors. By blocking specific types of cell surface receptors with monoclonal antibodies, Fc binding can be distinguished from F(ab')₂ binding or binding via immune complex fixed C3b and/or C4b.

The availability of preparative techniques providing a partially purified material allows further immunochemical and biochemical characterization of the complexes. Several techniques make the study of molecular composition of immune complexes for clinical research possible: direct electron microscopic examination of precipitates obtained by polyethylene glycol or cold precipitation; dissociation of complexes at acid pH (Figures 1 and 2), by urea or heat; *in vitro* manipulation of complexes by adding suspected antigen, with a possible shift of the antigen: antibody ratio towards antigen excess so that loss of their complement fixing ability can be ascertained; and absorption of immune complexes on concanavalin-coated polystyrene beads allowing their further purification and study. Using this type of technology, it is possible to obtain information on the composition of immune complexes in disease (Table 1).

Physiology of immune complex formation and experimental immune complex disease

Study of antibody-antigen interactions can now be modeled at the atomic level. For example, with viral peptide epitopes, the epitope may bind in a shallow crevice on the Fab surface with, sometimes, only portions of the epitope deeply penetrating into the Fab (Figure 3). Numerous hydrogen bonds, salt bridges and electrostatic forces now come into action to hold together what we designate an immune complex.

Under physiological conditions, the transient formation of immune complexes is normal and will remain undetected by usual methods because their concentration does not reach the sensitivity of the detection techniques. The physiological relevance of immune complex formation may be summarized as follows.

1. Through their binding to antigen, antibodies present antigen to the removal systems of the body, by a phenomenon called opsonization, which leads to elimination and destruction of the antigen. Monocytes, macrophages and granulocytes, as part of the reticuloendothelial system in liver, spleen, bone marrow and skin, function as immune complex-retaining filters, their performance possibly deciding whether antigen removal is successful or turns into chronic inflammatory disease.
2. Antibodies will carry antigen to the lymphoid tissues and keep it fixed there on the surface of follicular dendritic cells, which allows optimal immune responses following prolonged antigen recognition by specific lymphocytes.

Upon injection of preformed complexes of known composition into animals, large complement-fixing immune complexes immediately bind to circulating cells bearing the CR1 receptor (CD35) for C3b.

Table 1 Selected examples of established immune complex diseases

<i>Nosological entity</i>	<i>Involved antigen</i>
<i>Serum sickness, iatrogenic</i>	Animal antitoxins, antisera, drugs, antilymphocyte globulin, HgCl ₂ , gold, D-penicillamine
<i>Infectious agents</i>	
Glomerulonephritis of infected atrioventricular shunts	<i>Staphylococcus epidermidis</i>
Glomerulonephritis of malaria	<i>Plasmodium malariae</i>
Glomerulonephritis of schistosomiasis	<i>Schistosoma mansoni</i>
Immune complex disease of hepatitis B infection	Hepatitis B virus
Cryoglobulinemia/vasculitis of hepatitis C infection	Hepatitis C virus
<i>Autologous antigens</i>	
Systemic lupus erythematosus (SLE)	DNA, nucleoproteins
Rheumatoid arthritis	IgG as an antigen for rheumatoid factors
Mixed cryoglobulinemia	IgG as antigen (for anti-idiotypic antibodies?)
Glomerulonephritis	Renal tubular antigens
Goodpasture's syndrome	Glomerular basement membrane
Thyroiditis with thyroid carcinoma	Thyroglobulin

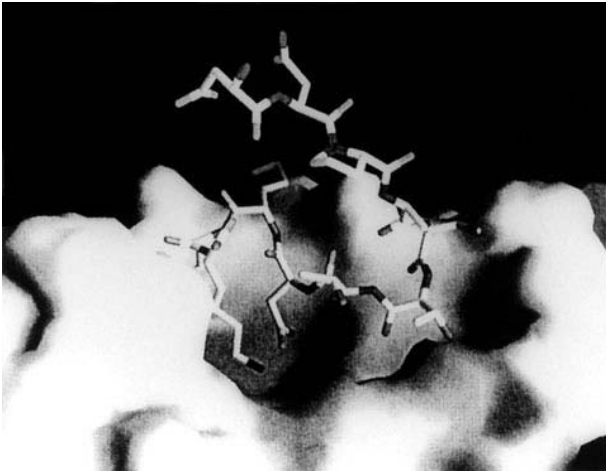


Figure 3 Complex between a viral peptide epitope of poliovirus and a neutralizing antibody Fab (as displayed by computer modeling). The structural and chemical complementarity between antigen and antibody shows clearly, and negative charges in the antigen binding pocket evidently contribute to binding. (Reproduced with permission from Wien et al (1995).)

These cells are erythrocytes in human and nonhuman primates and platelets in various rodents and subprimates – including mice, guinea pigs and rabbits. Studies in primates have shown that circulating erythrocytes rapidly interact with exogenously infused or *in vivo* formed immune complexes and carry them to the hepatic and splenic reticuloendothelial system, where the complexes are stripped from the erythrocytes and further processed by the mononuclear phagocytes. Decomplementation of the animals, thereby preventing association of C3b to immune complexes, makes immune complex fixation to erythrocytes impossible, facilitating diffuse deposition of immune complexes outside the reticuloendothelial tissues. CR1 in humans is found not only on erythrocytes, from which it was isolated in the late 1970s by Fearon, but also on neutrophils, monocytes and lymphocytes. The presence of such receptors on lymphocytes provides an immunoregulatory function of immune complexes; their fixation to lymphoid tissues permitting prolonged immune recognition of the antigen and, perhaps, the maintenance of immunological memory.

The interaction of immune complexes with complement leads to ionic fixation of the globular domains of C1q to the C γ 2 domain of IgG, or the C μ 3 domain of IgM after these antibodies have formed immune complexes. The IgG- and IgM-binding site on the C1q globular domains is strongly electrostatic. The IgG residues involved in the binding to C1q are charged residues, Glu318, Lys320 and Lys322. Subsequent C4b and C3b binding further contribute to coating of immune complexes by

complement. In the circulation at any one time, the proportion of immune complexes bound to erythrocytes is dependent not only on the rate of binding but also on inactivation of CR1-bound C3b, which leads to release of the immune complexes from the erythrocyte surface. The released complexes remain coated with iC3b and C3dg and are incapable of activating complement any further but maintain a capacity to cause an inflammatory response. The enzyme responsible for C3b inactivation is factor I: its concentration and activity determine release reactions and hence influence the kinetics of the exchange of immune complexes between erythrocytes and plasma.

It is at the level of the reticuloendothelial system – mainly in liver and spleen – that immune complexes become discharged from the erythrocytes in transit through the circulation. The iC3b- or C3dg-bearing immune complexes remain in the reticuloendothelial system, where they interact with iC3b receptors (CR3, CD11b) or C3dg receptors (CR4, CD11c) that are expressed on phagocytic cells. Failure of the erythrocyte shuttle transport system or dysfunction of the different processing steps of immune complexes in the reticuloendothelial system may lead to a chronic persistence of complexes in the circulation, thereby predisposing to their entrapment in kidney, skin, lungs, retina and other organs.

The complement system thus emerges as the major immune complex-processing protein system that confers upon a variety of circulating and tissue fixed cells the potential to dispose of these complexes. Human erythrocytes are now known to autonomously inhibit the complement-mediated solubilization of immune complexes bound to their surface, suggesting that these cells would control some physicochemical properties of the complexes they carry.

At the beginning of the twentieth century, Arthus described a clinical syndrome that developed after the injection of horse serum into rabbits, and Clemens von Pirquet recognized that antigens not only induce antibodies but are also responsible for serum sickness. Immune complexes were identified as the toxic products responsible for tissue injury of individuals with serum sickness. We now know that, for the Arthus reaction to occur, relatively large amounts of immune complexes have to be localized in the vessel walls and in perivascular areas. Both passively adsorbed and locally formed immune complexes will react with complement and induce release of anaphylatoxins, such as C3a and C5a, and cytokines, which enhance vascular permeability and generate other chemotactic factors, attracting polymorphonuclear leukocytes to the site of the lesion. An increase in

vascular permeability evidently favors the leakage of circulating immune complexes into the extravascular compartment. *In vitro* studies further suggest that the binding of immune complexes to tissue components may cause direct, complement- and inflammatory cell-independent tissue injury by stimulating oxygen radical release.

At this stage, inflammation becomes largely dependent on interaction of immune complexes with the Fc receptor apparatus. In fact, FcR signaling on such cell types as macrophages, neutrophils and natural killer (NK) cells recruits cellular tyrosine kinases, followed by elevation of intracellular calcium and release of inflammatory leukotrienes, prostaglandins and hydrolases and the transcription of genes encoding for cytokines.

Immune complexes containing IgG suppress interferon γ (IFN γ)-induced activation of the Fc γ RI gene by preventing tyrosine phosphorylation, a finding that explains the impairment of tumoricidal activity and enhancement of major histocompatibility complex (MHC) class II expression in macrophages by immune complexes. A considerable diversity of cellular responses to immune complexes thus emerges, which is largely due to actions at different types of FcR. In turn, FcRs are themselves under control of various cytokines. Thus, a network of regulatory factors exists which can become disturbed by immune complexes. Furthermore, immune complexes are now known to induce and stimulate interleukin 10 (IL-10) production from macrophages, thereby inhibiting antimicrobial responses (e.g. against *Listeria monocytogenes*). In turn, immune complexes induced IL-10 production when experimentally used to provoke acute alveolitis, an activity that is favorable for the host, because IL-10 decreases tumor necrosis factor α (TNF α) production, thereby attenuating injury. IL-10 thus downregulates not only inflammation directed at foreign invaders but also at self constituents (Figure 4).

Since the original definition of serum sickness, a great variety of diseases have been found to resemble the immune complex model described in rabbits. Particularly well studied are the autoimmune hemolytic disease and chronic membranous glomerulonephritis in New Zealand black mice. Further immune complex-type diseases in animals involve viral antigens such as Aleutian disease of mink, lymphocytic choriomeningitis, in which immune complex-induced lesions develop, and lactic dehydrogenase-elevating virus (LDV) infection in mice, with the production of often lifelong levels of circulating immune complexes.

Augmented production of immune complexes, and/or their reduced removal, plays a crucial role in

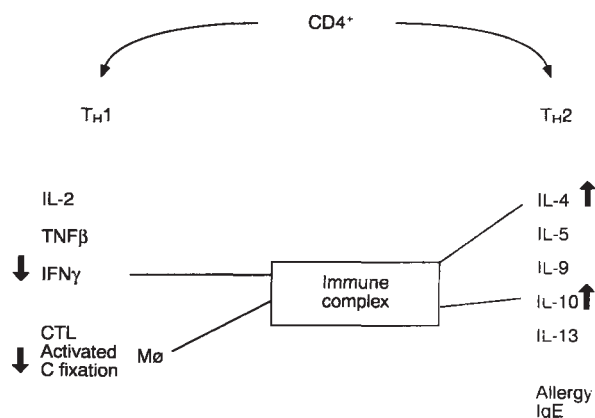


Figure 4 Intervention of immune complexes with opposing sets of cytokines. Immune complex-induced increased production of IL-4 and IL-10, and immune complex-induced suppression of IFN γ . IL-10 suppresses IFN γ -production by helper T lymphocytes and NK cells. In addition, IL-10 suppresses the production of several proinflammatory, macrophage (M ϕ)-derived cytokines, including TNF α , IL-1 α , IL-1 β , IL-6, IL-8, granulocyte-macrophage and granulocyte colony-stimulating factors. IL-10 also inhibits expression of MHC class II antigen on monocytes and upregulates inducible nitric oxide synthase in macrophages. Exogenously administered IL-10 has been shown to protect rats from lung injury after intrapulmonary deposition of IgG immune complexes. CTL, cytotoxic T lymphocyte.

the pathogenesis of many immune complex-related diseases in humans, as illustrated by systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and HIV infection. **Table 1** lists a number of nosological entities in which immune complexes play a relevant immunopathological role and in which their therapeutic removal may improve the patient's condition.

Because complement is so important to immune complex processing, adverse consequences with regard to humoral immune defense are understandable in hypocomplementemic patients. In patients with inherited complement deficiencies, increased serum concentrations of immune complexes are found associated with deficiencies of components of the classical pathway of complement activation C1, C4 and C2. In acquired complement deficiency, however, low levels of these and other components (C3, C5, C5b-9) may be a result of chronic complement activation either by the inflammatory process or directly by immune complexes. This is mostly reflected by the demonstration of low levels of C3, C4 and other components and high levels of the complement component breakdown products C3dg, C3a and C5a. In many patients, the levels of circulating immune complexes and those of complement breakdown products correlate significantly.

Clinical aspects in human medicine

Immune complexes are found in the circulation and/or other biological fluids of patients with a large spectrum of diseases not resembling each other, such as autoimmune, infectious and neoplastic disorders. This proves that elimination of antigens in the stage of immune complexes is a common feature to many diseases, and symptoms may not necessarily result from their presence. In fact, in many circumstances, it is not possible to differentiate between a role for immune complexes in physiological antigen removal and a deleterious pathogenic factor. In glomerulonephritis the pathogenic role of immune complexes is generally accepted. The following immune complex diseases are frequently associated with nephritis: SLE, polyarteritis, cryoglobulinemia and many microbial diseases such as bacterial endocarditis, leprosy, malaria, trypanosomiasis, hepatitis B and C infection and dengue hemorrhagic fever. In contrast, RA, polymyositis, dermatomyositis, cutaneous vasculitis, fibrosing alveolitis and the acquired immune deficiency syndrome (AIDS) typically do not involve the kidneys. The antigens that have been identified in immune complex-mediated glomerular disease include endogenous antigens (nuclear, brush border, thyroglobulin, immunoglobulin, carcinoembryonic and other tumor antigens) and exogenous antigens (bacterial, viral, parasitic, fungal) and others such as drugs and toxic agents. In RA, levels of immune complexes roughly correspond to clinical severity and/or progression of the disease. In SLE, no constant correlation between levels of circulating immune complexes and disease activity and kidney or skin involvement is found. In addition, a range of drugs may interfere with immune complex-complement interaction; examples here are hydralazine, isoniazide and penicillamine, which all inhibit covalent binding of C4 to complement-activating surfaces, such as provided for by immune complexes. Chronic circulation of immune complexes over prolonged time may reflect insufficient management of infectious attacks in certain bacterial and viral diseases, for example infective endocarditis or hepatitis B virus infection. Very high concentrations of immune complexes are seen in AIDS and hepatitis C cryoglobulinemia. They have been related to the occurrence of complications found in patients exposed to HIV and hepatitis C, such as vasculitis and thrombocytopenia.

At this point it should be noted that the quality of antimicrobial antibodies is crucial in determining the host's capacity to eliminate immune complexes; thus, antibodies may bind to viruses without necessarily mediating their neutralization and/or removal. Such a binding may even be deleterious, as it may facilitate entry of viruses into cells richly endowed with FcR through FcR-dependent mechanisms – one example being the colonization of macrophages by HIV.

See also: Complement, classical pathway; Cytokines; Fc receptors; Idiotype network; Immunoglobulin, cell surface.

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IMMUNE RESPONSE

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The immune response involves a coordinated set of interactions among host cells and the protective molecules they produce upon encountering a pathogen or antigen, the purpose of which is to prevent dangerous incursions and then to restore homeostasis. The immune system must not only confront external, invasive challenge from microorganisms but also the possibility of internal attack from tumor cells, and occasionally autoaggressive attack from its own defensive forces (autoimmunity). The required complexity of this response is illustrated by the fact that throughout evolution, viruses, bacteria and other parasites have accommodated to the host response by developing their own unique strategies designed to circumvent the host defense system. Likewise, appropriate responses in the host have evolved to cover these contingencies, with some accompanying redundancy. Thus, there appear to be many nodal points throughout the system where a choice of reactive pathways is available. Indeed, the host immune response consists of two interacting systems, one innate and one adaptive.

Innate system versus adaptive system

When it is realized that some microbial populations can double in 20 minutes under ideal circumstances, it is evident that the specific immune system can only protect effectively in the case of aggressors for which it has previously developed memory. Therefore, the innate immune response has developed into a system of a generic sort, always in place, capable of immediate responsiveness to foreign encroachment. It is the consequence of long-term evolution which has its origins in the invertebrate phyla and includes mechanisms of phagocytosis, chemotaxis, cellular mobilization, pattern recognition and coordinated action. The mammalian innate system comprises various features, including protective plasma proteins, phagocytic cells (granulocytes) and physical barriers.

One interesting feature is the way in which aspects of innate and adaptive systems interact. Let us consider two such important cellular components of the innate system: the dendritic cells (DCs) and the natural killer (NK) cells. The dendritic cells perform a guarding function, by which they are stationed at all outposts of the body – in the skin, where they are

called Langerhans cells, or in various organs such as the lungs, liver and kidneys, where they bind and ingest foreign or intrinsic invaders. Entry of an infectious agent induces an inflammatory response, largely carried out by other members of the innate system, which leads to activation of DCs. This enables the DC to carry antigen, which it has taken up and processed, to the peripheral lymphoid organs where it is presented to specific T and B lymphocytes to start the specific immune response.

The NK cells also play a dual role and provide molecules known as cytokines to modulate the activities of other cell types. NK cells can be effector cells, killing virus-infected or transformed cells, and like the DCs, are vital intermediaries with the adaptive immune system.

The principal effector cells of the adaptive system are T and B lymphocytes. Unlike the innate system, the adaptive system develops in response to antigenic challenge. The specificity of this system requires the evolution of antigen receptors with the capacity to recognize millions of distinct molecular structures; however, each antigen specificity is represented by only a limited number of lymphocytes. Therefore, two key features of this system are the enormous expansion of specific lymphocytes following antigen exposure and the storage of specific memory in 'memory cells', which upon subsequent challenge are able to respond rapidly. Most importantly, the system is a migratory one, in which responsiveness occurs in defined locations. Evolution has finely tuned the homing mechanism for lymphocytes and antigen-presenting cells (APCs) so that their meeting will lead to productive interactions.

In the rest of this entry, we describe the intricate and interactive maneuvers of subspecialized groups of cells involved in the defensive function.

Elements of the adaptive defense system

The humoral and the cell-mediated immune responses are the two major components of the acquired immune response. Its components are principally the B lymphocytes (B cells) and the T lymphocytes (T cells), and their interaction is required for a successful response. These lymphocytes express highly specific antigen receptors on their surface in a

clonally restricted manner such that each lymphocyte bears fine specificity for a given structural motif. The antigen-specific receptor present on the surface of B cells is the immunoglobulin (Ig) molecule in its membrane-bound form. When a B cell encounters an antigen that interacts with its surface immunoglobulin, provided it receives the necessary T cell signals, it proliferates and differentiates through several generations into immunoglobulin-secreting plasma cells and memory cells. Antigen-specific immunoglobulins or antibodies detected in the serum or other bodily fluids are indicative of antigenic encounter and an immune response. Monomeric antibody molecules, comprising two identical heavy chains (α , δ , γ , ϵ or μ) and two identical light chains (κ or λ), can be described functionally as having a 'Y' shape. Sequences in the arm are highly variable among molecules and form two identical antigen-binding sites. The stem of the 'Y', or constant region, contains more conserved sequences and endows the five distinct antibody classes (IgA, IgD, IgG, IgE and IgM) with functional properties. During an immune response, the first class of antigen-specific antibody detected in the serum is the pentameric IgM molecule, but a switch then occurs to IgG, IgA or IgE. In T cell-dependent antibody responses, switching is accomplished in conjunction with T cell and cytokine signaling.

The functions of the humoral or antibody response include neutralization of a pathogen and/or its toxic products by antibody binding, to prevent infection or toxic damage, respectively. Additionally, antibody can act as an 'opsonin' to facilitate the phagocytosis of pathogens by macrophages, which in turn present parts of the microbe to specific lymphocytes. A third function is activation of the complement cascade. Complement is a group of plasma proteins that bind to antigen-antibody complexes, enhancing opsonization or, in some cases, directly killing the bacterium or the infected cell.

T cells also respond to stimulation of their unique antigen receptors (T cell receptor, TCR) by proliferating, differentiating and secreting cytokines. The majority of T cells express a TCR composed of an α and a β chain, while a minority of cells express a heterodimer of γ and δ chains. Unlike B cells, T cells cannot recognize native antigens but require short peptides derived from them to be displayed on the surface of an APC in association with molecules of the major histocompatibility complex (MHC). Dendritic cells, macrophages and activated B cells are professional APCs which capture antigen by endocytosis, then digest or process the antigen so that it can be placed within a binding groove in MHC class II molecules for presentation to T helper cells

(T_H) carrying the CD4 coreceptor which contacts an invariant region of the class II molecule.

The other type of MHC molecule, the class I, is present on all nucleated cells and binds peptides from endogenous self proteins and intracellular pathogens, such as viruses and some bacteria. Destruction of such pathogens is accomplished through killing the host cell by cytotoxic T lymphocytes (CTLs). Cytotoxic T cells that recognize peptides in association with MHC class I molecules bear the CD8 molecule on their surface, which acts as a coreceptor and contacts an invariant region of the class I molecule.

TH_1 versus TH_2 : the importance of an appropriate response

Antigen-specific activation of T cells results in the clonal expansion of specific lymphocytes and the secretion of cytokines. The T cells can be classified into two major subsets according to their pattern of cytokine secretion: type 1 and type 2 T cells. T helper type 1 cells (Th_1) secrete interferon γ ($IFN\gamma$), interleukin 2 (IL-2) and tumor necrosis factor β ($TNFB$), while the type 2 T helper cells (Th_2) secrete IL-4, IL-5, IL-6 and IL-10, among others. Differentiation down the Th_1 pathway is regulated by the monocyte-derived cytokine IL-12, while Th_2 development is governed by IL-4. Most importantly, the Th_1 and Th_2 pathways are mutually antagonistic, so that progression in one direction tends to be persistent. The pattern of $CD8^+$ T cell cytokine secretion is not as clear but there appears to be a similar polarization.

Type I cytokines help to control viral infections by activating NK cells and CTLs. The type II cytokines, especially IL-4 and IL-5, are relevant to immune responses to extracellular parasites and the development of allergies by virtue of their ability to induce IgE production in B cells and enhance the growth of mast cells. The disease leishmaniasis illustrates the importance of activating the appropriate T helper subset. Protection from the disease is dependent upon the activation of the Th_1 pathway which leads to $IFN\gamma$ production, macrophage activation and parasite killing, and inhibits IL-4 production. In experimental mice, deviation towards a Th_2 response can result in death. Multiple factors can influence T cell function and cytokine polarization, including the genetic background of the host, the type of APC, the nature of the costimulatory and accessory molecules, the affinity of the antigen for the MHC and the antigen-MHC complex for the TCR.

Antigenic determinants (dominance versus crypticity)

The processing of antigens into peptides capable of stimulating T cells, when associated with MHC molecules, is a vital part of the immune response. The specific immune response to an antigen is directed to only a limited number of all possible peptides that can theoretically be generated from this protein, the so-called 'immunodominant determinants'. This phenomenon has been studied classically using the lymph node proliferation assay. 'Dominant' determinants are capable of inducing *in vitro* proliferation of lymphocytes ('recall') that have been primed *in vivo* with the whole protein. When 'dominant' determinants are used to immunize, they can raise a response which can be recalled by the whole protein antigen. 'Cryptic' determinants are only capable of inducing a response when in the peptide form, and this response is not recalled by the whole protein. A large repertoire of T cells directed against self remains available for reactivity, as only dominant determinants induce thymic tolerance.

Activation versus tolerance

While antigen recognition is an essential feature in immune activation, it also is critical for the development of immunological self tolerance in the host. According to the two-signal model of T cell activation, TCR ligation by peptide-MHC complexes provides a primary signal that has to be complemented by a second signal for a naive T cell to become fully activated. Costimulatory molecules and their receptors, such as B7 and CD28, respectively, provide this second signal, while other pairs of accessory molecules function to stabilize the interaction between the two cells. Shortly after T cell activation, expression of an additional B7 interacting molecule, CTLA-4, may be induced, which can downregulate further T cell immune expansion.

The interaction between the accessory molecule CD40 on B cells and CD40 ligand (CD40L, gp39) on T cells also functions as a costimulatory signal for both the T cell and the B cell. Ligation of the TCR by a peptide-MHC complex on an APC such as a B cell leads to the upregulation of CD40L expression on T cells, which mediates a signal via CD40 on the B cell, leading subsequently to increased expression of B7 molecules. The enhanced expression of B7 on the B cell allows full activation of the T cell through CD28, which then secretes cytokines that induce the differentiation of the B cell. Thus, CD40 plays a role in the regulation of both humoral and cell-mediated immune responses.

While orchestrating its defenses, the immune system must also take steps to prevent cellular damage which accrues via friendly fire (autoimmunity). In contrast to activation, which leads to the stimulation and expansion of certain lymphocytic clones, immunological self tolerance is achieved by the selective elimination from its armament of the lymphocytes which are reactive with dominant determinants on self antigens. At least three mechanisms have been described for the induction of tolerance: clonal deletion, clonal anergy and active suppression via cytokines or regulatory lymphocytes which recognize certain immunoglobulin or TCR molecules. Clonal deletion is accomplished in part during the establishment of central tolerance, where high-affinity, autoreactive T and B cells are physically removed during thymic and bone marrow selection, respectively, via antigen-induced apoptosis. However, negative selection in the primary lymphoid organs alone is not sufficient for the maintenance of self tolerance as many potential target antigens are not expressed in the thymus and bone marrow and others are developmentally regulated and appear after the repertoire has been established. Autoreactive T cells which escape the thymus are rendered 'unresponsive' when they are stimulated in the periphery by nonprofessional APCs which lack costimulatory molecules (naive B cells and parenchymal cells). In fact, successful T cell clonal expansion requires the presence of activated B cells, which in turn are dependent upon T cell help. Thus, the discrimination between activation and tolerance induction is the product of several factors, including the nature of the APC and the local environment.

Hyper- and hyporesponsiveness: examples of dysfunctional immune responses

Clearly, T cells bearing TCRs capable of recognizing self antigen-MHC complexes are present in healthy individuals, yet autoimmune disease does not normally occur. However, if a self reactive T cell population becomes activated, a vigorous inflammatory response can be created, spreading to other previously cryptic determinants on the self antigen ('intramolecular spreading'). In the absence of regulation, such an incident may perpetuate into a chronic, autoimmune condition. Conversely, the recognition of a self determinant can be beneficial, as when a TCR peptide on an autoimmune effector T cell is targeted by a regulatory T cell.

On the other hand, immunodeficiency brings equally serious problems as hyper-responsiveness. The devastation induced by the human immunodeficiency virus (HIV) is a stark example of this.

ciency virus (HIV) in targeting CD4-bearing cells of the immune system is well known. When the loss of CD4 cells becomes acute, most immune responsiveness fails, as these cells are crucial for the induction of other activities in the system. One additional example of an inadequate immune response may be the outgrowth of malignant tumors in otherwise healthy individuals. Many experimentally induced tumors are quite immunogenic, e.g. immunization with a small or attenuated tumor dose protects from further challenges with the same tumor, indicating that the immune system is capable of recognizing and killing the tumor cells. Unfortunately, this is not the case for most spontaneous tumors which are non-immunogenic (hence their spontaneous appearance). They are, however, antigenic – capable of inducing a specific immune response if appropriate costimulatory signals are provided. Nonimmunogenic tumors transfected with mixtures of cytokines (e.g. IFN γ) and/or with costimulatory molecules (B7) can attain the capacity to induce an immune response: they are efficiently rejected and subsequent long-term protection against the parental tumor becomes established ('memory'). Another way to efficiently prime the immune system to specific tumor antigens is to present them via a professional APC, such as a DC. Both approaches have been effective in inducing a protective immune response to a subsequent challenge with the same tumor, when combined with cytokine administration.

The previous sections describe how the immune system constantly accumulates information to fine tune the type of response that adequately meets the challenge. Although we and others have used a military analogy to describe the system, it can also be viewed as expending continuing effort throughout life 'to know itself', and to maintain the organism's self integrity.

See also: **Acquired immune deficiency syndrome (AIDS); Autoimmunity; Bacteria, immunity to; Cell-mediated immunity; Fungi, immunity to; Humoral immunity; Hypersensitivity reactions; Immune response in vitro; Innate immunity; Parasites, immunity to; Tumors, immune response to; Viruses, immunity to.**

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IMMUNE RESPONSE (Ir) GENES

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Immune response (Ir) genes can be defined as any gene locus at which different alleles affect the magnitude of an immune response. Thus, Ir genes were identified on the basis of a complex phenotype and, not surprisingly, comprise a heterogeneous group of structural genes. Nevertheless, genes encoded within the major histocompatibility complex (MHC) clearly have the most dramatic effect on the immune response to a variety of antigens, and thus became the first well-characterized Ir genes.

Historical background

From the beginning of this century, immunologists had noted that there were differences in the ability

of individual animals to mount an immune response to an antigen, and that these differences were heritable. Because the animals used in early experiments were outbred, and the antigens generally complex proteins or whole organisms, the genetic influence was complex and involved a large number of genes. In an attempt to simplify the complexity of antigens, immunologists turned to the use of synthetic amino acid copolymers (two or more amino acids linked in linear or branched chains). In 1963, the laboratories of Maurer and of Benacerraf reported that the immune response of guinea pigs (both antibody production and delayed-type hypersensitivity (DTH)) to synthetic copolymers such as poly-(Glu,Lys), or poly-L-Lysine was under genetic control, and that the

response segregated as a single autosomal dominant gene. McDevitt and Sela also demonstrated that the immune response of mice to branched-chain copolymers such as (T,G)-A-L was under single gene control and designated this the immune response 1 or Ir-1 gene. Using inbred mouse strains, they subsequently mapped the Ir-1 gene to the H2 complex, in a region termed the I-region. Very shortly thereafter, a number of antigens were shown to be under Ir gene control. While these genes all mapped in the H2 complex, intra-MHC recombinant mice revealed the existence of multiple loci. The Ir-1 locus was soon renamed Ir-1A. A distinct locus, Ir-1B, was defined by the antibody response to IgG2 allotypes. Ir-1C was defined by its ability to complement with Ir-1A in the response to the synthetic copolymer GLT.

Independently, the groups of David, Frelinger and Shreffler, and Hauptfeld and Klein, identified lymphocyte cell surface antigens that mapped within the I-region of the H2 complex. These antigens were designated class II MHC molecules (the class I antigens were first identified by Peter Gorer in the late 1930s) and because they mapped within the I-region, they were referred to as immune-associated, or Ia antigens. Shortly thereafter, the phenomenon of MHC restriction was discovered by Doherty and Zinkernagel, and it became apparent that both class I and class II MHC molecules were 'restriction elements' in the recognition of antigen by T cells.

As more and more evidence accumulated that the Ir genes originally identified were identical with Ia antigens, and that class I molecules could also manifest Ir gene effects, a generally accepted model emerged. Ir gene effects (at least those mapping within the H2 complex) could be explained as the consequence of MHC restriction and variability of different polymorphic alleles of class I and class II MHC antigens to 'present' antigen to T cells. Finally, in the last 10 years, we have developed a detailed understanding of the mechanism of MHC restriction and can explain in considerable detail, the molecular mechanism of MHC-linked Ir gene effects.

MHC-linked Ir genes

The original Ir genes defined by the response to synthetic copolymers are, in fact, the structural genes for the Ia antigens, referred to as class II MHC genes. The Ia antigens in the mouse consist of two similar cell surface glycoproteins, I-A and I-E, each of which is composed of an α and a β chain. The four genes encoding these molecules are A α , A β , E α and E β . The genes are highly polymorphic (except for E α) and exist in multiple allelic forms. They are also

codominantly expressed such that, generally, both alleles at each locus are expressed in a heterozygous individual. In heterozygous individuals, codominant expression also allows for the pairing of an α polypeptide chain encoded on one chromosome with a β polypeptide chain encoded on the other chromosome, forming a 'hybrid' Ia molecule that is not found in either parent. The existence of hybrid Ia molecules explains the complementation of Ir genes observed by several investigators. Additional β chain genes exist, and some appear capable of coding for protein, however protein products have not been identified, and their role in MHC restriction and Ir gene control remain unknown.

The three-dimensional structure of MHC class I and class II molecules has been solved by X-ray crystallography. Both of these structures reveal a cleft formed by two α helices, and it is clear that peptide fragments of antigen occupy these clefts. In order for antigen to be recognized by the T cell, it must first be degraded and presented as a peptide bound in the groove of an MHC molecule. The T cell receptor then recognizes the antigen, as well as a portion of the MHC molecule. The specificity for interaction of the T cell receptor with a particular MHC molecule is determined during T cell development in the thymus by a process called positive selection. This dual specificity of the T cell receptor for peptide and a specific allele of MHC antigen is called MHC restriction. The MHC restriction specificities of the T cells are dictated by the MHC antigens expressed in the thymus during T cell development. Thus for a T cell to recognize and respond to antigen, two criteria must be fulfilled: the proper antigenic peptide must be bound in the groove of an MHC molecule on an appropriate antigen-presenting cell (APC), and the MHC molecule to which the antigen is bound must be the proper restriction element for that T cell. Finally, the T cell repertoire is influenced by negative selection in the thymus, where T cells reactive to self antigen plus self MHC are clonally deleted. The result of this type of selection is that there can be 'holes' in the T cell repertoire that vary, depending on the expression of MHC and polymorphic self antigens in different individuals.

The mechanisms described above led to the development of two general models to explain Ir gene control of the immune response. One model, referred to as the determinant selection model, states that different polymorphic variants of Ia molecules differ in their capacity to bind and present particular peptide antigens. Therefore, non- or low-responsiveness would be due to the relative inability of certain MHC molecules to bind a particular antigen peptide. The alternative, although not mutually exclusive, expla-

nation is that the low response phenotype is due to a lack of specific T cells for that particular antigen, the so-called 'hole in the repertoire' model. Since the T cell repertoire is, to a large extent, dictated by the polymorphic MHC alleles expressed in the thymus during ontogeny, this phenotype would also be controlled by the MHC class I and class II antigens. There is now strong experimental evidence indicating that both models are correct. The ability of MHC molecules to discriminate between different peptides was demonstrated by peptide-binding studies using solubilized MHC molecules and labeled peptides of known MHC restriction. Thus, the peptide fragment HEL 46-61 of the hen egg lysozyme molecule is the immunodominant peptide in I-A^k-positive mice, and is presented by the I-A^k molecule. The I-A^d molecule does not present this peptide and mice with this allele are nonresponders to this peptide. *In vitro* binding assays revealed that peptide HEL₄₆₋₆₁ binds to the I-A^k but not the I-A^d molecule, suggesting that the nonresponsiveness of I-A^d mice is due to the inability of the I-A^d molecule to bind and present this antigen to T cells. The binding of HEL₄₆₋₆₁ can be inhibited by competition with some unlabeled peptides but not others. There is a direct correlation with the ability to compete for binding and the ability to be recognized by the relevant T cell. Thus, if a peptide cannot bind, it cannot stimulate a T cell, establishing that Ir gene control does operate at the level of peptide binding. However, not all peptides that bind are capable of stimulating T cells. For example, the autologous mouse lysozyme sequence binds quite well to the I-A^d molecule, but does not stimulate mouse T cells, presumably due to self-tolerance.

The finding that autologous lysozyme peptides bind to I-A^d molecules suggests that peptide binding to MHC molecules is not the sole factor in determining immune responsiveness. There are now many examples of peptides that bind to particular I-A or I-E molecules, but do not stimulate T cells. For example, the lambda repressor protein, cl₁₂₋₂₆, binds equally well to I-A^d and I-E^k, but only stimulates T cells when associated with I-A^d. Since the peptide is capable of being recognized by the T cell, and does bind to I-E^k, these results indicate that the nonresponsiveness to cl₁₂₋₂₆ in association with I-E^k is due to a lack of cl₁₂₋₂₆-specific T cells restricted to this MHC class II molecule.

The class I molecules are also capable of functioning as Ir gene products in a manner analogous to the class II molecules. There is, however, a qualitative difference due to the T cell subsets (CD8⁺ cells as opposed to CD4⁺) that respond to class I plus antigen, and the types of antigens that become associated with the class I molecule on the cell surface. In gen-

eral, class I molecules preferentially associate with endogenously synthesized antigens, while class II molecules associate with exogenously added antigens. Nevertheless, clear Ir gene effects are associated with class I molecules. Analogous to the results with class II antigens, Townsend and colleagues demonstrated that endogenously synthesized viral gene products are processed and become associated with class I MHC molecules as peptide fragments. They further demonstrated that peptide binding is selective and usually depends on the appropriate responder class I allele. As with the class II molecules, peptide binding to class I antigens is required for immune responsiveness, however, not all peptide-class I combinations will stimulate T cells, presumably due to a lack of specific T cells in those instances.

We can now explain the basis for MHC-controlled, Ir gene phenomena. The first requirement for an immune response to antigen is that a peptide fragment be bound to a particular MHC molecule. If a peptide cannot bind to a particular MHC molecule, then there will be no response to that particular peptide-MHC combination, and that allele will be a nonresponder allele for that particular peptide. On the other hand, it is possible for a peptide to bind perfectly well to a particular MHC molecule, but not elicit a response due to a hole in the T cell repertoire. Since the T cell repertoire is primarily controlled by the MHC molecules expressed in the thymus during T cell ontogeny, this Ir gene effect also maps to the MHC structural genes.

In spite of these restrictions on the immune response, most individuals respond quite well to a variety of antigens. In mice of I-E⁻ haplotypes, like C57BL/6, only one class II molecule, the I-A^b molecule, is available for presentation of antigens, and yet these strains are fully immunocompetent. The reasons for this are several-fold. First, peptide binding to any given MHC molecule is extremely nonspecific, so that a wide variety of different peptides are accommodated. Second, since most antigens are complex proteins, there will usually be some peptides from any given protein that are capable of binding to a particular MHC molecule. Thus, Ir gene effects are most apparent when the number of antigenic determinants is limited, as in the case of synthetic copolymers, or proteins that differ from the responding species by a very few number of amino acids.

Non-MHC-linked Ir genes

A number of non-MHC-linked genes have also been shown to affect the magnitude of the immune response to particular antigens, and therefore fulfill

the requirements for Ir genes. However, unlike the MHC genes, the mechanisms of action are poorly understood. The Ir-2 locus controls the antibody response to the blood group antigen Ea-1. Ir-2 appears to be a single locus, mapping to chromosome 2 of the mouse, for which nonresponse is dominant. The Ir-3 locus controls the response to the Pro-Lys backbone of the synthetic copolymer (Phe,G)-Pro-L. This antigen is also under MHC-linked Ir gene control, making Ir-3 difficult to study. The Ir-4 locus controls the antibody response to the H2.2 specificity of the H2D^b molecules. The Ir-5 locus controls the antibody response to the mouse Thy-1 antigen and maps approximately 17 cM telomerically from H2. As with Ir-3, Ir-5 interacts with H2 in the response. The Ir-7 locus controls the response to the cell wall polysaccharide of group A streptococci. Three alleles have been described that interact in a complex fashion.

Genetic analysis of Ir phenotypes indicates that there are many additional loci that can manifest Ir gene effects. In fact, any polymorphic self protein, in theory, can influence the immune response to foreign antigen through the process of negative selection in the thymus. This is most dramatically seen in the case of the endogenous retroviral superantigens. Although not strictly a self molecule, these integrated defective retroviruses show mendelian inheritance and produce protein products that are indistinguishable from other self proteins. Mouse mammary tumor virus 7 (MMTV-7), previously known as Mls-1^a, produces a superantigen that interacts with all T cells bearing V_β 6, 7, 8.1 and 9 T cell receptors. Consequently, mice carrying MMTV-7 clonally delete most, if not all T cells bearing these particular V_β T cell receptors (TCR), resulting in a large 'hole' in the T cell repertoire. Finally, the genes encoding the immunoglobulin and T cell receptor V regions are technically Ir genes since the presence of particular V genes varies between haplotypes. Many haplotypes have large deletions of TCR or Ig V genes, significantly altering the T or B cell repertoire in these mice.

Human Ir genes

A number of studies have demonstrated HLA associations with the immune response to different antigens. While it is more difficult to formally demonstrate Ir genes in humans, it is clear that the human MHC gene products function in an analogous manner to the mouse. One example where Ir gene effects in humans most likely play a role is the association of HLA alleles with autoimmune diseases such as

ankylosing spondylitis associated with HLA-B27, and rheumatoid arthritis and juvenile onset diabetes associated with HLA-DR4. The HLA association is with susceptibility, not resistance, suggesting that the associated HLA antigens may be Ir genes controlling the response to some unknown antigen, either foreign, or self.

Perhaps the most striking and relevant example of Ir gene effects in humans is the demonstration by Hill and colleagues that the HLA-B*5301 allele controls the binding of a particular peptide from the liver stage-specific antigen of the malarial parasite, *Plasmodium falciparum*, thus making B*5301 an immune response gene for this peptide antigen. Most importantly, this peptide represents a critical cytotoxic T lymphocyte epitope and the B*5301 allele (which is present at greatly increased frequencies in West African populations) confers a high degree of resistance to malaria. Thus, immune response genes in the form of MHC polymorphisms can indeed play a pivotal role in the response to complex antigens such as the malarial parasite, and appear to be capable of evolving due to the influence of natural selection by the pathogen.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Clonal deletion; Congenic mice; H2 class I; H2 class II; HLA class I; HLA class II; Inbred strains; MHC disease associations; MHC, functions of; MHC restriction; Thymus; T lymphocyte differentiation.

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IMMUNE RESPONSE IN VITRO

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Immune responses *in vitro* and *in vivo* are closely related and interdependent, their components being effectively the same. Formulating *in vitro* methodologies is dependent on the level of understanding of immune responses *in vivo*, while insight into the *in vivo* environment can be assisted by *in vitro* experimentation. As *in vitro* studies have progressed, further clarification of leukocyte interactions has been forthcoming, giving us the *in vitro* immune response systems employed today. These methodologies have found a number of applications. One of the more recent is the analysis of vaccine efficacy, allowing the differentiation of vaccinated from non-vaccinated animals. A more widely applied use is in hybridoma technology: *in vitro* immune responses have been used to produce the antigen-specific lymphoblasts required for hybridoma generation. An area which has seen considerable application of immune responses *in vitro* is fundamental immunological research. It is now possible to analyze, in a detail not otherwise possible, how different cell populations communicate and interact with each other, and the means by which lymphocytes recognize and react with antigen, to name just two widely studied examples. The following sections look in more detail at the methodologies employed, and how variations therein have been applied to particular analyses.

The basis of the immune response *in vitro*

Immune responses *in vitro* can be effected as either primary or secondary responses. The former make use of leukocytes from naive (nonimmunized) animals, the latter from animals previously immunized with the antigen in question. Both types of response require the same basic components of lymphocytes, monocytes and intercellular communications, including cytokine activity (Table 1).

Cellular requirements

As with responses *in vivo*, B lymphocytes react directly with the antigen for which they are specific, but complete differentiation into antibody-producing plasma cells requires immunological help from helper T (T_H) lymphocytes (Figure 1). A few antigens can stimulate B lymphocytes independently of T_H lymphocytes, but the majority of antigens are T_H

lymphocyte dependent. Antigen-stimulated precursor cytotoxic T (T_C) lymphocytes also require immunological help from T_H lymphocytes (Figure 1). In contrast to the B lymphocyte, both T_H and T_C lymphocytes require antigen to be processed and presented in peptide form associated with major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (Figures 1 and 2). When the peptide is associated with MHC class II, T_H lymphocytes can be stimulated; when associated with MHC class I, T_C lymphocytes can be stimulated (Figure 2). Monocytes, the related dendritic cells and, to a lesser degree, macrophages appear to be the main antigen-presenting cells. B lymphocytes can also present antigen, but apparently only to memory T lymphocytes; the monocytic antigen-presenting cells present to both naive and memory T lymphocytes. Accessory cells, such as endothelial cells and fibroblasts, play important additional roles in intercellular signaling (Figure 3), and are important for maintaining viability in a culture.

Cytokine requirements

The cytokines of the *in vitro* immune response are linked to the T_H lymphocytes and monocytes which produce them (Figure 1). Table 2 shows examples of those which have been most extensively studied and applied to *in vitro* immune responses. Whether exogenous cytokines are to be applied, or only the stimulation of the cytokine-producing cells within a culture, will depend on the type of *in vitro* immune response under study, and the objectives of the experiment. Nonetheless, it is the cognate interactions between helper cells and effector cells, or their precursors, which are essential for optimum immune responses *in vitro*.

Antigenic requirements

Antigen requirements *in vitro* will not necessarily relate to those *in vivo*, and are normally determined by experimentation. Immunogenicity *in vivo* is not a guarantee that an *in vitro* response can be stimulated. Generally, the more complex an antigen – in terms of structure and topography – the more efficient will be the induction of an immune response (see below under the topics of initiation of primary and secondary immune responses *in vitro*).

Table 1 The basic requirements for *in vitro* immune responses

Component	Conventional sources	Use
Monocytic APC	Lymphoid organs, blood	Antigen presentation; intercellular signaling; endogenous cytokine production
Dendritic cell APC	Lymphoid organs, blood, skin	Antigen presentation; intercellular signaling
B lymphocytes	Lymphoid organs, blood	Antigen-specific antibody production; antigen presentation to memory T _H lymphocytes (secondary immune responses)
T lymphocytes	Lymphoid organs, blood	Antigen-specific immunological help (T _H lymphocytes, via their cytokine production); endogenous cytokine production (T _H lymphocytes); antigen-specific cytotoxicity (T _C lymphocytes)
Endothelial cells	Lymphoid organs, tissues/organs containing microvascular capillaries	Intercellular signaling; endogenous cytokine production
Fibroblast accessory cells	Lymphoid organs, lung, skin	Intercellular signaling; endogenous cytokine production
Cytokines	Recombinant biotechnology, mitogen-activated mononuclear cell cultures	Lymphocyte proliferation and differentiation; antigen processing (activity depends on the cytokine or mixture of cytokines)
Antigen	As required for the study	Stimulation of the lymphocyte proliferation, either directly (for B lymphocytes) or indirectly after processing and presentation by APC (for T lymphocytes)

APC, antigen-presenting cell.

Establishment of the immune response *in vitro*: practical considerations

Sources of cells for immune responses *in vitro*

Mononuclear cells (lymphocytes and monocytes) from secondary lymphoid organs (lymph nodes, spleen or tonsils), as well as peripheral blood have been successfully applied to *in vitro* cultures (Figure 4; Table 1). For lymphoid organ cell preparations, mild enzymatic treatments of collagenase plus DNAase are preferred (Figure 4); preparation of blood cells is by centrifugation through density gradients of Ficoll-Diatrizoate (e.g. Ficoll-Hypaque) (Figure 4). Accessory endothelial cells can be obtained from lymph nodes and microvascular areas (such as umbilical cord, skin and lung tissue), while fibroblast cultures can be prepared from lungs and skin (Table 1), again using collagenase digestion or trypsinization (Figure 5).

In vitro deviations from *in vivo*

The cellular component of an *in vitro* immune response (Table 1) must contain viable and potentially active leukocytes. Problems with respect to this essential requirement can arise due to differences between the *in vitro* and *in vivo* immune response systems. Within secondary lymphoid organs, in which immune responses are initiated, there is a high cell concentration and a cellular network permitting

efficient interaction. These are essential for the maintenance of the cells in a viable and responsive state. When the cells are removed for *in vitro* culture, both the network of interactions is broken and the cell concentration reduced. Although preparatory procedures for *in vitro* culture may result in cell damage, modern methods render this minimal and of minor consequence compared to the reduced intercellular communication and signaling *in vitro*.

The problem of cell death *in vitro*

The loss of the *in vivo* intercellular interactions abrogates the delivery of signals required to control programmed cell death (also referred to under the morphologic term of apoptosis). This is particularly the case with T and B lymphocytes, but not with monocytic cells, endothelial cells or fibroblasts, which can be cultured without much problem. The majority of T and B lymphocytes may enter programmed cell death within the first 3 days of culture, in the absence of stimulation and cosignaling. Stimulation of the lymphocytes *in vitro* with antigen can prevent programmed cell death, providing that the correct cosignaling is also present (Figures 1 and 3, Table 1). Accessory cells can be employed to this end. However, their presence in conventional preparations of cells for *in vitro* immune responses is often inadequate, due to the reduced cell concentration employed with *in vitro* cultures. Consequently,

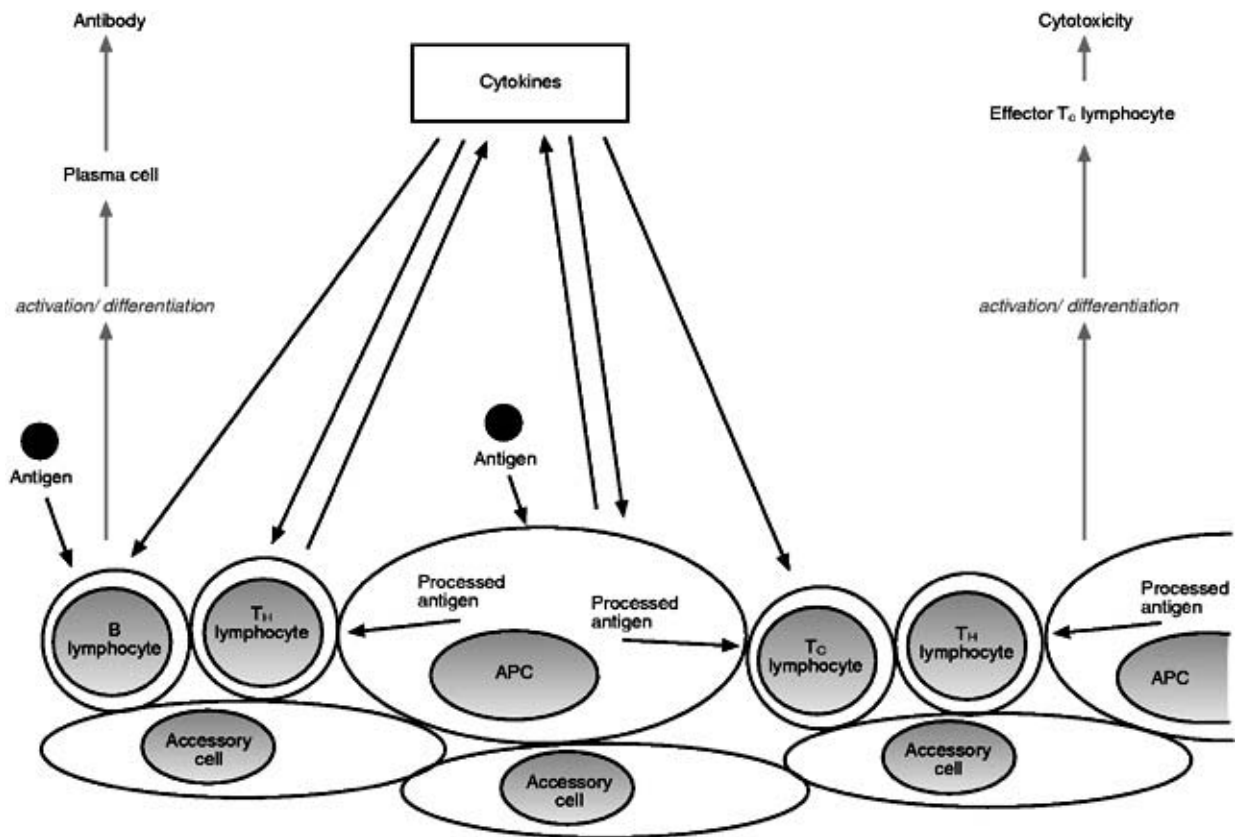


Figure 1 The principal components required for the immune response *in vitro*. T_H lymphocytes and antigen-presenting cells (APCs), which are usually the monocytic or dendritic cell APCs, are essential for all immune responses. The T_C lymphocytes will be studied in the context of antigen-specific cytotoxicity, while the B lymphocytes will be of interest for analyses of antigen-specific antibody production. Although the T_H lymphocytes and APCs produce cytokines, it may also be necessary to supplement certain cultures with exogenous cytokines, particularly at the initiation of the response. Accessory cells, such as endothelial cells and fibroblasts, may be useful constituents for the maintenance of the correct intercellular communications required by the immune response.

accessory cells can be pre-prepared as a monolayer (Figures 4 and 5), when their application in cocultures with lymphocytes can reduce programmed cell death to <20%.

Nevertheless, immune responses *in vitro* can be effected without accessory cells. The key to success is to ensure efficient interaction between the appropriate mononuclear cell subpopulations shown in Figure 1. How this can be achieved will be dealt with in the following sections.

Enrichment of cells for immune responses *in vitro*

Separation of mononuclear cell subpopulations has been successfully applied to increasing the efficacy of immune response systems *in vitro*. The simpler methods make use of the differential adherence of leukocyte populations. T lymphocytes can be separated by the preferential binding of B lymphocytes and monocytes to nylon wool, although some T lymphocytes may also display a degree of adherence.

Antigen-presenting cells (APCs) can be enriched from lymphocytes by their differential adherence to plastic or Sephadex G10 (there may be some retention of B lymphocytes by such resins). The antigen-presenting dendritic cells can be selected from other monocytic cells through their differences in both adherence and density.

These relatively simple methods are not absolute for separating leukocyte populations. In contrast, the T cell rosetting method can enrich to a higher degree, although not all animal species possess T lymphocytes capable of binding sheep erythrocytes, via the CD2 molecule on the lymphocyte surface. Particularly specific enrichment or removal of leukocyte populations employ monoclonal antibodies against markers on leukocytes, in association with panning or cell sorting. For panning, antibody specific for a certain marker is bound to a plastic surface or Sephadex column, to which cells carrying the marker become immobilized. With cell sorting, the cells are first labeled with the antibody, followed by a magnetic bead or fluorochrome-labeled anti-mouse

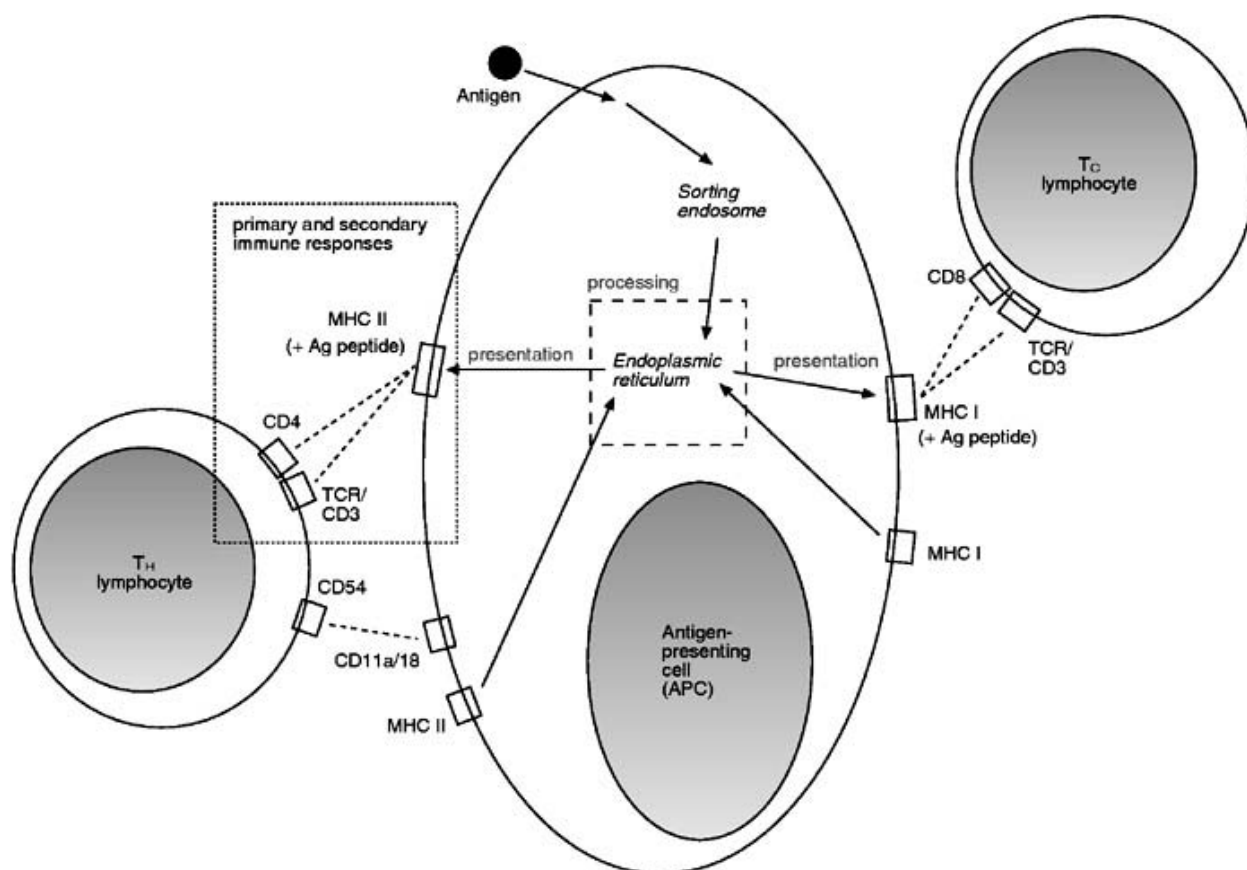


Figure 2 Antigen presentation by the APC to T_H and T_C lymphocytes, showing the relevance of MHC class I and class II expression, as well as adhesion molecule interactions, in these processes.

Table 2 Examples of cytokines which have been successfully employed for *in vitro* immune response systems, either individually or in combinations, such as in 'cytokine soups' from mitogen-activated mononuclear (lymphocytes plus monocytes) cultures

Cytokine	Common sources	Known influences with <i>in vitro</i> immune responses
IL-1	Activated monocytic cells, fibroblasts, endothelial cells	Cofactor for T lymphocytes and antigen-stimulated B lymphocytes
IL-2	Activated T _H lymphocytes	Differentiation factor for antigen-stimulated B lymphocytes; T lymphocyte growth factor
IL-4	Activated T _H lymphocytes	Cofactor for T lymphocytes; differentiation factor for B lymphocytes (important in isotype switching); enhances APC activity
IL-5	Activated T _H lymphocytes	Differentiation factor for B lymphocytes (important in isotype switching); cofactor with IL-2 for B lymphocytes
IL-6	Activated monocytic cells, T _H lymphocytes	Cofactor for B and T lymphocytes; differentiation factor for B lymphocytes (differentiation into plasma cells)
Interferon γ	Activated monocytic cells, T _H lymphocytes	Cofactor with IL-2 for antibody secretion by B lymphocytes; regulator of T lymphocyte growth

conjugate; separation from unlabeled cells employs a magnet or a fluorescence-activated electrical field. Examples of markers commonly used are CD14 on myeloid cells (not all dendritic cells are CD14⁺), surface immunoglobulin on B cells, and CD2, CD4 or

CD8 on T lymphocytes (the latter two will be useful for separating T lymphocyte subpopulations); the T lymphocyte CD3 is not employed, because antibody reaction with this marker can stimulate the cells. An additional modification to the procedure employs

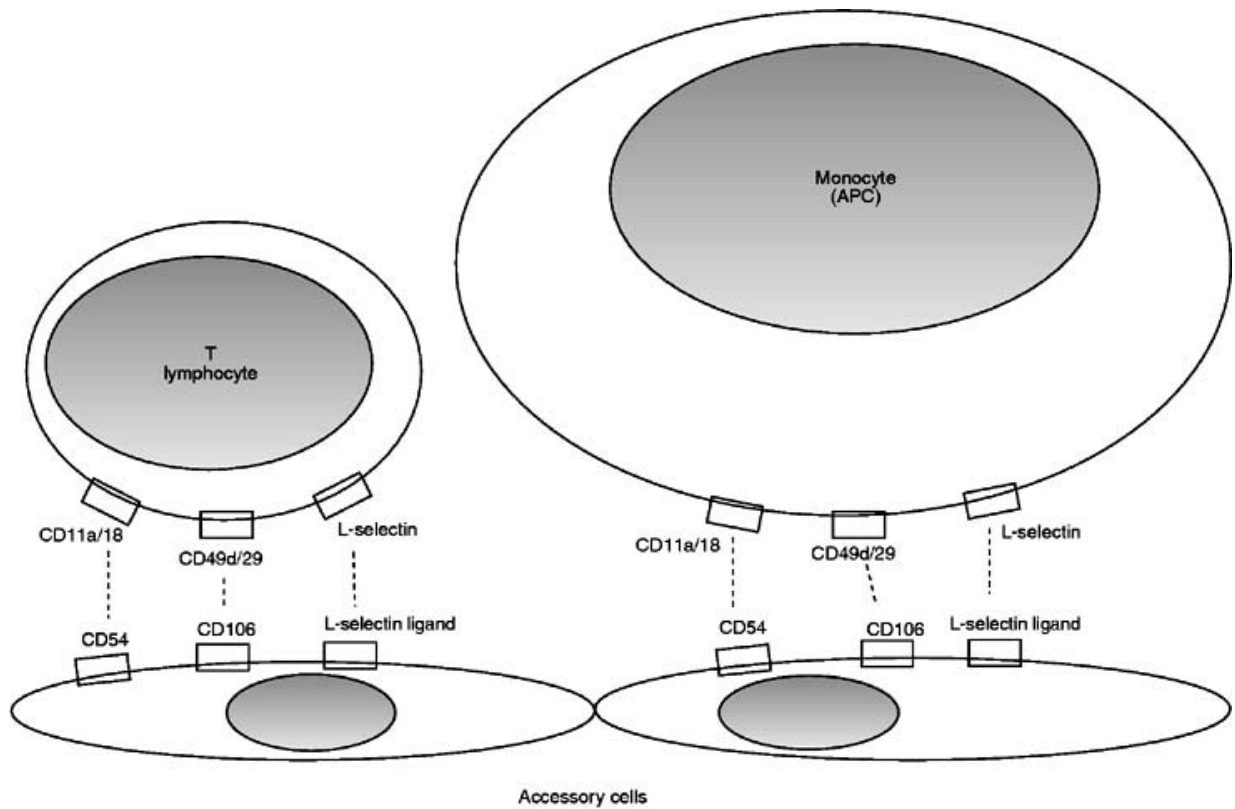


Figure 3 Accessory endothelial cell interaction with T lymphocytes and monocytic APC, via adhesion molecules and their ligands.

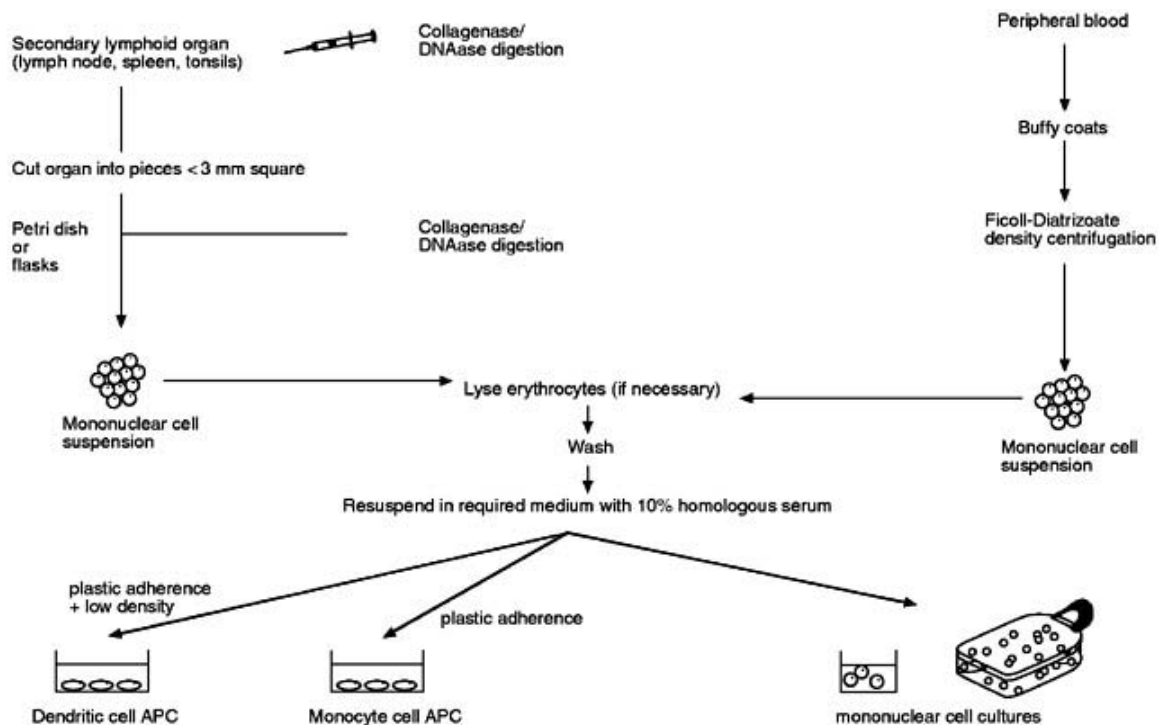


Figure 4 Summary of the methods employed for the preparation of mononuclear cells from lymphoid organs and the blood. These sources of mononuclear cells can be used for the preparation of APCs, or for the cultures to be stimulated by antigen in the immune response *in vitro*.

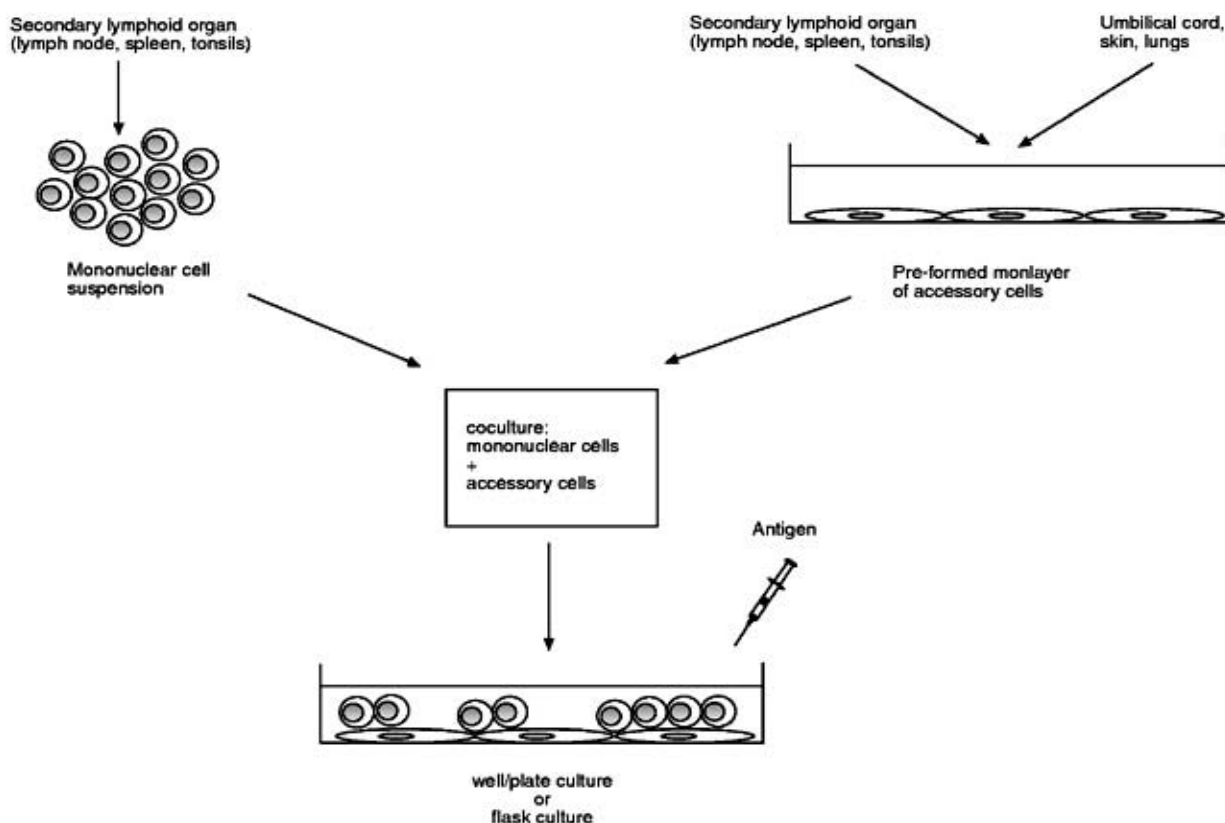


Figure 5 Summary of the preparation of preformed monolayers of accessory cells, and their application to coculture with mononuclear cells in immune response systems *in vitro*.

complement-mediated lysis to deplete unwanted cells: the antibody-labeled cells are treated with rabbit anti-mouse immunoglobulin and complement to induce the lysis.

A decision about whether or not to employ enrichment will depend on the required use of the *in vitro* immune response. Enrichment is not always essential. Its application should be carefully considered in the context of how beneficial it would be to the objectives of the experimentation.

Primary immune responses *in vitro*: general considerations

Mononuclear cell cultures derived from secondary lymphoid organs are preferable, in general, to blood cell cultures. Within the former, the cell populations required for an immune response are correctly represented, whereas blood mononuclear cells have a greater dominance of T lymphocytes, and an important population of suppressor cells. The latter can be impaired, unless they are the subject of interest, using agents such as leucyl methyl esters,

although not all species possess cells with equal sensitivity to such treatments.

The expected concentration of antigen-reactive lymphocytes within a primary immune response *in vitro* will be not more than $1 \text{ in } 10^6$. Considering the required cellular interactions, and that conventional cultures employ between 2×10^6 and 2×10^7 leukocytes ml^{-1} , this low concentration of potentially reactive cells does not lend itself to success. Enrichment procedures have been particularly useful in circumventing this problem. The potential also exists to apply hollow fiber or similar capillary culture vessels, wherein cell concentrations can be maintained at 10–1000 times that in conventional culture systems. Nevertheless, the methods which have found the widest application are the use of exogenous cytokines and the preprocessing of antigen. These techniques, which will be dealt with in the next section, have the advantage over enrichment procedures in that the balance of leukocyte populations within a mononuclear cell preparation has not been modified.

Initiation of the primary immune response *in vitro*

Cytokine application

With the immune response *in vitro*, B lymphocytes will be directly stimulated by antigen, whereas T_H lymphocytes must await antigen processing. This can result in stimulated B lymphocytes requiring T lymphocyte help before it is available. A relatively simple solution is to apply, at the moment of immunization, the cytokines required by the B lymphocyte differentiation pathway (Figure 6; Table 2), the stimulated T_H lymphocytes providing cytokines later in the response. Exogenous cytokines can be in two forms: recombinant cytokines (Table 2), or a 'cytokine soup' derived from a mixed leukocyte reaction (MLR) or mononuclear cell cultures stimulated with mitogen. Not all cytokines are available in recombinant form, nor for all animal species. Furthermore, cytokine soups should provide all the required factors, not just some of them. The drawback with the

cytokine soups is the variation between batches, due to variations in the responsiveness of different batches of mononuclear cells. Consequently, large batches of cytokine soups are normally prepared and pretested for their capacity to support an immune response.

Additional enhancement factors may be employed, such as β_2 -mercaptoethanol. This appears to function by interacting with serum components, which in turn have an influence on the B-T lymphocyte interaction. An important overall effect is enhancement of the generation of antibody-producing cells.

Antigen processing and presentation

An alternative approach would be to equilibrate the initiation of the B and T_H lymphocyte stimulations by preprocessing antigen. This uses cultures of APCs, MHC matched with the mononuclear cells to be employed. The APCs can be enriched (Figure 4) or irradiated mononuclear cells from secondary lymphoid organs without enrichment, the latter at high cell

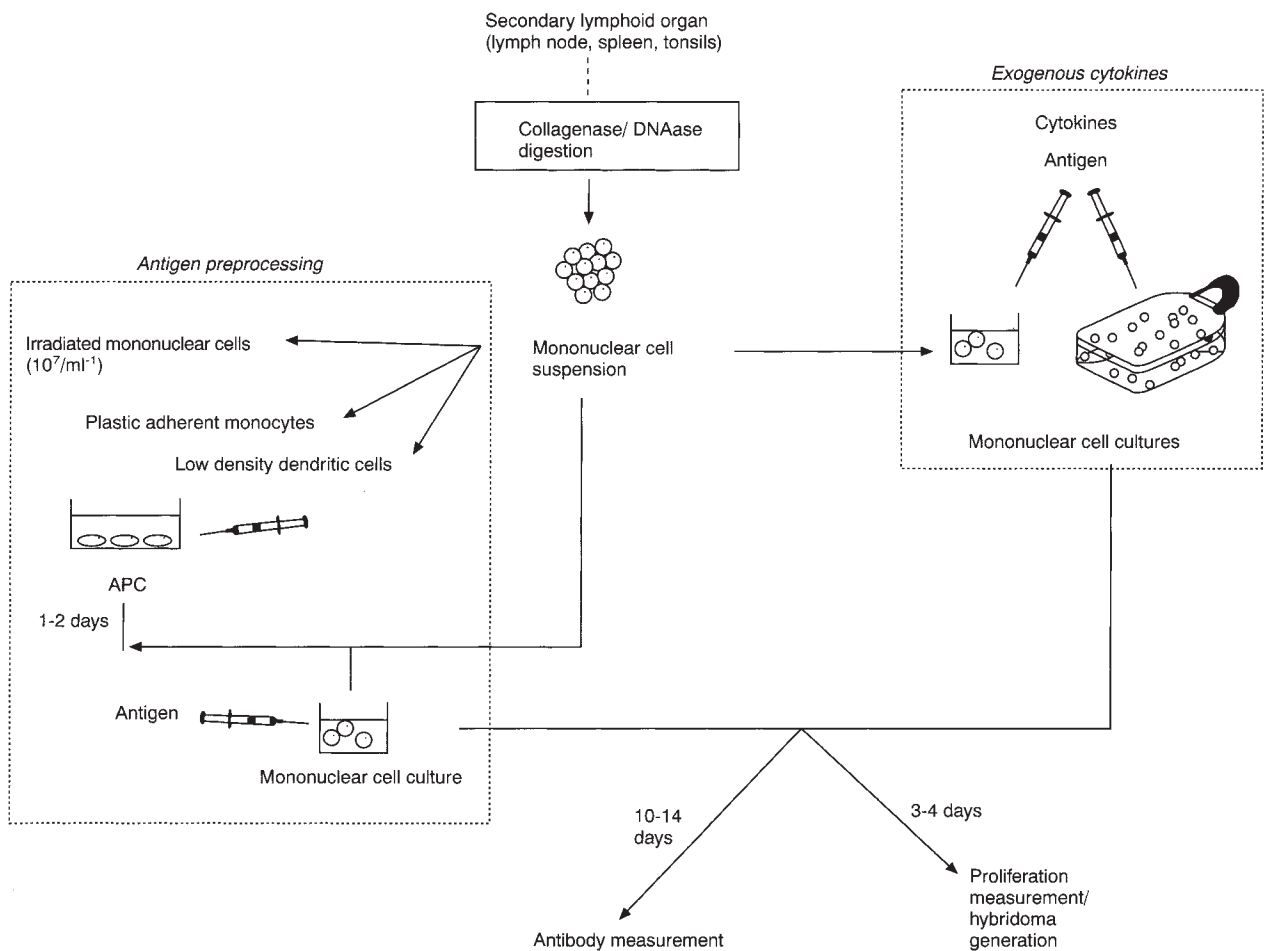


Figure 6 The primary immune response system *in vitro*, showing the application of exogenous cytokines and the use of antigen preprocessing. The source of the mononuclear cells is usually a secondary lymphoid organ.

concentrations (10^7 ml^{-1}) to ensure the availability of the APCs. Antigen is fed to the APCs, usually 24–48 h before addition of the mononuclear cells to be stimulated plus fresh antigen (Figure 6). The T lymphocytes will be stimulated by the processed antigen on the APCs, and B lymphocytes by the fresh antigen (Figure 6). It is also possible to mix enriched T lymphocytes with APCs at the time of antigen addition, followed after 3–6 days with freshly prepared B lymphocytes, enriched or not. With such methodologies, it may be necessary to ascertain that the presence of the T lymphocytes in culture before APCs have processed the antigen will not be detrimental to these lymphocytes. Both techniques involving antigen processing have the advantage that exogenous cytokines are no longer necessary. Measurement of primary immune responses *in vitro* most often employs lymphoblast proliferation, although antibody production by B lymphocytes may be detectable if the antigen is particularly potent (Figure 6).

Choice and use of antigen

Primary immune responses *in vitro* require antigen to be particularly immunogenic. Examples are hemocyanin, ovalbumin and sheep erythrocytes, all of which have proved useful for studying aspects of the immune response *in vitro*, as opposed to specific antigen-related topics. Other antigens, such as viruses and bacteria, often require the assistance of exogenous cytokines or antigen processing before a primary response *in vitro* can be induced (Figure 6). Such procedures have found favor with *in vitro* primary immunization towards lymphoblast production for hybridoma generation. The preprocessing of antigen enhances lymphoblast differentiation and survival, while exogenous cytokines influence the isotype of subsequently produced antibody. It is currently considered that the employment of both of these events would be beneficial to the generation of antigen-specific hybridomas from *in vitro* immune responses.

Secondary immune responses *in vitro*: general considerations

Both secondary lymphoid organs and blood can be sources of mononuclear cells for secondary immune responses (Figure 7). The suppressor cell problem within blood mononuclear cells is less apparent, or critical, with secondary responses, and may not require prior inhibition. Nevertheless, lymphoid organ cells have been the more widely applied in secondary immune responses *in vitro*.

Not all problems encountered with the primary response *in vitro* are found with secondary response

Table 3 Advantages of employing secondary compared to primary immune responses

Increase in relative concentration of antigen-specific lymphocytes
More rapid induction of T_H lymphocyte proliferation
More rapid induction of T_H lymphocyte cytokine production
Enhanced efficiency of T_H lymphocytes in delivering their immunological help
Reduced obligation for preprocessing of antigen
Reduced obligation for exogenous cytokine addition
Requirements for 'enhancement factors' less likely
Absence of accessory cell function less of a problem
<i>In vitro</i> immune response system can be simple – mononuclear cells plus antigen
Increased manipulability of the immune response system, facilitating studies on accessory cell and cytokine involvement in the immune response

systems. Secondary response systems have a number of advantages (Table 3) relating to memory cell development, wherein memory antigen-specific lymphocytes can be of the order 1 in 10^3 to 1 in 10^4 within a lymphocyte preparation. Nevertheless, the cell concentration – $1\text{--}4 \times 10^6$ lymphocytes ml^{-1} – in the secondary immune response *in vitro*, and thus programmed cell death, are still potential problems. Accessory cells can assist in this direction (Figures 1, 3 and 5), but the secondary response can be effected in their absence. In contrast, application of accessory cells to general analyses of the secondary response can be informative, especially in the area of intercellular communication. Enrichment of cell subpopulations can also be applied to secondary immune responses, often but not always to study the roles played by the enriched cells (Figure 7). However, secondary immune responses *in vivo* do not require such manipulations.

Initiation of the secondary immune response *in vitro*

Cytokine application

Neither exogenous cytokines nor preprocessed antigen are obligatory for secondary immune responses *in vitro* (Figure 7). When applied, it is usually to study their influence on the immune response. These characteristics relate to the more efficient capacity of memory T lymphocytes to respond after antigenic stimulation, produce cytokines, and interact with B lymphocytes. The response is further assisted by the B lymphocytes presenting antigen to memory T lymphocytes.

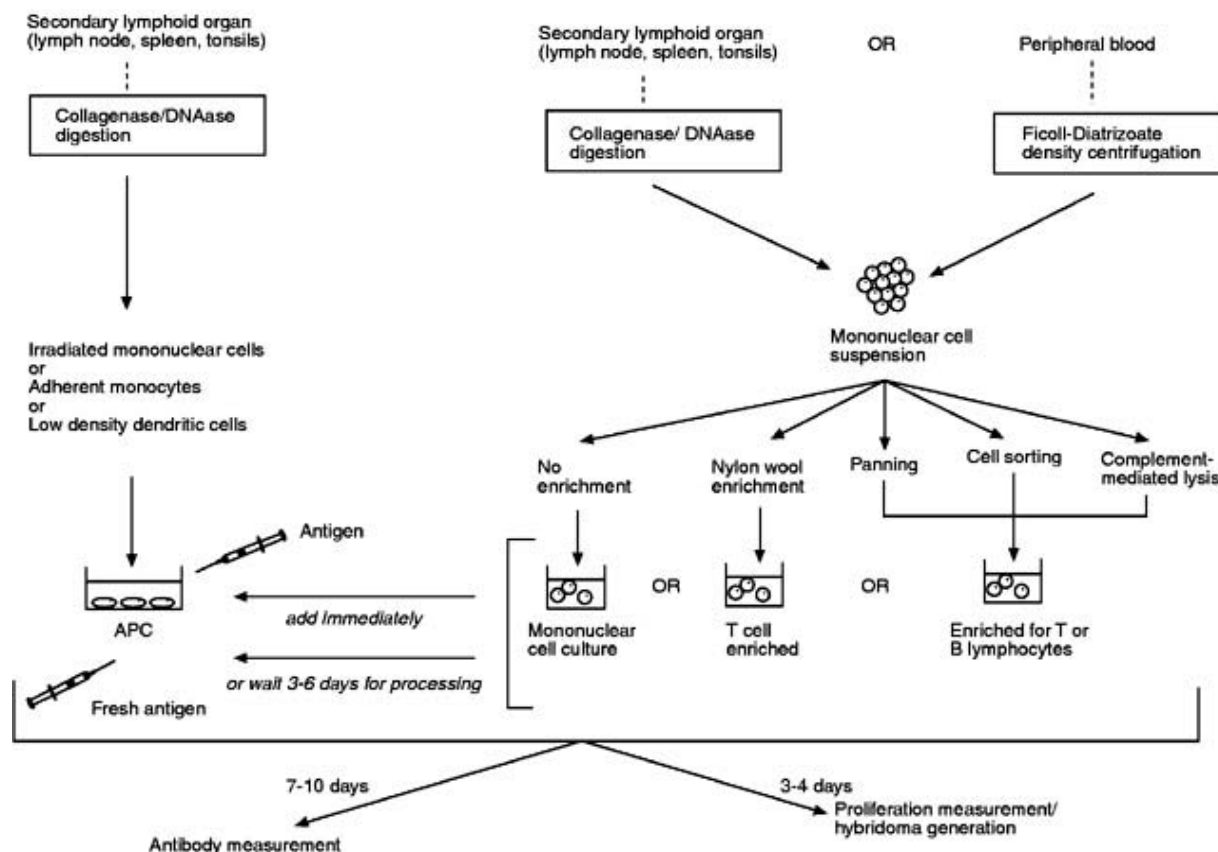


Figure 7 The secondary immune response system *in vitro*, showing that antigen preprocessing by APCs may be employed, but is not essential. The mononuclear cell preparations can be from lymphoid organs or blood, and enrichment of particular populations may also be employed.

Antigen processing and presentation

Despite the more efficient capacity of lymphocytes to respond during a secondary immune response *in vitro*, preactivation of T lymphocytes is a commonly employed procedure. Cultures may simply be mononuclear cells, or alternatively enriched populations. With enrichment, a source of APCs must be ensured: irradiated mononuclear cells or enriched dendritic cells can be employed (Figure 7), although the capacity of memory B lymphocytes to function as APCs should also be considered. In experiments employing enrichment, antigen is often applied 3–6 days before B lymphocytes are added (Figure 7); fresh antigen may be unnecessary, dependent on the antigen and characteristics of the memory lymphocytes in the culture. When nonenriched mononuclear cell cultures are employed, antigen is added at the initiation of the experiment (Figure 7). Measurement of secondary immune responses *in vitro* often employs T lymphocyte proliferation or antibody production by activated B lymphocytes (Figure 7).

Choice and use of antigen

Secondary immune responses *in vitro* have been applied to studies employing antigens of, for example, viruses, bacteria and peptides of known sequence or structure. The applicability of the procedures is evident from the studies on peptide antigens, which have been applied to analyses of B lymphocyte and, in particular, T lymphocyte responses. Not all peptide antigens are equally immunogenic. Certain may require costimulation with interleukin 2 (IL-2), followed by restimulation with the peptide antigen before lymphocyte proliferation is detectable. Similar restrictions may apply to certain more complex antigens, but will depend on both the antigen and the capacity of the constituent mononuclear cells in an *in vitro* culture to respond.

Additional applications particular to secondary immune responses *in vitro*

Secondary immune responses *in vitro* have also been applied to studies on intercellular communications.

Cultures of mononuclear cells have been employed for analyses of lymphocyte/monocyte and lymphocyte/lymphocyte interactions. Cocultures of mononuclear and endothelial cells are often used to study the role of the endothelial cells. Both types of culture, as well as those with enriched subpopulations or cell lines, have assisted in the identification of adhesion molecule and cytokine activity. Cultures employing enriched subpopulations have been particularly useful in identifying the roles of these subpopulations in the immune response, especially with respect to intercellular communications and interactions.

Concluding remarks

The potential to effect immune responses *in vitro* has advanced rapidly over the past 25 years. Analyses have demonstrated the complexity of the immune response, studies having taken two main forms: initial *in vitro* experimentation, from which subsequent *in vivo* analyses were designed; observations from *in vivo* experimentation studied further by *in vitro* means, in a detail not otherwise possible. The *in vitro* immune response systems have permitted a controlled analysis of the compartments and factors involved *in vivo*. Application of *in vitro* systems has also permitted a reduction in the usage of animals. The areas which have profited most from immune responses *in vitro* have been the generation of hybridoma and T lymphocyte cell lines, and the studies on immune responses *per se*. These latter studies have increased and will continue to increase the level of understanding of the immune system and the constituent responses.

See also: Adhesion molecules; Antigen-presenting cells; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Apoptosis; B lymphocyte activation; B lymphocyte differentiation; CD antigens; Helper T lymphocytes; Cell separation techniques; Cytokines; Dendritic cells; Germinal center; Immune response; Interferon γ ; Interleukin 1 and its receptors; Interleukin 2; Interleukin 4; Interleukin 5 and its receptor; Interleukin 6; Interleukin 10 and its receptor; Leukocyte culture; Lymph nodes; Rosetting techniques; T cell receptor, recognition by; T lymphocyte activation; T lymphocytes.

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IMMUNE SURVEILLANCE

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Evolution has supplied us with an immune system capable of dealing with pathogenic microorganisms. Neoplasms may be recognized by the immune system either because the molecular mechanisms evolved for recognition and elimination of pathogens are also coincidentally effective in the elimination of tumor cells or, because a primary function of the immune system is to survey the body for arising neoplasms. The beginning of this immune surveillance theory dates from 1957 when Burnet wrote: 'It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence'.

The immune surveillance theory states that the immune system is constantly monitoring the body for the appearance of tumor cells, and that the majority of these aberrant cells are destroyed by the immune system before giving rise to clinically manifest tumors. If the concept of immune surveillance is correct and fully applicable, all tumor cells which arise will be immunogenic and the immune reaction induced by these cells prevents the outgrowth of a tumor. The presence of clinically detectable tumors in immunocompetent individuals, however, indicates that mechanisms have evolved to escape elimination. Examples of escape mechanisms are: antigenic modulation (e.g. HLA downregulation), antigen masking, production of soluble tumor antigens or other tumor products (e.g. transforming growth factor β , TGF β), induction of tolerance and weakening of the defense system itself.

History of the immune surveillance theory

At the end of the nineteenth century, tumor biologists started to study allogeneic tumor growth in animals upon transplantation. Observations in 1902

by Jensen and Ehrlich on tumor rejection tempted Ehrlich to suggest a role for the immune system in the control of tumor growth. However, attention for this idea soon faded as it became known that transplant rejection or acceptance was merely a consequence of genotypic variation between host and donor rather than of a specific antitumor response. The interest in tumor immunology revived around 1950 with the introduction of inbred animal strains creating a tool to study tumor rejection in genetically identical animals. From then on proof accumulated for a role of the immune system in the eradication of tumors. In 1959, stimulated by the ideas of Thomas and others, Burnet postulated the immune surveillance theory.

At present, malignant tumors are believed to arise as a result of a series of specific genetic events which include the activation of oncogenes and the loss of function of tumor suppressor genes. A large array of control mechanisms are operative within a cell to prevent abnormal growth (e.g. DNA repair mechanisms, tumor suppressor genes and apoptosis of irreversibly damaged cells). If these control mechanisms are no longer operative, cells continue to proliferate. Subsequently, a dynamic interaction of the malignant phenotype with its microenvironment (including the immune system) occurs, which may result in tumor progression. Thus, in addition to the genetic properties of aberrant cells, the microenvironment will also determine survival or death of malignant cells (Figure 1).

It is assumed in the immune surveillance theory that immunocompetent individuals eradicate tumor cells before tumors become clinically detectable. Therefore, it is difficult to obtain indisputable evidence regarding the validity of the immune surveillance theory. One should always be aware that the arguments either in favor or opposing the theory (Table 1) are extrapolated from observations of established tumors.

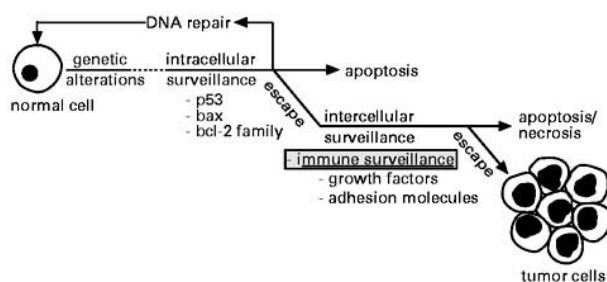


Figure 1 Schematic representation of the development of a normal cell into a tumor cell.

Experimental and clinical observations supporting the immune surveillance theory

Primary immunodeficiency

In all species cancer is mainly associated with old age, being from about 2 years in mice to about 70 years in humans. Older mice have been found to be less resistant to xenogeneic, allogeneic and syngeneic tumor cells than younger mice. These observations may indicate involvement of the immune system in

prevention of tumor development, as the immune system becomes less effective with increasing age. Immunodeficient animals show a higher frequency of virus-induced and chemically-induced tumors.

Natural killer (NK) cells, part of the first line of defense as they are able to kill cells without prior sensitization, are presumed to play an important role in immune surveillance. Mice with genetically determined low levels of NK cells, beige mice, display an increased incidence of spontaneous tumors and a lower natural resistance to syngeneic leukemias. Furthermore, nude mice which have no functional T cells but normal NK cells do not show an increased incidence of tumors in general, but only of virus-induced tumors, indicating a role for T cells in immune surveillance of the latter tumor types and for NK cells in the other types of tumors.

In agreement with the observations in immunodeficient animals, in immunodeficient patients a higher cancer incidence is also observed. Humoral deficiencies, such as X-linked agammaglobulinemia and common variable immune deficiency, and cellular immunodeficiency syndromes, such as Wiskott-Aldrich syndrome, ataxia telangiectasia, the X-linked

Table 1 Clinical observations with explanations related to the immune surveillance theory

Observation	Explanation	
	In favor	Opposing
Tumors are clinically present	Tumor cells escape from immunological control	Tumor cells are not immunogenic
Increased tumor incidence in immune deficiency, especially non-Hodgkin lymphomas and squamous cell carcinomas	Impaired immunological control Accumulation of multiple hits in common tumors is a slower process as compared to tumor induction in virus-associated tumors	The presence of malignancies is due to (genetic) dysregulation of the cells, carcinogenic properties of immunosuppressive drugs, transformation of cells due to viruses, or predisposition of cells due to increased incidence of viral infection
Spontaneous regression of tumors	Elimination of tumor cells by the immune system	Destruction of tumor cells due to other than immunological causes (e.g. insufficient blood supply)
Regression of non-Hodgkin lymphoma after drastically decreasing immunosuppressive therapy	Recovery of a functional immune system resulting in elimination of tumor cells	Immunologic destruction due to the presence of EBV in the majority of these tumors
Increased tumor incidence with age	Decreased T cell function (coinciding with involution of the thymus)	Carcinogenesis requires multiple genetic hits which accumulate with age
Long latency in the appearance of secondary tumors	Struggle of the immune system with tumor cells	Latency due to environmental factors or necessity for additional genetic hits
Immune cells are associated with tumors	Tumor cells induce an immune response	Dead tumor cells evoke a chronic inflammatory response
Tumor regression after immunotherapy	Tumor cells induce an immune reaction	Tumor cells occasionally induce an antitumor reaction, depending on the antigens locally presented

lymphoproliferative syndrome, and the Chédiak-Higashi syndrome, are associated with an increased chance of developing cancer, especially non-Hodgkin lymphoma.

Secondary immunodeficiency

Among the acquired immunodeficiencies, information regarding immune surveillance can be obtained from those instances where the immune system is depressed either due to drugs (azathioprine, corticosteroids or anti-T cell antibodies (OKT3)) used in tissue transplantation, or due to infection with microorganisms (e.g. human immunodeficiency virus (HIV) or malaria). An increased incidence in malignancies has been reported in kidney, liver and heart transplant patients. Most data are available from kidney transplant patients. In these patients generally skin cancers (squamous cell carcinomas) and non-Hodgkin lymphoma have been reported to be increased. Interestingly, if malignancies develop in patients with tissue transplants, a drastic reduction in the administered dose of the immunosuppressive drug results in a clinical remission in approximately 25% of the cases, suggesting that recovery of immune function results in eradication of tumor cells.

In immunosuppressed patients due to malaria an increased incidence of Burkitt's lymphoma has been observed. In patients with the acquired immune deficiency syndrome (AIDS), primarily Kaposi's sarcoma and non-Hodgkin lymphoma are present, whereas among women with AIDS an increase in cervical carcinoma is noted.

Spontaneous regression of tumors

The clinical observation of spontaneous regression of tumors also supports the immune surveillance theory. An inverse relationship between the prevalence of the tumor type and the probability of spontaneous regression is observed. Of all the cases reported, the majority comprise malignant melanoma, renal cell carcinoma, lymphoma, leukemia, neuroblastoma and retinoblastoma. The reports on spontaneous regression also include the observation that metastases might disappear after resection of the primary tumor, a phenomenon called concomitant immunity. Remissions have been associated primarily with infection, surgery or trauma. These impairments may have (re)activated the immune system, resulting in tumor regression.

Also the observation that metastases reappear after long latent periods, e.g. patients with breast cancer, malignant melanoma, neuroblastoma and leukemia, may support the immune surveillance theory. The latency in tumor growth observed can

be explained as a struggle between the immune system and tumor cells.

Tumor-infiltrating cells

Microscopical examination of tumor biopsies shows that tumors are surrounded or infiltrated by immune cells. Generally, T cells and macrophages are abundantly present in human tumors, suggesting an immune response directed against the tumor cells. Indeed T cells directed against autologous tumor cells have been isolated from several tumor types, e.g. malignant melanoma, renal carcinoma and cervical carcinoma. These T cells either recognize peptides presented in the context of HLA class I from genes which are silent in most other cell types, like MAGE in malignant melanoma, overexpressed proteins (e.g. p53), or peptides derived from mutated (e.g. ras) or truncated proteins. Also, antibodies directed against tumor-associated antigens can be demonstrated in cancer patients. Thus, there is strong evidence that tumor cells can induce an immune response *in vivo*.

Immunotherapy of cancer

Infusion of cytokines, autologous *in vitro* IL-2 stimulated lymphocytes obtained from the peripheral blood of cancer patients, or tumor-infiltrating lymphocytes occasionally results in a complete remission, notably in patients with neuroblastoma, renal cell carcinoma, or malignant melanoma. This observation suggests that an immune response can be elicited against these tumor types.

In summary, as stated by Burnet, it is conceivable that small accumulations of tumor cells develop and that aberrant tumor cells evoke an immune response resulting in the eradication of these cells. However, it is at present neither clear whether *all* tumor cells are able to evoke an immune response nor whether the presence of established tumors represents escape from immunological control.

Alternative explanations for the phenomena assigned to the immune surveillance theory

Those being skeptical with regard to the immune surveillance theory refer to the alternative explanations for phenomena such as increased tumor incidence in immunodeficiency, spontaneous regression of tumors, and the association of tumor cells with cells from the immune system.

Tumor incidence associated with immunodeficiency

Alternative explanations regarding the increased

tumor incidence in immunodeficiency are also entirely plausible. The increased tumor incidence in immunodeficient renal transplant patients is primarily due to an increase in skin cancer (squamous cell carcinoma) and non-Hodgkin lymphoma (the majority being of B cell origin), whereas a substantial increase in the more common cancer types, such as breast cancer, cancer involving the respiratory system, and gastrointestinal cancer, is not observed in these patients. Non-Hodgkin lymphoma and skin cancer in immunodeficiency are associated with a viral etiology (Epstein-Barr virus (EBV) and human papillomavirus (HPV), respectively). It is therefore conceivable either that these viruses are carcinogenic themselves or that the increased frequency of viral infection acts as a cofactor, making the infected cell vulnerable to becoming a tumor cell. With regard to immunosuppression, it should be noted that only parts of the immune system are immunosuppressed (generally the T cell compartment). Similarly, the increased incidence of non-Hodgkin lymphoma in patients with congenital immune deficiencies might be due to an abnormal proliferative response (regulation) of the immune cells, due to genetic aberrations in these patients.

Immune suppressive drugs may also cause the anomalies observed. Patients treated for longer periods with cyclosporine or azathioprine were shown to have chromosomal abnormalities. Immunosuppressive drugs may also have synergistic effects to other carcinogens. Metabolites of azathioprine cause chemical photosensitization. A synergistic effect of azathioprine with ultraviolet light or HPV may explain the high incidence of skin cancer in allograft recipients.

Spontaneous regression of tumors and latency

Because of a lack of knowledge regarding the etiology of spontaneous remissions, such remissions could be associated with a variety of causes (e.g. vascular insufficiency, tumor differentiation or psychosomatic mechanisms) in addition to immunological phenomena. The presence of long latency periods before metastases appear may equally well be due to environmental factors (e.g. lack of adhesion molecules or growth factors), the absence of additional genetic 'hits' required for tumor progression, as to immune mechanisms.

Tumor-infiltrating cells

The observation of an infiltrate of immune cells surrounding tumor cells might be due to a chronic inflammatory response initiated and sustained by cell death. During this process, only occasionally (depending on the proteins expressed by the tumor

cells) does an antitumor response occur. This occasional immunogenicity of the tumor cells may also explain the limited successes of immunotherapy of cancer.

In conclusion, inherent with the nature of the theory that neoplasms are eradicated before becoming clinically evident, formal proof either in favor or against the immune surveillance theory is difficult to obtain. It is proven beyond doubt that the immune system is able to mount an immune response against particular tumor cells. However, one can still argue whether all tumor cells which arise are immunogenic, and if an immune response occurs each time tumor cells arise.

See also: Immune surveillance; Immunodeficiency, primary; Immunodeficiency, secondary; Immunosuppression; Immunotherapy of tumors; Tumor antigens; Tumors, immune response to; Tumors, immunological escape of.

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IMMUNE SYSTEM, ANATOMY OF

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Cells participating in the immune response are organized into discrete lymphoid tissues and organs and spread through the connective tissues of non-lymphoid organs. The cell type responsible for the specificity of the immune response is the lymphocyte. Approximately 2×10^{12} lymphocytes constitute the mature lymphoid system in humans together with a variety of 'accessory' cells which include epithelial cells, monocyte/macrophages and other antigen-presenting cells. Accessory cells are required both for the maturation and for the effector functions of lymphocytes.

Lymphocytes are derived from hematopoietic progenitors which mature within the bone marrow (and the fetal liver) giving rise to B lymphocytes, or seed to the thymus where they differentiate into T lymphocytes. The bone marrow and the thymus are therefore the primary or central lymphoid organs. Mature lymphocytes leave the central organs via the blood vessels, and migrate to secondary or peripheral tissues and organs where they exert their effector functions in response to antigen. Peripheral lymphoid tissues include the spleen and lymph nodes (Figure 1) and MALT (mucosa-associated lymphoid tissue) which is associated with the respiratory, genitourinary and gastrointestinal tracts (Figure 2), accounting for approximately 50% of the total lymphoid cells in the body.

Central lymphoid organs

The bone marrow, and earlier in fetal life, the liver, and the thymus provide the microenvironments for the differentiation of B and T lymphocytes respectively, and for the production of mature cells responsible for humoral or cellular immune responses. During B and T cell maturation, similar events occur in the bone marrow and in the thymus, namely the rearrangement of the genes encoding antigen receptors (immunoglobulin (Ig) or T cell receptors (TCRs)) and the expression of these receptors on the

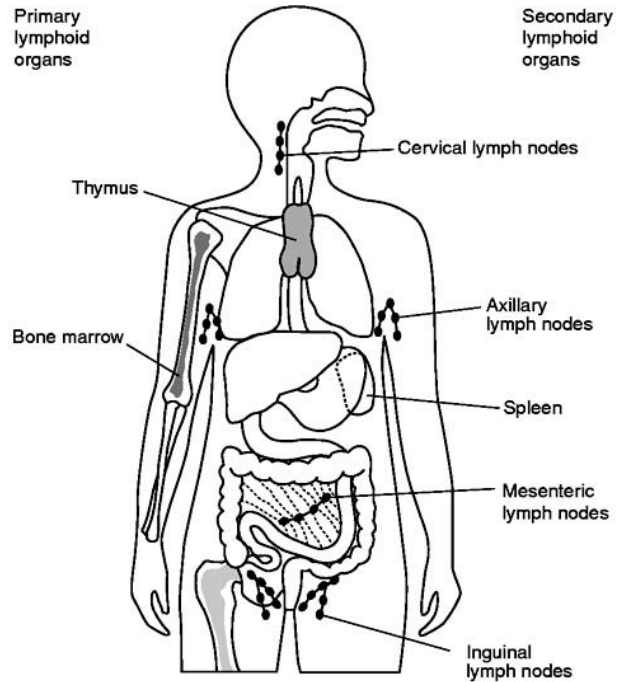


Figure 1 Major lymphoid organs and tissues.

cell surface. A variety of other cell surface 'marker' molecules appear in an orderly sequence which defines the maturational stages of both B and T cells.

The bone marrow provides the microenvironment for B lymphocyte differentiation. This process takes place through the direct interaction of B cell progenitors with ill-defined 'stroma' cells and with the help of soluble factors, notably interleukin 7 (IL-7), which supports the growth of pre-B cells and possibly of their progenitors.

The thymus provides the microenvironment for differentiating bone marrow-derived precursors seeding into the epithelial rudiment during ontogeny. The complete sequence of maturational events leading to the production of mature T cells takes place within this organ. Maturing T cells interact with nonlymphoid thymic cells, namely epithelial cells and

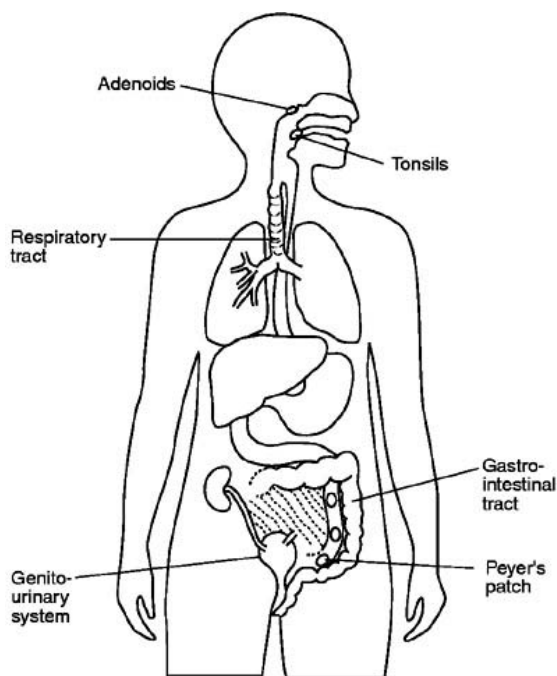


Figure 2 The mucosa-associated lymphoid system.

macrophages/interdigitating cells. Intrathymic T cell maturation results from these cellular interactions and from the resulting cytokines (IL-1, IL-2, IL-6) produced by them. The major events that occur in the thymus are proliferation and the positive and negative selection of T cells. Thymocyte proliferation leads to polyclonal expansion of T cells and is the result of a direct interaction between thymocytes and epithelial cells. Positive selection favors the expansion of those cells equipped with TCRs that have proven able to bind self MHC molecules weakly, even in the absence of antigen. Those developing T cells which display the best fit between their TCRs and class I and class II MHC molecules expressed by nonlymphoid thymic epithelial cells have a growth advantage. Negative selection implies the elimination of self-reactive T cell clones upon encounter with self antigens during intrathymic maturation. It is generally believed that negative selection takes place following interaction of the more mature thymocytes with interdigitating cells present in the thymic medulla.

Mature B and T cells leave the central organs and migrate to the peripheral lymphoid tissues.

Peripheral lymphoid organs

The effector functions of B or T cells responsible for humoral or cell-mediated immune responses are carried out in the lymphoid periphery which consists of discrete organs or foci of lymphoid tissue, and of

lymphocytes dispersed in the interstitial areas of non-lymphoid organs (e.g. liver, lung and the lamina propria of mucosae). The peripheral lymphoid tissues possess a vascular and cellular organization which favors the encounter the antigens and antigen-presenting cells with mature B and T lymphocytes. The frequency of appropriate encounters between antigens and cells bearing specific antigen receptors is increased by the active recirculation of lymphocytes via blood and lymph vessels. Thus, the structural organization of peripheral lymphoid tissues is dynamic, with actively renewing mature cell populations supported by the thymus and bone marrow.

In addition to the lymphocytes which constitute the major cell component of the lymphoid tissues, monocyte/macrophages and other antigen-presenting cells of the same lineage (such as dendritic cells, interdigitating cells, veiled cells, etc.) play an essential role in the initiation and development of the immune response. Many of these 'accessory' cells are also able to migrate from skin and mucosae to draining lymph nodes in order to accomplish their function as antigen 'carriers' and 'presenters'.

Mature B and T lymphocytes migrate from the central organs into distinct areas of the peripheral lymphoid tissues, namely the B-dependent and T-dependent zones. Encounter with antigen initiates proliferation, activation and differentiation events leading to the generation of effector cells and memory cells. The effector cells for humoral immune responses are the antibody-secreting plasma cells. T helper and T cytotoxic cells are the effector cells for cell-mediated immune responses. Effector and memory cells are found in distinct areas within the peripheral lymphoid tissues. For example, plasma cells are present in the spleen red pulp and in the lymph node medullary cords, whereas memory B cells are located in the germinal centres of the secondary lymphoid follicles in splenic white pulp, lymph node cortex and MALT.

Antigens can penetrate the natural barriers provided by the skin and mucosal surfaces, or they can enter the blood circulation directly. To ensure appropriate protection, peripheral lymphoid organs (lymph nodes) are strategically located along the lymphatic vessels draining the skin and mucosal areas of the body and defend the external and internal body surfaces. The spleen combats blood-borne antigens. Antibodies produced in the spleen and lymph nodes are usually detectable in the serum. The type of protection which is provided by these organs is usually referred to as systemic immunity.

The mucosa-associated lymphoid tissue forms a functionally distinct line of defense (see **Figure 2**). The MALT consists of aggregates or foci of

lymphoid tissue which are found in the lamina propria and in the submucosa of several mucosal membranes of the digestive, respiratory, urinary and genital tracts. The size of these lymphoid aggregates varies from that of microscopically detectable solitary lymphoid nodules to larger accumulations such as those of the tonsils, Peyer's patches and appendix. The same compartmentalized B and T cell-dependent zones described for the spleen and lymph nodes occur in the MALT. However, one notable feature of the latter is the close association of the lymphoid tissue with the mucosal epithelium. Epithelial cells overlaying the lymphoid aggregates can take up antigens present in the lumen (or adhering to the mucosal surface) and transport them to the underlying lymphocytes. Furthermore, epithelial cells play an active role in the transport and secretion of locally produced IgA antibodies into the lumen. In fact, antibodies produced at the mucosal level are predominantly of the secretory kind.

Another important feature of the MALT is the frequent presence of lymphocytes within the epithelium (intraepithelial lymphocytes, IELs). Most IELs are of T lineage and some of them express $\gamma\delta$ TCRs. It is possible that these cells provide a line of defense at the epithelial level through cell-mediated immune responses. Therefore, it appears that the MALT represents a system which is quite distinct from that responsible for the systemic immunity and which accounts for mucosal immunity.

The distinction between systemic and mucosal immunity is further reinforced when certain features of lymphocyte trafficking are considered in the two systems. Lymphocytes circulate via blood and lymph vessels which provide the major routes for their seeding from central to peripheral organs and for their migration amongst the peripheral lymphoid organs, respectively. Circulation and homing of lymphocytes is determined and regulated by their ability to interact with endothelial cells. This interaction occurs primarily with the endothelium of high endothelial venules (HEVs), a special type of postcapillary venules. These vessels are found mainly in the T-dependent areas of the peripheral lymphoid tissues, but also in other tissues including the MALT and thymus. Endothelial cells may be activated and alter their shape, structure and receptor expression to become high endothelial cells in sites of inflammation

such as the synovium in patients with rheumatoid arthritis. A number of molecules are expressed by activated endothelial cells and include members of the immunoglobulin superfamily, e.g. ICAM-1 (CD54) and VCAM-1 (CD106), and selectin family, e.g. E-selectin (ELAM: CD62E) and P-selectin (CD62P). Lymphocytes bind to endothelial cells through surface molecules collectively grouped in the CD44 antigen family (previously designated as Hermes, Pgp-1, gp80–95). Integrins expressed by lymphocytes (such as LFA-1) are also involved. Interactions between lymphocyte CD44 or integrins and adhesion molecules on endothelial cells trigger the subsequent events that regulate the migration of lymphocytes between adjacent endothelial cells.

There appears to be some degree of selectivity in the interaction between lymphocytes and endothelial cells. Adhesion molecules expressed by some lymphocytes ($\alpha_4\beta_7$ -integrins) will be recognized and interact with 'addressins' (e.g. MAdCAM-1) expressed by the HEV endothelial cells in Peyer's patches. MAdCAM-1 is not expressed by endothelial cells in spleen and lymph nodes. It therefore follows that migration of lymphocytes from the circulation into the peripheral lymphoid tissues is not just random but is specifically directed. In support of this notion it has been shown that B lymphocytes primed in the intestine can migrate to other regions of the MALT. These cells can differentiate into plasma cells and produce antibodies detectable in the saliva or in the milk produced by the lactating mammary gland. Thus, the selective trafficking of lymphocytes appears to provide additional support for the distinction between a systemic and a mucosa-associated immune system.

See also: Antigen-presenting cells; B lymphocyte differentiation; Bone marrow and hematopoiesis; High endothelial venules; Lymph nodes; Lymphocyte trafficking; Lymphocytes; Mucosa-associated lymphoid tissue (MALT); Spleen; Thymus; T lymphocyte differentiation.

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IMMUNOADSORBENTS

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Immunoabsorbents are specific antibodies attached chemically to solid supports. The antigen of interest contained in a crude mixture is bound noncovalently to the immunoabsorbent, after which it can either be discarded (when unwanted materials must be removed) or isolated after treatment of the sorbent–ligand complex with a denaturing agent. Properties of immunoabsorbents depend critically on the methods used to prepare them. Both soluble antigens and intact cells can be bound and suitable sorbents are commonly employed in research and clinical treatment.

Solid supports

Antibodies can be immobilized simply by treating them with a cross-linking agent such as glutaraldehyde, but the resulting agglutinated material is not homogeneous and many of the reactive sites are either damaged or hidden. A more efficient method employs a solid support to which antibodies are chemically attached. Glass, silica, plastic and cellulose have been used but the most generally satisfactory support material is agarose, a polysaccharide prepared from uncharged fractions of agar. Agarose has also been used to encapsulate immunoabsorbents made from other materials as it is highly biocompatible. It is supplied in the form of hydrated porous spheres 40–300 μm in diameter containing 2–8% agarose by weight in aqueous solution. Agarose is resistant to degradation by enzymes from most bacterial strains and by chemical agents, and after internal cross-linking by treatment with epichlorohydrin or similar agents it can be autoclaved in 1 N

sodium hydroxide allowing for both sterilization and depyrogenization. Agarose can be hydrolyzed to simple sugars by overnight incubation in 3 N HCl at 80°C but is resistant to 1 N acids at ambient temperature.

Immunoabsorbents are traditionally constructed from high resolution small agarose beads approximately 100 μm in diameter, such as Sepharose 4B (Pharmacia-LKB). However, for use in immunoabsorbents larger beads of 212–300 μm work equally well and have much better flow properties, being very resistant to plugging and useful even with undiluted plasma, whole blood or dispersed cells. The sorbent capacity is a function of the percent agarose content and is highest between 2 and 6% agarose; some ligands may experience greatly increased binding on immunoabsorbent beads of 2% agarose.

Coupling antibody to agarose

Sorbent beads must be activated in order to bind antibody molecules. Generally the agarose is first reacted with a bifunctional cross-linking reagent yielding an agarose linker with one remaining functional group. This activated agarose is then added to a solution of the antibody to be coupled. Table 1 summarizes the results obtained with various coupling chemistries applied to one antigen–antibody system. While all methods tested give good coupling of antibody to agarose, there are large differences in the ability of the immunoabsorbents to bind antigen. The highest activity was achieved with the cyanogen bromide technique, a finding noted with other antigen–antibody systems which has caused it to remain popular despite the toxicity of its reagents and the

Table 1 Comparison of methods for preparing immunoabsorbents. Activated gels of 4% cross-linked agarose were reacted with saturating amounts of affinity-purified goat antihuman low density lipoprotein antibody, and the amount of antibody coupled to agarose is shown in column 3. The immunoabsorbents were then reacted with a saturating amount of low density lipoprotein and the amount of low density lipoprotein reversibly bound and eluted by 1 M acetic acid is shown in column 4

Agent used to couple antibody to agarose	Stability	Maximum amount of antibody coupled (mg ml ⁻¹ agarose gel)	Maximum amount of ligand bound (mg ml ⁻¹ sorbent)
Cyanogen bromide	Moderate	4.9	4.3
2-Fluoro-1-methylpyridinium <i>p</i> -toluene sulfonate	Excellent	6.0	2.6
<i>N</i> -Hydroxysuccinimide	Moderate	10.1	2.5
Glutaraldehyde	Excellent	6.3	1.6
Trichlorotriazine	Excellent	12.0	0.7

relative instability of the linkage produced at extremes of pH. The instability is reduced considerably if internally cross-linked agarose is used and if the immunoadsorbent is washed with detergent, so that it is rarely a critical limitation.

The physical separation of agarose beads from chemically attached antibodies by interposition of long hydrophilic spacer arms theoretically allows greater conformational movement by the antibody, but in practice these spacers often make little difference to the performance of immunoadsorbents.

After coupling is complete unreacted sites are blocked by overnight incubation with a large concentration of a small molecular weight agent such as ethanolamine or glycine. Nonspecifically adsorbed antibody can be removed in two steps. First, ionically bound material is removed using several alternating pH 4 and pH 9 washes with buffer containing 0.5 M salt. Then hydrophobic bonds are disrupted by incubation in 1 M acetic acid for several hours, followed by treatment with neutral buffer containing a detergent such as 1% Liquinox or Triton X-100. Immunoadsorbents are usually stable to detergent treatment and this process will reduce leaching of noncovalently attached antibody from the sorbent during use. Much of the antibody release from immunosorbents originally thought to be due to chemical bond lysis is actually desorption of hydrophobically bound material, which can be reduced greatly by detergent treatment.

Use of the immunosorbent

The sample is best applied to the immunosorbent in batch by rotating end-over-end for approximately 2 hours. Washing and elution of the desired material is most conveniently done by pouring the gel into a column. The best general eluant is 1 M acetic acid (pH 2.3) applied with sufficient forward linear flow to elute the column over 0.5–3 hours. Elution of ligand in a batch process is sometimes ineffective. Alternative methods of immunosorbent elution use

alkaline buffers (pH 11) or chaotropic agents such as 3 M potassium thiocyanate.

Several factors limit the separations which can be achieved with immunoadsorbents. Although in theory a single substance will be isolated, immunoadsorbents are necessarily ion exchangers because of the charges on antibody protein and are also capable of hydrophobic interaction with unwanted components of the sample. These contaminants are released along with the desired antigen when the immunoadsorbent is exposed to denaturant. The separation achieved therefore depends on the concentration of the antigen of interest relative to contaminants prevailing before application to the immunoadsorbent. For example, specific antibodies isolated by immunoadsorbent from high-titer immune serum (which may contain 20% of the specific antibody) are nearly homogeneous, whereas immunoadsorbent preparations of nonabundant proteins from crude cell or tissue extracts are composed principally of contaminants. Thus, immunoadsorbents are useful as a single step in the preparation of abundant proteins, or helpful in conjunction with several other independent separation techniques in the multistep purification of nonabundant proteins. Antibodies may lose avidity and capacity for antigen after immobilization and this tends to accentuate the limitations noted.

Monoclonal antibodies

Effective sorbents can be made from monoclonal antibodies, but the antibody to be used needs careful selection, as illustrated in Table 2. Data are presented for two monoclonal antibodies, both of which bind efficiently to antigen in solution or on ELISA plates and are also coupled efficiently to agarose beads. However, immunoadsorbents made from the first antibody have ten times the antigen-binding capacity of those made from the second. In fact, immunoadsorbents prepared from the second antibody are nearly useless because of the small amount

Table 2 Example of variation in the suitability of monoclonal antibodies for use in immunoadsorbents. Anti-low density lipoprotein (LDL) antibody B1B3 was superior to antibody C3D1 in ELISA assays and in coupling to agarose beads. However, the C3D1-agarose immunoadsorbent was ten times more effective at binding LDL (right column)

Anti-LDL monoclonal antibody	LDL binding in ELISA assay ^a (mg LDL mg ⁻¹ antibody)	Antibody coupled to agarose beads ^b (mg ml ⁻¹ agarose)	LDL bound to immunoadsorbent (mg ml ⁻¹ sorbent)
C3D1	1.2	4.1	8.7
B1B3	3.5	5.1	0.7

^aData from Tikkanen MJ, Dargatzis R, Pfeiffer B, Gonen B, Davie JM and Schonfeld G (1992) Antigenic mapping of human low density lipoprotein with monoclonal antibodies. *Journal of Lipid Research* 23: 1032–1038.

^bCoupling of antibody to 2% agarose beads by means of cyanogen bromide.

of specific antigen binding. There is no general method for selection of antibodies for immunoadsorbents, but the potential for improved performance is clear.

Clinical applications

Immunoadsorbents are currently used in the preparation of drugs and biologicals and must not convey undue risk to humans who use the end-products. Disinfection, the elimination of vegetative bacteria, can be achieved with 1 M acetic acid or 70% ethanol used at room temperature for several hours, with retention of immunoactivity. In fact, in some cases treatment of agarose-immobilized immunoglobulins with ethanol actually increases the sorbent capacity. Immunoadsorbents become inactivated when autoclaved or exposed to ethylene oxide gas, but they retain 30–100% of activity after treatment with 1% peracetic acid, 2% glutaraldehyde, or 0.34% phosphoric acid in 80% ethanol, all of which are true sterilizing agents. Immunoadsorbents should be free of DNA for human use applications. Pyrogens can usually be removed by extensive detergent washing.

It is possible to use immunoadsorbents as artificial organs in clinical applications. For example, patients with homozygous familial hypercholesterolemia have extreme elevations of plasma low density lipoprotein (LDL) which can be removed extracorporeally by pumping plasma or even whole blood over sterile columns of anti-LDL agarose. This process is termed LDL-apheresis. Although the antigen–antibody complexes formed in the sorbent column activate complement, this can be suppressed by the use of citrate, a calcium complexing agent, as the anticoagulant.

Immunoglobulin-binding protein A sorbent columns have been used to treat a variety of immunological disorders. Bone marrow stem cells have been selected and purified by binding to immunoadsorbents prior to transplantation, demonstrating the feasibility of selecting for or excluding specific cells in a clinical setting.

See also: Affinity chromatography; Antibody-antigen intermolecular forces; Plasmapheresis; Protein separation techniques.

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IMMUNOASSAYS

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Immunoassays fall within the broader class of techniques – frequently described as ‘binding assays’ – that rely on observation of the binding reaction between the analyte and a specific binding substance, the latter being generally of biological origin and typically comprising a specific binding protein. Antibodies constitute an especially important class of protein in this context, as they can be generated conveniently and in large amount (either by conventional *in vivo* methods or, more recently, by the *in vitro* techniques pioneered by Milstein and Köhler)

against an extensive range of substances of biological interest, including high molecular weight substances (e.g. proteins, polypeptides, viral particles, oligonucleotides, etc.) as well as substances of relatively small molecular size not themselves intrinsically immunogenic (‘haptens’), such as drugs, steroid and thyroid hormones, vitamins, etc. Naturally-occurring specific binding proteins nevertheless continue to be used in ‘protein binding’ assays, e.g. in assays for vitamin D and its metabolites, vitamin B₁₂, folic acid and (occasionally) thyroxine, albeit the availability

of specific antibodies against most of these substances has resulted in the progressive abandonment of the binding proteins as analytical reagents. More recently, binding assays essentially identical in principle to immunoassay, but relying on DNA 'probes' as binding reagents, have been introduced, although these are restricted in application to the detection and/or measurement of complementary nucleotide sequences, and have not as yet achieved the widespread usage or commercial importance of antibody-based assay methodologies.

The binding reaction between high concentrations of analyte and antibody may be observed 'directly' in consequence, for example, of the formation of a visible precipitate (comprising the analyte-antibody complex), whose amount is indicative of the amount of analyte in the test sample (assuming a sufficiency of antibody to be present). This approach formed the basis of traditional methods used to quantify both antigens and antibodies, such as the immuno double-diffusion and immunoelectrophoresis techniques. However, for greater sensitivity, either of the reactants may be labeled using a marker more readily detectable than the reactants or reaction products themselves, enabling observation of binding reactions between lower reactant concentrations. One of the original labels used for this purpose comprised red cells, to whose surface antigen was linked. The presence of antibodies in a test sample was detected by cell agglutination (arising from cross-linkage by the antibody), resulting in a characteristic layer of agglutinated cells at the bottom of the incubation tube or well. This technique was also used to detect specific antigens in test samples by their inhibitory (competitive) effect on the agglutination reaction between antibody and (identical) antigen on the red cell surface. Such techniques were semiquantitative in that they yielded a somewhat imprecise measure of the maximal dilution of test samples causing visible agglutination.

The emergence of far more precise and sensitive immunoassays followed the adoption of radioisotopic labeling techniques for the observation of antibody-antigen reactions. The original methods of this genre relied on the use of labeled antigens (used essentially as tracers of unlabeled antigen present in test samples), and are now commonly known as radioimmunoassays (RIAs). The term is occasionally applied to methods relying on isotopically-labeled antibodies; however, the term immunoradiometric assay (IRMA) is conventionally applied to methods based on the latter approach.

Immunoassays characteristically display high structural specificity, reflecting the ability of appropriately selected antibodies to distinguish and bind

to antigenic sites (epitopes) on particular analyte molecules. The use of high specific activity isotopes such as ^{125}I or ^3H (or, in the case of DNA probes, ^{32}P) to label either the antibody or (tracer) analyte permits binding reactions between small numbers of molecules to be readily monitored, endowing these methods with exceedingly high sensitivity. These attributes, coupled with their relative simplicity, underlie the widespread use of isotopically-based immunoassays in medicine and biology and increasingly in many other related fields (e.g. the food industry, agriculture, forensic science, environmental monitoring, etc.). Since the early 1980s, however, increasing interest has focused on so-called alternative, nonisotopic techniques based on principles identical to those of RIA and IRMA, albeit differing in the reagent labels used. Various environmental, legal, economic and practical considerations underlie this development (e.g. the limited shelf-life of isotopically-labeled reagents, the problems of radioactive waste disposal, the complexity of radioisotope counting equipment, the demand for diagnostic kits for home use, etc.), most of which are primarily of social or logistic significance. Other factors are more fundamental, relating to the development of methodologies of greater sensitivity than the isotopic methods or which address other analytical objectives unattainable using isotopic labels. Such objectives include the development of transducer-based immunosensors for directly measuring analyte concentrations in biological fluids, and multianalyte assay systems capable of measuring multiple analytes in the same sample.

A wide range of nonisotopic labels have been employed in this context, of which the most commonly used are enzymes, and chemiluminescent and fluorescent substances. Regrettably, the diversity of different labels now in use has given rise to a confusing range of acronyms by which the differing assay formats developed by individual researchers or immunoassay kit manufacturers are known. The conventional terminology distinguishing the original, isotopically based methods is often used; for example, methods relying on fluorescent labels are referred to either as fluoroimmunoassay (labeled antigen) or immunofluorometric assay (labeled antibody), on enzyme labels as enzymeimmunoassay and immunoenzymometric assay, etc. However, other terms – some of which depart from this convention – are also in common use, including, for example, enzyme-linked immunosorbant assay (ELISA; generally signifying the use of an enzyme labeled antibody and solid-phase antigen), time-resolved fluoroimmunoassay (TR-FIA; based on time-resolution methods of fluorescence measure-

ment), and competitive enzyme-linked immunoassay (CELIA; relying on the use of enzyme-labeled antibodies in a competitive assay mode).

The terms 'competitive' and 'noncompetitive' frequently feature in descriptions of immunoassay and other binding assay methodologies, as exemplified by CELIA and competitive protein-binding assay (CPBA). These terms (and similar descriptions, such as 'inhibition' assay) derive from the historical portrayal of certain binding assay methods (e.g. RIA) as dependent on competition between unlabeled and radiolabeled (tracer) analyte for a limited number of antibody (or protein) binding sites. For this reason, labeled antibody methods are frequently viewed as inherently noncompetitive, the terms being used virtually synonymously. However, certain labeled antibody methods are also described as competitive (e.g. CELIA), evidently on the grounds that, as with labeled analyte methods, maximal sensitivity is achieved using restricted amounts of antibody (see below).

The underlying reasons for the existence of these two broad types of assay may be clarified by brief consideration of the basic principles governing immunoassay. All such assays essentially rely on the measurement of (fractional) antibody binding site occupancy by analyte following reaction between the two. However, binding site occupancy may be determined either by *direct* measurement of *occupied* sites (noncompetitive assay), or by *indirect* measurement, i.e. by the measurement of *unoccupied* sites, from which binding site occupancy is inferred (competitive assay). The optimal amount or concentration of antibody yielding maximal assay sensitivity largely depends on which of these alternative strategies is adopted, tending to infinity in the first case, and to zero in the second. This difference in assay design – which is essentially unrelated to which component, if any, of the assay system is labeled – underlies the distinction between, and differing performance characteristics of, noncompetitive and competitive methods. For example, introduction of labeled analyte into the assay system either following, or during, the reaction between antibody and analyte provides a method of estimation of unoccupied antibody binding sites, implying that all labeled analyte methods may be classed as competitive. In contrast, if labeled antibodies are employed, the assay may be classed as competitive or noncompetitive, depending on the approach used to separate and measure binding sites occupied by analyte. Thus, if an immunosorbant is used to sequester unoccupied sites, and labeled antibody *attached to the immunosorbant* is determined, the assay is competitive. Conversely, if labeled antibody *remaining in solution* (i.e. antibody bound to analyte) is measured, the assay

is noncompetitive. Assuming an efficient method of separation of occupied from unoccupied sites, the optimal antibody concentrations yielding maximal sensitivity will significantly differ, depending on which of these alternative approaches is adopted.

Irrespective of whether (tracer) analyte or antibody is labeled (but assuming the use of a label of infinite specific activity), the maximal sensitivity attainable using a competitive assay may be shown to be ϵ/K , where ϵ represents the experimental error (cv) in the measurement of the assay response variable (e.g. fraction of labeled analyte bound to antibody), and K = the antibody affinity constant *vis-à-vis* analyte. For example, if experimental errors in the measurement of the assay response approximate 1% (i.e. $\epsilon = 0.01$), and $K = 10^{12} \text{ l mol}^{-1}$, the maximal sensitivity attainable using a competitive approach is about $10^{-14} \text{ mol l}^{-1}$, i.e. about 6×10^6 molecules ml^{-1} . The use of radioisotopic labels (such as the iodine isotope ^{125}I) of finite, albeit high, specific activity, does not, in practice, significantly reduce assay sensitivity (assuming use of an antibody of affinity constant about $10^{12} \text{ l mol}^{-1}$ or less). This implies that no significant sensitivity improvement can result from the substitution of other labels in immunoassays of competitive design. However, in the case of noncompetitive immunoassays, similar theoretical analysis reveals that (assuming efficient separation of occupied from unoccupied labeled antibody binding sites), sensitivities of about 10^2 – 10^3 molecules ml^{-1} are, in principle, attainable, albeit only using labels of much higher specific activity than the commonly used radioisotopes. These conclusions underlie the current development of a variety of ultrasensitive, noncompetitive, labeled antibody methods relying on, for example, lanthanide chelate fluorophors, chemiluminescent substances such as the acridinium esters, and various enzyme labels.

A disadvantage of the use of high antibody concentrations in noncompetitive assay designs is a concomitant loss in assay specificity, arising from the increased relative potency of substances other than the analyte as the antibody concentration is raised. (Note that all substances capable of reaction with an antibody become equipotent in an immunoassay when the amount of antibody is infinite.) This problem is minimized in so-called 'sandwich', or 'two-site' assays, relying on dual antibodies. The first, linked to a solid support – often termed the 'capture' antibody – is generally directed against one epitope characterizing the analyte molecule and is used to isolate molecules bearing the epitope from the medium. The second (labeled) antibody, directed against a second epitope, enables determination of the number of captured analyte molecules adhering

to the solid support which carry *both* epitopes. Sandwich assays thus combine the sensitivity of the non-competitive approach with the specificity improvement inherent in dual-epitope recognition, and – subsequent to the development of *in vitro* techniques of monoclonal antibody production – have emerged as the dominant methodology for the assay of analytes of large molecular size. (Note, however, that isoforms of many analytes (e.g. erythropoietin) differ in both structure and biological activity, notwithstanding their possible possession of many identical epitopes. Thus, sandwich immunoassays cannot be assumed to be totally specific for analyte molecules of a single, unique, structure, and may therefore be analytically invalid. This phenomenon contributes to the problems of immunoassay standardization.)

For the two-site dual antibody approach to be successful, the analyte molecule must be of sufficient size to permit its simultaneous binding to two different antibodies without steric impediment. Attempts to develop sandwich assays for analytes of small molecular size (such as steroid hormones) by generating antibodies reacting exclusively with occupied capture-antibody binding sites have so far failed, largely because of the propensity of antibodies generated against occupied sites also to bind to unoccupied sites. However, a recently described technique relies on blocking of unoccupied sites by an anti-idiotypic antibody prior to exposure of capture antibody–analyte complexes to labeled antibody. The latter – prohibited from binding to unoccupied sites – thus binds only to capture antibody–analyte complexes. The technique thus constitutes a noncompetitive assay for analytes of small molecular size, and is thus potentially capable of yielding greater sensitivity. However, it remains questionable whether the approach provides the specificity advantages of a genuine sandwich assay, and overcomes the loss in specificity that the use of large amounts of labeled antibody to maximize sensitivity implies.

A further reason for the adoption of nonisotopic labels is the necessity, in the case of the isotopically-based methods, for physical separation of the immunological reaction products, whichever is labeled. Certain nonisotopic labels permit the reaction products to be distinguished without such separation, methods based on their use being generally described as homogeneous. In practice, such methods have in the past been largely confined in their application to analytes of small molecular size, relying on a labeled form of the analyte, the signal from which is affected by its binding to antibody. Certain commercial assay kits rely on the use of enzyme labels, whose activity is either restricted or enhanced when the labeled analyte is antibody bound. Another tech-

nique relies on fluorescent labels, and exploits alterations in the plane of polarization of the fluorescent emissions when the labeled antigen molecule is in free or antibody-bound states. Such techniques are of relatively low sensitivity, and their use has been confined to the assay of analytes present in biological fluids at relatively high concentration, such as therapeutic drugs. Recently, however, homogeneous sandwich methods of high sensitivity for the assay of analytes of large molecular size have been developed by various manufacturers and are beginning to appear on the immunodiagnostic market. One such method relies on a time-resolved fluorescence detection system involving the transfer of laser excitation energy from one antibody (labeled with a fluorescent europium cryptate) to the second, the latter (labeled with the fluorophor allophycocyanine) forming the complementary part of the sandwich. Such energy transfer essentially only occurs when both antibodies are simultaneously bound to individual analyte molecules and are therefore in close proximity. Thus only analyte molecules simultaneously bound to the two antibodies generate a detectable signal.

Neither analyte nor antibody is labeled in a conventional sense in the case of the newer immuno-sensor technologies now under development. Such devices comprise a solid probe on whose surface antibody is coated; antibody occupancy following exposure to an analyte-containing medium is monitored by an internal transduction system, yielding an observable electronic or optical signal. A number of such transduction systems, such as field-effect transistors (FETs), have been proposed, but most have proved either too low in sensitivity or vulnerable to nonspecific effects to be of practical use. A promising contender in this field relies on surface plasmon resonance (SPR), an electronic effect generated in an ultrathin conducting metal (e.g. gold or silver) film on glass (or quartz) when light impinging at a particular angle on the glass–air interface is internally reflected. The resonance angle depends on the mass of material present at the interface; it thus alters in response to changes in the occupancy of antibody (or antigen) molecules coated to the metalized/glass surface. Instruments relying on these principles are now commercially available, albeit they are primarily intended for research studies (such as epitope mapping) rather than for routine immunoassay use. Although likely to prove of great value in certain situations, the transducer-based sensor approach to antibody-occupancy measurement possesses a number of potential drawbacks which makes it uncertain that it will substantially replace conventional label-based methodologies in the near future. For example, the specificity improvements offered by two-site

sandwich assays (particularly in the context of high-sensitivity, noncompetitive assay designs) makes large-scale reversion to single-site immunoassays unlikely. Although sensor techniques such as SPR are capable of detecting second (i.e. sandwich-forming) antibodies bound to analyte molecules adhering (via capture antibody) to a glass surface (particularly if the second antibodies are labeled with, for example, colloid metal particles to increase their mass), the inclusion of second antibodies in an assay system narrows the distinction between sensor-based and conventional label-based methods of antibody-occupancy measurement. Thus the future impact of SPR and other transducer-based techniques is likely to depend largely on the reduction in operational costs, if any, that they offer as compared with current methodologies. Meanwhile, the slow rates of association and dissociation of antigen-antibody complexes imply that the ultimate goal of immunosensor development – the ability to monitor changing analyte concentrations in a medium – remains far from fulfillment.

Another development – that of multianalyte immunoassay systems – reflects an increasing need to measure many different analytes in the same sample, both in medicine and in many biologically-related fields, e.g. the food industry. The possibility of developing such technologies also emerges as a consequence of the availability of very high specific activity, nonisotopic, reagent labels, e.g. fluorescent labels. This permits the development of high sensitivity 'microspot' immunoassays in which a capture antibody is located on the surface of a probe within an area no more than 50–100 μm^2 . This in turn permits construction of multimicrospot arrays, each microspot directed against a different analyte. Provided the total number of capture antibodies located within each microspot is 'small' (i.e. about 0.05 ν/K or less, where ν = the volume of the analyte-containing medium to which the microspot is exposed), fractional occupancy of capture antibody binding sites is solely dependent on the analyte concentration in the medium. Moreover, by labeling both capture antibody and a second 'recognition' antibody (directed against either occupied or unoccupied sites) with different labels, e.g. two fluorescent labels, the analyte concentration can be derived by observation of the ratio of signals emitted from the microspot when exposed, for example, to laser illumination. Thus, by utilization of laser scanning techniques comparable to those employed in other fields, arrays of many different antibody microspots may be rapidly examined, thus permitting the development of simple multianalyte immunoassay methodologies capable of

measurement of many different analytes in the same small biological sample.

Microarray techniques of this kind (but relying on oligonucleotide arrays rather than antibody arrays) are also applicable to DNA analysis; such development constitutes the objective of the US Genosensor Project, established in 1992.

Antibody microspot arrays may be constructed on a commercial scale using, for example, ink-jet spotting techniques. Researchers at Boehringer Mannheim GmbH have described the use of this approach to construct arrays comprising 196 microspots located on the flat circular base (3 mm in diameter) of a plastic sample container, these being produced at a rate of approximately 5000 chips an hour. A somewhat different approach has been adopted by the Californian company, Affymetrix Inc., for the construction of oligonucleotide arrays for use in DNA analysis. This relies on the use of so-called combinatorial chemical techniques to construct (*in situ*) the different oligonucleotide sequences within the individual spots that make up the oligonucleotide array. The technique involves sequential masking and exposure of selected areas on a silicon base using photolithographic techniques similar to those used in the electronics industry for the construction of semiconductor chips. Such technology – although applicable to binding agents such as oligonucleotides and polypeptides which can be built up sequentially – are obviously not applicable to antibodies, which cannot be synthesized in this fashion.

The current intense interest in microarray technologies being displayed both in Europe and in the USA, and in the development of both manufacturing techniques and appropriate instrumentation, suggests that antibody microspot arrays will form the basis of the next major revolution in the field of immunoassay. But whether or not this prediction is fulfilled, binding assay methodology of one form or another is clearly likely to retain a dominant position in the microanalytical armamentarium for the foreseeable future. *In vitro* techniques of monoclonal antibody production have removed much of the element of chance associated with *in vivo* antibody production methods, and increased control over the nature, properties, and long-term availability of binding reagents. The recent introduction of single domain antibodies (dAbs) by Ward and colleagues, which rely on genetic engineering techniques for the *in vitro* production of the heavy chain variable (V_{H1}) domain of selected antibody molecules, represents a further step along a path leading ultimately to the synthesis of entirely artificial analyte-recognition binding molecules.

See also: Affinity; Agglutination; Antibodies, detection of; Antibody-antigen intermolecular forces; Antiserum; Enzyme labeling of antibodies and antigens; Enzyme-linked immunosorbent assay (ELISA); Fluorochrome labeling; Immunocytochemistry and enzyme markers; Monoclonal antibodies (mAbs); Precipitation reaction; Radiolabeling; Surface plasmon resonance; Valency of antigens and antibodies; Western blotting.

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IMMUNOCONGLUTININS

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Bovine serum normally contains a protein known as 'conglutinin', which is responsible for the tight aggregation ('conglutination') of complement-coated erythrocytes. Conglutinin is not convincingly found in the serum of species other than Bovidae but a similar activity can be generated in other species, either by immunizing them with complement-coated material or spontaneously after *in vivo* complement activation. These factors are called the immunoconglutinins and are autoantibodies to neoantigens exposed in fixed complement components but not in free complement components. C3 and C4 are the major complement components that become fixed at complement fixation sites and most immunoconglutinins react with hidden determinants in bound C3 and C4. Although neoantigens are known to occur in the membrane attack complex, autoantibodies to these neoantigens following *in vivo* complement activation have not so far been described.

Although immunoconglutinins of all immunoglobulin classes can be detected, those that are measured by the usual agglutination techniques are predominantly immunoglobulin M (IgM) and they are the equivalent for the complement system of the 'rheumatoid factors' which react with bound immuno-

globulins. Human sera all contain low levels of immunoconglutinin but elevated levels are diagnostic of *in vivo* complement activation and thus are found in acute and chronic infections and in chronic inflammatory diseases. They are particularly high in intravascular infections which give rise to high IgM antibody formation (e.g. African trypanosomiasis), but high levels are also encountered in chronic bacterial infections and in chronic viral infections. In minimal change nephritic syndrome, raised immunoconglutinin titers are the only evidence that complement activation is involved. Immunoconglutinins have a fairly limited role in diagnostic immunopathology, most recent publications being concerned with their levels in parasitic infections.

A special example of immunoconglutinins are the so-called nephritic factors which are autoantibodies to the alternative (but very rarely the classical) pathway C3-convertases. These antibodies stabilize the C3 convertases and thereby derange complement homeostasis.

Nephritic factor (Nef) is concentrated in the IgG3 subclass and is larger than the usual IgG molecule by virtue of increased glycosylation. The stimulus for forming Nef is unknown, but it is more commonly found in subjects carrying the C3F allele. Subjects

with Nef have low plasma C3 levels. Nef was first described by Spitzer and colleagues in 1969 in patients with mesangiocapillary glomerulonephritis, but there is a stronger association with partial lipodystrophy. A few subjects with Nef have systemic lupus erythematosus (SLE) and occasionally there is no associated disease.

See also: **Agglutination; Autoantigens; Complement, alternative pathway; Complement, classical pathway.**

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IMMUNOCYTOCHEMISTRY AND ENZYME MARKERS

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Immunocytochemistry (ICC) involves the visual identification of target molecules in tissues and cells through the specific interaction of antibody(s) with antigen by using antibodies labeled with indicator molecules. The antibody is tagged with a specific signal generator (indicator), e.g. a fluorochrome, color-producing enzyme or metallic particle. The sensitivity, or strength of the signal, can be enhanced by increasing the indicator/antigen ratio; this is done through a variety of indicator-amplifying mechanisms.

The essential requirements of ICC are: a) production of sensitive and specific antibodies to a selected antigen and marker reagents, b) optimal treatment of tissues in terms of fixation and histopreparation to promote optimal interaction between target molecules and reagents, and c) appropriate identification (quantification) of the indicator molecule, all with optimal tissue preservation. ICC reagents can nonspecifically stain the tissues in a similar fashion to that of standard histochemical stains, thus appropriate controls are essential.

ICC is essentially simple in concept, however there is a considerable degree of technical knowledge and expertise needed to adequately perform the assay, and it can be difficult to interpret. Many different approaches and marker systems now exist and the overall sensitivity of ICC has been much improved in recent years. This has been due in no small part to an increase in the specificity of antibodies used, brought about by monoclonal antibody (mAb) technology, as well the development of more refined tissue fixation for use in ICC, automated approaches that standardize procedures, as well as more sensitive quantitation systems.

The first labeling of antibodies was achieved by John Marrack in 1934. This was taken up by the major pioneer of immunocytochemistry, Albert Coons, who with advice from Creech and Jones used isothiocyanate methodology to couple a fluorescent label to rabbit antisera directed against pneumococci. Other names that deserve mention in developing ICC include Kunz, who in 1941 began working towards a new way of localizing tissue constituents, Nakane who introduced enzymes to produce colored reaction products as markers, as well as Sternberger and Mason who independently developed methods for amplifying enzyme systems. In recent years there have been advances in the chemistry of fluorochromes and in development of sensitive systems to assess and quantify these useful labels.

ICC systems

ICC methods can be divided into four groups:

The direct method (see Figure 1) Here a labeled antibody (known as the primary antibody) attaches directly to the target antigen in the tissues. An inherent problem with this system is that unwanted antibodies directed against tissue antigens are also labeled and can attach to the tissue section thus producing high backgrounds. The use of mAbs can reduce this contamination.

Indirect methods Here the marker molecules are attached to a secondary antibody applied to recognize the presence of a primary antibody on the target antigen. Indirect methods enhance stain strength and

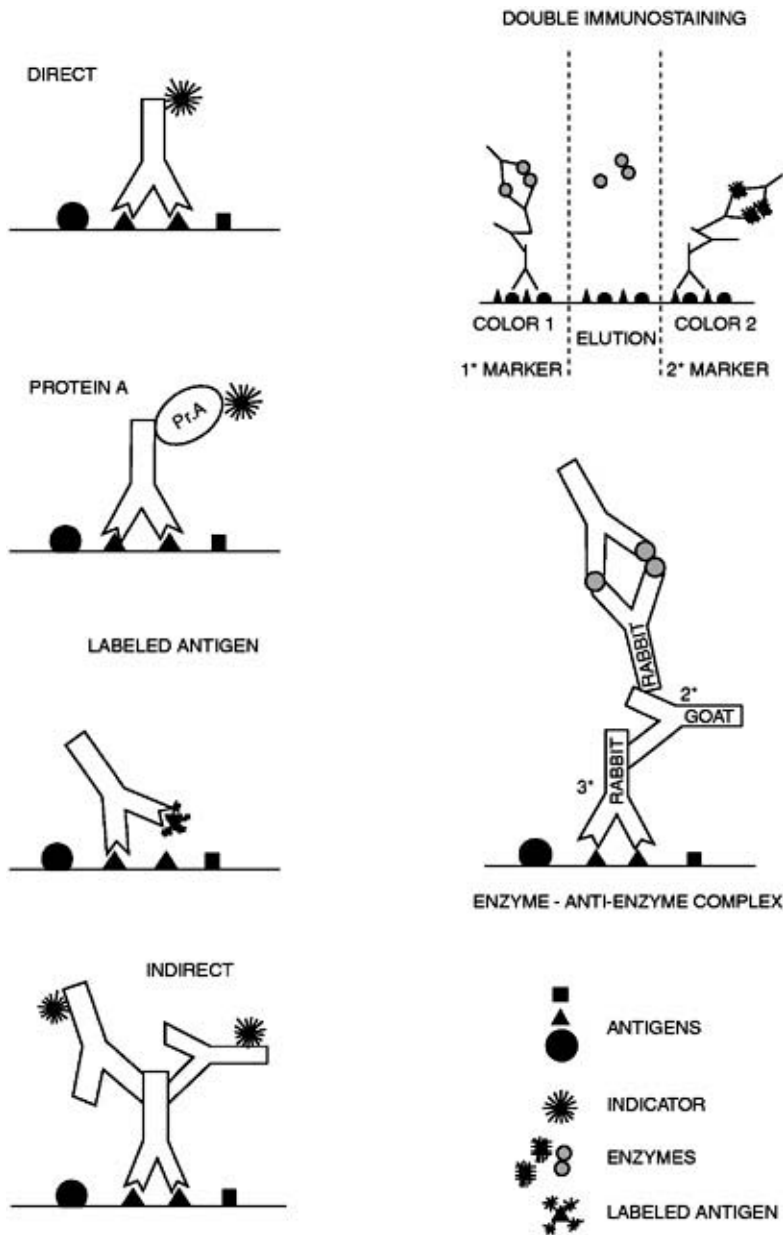


Figure 1 The basic forms of immunocytochemical reactions.

use less reagent but problems still exist with regard to the need to block unwanted couplings.

Bridge indirect methods The bridge principle involves an antibody lying between the primary antibody and the antibody carrying the indicator molecule. A number of variations on this technique exist, including some that involve the use of enzyme-antibody complexes. Commonly used examples are peroxidase-antiperoxidase (PAP) and alkaline phosphatase-antialkaline-phosphatase (APAAP) complexes. All bridge procedures can be intensified by repetition of the staining sequences thus producing a 'branching' effect.

Double staining methods These are used to identify more than one antigen in a single tissue section. In this approach the first immunoreagent and its marker is fully developed, then a prolonged rinse in an acid buffer removes this first antibody leaving only the reaction product behind, and finally the second reaction proceeds superimposed on top of the first. A problem with double staining lies in the fact that the marker antibody used for the first antigen may still have sites available for the second antibody, and this results in an unclear color reaction produced by the intermingling of the two colors.

Antibodies

Most of the antibodies used in ICC to date are immunoglobulin G (IgG), with a minority being IgM. The species of animal used for the production of antibodies should be genetically as far removed as possible from that of the test reagent. Two major concerns are the sensitivity and the specificity of the antibodies used. As a general rule, an increase in one of these is usually associated with a reduction in the other. The specificity of ICC lies largely with the antibody.

The strength (or avidity) of the binding between antigen and antibody is important in ICC. Antibodies should be of high avidity, as low-avidity antibodies will become detached during washing or may themselves block the attachment of the preferred high-avidity antibody. Repeated incubations in anti-serum will increase the chances of high-avidity antibodies refilling any epitopes freed of low-avidity molecules during the washing steps, thus resulting in increased detection efficiency and improved sensitivity. This also allows for the use of more diluted antibodies and thus a reduction in cost. The extent of dilution used for ICC assays is also important as it is possible to dilute out contaminating antibodies, as well as interfering proteins and enzymes.

Nonspecificity can be due to heterogeneous antibody reagents and to shared amino acid sequences (i.e. antigen cross-reactivity). A remedy for this problem is purification of the antibody reagent. However this often causes a reduction in the important highly avid antibody forms. Other techniques adopted include the use of high dilutions of antibodies, absorption by 'common' tissue preparations (e.g. liver powder), staining first with nonimmune serum from the same species as the primary antibody was derived. The carrier proteins (such as serum albumin) used in the preparation of antihapten antibodies for ICC are sometimes impure and can lead to cross-reactivity.

The size of the antibody (and its attached marker molecules) affects its ability to penetrate into tissues and reach the target antigens. One approach taken is to use fragments of antibodies, such as Fab fragments, where penetration into the tissues is a critical factor, e.g. with pre-embedding staining.

The preparation of antibodies is therefore a primary concern in ICC and the removal of contaminating antibodies is a major requirement. Purification of polyclonal antibodies by standard immunochemical techniques often is inadequate for ICC. Affinity purification, it must be remembered, works well for antibodies of low or medium avidity but not

for those of high avidity (i.e. the type needed for ICC).

A technique useful for isolating mAbs from polyclonal sera involves blot affinity purification. Here a nitrocellulose filter containing antigen is treated as one would a tissue, i.e. vapor fixed, incubated with polysera, washed and eluted to produce antisera free of nonspecific reactions. Techniques that specifically purify the IgG out of immune sera should only be employed if it is necessary to remove other serum components or enzymes. In fact, crude serum may be as good as, or even better, than purified reagents in many instances.

The testing of an antibody for ICC is best carried out using a known positive tissue. This will not only determine the background but also the avidity, and show that the antibody will resist the washing process. In general terms other techniques are inferior, although the ELISA assay is closer in relevance to ICC than radioimmunoassay (RIA). Cytochemical paper (nitrocellulose) dot blots and artificial tissue sections are useful where filter paper is treated as for tissue sections (e.g. vapor fixed, embedded and sectioned) and tested for different levels of antigens.

The size and complexity of the antigen to which the antibody is directed influences the effectiveness of the reagent. Antibodies directed against certain areas of a molecule are advantageous; however the smaller the area the more the chance of cross-reaction with other molecules. Thus, in peptide immunocytochemistry it is often essential to stain sections with antibodies to several different regions of the molecule, e.g. the N-terminal, the mid-portion and the C-terminal amino acid sequences. Consequently, it is often useful to use a cocktail preparation of monoclonals.

Monoclonal antibodies have now been developed that are more functional in ICC and which identify embedding and fixation resistant epitopes, e.g. those which recognize CD45 (leukocyte common antigen). Anti-idiotypic antibodies have been used for cytochemical identification of peptide receptors in the CNS.

The localization of indicator molecules in tissues by using technique systems based on chemistry other than antigen-antibody interactions is fruitful in some circumstances. The *Staphylococcus aureus* component protein A links specifically to the Fc portion of IgG and can be labeled with standard ICC markers. The avidin-biotin linking system is popular. Avidin, which can be linked directly to indicator enzymes, is a large glycoprotein (mol. wt 68 000) and has four binding sites for the low molecular weight (mol. wt 242) vitamin, biotin; probing antibodies are linked to biotin (biotinylated). Antibodies directed against

the biotin label on biotinylated entities are now used to replace the biotin-avidin link.

A problem accompanies the use of biotin as a marker molecule due to the presence of natural levels of this vitamin in the tissues. Thus alternatives such as the steroid hapten digoxigenin (DIG) has been developed as a marker system because it is absent from animal tissues and cells; DIG occurs in plants of the *digitalis* family.

Antigens

The tissue component detected by ICC is an antigen, or a complex of antigens, located in a structurally and biochemically complex microenvironment. The conditions needed for successful ICC include an insoluble antigen that remains *in situ* and preservation of the tissue architecture to allow adequate morphological identification.

Fixation immobilizes the antigen and preserves the tissue architecture; however this process can reduce antigenicity and interfere with the passage of reagents to the sites of the tissue entities. Thus fixation is one of the most important considerations in ICC and is responsible for many of the comparative discrepancies and limitations of the technique as a whole. Antigens can be extracted by the fixative and lost in some instances. This can be influenced by the antigen's locality (e.g. extracellular spaces extract quickly), and the antigen's inherent properties (e.g. those lacking primary amino and sulfhydryl groups are extracted by aldehyde fixatives).

Two basic forms of fixation are used in ICC: precipitating and cross-linking fixatives. Precipitating fixatives remove lipid but do provide comparatively good penetration by removing the lipid component of membranes. Such fixatives (e.g. ethanol, methanol and acetone) act by precipitating large protein molecules and were commonly used in the early days of immunocytochemistry; the protein denaturation they cause is largely reversible. They are not suitable for electron microscopy as they cause tissue shrinkage and extraction of too many antigens. Nevertheless they may be useful for certain specific purposes (e.g. immunoglobulin localization). However, precipitating fixatives extract rather than fix low molecular peptides.

The cross-linking aldehyde fixatives are the backbone of modern tissue preparation. Formalin and glutaraldehyde are the basic cross-linking fixatives; these are useful for preserving low molecular weight antigens, such as peptides, but less valuable for larger protein antigens, such as cytofilaments and IgGs (where it is better to use the precipitating fixatives). Chemical cross-linkers disturb antigenicity in four

ways: changing the conformation and thus masking epitopes, chemically modifying the reactive amino acids of the epitope, causing steric hindrance, and promoting extraction of the antigens during the washing steps or the embedding procedures. Cross-linkers may also produce artificial barriers within cells to reagents by fixing membranes and cytosolic proteins.

Formaldehyde fixation is not well understood as far as its effect on antigenicity. However, formalin is used widely (particularly in pathology) and is regarded as a relatively mild fixative. Paraformaldehyde (PF), as a source of fresh formaldehyde, has been commonly used in recent years for ICC, particularly in combined fixative solutions. Formaldehyde-amine fixatives have proved useful for ICC. In order for formaldehyde to cross-link proteins it must first react with a primary amino group on one of the proteins. If an exogenous primary amine is present then formaldehyde can cross-link proteins that lack primary amine groups. It has been found that better structural preservation is achieved with an amine formaldehyde combination. Cyclohexylamine-formaldehyde has been used successfully for both electron microscopy (EM) and light microscopy.

It should be noted that washing of formaldehyde- and paraformaldehyde-fixed tissues can lead to the reversal of lost antigenicity and this is especially useful for treatment of pathologic material; washing overnight under running tap water produces satisfactory results for retrospective studies. More recent approaches have involved 'antigen retrieval procedures' which use high temperatures (usually through microwave heating) to re-expose antigens.

Glutaraldehyde is a very effective cross-linker and produces much more permanent states than does formaldehyde; it is also a preserver of enzyme activities working through as yet unknown mechanisms. It penetrates tissues more slowly than formalin and therefore smaller tissue samples must be used.

The use of combination fixatives is now commonplace in immunocytochemistry. This approach was first taken by Karnovsky who perfused organs etc. with a mixture of glutaraldehyde and paraformaldehyde followed by brief immersion of tissues in the fixative at 4°C: the formalin penetrates quickly and protects some of the reactive elements from the more severe effects of the glutaraldehyde. The 'reversible' nature of formalin fixation insures that sufficient sites remain available to react with glutaraldehyde. This combination is suitable for small diffusible antigens; however, some antigens do not suit aldehyde fixation and in these cases it is recommended that parabenzoquinone (PBQ) be used with glutaraldehyde or with paraformaldehyde. Success has also

been achieved with picric acid used with either glutaraldehyde or formaldehyde.

Penetration by a fixative may be slow and this can be overcome or addressed by using small blocks, using vapor fixation of freeze-dried tissue blocks, or by perfusion. A method used successfully for ICC by many is to make cryostat sections of snap-frozen unfixed tissues and then thaw them in the fixative.

Freeze-drying provides optimal enzyme preservation by turning ice into vapor without liquefying the water – the liquid phase of water causes distortion. Rapid freezing produces small ice crystals and minimizes diffusion processes as well as avoiding crystal growth. Freeze-drying and vapor fixational gaseous paraformaldehyde or PBQ is a highly sensitive approach for immunohistological localization of antigens.

Tissue sectioning

The preparation of tissue sections that are both suitable for sufficient entry of ICC reagents and retain the morphology for microscopy requires careful consideration. Paraffin-embedding techniques have caused problems but with improved reagents this has lessened. Nevertheless, cryostat sectioning remains an important approach, although it is necessary to protect the tissues against freezing damage. Cryoprotectants commonly used include dimethyl sulfoxide (DMSO), glycerol and sucrose, with sucrose causing the least blocking of antigenicity. The disadvantages of cryostat preparations include morphological deterioration, diffusion of cellular antigens and the problems of storage and repeated sectioning inconvenience.

Preparation of tissues for EM immunocytochemistry is carried out through three different sequences: 1) No embedding: fixing, freezing, sectioning, and reacting. 2) Pre-embedding: fixing, slicing, reacting, embedding, and sectioning. 3) Postembedding: fixing, embedding, sectioning, reacting. The postembedding technique was given a major boost through the development of colloidal gold linked to protein A. Various sizes of immunogold particle are now available to allow double or even triple labeling.

Permeabilization

It is necessary in most instances to increase the ability of reagents to enter tissues and reach the target sites. In general, cross-linking fixation is preferred to precipitating fixatives but this approach sometimes requires 'unmasking' of antigens. Most commonly used reagents are proteolytic enzymes such as pro-

tease, trypsin, pepsin and pronase. Penetration of reagents can also be achieved by rapid freezing and thawing, which microfractures cells allowing antibody penetration without substantial morphological compromise. Detergents solubilize the membrane lipids but with a lack of predictability of these effects. Cryostat sections themselves have improved antibody penetration since the action of freezing and thawing opens the cells. Membrane permeabilizing agents include sodium deoxycholate, digitonin, saponin and Triton X-100. Saponin is the most useful for EM where the membranes contain cholesterol. As described above microwave heating enhances antigenicity.

The stronger the cross-linking fixative in the primary fixation, the less the tissues and cells are permeable to antibodies. One can use Fab fragment reagents and small conjugates in these cases; in contrast, colloidal gold conjugates are often too large and have poor penetration. Postembedding techniques pose no problem with regards to penetration except where epon resins are used and then etching systems must be employed; polar resins get around some of these problems.

Paraffin embedding may damage through many of its steps such as the decalcification step, the heat treatment in wax melting, the effects of the hydrophobic properties of xylene and paraffin which can be detrimental to the proteins and other antigens under question. This preparative procedure is theoretically far from ideal but due to the universality of the technique antibodies that detect antigens in such material are most useful.

Plastic embedding techniques are of two basic forms, i.e. Aroclor and the polar Lowicryl-LR white. ICC sections require the removal of the plastic, removal of the osmic acid and the unmasking of antigens. Plastic materials are therefore not the first choice in ICC although LR white is successfully used for immunogold labeling. Non-embedding approaches for EM ICC have centered around ultracyromicrotomy of lightly fixed or unfixed tissues or cells that are cryoprotected by the infusion with sucrose and are snap frozen. Pre-embedding methods are the choice for cell surface antigen and receptor immunolocalization and the detection of antigens prone to masking by preparative reagents.

Indicator molecules

The earliest labels developed were the fluorochromes, followed by the use of enzymes that produce colored reactions, the first being horseradish peroxidase (HRP). Simple enzyme labels are generally less sensitive than fluorochromes but they have

Table 1 Examples of fluorochrome and enzyme markers used in immunocytochemistry

Fluorescein
Horseradish peroxidase
Alkaline phosphatase
R-phycoerythrin
Rhodamine (tetramethylrhodamine)
Image-blue
Texas Red
Syntex Green
Allophycocyanin
Propidium iodide

the advantage of being able to be observed at leisure under ordinary bright-field microscopy, and now can in very many cases utilize paraffin-embedded tissues. The enzymes commonly used include HRP, glucose oxidase or intestinal alkaline phosphatase (Table 1). Immunoenzyme techniques allow a wide range of colors ranging from the red of amine ethyl carbazole as a chromogen for HRP, the black reaction product for Fast Blue RR used with alkaline phosphatase, to the blue or purple of the formazan reaction product formed by glucose oxidase. Immunoenzyme techniques can be improved in sensitivity through bridging techniques in which relatively large numbers of indicator molecules (enzyme) are incorporated. Penetration of the comparatively large enzyme labeled antibodies into tissues and cells can be a problem. In the early days enzyme antibodies only reached two or three cells deep into the tissues, but this became less of a problem when the membrane permeabilizing agents were introduced in the late 1970s.

An example of the usefulness of enzyme ICC is the fact that in biology today enzymes are often localized by their immunocytochemical reactivity rather than by biochemical reactions.

Peroxidase is most commonly used to produce permanent brown stain, which can also be developed as either red, blue or even green, with different substrates. Endogenous peroxidase can be a problem and this must be blocked by treatments with agents such as hydrogen peroxide and methanol, periodide and borohydride, sodium nitroferricyanide or phenylhydrazine. Enzyme complex methods such as PAP are 100 times more sensitive than the enzyme used alone; since the peroxidase molecules are immunologically bound and not chemically bound, and their enzyme activity is not compromised.

As the diamino-benzidine used to develop the brown peroxidase product is carcinogenic it is important to consider using alternatives such as:

- Haker-Yates reagent (paraphenylenediamine-pyrocatechol) to produce a blue/black end product;
- 4-chloro-1-naphthol method giving a dark blue purple reaction product; or
- 3-amino-9-ethylcarbazol (AEC) method which produces a red product. This is usually counterstained with hematoxylin.

Sternberger combined peroxidase with antiperoxidase to form a stable cyclic complex with three peroxidase molecules to two antibody molecules (Figure 1). Some examples of immunoperoxidase staining are shown in Figure 2.

Alkaline phosphatase is being used increasingly as a label, despite the fact that many mammalian tissues contain these enzymes. The commonly used substrate is naphthol-AS-phosphate or Fast Blue BB. The problem of endogenous alkaline phosphatase may be resolved by the use of specific fixatives, or inhibitors such as levamisole, and with 20% acetic acid. Alkaline phosphatase-anti-alkaline phosphatase complexes (APAAP) are useful in cases where there is a problem with using PAP because of endogenous peroxidase, e.g., erythrocytes and eosinophils.

Glucose oxidase (GO) is an under-utilized enzyme marker system. Its strength lies in that this enzyme is absent from mammalian tissues, being a bacterial enzyme (from *Aspergillus niger*), and in that it is four times larger than HRP with many more amino groups, and therefore in theory it can generate a larger reduction product. The presence of the GO label is demonstrated by the oxidation of its substrate (i.e. glucose) and the reduction of a tetrazolium salt to form a colored deposit, a formazan. Nitro blue tetrazolium is commonly used in this reaction because of its rapid and strong reaction as well as the fact that its formazan permits precise localization as its blue color contrasts well with peroxidase brown. Glucose oxidase has been used in avidin-biotin immunoglucose oxidase system and with a glucose oxidase-anti-glucose oxidase technique.

Metallomarkers are popular for EM localization of antigens but are also now increasingly common in light microscopy. Gold as a second-level antibody label is also useful and can be built up in a complexed manner thus strengthening the response. Gold particles can be made to any size and have become the standard marker for EM immunocytochemistry. Gold labeling can be greatly enhanced by silver enhancement systems.

Colloidal gold is useful for light microscopy where gold particles backscatter light under dark field optics; this approach is useful for paraffin sections; epipolarization optics are best used for observing cell

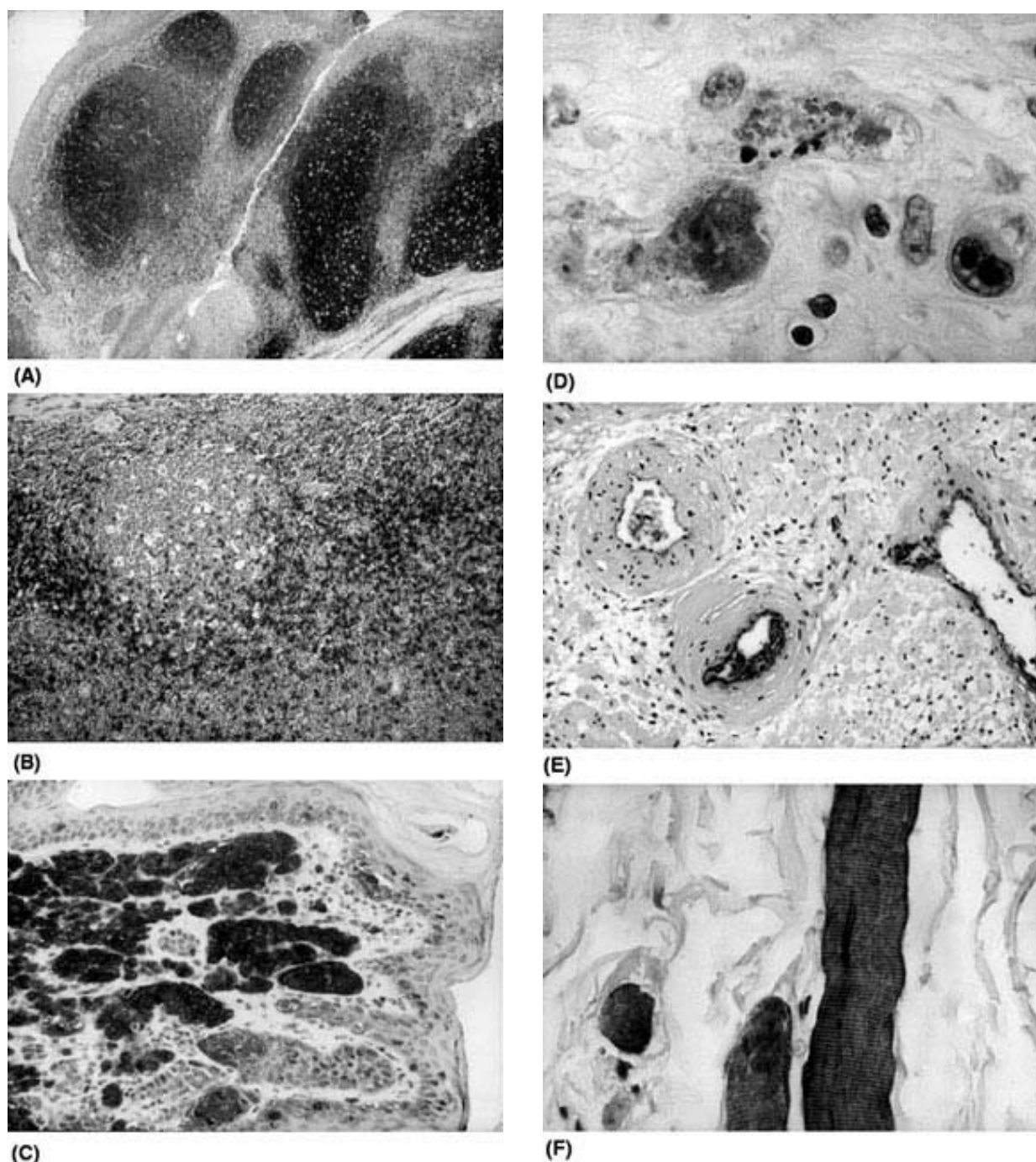
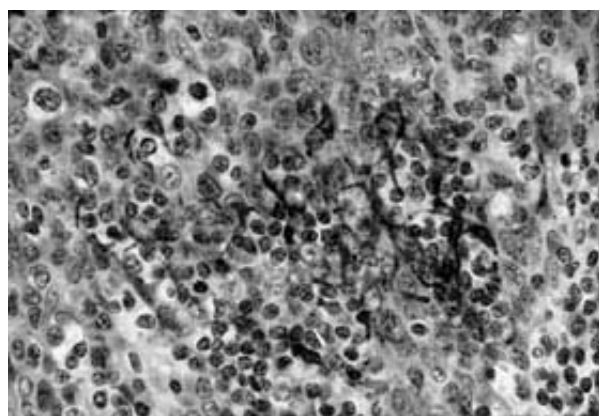


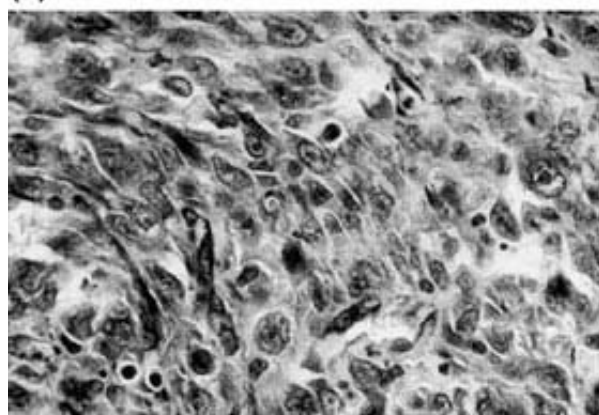
Figure 2 (A) DAB peroxidase-labeled anti-B lymphocyte antibody in human tonsil tissue. (B) DAB peroxidase-labeled anti-T lymphocyte antibody in human tonsil tissue. (C) Peroxidase-labeled anti-S100 antigen antibody in a skin sample. (D) AEC peroxidase-labeled anti-cytomegalovirus antibody in reproductive tract. (E) Antibody against factor VIII-vWB antigen labeled with AEC peroxidase. (F) DAB peroxidase labeled antidesmin in human muscle tissue. (G) Anti-EMA antigen in a case of cervical cancer. (H) Antibodies against vimentin in a human sarcoma tissue. (I) Anti-AE1/AE3 (cytokeratin) antibody labeled with AEC peroxidase in human tissue. (See also color **Plate 6**.)

suspensions. The indirect immunogold silver staining method for the detection of leukocyte and other cell surface antigens in cell smears involves the use of bright-field microscopy and counterstaining with

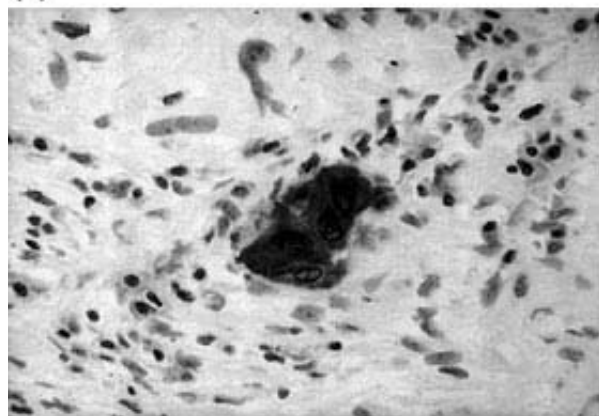
May–Grunwald–Giemsa stain; this reveals gold as black granules on the surface membrane of the cells. Alternatively, epipolarization microscopy reveals bright granules on the dark background and the



(G)



(H)



(I)

Figure 2 Continued.

increase in contrast compared with bright-field microscopy makes this a more sensitive approach.

Gold linked to immunoglobulins, toxins, antigens or protein A have all been used for staining of EM sections on grids (i.e. postembedding procedures).

Immunogold for light microscopy usually uses a more concentrated primary antibody solution than used with the PAP method. It is probably less sensi-

tive than DAB, but is reliable and gives a good color contrast by producing a red-colored gold reaction.

The availability of 1 nm gold particles now permits the use of a particulate label with standard pre-embedding EM techniques for the first time. These small particles must be enlarged, however, for transmission electron microscopy.

Nonspecific binding of colloidal gold in cytochemical reactions can usually be prevented by the addition of macromolecules such as protein solutions (BSA, ovalbumin, gelatin and milk proteins) as quenching agents. It is believed that electrostatic charges are the basis of the nonspecific binding properties of colloid gold complexes.

Controls

It is essential with such a sensitive and variable technique as ICC that a high standard of controls must be maintained. Various levels and types of controls can be used:

Method controls

1. A known positive tissue.
2. A test tissue stained with nonimmune primary layer, inappropriate antiserum, and no primary antisera (these should all be negative).

Antiserum specificity controls

1. Antibody preabsorbed with the specific antigen.
2. Antibody preabsorbed with inappropriate antigen.
3. Antibody absorbed with antigen fragments or structurally related antigen. This approach gives information on epitope specificity.

Much care must be taken in the use of controls both in carrying out those that are appropriate, and in their interpretation. First-level controls indicate the specificity and validity of the primary antibody-antigen reaction. Second-level controls relate to the tissue quality as well as the staining quality (e.g. the presence of endogenous marker interference such as enzyme or autofluorescence). These must be negative before one can make any valid assessment of the tissues in terms of a specific ICC reaction.

False-positive adsorption controls can be caused by the presence of immune complexes which may bind to, for example, Fc receptors. If binding sites for the antigen in question still remain in the tissues then the complexes may bind to these. False observations may arise because there may be enzymes in the antisera that destroy the antigen and there may be contamination of the antibodies.

Interpretation

The sensitivity of the immunocytochemical method is not evaluated by staining intensity, rather it is measured by the ratio of specific staining intensity to the amount of background staining intensity. It is important to differentiate between the type of staining in a cell and not simply whether it is positive or negative. The morphology of staining also is important: a truly positive reaction is generally heterogeneous in distribution either within a cell, amongst a group of cells or throughout a tumor; diffuse monotonal staining is in all likelihood nonspecific. For example, with lymphocytes the uptake of antigens is seen as diffuse cytoplasmic staining, usually most marked at the periphery of cells and has a smooth pattern. Synthesis of the target antigen by the cells will show a more intense staining around the nucleus and less at the periphery of the cell and it will appear as a granular pattern. A conservative approach to interpretation of results from lightly fixed or unfixed tissues is prudent.

Other labeling systems

Radio-immunocytochemistry (RICH), where the marker molecule is a radiolabel detected through autoradiography is generally too slow a procedure and is not commonly used although it is still used for *in situ* hybridization.

Optical systems

The optical system utilized for immunocytochemistry should be of sufficient quality to allow the clear distinction between a positive stain and the negative background. The use of differential interference contrast optics (DIC or Nomarski) is highly suitable for immunocytochemistry. These optics are particularly helpful in visualizing the fine deposits of immunoperoxidase. Fluorescence ICC requires more expensive microscopic equipment.

Current uses for ICC

The *in situ* visualization of antigens in tissues and individual cells has been a major addition to the technical armamentarium of pathologists, immunologists, histologists and anatomists (especially neuro-anatomists), plant biologists and more recently molecular biologists.

Immune markers have been used to investigate leukemias, tissue neoplasms (for example, antikeratin staining for thymomas and epithelioma) and the localization of peptide hormones in tumors. Carcino-

embryonic antigen (CEA) is used for colonic adenocarcinomas and an epithelium membrane antigen (EMA) is used for breast carcinomas, as are estrogen receptors.

In recent years immunocytochemistry has become an important addition to diagnostic cytology, however the indications for ICC are not at present as broad in this area as they are for histopathology. As the available cell markers increase in number the use of ICC for cytology will also increase. A primary need is an ICC marker that defines a difference between benign and malignant cells; a combination of immunocytochemistry and DNA hybridization technology may provide this first.

Quantitation

Most of the information generated with immunocytochemistry involves some form of quantitative statement. Although these are usually highly subjective judgements there is a need for more quantitative information. Theoretically three forms of information can be derived: 1) number of immunopositive elements in a defined area; 2) the amount of staining present; and 3) the amount of antigen present in a defined compartment. Microfluorimetry and microdensitometry have been attempted. Problems that can arise with quantifying antigen in tissues result from: 1) the difficulty in preserving all of the epitopes; 2) the inadequate penetration into the tissues of the antibodies used in the detecting agents; 3) steric hindrance reducing the efficiency of the detection procedures. It must be remembered it is only the top of a section that is stained; this affects quantitation. New laser-equipped fluorescence analysis systems which generally use argon ion lasers can analyze with remarkable accuracy the emissions from proteins antibody labeled with fluorescein isothiocyanate (FITC) and R-phycoerythrin. These newer techniques suggest that highly accurate quantification can be achieved with immunocytochemistry, and this area of immunology may be quickly transformed into a truly quantitative system.

In situ hybridization techniques

Immunocytochemistry does not provide detailed information about expression of a protein since rapid secretion or intracellular degradation may cause negative immunocytochemical results despite high levels of protein synthesis; *in situ* nucleic acid hybridization corrects some of these problems. The combination of *in situ* hybridization and immunocytochemistry permits further insights into the mechanisms of regulation of gene expression at the single

cell level and should find application in both research and in diagnostic histopathology.

See also: Antiserum; Autoantibodies, tests for; Electron microscopy, immunological applications; Enzyme labeling of antibodies and antigens; Flow cytometry; Fluorochrome labeling; Leukemia; Monoclonal antibodies (mAbs); Bacterial immunoglobulin-binding proteins; Tumor antigens; Valency of antigens and antibodies.

Further reading

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IMMUNODEFICIENCY, ANIMAL MODELS OF

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The effects of mutations at specific genetic loci give considerable insight into many areas of biology. This is particularly true for the genetically determined syndromes of human immunodeficiency which can cause partial to severe impairment of immune function, often with a lethal outcome. While important information has come from analyzing tissues from these patients, animal models of immunodeficiency are crucial for understanding how genetic abnormalities affect immune competence. Studies on the laboratory mouse have provided much of our knowledge on the normal function of the immune system, so it is not surprising that this species also provides the most widely used animal models of immunodeficiency. This system allows for the production of large numbers of genetically identical animals bearing distinct defects in immune responsiveness.

In the past, most of these models came from spontaneously occurring mutations. Fewer examples derive from selected breeding of an outbred stock to obtain alterations in particular immune responses. The most well studied of these are the Biozzi lines originally selected for high and low antibody responses to sheep erythrocytes, and lines selected for high- versus low-affinity antibodies to protein antigens. Other examples of altered immune competence occur in strains such as the NZB, which has severe autoimmune and immunodeficiency characteristics. However, such systems based on polygenic differ-

ences from normal inbred mouse strains are difficult to study due to the magnitude of the genetic changes involved. On the other hand, spontaneous mutations can generate immune abnormalities due to single mutant genes which are generally easier to identify and backcross on to other genetic backgrounds. These offer the greatest opportunity of identifying the gene or gene product involved and the possible identification of homologous loci in humans.

Among the hundreds of known mouse mutants, many affect immune competence. In some, the primary manifestation is in the immune system, but the majority involve changes in other organs which were the basis for their original isolation (e.g. coat color). Only two (*nu* affecting the thymus and *Dh* affecting the spleen) actually result in the absence of a lymphoid organ. The remainder are the result of intrinsic stem cell abnormalities (e.g. *scid*) or of defects in essential growth factors (e.g. *Sl* or *op*). Alternatively, defects in particular cell functions can cause abnormal responses to certain stimuli (e.g. *lps*), while others can be secondary to hormonal alterations in the animal (e.g. *ob*). Finally, deficiency in the complement components C5 and C4 can occur in some mouse strains due to genes encoded on chromosome 2 (*Hc*) and 17 respectively.

This entry will briefly review the genetically determined animal models of immunodeficiency. More detailed descriptions of these and other mutants can be found in the Further reading listed

below. Details of the athymic nude mutant and immunodeficiency associated with autoimmune diseases will be discussed elsewhere. It is important to note that, apart from the inherited defects presented here, immunodeficiency can be acquired by infection of the host with a variety of microorganisms. This can extend to responses against heterologous antigens as well as those of the infecting organism and is seen particularly in infection with the parasitic protozoa such as *Trypanosoma cruzi* and *Plasmodium* as well as of course in the context of human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS). Indeed, there is growing interest in generating models of AIDS by infection of experimental animals with various retroviruses. Retroviruses of both the onco- and lentivirus subfamilies can induce immunodeficiency syndromes in mice (e.g. MAIDS), cats (feline leukemia virus), chickens (avian leukosis virus) and monkeys (simian immunodeficiency virus). Furthermore, some murine models of immunodeficiency are being adapted to develop systems for modeling HIV infection *in vivo* (e.g. SCID-hu chimeras) and to study the opportunistic infections which ultimately are responsible for mortality in these patients. Finally, two important advances have occurred over the last few years. Firstly, with the increased application of molecular methods, many of the naturally occurring mutations in mice which affect immune competence have been localized to a specific gene. Structure-function studies of the proteins they encode have now been performed and, in some cases, human homologs of these genes and their defects have been found. Secondly, there has been a revolution in the development of novel, immunodeficient mice by the use of transgenic and in particular 'knockout' technology. These techniques have radically altered our approaches to the generation and use of immunodeficient animal models and hold the key to developing ever more useful and clinically relevant models of human disease.

Primary defects in immune competence

Severe combined immune deficiency

Mice homozygous for the autosomal recessive mutation 'severe combined immune deficiency' (*scid*) are the result of a random mutation occurring in the CB-17Icr strain. This was first identified by the absence of serum immunoglobulins in affected animals and maps close to the mahagenoid locus and centromeric to the immunoglobulin λ light chain locus on chromosome 16. Adenosine deaminase (ADA) levels are unaffected by the mutation (the gene encoding ADA is on chromosome 2) and *scid*

mice share some characteristics with human SCID patients with normal ADA. Further analysis of this mutant showed that both T and B lymphocyte lineages were functionally deficient in *scid* mice. In addition to hypogammaglobulinemia, *scid* mice do not mount humoral immune responses to either T cell-dependent or independent antigens *in vivo* and are unable to reject histoincompatible skin grafts. In culture, spleen or lymph node cells from *scid* mice do not proliferate in response to T or B cell mitogens and do not develop proliferating or cytotoxic T cells during one-way mixed lymphocyte reactions.

The absence of functional responses from both T and B cell compartments is reflected in the lymphoid organs *per se*. T cell development is arrested at a point equivalent to 14–15 days of gestational age, with 98% of thymocytes being CD4⁺/CD8⁺ (compared to only 2% in immunocompetent CB-17 controls), lacking CD3 and expressing low levels of CD2. In the spleen, a few Thy-1⁺ cells are present but again CD4- or CD8-bearing cells are virtually absent. Development of the B cell lineage is arrested at the pro-B cell stage prior to the expression of cytoplasmic or surface immunoglobulin. However, while pre-B cells bearing cytoplasmic μ chain are undetectable *in vivo*, they can be generated from *scid* bone marrow *in vitro*, albeit at a reduced frequency. Interestingly, natural killer (NK) cell differentiation does occur in *scid* mice, with normal numbers of both bone marrow progenitors and functional cytotoxic cells in the periphery. The latter express the NK cell makers NK2.1 and ASGM1 but not T cell subset markers such as CD4/CD8, again suggesting that NK cell development is independent of mature T cell differentiation. NK cells in *scid* mice provide an alternate (i.e. non-T cell) source of interferon γ (IFN γ) which can mediate macrophage activation and partial resistance to infection with intracellular pathogens such as *Listeria monocytogenes* and *Toxoplasma gondii*. This natural resistance pathway may help to explain why, despite the loss of T and B cell function, *scid* mice can usually be maintained under pathogen-free conditions for over a year.

The abrogation of cellular development in *scid* mice is restricted to cells of the lymphocytic series. Myeloid and erythroid lineages are apparently unaffected by the mutation and mast cell progenitors are present in the bone marrow of homozygotes. In contrast, *scid* mice do not develop increased mast cell numbers *in vivo* following challenge with *Nippostronglyus*, presumably due to a defect in cytokine production as a result of their T cell deficiency. Normal numbers of macrophages are found in the peritoneal cavity, spleen and liver which produce interleukin 1 (IL-1) and tumor necrosis factor α

(TNF α) *in vitro* and can be activated for tumoricidal activity and enhanced expression of class II histocompatibility molecules when incubated with cytokines such as IFN γ . The normal expression of class II histocompatibility antigens in *scid* tissues differentiates this defect from a subset of human SCID patients where deficiency of a DNA binding protein for the X box HLA class II promoter region abrogates expression of these molecules.

The *scid* mutation arrests the development of T and B lymphocytes while maintaining the normal stroma and environment of the lymphoid organs. Bone marrow stem cells from *scid* mice are unable to restore lymphoid function in lethally irradiated normal CB-17 recipients, but, conversely, CB-17 stem cells can reconstitute the lymphoid defect in the *scid* mouse. The *scid* defect therefore results in an intrinsic abnormality in lymphoid stem cells. The molecular hallmark of the *scid* mouse is the absence of lymphocytes bearing antigen-specific receptors. Abnormal T cell receptor or immunoglobulin gene rearrangements are observed in transformed lymphoid cells, spontaneous thymomas or long-term bone marrow cultures derived from *scid* mice. The joining of appropriate coding regions is either undetectable or occurs with massive deletions involving V(D)J gene segments. This suggests that *scid* mice have an active but defective system for the recombination of antigen-specific receptor genes. The inability to express a productively rearranged receptor would presumably be lethal for developing cells, accounting for the selective arrest in lymphocyte development. However, if this process is bypassed by transgenic introduction of already rearranged T cell receptor or immunoglobulin (Ig) genes, mature T and B cells can now be detected, although in the case of the B cell compartment their expression in peripheral lymphoid tissue is still reduced. Recently, the molecular basis of the SCID mutation has been identified as a deficiency in activity of a DNA-dependent protein kinase (DNA-pk). This is a member of the PI3 kinase family which serves in V-D-J recombination and double stranded DNA break repair. It is currently believed that SCID cells have a nonsense mutation in the catalytic subunit of this enzyme, resulting in normal protein expression but impaired function. However, this defect may not account for the increased radiosensitivity of human autosomal SCID cells which can express normal DNA-pk activity.

Motheaten

Mice homozygous for the 'motheaten' (*me*) and 'viable motheaten' (*me^v*) genes have proved difficult to study due to their short lifespan (3–6 weeks). Both strains usually die from pneumonitis characterized

by infiltration with macrophages, neutrophils and lymphocytes. However, the earliest features of the mutation are observed in the skin, where aggregates of neutrophils result in the characteristic motheaten appearance. The skin is also involved later when there is a reduction in the number of major histocompatibility complex (MHC) class II⁺ Langerhans cells. Although the extent of immunodeficiency shows some minor differences between *me* and *me^v* strains, motheaten mice in general show severely reduced responses to T cell-dependent and -independent antigens, T and B cell mitogens as well as impaired levels of cytotoxic T cell and NK cell activity. Thus, unlike *scid* mice, affected animals have loss of B, T and also NK cell function and represent one of the most severe animal immunodeficiency models.

A prominent histological feature of this mutant is the involution of the thymus and depletion of cortical thymocytes at approximately 4 weeks of age. Cell transfer studies show that prothymocytes are present in normal numbers in the bone marrow of *me^v* mice but are unable to migrate to the thymus and have impaired chemotactic responses to thymic culture supernatants *in vitro*. In contrast to atrophy of the thymus, splenomegaly occurs due to a shift in hematopoiesis from the bone marrow to the spleen. This is mediated by an increase in myelo- and erythropoietic activity at the expense of normal lymphoid development with depletion of the periarteriolar T cell areas.

Although B cell numbers are reduced in the spleen, those remaining are abnormally enriched for cells of the Ly-1 phenotype. This lineage is associated with the production of autoantibodies (such as in the NZB strain) and represents only a minor fraction of B cells in the normal host. In contrast, greater than 50% of hybridomas derived from *me^v* spleen cell show autoreactivity to self antigens on thymocytes, erythrocytes and skin, consistent with the immune pathology observed in the mutation. Motheaten mice also exhibit hypergammaglobulinemia with a skewed B cell repertoire and raised circulating levels of B cell maturation promoting cytokines.

Motheaten mice show profound changes in bone marrow function and do not produce lymphocytes under Whitlock–Witte culture conditions, nor stem cells or neutrophils under Dexter culture conditions. In fact, adherent stromal cells from motheaten mice actively inhibit normal bone marrow cultures by direct cell contact, suggesting that the *me^v* defect is primarily due to alterations in the lymphoid/myeloid microenvironment. However, this has recently been contradicted by the finding that aberrant lymphoid development can occur in normal mice given *me^v*

bone marrow, suggesting an additional intrinsic T and B cell defect.

Molecular examination of cells from motheaten mice has helped to explain this complex phenotype and identified the absence of the tyrosine phosphatase SHP-1 (previously known as PTP-1c, SHPTP1 or HCP) as the primary defect in this mutant. SHP-1 is a key negative regulator of tyrosine phosphorylation events which drive receptor-mediated signaling in many immune cells, including macrophages and lymphocytes. Cells from motheaten mice have a frameshift mutation in SHP-1 resulting in a null phenotype associated with uncontrolled tyrosine phosphorylation and activation of key signaling molecules, including the CSF-1 receptor in macrophages and the src family kinases lck and fyn in T cells. As a consequence, motheaten T cells show a defect in apoptosis which may underlie the pneumonitis observed in these mice which includes actively cycling T cells. However, the multiple tissue pathologies seen in these mice is not solely due to a T and B cell-mediated autoimmunity, as previously suggested. Breeding of the motheaten strain on to the RAG-1 background deletes all antigen-specific lymphocytes but does not abrogate the inflammatory lesions, splenomegaly and elevated hematopoiesis which characterizes the motheaten mutant *in vivo*.

X-Linked immunodeficiency

Genes localized to the X chromosome are essential to the development of the immune system, and in humans at least six immunodeficiency syndromes have been mapped to this chromosome. In mice, the most established X-linked abnormality is seen in the CBA/N strain which bears a recessive mutation at the *xid* gene. This results in abnormal B cell development seen in homozygous female or hemizygous male mice. CBA/N mice have a reduced number of B cells and lack a mature population of Lyb-3⁺5⁺7⁺ cells expressing sIgM^{lo}/sIgD^{hi} and peritoneal Ly-1⁺ B cells. As a result, serum levels of IgM and IgG3 antibodies are reduced, and, most characteristically, CBA/N mice are unable to respond to Ti-2 antigens such as pneumococcal polysaccharide. CBA/N B cells can be activated *in vitro* with soluble, mitogenic preparations of anti-Ig antibodies to enlarge and increase expression of MHC class II antigens, but will not proliferate. Furthermore, these cells do not respond appropriately to IL-5, IL-10, lipopolysaccharide (LPS) and cross-linking of either CD38 or CD40. Long-term cultures of CBA/N bone marrow show normal numbers of myeloid and B cell lineage colonies, which show enhanced kinetics of development. Contrary to earlier hopes, the residual B cells present

in CBA/N mice may not be analogous to the immature B cells seen during normal neonatal development. B cell development is unusually T cell dependent as a result of the *xid* mutation and few B cells are found in athymic *nu/nu-xid* double congenics, giving a phenotype much more akin to human X-linked agammaglobulinemia.

In addition to affecting humoral immune competence, the CBA/N defect may also have more subtle effects on other cell types. Macrophage and NK cell-mediated cytotoxicity are normal, but altered T cell and accessory cell functions have been described. CBA/N mice exhibit several functional consequences as a result of their humoral immunodeficiency. Affected animals have low levels of naturally occurring tumor-specific antibodies and show enhanced susceptibility to inoculation with transplantable tumors. They are also more susceptible to infection with organisms such as *Salmonella typhimurium*, *Plasmodium* and *Giardia*.

The *xid* locus was originally linked to at least one of the XLR (X-linked, lymphocyte-regulated) sequence family which encode RNA transcripts expressed only in T and B lymphocytes. Evidence that changes in intracellular signaling account for the B cell abnormalities observed in affected mice began with the finding that CBA/N cells have reduced inositol phospholipid metabolism and Ca²⁺ mobilization following triggering with anti-Ig. CBA/N B cells also differ in the expression of glycoproteins noncovalently linked to surface IgM and IgD. Together with the finding that these cells respond normally to agonists which act further down the signaling cascade (such as calcium ionophore and phorbol esters), the functional B cell defect in CBA/N mice was suggested to act at a relatively early process in B cell membrane signaling. This has been borne out by the demonstration that *xid* mice exhibit a point mutation in Bruton's tyrosine kinase (Btk), a member of the Btk/Tec family of nonreceptor tyrosine kinases. The *xid* mutation occurs in the pleckstrin homology (PH) domain of the enzyme which is likely to mediate the interaction of Btk with other signaling molecules such as PI3, PIP2 and protein kinase C. Mutations in Btk form the structural basis for the human XLA phenotype but these can also occur at other sites in the molecule such as in the kinase domain *per se*.

Another mutation at the X chromosome, *scurfy* (*sf*), results in dermatological and hematological abnormalities as well as X-linked immunoproliferative disease. The *scurfy* locus occurs in an X chromosome segment which is homologous to the human chromosome region encoding the gene for the Wiskott-Aldrich syndrome protein (WASP) and is inseparable from the murine WASP locus by backcross

analysis. Wiskott–Aldrich syndrome is the result of a mutation in human WASP, a proline-rich protein involved in regulation of the actin cytoskeleton and possibly also tyrosine kinase-dependent cell signaling events. Together, the *scurfy* and *xid* mutants share some phenotypic features with this syndrome which is characterized by impaired responses to polysaccharide antigens along with eczema and platelet abnormalities.

Responsiveness to bacterial lipopolysaccharide

Lipopolysaccharide (LPS) derived from the outer membrane of gram-negative bacteria has profound effects on mammalian inflammatory and immune responses. This is primarily mediated by the lipid-A component which has pleiotropic actions ranging from initiation of fever and endotoxic shock to the activation of host macrophages, lymphocytes and fibroblasts. Mutation at the *lipopolysaccharide response* (*Lps*) locus on chromosome 4 has occurred independently in C3H/HeJ and C57BL/10ScN mice, resulting in impaired immunological responses to LPS. Thus, C3H/HeJ (*lps*^d) mice have increased resistance to endotoxin shock induced by administration of LPS *in vivo*, correlating with the impaired generation of inflammatory cytokines such as IL-1, IL-6 and TNF α . Based on these results, one would predict increased resistance of nonresponder mice to the lethal effects of infection with gram-negative bacteria. In contrast however, C3H/HeJ mice have greater susceptibility to infection with *Escherichia coli* and *Salmonella* and can be protected from lethal infection by exogenous administration of purified TNF α and IL-1. Thus, early production of these cytokines is critical for host resistance and activation of macrophages for microbicidal activity.

Numerous studies have also demonstrated the effect of this mutation *in vitro*, with particular emphasis on the impaired mitogenic responses by C3H/HeJ B cells. Macrophages from these mice also have multiple defects in LPS-mediated responses, including induction of tumoricidal and microbicidal activities. Unresponsiveness is specific for LPS, in that C3H/HeJ macrophages respond normally to other stimuli such as the combination of phorbol myristate acetate and calcium ionophore or gram-positive bacteria. C3H/HeJ mice have a higher incidence of mammary tumors and increased susceptibility of their macrophages to virus infection *in vitro* due to impaired endogenous interferon production. Recently, the structural gene cluster for IFN α (*Ifa*), which was also mapped to chromosome 4, has been segregated from the *Lps* locus using recombinant inbred mice. This suggests a causal link between impaired LPS responsiveness and interferon pro-

duction rather than the presence of the *lps* gene as part of the *Ifa* gene cluster.

While the functional effects of the *Lps* gene have been extensively documented, the biochemical basis for the defect is still unclear. Studies with radiolabeled LPS demonstrate equivalent binding to cells of both LPS responder and nonresponder mice, and normal expression of the 80 kDa LPS binding receptor, suggesting a deficit in signal transduction (rather than ligand binding) in the *lps* mutant. This is supported by observations that, unlike normal cells, C3H/HeJ macrophages do not undergo intracellular translocation of protein kinase C from the cytoplasm to the actin cytoskeleton following interaction with LPS. In addition, LPS is also unable to induce extracellular signal regulated kinase (ERK2) activity in nonresponder macrophages, and thus triggering of the ras/MAPK signaling pathway for cell activation does not occur. Finally, it is important to note that mice bearing the *lps*^d allele can respond to LPS derived from some rough strain mutants of *Salmonella* and *E. coli* and also lipid-A-associated proteins. The presence of the latter depends upon the method of LPS purification and may account for responses of C3H/HeJ mice to some LPS preparations.

Immunodeficiency with defects in other organ systems

Among the large number of mutations affecting coat color in the mouse, several have been found to alter immune competence. The most extensively studied of these are a series of mutations at the 'beige' (*bg*) locus on chromosome 13. A primary histological feature of *bg/bg* mice is the presence of abnormal lysosomal granules in several cell types and lack of elastase and cathepsin D. This is associated with defective vesicular transport and fusion between lysosomes and late endosomes. This mutation is considered a model of the Chediak–Higashi syndrome in humans which shares the enzyme and lysosomal granule abnormalities and is predisposed to lethal pyogenic infection. Indeed, a candidate gene for the beige mutant (*Lyst*) has been identified on mouse chromosome 13, which has a closely related human homolog on the syntenic region of human chromosome 1q. *Lyst* has been found to contain a frameshift mutation in a child affected with Chediak–Higashi syndrome, whereas a 5 kb deletion within the gene is observed in the beige mutant mouse.

The beige mutation provided the first experimental model of impaired NK cell function. NK cells are present in normal numbers in *bg/bg* mice but are unable to kill susceptible tumor target cells due to a defect in the postrecognition process of target lysis.

These mice have decreased resistance to a variety of infections and transplantable tumors and are frequently used to determine the importance of NK cells in host resistance. Unfortunately, while initial studies described this mutation to be selective for NK cells, more recent work has identified abnormalities in a variety of other immune parameters including development of cytotoxic T cells, macrophage tumoricidal activity and neutrophil chemotaxis and microbicidal function. Furthermore, the production of IFN γ by NK cells in these mice is reportedly normal. Thus, the *bg* mutation offers a model for the study of NK cell cytotoxic (but not secretory) activity, but the other changes in immune function must be considered before ascribing resistance solely to NK cells.

Two other independent mutations also affecting both lysosomal function and NK cell activity have been described, 'Pale ear' (*ep*) mice can halt the progression but are unable to eradicate infection with *Leishmania donovani*, the causative agent of visceral leishmaniasis, and show defective granulomatous responses in infected tissues. 'Pallid' (*pa*) mice spontaneously develop emphysema and have an inherited deficiency in serum α_1 -antitrypsin and defective lysosomal secretion.

Mutation at the 'agouti' locus (*A*) on chromosome 2 also determines coat color and these mice show enhanced susceptibility to neoplasia, along with reduced humoral and phagocytic functions. These defects correlate with the degree of obesity and raised blood insulin levels in mice heterozygous for the dominant alleles *A^y* and *A^{vy}*.

Mutations at the 'dominant white spotting' (*W*; chromosome 5) and 'Steel' (*Sl*; chromosome 10) loci have multiple effects, including anemia and defects in gametogenesis and pigmentation, but are particularly useful in analysis of mast cell development. Homozygotes or mice bearing compound alleles (e.g. *W/W^v* or *Sl/Sl^d*) have less than 1% of the normal number of tissue mast cells. Passive cutaneous anaphylaxis reactions are absent in *W/W^v* mice but they retain systemic anaphylactic responses, possibly via basophil degranulation. The expulsion of intestinal parasites can be normal or delayed depending on the organism studied. Humoral immune responses are unaffected, but evidence for the predicted effects of mast cell depletion on development of delayed-type hypersensitivity responses is contradictory. Cell transfer studies and the growth of mast cell precursors *in vitro* have suggested two distinct mast cell defects in these strains. *W/W^v* mice lack stem cells which can proliferate and differentiate into mast cell colonies in response to fibroblast-derived growth factors. Similar results have also been described for the mast cell-deficient *mi/mi* mutation (see below). In

contrast, mast cell progenitors are present in *Sl* mutants but connective tissue cell monolayers from the latter are unable to support their development *in vitro*. The *W* locus is now known to encode the receptor tyrosine kinase c-kit, whereas the *Sl* locus encodes its ligand Steel factor (c-kit ligand or mast cell growth factor). In normal mast cells, binding of Steel factor results in autophosphorylation of the c-kit receptor, allowing its subsequent interactions with PI3 kinase and phospholipase C γ 1, two essential mediators of intracellular signal transduction. However, in the *W* mutant this phosphorylation does not occur, preventing engagement of downstream signaling pathways and appropriate cell activation. Mutant mice also display an increased sensitivity to lethal doses of ionizing radiation which may be related to the apoptosis suppressing properties of Steel factor *in vitro*. Interestingly, Steel factor is induced in the gut following challenge with *Salmonella* or cholera toxin whereas *W* mutant mice have increased susceptibility to oral salmonellosis, suggesting that this ligand-receptor interaction is also involved in local protective mechanisms against infection in the intestinal tract.

Immunodeficiency also occurs with genetic abnormalities in other organ systems. These include mutations affecting neurological, skeletal and endocrine development. Mice homozygous for the recessive mutation 'wasted' (*wst*) on chromosome 2 show progressive vacuolar degeneration of neural tissue leading to paralysis and death. This is associated with pronounced immunological changes, include thymic atrophy and reduced numbers of B cells and IgA at mucosal sites. These mice also express altered levels of endogenous cytokines, transforming growth factor β 1 (TGF β 1), IFN γ and IL-2, compared with controls. Presence of the *wst* allele has been correlated with enhanced expression of endogenous murine leukemia virus RNA. However, it is unclear whether this represents a causal or secondary effect of the immune deficit. Also of interest is the abnormally high incidence of chromosomal damage and poor DNA repair seen in lymphoid tissue of *wst/wst* mice. Studies on the *wst* mutation may thus help understanding of the triad of immunodeficiency, neuropathology and abnormalities of DNA repair also characteristic of the human disease ataxia telangiectasia. T cells from these mice exhibit high spontaneous and radiation-induced apoptosis. Other recessive mutations affecting both immune and neurological function include 'lethargic' (*lh*) on chromosome 2 and 'motor end-plate disease' (*med*) on chromosome 15.

Autosomal recessive mutations affecting bone resorption can also impair immune function. Mice

homozygous for the 'gray-lethal' (*gl*), 'oseopetrosis' (*op*), 'osteosclerosis' (*oc*) and 'microphthalmia' (*mi*) mutations (the last also affecting pigmentation and eye size) show alterations in osteoclast numbers or morphology and a range of immune deficits affecting both humoral and cellular responses. Mice homozygous for the *op* mutation also have reduced numbers of macrophages and are unable to synthesize macrophage colony-stimulating factor (M-CSF). Both the osteoclast and macrophage deficit can be partially restored by administration of exogenous M-CSF, suggesting that absence of this growth factor is the primary defect in these mice.

Finally, several mutations causing obesity and noninsulin-dependent diabetes in mice have been examined for immune responsiveness. In particular, the 'diabetes' (*db*) and 'obese' (*ob*) mutants have been demonstrated to have impaired cell-mediated immune responses *in vivo*, possibly associated with increased corticosteroid levels. These deficits are not manifest when lymphoid cells are tested *in vitro*, suggesting a reversible abnormality associated with metabolic changes *in vivo*.

Models of immunodeficiency in other species

In comparison to the murine system, relatively few models of genetically determined immunodeficiency exist in other species. The autosomal recessive *nude* gene has been identified in laboratory rats, resulting in loss of both thymic development and hair. This gene has been backcrossed on to several genetic backgrounds and nude rats have many uses in parallel to studies in athymic mice. Also, the BB rat is an experimental model for insulin-dependent diabetes and one of the sublines from this strain (BB-DP) has a profound reduction in T lymphocytes. These animals have reduced numbers of both CD4 and CD8 lymphocyte subsets and the absence of T cells bearing the RT6 marker. Transfer of BB-DP bone marrow to irradiated normal recipients does not result in development of RT6 positive cells, suggesting an intrinsic defect in T cell development. Consequently, BB-DP lymphoid cells show abnormally low proliferative and cytotoxic T cell responses. The latter may also be explained by subnormal levels of CD8 antigens present on the residual antigen receptor bearing T cells in these animals.

A model of severe combined immunodeficiency (SCID) also occurs in Arabian horses as an autosomal recessive trait. Death due to secondary infection usually occurs within 6 months of age and affected animals show increased susceptibility to opportunistic pathogens such as *Pneumocystis* and

Cryptosporidium. This defect is characterized by lymphoid hypoplasia, hypogammaglobulinemia and the absence of functional T and B cells. Some prothymocytes and phenotypically mature thymocytes can be detected in the thymic remnant, but cortical thymocytes are almost absent. Large granular lymphocytes can be detected in the peripheral blood and develop NK-like cytotoxic activity following incubation with IL-2. Finally, X-linked SCID has also been reported in dogs and is associated with a four base pair deletion in the IL-2 receptor γ gene. Thus these animals share some phenotypic and functional characteristics with the murine and human SCID defects.

Maintenance and use of immunodeficient animals

While some of the immunodeficient mouse strains described above are kept under normal housing conditions, those with severe abnormalities (e.g. *scid*) are frequently kept in sterile or aseptic environments. This can be achieved by providing autoclaved food, bedding and water and covering the sterilized cages with filter bonnets. Alternatively, sterile air laminar flow racks or flexible film isolators can be used for more stringent control of the environment. Infection of immunodeficient mouse colonies with opportunistic pathogens is a serious problem. Of these, mouse hepatitis virus and *Pneumocystis carinii* are particularly severe. Some protection against *Pneumocystis* can be achieved by prophylactic administration of trimethoprim-sulfamethoxazole but this is microbistatic and not microbicidal and therefore requires continual use.

Variation in the extent of immune deficiency can occur among mice of a particular strain. For instance, T cell function can be readily demonstrated in older athymic nude mice or following infection with experimental pathogens. In the case of *scid* mice, some 15% of offspring derived from homozygous *scid/scid* mating will show some serum immunoglobulins, usually accompanied by partial restoration of T cell function. The frequency of such 'leaky' *scid* mice increases with age and the degree of environmental stimulation (i.e. higher in normal versus specific pathogen-free conditions) and is overcome by routine screening of serum immunoglobulin levels prior to use.

As seen above, several animal immunodeficiency models share characteristics with selected human immunodeficiency syndromes (Table 1). Others have no direct homology to specific human diseases but instead provide valuable insight into the processes controlling normal immune function. In addition to providing information on the basic mechanisms of

Table 1 Common murine models of immunodeficiency

Name	Gene symbol	Chromosome	Primary characteristics	Similar to human
SCID	<i>scid</i>	16	T/B cell deficiency	ADA-positive SCID
Mothaten	<i>me</i>	6	Impaired T/B/NK cell function and hypergammaglobulinemia	
Viable motheaten	<i>me^v</i>			
Nude	<i>nu</i>	11	Absence of thymus	DiGeorge syndrome
Lipopolysaccharide	<i>lps</i>	4	Impaired responses to LPS	
X-linked immunodeficiency	<i>xid</i>	X	Absence of mature B cells; impaired response to T1-2 antigens	Wiskott–Aldrich syndrome
Complement deficient	<i>Hc</i>	2	Absence of C5	
Beige	<i>bg</i>	13	Impaired NK cell function	Chediak–Higashi syndrome
Dominant spotting	<i>W</i>	5	Mast cell deficiency/anemia	
Steel	<i>Sl</i>	10	Mast cell deficiency/anemia	
Wasted	<i>wst</i>	2	Thymic atrophy, abnormal DNA repair	Ataxia telangiectasia

immune development (e.g. the recombinase system in *scid* mice), these models have been used to address many other questions. For instance, animals with selected deficiencies can replace the experimental ablation of particular lymphoid populations in normal animals. Thus the *scid* mutation may provide a more defined model of B cell deficiency than normal mice treated with anti-IgM antibodies from birth.

A common use of immunocompromised animals has been in the analysis of resistance to neoplasia or infection. The original finding that human or murine tumors could be grown in athymic nude mice has provided an enormous literature on the tumorigenicity and metastatic potential of various tumors *in vivo*, as well as models to assess the efficacy of new chemotherapeutic agents. Experimental infection of normal versus immunodeficient animals can identify the pathways of immunity relevant for resistance to particular pathogens. For instance, studies in nude or *scid* mice have demonstrated the role of T cells in development of sterilizing immunity to many facultative or obligate intracellular pathogens such as *Listeria* and *Leishmania*. Immunodeficient animals also allow the study of opportunistic pathogens which do not infect the immunocompetent host. For instance, new models of infection with organisms including *Pneumocystis* (an important AIDS-related pathogen), *Borrelia burgdorferi* (the causative agent of human Lyme disease) and reovirus have been developed in immunodeficient *scid* mice. In view of the life-threatening opportunistic infections seen in AIDS patients and other immunodeficiency states, these may provide models for screening of new therapeutic agents as well as for studying the pathogenesis of infection in the immunocompromised host.

Immunodeficient animals also provide ideal recipi-

ents for *in vivo* reconstitution with different lymphoid or myeloid cell types. These range from the routine *in vivo* passage of hybridomas to more complicated transfers of specific cell populations and their effects on resistance to infection or antigenic challenge. The inability of some immunodeficient animals to reject foreign tissue has been taken to its logical extreme by the passage of human cells into immunodeficient mice. This was originally attempted over a decade ago in athymic mice given grafts of human skin. More recent interest has come from reports of the maintenance and growth of human lymphoid cells in immunodeficient *scid* mice. The presence of viable T lymphocytes in such *scid* hu chimeras has been used to set up experimental models of HIV infection. The similarity of this system to the human disease is still under scrutiny, but is being used to assess the *in vivo* efficacy of anti-HIV drug treatments. Chimeric *scid*-hu mice are also now susceptible to infection with Epstein–Barr virus (EBV) and may provide a unique opportunity to study the mechanisms of EBV-induced lymphoproliferative disease in an experimental animal.

Targeted generation of novel immunodeficiencies

Since the early description of the athymic nude mutation, animal models of immunodeficiency have continued to provide important insights into the immune system. Most have arisen by spontaneous mutations which have then been tested for their effects on immune function. In addition to the new mutants which will undoubtedly be discovered, it is now possible to selectively induce genetic immunodeficiencies *in vivo*. Methods to achieve this fall into

three major groups. The first example is the backcrossing of mouse strains with independent mutations to achieve double (e.g. *nu/xid*) or triple (e.g. *nu/xid/bg*) congenic strains. This can result in either additive effects of each independent characteristic or apparent synergy in which more severe defects are observed. In addition, existing mutants can be treated to delete residual immune responses for specific tasks. Thus the ability of SCID mice to permit growth of human cells *in vivo* can be increased by antibody-mediated depletion of NK cells prior to engraftment.

By far the most important advances have come from molecular manipulation of the mouse genome either by direct transgenesis or gene deletion (knockout) technology. Transgenic insertion of new or modified genes allows removal of specific cell types or neutralization of function of key molecules in the immune system. Thus, the herpes simplex type 1 thymidine kinase gene (HSV-1-tk) can be transgenically introduced in the mouse genome using lymphoid cell specific promoters. Dividing lymphoid cells expressing HSV-1-tk are then selectively ablated in adult mice by administration of nucleoside analogs (such as ganciclovir) which are converted to toxic metabolites by viral but not host thymidine kinase. This procedure has been used to delete T and B cells in lymphoid organs of transgenic mice *in vivo*. Other methods using constitutive transgenic expression of the A chain of ricin or diphtheria toxins are also being examined, but the ability to control when cell ablation occurs by altering the dose and timing of analog delivery has multiple advantages. Alternatively, overexpression of normal genes by transgenesis can result in novel models of immune deficiency. For example, mice expressing a human CD3 ϵ transgene show ablation of both T lymphocytes and NK cells *in vivo*, while overexpression of the human IL-2 receptor γ chain abolishes NK cells and epidermal dendritic cells in homozygous mice. While these methods rely on insertion of functional genes, the alternative approach of overexpressing functionally inactive genes gives rise to 'dominant negative mutants'. This allows selected loss of function mutants where, for example, mice expressing an inactive IFN γ receptor α chain under a macrophage-specific promoter have a functional deficit in interferon responsiveness in this cell type but not others.

However, by far the most powerful models have come from the generation of targeted gene deletion or knockout mice. Here, point mutations, or more usually larger sequences of nonsense DNA (such as the selectable marker conferring resistance to neomycin), are targeted into particular genes in pluripotent mouse embryonic stem cells, thus entering the

germ line. New methods for selecting homologous recombination events rather than simply random insertion of this material (which is the most frequent result of transgenesis) now allows the potential for selective disruption of almost any mouse gene of interest. This approach was first used in the immunological setting to ablate expression of β_2 -microglobulin *in vivo* resulting in loss of CD4 $^+$, CD8 $^+$ T cells. Since the early 1990s the number of genes deleted in this manner has increased dramatically. Improved methods for growth and manipulation of embryonic stem cells *in vitro* and selection of targeted events has meant that this technology is becoming more feasible for many laboratories. Nevertheless, this approach still requires considerable investment in both molecular expertise and animal husbandry to generate founder lines and sufficient stock animals for experimentation. An increasing trend is for new knockout strains to be donated to public repositories, such as the Induced Mutant Resource in the Jackson Laboratories, Bar Harbor, USA, allowing access for investigators who are unable to generate their own animals.

The knockout approach has several distinct advantages over previous methods of studying immune deficiency. Firstly, it is no longer necessary to rely on identification of spontaneously occurring mutants or selective breeding. Secondly, it affords complete ablation of the encoded protein, compared with the risk of incomplete removal of particular cell types or cytokines in antibody-mediated depletion studies *in vivo*. Furthermore, it allows functional deletion of key intracellular proteins (such as kinases or phosphatases involved in lymphocyte activation) which cannot be inhibited by antibodies *in vivo*. Finally, it allows a much more responsive approach to studying the molecular basis of clinical immunodeficiency (Table 2). Thus genes which have been implicated in human syndromes such as p47phox and ZAP-70 (identified as the molecular basis of CGD and a rare form of autosomal SCID, respectively) can be deleted

Table 2 Examples of targeted gene deletions in mice which mimic primary human immunodeficiencies

Deleted murine gene	Human disorder
Bruton's tyrosine kinase	Bruton's X-linked agammaglobulinemia
CD40 ligand	X-linked hyper-IgM syndrome
Janus kinase (JAK-3)	Autosomal recessive SCID
ZAP-70 kinase	
IL-2 receptor γ chain	X-linked SCID
p47 phox	Autosomal recessive CGD
gp91phox	X-linked CGD

from the mouse genome to ask whether this gives the same phenotype as the human disease. This in turn generates a novel animal model for the study of immune development, susceptibility to infection and new therapeutic strategies. Conversely, the phenotype of knockout mice can give new insights into the molecular basis of clinical immunodeficiency. This is illustrated in the contrasting phenotypes following deletion of the IL-2 (normal T cell development) versus IL-2 receptor γ chain genes (resulting in murine SCID) and the identification of the latter as the basis of human X-linked SCID.

Nevertheless, the knockout approach has several inherent disadvantages. Genes which are transcriptionally suppressed or essential for early events in embryogenesis may be difficult to inactivate with this approach. Fortunately, this is less of a problem in studies of the immune system because, if shielded from the risks of infection, most animals with even massive defects in immune competence can mature to adulthood to allow subsequent experimentation. The most commonly cited problem of knockout mice is the issue of compensation. Thus, animals which have developed entirely without a particular component often show overactivity of other immune functions to 'compensate' for this defect, either spontaneously or in response to microbial challenge. In contrast, selective depletion by antibodies in adult mice results in an immediate loss of function and there is usually insufficient time to generate alternative immune pathways. In truth, while compensatory responses may make interpretation of experimental data more difficult, they actually give valuable insights into the plasticity of the immune system which can be relevant to the problems of primary immune deficiencies in humans.

Future perspectives

The study of animal models of human immunodeficiency is undoubtedly in its most exciting phase. The ability to generate genetically targeted deficiencies in

mice has revolutionized this field and provided critical models for other immunologists interested in the molecular basis of normal immune function. One of the most promising new areas is in the generation of inducible knockouts (such as with the recently described Cre-lox system) in which normal development will occur but, at a selected time in ontogeny, the gene of interest can be functionally ablated. Also, transgenic mice expressing specific antisense RNA constructs may allow direct inhibition of a selected gene product in a controlled manner. Finally, as new human genes involved in host immunity are discovered, they can be tested directly for their biological role in transgenic and knockout mouse systems. Hopefully, these new approaches, as well as further investigations of existing mutants, will increase our understanding of normal immune function as well as assist in the development of effective therapies for patients who are afflicted with these defects.

See also: Autoimmune diseases; Congenic mice; Immunodeficiency, primary; Immunodeficiency, secondary; Mouse inbred strains; Nude (athymic) mice.

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IMMUNODEFICIENCY, PRIMARY

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The first patient with hypogammaglobulinemia was diagnosed by Bruton in 1952 soon after the introduction of serum protein electrophoresis. At about the same time, severe combined immunodeficiency (SCID) was described in Switzerland and

has subsequently been subclassified into a number of different disorders. In 1965, DiGeorge showed that the thymus was important in humans by describing an athymic infant with recurrent infections.

The term 'primary' is taken to mean that the immunodeficiency is not secondary to any known infection, lymphoreticular malignancy, or drugs. Whilst most 'primary' disorders are due to single gene defects, 'common variable' immunodeficiency and selective immunoglobulin A (IgA) deficiency, which represent the most common disorders, are probably based on a polygenic mechanism. There are also some metabolic disorders where a variety of cell types, including lymphocytes, are affected. In such cases, the distinction between 'primary' and 'secondary' is somewhat arbitrary.

Selective T cell deficiencies

Thymic aplasia

In 1965, DiGeorge described a child without a thymus or parathyroid glands due to congenital malformation of the third and fourth pharyngeal pouch. Most patients have microdeletions at chromosome 22q11, or less frequently at 10p13, where there are probably genes which control the development of the neural crest, from which cells migrate to the pharyngeal pouches. The disorder is familial in a few cases and the prevalence is about 5 per 100 000 children. A spectrum of severity has been recognized, with some children lacking all thymic and parathyroid tissue, sometimes with additional abnormalities in midline structures such as the lips, palate, heart and major thoracic vessels, while others retain some thymic tissue and have mild symptoms. In the severe type, there is a virtual absence of circulating T lymphocytes, sometimes with a relative expansion of B cells. These patients are capable of making antibodies after immunization, although it is unclear whether the quality or quantity of antibodies produced is normal. In general, there is a raised incidence of chronic cytomegalovirus and varicella infection, and some affected children have developed systemic vaccinia infections after immunization. Recurrent bacterial infections are unusual. Autoimmune phenomena, such as hemolytic anemia and vitiligo, may occur.

Early attempts at therapy focused on repleting thymic hormone with fetal thymic grafts, and more recently purified thymic hormone; there is no good evidence that either of these strategies work. Fortunately, most patients have a remnant of thymic tissue left, and the numbers of circulating T cells gradually rise to normal during the first few years of life. There is a possibility that the majority of these lymphocytes are derived from the gut-associated lymphoid tissue.

CD3 defects

Two siblings with autosomal recessive inherited

mutations in the CD3 γ chain gene have been reported, one dying from severe autoimmune disease in infancy, while the other remains healthy at 10 years; this was associated with about 50% of normal expression of the CD3/TCR complex. One child with recurrent lung infections is reported with inherited defects in the CD3 ϵ gene. Since the CD3 ϵ chain is crucial for normal T cell development, the residual 10% of normal expression of the CD3/TCR complex in this patient was presumably enough to provide partial T cell immunity.

Peptide transporter (TAP) defects

A few patients, some apparently not prone to infection, have been reported with very low expression of HLA class I on lymphocytes. This disorder was originally called the 'bare lymphocyte syndrome', but this term should be abandoned because it is sometimes confused with major histocompatibility complex (MHC) class II deficiency (see below). Two siblings with bronchiectasis were recently found to have mutations in the TAP2 gene, causing inefficient transportation of class I to the trans-Golgi compartment. Both had deficient natural killer (NK) cell activity, but no obvious predisposition to viral infection.

Severe combined immunodeficiency (SCID) (Figure 1)

The first cases of SCID were described in Switzerland in the 1950s, hence the original term 'Swiss type agammaglobulinemia', although later it became apparent that cellular immunity was severely compromised. A better understanding of the condition had to await the discovery of T and B lymphocyte markers in the 1970s. Since then the condition has been subclassified, and in most cases the disorder is understood at a molecular level. SCID occurs in about 1:50 000 live births. The syndrome is characterized by severe opportunistic fungal, viral, protozoal and bacterial infections. Affected infants fail to thrive, have chronic diarrhea due to enteric viruses and parasites, have extensive mucosal candidiasis, and develop *Pneumocystis carinii* pneumonia. These clinical features, together with the finding of very low numbers of circulating T cells, negative delayed hypersensitivity skin reactions, poor or absent T lymphocyte transformation *in vitro* with mitogens and thymic dysplasia (without Hassall's corpuscles), is virtually diagnostic. The exceptions are MHC class II deficiency, ZAP-70 defects and Omenn's syndrome, where there are often normal numbers of circulating lymphocytes. Circulating B cell numbers in SCID are variable and sometimes there is polyclonal expansion

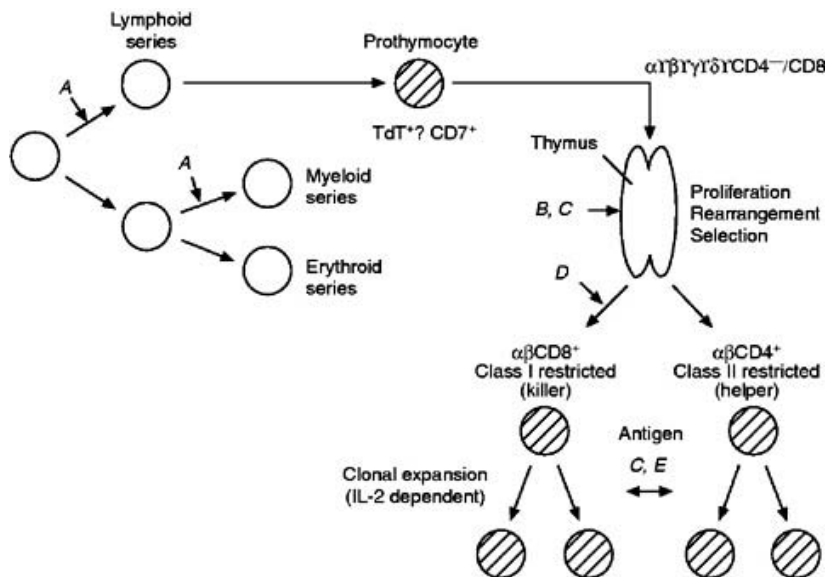


Figure 1 Ontogeny of T lymphocytes. The blocks in differentiation/maturation in the primary immunodeficiencies are as follows: A, reticular dysgenesis; B, thymic aplasia/X-linked SCID/PNP deficiency/RAG and JAK3 kinase defects; C, ADA/class II deficiency; D, leaky types of JAK3 and ZAP-70 kinase deficiency; E, some CVID patients, CD3 γ chain defects, and leaky types of CD3 ϵ chain defects.

of one isotype with hyperimmunoglobulinemia. However, functional antibodies are rarely detected. Most affected children die within 1 year of diagnosis unless they receive a successful bone marrow transplant.

X-linked SCID

Affected infants have inherited defects in the common γ chain for interleukin 2 (IL-2), IL-4, IL-7, IL-9 and IL-15 receptors; the defect accounts for about one-third of all SCIDs. Involvement of multiple cytokine receptors explains the severity of this condition.

Adenosine deaminase (ADA) deficiency

The disease was discovered by chance during the routine analysis of erythrocyte isoenzymes in a SCID infant. ADA has a central role in the interconversion pathways of purine metabolism, a defect in the enzyme leading to the accumulation of deoxyadenosine. This accumulates in the plasma and its appearance in the urine is a useful diagnostic marker. Deoxyadenosine is toxic to both T and B lymphocytes, as well as macrophages, which accounts for the very poor prognosis when compared to PNP deficiency (see below). There is a spectrum of clinical severity, probably reflecting different mutations in the ADA gene. Regular subcutaneous injection of calf ADA linked to polyethylene glycol (PEG-ADA) can restore immunocompetence. However bone marrow transplantation provides the only definitive cure. The ADA gene on chromosome 20 has been cloned, and transfection of the gene into bone marrow stem cells or peripheral lymphocytes of patients has been achieved, but so far not enough ADA has been produced to restore immunocompetence.

Purine nucleoside phosphorylase (PNP) deficiency

In 1975, Giblett and colleagues discovered PNP deficiency when screening for purine metabolic defects in patients with T cell deficiencies. The enzyme is important for the interconversion of purines in cells, although its absence only compromises the function of T cells, and in some patients the central nervous system (CNS) and/or neutrophil and erythrocyte development. The substrates for PNP are guanosine and deoxyguanosine, which accumulate in the serum and appear in the urine, the latter being a useful diagnostic marker for the disease. The clinical features are heterogeneous, probably reflecting different mutations in the PNP gene on chromosome 14; some patients present with predominantly CNS features of hypotonia or spasticity, and some have neutropenia or megaloblastic anemia. The numbers of circulating T cells are profoundly depressed (<10%) in severe cases who suffer from the same complications as patients with thymic aplasia. The thymus gland is sometimes atrophic at birth or may gradually atrophy in the first few years of life in those with a delayed onset of the immunodeficiency.

Attempts to block the accumulation of guanosine or deoxyguanosine with purine analogs have been unsuccessful. Bone marrow transplantation remains the treatment of choice in severe cases, although this will not reverse the CNS disease.

JAK3 kinase deficiency

Mutation in this important kinase in the T cell signal transduction pathway is a rare cause of SCID.

Reticular dysgenesis

This is the most severe type of SCID, due to an inherited defect in a pluripotential bone marrow stem cell from which lymphocytes, neutrophils and monocytes are derived. In one reported case there was normal monocyte development. Affected infants only survive a few weeks after birth unless they receive a successful bone marrow transplant.

RAG defects

Mutations in the recombination-activating genes (RAG-1 and -2) account for about a third of T⁻B⁻ SCID, preventing VDJ rearrangement of immunoglobulin and T cell receptor genes.

Defects in NF-AT transcription factor

This rare defect was initially thought to be due to a specific deficiency of IL-2 and was then found to involve NF-AT which controls the transcription of many cytokines.

MHC class II deficiency

This rare autosomal recessive disorder is caused by genetic defects in transcription factors which regulate the MHC class II gene, resulting in failure to express class II on lymphocytes and macrophages; many patients are of North African descent. There is usually a moderate CD4⁺ T cell lymphopenia; lymphocytes proliferate normally *in vitro* with mitogens, but not with specific antigens, and fail to stimulate or respond in a mixed lymphocyte reaction. Molecular defects have been found in three different transcription factors (RFX5, RFXAP and C11TA), and there is a fourth type to be identified.

Omenn's syndrome (familial reticuloendotheliosis)

This unusual form of SCID is characterized by thickened eczematous skin, massive lymphadenopathy, and hepatosplenomegaly due to infiltration of polyclonal lymphocytes, histiocytes, and eosinophils. Circulating B cells may be present at birth, then gradually disappear as the patient becomes severely hypogammaglobulinemic. Until recently many thought that this condition represented a chronic GVH (graft-versus-host) disease due to maternal engraftment of an SCID fetus, but there is now good evidence that it is a distinct disorder.

ZAP-70 defects

A rare genetic cause of SCID involves a tyrosine kinase which interacts with the ζ chains of the CD3 complex, causing a severe functional T cell defect with absence of CD8⁺ cells.

Cellular immune defects and dwarfism

There is a distinct disorder characterized by short limb dwarfism, thin hair and variable T cell deficiency, sometimes with severe hypoplastic anaemia. Relatively common in the Old Order Amesh, over a hundred cases have now been reported in Finland and sporadically elsewhere. SCID occurs in only rare cases. The relevant gene has been localized to chromosome 9p12. There is another, much rare condition characterized by redundant skinfolds and hair loss due to ectodermal dysplasia, dwarfism and SCID.

Antibody deficiency syndromes (Figure 2)

Following Bruton's first description of agammaglobulinemia in 1952, a large number of patients have been described with various patterns of antibody deficiency, ranging from an almost total absence of all isotypes to selective isotype and subclass deficiencies. Now that test immunization is becoming a routine diagnostic procedure, patients are being recognized with depressed antibody responses despite normal serum immunoglobulin levels. Some of the rare conditions are due to genetic defects on the X chromosome, while others are either single-gene or multigene autosomal defects.

The treatment is regular intramuscular, subcutaneous or intravenous immunoglobulin replacement therapy and antibiotics for infections.

X-linked agammaglobulinemia (XLA)

This disease is caused by mutations in a cytoplasmic tyrosine kinase (Bruton's tyrosine kinase – Btk) which is critical for the differentiation/proliferation of pre-B cells in the bone marrow. There are virtually no circulating surface positive IgM B lymphocytes and there is no antibody production, although cellular immunity remains intact. Autosomal recessive inheritance of mutations in the immunoglobulin μ -chain can cause a similar phenotype in females. The patients present with upper and lower respiratory tract infection, usually due to nontypable *Haemophilus influenzae*, in the first 2 years of life. Pneumococcal septicemia and meningitis may occur, and in the gut *Campylobacter* and *Giardia* infections are common; mycoplasma arthritis is an important but less common complication. There is usually an uneventful recovery from common viral infections, although enteroviruses, particularly echoviruses, may cause an ultimately fatal chronic meningitis. A few patients with X-linked agammaglobulinemia and growth hormone deficiency have been reported, but

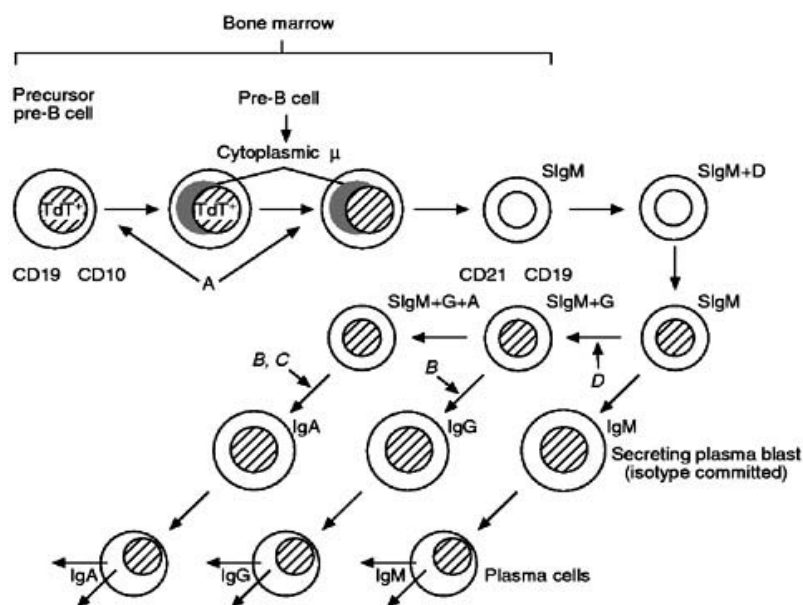


Figure 2 Ontogeny of B lymphocytes. The blocks in differentiation/maturation in the antibody deficiency syndromes are as follows: A, X-linked agammaglobulinemia; B, common variable immunodeficiency μ -chain defects; C, selective IgA deficiency; D, X-linked immunodeficiency with hyper-IgM.

there is no molecular explanation for this and the growth hormone deficiency is probably secondary to hypothalamic/pituitary damage from viral infection.

X-linked immunodeficiency with hyper-IgM (X-HIM)

This is a very rare condition caused by mutations in the gene for CD40 ligand, a molecule which is expressed on the surface of activated $CD4^+$ T cells and which interacts with CD40 on B cells to enable switching from IgM to IgG/IgA production. Affected patients may also have neutropenia, and unlike XLA patients, are prone to opportunistic viral and fungal infection, sclerosing cholangitis and liver cancer.

'Common variable' immunodeficiency: CVID (synonym 'common variable' hypogammaglobulinemia: CVH or CVI)

This is usually a nonfamilial sporadic condition presenting at any age, particularly in the first few years of life and toward the end of the second decade. The criteria for diagnosis are not well defined, although in practice most patients have a serum IgG below 4 g l^{-1} , an IgA level below 0.1 g l^{-1} , and a serum IgM level below 0.2 g l^{-1} ; however some patients with less severe symptoms have IgM levels at the lower limit of the normal range and make significant amounts of specific IgM antibodies.

The circulating T lymphocytes in most patients fail to proliferate with antigens following immunization, and delayed hypersensitivity skin reactions cannot be induced *in vivo*. Many patients have a $CD4^+$ lymphopenia but opportunistic viral, fungal or protozoal infections are rare. There are genetic predisposing

factors in at least 20% of patients who have a first-degree relative with either CVID or IgA deficiency (IgAD), suggesting an autosomal dominant inheritance with incomplete penetrance. About 50% of patients share a common haplotype in the MHC complex on chromosome 6 (HLA-DR3⁺, C4A null, and a particular TNF polymorphism), but attempts to formally link genes to the MHC complex have so far failed. Variable blocks in B cell differentiation can be demonstrated *in vitro*, with most patients having normal numbers of circulating B cells expressing surface IgM, IgG and IgA. There is a defect in terminal differentiation of committed B cells into plasmablasts/plasma cells in the majority of patients *in vivo*, which can be partially overcome *in vitro* by adding recombinant IL-2, CD40 ligand and IL-10. It is unclear whether CVID is a group of separate disorders or a spectrum of a single disease, but the underlying defect seems to be in the $CD4^+$ (helper) T cell.

The pattern of infection is similar to that seen in XLA, but there are special features which include autoimmune disease, granulomatous splenomegaly and lymphadenopathy, lymphoid hyperplasia in the bowel, and a raised incidence of lymphoid and stomach malignancy.

X-linked lymphoproliferative syndrome (XLPS) (Duncan's disease)

The syndrome is due to an underlying inability to control Epstein-Barr virus replication, the gene being localized to Xq25 on the X chromosome. The clinical features vary, even within members of a single family, with some developing hypogammaglobulin-

emia, others Burkitt's-like B cell lymphomas, and some dying in childhood from fulminant infectious mononucleosis. No consistent cellular defect has been found to explain the disease. The obligate healthy heterozygotes usually have high levels of serum antibodies to EB viral capsid.

Thymoma and hypogammaglobulinemia

This nearly always affects patients over 40 years of age and is not familial. The severity of the hypogammaglobulinemia is variable, and is not reversed by removal of the tumor, which usually consists of spindle-shaped cells and is well encapsulated. Some patients lack circulating B lymphocytes and pre-B cells in the bone marrow, suggesting an effect on an early precursor, possibly due to an autoimmune reaction. Other associated autoimmune phenomena occur, such as myasthenia gravis, thrombocytopenia, neutropenia and red cell aplasia.

Selective immunoglobulin deficiencies

IgA deficiency (IgAD)

Defined as a serum IgA level of $<0.01 \text{ g l}^{-1}$. There are interesting geographical differences in prevalence, the deficiency occurring in 1 in 700 of Caucasians, but in only 1 in 18 000 Japanese. Most cases are sporadic, and in very rare cases deletions in the IgA heavy-chain gene cause the deficiency. In about 20% of IgAD cases, a first-degree relative has IgAD or CVID, suggesting a dominant inheritance and a mechanism similar to that of CVID. There is also an association, but no formal linkage, with the same haplotype in the MHC region which is associated with CVID. The circulating B cells in these cases express surface IgA but cannot differentiate into secreting IgA plasma cells.

Some patients suffer from recurrent respiratory infections, although usually they will also have poor IgG antibody responses following test immunization. The high incidence of serum antibodies to milk and other food proteins in affected adults seems to have no clinical significance, although there is evidence that low serum IgA levels in infants and young children predispose to symptomatic cows' milk allergy. There is an association between IgA deficiency and autoimmune/chronic inflammatory disorders, such as Still's and celiac disease.

IgG subclass deficiencies

IgG2 deficiency is the most common, and like IgA is normally late to develop in infants and young children. IgG2 deficiency is sometimes associated with IgAD, suggesting a similar mechanism. IgG1 tends to

compensate in the first few years of life, but this may fail temporarily and cause 'transient hypogammaglobulinemia of infancy' and recurrent infections. IgG2 deficiency may occur in about 1 in 1000 of the 'healthy' adult population, and can be associated with failure to respond to carbohydrate antigens, theoretically leading to a predisposition to infection with encapsulated organisms (e.g. pneumococci). IgG1, IgG3 and IgG4 deficiencies, either alone or in various combinations, have been described in association with recurrent infections. However, in general, there appears to be considerable cross-compensation within the subclasses; IgG4 probably has no clinical significance. Mutations in the relevant heavy-chain genes have been described in a few families, but usually the defect is due to a failure of terminal B cell differentiation for the particular subclass.

Selective IgM deficiency

This is very rare as a primary disorder, and most cases have been found by chance when screening hospitalized patients with a variety of conditions. Excess suppression of IgM synthesis *in vitro* by T lymphocytes has been shown in some patients, possibly triggered by infection or drugs. Recurrent infections are unusual since IgG and IgA compensates.

κ chain deficiency

A rare defect due to mutations in the κ chain genes which has no clinical significance.

Disorders associated with partial immunodeficiency

Wiskott-Aldrich syndrome (WAS)

This X-linked disorder is characterized by thrombocytopenia, eczema, recurrent infections, and a high incidence of B cell lymphomas at an early age. The WAS gene has been cloned and its product (WASP) probably has a role in cytoskeletal organization and glycosylation of a number of surface proteins; absence of lymphocyte sialophorin (CD43) is a useful screening test. Patients have a very poor antibody response to polysaccharides, frequently have depressed IgM but high serum IgE levels, and a progressive functional defect in T cells.

Chediak-Higashi syndrome

This is an autosomal recessive condition characterized by albinism and large cytoplasmic lysosomal granules in the white cell series which compromise neutrophil movement and phagocytosis. T and B lymphocyte function is not significantly impaired, but severely depressed NK activity may account for

the pancytopenic crises which are thought to be caused by viral infection.

Mucocutaneous candidiasis

Although this can occur in SCID, there is a rare distinct disorder, which is sometimes familial, where there is a selective failure to control *Candida* infection. Patients have variable abnormal immune responses to *Candida* antigens, but the basic mechanism is not understood.

DNA repair defects

There are a variety of autosomal recessive genetic diseases caused by defects in the excision/repair of DNA which has been damaged by chemicals, ionizing and UV irradiation. Cell lines from such patients show chromatid damage when exposed to these agents *in vitro*. The common theme is a high incidence of malignancy at a young age, particularly lymphomas.

Ataxia telangiectasia (AT)

Patients suffer from infection, premature aging of the skin (pigmentation, telangiectasia), and severe ataxia due to cerebellar degeneration. The AT gene has been cloned on chromosome 11 and is a kinase (with homology to phosphatidylinositol 3-kinases) which is involved in DNA repair and cell cycle regulation. Lymphoma and other solid tumors are common; heterozygotes, which account for about 2% of the population, are also predisposed to malignancy. There are variable immunoglobulin deficiencies, selective IgA deficiency being common. There are functional T lymphocyte defects, the most consistent being a failure to generate cytotoxic T cells to viral-infected autologous cells.

Nijmegen-Breakage syndrome

A similar but distinct disease to AT, but without ataxia and associated with microcephaly, 'bird-like' facial features, mental and growth retardation. The immunological defects are similar to those of AT.

Ligase I defect

Only one patient has so far been reported with inherited mutations in the ligase I gene. She had severe growth retardation, recurrent infections due to partial cellular and humoral immunodeficiency and skin sensitivity to sunlight; she died from lymphoma at 19 years.

Bloom's syndrome

This is characterized by growth retardation, skin sensitivity to UV light and cancers in childhood or adolescence. The relevant gene has homology with RecQ helicases. There are variable defects in immunity, but frequently poor or absent *in vitro* lymphocyte proliferation with a variety of stimuli, and low levels of IgM.

Xeroderma pigmentosum (XP)

There is a defect in excision repair of UV-damaged DNA, associated with skin pigmentation and a high incidence of cutaneous malignancies. Affected patients have subtle immune defects with depressed NK lymphocyte activity, possibly due to depressed interferon γ production; they are not particularly prone to infection.

See also: Ataxia telangiectasia; Bare lymphocyte syndrome; Bruton's agammaglobulinemia; Hematopoietic stem cell transplantation; Chédiak-Higashi syndrome; Chronic granulomatous disease; Common variable immunodeficiency; Complement deficiencies; DiGeorge syndrome; Gammaglobulin; Gene therapy; Genetic analysis at the molecular level; Genetic analysis at the phenotypic level; Hyper-IgM syndrome; Immunodeficiency, animal models; Immunodeficiency, secondary; Immunopotential; Leukocyte adhesion deficiency; Nude (athymic) mice; Opportunistic infections; Retrovirus, infection and immunity; Severe combined immunodeficiency.

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IMMUNODEFICIENCY, SECONDARY

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Although the term 'secondary' is unsatisfactory and in the future is likely to be replaced by a more descriptive classification, secondary immunodeficiency is used to describe most of the immunodeficiency disorders that are not due to inherited single gene defects of lymphocyte function, with the important exception of 'common variable' immunodeficiency (CVID) and selective immunoglobulin A (IgA) deficiency, where the mechanism remains obscure. Secondary immunodeficiency embraces a wide range of lymphocyte disorders, from those associated with lymphoid malignancy, to those induced by viruses and the immunodeficiency related to prematurity and old age (Table 1). There are a large number of drugs which can cause partial immunodeficiency; this has been difficult to study because the drugs are often given against a background of existing immunodeficiency associated with malignant or autoimmune diseases.

In contrast to the 'primary' immunodeficiencies, secondary immunodeficiency cannot be conveniently subclassified into those affecting either antibody production or cellular immunity, since there is usually a mixed picture. It is more useful to subclassify the disorders according to the type of disease with which they are associated (e.g. viral infections, malignancy). Most of the disorders described here are associated with *in vitro* functional abnormalities of T lymphocytes, usually depressed T cell proliferation with mitogens; depressed delayed hypersensitivity skin reactions to recall antigens (tuberculin, candida) is also common. Throughout this article, the term 'functional T cell defects' will be used to describe these phenomena.

Secondary immunodeficiency and malignancy

Chronic lymphatic leukemia (CLL)

This is one of the most important causes of immunodeficiency because survival is usually prolonged and correction of the immunodeficiency improves the overall prognosis. More than half of affected patients develop severe antibody deficiency and hypogammaglobulinaemia, which tends to become more severe as the disease progresses. The mechanism is unclear, with some workers suggesting that the expansion of the malignant lymphocyte clone, which is usually a

Table 1 Secondary immunodeficiency

	Antibody	Cellular immunity
<i>Lymphoreticular malignancy</i>		
Chronic lymphatic leukemia	++	—
Multiple myeloma	++	—
Lymphomas	+	+
<i>Malnutrition</i>		
Protein calorie	+	++
<i>Vitamins and trace elements</i>		
B ₁₂ (transcobalamin II deficiency)	+	+
Vitamin A	—	+
Zinc (acrodermatitis enteropathica)	—	+
Selenium	—	+
<i>Physiological</i>		
Prematurity	++	+
Aging	+	+
<i>Drug-associated</i>		
Steroids	+	+
Cyclophosphamide	+	+
Azathioprine	+	+
Cyclosporine	+	+
Gold	+	—
Sulfasalazine	+	—
Penicillamine	+	—
Phenytoin	+	—
<i>Bone marrow transplantation</i>	+	+
<i>Viral-associated</i>		
HIV-1 and HIV-2	+	++
Rubella	+	—
Cytomegalovirus	+	+
<i>Trauma/surgery/thermal injury</i>	+	+
Splenectomy	+	—
<i>Metabolic disorders</i>		
Renal failure	+	+
Liver failure	+	+
Biotin-dependent carboxylase deficiencies	++	++
<i>Increased loss/catabolism</i>		
Nephrotic syndrome	+	—
Protein-losing enteropathy	+	—
Intestinal lymphangiectasia	+	+
Dystrophia myotonica	+	—

Moderate (+) or severe (++) deficiency.

B cell, disorganizes the architecture of lymph nodes, spleen and bone marrow, thus preventing cellular interactions needed for the induction of antibody responses. Others have shown that either the B cell clone and/or natural killer (NK) cells from affected patients will suppress *in vitro* immunoglobulin production by normal B cells, although this may not be

a direct effect. The antibody deficiency is of clinical importance, since early treatment with immunoglobulin replacement therapy will improve the quality of life by preventing chronic bronchitis and bronchiectasis.

Multiple myeloma

Most patients have a clinically significant depression of polyclonal antibody production which predisposes to pneumonia and septicemia with capsulated organisms (e.g. pneumococci). Although patients with myeloma can survive some years with modern chemotherapy, recent trials suggest that immunoglobulin replacement therapy neither influences morbidity nor survival, possibly because antibody production tends to normalize during remission. The mechanism of the antibody deficiency is unclear, but there is evidence that the malignant plasma cells, as well as monocytes from myeloma patients, will suppress normal B cell proliferation *in vitro*, possibly through depression of interleukin 1 (IL-1) and IL-2 synthesis. Light chain isotype suppression, whereby polyclonal B cells carrying the same light chain as the malignant clone are suppressed, is also not understood but is thought to reflect a partially successful control mechanism for the malignant clone itself.

Lymphomas

Mild to moderate defects in both cellular and humoral immunity are associated with a variety of lymphoreticular malignancy and lymphocytic leukemias, functional T cell defects being particularly well described in Hodgkin's disease where survival may be prolonged. However, no clear patterns of immunodeficiency in the various lymphomas have been documented, and the picture is usually confused by concomitant chemotherapy.

Nonlymphoid tumors

There is much interest in defects in 'immune surveillance' which may precede or are secondary to a variety of malignant tumors. Suppression of IL-2 synthesis may be important, and a number of therapeutic trials with recombinant IL-2 are in progress. There is still no clear evidence that immunodeficiency contributes either to the development of cancer or a poor prognosis.

Nutritional deficiencies

Protein calorie malnutrition

Malnutrition in the Third World is an important predisposing factor for infectious disease, which is the

major cause of death, particularly in young children. Susceptibility to tuberculosis and recovery from measles infection are particularly influenced by nutritional status, probably through an effect on cellular immunity. Vitamin deficiencies are an important cofactor. There is evidence that vitamin B₁₂ deficiency may be a predisposing factor for tuberculosis in Asian communities in the West, and studies in the Third World suggest that vitamin A supplementation reduces the infection rate in children.

Severe deficiency of vitamin B₁₂, due to an autosomal recessive genetic deficiency of transcobalamin II, a transport protein, affects a variety of bone marrow-derived cells, causing neutropenia, B cell abnormalities with hypogammaglobulinemia, and megaloblastic anemia. Vitamin B₁₂ injections will correct the deficiency.

Metals and trace elements

Iron deficiency is common and not usually associated with a predisposition to infection. However, there is a link with chronic mucocutaneous candidiasis where it appears to be a cofactor for infection, sometimes with endocrine abnormalities. A deficiency of zinc, critical for many enzyme functions, will compromise T cell function and predispose to opportunistic infections. Acrodermatitis enteropathica is a rare autosomal recessive genetic disorder causing a partial defect in zinc absorption from the gut; affected patients suffer from dermatitis, malabsorption and infections, which can be corrected by zinc supplements. Selenium may be an important trace element for T cell function, and it is claimed that a partial deficiency in children may predispose to infections.

Bone marrow transplantation

B lymphocyte function in allogeneic bone marrow transplantation is slow to recover, and patients may need immunoglobulin therapy for many months. Autologous T lymphocyte-depleted transplantation (e.g., for leukemia, scleroderma) is followed by a profound lymphopenia which recovers after a few months. Because the thymus is relatively inactive in transplanted adults, it is possible that the T cell repertoire is reconstituted by CD4 and CD8 T cells from the gut-associated lymphoid tissue.

Prematurity and aging

There are two similarities in the immunodeficiency associated with the two extremes of life. Premature low birthweight infants are prone to fatal infections

with a variety of bacteria, particularly gram-negative gut commensals with their endotoxins. Although the defects may involve a variety of cell types, including neutrophils, antibody deficiency is important, particularly the inability to produce antibodies against bacterial capsular polysaccharides and endotoxins. Even full-term normal weight infants usually fail to make antibodies of the IgG2 subclass against polysaccharides, and many will also fail to compensate with an IgG1 response. This explains the failure of polysaccharide vaccines to meningococci and *Haemophilus influenzae* type B to stimulate protective antibodies in the first 2 years of life. Antibody production to polysaccharides is also depressed in old age, probably explaining the increased susceptibility to pneumococcal septicemia in those over 80 years.

Many studies have demonstrated minor changes in T cell subsets and function in aged subjects. It is unclear whether these changes have any relevance to the predisposition to malignancy in this age group. However, subclinical immune dysregulation is common, as shown by the frequency of serum autoantibodies and paraproteins.

Drug-induced immunodeficiency

Most cytotoxic drugs used in the treatment of malignancy depress cellular and humoral immunity but this tends to be overridden by the neutropenia which often occurs with prolonged therapy, and which predisposes to gram-negative bacterial septicemia and fungal infections. Immune defects due to these drugs may become of greater clinical significance now that hematopoietic growth factors can be used to prevent the neutropenia. Steroids, cyclophosphamide and azathioprine commonly depress cellular immunity, and can depress immunoglobulin levels after prolonged usage. The clinical significance of these defects is difficult to determine, since the drugs are usually given for conditions (vasculitis, autoimmune diseases) where there may be an underlying defect in immune regulation. However, long-term treatment, particularly with azathioprine, may predispose to fatal opportunistic infections. Cyclosporine, now being used in autoimmune diseases as well as to prevent graft rejection, suppresses antigen receptor triggering of T lymphocytes. This, and other drugs used to prevent transplant rejection can also depress immunoglobulin levels, and some clinics are routinely using immunoglobulin replacement therapy following organ or bone marrow transplantation.

There is evidence of permanent CD4 T cell depletion in adolescents and adults given chemotherapy for leukemia, although CD8 T cells recover.

The later the chemotherapy is given in life, the more profound the CD4 T cell lymphopenia, which is explained by the relative failure of thymic activity with increasing age.

Gold, sulfasalazine, penicillamine, and phenytoin have each been implicated in the development of selective IgA deficiency, and in some cases panhypogammaglobulinemia. Discontinuing the drug usually leads to recovery of immunoglobulin levels, although this may take some years. Spontaneous recovery from CVID, which is the differential diagnosis, is extremely rare, a fact which strongly supports the view that these drugs can cause hypogammaglobulinemia. The mechanism is obscure, and no direct effect on B lymphocyte proliferation or differentiation *in vitro* can be demonstrated with doses of the drug likely to be achieved *in vivo*. The effect is idiosyncratic, since less than 1% of patients receiving these drugs develop any sign of immunodeficiency.

Drug-associated immunodeficiency is partly responsible for the raised incidence of selective IgA deficiency in juvenile rheumatoid arthritis and systemic lupus erythematosus, but there may be other factors which link immunoglobulin deficiencies with autoimmune disease in general. This link may lie in the major histocompatibility complex (MHC) region on chromosome 6, since patients with these diseases, and with CVID, frequently share a common haplotype.

Viral-induced immunodeficiency

Human immunodeficiency virus

HIV-1 and HIV-2 are human retroviruses which replicate in T cells and macrophages, causing gradual attrition of cellular immunity, and depression of antibody production in the terminal stages of the disease. The opportunistic bacterial, fungal, and viral infections associated with the acquired immune deficiency syndrome (AIDS) are probably due to functional defects in macrophages, although the mechanism is still unclear.

Rubella virus

Some infants with the congenital rubella syndrome do have persistently low serum IgG and IgA levels, associated with chronic excretion of rubella virus after birth and the presence of specific IgM anti-rubella antibody. The hypogammaglobulinemia may persist after the virus is eliminated. The mechanism is unclear.

Herpes viruses: cytomegalovirus (CMV)

There is no evidence that the Epstein-Barr virus (herpesvirus 4) causes immunodeficiency, apart from the hypogammaglobulinemia which occurs in the X-linked lymphoproliferative syndrome. However, cytomegalovirus (herpesvirus 5) does itself cause significant immunodeficiency when it becomes reactivated in immunocompromised patients, particularly following bone marrow or organ transplantation. CMV replicates in macrophages and stimulates the proliferation of CD8⁺ T cells which may suppress both cellular and humoral immunity. There is evidence suggesting that IL-1 and IL-2 secretion is depressed in monocytes and lymphocytes respectively, although the mechanism is unknown. Furthermore, CMV itself may bind to β_2 -microglobulin and could dysregulate the immune response through an effect on class I molecules.

Trauma/surgery/thermal injury

Severe trauma is usually associated with significant immunosuppression which may determine prognosis. Prophylactic antibiotics usually prevent bacterial sepsis, but CMV reactivation and fungal pneumonia may occur due to depression of cellular immunity. A variety of mechanisms are involved, with prostaglandin release from monocytes downregulating T cell function, together with other factors causing suppression of IL-1 and IL-2 synthesis. Antibody production may also be depressed but this is rarely clinically important.

Relatively minor (<10% surface area) thermal injury in young children selectively depresses IgG2 and IgG3 serum immunoglobulin levels, although more severe burns in both children and adults suppress all antibody responses. It is not clear whether this is due to decreased synthesis or increased catabolism. The clinical significance is uncertain and cannot be tested in the West because of the effective use of prophylactic antibiotics. However, fatal endotoxemia following burns in the Third World is common, and can be prevented with passively administered antibodies.

Splenectomy/thymectomy

Splenectomy predisposes to septicemia/meningitis with pneumococci, meningococci, or *Haemophilus influenzae* bacteria. Following post-traumatic splenectomy, <5% of patients will develop septicemia, but this rises to 25% when splenectomy is carried out for other reasons (e.g. Hodgkin's disease, autoimmune hemolytic anemia). Congenital asplenia and multiple splenic infarction in sickle cell anemia car-

ries a similar risk, and all patients are advised to take prophylactic penicillin. The mechanism is not clear but is probably due to a combination of a failure to trap bacteria within the splenic reticuloendothelial system and the consequent failure to generate specific antibodies fast enough to limit the infection. Vaccination of affected patients with polysaccharides from the relevant organisms is theoretically justified, preferably before splenectomy.

There are no good data on the effect of thymectomy in infancy, but this is unlikely to be clinically significant. Most of the studies have been done on adults thymectomized for myasthenia gravis when there is a small fall in the numbers of circulating T cells, with no effect on the level of antibodies to common organisms.

Metabolic disorders

It is not surprising that severe metabolic disorders such as renal and liver failure are associated with depressed immunity, with pneumonia being a common terminal event. Death from infection in infancy is also common in many of the severe inherited metabolic defects, although it is usually inappropriate to investigate this in any detail. In some cases, a metabolic defect may retard the normal development of immunity in infants, such as the reported hypogammaglobulinemia in a few infants with uridine monophosphate synthase deficiency (orotic aciduria). On the other hand, genetic defects in enzymes involved in the synthesis of biotin, an important cofactor for a variety of carboxylases, leads to a moderately severe combined immunodeficiency affecting both cellular and humoral immunity, with mucocutaneous candidiasis being a characteristic clinical problem.

Increased loss/catabolism of immunoglobulins

Nephrotic syndrome/protein-losing enteropathy

Loss of proteins through the kidneys in the nephrotic syndrome often causes moderate hypogammaglobulinemia, usually with a low serum IgG and IgA; the IgM level remaining normal presumably due to its higher molecular weight. However, not all the IgG subclasses are equally affected, with IgG3 and IgG4 remaining at normal levels. This cannot be explained by differences in the molecular weights of the IgG subclasses and suggests that factors other than glomerular leakage play a part. The antibody deficiency is not usually severe, but is probably responsible for the predisposition to pneumococcal septicemia described in early textbooks.

Protein-losing enteropathy is a rare complication of chronic inflammatory bowel disease (e.g. Crohn's) or chronic bacterial infection (e.g. Whipple's disease). Another cause is intestinal lymphangiectasia where there is a dilatation of the mucosal lymphatics with loss of protein and lymphocytes. This is caused by lymphatic obstruction due to infection (e.g. tuberculosis), malignancy (e.g. lymphoma), or increased hydrostatic pressure in severe congestive heart failure. There is also a primary genetic disorder of lymphatic vessels which can affect the gut. IgG and IgA are usually moderately depressed and infections are unusual; the T cell lymphopenia and the associated functional defects in cellular immunity are clinically unimportant. A diet high in medium-chain triglyceride can reduce lymphatic flow and alleviate symptoms, but otherwise surgery may be necessary to remove the affected area of bowel.

Dystrophia myotonica is the only well-characterized condition where there is a selective increase in the catabolism of serum IgG, although the IgG level is only moderately depressed. Although this autosomal dominant condition primarily affects muscles, it must also affect the uptake and/or degradation of IgG by macrophages, although this has not been formally demonstrated.

See also: Acquired immune deficiency syndrome (AIDS); Aging and the immune system; Cytomegalo-

virus, infection and immunity; Human immunodeficiency viruses; Immunodeficiency, primary; Immunosuppression; Iron and the immune system; Leukemia; Lymphoma; Opportunistic infections; Togavirus, infection and immunity; Vitamin A and the immune system; Vitamin B group and the immune system; Zinc and the immune system.

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IMMUNODIFFUSION, SINGLE RADIAL

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As the term implies, immunodiffusion refers to an *immunological reaction* in which the reactants *diffuse* into a supporting medium, i.e. a gel. The reaction involved is that of precipitation, the objective being to visualize and immobilize an *in vitro* reaction between a precipitating antibody and a soluble antigen. Thus, immunodiffusion is a modification of the precipitin reaction first observed in fluid media by Kraus in 1897. As one of the reactants is placed in wells cut in the gel, the diffusion is radial.

As commonly practised, immunodiffusion is carried out in either of two forms: double diffusion and 'single radial immunodiffusion' (SRI; also RID, i.e. radial immunodiffusion). In double diffusion, both antigen and antiserum diffuse from wells in the gel, whereas in SRI one reactant – usually the antibody – is included in the gel and only the other diffuses in.

Thus, in double diffusion precipitation lines are formed in the gel where antigen and antibody from juxtaposed wells overlap; while in SRI the precipitate takes the form of a ring round the well.

SRI may be traced back to the observation of Petrie in 1932 that a ring of precipitate formed round a colony of an exotoxin-producing bacterium growing on agar which contained the specific antitoxin. In 1949, Ouchterlony and Elek independently adapted this observation to produce a double immunodiffusion technique. Wells were produced in an agar plate, using a template or cutter. Individual wells received either antigen or antiserum. As the two reactants diffused into the gel, a line of precipitate formed within the zone of overlap. This has become a most useful qualitative technique for demonstrating and comparing antigens and

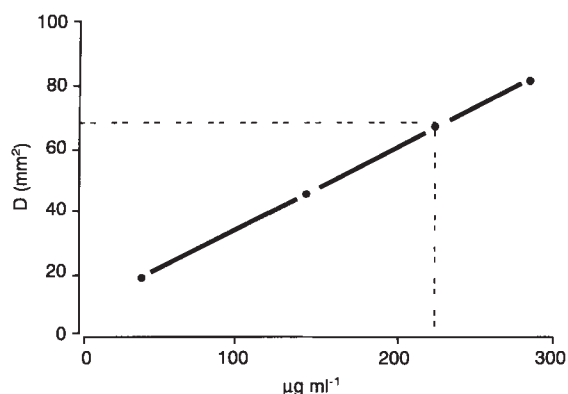


Figure 1 Assay of an antigen from the straight line plot of D^2 versus concentration on arithmetic graph paper.

antibodies, but proved inadequate for quantitative determinations.

Simultaneously, Oudin was transferring Petrie's observations to a tube, showing that rings of precipitate would form in a gel containing antiserum when it was overlaid with a solution of the corresponding antigen. This was *single* linear diffusion, as for the purpose of the method only the antigen diffusion was of consequence. It was a dynamic system, the precipitate rings migrating linearly down the gel with time; thus, single linear immunodiffusion. As the rate of the ring migration was determined by antigen concentration, it could be used (albeit not very conveniently) for quantification of antigens against known standards.

In 1957, to achieve a more convenient immunodiffusion assay system, Feinberg combined Oudin's tube single diffusion with the Ouchterlony-Elek plate to produce a *single radial immunodiffusion* system.

As in Oudin's technique, serum was incorporated in a molten gel, but as with Ouchterlony-Elek the gel was poured into a plate and allowed to set. With a specially designed gel cutter, uniform wells were cut in the antiserum-gel and filled with antigen solution, using standard concentrations of the antigen for comparison with test antigens. Diffusion of antigen and formation of the precipitate rings were allowed to go to completion, assay being based on a limiting dilution end-point, i.e. the lowest concentration of antigen showing a ring of precipitate.

In 1963, Tomasi and Zigelbaum modified the Feinberg technique, eliminating the need to achieve an end-point. More conveniently, they measured the diameters of the precipitate rings by placing a transparent ruler beneath the plate and plotting the diameters against antigen concentrations. With suitable standards they were able to assay secretory immunoglobulin A (IgA) in saliva, colostrum and

urine. In 1965, Fahey and McKelvey confirmed the validity of the system and used it to measure levels of immunoglobulins in serum.

In 1965, Mancini and coworkers extended observations to include a diversity of antigens, simultaneously observing the reliability of SRI assay and noting that it was not influenced by variations in temperature. However, in their estimation, merely recording ring diameters could give erroneous results. They advocated projection of the rings on to strong paper, outlining the projected rings with pencil, cutting the paper discs outlined and weighing them. This so-called 'Mancini technique' proved to be cumbersome, as subsequently acknowledged by Heremans. Because of this, in practice it has largely been abandoned, whereas the Tomasi and Zigelbaum modification of the Feinberg technique has generally been adopted as the method of choice and now forms the basis of commercially supplied immunodiffusion plates for the immunoassay of proteins in plasma and body fluids.

Though SRI is most widely used for the assay of antigens, Feinberg observed it could also be used for the assay of antibodies, for which purpose the placing of the reactants is reversed: the antigen is incorporated in the gel, while dilutions of antibody are placed in the wells, i.e. reversed single radial immunodiffusion (RSRI). This was confirmed by Vaerman and colleagues in 1969.

Mechanisms

When antigen diffuses radially into a gel containing antiserum, immune complexes are formed and a precipitate begins to deposit as a ring at the interface between the two reactants. The higher the concentration of antigen in the well, the more rapidly precipitation takes place, i.e. rings appear sequentially in direct relationship to antigen concentration. Ongoing diffusion of antigen from the well builds up the precipitate at the outer edge of the ring, where the antigen encounters additional antibody. Therefore, the system is initially in a dynamic state in which the rings increase in size with time. A static stage is reached when all the antigen has diffused into the gel and precipitation is complete. At this stage, the rings are most distinct and remain constant in size, so readings can conveniently be taken at any time thereafter.

The time to reach the static state and the ultimate size of the rings are dependent on the molecular size of the antigen, as well as the antigen concentration. The larger the molecule, the longer the time and the smaller the ring. As these are constants for any one antigen and apply equally to standard and test anti-

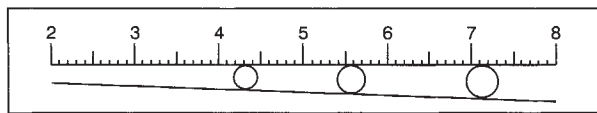


Figure 2 Measuring ring diameter with the convenient wedge-shaped rule.

gen, they do not ordinarily interfere with the validity and accuracy of SRI. However, they do become significant factors when assaying antigens which exist in different polymeric forms, or which tend to complex with other proteins, so one must remain aware of such possibilities.

At the static stage, the area circumscribed by the ring is proportional to the concentration of the antigen in the well. Since area is proportional to the square of the radius (and, hence, to the square of the diameter, D), then the square of the diameter (D^2) of the ring is proportional to the concentration of antigen. Such being the case, plotting D^2 against concentration on arithmetic graph paper will produce a straight line (Figure 1). Alternatively, to avoid the requirement of calculating D^2 , semilog graph paper may be used, plotting D itself on the linear scale against antigen concentration on the log scale.

Methodology

SRI is generally carried out in an agarose gel. The molten gel is cooled to $\sim 50^\circ\text{C}$ to avoid heat denaturation of the antiserum, which is warmed to a similar temperature and added to the molten gel in desired concentration. The two are mixed gently, but thoroughly, and poured into a dish, or on to a glass plate (e.g. a microscope slide), supported on a perfectly horizontal surface to ensure uniform gel thickness. When the gel has set, wells are cut using a sharp cutting instrument designed for the purpose. Alternatively, a plastic mask with a pattern of holes can be floated on the molten gel, which is then allowed to set to form a firm seal between mask and gel. In this case, wells need not be cut, the antigen solution being applied to the exposed areas of gel.

Three known standards, spanning the anticipated range of concentrations of the test samples, are used along with the test samples. Uniform measured volumes must be used throughout. Alternatively, when wells are used, comparable volumes can be achieved by filling each well until the meniscus just disappears;

this is preferable if the gel may not be of uniform thickness.

The plates are incubated in a humid atmosphere, preferably until ring formation is complete. Measurement of the ring diameters may be done with a transparent ruler or, more conveniently, with the wedge-shaped scale introduced by McSwiney (Figure 2). Mechanical aids for reading ring diameters are also available commercially. These include a modified jeweller's eye-piece with an in-built graduated scale. Most sophisticated and best suited for making large numbers of readings routinely is an electronic plate-reader.

For the purpose of assay, graphs are constructed, as described above, from three standard dilutions. The ring measurement of each test sample is then positioned on the graph and the corresponding concentration read off.

Investigating the sensitivity and accuracy of SRI in 1965, Mancini and colleagues reported that as little as $1.25\ \mu\text{g}$ of antigen could be measured when using $2\ \mu\text{l}$ samples of antigen solution. They found the standard deviation of SRI antigen assays to be less than 2% of the mean.

See also: Antibodies, detection of; Antibody-antigen intermolecular forces; Immune complexes; Precipitation reaction.

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IMMUNODOMINANCE

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Antigens are molecules recognized by specific T and B cells. In defining antigens two properties should be distinguished: immunogenicity, the capacity of a given antigen to induce an immune response, and antigenicity, the capacity of the antigen to be recognized by the antigen-specific receptors expressed by T and B cells. Therefore, every immunogen is also an antigen but not all antigens are immunogens. Antigens are constituted by antigenic determinants or epitopes. In proteins, which represent the vast majority of antigens, two types of antigenic determinants can be distinguished: continuous or segmental determinants, entirely contained in a continuous primary sequence of amino acid residues, and discontinuous or assembled determinants, formed by residues distant in the primary sequence but topographically assembled in the tertiary structure of proteins. These definitions have substituted the obsolete distinction between sequential and conformational determinants, since sequential determinants also interact with their specific receptor in a preferential conformation. The combining site of antigen-specific T and B cell receptors consists of a three-dimensional arrangement of amino acid residues, and therefore, among the many conformations of a polypeptide antigen, e.g. a synthetic peptide, some will be bound with higher affinity. Thus, the three-dimensional architecture of the combining site defines the conformation of the most complementary antigen and in this sense all antigenic determinants are conformational. Some antigenic determinants, either because of characteristics intrinsic to the antigen and/or to the immunized individual, become immunodominant, representing epitopes preferentially recognized by T and B cells of a given individual after immunization with the entire protein.

Antigen recognition by T and B cells

T and B cells recognize antigen in a fundamentally different way. Whereas antibodies (hence also antigen-specific B cell receptors) recognize soluble proteins in their native three-dimensional conformation, antigen-specific T cell receptors recognize a binary complex formed by an antigenic determinant bound to molecules encoded by the major histocompatibility complex (MHC). Antigen recognition by T cells is therefore not direct, as for antibodies, but takes place after protein internalization and partial proteolytic degradation in cells capable of antigen

processing and presentation (e.g. macrophages, dendritic cells and B cells), which then express on the cell surface complexes formed by T cell determinants bound to MHC molecules. These different recognition mechanisms explain why antibodies, interacting with native proteins, preferentially recognize assembled determinants, whereas T cells predominantly recognize sequential determinants of about 8–15 residues, derived from the limited degradation of protein antigens.

Immunodominant T cell determinants

T cells recognize antigens as peptides associated with self molecules encoded by the MHC, and therefore the binding of immunogenic peptides to MHC molecules is a prerequisite for T cell activation. Each MHC glycoprotein has a single binding site for peptides and, because the number of antigenic peptides to be presented to T cells is very large, each MHC molecule must have the ability to bind many peptides of unrelated sequences – for example, several of those generated by the processing of a single protein antigen. It would thus be expected that T cells respond to several different epitopes on a single foreign protein; however, T cell responses are generally specific for a few, and often only one, of the peptides derived by processing of a protein antigen. These T cell epitopes are referred to as immunodominant. Immunodominance of selected T cell epitopes has been demonstrated for several proteins, including cytochrome *c*, lysozyme, myoglobin, ovalbumin, influenza virus hemagglutinin, staphylococcal nuclease, ragweed allergen, bacteriophage λ repressor and acetylcholine receptor. Epitope immunodominance has been studied mostly in class II MHC-restricted T cell responses, but it is also present in class I-restricted cytotoxic T cell responses.

Thus, although many potential T cell determinants may exist within a given protein antigen, the T cell response of a given individual is focused on a few selected ones. Determinants eliciting the most vigorous immune responses following priming with protein antigen are defined as dominant, while those stimulating weaker responses are termed subdominant. In addition, a third class of T cell determinants exists, the cryptic epitopes. These determinants correspond to peptides which are themselves strongly immunogenic yet do not recall a significant response after immunization with the native protein, implying

inefficient generation of naturally processed epitopes corresponding to these determinants.

The molecular events involved in antigen processing are still poorly understood, but the emerging view indicates that, in general, protein antigens undergo a limited degree of proteolysis in an acidic intracytoplasmic compartment, the resulting peptides associate with MHC molecules and are transported to the cell surface complexed with these molecules. In antigen presentation by MHC class II molecules, the first event in antigen processing probably consists in unfolding of the native protein antigen. Unfolded polypeptide antigens bind to class II molecules. Polypeptide regions external to the MHC binding site are then trimmed by proteolysis, while the bound determinant remains protected. This view stresses the importance of intramolecular competition between determinants on the same polypeptide for binding to an MHC molecule and of competition between different MHC class II molecules, e.g. I-A and I-E, for binding to the unfolding protein antigen. Accordingly, several mechanisms can be anticipated to influence the immunodominance of T cell determinants: 1) availability of a given epitope after unfolding of protein antigen; 2) its ability to bind to MHC molecules; 3) competition between different epitopes from the same protein antigen (or other proteins) for binding to MHC molecules; and 4) presence in the repertoire of T cells able to recognize the peptide-MHC complex. All these mechanisms can operate during the T cell response to a given protein antigen, as exemplified by the emergence of T cell determinants in the response to hen egg-white lysozyme (HEL).

In this case, the immunodominance of T cell determinants was studied by analyzing the correlation between binding of a series of synthetic peptides encompassing the entire HEL sequence to class II molecules of the H-2^k haplotype, and T cell responsiveness to these peptides. After HEL priming, three immunodominant T cell epitopes were found: two were recognized in association with I-A^k molecules and one with I-E^k molecules. Accordingly, these peptides bound to the appropriate class II molecule. Several other HEL peptides, although capable of binding to class II molecules, were not immunodominant, and this could be accounted for by: 1) failure to generate the peptide during antigen processing; 2) competition by an immunodominant peptide for presentation *in vivo*; and 3) an inherently poor capacity of the T cell repertoire to respond to a particular peptide-MHC complex.

Therefore the immunodominance of T cell determinants results from: 1) intrinsic characteristics of the epitope, e.g. the presence of structural motifs

allowing binding to MHC molecules; 2) characteristics of the immunized individual, e.g. the presence of appropriate MHC molecules and T cell receptors; and 3) events related to antigen processing and presentation, e.g. competition between different epitopes of a given protein antigen for the available MHC binding sites. Immunodominance is not due to the pre-emption of the response by a single dominant T cell clone, and actually immunodominant epitopes are usually the focus of a polyclonal T cell response. The combination of all these levels of influence determines the final pattern of immunodominant T cell determinants, and a given protein injected in different individuals will give rise to distinct immunodominant epitopes. Understanding the mechanisms influencing immunodominance in protein antigens of pathogenic microorganisms is obviously critical in the development of effective vaccines.

Immunodominant T cell epitopes can be defined not only in the response to foreign proteins, but also in the anti-self response. During the response to foreign proteins immunodominant epitopes tend to remain constant, whereas they may change during the response to self antigens. A dynamic model of immunodominance in autoimmunity, based on the recognition by autoreactive T cells of novel immunodominant epitopes during the course of disease, has been proposed. This phenomenon has been referred to as determinant spreading. The emergence of novel immunodominant epitopes may be due to alteration in self antigen presentation that leads to the display of previously cryptic self determinants.

Immunodominant B cell determinants

Unlike T cells, B cells recognize many determinants as immunodominant on the surface of a protein antigen. Most determinants depend upon the conformational integrity of the native protein, and those to which an individual responds are dictated by the structural differences between the antigen and the host's self proteins and by the cellular regulatory mechanisms of the host, as well as by inherent properties of the protein molecule.

Thus, a nearly limitless spectrum of antibodies can potentially be induced, but in a given individual only some of the many specifically reactive B cell clones are activated. In the case of cytochrome *c*, a slowly evolving protein, the limited sequence differences between each cytochrome *c* molecule and that of the responding host restricts the number of determinants favoring the expression of immunodominant epitopes. However, the fact that most substitutions are immunologically detectable in some hosts indicates that all the surface protein is immunogenic in the

species. This is similar to the situation observed with T cell determinants. In any given individual few, and often only one, determinant becomes immunodominant for T cells, but at the species level (for example, considering all the available mouse strains with different MHC haplotypes) any protein determinant can be usually shown to be immunodominant in some hosts. However, as discussed before, immunodominance is defined by the immune response of an individual immunized with a given protein. Therefore, in this sense, immunodominant determinants can also be defined for B cells.

The interest in identifying immunodominant B cell determinants derives from their possible use in synthetic vaccines, which should be ideally composed of both T and B cell determinants, since in most cases these would not overlap. Besides many immunoregulatory mechanisms, largely T cell dependent, intrinsic characteristics of the antigenic determinant, such as accessibility, hydrophilicity and mobility, also influence the immunodominance of B cell epitopes. Although most B cell protein epitopes are discontinuous, the emphasis on continuous epitopes is due to the potential usefulness of predicting which linear peptide fragments are likely to possess cross-reactive antigenicity with the intact protein. Since for most proteins the only available structural information is their amino acid sequence, identification of short linear sequences, mimicked by synthetic peptides, representing immunodominant epitopes of the native antigen is currently the only practical approach.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Antigens; Epitopes; Hapten; Immunogen; MHC restriction; Vaccines.

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IMMUNOELECTROPHORESIS

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The term immunoelectrophoresis, or electroimmunoprecipitation, which is sometimes used synonymously, refers to any of a series of methods where immunoprecipitation is obtained after electrophoretic separation of proteins in an electric field. Based

on differences in antigenic composition and charge, these methods make possible identification and quantification of proteins with a molecular weight of 5–10³ kDa or more.

Immunoelectrophoresis was first coined by Grabar

and Williams in 1953. After electrophoretic separation of serum proteins in an agar gel, the serum proteins were allowed to diffuse against antibodies, leading to a pattern of precipitation arcs in the agar representing the major constituents among the serum proteins. Thus the globulin fractions known at that time, the α -, the β - and the γ -globulins, were found to contain several distinct proteins, the main components of which have since been distinguished as α_1 -antitrypsin, α_2 -macroglobulin, transferrin, C3, immunoglobulin G (IgG), IgA and IgM. Immunoelectrophoresis created a breakthrough in protein identification and in immunology.

Crossed immunoelectrophoresis

The disadvantage of the original immunoelectrophoresis was the long second diffusion step, whereby the primary separation of the various proteins was partially masked, leading to overlapping between molecular species with antigenic similarity and similar electric charge. This was overcome by development of crossed immunoelectrophoresis, where the primary electrophoretic step was followed by forcing the separated proteins by an electric field into a second gel containing antibodies against the various proteins. When protein antigens react with their corresponding antibodies in specific ratios, distinct and narrow precipitation arcs are formed. Crossed immunoelectrophoresis is a sensitive technique, making visible differences in charge and/or antigenic composition between proteins otherwise difficult to identify or separate. The method is valuable for the analysis of heterogeneous protein populations, for studies of genetic polymorphism, for identifying structural changes upon degradation of proteins and the identification of the fragments formed, and for analysis of protein-protein complexes and enzyme-inhibitor interactions. Thus, for instance, α_1 -antitrypsin variants are recognized, the presence of cleavage products of C3 – C3b, C3c and C3d – can be shown, and enzyme-inhibitor complexes such as those containing antithrombin III or α_1 -antitrypsin or C1 inhibitor (C1 inactivator) can be analyzed.

The composition of the C1 complex of the complement system can be demonstrated by crossed immunoelectrophoresis with monospecific antibodies against the subcomponents C1q, C1r and C1s, respectively. When fresh normal serum was run with Ca^{2+} present in both electrophoretic steps, the native C1qrs complex remains at the application site and no visible precipitate appeared with anti-C1q, anti-C1r or anti-C1s in the second gel. When the second electrophoretic step was done with EDTA present, two separate precipitation arcs appeared at the appli-

cation site, representing C1r and C1s which have dissociated from C1q. The dissociated C1q subcomponent migrated towards the cathode and gave rise to a cathodic precipitate when anti-C1q was used in the second electrophoretic step. A diffuse precipitation arc was shown in the α region, which on further analysis was found to contain C1r, C1s and C1 inhibitor.

Crossed immunoelectrophoresis has been used to study C1 and C1 activation in various diseases with an immunological background. Figure 1 shows the findings with serum from a patient with chronic urticaria and angioedema. Electrophoresis was run with monospecific antisera against the C1 subcomponents and with Ca^{2+} present in the first step and with EDTA in the second step. Two separate precipitation arcs appeared at the application site, representing C1r and C1s of the native C1 complex. Two separate precipitation arcs appeared also in the β region, caused by C1r and C1s, which were present in the serum as a complex of 'free' C1r–C1s in zymogen form. The third precipitate appearing in the α region was caused by increased amounts of complexes between C1r–C1s and C1 inhibitor. Performance of both the electrophoretic steps in the presence of Ca^{2+} resulted in a depressed, 'filled' precipitation arc in the β region, indicating the dependence upon Ca^{2+} for the complexing between C1r and C1s.

Such patterns are often found in sera from patients with rheumatoid arthritis, systemic lupus erythematosus, chronic urticaria or primary biliary

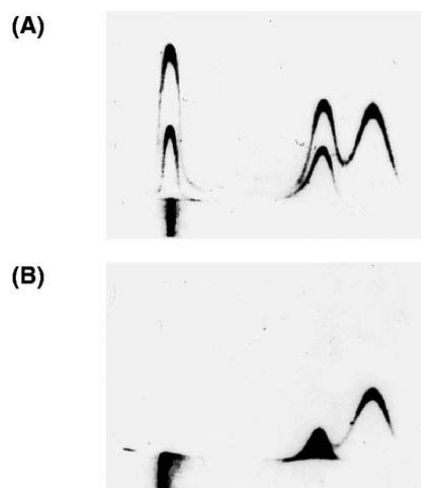


Figure 1 Crossed immunoelectrophoresis of serum from a patient with chronic urticaria and angioedema. The separating electrophoretic step was done in the presence of Ca^{2+} , and the second electrophoretic step was performed with a mixture of anti-C1s and anti-C1r in the gel and with (A) EDTA or (B) Ca^{2+} in the buffer. (Reproduced with permission of Georg Thieme Publishers, Stuttgart, from Opferkuch W, Rother K and Schultz DR (eds) (1978) *Clinical Aspects of the Complement System*.)

cirrhosis. Sera from children with recurrent pneumococcal otitis media often contain excessively high levels of zymogen C1r–C1s complexes, which disappear on recovery.

By means of crossed immunoelectrophoresis, aberrations within the immunoglobulins can be visualized, thus revealing deficiency states or myeloma and macroglobulinemia, even though other methods are now available that are generally used for this purpose.

Crossed immunoelectrophoresis is very useful in complement research, making possible the analysis of the activation of the classical and alternative pathways. It is an important clinical tool for obtaining information on the involvement of complement in the pathogenesis of various immunological and inflammatory diseases.

Electrophoresis-immunofixation

With these techniques protein mixtures are separated by electrophoresis in agarose, polyacrylamide gels, on cellulose acetate strips or by isoelectric focusing, followed by overlaying with monospecific antiserum. After incubation and washing, the gel is stained for precipitated proteins. The method is used for phenotyping, for instance of the C4 complement component. It is also widely used for identification of the abnormal proteins in myeloma, Waldenström's macroglobulinemia and heavy and light chain diseases.

Electroimmunoassay (rocket immunoelectrophoresis)

When electrophoresis of an antigen is performed from a punched well in an agarose gel containing the corresponding antibody, a precipitate is obtained with a rocket-like appearance, the height of which is proportional to the amount of antigen in the gel. The shape of the precipitate is dependent on the antigenic content of the molecule studied and on the specificities of the antibody used in the agarose gel. Therefore, visual examination of the shape of the precipitate is very important and must be compared with the shape of the precipitate obtained with a standard preparation of the relevant antigen. Thus C1s rockets obtained with normal plasma are clear and distinct, whereas in many pathological sera diffuse precipitates are obtained, indicating changes in the antigenic reactivity of C1s, usually due to masking of antigenic determinants by complex formation with C1 inhibitor or by conformational changes during activation of C1. A similar course of events may be of relevance for several plasma proteins. Findings of aberrations by electroimmunoassay may provide

a basis for further analysis of, for example, complex formation, cleavage of the molecule or genetic antigenic variation.

Investigation of the C1 inhibitor by the rocket technique in a patient with hereditary angioedema using anti-C1 inhibitor in the gel showed a very high and thin precipitate, indicating a molecular structure partially different from that of normal C1 inhibitor (Figure 2). Further analysis showed that the nonfunctioning inhibitor of the patient was complexed with albumin. The high, rather thin rockets can be explained by masking of antigenic determinants on the C1 inhibitor molecule, resulting in fewer sites able to combine with the antibody.

The electroimmunoassay allows specific and quantitative determination of antigen in mixtures of proteins, such as serum, plasma, synovial fluid, urine, cerebrospinal fluid and so forth. It is an easy and fairly rapid method, taking between 2 and 16 hours, depending on the electrophoretic mobility of the molecules under investigation and the field strength used. Electroimmunoassay has been widely used for quantification, particularly of plasma constituents such as acute phase proteins (e.g. C-reactive protein (CRP), α_1 -antitrypsin, α -antichymotrypsin) and complement components (for diagnosing complement deficiency and hypo- or hypercomplementemia).

The electroimmunoassay is an easy and rapid laboratory method for tracing the individual proteins in chromatographic separation studies and possible changes in their structure during purification procedures. The morphology of the precipitate obtained is of importance in assessing any denaturation of the molecules under study. In this respect both electroim-

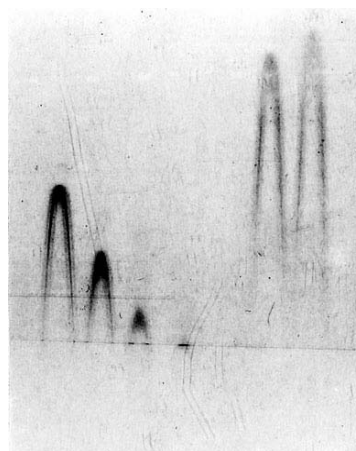


Figure 2 Rocket electrophoresis of C1 inhibitor in serum from a patient with hereditary angioedema. Anti-C1 inhibitor was present in the agarose gel. The four holes to the left contained normal serum in dilution 1/10, 1/20, 1/40, 1/80. The two holes to the right contained serum from the patient diluted 1/20.

munoassay and crossed immunoelectrophoresis may provide further information which complements results obtained with other techniques.

Modifications of electroimmunoprecipitation methods

Techniques using intermediate gels

The components of molecular complexes precipitating in the electroimmunoassay may be identified in greater detail by using an intermediate gel, cathodal to an anodal gel containing the discriminating antibody. Molecular complexes containing a component corresponding to the antibodies in the intermediate gel will be trapped there, resulting in smaller or no precipitates in the anodal gel, which contains the antibodies identifying the other molecular components of the complex. This variation of the electroimmunoassay and crossed immunoelectrophoresis has been often used, for example for the detection and measurement of C3d in plasma and synovial fluid ('double decker electrophoresis') (Figure 3). A cathodal intermediate gel incorporates antibodies which react with epitopes common to native C3, C3b and C3c, but absent in C3d. C3, C3b and C3c molecules are precipitated by anti-C3c and do not reach the anodal gel, which contains antibodies specific for C3d. C3d passes freely into the anodal gel and forms a precipitate with anti-C3d.

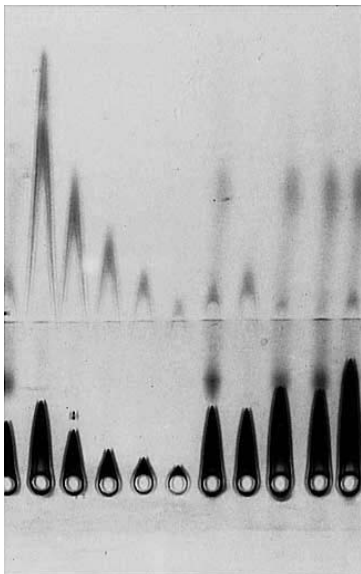


Figure 3 Rocket immunoelectrophoresis ('double decker electrophoresis') for quantitation of C3d. Samples were applied in holes in a cathodic gel containing antibodies reacting with C3, C3b and C3c, but not with C3d. Anodal to this gel, a gel with antibodies to C3d was moulded. The four holes to the left contained dilutions of normal serum treated with inulin to convert C3 to C3d.

A similar simplified procedure for trapping protein-protein complexes is obtained by filling a small basin – anodal and near to the antigen application well – with antibodies against one of the components of the complex under study. In electroimmunoassay the height of the rocket will be less than that of the rocket in a parallel run where the anodal basin contains buffer or an irrelevant antibody.

Fused rocket immunoelectrophoresis

This modification of the rocket technique can be utilized to visualize the distribution profile of individual proteins in mixtures, and has proved valuable in the analysis of fractions obtained in separation and purification experiments. The samples are applied to a row of wells in an antibody-free agarose gel, adjacent to an agarose section containing antibodies to the various proteins that may be present in the samples. After diffusion into the antibody-free gel, the proteins are induced by electrophoresis to migrate into the gel containing antibody. An undulating precipitation line will be formed showing the quantitative distribution of each antigen.

Tandem crossed electrophoresis

In this modification of crossed immunoelectrophoresis the first electrophoretic step is run with an antigen mixture applied in adjacent wells in the agarose gel, followed by electrophoresis into the second gel containing appropriate antibodies. The resulting precipitates help to reveal identity, partial identity and nonidentity between the proteins used in the experiment.

Further variants are line immunoelectrophoresis and rocket line immunoelectrophoresis, which have been used for comparing the antigenic composition of unknown proteins with mixtures of standard antigens.

Counterimmunoelectrophoresis or immunoelectro-osmophoresis

This method is utilized to accelerate and accentuate immunoprecipitation. Most antigens are negatively charged and move towards the anode in an electric field, whereas IgG immunoglobulins in buffers of pH 8.2–8.6 are fairly stationary owing to the electroendosmotic flow in the agarose matrix. The antigen is applied in a well at the cathodal end of the gel and the antibodies in a corresponding anodal well. On electrophoresis the antigen migrates towards the antibody, a precipitate being formed where they meet. The technique is widely used in microbiology for the detection of bacterial carbohydrate antigens, especially in cerebrospinal fluid, and for detection of

viral antigens in body fluids and in screening for viral antibodies.

Affinity electrophoretic method(s)

This procedure is used to study interactions between lectins and glycoproteins. The principle is the same as in immunoelectrophoresis using an intermediate gel containing antibody. However, instead of antibodies, lectins are incorporated into the intermediate gel and the second step is then performed in the usual way. Components binding to the lectin will be trapped in the intermediate gel and lower or no precipitation arcs will appear, as compared with the control experiment. This method and its variants allows identification of ligand-binding proteins and characterization of reactive sites.

Amplification methods

Sandwich technique using enzyme-labeled anti-Ig antibodies

After ordinary performance of crossed immunoelectrophoresis or electroimmunoassay, the gel is pressed, washed and then overlaid and incubated with enzyme-labeled anti-Ig antibodies directed against the antibodies used in the agarose gel. After washing, the precipitates in the gel are visualized by staining with the substrate of the enzyme label on the anti-Ig. As compared with protein staining with Coomassie brilliant blue, there is a 25–50 fold increase in sensitivity. This method is of value in studies of plasma proteins present at low concentrations. Factor D of the alternative pathway of complement, with an average plasma concentration of 1.6 mg l^{-1} can be determined by this sandwich variant of electroimmunoassay, and its relationship to and reactivity with other plasma components can be studied.

Radioimmuno-electrophoresis

The sensitivity of crossed immunoelectrophoresis and of rocket immunoelectrophoresis can be increased by the addition of radiolabeled anti-Ig antibodies after completion of the electrophoresis, as described above for enzymatic amplification of the methods. After washing of the gel, the radiolabeled anti-Ig antibodies will bind to the trace immunoprecipitates present in the gel. With autoradiography the precipitation lines can be determined. The method has been used for identification of allergens and determination of the specificity of patients' IgE.

A rocket electrophoresis variant consists of incorporation of a radiolabeled antigen in an antibody-free gel section at the site of sample application.

Adjacent to this section, the gel containing the relevant antibodies is moulded. As with the enzyme-labeled methods, the techniques using radiolabeled antigens in the gel have been used for the study of proteins present at low concentrations in body fluids. Examples include α -fetoprotein and certain pregnancy-associated proteins such as chorionic gonadotropin.

Electrofocusing-immunoelectrophoresis

The isoelectrophoretic separation of protein mixtures – most commonly by polyacrylamide electrophoresis – is followed by electrophoresis in a gel containing antibody. Thus the proteins can be characterized by their isoelectric point and the antigenic composition can be determined. This gives high resolution of identity, partial identity and nonidentity of various antigens, of genetic variants of single antigens, and of isoenzymes.

Electroimmunoprecipitation and electroimmunofocusing are also suitable for analysis of solubilized membrane proteins. For example the terminal C5b–C9 complement complex was studied and could be shown to be integrated in the erythrocyte membrane. Analysis by these methods of membranes of lymphocytes and thrombocytes as well as of membranes of microorganisms have proved valuable in the study of membrane proteins.

See also: Acute phase proteins; Antibodies, detection of; C-reactive protein; Enzyme labeling of antibodies and antigens; Gammaglobulin; Immunodiffusion, single radial; Isoelectric focusing; Lectins; Precipitation reaction; Radiolabeling.

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IMMUNOGEN

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Immunogen is a substance (molecule) capable of provoking an immune response. This immune response may be strictly cellular, leading to a specific T cell response, or it may lead to specific antibody formation. The notion of immunogen should be distinguished from that of *antigen*, which encompasses both the properties of an immunogen but also that of a substance which cross-reacts immunospecifically with antibodies or immunocytes but is incapable of triggering an immune response. It should also be distinguished from a *tolerogen*, which is a substance (molecule) inducing immunological tolerance.

Immunogenicity is the capacity to provoke an immune response. When one refers to an immunogenic molecule, this means that this molecule is capable of triggering an immune response by itself, not when conjugated to another macromolecule. Thus, an immunogenic peptide, for example, refers to a peptide immunogenic by itself, whereas a peptide which serves as an important epitope while attached to a macromolecule, should be referred to as *immunopotent* or, if it is more immunopotent than related peptides, as *immunodominant*.

A chemical or physical change in a molecule that will result in an increased immune response will increase its immunogenicity, even though it may or may not have changed its *antigenic specificity*.

The immunogenicity of a substance will depend on the manner in which it is administered, on the amount administered, and on the genetic make-up of the animal or human being immunized. Defining a substance as immunogenic will depend on the sensitivity of the method used to detect the immune response.

See also: Adjuvants; Antibodies, specificity; Antigens; Autoantigens; Cytotoxic T lymphocytes; Immune response; Immune response (Ir) genes; Immunodominance; Immunopotential; Tolerance, peripheral; Vaccines.

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IMMUNOGLOBULIN, CELL SURFACE

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It was Ehrlich in 1900 who first postulated that lymphocytes recognized antigen via receptors on their cell surface which were structurally similar to the effector molecules that they secreted. This notion was later developed by Burnet into the clonal selection theory to account for specificity of the inductive and effector phases of the immune response. Subsequently, immunofluorescence studies by Coon and Möller established that B lymphocytes expressed immunoglobulin (Ig) molecules on their surface (sIg). This observation was quickly followed by the findings that these molecules were the antigen recognition structure, or antigen receptor, on the B lymphocyte.

Since these initial observations, all immunoglobulin isotypes have been found on the surface of individual B cells. In general, a given B lymphocyte expresses immunoglobulin of only one isotype at any given time. The exception to this rule are unstimulated, mature B lymphocytes which coexpress surface IgM (sIgM), as well as surface IgD (sIgD).

Function of surface immunoglobulin

Membrane-associated immunoglobulin functions as a receptor on the surface of B cells which recognizes and binds antigen. Upon binding antigen, the sIg-antigen complex is endocytosed, processed and represented on the B cell surface in association with class II major histocompatibility antigens (MHC class II) for presentation to class II-restricted T helper cells. This interaction serves to facilitate activation of the T cell, which in turn delivers activation and differentiation signals to the B cell. In fact, it has been argued that the only signals necessary for B cell activation are derived from this B-T cell interaction; sIg, in this case, plays an insignificant role in generating activation signals for the B cell. In this regard, antigen processing and presentation by B cells has been found to be independent of sIg signaling. Monovalent immunoglobulin-specific antibodies (anti-Ig) that would not be expected to elicit an activation signal because they do not cross-link the receptor, nonetheless are presented as efficiently as divalent anti-Ig to Ig-specific T cell lines. This interaction generates the necessary signals for the activation of the T cell, which in turn can provide all the required signals for stimulation of the B cell to proliferate and secrete immunoglobulin. In an apparent contradic-

tion of this conclusion, other studies clearly establish that signals generated by sIg cross-linking do potentiate the activation of the B cell. In fact, under conditions of optimal antigen receptor cross-linking (i.e. by divalent anti-Ig or thymus-independent antigens) these signals are sufficient to trigger entry into cell cycle and proliferation by the B cell.

To reconcile these apparently contradictory requirements for triggering B cells, it is perhaps informative to consider that antigen-specific activation of the B cell requires that the B cell achieve a certain threshold stimulation. Superoptimal T cell help and sIg cross-linking are by themselves each able to facilitate at least the initial stages of B cell activation. However, in most cases, the initial sIg signal is suboptimal and the antigen-reactive T helper cells at the time of initial interaction with the B cell are not optimally activated. In this more typical case, multiple signals generated through sIg and as a consequence of B cell presentation of antigen to the T cell are necessary to achieve activation. This idea is consistent with the observation that sIg-stimulated B cells express higher levels of class II antigen and surface molecules involved in costimulation of T helper cells.

Structure and composition of the B cell antigen receptor (BCR)

The basic monomeric structure of sIg is identical to that of the secreted form of this molecule. Each molecule possesses two light chains disulfide coupled to two disulfide-linked heavy chain molecules. The membrane form of immunoglobulin differs from the secreted form with respect to the carboxy end of the molecule. The membrane form includes additional amino acid residues comprising a spacer, transmembrane and cytoplasmic region. This material is encoded by two additional exons, M1 and M2, which differ to a greater or lesser extent for each immunoglobulin. Production of the membrane form as opposed to the secreted form is determined post-transcriptionally through differential RNA processing.

The basic structure of the membrane form of sIgM is illustrated in **Figure 1**. The spacer region is composed of 12 residues. This region is highly hydrophobic, containing six glutamic acid residues per chain. The spacer region is followed by a highly con-

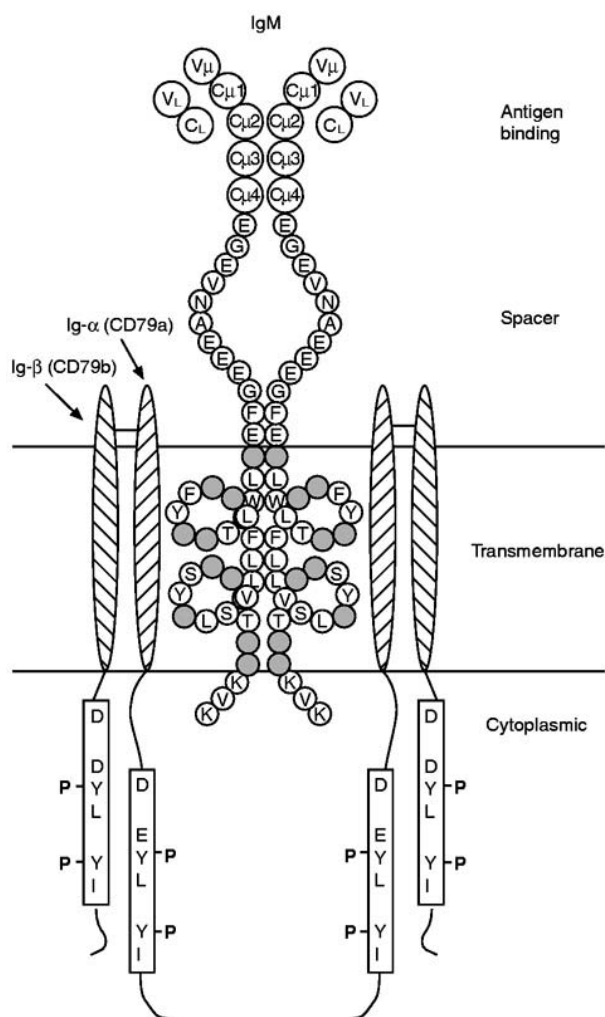


Figure 1 Structure of membrane-associated IgM. Shaded amino acid residues depict those of high variability between immunoglobulin isotypes. General structure and postulate stoichiometry of Ig- α and Ig- β molecules highlighting ITAMs.

served hydrophobic sequence of 25 amino acids which comprises the transmembrane region. Homology within this region is high among different iso-types, with differences tending to cluster to specific subregions. Interestingly, imposition of an α -helical configuration to the transmembrane region clusters these nonhomologous residues to the outside of the structure, leaving the most highly conserved residues at the point of interaction between the two heavy chains.

The C-terminal segment of the molecule is hydrophilic, beginning with lysine-valine-lysine for μ , δ , and γ . This sequence constitutes the entirety of the cytoplasmic domain for μ and δ . The γ isotypes possess an additional 25 amino acids. The membrane form of the α isotype does not contain the characteristic lysine-valine-lysine cytoplasmic sequence but, rather, possesses a unique 14 amino acid stretch.

The BCR exists as a complex in the plasma membrane of the B cell, consisting of the slg antigen recognition structure together with the Ig- α (CD79a) and Ig- β (CD79b) proteins. These latter molecules are products of the mb-1 and B29 genes, respectively, and constitute the signaling components of the B cell antigen receptor. Both the Ig- α and Ig- β proteins possess extensive cytoplasmic domains which contain a region called an immunoreceptor tyrosine-based activation motif (ITAM) homologous to other signaling molecules in the immune system, including components of the T cell antigen receptor. Tyrosine residues associated with these ITAMs become phosphorylated following slg cross-linking and are important in initiating biochemical signaling pathways associated with antigen-driven B cell activation.

slg transmembrane signaling

Cross-linking sIg causes a rapid activation of receptor-associated tyrosine kinases. These kinases, *fyn*, *lyn*, *blk*, and in some reports *lck* belong to the *src* family of tyrosine kinases and are associated with the receptor in the unstimulated B cell. It is currently believed, although not formally proven, that these receptor-associated kinases are responsible for the tyrosine phosphorylation of the ITAMs of Ig- α and Ig- β described above, as well as a number of other protein substrates responsible for initiating defined biochemical signaling pathways associated with sIg-induced activation. These substrates and signaling pathways are shown in Figure 2. So far, three signaling pathways have been shown to be associated with BCR signal transduction: phosphatidylinositol (PI) hydrolysis, *ras*/MAPK and phosphatidylinositol-3-kinase (PI3K) pathways. Association with multiple signaling pathways might at first be unexpected but probably reflects the fact that antigen stimulation through sIg must initiate a number of diverse cellular responses (i.e. ligand internalization and processing, changes in cell adhesion and localization, proliferation, differentiation). Regulation of these pathways is complex and there is evidence for cross-talk occurring between them.

Phosphatidylinositol hydrolysis (PI hydrolysis) has been shown to be important in linking BCR signaling to cell cycle entry and proliferation. Phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) results in the generation of diacylglycerol (DAG) and inositol triphosphate (IP₃), which activate the serine/threonine kinase PKC and cause a release of calcium from stores in the endoplasmic reticulum, respectively. Protein kinase C (PKC) and Ca²⁺ act as intracellular second messengers to propagate the BCR-generated signals through

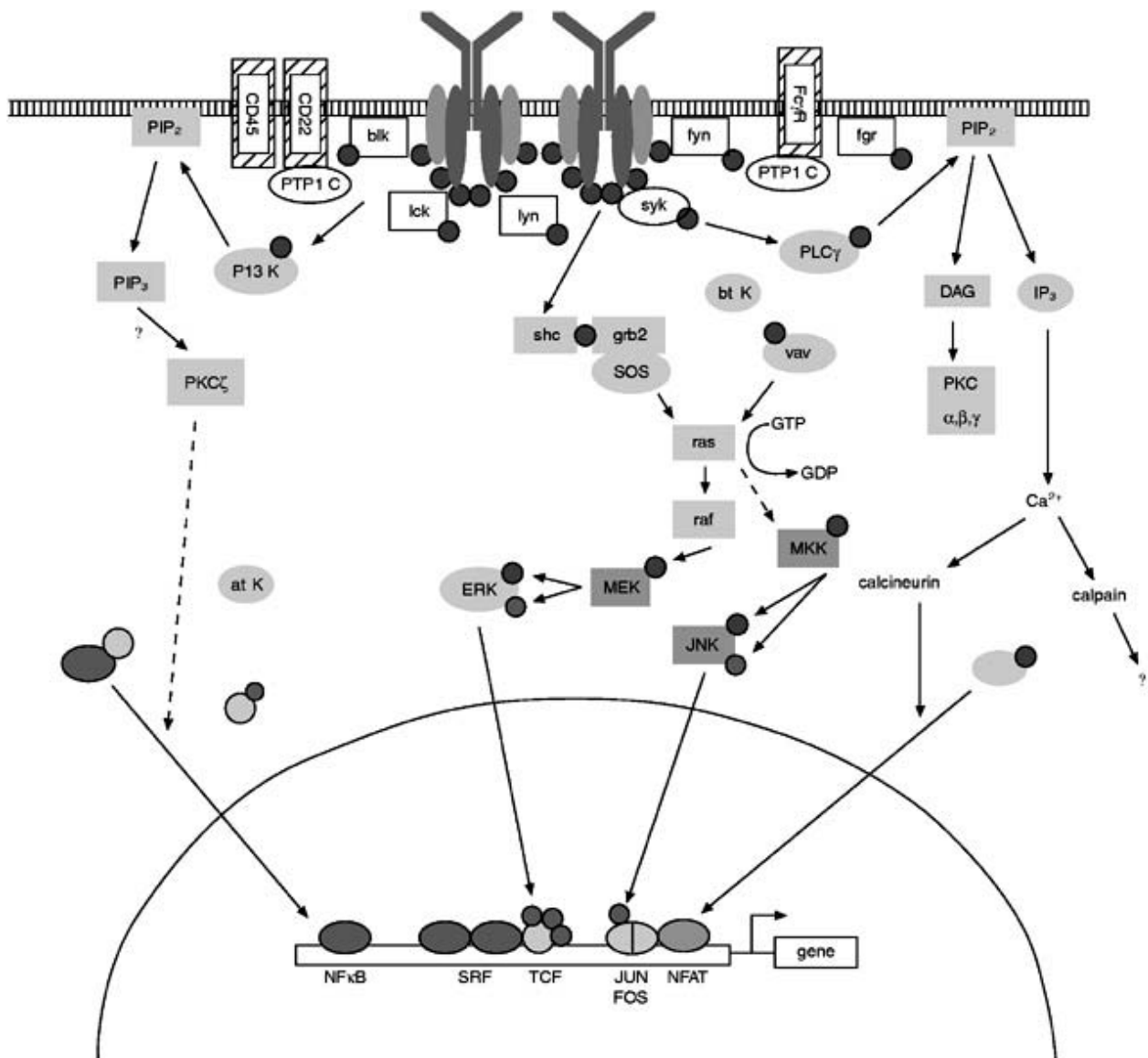


Figure 2 Signal transduction pathways associated with slg in mature-stage B cells. See text and references for details.

the cytoplasm. As demonstrated first in the platelet-derived growth factor receptor system and later in B cells, tyrosine phosphorylation of PLC γ 1 and γ 2 is necessary for the receptor-induced activation of their enzymatic activity. Therefore, the BCR-induced tyrosine phosphorylation of PLC γ 1 and γ 2 observed in B cells is likely to be an important event linking slg cross-linking to downstream signaling events. Furthermore, PI-pathway linked activation of PKC may be involved in the regulation of other signaling pathways in B cells. Through its ability to activate *vav* and SOS, PKC may be involved in regulating the activation of p21^{ras}.

p21^{ras} is a membrane-associated guanine nucleotide-binding protein whose activation has been as-

sociated with receptor-mediated activation in a number of cell types, in addition to BCR-stimulated B cells. The regulation of *ras* during receptor signaling is complex, involving a number of cytoplasmic signaling processes. Positive activation of *ras* in BCR-stimulated B cells has been shown to involve the sequential recruitment of shc, grb2 and the guanine nucleotide exchange factor SOS to the phosphorylated ITAMs of the Ig- α and Ig- β chains of the BCR. Recruitment of SOS facilitates the exchange of GTP for GDP at the nucleotide-binding pocket of *ras*, resulting in a catalytically active molecule. Negative regulators of *ras* activity also exist. Evidence argues that GAP, another substrate of tyrosine kinases in B cells, reduces activity of *ras*, a regulatory process that

is reversed upon tyrosine phosphorylation of GAP. Although a target for tyrosine kinases in B cells, the limited experimental evidence available does not support a role for GAP in the regulation of *ras* in BCR-stimulated B cells. However, another GAP-like protein, neurofibromin, may play a role. Finally, PKC has also been shown to upregulate *ras* activity, although the mechanism by which this regulation is accomplished is not fully understood.

Recently, it has been observed that PKC may activate *vav*, and *vav* has been shown to be able to affect *ras* activation, presumably due to its ability to accelerate the GDP–GTP exchange associated with the activation of *ras*. Thus, the *ras* signaling pathway can be directly activated by tyrosine kinase activation (in the case of *shc/grb2/SOS*) as well as indirectly by the PI-hydrolysis pathway (in the case of PKC activation of *vav*), demonstrating both the redundancy inherent in many signal transduction pathways as well as the cross-talk that can occur between different second messenger pathways.

The PI3K pathway is the most poorly understood signaling pathway associated with BCR signaling. Phosphorylation and activation of PI3K has been shown to occur following BCR cross-linking. The target of PI3K is the phospholipid PIP₂, which is phosphorylated to PIP₃. The relevance of PIP₃ in signal transduction is not well-defined, although it has been implicated in the activation of the nonconventional protein kinase C isozyme, PKC ζ . In *Xenopus*, PKC ζ has been implicated in oocyte maturation and in responses to the PDGF receptor in fibroblasts. However, the role of PKC ζ and the PI3K pathway has not been studied in BCR-stimulated B cells.

Developmental regulation of BCR signaling

Immunoglobulin heavy chain genes are rearranged and expressed during the late pro-B cell stage. Recently it has been shown that these are expressed on the surface of pre-B cells. However, in contrast to mature-stage B cells the pre-B cell form of sIg is not associated with the conventional κ or λ light chains. Rather, the μ heavy chain is complexed with the $\lambda 5$ and VpreB proteins which form the surrogate

light chain. The $\mu\lambda 5$ /VpreB complex, like the mature form of the antigen receptor, is associated with Ig- α and Ig- β and there is some preliminary biochemical and functional evidence to suggest that it generates a signal. The ligand for the pre-B cell form of sIg and the role of this receptor in B cell biology is under investigation.

The mature form of sIg is first expressed at the immature stage of B cell development. Interestingly, while cross-linking sIg on mature, immunocompetent B cells initiates activation, sIg cross-linking on immature-stage B cells leads to negative selection and tolerance. Although there are a number of possibilities that could account for differences in complex processes involving activation and tolerance induction of B cells, there is evidence to suggest that the mechanism of sIg signal transduction in immature and mature B is different.

See also: B lymphocyte activation; B lymphocyte, antigen processing and presentation; B lymphocytes; Clonal selection; Immunoglobulin, functions; Immunoglobulin structure.

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IMMUNOGLOBULIN CLASS SWITCHING

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The class of an antibody determines its functional properties and thus the kind of humoral immune response against a particular antigen. In 1964, Nossal and collaborators observed for the first time that individual B cells can switch the class of the immunoglobulin (Ig) they produce, while retaining the antigenic specificity. The molecular basis for this process, termed isotype or class switching, is a recombination between highly recombinogenic DNA sequences, so-called switch (S) regions. The switch recombination machinery is induced in naive and memory B cells upon activation by antigen or mitogen and targeted to distinct S regions by cytokines. These cytokines target recombination to particular S regions by inducing transcription of the respective S regions prior to recombination and this transcription is somehow functionally linked to the recombination event.

Molecular basis of immunoglobulin class switching

Mice have antibodies of the classes IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE and IgA, and humans of the classes IgM, IgD, IgG3, IgG1, IgG2, IgG4, IgE, IgA1 and IgA2. Antibody classes differ in the Ig heavy chain constant region gene (C_H gene) that is expressed. The C_H genes are clustered at the 3' end of the Ig heavy chain variable region genes (V_H genes). The entire murine C_H gene locus spans approximately 200 kb with eight C_H genes that are arranged in the order 5' C_{μ} - C_{δ} - $C_{\gamma 3}$ - $C_{\gamma 1}$ - $C_{\gamma 2b}$ - $C_{\gamma 2a}$ - C_{ϵ} - C_{α} 3'. The human C_H gene locus extends over more than 300 kb with nine functional genes and two pseudogenes (ψ) that are ordered 5' C_{μ} - C_{δ} - $C_{\gamma 3}$ - $C_{\gamma 1}$ - ψ_{ϵ} - $C_{\alpha 1}$ - ψ_{γ} - $C_{\gamma 2}$ - $C_{\gamma 4}$ - C_{ϵ} - $C_{\alpha 2}$ 3'.

Coexpression of C_{μ} and C_{δ} with the same VDJ gene on mature naive IgM⁺ IgD⁺ B cells is due to differential termination of transcription and differential splicing of transcripts encompassing both constant region genes.

The molecular basis of antibody class switching to the expression of C_{γ} , C_{ϵ} , and C_{α} genes in activated B cells is a recombination which positions the new C_H gene 3' next to the VDJ gene. The apparent sites of Ig class switch recombination are located within the S regions, highly repetitive DNA sequences which are present 5' of each C_H gene, except C_{δ} .

All murine and most humans S regions are sequenced at least partially. They are 1–10 kb in length, highly repetitive and GC-rich. Murine and human S_{μ} are almost homogeneously composed of the two pentamer sequences GAGCT and GGGGT and the heptamer sequence (C/T)AGGTTG. All other S regions also contain multiple copies of the pentameric sequences. All murine S regions except S_{μ} are composed of tandem repeats that vary both in sequence and in length, with 49 bp repeats for $S_{\gamma 1}$, $S_{\gamma 3}$ and $S_{\gamma 2b}$, 52 bp repeats for $S_{\gamma 2a}$, 80 bp repeats for S_{α} and 40 bp repeats for S_{ϵ} . Both human and murine S_{μ} are more homologous to S_{ϵ} and S_{α} than to the S_{γ} regions, which have considerable homology among each other. The homology of murine S_{γ} regions to S_{μ} decreases with the distance on the chromosome ($S_{\gamma 3} > S_{\gamma 1} > S_{\gamma 2b} > S_{\gamma 2a}$). The S regions are sufficiently conserved between human and mouse to allow human S regions to be used as substrate for switch recombination in murine cells. The S_{μ} , S_{ϵ} and S_{α} regions are more homologous between the two species than the S_{γ} regions. The length of the S regions is subject to considerable allelic variation (length polymorphism) indicating that there is no functional requirement for a particular size of a given S region.

S regions are preferred targets not only for the switch recombination machinery but also for other bacterial and eukaryotic recombination systems. The extraordinary recombinogenic potential might be due, in part, to the formation of unusual DNA structures promoted by the repetitive character, the high degree of homology and the high GC content of S regions.

The primary mechanism for switching that relocates a new C_H gene next to the VDJ gene is an intrachromosomal recombination between a donor and an acceptor S region, with looping out and deletion of the intervening DNA as a circle, that can be isolated from switched cells. In rare cases the deleted DNA is reintegrated in inverted form either back into the C_H gene locus or even into other regions of the genome.

More than 150 DNA sequences at S region recombination sites have been analyzed. Within S regions, switch recombination breakpoints are evenly distributed, without apparent preference of a particular area within the S region and without specific signal

sequences present at or juxtaposed to all switch recombination sites. Some recombination sites reveal a few base pairs of homology between donor and acceptor S region. Analyzing switch recombination sites is difficult and results have to be taken with care due to the possibility that during amplification and cloning of recombined switch sequences, additional recombinations might take place in bacteria used for cloning and the sites identified may be not the sites of the primary switch recombination event in B cells.

Mitotic switch recombination by unequal crossing-over between sister chromatids may occur occasionally. It results in the asymmetric segregation of C_{H1} genes, giving rise to one daughter cell with a deletion of C_{H1} genes and another with a duplication.

Switch recombinations normally occur in *cis*, but S regions can also be recombined in *trans*. This has been demonstrated in several B cell lines, for normal B cells of transgenic mice with an additional S_{μ} region integrated outside the C_H gene locus, and at a surprisingly high frequency for rabbit B cells. Transchromosomal recombination is also responsible for some *c-myc* gene translocations, in which only one of the recombination partners is an S region.

Coexpression of IgM along with IgG, IgA or IgE on the surface, with all C_{H1} genes still in germline configuration, has been reported in both normal and transformed B cells. However, in most cases it has not been ruled out that Ig of one of the classes had been passively picked up, e.g. by class-specific Fc receptors. If cells expressing IgM together with other, non-IgD isotypes exist, they might do so either by differential processing of long transcripts spanning the VDJ, C_{μ} , C_{γ} , C_{α} and C_{ϵ} genes or by trans-splicing of the VDJ exon from primary VDJ- C_{μ} transcripts to C_{γ} , C_{α} or C_{ϵ} exons from C_{γ} , C_{α} or C_{ϵ} switch transcripts (see below). Both models have been proposed, but so far clear evidence showing that either or both of them are of biological relevance has not yet been provided. Instead, it has been shown that surface IgA1-bearing B lymphocytes from human peripheral blood, i.e. IgA1⁺ memory B cells, express only IgA1 and have already performed switch recombination, as seen in IgA-secreting plasma cells. This leaves little room for a physiological role of Ig class switching without switch recombination, except for IgD expression.

Induction of switch recombination

The switch recombination machinery can be induced in naive IgM- and IgD-expressing B cells and in memory B cells *in vitro*, by cross-linking surface Ig with mimics of T cell-independent type 2 (TI-2

antigens) such as anti-Ig antibody conjugated to dextran, by B cell mitogens (TI-1 antigens) such as bacterial lipopolysaccharide (LPS), or by activated T cells. Triggering of CD40 on B cells by its ligand (CD40L) on activated T cells, appears to play a crucial role for switch induction in the process of T cell-dependent B cell activation. Point mutations or deletions in the gene encoding the CD40L are responsible for the X-linked hyper-IgM syndrome, which is characterized by dramatically reduced levels of serum Ig of classes other than IgM, reflecting a severe defect in T cell-dependent immune responses and Ig class switching. Similarly, CD40- and CD40L-deficient mice show normal IgM but no detectable IgG, IgE and IgA antibody responses to T cell-dependent antigens, while antibody responses to type II T cell-independent antigens are not affected.

Within a period of about 2 days after onset of B cell stimulation, activated B cells begin to undergo class switching at high frequency. Frequencies of switched cells can be as high as 10% per generation. Switch recombination is not necessarily a one-hit event in individual cells. Usually activated B cells undergo switch recombination not only on the expressed but also on the allelically excluded IgH allele.

Intra-S region recombinations can also occur in switching cells, especially involving sequences in the area of the S_{μ} pentameric repeats and 5' thereof, which are frequently deleted on the chromosome after the switch recombination between two different S regions has taken place. Intra-S region recombinations are also observed on S regions of non-switched excluded IgH alleles, in particular on S_{μ} , and on downstream S regions, the latter in the case of S_{μ} -inactivated cells. Secondary or even tertiary switches involving two or more S regions downstream of S_{μ} on the chromosome can also occur, allowing B cells to switch sequentially to different isotypes. This seems to be the dominant although not obligatory pathway for switching to IgE, which often occurs via IgG1 in mice or IgG4 in humans.

All switch recombinations inducing B cell differentiation signals also induce DNA synthesis and proliferation in resting B cells, but it is not clear whether replication of DNA is required for switch recombination. Several groups have reported evidence that DNA synthesis is associated with switch recombination since inhibition of DNA synthesis in B cells also inhibits switching. Small mutations (substitutions, insertions and deletions), indicative of error-prone DNA synthesis, have been identified near the sites of switch recombination.

In hybrid cells of class switch-performing activated B cell blasts and immortalized plasma cells, switch

recombination is arrested indicating that the switch recombination machinery may be actively turned off by an inhibitor in the plasma cell stage.

Directing switch recombination to distinct switch regions

The choice of the S regions involved in class switch recombination in individual cells is not random. This first became evident from the observation that switch recombinations involving S regions of the same class of Ig occur on both IgH alleles of individual switched cells. Since those B cells could hardly have been selected for a particular switch recombination on a non-expressed IgH allele, this analysis has shown that activated B cells expressing particular isotypes are committed to switch to particular classes of Ig.

When and how is this commitment induced? It is clear that, depending on the mode of activation of the B cell and dependent on cytokines produced by T cells or other cells, including natural killer (NK) cells, mast cells, basophils and macrophages, distinct S regions are targeted for recombination in activated B cells. In the mouse, three cytokines, interleukin 4 (IL-4), interferon γ (IFN γ), and transforming growth factor β (TGF β), have been shown to play pivotal roles in this process; others may be identified in the future. IL-4 targets the S $_{\gamma 1}$ and, at higher concentrations, also the S $_{\epsilon}$ region for switch recombination, IFN γ targets the S $_{\gamma 3}$ and S $_{\gamma 2a}$ regions for switch recombination, and TGF β targets the S $_{\gamma 2b}$ and S $_{\alpha}$ regions for switching. Thus, each of the three cytokines regulates two of the six non-IgM, non-IgD murine Ig isotypes in a positive manner. Furthermore, in the mouse, IFN γ suppresses IL-4-induced switching to IgG1 and IgE, while IL-4 suppresses IFN γ -induced switching to IgG3 and IgG2b. Neither IL-4 nor IFN γ are obligatory for switch induction. IFN γ receptor-deficient mice can still produce IgG3 and IgG2b, and IL-4-deficient mice can still produce IgG1, albeit in reduced amounts. For human B cells, less is known about the signals that induce or suppress certain S regions for recombination, except that both IL-4 and IL-13 target the S $_{\gamma 4}$ and S $_{\epsilon}$ regions for switch recombination, probably by signaling via a shared receptor component of the IL-4 and IL-13 receptors.

At the molecular level, targeting of certain S regions for switch recombination is strictly correlated with induction of transcription of those S regions. This has been well established for cell lines which are stably committed to switch to particular Ig isotypes, as well as for murine and human B cells stimulated by switch-targeting cytokines. Human and murine S region transcripts (switch transcripts)

initiate at multiple initiation sites of a promoter, the I $_{\text{H}}$ promoter, located upstream of the respective S region, proceed through the S region and terminate at the normal polyadenylation sites downstream of the respective C $_{\text{H}}$ gene. The primary transcripts are processed into mature switch transcripts and transported into the cytosol. The processed switch transcripts, also called 'sterile' or 'germline' transcripts, differ from the productive VDJ C $_{\text{H}}$ mRNAs by an exon, the I $_{\text{H}}$ exon, encoded just 5' of the S region that, instead of the VDJ exon, is spliced to the first C $_{\text{H}}$ exon. Although transcription from the V $_{\text{H}}$ promoter proceeds through the S $_{\mu}$ region after VDJ recombination, in addition switch transcripts containing an I $_{\mu}$ exon initiate from the I $_{\mu}$ promoter. After switch recombination, transcription from this I $_{\mu}$ promoter proceeds through the hybrid S region and hybrid switch transcripts containing the I $_{\mu}$ exon and the exons of the switched C $_{\text{H}}$ gene are generated. Transcription of hybrid S regions might be required for sequential switching to downstream isotypes. Due to stop codons in the I $_{\text{H}}$ exons, switch transcripts are not translated into proteins (i.e. they are sterile). At most, short sequences may be translated into peptides.

The functional dependency of switch recombination on S region transcription has been effectively demonstrated by targeted deletion or replacement in murine genomes of the I $_{\gamma 1}$ and I $_{\gamma 2b}$ exons together with their cytokine-responsive promoters, which results in an almost complete and selective deficiency in germline transcription of the adjacent S regions as well as switch recombination to S $_{\gamma 1}$ and S $_{\gamma 2b}$, respectively. Deletion of the J $_{\text{H}}$ gene segments and the I $_{\mu}$ promoter leads to a drastic reduction of switch recombination involving S $_{\mu}$.

Along with transcription of S regions, cytokines such as IL-4 have also been shown to induce selective hypomethylation of previously hypermethylated CpG dinucleotides and to induce DNAase I hypersensitive sites at the regions of initiation of germline transcription. In general, induction of demethylation and of DNAase I hypersensitivity has been shown to reflect binding of inducible transcription factors to promoter and/or enhancer DNA sequences. Identification and characterization of the cytokine-responsive transcription elements that control S region transcription and cloning of the cytokine-inducible nuclear transcription factors that bind to these elements is just in its infancy. At present most information is available on the murine I $_{\epsilon}$ promoter. A minimal 179 bp I $_{\epsilon}$ promoter sequence was shown to impart IL-4-inducible and IFN γ -suppressive transcription to a heterologous reporter gene. Using electrophoretic mobility shift assays (EMSA), several

nuclear protein complexes have been demonstrated to bind to this region, but only two of the proteins involved have been cloned so far, STAT6 (STF-IL4) and BSAP. STAT6 is a member of the signal transducer and activator of transcription family of proteins that is activated by tyrosine phosphorylation in response to IL-4. STAT6-null mice are deficient in class switching to IgE. BSAP, also referred to as α -BP or NF- κ B1, is an early B cell lineage-specific activator protein which binds to an indispensable DNA element of the I ϵ promoter, but also to several other regions within the murine IgH locus, including sites 5' of the S α and S γ 2a region, repetitive sequence

motifs of the S μ region and sequences within the Ig 3' C α enhancer.

Molecular components of the switch recombinase

So far, efforts to identify components of the switch recombinase have focused on S region-binding proteins. A few such proteins such as LR1 and NF κ B or SNUP, SNAP and SNIP have been cloned, but so far no functional role in switch recombination could be attributed to any of them unequivocally (Figure 1).

An interesting observation is that transcription of

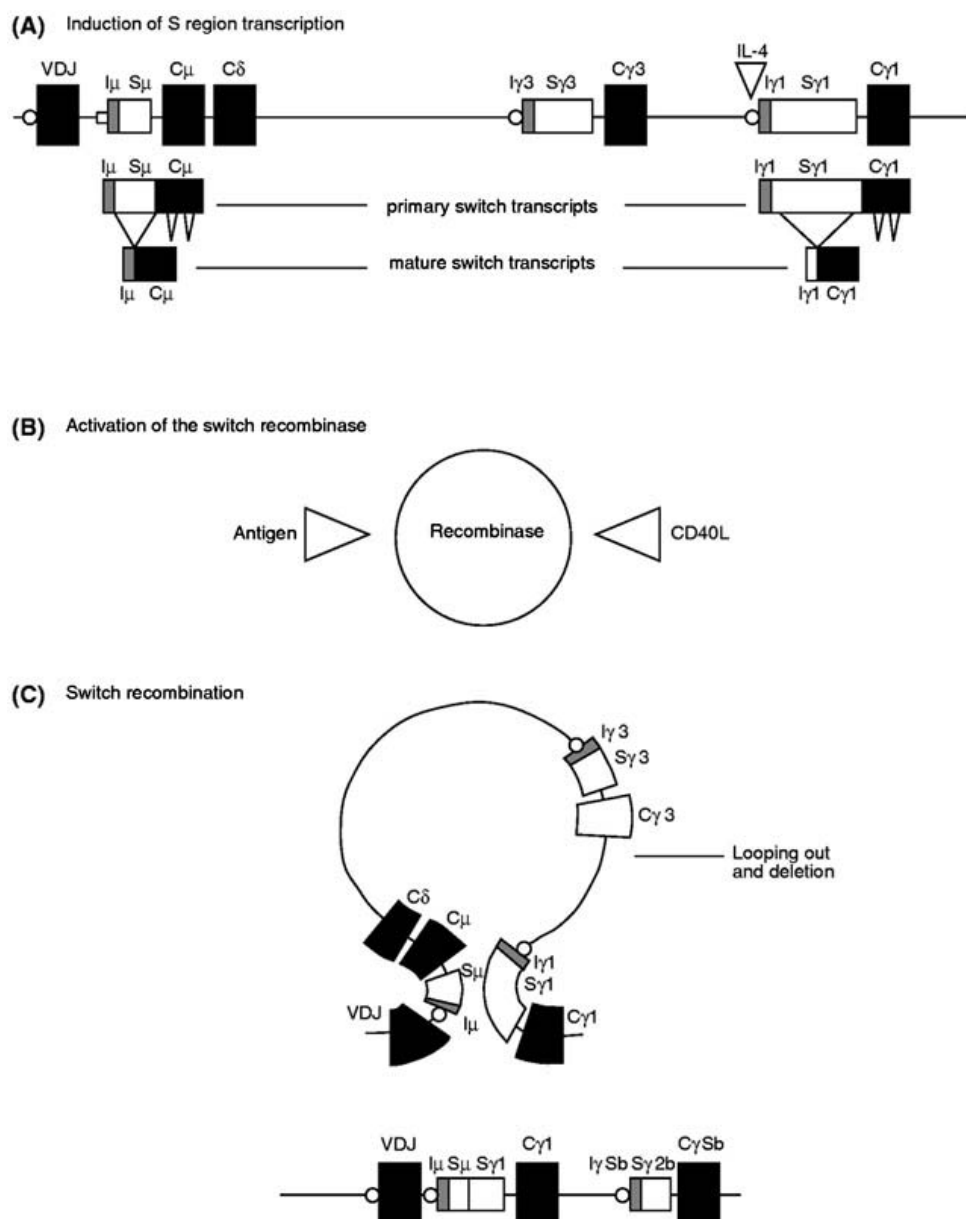


Figure 1 Mechanism of class-switch recombination.

S regions as such is insufficient to target switch recombination to particular S regions. Replacing the entire sequence of the I_ε exon together with its IL-4-inducible promoter by sequences of the IgH intron enhancer and a V_H promoter, substantial transcription through the S_ε region, but very little switch recombination to S_ε, is induced by LPS. Even more drastically, replacement of the I_γ1 promoter together with the entire I_γ1 exon by a human metallothionein (hMT) promoter completely shuts down switch recombination of S_γ1, although the S_γ1 region is constitutively transcribed. However, replacement of the I_γ1 promoter with the hMT promoter, leaving the splice donor site of the I_γ1 exon intact, targets switch recombination to S_γ1 at high frequency. These results strongly suggest that the process of splicing of switch transcripts, the spliced intron with S region sequences, or the spliced switch transcript play a functional role in targeting of switch recombination. Indeed, the primary switch transcripts or their splice products are obvious candidates for a class-specific component of the switch recombinase.

See also: B lymphocyte differentiation; Helper T lymphocytes; Cytokines; Effector lymphocytes; Immunoglobulin, cell surface; Immunoglobulin, functions; Immunoglobulin genes; Immunoglobulin structure; Interferon γ ; Interleukin 4; Interleukin 13 and its receptor; Isotype; Polyclonal activators; Transforming growth factor β (TGF β).

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IMMUNOGLOBULIN, EVOLUTION OF

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The first studies of antibody production in lower vertebrates were carried out between 1897 and 1903 by Mechnikoff, and Widal and Sicard in Europe and Noguchi in the USA. Mechnikoff emphasized the importance of comparative studies, showing that alligators produced antitoxins. Widal and Sicard showed that frogs made antibodies in response to inoculation with living typhus bacteria. Noguchi reported that turtles produced antibodies following challenge with whole blood from frogs, fish or other turtles. Studies of lower animals were carried out sporadically during the next 50 years, but there was a burst of interest in evolutionary studies in the 1960s. Good and his collaborators studied antibody production in nonmammalian vertebrate species ranging from cyclostomes to birds. In 1965, Marchalonis and Edelman first isolated and partially characterized immunoglobulins of a primitive vertebrate, the dogfish shark. They found that shark antibodies resembled mammalian immunoglobulins in possessing light and heavy polypeptide chains expressing charge diversity. Shark immunoglobulin most closely resembled mammalian IgM. Similar results were obtained for another shark, the lemon shark, by Clem and Small in 1967.

Although the fundamental similarities between immunoglobulin chains of lower vertebrates and their homologs in mammals was strongly indicated by the studies carried out in the late 1960s and early 1970s, definitive characterization of these molecules only became possible more recently. Initial strides in studies of immunoglobulins and their genes in human and mouse were facilitated by the fact that these mammalian species are subject to multiple myeloma, a cancer of the lymphoid system that results in the elaboration of monoclonal lymphocytes or plasma cells that secrete monoclonal immunoglobulins. No comparable monoclonal gammopathies occurred in primitive vertebrates. Therefore, the application of recombinant DNA technology which allowed genes to be cloned, sequenced and analyzed for their arrangements catalyzed a breakthrough in this area. Litman and his associates found that a murine V_H probe (S107) could be used to screen genomic libraries of elasmobranchs and reptiles with the detection and subsequent characterization of V_H genes showing greater than 60% identity to the mammalian prototype. In other instances, as in the

case of light chains of sharks, heterologous mammalian probes did not allow isolation of homologous genes and it was necessary to use the approach of isolating the shark immunoglobulins, making potent antiserum to them, and using these to screen cDNA libraries packaged in expression vectors. This approach was successfully used by Shambloott and Litman and Schluter and colleagues to obtain sequences of light chains of horned shark and sandbar shark respectively. Overall, there has been an explosion of knowledge regarding sequences and arrangements of immunoglobulin genes of lower vertebrates. Exciting work characterizing heavy chain genes of amphibians, teleosts and elasmobranchs by the above workers and others including Steiner, Du Pasquier, Schwager, Warr and Cuchens is currently being reported with increasing frequency. A fascinating development that emerged is that, despite the clear and strong homology in comparison among the corresponding chains of lower vertebrates and mammals, gene arrangements can vary considerably when the system specifying the homologous molecules are compared among sharks, chickens and mammals.

Immunoglobulin classes of vertebrates

All vertebrates can make antibodies that exhibit classic immunoglobulin structure consisting of two types of polypeptide chains that are polydisperse in charge and are usually linked covalently by disulfide bonds. Gene and protein sequence data are now available for immunoglobulins of all classes of vertebrates derived from the primitive jawed fish, the placoderms. These include elasmobranchs (e.g. sharks and rays), the ancestors of which diverged from those of mammals more than 400 million years ago, as well as teleost fish (e.g. goldfish), amphibians (e.g. bullfrogs and *Xenopus*), reptiles (e.g. the caiman), and birds (e.g. chickens). Based upon general structural properties, all placoderm-derived vertebrates possess antibodies comparable to the IgM (immune macroglobulin) isotype of mammals. The heavy chains are the same size as mammalian μ chains (70 kDa) and serological cross-reactions between the mammalian and shark chains have been found. The antibody molecules of sharks occur in two forms in serum: a 7S 'monomer' consisting of two light and two heavy chains of the form $(L\mu)_2$ and a pentamer of the form $(L\mu)_{10}$ comparable to the

predominant form of human IgM. The intact IgM molecules of bony fish teleosts occur in serum as tetramers instead of the pentamers found in other vertebrates.

Table 1 compares sequence alignments of the μ chains of horned shark, clawed toad, chicken and human. Overall, the sequence identity in the constant region domains is approximately 30%. The identity in the framework sequences of the variable regions ranges from 40 to 75%, illustrating strong evolutionary conservation of this portion of the molecule.

Other classes of immunoglobulin characterized by distinct heavy chains are present in lungfish (Dipnoi), amphibians, reptiles and birds. These resemble γ chains in consisting of multiple C-region domains, but are distinct from IgG immunoglobulins. All mammals, eutherians, marsupials and monotremes have 7S antibodies (apparent mass 150 000) having heavy chains of mass 50 000 that are serologically cross-reactive with human IgG. The distinct immunoglobulin monomers of amphibians, reptiles and birds have larger heavy chains than the mammalian γ chain and most probably represent the

products of heavy chain gene duplications distinct from the one which generated the γ chain. These non-IgM immunoglobulins have been termed IgY (amphibians, reptiles, chickens). In addition, other distinct immunoglobulin classes have been found in amphibians (IgX), lungfish (IgN, intact mass 120 000; heavy chain 40 000), reptiles (IgN in the turtle) and birds (IgN in the duck). These distributions are listed in Table 2.

Evolution of light chains

Light chains of mammals are classed as either κ or λ chains and their relative frequencies vary from species to species, e.g. in humans the ratio of κ to λ is 2 : 1, in the mouse it is 95% κ , 5% λ , and in pigs it is 5% κ , 95% λ . Comparison of gene and protein sequence data shows that the distinction between κ and λ chains is a phylogenetically ancient one that was established by the divergence between the ancestors of sharks and man. Table 3 compares the sequences of a light chain (cl 5.1) cloned from the sandbar shark (*Carcharhinus plumbeus*) with those of the characterized human λ Bence Jones protein Mcg, the mouse (MOPC 315) and the chicken. In many comparisons with mammalian λ chains, the C regions of the sandbar shark light chains characterized (six independent clones) show approximately 40% identity to the human, V regions >50% identity, and FR4 (encoded by the J or joining segment) about 75%. Figure 1 is a dendrogram depicting the evolution of light chain constant regions. The constant region of chicken light chains assorts with λ chain and that of the bullfrog clusters with κ chains.

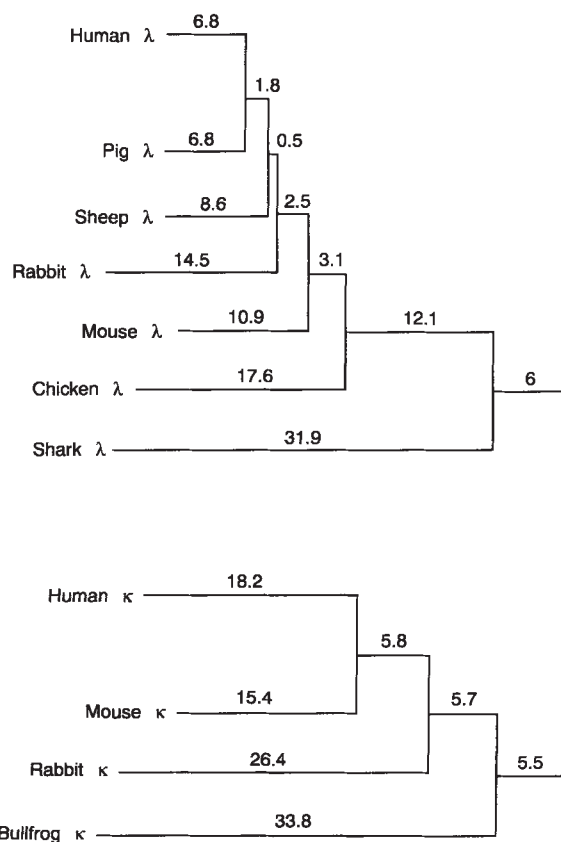


Figure 1 Phylogenetic tree for immunoglobulin light chains derived from analysis of constant region sequences using the method of Feng and Doolittle (*Journal of Molecular Biology* 1987; 25: 351–360).

Comparative arrangement of immunoglobulin genes

Although light chains and heavy chains of placoderm-derived vertebrates (gnathostomes) are clearly homologous to one another and are specified by variable (V), diversity (D in heavy chains), joining (J) and constant (C) gene segments, the arrangement of these can differ markedly in different species. Figure 2 illustrates the arrangement of the λ light chain genetic loci (translocons) in humans, the chicken and the shark (sandbar and horned). Figure 2 also depicts the arrangement of the heavy chain translocons in humans and in the horned shark. It is the definitive characteristic of immunoglobulins (and antigen-specific T cell receptors) that at least two gene rearrangements are required to form functional genes with clonal restriction of the antigen-specific B cells (and T cells), but the fundamental or prototypic

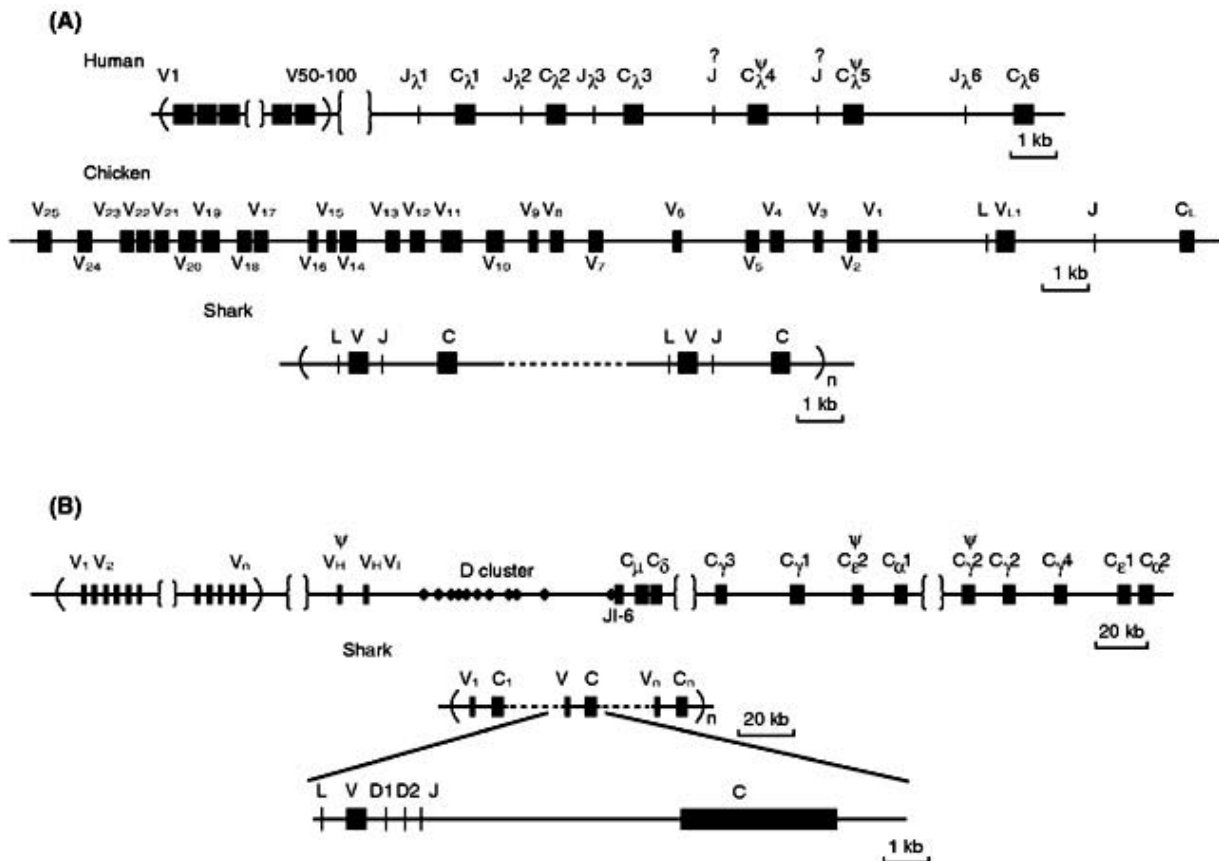


Figure 2 Physical maps of (A) λ light chain and (B) μ heavy chain loci of human, chicken and shark. Curly brackets { } indicate unknown distances between gene segments on the same chromosome. Dotted lines indicate that it is not even known if the gene segments are on the same chromosome. The organization and distances of gene segments within parentheses () is unknown. ψ indicates a pseudogene. The maps are adapted from Lai *et al* (1989), McCormack and Thompson (1990), Shambloott and Litman (1989) and Hohman, Schluter and Marchalonis (1995).

The basic gene arrangement as illustrated by the human λ and μ chains is the type of configuration adopted by mammals, amphibians (frogs) and teleosts (fish). For each gene cluster, a large number of V regions is associated with a limited number of C regions. The whole V region repertoire is available for each C region. There are also numerous J and D (for μ chain) segments. Diversity is generated by randomly combining together through DNA rearrangement events single V(D)J elements with a C region to form a functional gene. In the chicken, there is only a single set of functional genes and all antibodies are derived from the same rearrangement event. Diversity is generated by gene conversion events between the single functionally rearranged V region and numerous V region pseudogenes. The arrangement in the shark is again quite different. Whereas in the situations described above diversity is generated by having many V regions or pseudo-V genes associated with a few C regions, in the shark

Unexpected multiplicity of immunoglobulins in lower vertebrates

Table 1 Comparison of amino acid sequences of heavy chain variable and μ chain constant regions of human, chicken, clawed toad and shark. Identities are shown boxed or circled. The alignments were carried out according to the procedure of Feng and Doolittle, with sequence data referenced in Marchalonis and Schluter (FASEB Journal, 1989). V_H and C_H domains are placed with reference to the human sequence according to Kabat. Sources of sequences: a) human - myeloma protein gel from the Genbank database; b) chicken - V region is cDNA clone 3W-25 from Reynaud *et al* (1989). C region is from the Genbank database; c) clawed toad (*Xenopus*) - from Schwager *et al* (1989); d) horned shark - V region is genomic clone 2809, DJC region is cDNA clone 12622 from Kokubu *et al* (1988). Citations are given in Marchalonis and Schluter (1989). Stars denote complete conservation among the four species

	FR1	CDR1	FR2	CDR2	FR3
Human	E V I Q L V E S G G D L V Q P G R S L R L S C A A S G F B E B B L G M T W V R Q A P G K G L E W V A N I K Z B G S Z Z B B Y V V D S V K G R F T I S R D N				
Chicken	A V T L D E S G G L Q T P G G G L S L V C K A S G F T S S Y N M M G W R Q A P G K G L E F V A G I G N T G S Y T A Y G S A V K G R A T I S R D N				
Toad	Q T L Q E S G P G T V K P S E S L R L T C T V S G F E L T S Y Y V V W I R Q P P R K T L E W I G V V R T D G S T A I A D S L K N R V T I T K D				
Shark	D I V L T Q P E A E T G K P G G S L R L T C K T S G F D L G S H W I C W V R Q V P G Q E L E W L V G Y Y G L F S S F Y A P G I K D R F T A S K D I T				
	FR3	CDR3	FR4	CH1	
Human	N A K N S L Y L Q M N S L R V E D T A L Y Y C A R G				
Chicken	N G N S T V R L N L N N L R A E D T A T Y Y C A K T T G S A Y Y W D A D T A V S I D A W G H G T E V I V S S A S A S P S P P R L F P L V L C S P S				
Toad	N G K K Q V Y L Q M N G M E V K D T A M Y Y C I T S T				
Shark	T S N N I F T L D M K N L K T E D T A I Y F C R R				
	FR3	CDR3	FR4	CH1	
Human	B P S S T V A V G C L A Q D F L P D S I T F S W K Y K N N S D I S S T R G F P S V L R G G K Y A A T S Q V L L P S K D V M Q G T N E H V C K				
Chicken	D S V Y V G C A F D F Q P S S I A F T W F D S N N S S V S G M D V I P K V I S G P P Y R A V S R I Q M N Q S E G K E K Q P F R C R				
Toad	M V P B I G C L A K E F L P E T I S F T W G D K N N A S Y S T G L K S Y K P V M Q S S G T Y S A S S Q E N V A S A V M D N I V Q F Y C N				
Shark	N T D G S L A Y G C L A M D Y S P E I T S I T W K K D K E P I T G L K I Y P S V L N K K G T Y T R S S Q L T I T E S E V G S S K T Y C E				

Table 1 Continued

	CH3	CH4	
Human	G E R F T C T V T H T D L P S P L K Q T I S R P K G V A L H R P D V Y L L P P A R E Q L N L R E S A T I T C L V T G F S P A D V F V Q W M Q R G Q P		
Chicken	G D G Y V C K V N H P D L L F P M E E K M R K T K A S N A R P P S V Y V F P P P T E Q L N G N Q R L S V T C M A Q G F N P P H L F V R M M R N G E P		
Toad	N D K F V C K V E H T E L A S M K E V F L G K E G E Y N T P S V Y V F P P P L E E L S K R E T A T L T C L V K G F S P S E I F V K W L H K N E A		
Shark	G A E F Y C V V S H Q D L P T P L R A S I H K E E V K D L R E P F V S V K K O O A E D V S A Q R F K S K T C L V R G F S P R E I F I K W T V M D K S		
			SECRETORY
Human	L S P E K Y V V T S A P		
Chicken	L P Q S Q S V T S A P		
Toad	V P K Q N Y I N T S I N V E L L P K G Q K S G K F F L Y S L H T D I K D W D A G D S F C C V V G H E S L P L Q L T Q R S I D K S S G K P T N V N		
Shark	V N P G N Y K N T E V		
Human	M E P Q A P G R V F A H S I L T V S E E E W N T G E T Y T C V A H D A L P N R V T E R T V D K S T G K P T L Y N		
Chicken	M A E N P E N E S Y V A Y S V L G V G A E E W G A G N V Y T C L L V G H E A L P L Q L A Q K S V D R A S G K A S A V N		
Toad	V P K Q N Y I N T S I N V E L L P K G Q K S G K F F L Y S L H T D I K D W D A G D S F C C V V G H E S L P L Q L T Q R S I D K S S G K P T N V N		
Shark	M A E N D N R S F F I Y S L L S I A A E E W A S G A S Y C C V V G H E A P L K I I N R T V N K S S G K P S F V N		
Human	V S L V M S D T A G T C Y		
Chicken	V S L V L A D S A A C Y		
Toad	V S L V L S D T C		
Shark	I S L A L L D T V N S C Q		

Table 2 Distribution of immunoglobulin classes among vertebrate classes

<i>Vertebrate class</i>	<i>Immunoglobulins</i>	<i>Light chain types documented</i>
Mammals	IgM, IgG, IgA, IgD, IgE	κ , λ
Birds	IgM, IgA, IgY, IgN	λ
Reptiles	IgM, IgY, IgN	Light chains present but uncharacterized
Amphibians	IgM, IgY, IgX	Three light chain types present
Lungfish	IgM, IgN	Light chains present but uncharacterized
Bony fish	IgM	Light chains of teleost type
Sharks/rays	IgM, IgW/NARC	λ , κ , other
Lampreys/hagfish	IgM(?)	Light chains present but uncharacterized
Protochordates	No 'classical immunoglobulin' but proteins cross-reactive with antibodies of shark and lampreys occur	

heavy chain containing one variable and six constant domains and may represent the primitive immunoglobulin in evolution. IgW variable domains resemble those of heavy chains, but are significantly different and show substantial variation in sequence consistent with the diversity to recognize a large number of antigens. Furthermore, major diversity both in isotypes and variable domain sequences has been found among light chains of cartilaginous fishes. The dominant family in carcharhine sharks (e.g. the sandbar shark) is a family related to the λ light chains of higher vertebrates, and this group itself consists of at least three major families containing more than two hundred separate genes. The dominant class of light chains in the nurse shark resembles mammalian κ chain and that in the horned shark is a separate type which appears to be restricted to the sharks.

Origin of variable region diversity

Investigations of mammals, birds and the *Xenopus* have indicated that two genes are required early in the development of T and B cells in order for recombination of variable, diversity and joining segments of immunoglobulin light and heavy chains and T cell receptor chains to occur. These have been termed RAG-1 (recombination activating gene 1) and RAG-2 (recombination activating gene 2). Although their mechanism of action is currently unknown, their presence is required in early T and B cells to allow recombination. Genes unequivocally homologous to the human RAG-1 gene occur in all jawed vertebrates including sharks, paddlefish, goldfish, *xenopus* and the axolotl. Comparison of the sequence of the complete 1113 amino acid shark RAG product with the human gene product shows regions of greater than 85% identity as well as segments of approximately 20–50% identity. All RAG-1 genes have homology to DNA-processing enzymes, expressing among other features the so-called RING

finger Zn-finger motif expressed by the yeast excision repair enzyme RAD18. Analysis of the shark sequence indicates that RAG-1 genes are members of the integrase family of site-specific DNA-rearrangement enzymes that have been studied in bacteria. Comparable analyses of sequence homology suggests that the vertebrate RAG-2 molecule is homologous to integration host factors that in bacteria act in concert with the integrases.

Origins of immunoglobulins

The evidence is now conclusive that the most primitive living vertebrates, the cyclostomes hagfish and lampreys, make antibodies comparable to those of higher vertebrates in charge dispersity and in consisting of two types of polypeptide chains (light and heavy) that can associate covalently by disulfide bonds. These molecules are serologically cross-reactive with those of elasmobranchs, but their protein and gene structure remain elusive at this time. The question of the origin of the rearranging immunoglobulin system likewise is a challenging contemporary problem. It is likely that this system arose in the deuterostome ancestors of vertebrates. Consistent with this hypothesis, molecules found in some protochordate species (tunicates) are antigenically related to heavy chains of sharks and lampreys and have sufficient mass (25–30 kDa) to contain the product of V and C gene segments. Whether these molecules prove to be the protoimmunoglobulin 'missing links' remains to be determined, but the search for the first vertebrate-type antibody continues to be a major issue in immunology.

See also: Amphibian immune system; Avian immune system; Fish immune system; Immunoglobulin genes; Immunoglobulin structure; MHC, evolution of; Ovine immune system; Phylogeny of the immune response; Porcine immune system; Rabbit immune

Table 3 Comparison of λ light chain sequences of the sandbar shark (clone 5.1), mouse (MOPC 315), human (Mcg) and chicken

	VARIABLE																																																																																																																																																					
	*****CDR1*****										*****CDR2*****																																																																																																																																											
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Shark	D	P	V	L	T	Q	P	G	S	I	S	S	P	G	K	T	V	T	I	T	C	T	M	S	G	G	T	I	S	Y	W	A	-	S	W	Y	W	Q	K	P	D	S	A	P	V	F	V	W	S	E	S	D	R	M	A	S	G	I	P	N	R	F	A	G	S																																																																																					
Mouse	Q	A	V	V	T	Q	E	S	A	L	T	I	S	P	G	G	T	V	I	L	T	C	R	S	S	T	G	A	V	T	T	S	N	Y	A	N	I	Q	E	K	P	D	H	L	F	T	G	I	G	T	S	D	R	A	P	G	V	P	V	R	F	S	G	S																																																																																						
Human	Q	S	A	L	T	Q	P	P	S	A	S	G	S	L	G	Q	S	V	T	I	S	C	T	G	T	S	S	D	V	G	G	Y	N	Y	V	S	W	Y	Q	Q	H	A	G	K	A	P	K	I	I	Y	V	N	K	R	P	S	G	V	P	D	R	F	S	G	S																																																																																					
Chicken	A	L	T	Q	P	A	S	V	S	A	N	P	G	E	T	V	K	I	T	C	S	G	G	S	G	-	-	G	S	Y	-	-	-	G	W	Y	Q	Q	-	P	G	S	A	P	V	T	V	I	Y	S	N	D	K	R	P	S	D	I	P	S	R	F	S	G	S																																																																																					
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Shark	V	D	S	S	N	K	M	H	L	T	I	T	N	V	Q	S	E	D	A	T	D	Y	Y	C	A	A	A	S	R	S	P	Y	R	S	I	F	G	S	G	T	K	L	N	V	L	G	N	P	R	S	P	T	M	S	L	L	P	P	S	S	D	Q	I	T																																																																																						
Mouse	L	-	-	I	G	D	K	A	L	T	I	T	G	A	Q	T	E	D	D	A	M	Y	F	C	-	-	-	A	L	W	F	R	N	H	F	V	F	G	G	T	K	V	T	V	L	G	Q	P	K	S	S	P	S	V	T	L	F	P	P	S	S	E	E	L	E																																																																																					
Human	-	-	K	S	N	S	A	S	L	T	I	S	G	L	Q	T	E	D	E	A	D	Y	Y	C	-	-	-	Q	S	Y	D	R	D	H	W	V	F	G	G	T	K	L	T	V	L	C	Q	P	K	A	M	P	T	V	T	L	F	P	P	S	S	E	E	L	Q																																																																																					
Chicken	-	-	K	S	G	S	T	A	T	L	T	I	T	G	V	Q	A	E	D	E	A	V	Y	F	C	-	-	-	G	S	Y	D	S	Y	V	G	I	F	G	A	G	T	T	L	T	V	L	G	Q	K	V	A	P	T	I	T	L	F	P	P	S	K	E	E	L	N																																																																																				
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system; Reptilian immune system; T cell receptor, evolution of.

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IMMUNOGLOBULIN, FUNCTIONS

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The function of immunoglobulin is in essence to link foreign material (antigen) to its means of elimination (effector system). Antigens come in all shapes and sizes, whereas the number of effector systems is small. This is reflected in the organization of the antibody molecule into variable (antigen recognition) and constant (effector recognition) parts. It is also reflected in the unusual degree of molecular flexibility found in antibodies and illustrated in Figure 1 for the major antibody in serum, IgG. Fab arm flexibility about the hinge (Y to T shape changes, Fab arm wagging) allows 'variable reach' for the antibody and therefore bivalent recognition of differently spaced antigens on, for example, a cell surface. The other Fab motions similarly should reduce the steric requirements in antigen conformation for antibody binding. Motions of Fab and Fc relative to one another should, in principle, assist in the simultaneous

binding of antigen and effector molecule and may in some cases be crucial.

The principal effector functions triggered by antibody are complement activation and cellular elimination mechanisms such as phagocytosis and antibody-dependent cellular cytotoxicity (ADCC). These functions are generally triggered only by antibody molecules clustered together as on a foreign cell surface and not by free unliganded antibody. This is crucial considering the high serum concentration of some antibodies. Effector molecule binding appears concentrated in the C_H2 domain of IgG or its equivalent in other antibody molecules.

Complement activation

The classical pathway of complement is triggered by the interaction of the first complement component, C1, either with IgG in an associated state (e.g.

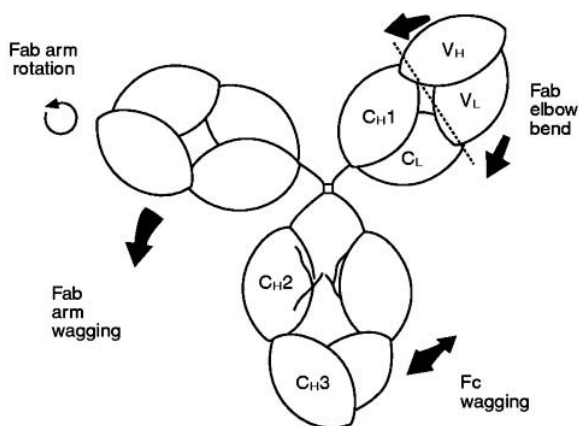


Figure 1 Flexibility of the IgG molecule.

coating a target cell or aggregated by antigen in an immune complex) or with IgM bound to antigen.

C1 is a complex consisting of the subcomponents: C1q, C1r and C1s. It is C1q which interacts with antibody to initiate the enzymatic process of the pathway. This hexameric molecule, having the appearance of a 'bunch of tulips', is multivalent in its binding to antibody (**Figure 2**). This multivalency, rather than any conformational change, is probably the key to why complement is only triggered by IgG in an associated form. Binding of C1q to monomeric IgG is only weak ($K_a \sim 10^4 \text{ M}^{-1}$), whereas binding to associated IgG and the consequent use of two or more 'tulip heads' makes binding much tighter (K_a

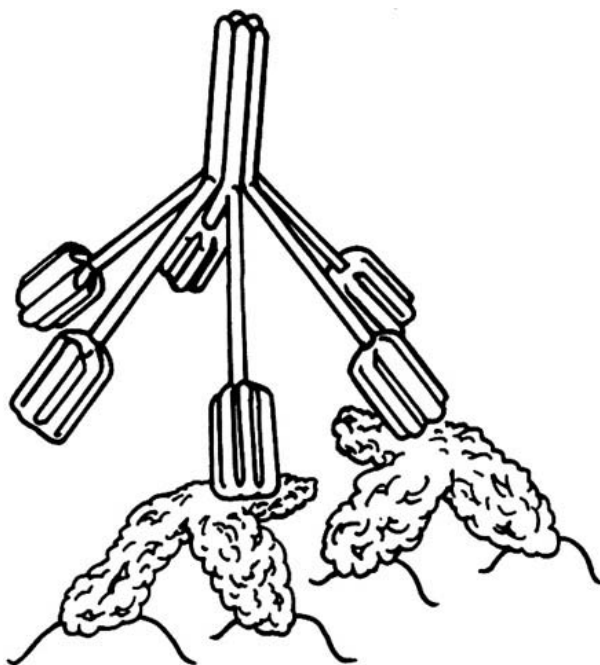


Figure 2 Schematic representation of complement C1q interacting with two IgG molecules bound to antigen on a cell surface.

$\sim 10^8 \text{ M}^{-1}$), and allows the activation process to proceed. On the other hand, IgM is polymeric even in the absence of antigen and since one complexed IgM molecule will activate complement, it appears that binding to antigen does induce a conformational change in the antibody. It is suggested from electron microscope studies that this may involve movement of Fab arms relative to Fc in a 'star' to 'staple' transition. This process has been termed antibody dislocation and may also be important for the function of IgG.

The binding site for C1q on IgG includes a simple motif, Glu-X-Lys-X-Lys (318–322) located on the final β strand of the C_H2 domain and at least one residue from a nearby loop (Pro331). The charged residues in the linear sequence are directed out into the solvent, constituting a contiguous site, and the alternate residues (X) into the interior of the domain. The charged motif is one that is found in many proteins and its recognition in bacterial or viral arrays may have constituted an ancient defense mechanism. Later in evolution, the antibody may have appeared as an adaptor avoiding the requirement for the motif to be written directly on the pathogen.

Complement activation is strongly isotype-dependent. In any comparison it is important to distinguish C1q binding, C1 binding, C1 activation, and whole complement activation. The most widely used assay is the measurement of the end-product of the whole complement cascade, i.e. cell lysis. The inability of an IgG to promote efficient lysis does not necessarily indicate an inability to bind C1q; a later stage may be implicated. For example, C1q binding is not always related to C1 activation and later components of complement (e.g. C4b, C3b) also interact with IgG. Further, the source of complement can be an important factor in describing the ability of an isotype to activate the cascade. The human IgG subclasses as monomer bind C1q with measurable affinity in the order IgG3 > IgG1 > IgG2. IgG3 and IgG1 activate C1 and whole complement efficiently. IgG2 generally does not activate human complement. IgG4 does not bind C1 and does not activate complement. In other species, mouse IgG3, IgG2a and IgG2b, guinea pig IgG2, rabbit IgG, rat IgG2b, IgG1 and IgG2a bind C1q and activate complement. Mouse IgG1, guinea pig IgG1 and rat IgG2c bind C1q weakly, if at all, and do not significantly activate complement.

The alternative pathway of complement is activated weakly by associated IgA as immune complexes or coated on to microtiter plates. For human IgA, both subclasses and secretory IgA activate. An F(ab')₂ fragment is functional but isolated Fc or Fab fragments are not.

Fc receptor binding

A major effector system is the recognition of antibody-coated targets by cells bearing Fc receptors. These are found on a number of cell types and are associated with a number of functions including phagocytosis (monocytes, macrophages and granulocytes), ADCC (monocytes, macrophages, neutrophils and lymphocytes), anaphylaxis (mast cells) and maternofetal transport (trophoblasts). Receptors for all the classes of immunoglobulin have been described and those for IgG, IgE and IgA cloned.

Human leukocyte IgG Fc receptors can be grouped into three classes: FcγRI, FcγRII and FcγRIII. FcγRI (CD64) is found on monocytes, macrophages, dendritic cells and stimulated neutrophils and is often described simply as the 'high-affinity Fcγ receptor' because it binds monomeric human IgG1 and IgG3 with high affinity ($K_a \sim 10^8 \text{ M}^{-1}$). IgG4 is bound 10-fold more weakly and IgG2 not at all. Three very similar FcγRI genes have been identified although only one codes for the receptor which binds IgG. FcγRI can trigger a number of functions including internalization of small immune complexes, phagocytosis of antibody-coated erythrocytes, ADCC, enhancement of antigen presentation, cytokine release from monocytes and superoxide production in neutrophils.

FcγRII (CD32) is the most widely distributed class of FcγR and is found on most types of leukocytes. It is often described as a low-affinity receptor as it interacts only very weakly with monomer IgG ($K_a < 10^6 \text{ M}^{-1}$) although its interaction with certain IgG isotypes in associated form is much stronger. Three different FcγRII genes have been identified, giving rise to FcγRIIa, FcγRIIb and FcγRIIc receptors. FcγRIIa is found in two allotypic forms, termed high-responder (HR) and low-responder (LR) forms. The HR form binds well to associated human IgG1 and IgG3 but not with IgG2 or IgG4. The LR form also binds human IgG2. FcγRIIb binds well to associated IgG1 and IgG3, IgG4 with intermediate affinity and IgG2 very poorly. FcγRIIc probably resembles FcγRIIb in its IgG-binding profile. FcγRII can mediate many of the functions described for FcγRI. In addition, FcγRIIb is preferentially expressed on B cells where it appears to have a role in downregulation of B cell activation upon cross-linking with surface Ig. It is also expressed on mast cells where it downmodulates cell activation on cross-linking to FcεRI.

FcγRIII (CD16) is expressed as two gene products. FcγRIIIa is a transmembrane protein found on macrophages and natural killer (NK) cells and subsets of T cells and monocytes. It is the sole FcγR

found on NK cells. FcγRIIIa shows moderate affinity for human IgG1 and IgG3 (K_a approx. $3 \times 10^7 \text{ M}^{-1}$) and does not bind IgG2 or IgG4. FcγRIIIb is a glycosyl phosphatidylinositol (GPI)-anchored protein expressed exclusively on neutrophils. It shows low affinity for monomeric IgG ($K_a \ll 10^7 \text{ M}^{-1}$). FcγRIII molecules can mediate a variety of functions. Cross-linking of FcγRIIIa on activated NK cells can trigger apoptosis.

The binding site on IgG for FcγRI is believed to contain residues from the lower hinge ($C_{H1}2$ domain exon) centered around Leu235. This is the region attacked by pepsin in the commonly used procedure to generate $F(ab')_2$ fragments from IgG and probably explains its preservation. Involvement of this hinge region suggests a bent or dislocated conformation for IgG as it links antigen and Fc receptor (Figure 3). The lower hinge region has also been implicated in the binding of IgG to FcγRII and III, although it is believed that the details of recognition differ.

There are two human Fc receptors for IgE. FcεRI is found on mast cells and basophils where it binds monomer IgE with high affinity ($K_a \sim 10^{10} \text{ M}^{-1}$). It is also found on Langerhans cells and monocytes. Cross-linking of receptor-bound IgE by antigen (now often termed allergen) results in cell degranulation

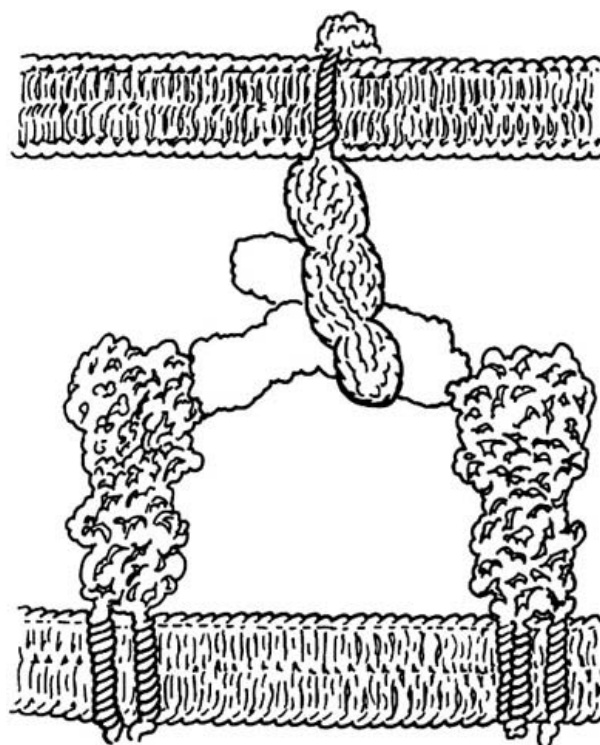


Figure 3 Schematic representation of IgG molecule (lower hinge region site) linking an effector cell (upper membrane) via its Fc receptor (FcRI domain 2) to a target cell via two antigen molecules.

and the release of chemical mediators such as histamine which produce the symptoms of allergy. The binding site on IgE for FcεRI has been mapped to three loops of notable charged character in the C_H3 domain of IgE. Since the C_H3 domain in IgE is the structural equivalent of the C_H2 domain in IgG, it appears that this domain is once again crucial for effector activity. FcεRII (CD23) is found on inflammatory cells such as monocytes where it is probably related to IgE-mediated phagocytosis and on lymphocytes where it may have a role in modulating immune responses. It has low affinity for monomer IgE and the receptor-binding site has been associated with the C_H3 domain of IgE.

An Fc receptor for IgA (FcαR; CD89) has been described on a variety of cell types including neutrophils, eosinophils and monocytes. The receptor binds human serum and secretory IgA1 and IgA2 with relatively high affinity ($K_a \sim 5 \times 10^7 \text{ M}^{-1}$) and actively mediates ADCC, endocytosis, phagocytosis, triggering of cytokine release and antigen presentation.

Virus neutralization

Binding of antibodies to virus particles can render the particles noninfectious *in vitro* in a process termed neutralization. This is considered an important phenomenon since it frequently correlates with protection against virus challenge *in vivo*. The mechanism(s) of antibody neutralization are much discussed. It seems that inhibition of viral attachment to the target cell is one mechanism but that inhibition of fusion of the viral membrane of enveloped viruses with the target cell membrane is another. The possibility of the inhibition of events following virus entry has also been raised. In many cases, virus neutralization is effected by Fab fragments almost as well as whole antibody molecules, implying that aggregation of virions or cross-linking of envelope spikes is not important in those cases. Antibody may also have a role in complement-mediated lysis of viral particles. In some cases, it appears that subneutralizing concentrations of antibody can potentiate viral infection by increasing uptake of virus through interaction of the antibody-coated virus with Fc receptors (or through complement-antibody-virus particles with complement receptors) or via other nondefined mechanisms.

Catabolism

The level of IgG in blood is tightly regulated, with the rate of catabolism being determined by IgG concentration. Catabolism is accelerated under conditions of elevated IgG and reduced under conditions

of low IgG levels. This has been explained in terms of a 'protective receptor' which binds IgG, protects the molecule from degradation and then recycles it to the circulation. Saturation of the receptor will lead to accelerated catabolism if IgG levels rise. Recent studies have shown that this receptor in rodents is identical to the neonatal Fc receptor (FcRn) which has homology to the major histocompatibility complex (MHC) class I molecule. The interaction site on IgG is at the C_H2/C_H3 domain interface, overlapping the bacterial Fc receptor protein A site, and involving residues His310 and His433.

Other functions of antibody

All the immunoglobulin classes can be expressed at the surface of B cells where they act as antigen receptors, although this is primarily a function of IgM and IgD. Surface immunoglobulin possesses an extra C-terminal sequence compared to secreted immunoglobulin containing linker, transmembrane and cytoplasmic segments.

A number of immune responses including polyclonal antibody production, T cell-mediated responses such as antigen and alloantigen-induced proliferation, and cell-mediated cytotoxicity can be mediated by subfragments of Fc of IgG. A peptide corresponding to residues 335–358 of human IgG1, a sequence which spans the C_H2 and C_H3 domains, has been shown to induce polyclonal antibody production.

Classical immunology explains the precipitin reaction in terms of the formation of lattices through the bridging of antibody-combining sites by multivalent antigens. However, it is found that IgG and its F(ab')₂ fragment behave very differently in immune complex formation, implying a role for Fc–Fc interactions.

Rheumatoid factors are autoantibodies directed against epitopes expressed in the Fc region of IgG. Although most patients with rheumatoid arthritis show a heterogeneous population of rheumatoid factors, a common specificity has been detected in most rheumatoid sera. This antibody is of the IgM class and reacts with the C_H2–C_H3 interface region, showing many features in common with protein A.

Six distinct bacterial IgG Fc receptors have been designated types I–VI and a number of bacterial Fc receptors binding other isotypes of Ig have been described. The interaction of IgG and type I receptor, commonly termed protein A, has been studied in detail by crystallography and the site of binding between the C_H2 and C_H3 domains described.

The binding sites on antibodies for certain antigens occur with a single type of V_H segment with little

conservation of other elements in the V regions. These antigens, such as protein A (which has an Fc-binding site and a V_H-binding site on human V_H3 family antibodies), have been termed unconventional antigens or B cell superantigens.

See also: Antibody-dependent cellular cytotoxicity; Antigen-binding site; Complement, classical pathway; Fc receptors; Idiotype network; IgA; IgD; IgE; IgG; IgM; Immune complexes; Immunoglobulin, cell surface; Immunoglobulin structure; Neutralization of biological reactions by antibodies; Opsonization; Phagocytosis; Bacterial immunoglobulin-binding proteins; *Staphylococcus*, infection and immunity.

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IMMUNOGLOBULIN GENE SUPERFAMILY

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Many genes and their protein products share features that suggest a common ancestral origin and allow them to be grouped together into 'superfamilies'. The immunoglobulin (Ig) gene superfamily (or 'immunoglobulin superfamily', IgSF, when referring to the protein products of these genes) takes its name from the first members of this family to be sequenced and is one of the numerically largest of the various protein superfamilies. Proteins bearing IgSF domains exhibit a wide range of activities (Table 1), many of them having functions unrelated to the immune system, for example in the nervous system. This diversity of function is a reflection of the versatility of the Ig domain, a structure of about 70–110 amino acids which comprises a series of nonparallel β strands that are folded into two β sheets. This folding pattern (the 'Ig fold' Figure 1) is stabilized by a hydrophobic interior, and usually, but not always, by the possession of a characteristic disulfide bond between the sheets. The domain provides a stable platform for the display of specific determinants on

the outer faces of the β sheets or on the loops joining the β strands.

The criterion for inclusion in the IgSF is the presence of one or more Ig-type domains (Ig homology units) which confer upon the polypeptide a globular tertiary structure. Many proteins will contain IgSF domains together with other types of domains. For example the extracellular portion of the CD130 molecule is composed of one IgSF domain, one cytokine receptor domain, and four fibronectin type III domains. Such proteins are best referred to as mosaic proteins rather than as belonging to a particular superfamily. Although confirmation of the presence of an IgSF domain in any given protein is dependent upon X-ray crystallographic analysis, nucleic acid sequence comparisons with IgSF members whose crystal structure is known (e.g. Ig V and C domains, MHC class I α_3 domain), and with other members can provide strong support for inclusion in the superfamily. Further evidence can be obtained using circular dichroism to ascertain β sheet and α helical

Table 1 Some examples of proteins which contain IgSF domains

Protein	Mol. wt ^a (kDa)	Ig domains			Human chromosome	Function
		C1	C2	V		
CD1	43–49	1	–	–	1q22–q23	Presentation of peptide and lipid antigens to T cells
CD2	45–58	–	1	1	1p13.1	Signal transduction Ligand for CD48 and CD58
CD3 γ chain	25–28	–	1	–	11q23	TCR signal transduction
CD3 δ chain	20	–	1	–	11q23	TCR signal transduction
CD3 ϵ chain	19–20	–	1	–	11q23	TCR signal transduction
CD4	55–59	–	2	2	12pter–p12	TCR coreceptor Ligand for nonpolymorphic region of MHC class II
CD7	40	–	–	1	17p25.2–q25.3	T cell activation
CD8 α chain	32–38	–	–	1	2p12	TCR coreceptor Ligand for nonpolymorphic region of MHC class I
CD8 β chain	30–34	–	–	1	2p12	Homodimer or heterodimer with CD8 β chain TCR coreceptor Forms heterodimer with CD8 α
CD16 (Fc γ RIII)	48–70	–	2	–	1q23	Low/medium-affinity Fc receptor for IgG
CD19	95	–	2	–	16p11.2	Part of signaling complex for B cells
CD22	130–150	–	6	1	19q13.1	Negative signaling in B cells
CD28	44	–	–	1	2q33–q34	T cell activation Homodimer Ligand for CD80 and CD86
CD31 (PECAM-1)	140	–	6	–	17q23–ter	Adhesion
CD32 (Fc γ RII)	40	–	2	–	1q23–24	Low affinity Fc receptor for IgG
CD33	67	–	1	1	19q13.3	Adhesion
CD47	47–52	–	–	1	3q13.1–q13.2	Adhesion
CD48 (Blast-1)	41	–	1	1	1q21–q23	Ligand for CD2
CD50 (ICAM-3)	130	–	5	–	19p13.3–13.2	Ligand for LFA-1 (CD11a/CD18)
CD54 (ICAM-1)	90	–	5	–	19p13.3–13.2	Ligand for integrins including LFA-1
CD56 (NCAM)	175–185	–	5	–	11q23.1	Homotypic adhesion
CD58 (LFA-3)	40–65	–	1	1	1p13.1	Ligand for CD2
CD64 (Fc γ RI)	72–75	–	3	–	1q21.1	High-affinity Fc receptor for IgG
CD66a (CEA)	160–180	–	3	1	19q13.1–2	Adhesion
CD66b (CEA)	95–100	–	2	1	19q13.1–2	Adhesion
CD66c (CEA)	90–95	–	2	1	19q13.1–2	Adhesion
CD66d (CEA)	30	–	–	1	19q13.1–2	Adhesion
CD79a	47	–	1	–	19q13.2	BCR signal transduction
CD79b	37	–	–	1	17q23	BCR signal transduction
CD80 (B7-1)	60	–	1	1	3q21	T cell activation Ligand for CD28 and CD152
CD83	45	–	–	1	6p23–p21.3	Dendritic cell function
CD86 (B7-2)	75	–	1	1	3q13–q23	T cell activation Ligand for CD28 and CD152
CD89 (Fc α R)	55–100	–	2	–	19q13.4	Fc receptor for IgA
CD90 (Thy-1)	17–35	–	–	1	11q23.3	T cell activation
CD96 (Tactile)	160	–	–	3	Not known	Not known
CD100	150	–	1	–	Not known	Lymphocyte activation Homodimer
CD101	140	–	–	7	1p13	T cell activation Homodimer
CD102 (ICAM-2)	55–65	–	2	–	17q23–q25	Ligand for integrins

Table 1 Continued

Protein	Mol. wt ^a (kDa)	Ig domains			Human chromosome	Function
		C1	C2	V		
CD106 (VCAM-1)	95–100	–	7	–	1p31–p32	Ligand for integrins
CD114 (G-CSFR)	130	–	1	–	1p35–p34.3	G-CSF receptor
CD115 (CSF-1R)	150	–	4	1	5q33.2–q33.3	CSF-1 (M-CSF) receptor
CD117 (C-kit)	145	–	4	1	4q11–q12	Role in hematopoiesis Ligand for c-kitL
CD121a (IL-1R type I)	80	–	3	–	2q12	IL-1 α and IL-1 β receptor
CDw121b (IL-1R type II)	60–70	–	3	–	2q12–q22	IL-1 α and IL-1 β receptor
CD126 (IL-6R α chain)	80	–	1	–	1q21	Low-affinity IL-6 receptor Heterodimer with CD130 forms high-affinity IL-6 receptor
CD130 (shared cytokine receptor β chain)	130	–	1	–	5q11	Common signal transducer component of several cytokine receptors
CD135	130–150	–	4	1	13q12	Role in hematopoiesis Ligand for FLT3 ligand
CD147	50–60	–	1	1	19p13.3	Adhesion
CDw150 (SLAM)	75–90	–	1	1	Not known	T cell activation
CD152 (CTLA-4)	44	–	–	1	2q33–q34	Negative signaling in T cells Homodimer Ligand for CD80 and CD86
CD158a (KIR)	50–58	–	2	–	19q13.4	Killer cell inhibitory receptor Ligand for MHC class I
CD158b (KIR)	50–58	–	3	–	19q13.4	Killer cell inhibitory receptor Ligand for MHC class I
CD166	100	–	3	2	3q13.1–13.2	Ligand for CD6
Ig heavy chain	55–73	3–4	–	1	14q32.33	Antigen binding
Ig κ light chain	28	1	–	1	2p12	Antigen binding
Ig λ light chain	28	1	–	1	22q11.1–q11.2	Antigen binding
pTCR α	33	–	1	–	6p21.2–p12	Pre-TCR
TCR α chain	45–60	1	–	1	14q11.2	T cell antigen receptor for peptide–MHC
TCR β chain	40–50	1	–	1	7q35	T cell antigen receptor for peptide–MHC
TCR γ chain	45–60	1	–	1	7p15	T cell antigen receptor for peptide–MHC
TCR δ chain	40–60	1	–	1	14q11.2	T cell antigen receptor for peptide–MHC
MHC class I α chain	44	1	–	–	6p21.3	Presents peptides to TCR on CD8 ⁺ T cells
MHC class II α chain	33–35	1	–	–	6p21.3	Presents peptides to TCR on CD4 ⁺ T cells
MHC class II β chain	28–30	1	–	–	6p21.3	Presents peptides to TCR on CD4 ⁺ T cells
Fc ϵ RI α chain	45–65	–	2	–	1q23	High-affinity Fc receptor for IgE
IL-1R accessory protein	70–90	–	2	1	Not known	IL-1 receptor accessory protein
IL-11R α chain	150	–	1	–	9p13	Low-affinity IL-11 receptor Heterodimer with CD130 forms high-affinity IL-11 receptor
Ly-9	90–120	–	2	2	1q22	Not known
MAdCAM-1	58–66	–	2	–	10	Lymphocyte homing Ligand for integrins
Sialoadhesin	185	–	16	1	20p13	Adhesion

^aRefers to apparent molecular weight on reduced SDS-PAGE. Note that primary function is not necessarily mediated by IgSF domain (for example, MHC–peptide-binding groove is formed by the non-IgSF domains of the MHC molecules).

content. The minority of Ig-type domains which lack disulfide bonds (e.g. the first domain of CD2 and the third domain of CD4) usually have hydrophobic amino acids which point inward and contribute to the hydrophobic interior between the β sheets. The

exon–intron gene structure can also be indicative of IgSF membership in that each Ig-type domain is usually encoded by one exon. Exceptions to this arrangement include the first domain of CD4. IgSF members are generally spread throughout the

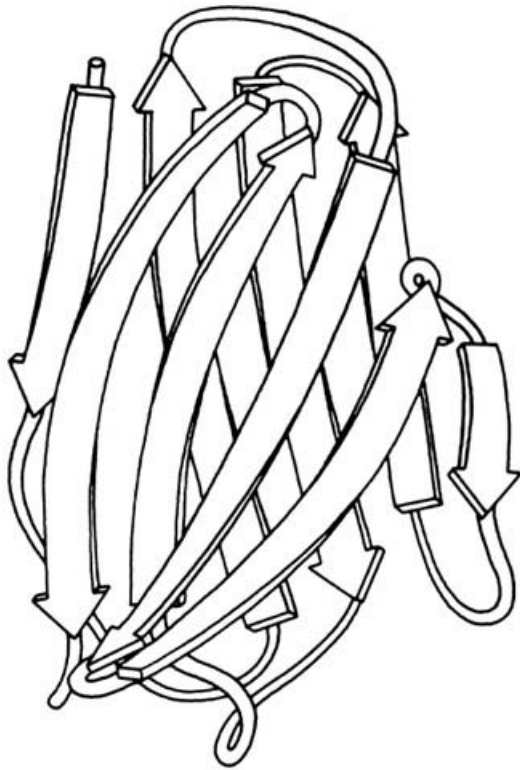


Figure 1 Schematic drawing of an immunoglobulin variable domain showing the characteristic 'immunoglobulin fold'. (Copyright by Jane S. Richardson, reproduced with permission.)

chromosomes, although some members are genetically linked together. For example CD90(Thy-1), CD-56(NCAM) and CD3 ϵ , γ and δ are all found on chromosome 11 at band position q23.

One commonly used approach for sequence comparisons with known members of the superfamily utilizes the Dayhoff ALIGN program which scores similarities between the candidate sequence and several sequences from accepted Ig gene superfamily members. The alignments used for such sequence comparisons need careful consideration, taking into account such factors as the β strand/loop organization and the repertoire of conserved amino acids. No residue is invariant in all Ig-related domains and the primary structure of the Ig-type domains can be very different. This is vividly illustrated by the Ig variable region genes where even proteins encoded by the same gene family are sometimes <20% similar. Three domain patterns have been identified in the Ig gene superfamily, namely the V-, C1- and C2-related domains. Each domain consists of two β sheets forming the characteristic sandwich structure of the Ig fold. The V domain structure has an additional loop which, in Ig, forms the second hypervariable region. The C2 type domain is more compact than the C1 and V domains and although it shares with

C1 the lack of the additional loop found in V domains it appears, overall, to have a relatively equidistant relationship to both V and C1 domain types. Some IgSF members possess Ig-type domains which exhibit features that make them somewhat difficult to assign to any one of the previously defined groups.

Since the members of the Ig superfamily are mostly located in environments in which proteases are common, it has been suggested that selection for resistance to proteolysis may be the key factor in conservation of sequence pattern yielding a compact globular structure. Indeed, the transmembrane and cytoplasmic regions of the Ig gene superfamily members are extremely diverse. For example, Thy-1 lacks a cytoplasmic domain and utilizes a glycosphospholipid membrane anchor, whereas the CSF-1 receptor (CD115) has a long cytoplasmic domain which exhibits tyrosine kinase activity.

Although molecules of the Ig superfamily have diverse biological roles, a common theme among them is that homophilic (e.g. CD56-CD56) or heterophilic (e.g. CD2-CD58) interactions between domains on different polypeptide chains are involved in their function. This has led to the suggestion that the superfamily has evolved from a single cell surface domain that was capable of homophilic interaction between cells. The existence of Ig superfamily members (fasciclin, neuroglian and amalgam) in insects and perhaps even in bacteria suggests that diversification of the domain occurred early in evolution. Gene duplication and subsequent divergence would allow the development of multidomain structures with diverse functions. Extensive diversification in the superfamily probably allowed heterophilic recognition between related molecules, leading to the sophisticated interactions seen in vertebrate immune systems.

See also: Adhesion molecules; CD2; CD3; CD4; CD8; CD28; Domains, immunoglobulin-type; Embryonic antigens; Fc receptors; H2 class I; H2 class II; HLA class I; HLA class II; Immunoglobulin structure; Inter-cellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Interleukin 6 receptor; Lymphocyte function-associated antigen 3 (LFA-3); Macrophage colony stimulating factor (CSF-1); Secretory component (the polymeric Ig receptor); T cell receptor, evolution of; Antibody-antigen complexes, three-dimensional structures; Thy-1; Tumor antigens; Cell surface receptors and adhesion molecules, three-dimensional structures.

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IMMUNOGLOBULIN GENES

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The structural diversity required for antigen recognition is encoded by a complex array of immunoglobulin genes. This entry will deal primarily with human immunoglobulin genes as they are the only ones to date that have been completely mapped and sequenced. These genes span approximately four million base pairs of DNA, over 0.1% of the estimated three thousand million base pairs in the human genome.

The heavy and light chains of the immunoglobulin molecule are derived from separate sets of immunoglobulin genes located on different chromosomes. Although the heavy chain (H) genes are located in a single locus on human chromosome 14 (mouse chromosome 12), the light chain genes can be derived from one of two loci: kappa (κ) on human chromosome 2 (mouse chromosome 6) or lambda (λ) on human chromosome 22 (mouse chromosome 16). The organization of the genes in each of these clusters mimics the domain structure of the heavy and light chain polypeptides. Thus, the heavy chain, κ light chain and λ light chain loci have variable region genes that encode the variable domain (responsible for antigen binding) and constant region genes that encode the constant domain (responsible for various effector functions, see below).

Constant region genes

In humans, there are nine functional heavy chain constant region genes (C_{μ} , C_{δ} , $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\alpha 1}$, $C_{\gamma 2}$, $C_{\gamma 4}$, C_{ϵ} and $C_{\alpha 2}$; **Figure 1A**), one κ light chain constant region gene (C_{κ} ; **Figure 1B**) and four functional λ light chain constant region genes ($C_{\lambda 1}$, $C_{\lambda 2}$, $C_{\lambda 3}$

and $C_{\lambda 7}$; **Figure 1C**) (see **Table 1**). The constant region genes expressed by a particular B cell determine the immunoglobulin's isotype. Initially each B cell expresses the C_{μ} gene in association with one of the five light chain constant region genes (producing the immunoglobulin M (IgM) isotype). Note that κ and λ light chains are rarely or never expressed in the same B cell. The mechanism that enables immunoglobulins other than the IgM isotype to be expressed is known as 'class switching'. This involves a recombination event at the DNA level between 'switch' sequences upstream of the C_{μ} gene and those upstream of the C_{γ} , C_{ϵ} and C_{α} genes, resulting in the expression of the IgG, IgE and IgA isotypes, respectively (this deletes the intervening region of DNA). In the case of the IgD isotype, the C_{δ} gene is sufficiently close to the C_{μ} gene to be spliced into the J_{H1} segment at the RNA level (see **Figure 1A**). Thus, IgD is an exception in that it can be coexpressed along with IgM by the same B cell.

Table 1 Number of functional variable and constant region genes

	Human			Mouse		
	κ	λ	H	κ	λ	H
Variable (V) segments	40	30	51	?	3	?
Diversity (D) segment	—	—	25	—	—	13
Joining (J) segments	5	4	6	4	3	4
Constant genes	1	4	9	1	3	8

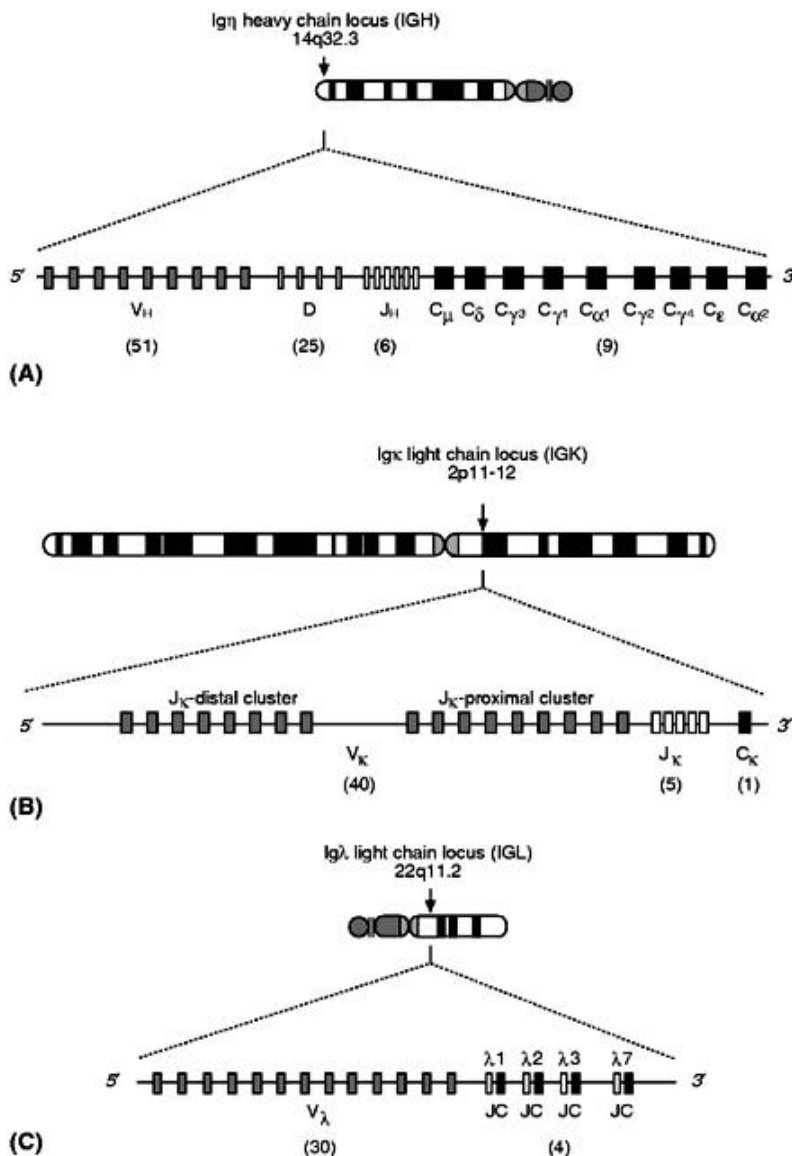


Figure 1 Organization of the human immunoglobulin genes. (A) The human immunoglobulin heavy chain genes (IGH) are located on chromosome 14q32.3. A schematic of the organization of the locus is shown (not to scale). Clusters of V_H, D and J_H segments are located upstream of the constant region genes (values in parentheses indicate the number of functional elements in each cluster). The entire locus including the constant region genes is approximately 1400 kb long. (B) The human immunoglobulin κ light chain genes (IGK) are located on chromosome 2p11-12. A schematic of the organization of the locus is shown (not to scale). Clusters of V_K and J_K segments are located upstream of the single constant region gene (values in parentheses indicate the number of functional elements in each cluster). The entire locus is approximately 1700 kb long. (C) The human immunoglobulin λ light chain genes (IGL) are located on chromosome 22q11.2. A schematic of the organization of the locus is shown (not to scale). Clusters of V_L segments are located upstream of the four functional J_L-C_L pairs (values in parentheses indicate the number of functional elements in each cluster). The entire locus including the constant region genes is approximately 900 kb long.

Somatic recombination of variable region genes

In contrast to the constant region genes, almost every B cell expresses a different pair of heavy and light chain variable region genes. This diversity is generated during B cell development by somatic recombination at the DNA level of a relatively small number of basic building blocks: V_H (variable), D (diversity) and J_H (joining) segments for the heavy chain variable region gene and V_K and J_K segments (kappa) or V_L and J_L segments (lambda) for the light chain variable region gene (the light chain does not use D segments). Each of these germline segments has a recombination signal sequence (adjacent to the 3' end of the V segment, both ends of the D segment and the 5' end of the J segment) that consists of a

conserved heptamer, a less-conserved nonamer and a 12 or 23 base pair spacer. The heptamer and nonamer sequences at the 3' ends of the V and D segments are complementary to those at the 5' end of the D and J segments, respectively. When recombination of the germline segments takes place, the complementary regions come together and the two segments fuse to form a rearranged variable region gene (see Figure 2 for details). Although the precise mechanism of recombination is unknown it must specifically recognize the recombination signal sequence, since a 12 base pair spacer will only pair with a 23 base pair spacer (the '12/23 rule'). The immune system uses this to great effect, in that V_H and J_H segments (that have 23 base pair spacers) cannot recombine with themselves or with each other, only with D segments (that have 12 base pair

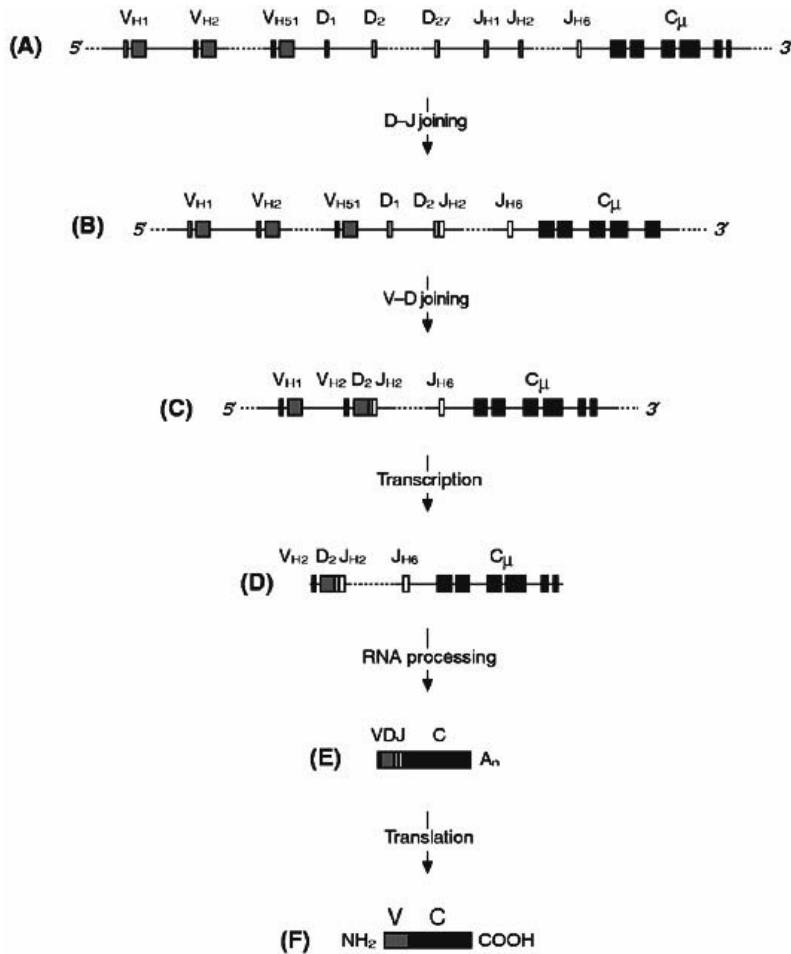


Figure 2 VDJ recombination. A vast repertoire of different heavy chains is created by the combinatorial rearrangement of a small number of variable (V_H), diversity (D) and joining (J_H) segments. In this example the hypothetical V_H segment 'V_{H2}' rearranges to the D segment 'D₂' and the J_H segment 'J_{H2}'. At the DNA level the heavy chain locus (A) first undergoes a D-J_H join, the intervening region of DNA being deleted to produce (B). This is followed by V_H-D joining to produce a rearranged V_H gene (C). The remaining J_H segments are removed after transcription (D) by RNA splicing that deletes the leader intron in the V_H segment, the J-C intron and the introns within the constant region gene (here the membrane form of IgM is produced that consists of six exons). Translation of the resulting mRNA (E) produces a functional heavy chain polypeptide (F). A similar rearrangement process occurs for the light chain polypeptide, except that there are no D segments (just a single V_κ-J_κ or V_λ-J_λ join) and the constant region is encoded by a single exon.

spacers). When recombination occurs between two segments in the same transcriptional orientation (both 5' to 3' with respect to the constant region gene/s) the intervening DNA is deleted from the genome and is lost in subsequent cell division (deletional joining). Occasionally (for example in the κ locus, see below) the two segments that recombine are in opposite orientations. In this case, the segments fuse as normal but the intervening DNA is inverted and maintained in the genome (invert joining).

In addition to enabling different V, D and J segments to come together (so-called combinatorial or germline diversity), the mechanism of recombination itself creates significant diversity due to imprecise joining and the incorporation of untemplated nucleotides at the join (junctional diversity). One consequence of this junctional diversity is that the D segment can be encoded in all three reading frames. Given that different heavy chains can also pair with different light chains the potential diversity created purely by somatic recombination is huge, although the actual diversity at any given time is limited by the number of B cells in the body (between 10^{11} and

10^{12} in humans). Antibodies selected from this 'primary repertoire' are believed to be able to bind antigen with at least moderate affinity.

Somatic hypermutation of variable region genes

The affinity of the antibody-antigen interaction is subsequently improved by somatic hypermutation of the rearranged genes followed by further rounds of selection (a process known as affinity maturation). These mutations are confined to the variable region gene and are primarily nucleotide substitutions, rather than insertions or deletions. The accumulation of multiple mutations occurs in a stepwise manner by the introduction of single nucleotide substitutions, followed by selection for and proliferation of B cells with higher affinity variants. Although the heavy and light chain variable region genes may only pick up a handful of mutations during this iterative process, mice immunized with various hapten antigens have been shown to express mutated antibodies with

affinities 10–100-fold higher than that of the unmutated clone.

Thus the structural diversity that forms the basis of antigen recognition is underpinned by a genetic diversity created in three ways: by the combinatorial rearrangement of different V, D and J segments (germline diversity), by junctional diversity produced during the recombination of these segments and later by somatic hypermutation that introduces point mutations into the rearranged heavy and light chain variable region genes. A prerequisite to dissecting the relative contributions of these three types of diversity is a complete description of the sequences and chromosomal organization of the germline V, D and J segments. Although the constant region genes were mapped and sequenced at the beginning of the 1980s, the mapping and sequencing of the human V, D and J segments have only recently been completed. The delay was mainly due to the number of segments and their homology to one another. This also confused early estimates of the total number of segments that suggested there may be as many as a thousand different V_H and V_κ segments.

Human germline V_H , D and J_H segments

The human V_H , D and J_H segments span 1100 kilobases (kb) of DNA upstream of the nine functional constant region genes (that span 300 kb) on chromosome 14q32.3 (Figure 1A). The transcriptional orientation of all segments in the locus (5' to 3' with respect to the constant region genes) is consistent with V_H –D and D– J_H joining occurring by a deletional mechanism (see above). There are a total of 51 functional V_H segments (Table 1), far fewer than originally anticipated, that can be divided into six main families (V_H1 to V_H6) on the basis of nucleotide homology of 80% or above. An additional family, V_H7 , is closely related but distinct from the V_H1 family. Functional members of the seven different V_H families are interspersed throughout the locus (along with at least 40 nonfunctional pseudogenes), in contrast to the organization of the mouse V_H locus, where family members are clustered. Interestingly, the genomic clone used to map the J_H -distal end of the locus was isolated by its ability to provide functional human telomere activity in yeast, indicating that the V_H locus is located within a few kilobases of the end of the long arm of the chromosome 14. Human D segments are also divided into six main families. These are arranged in four clusters (24 functional D segments) located between the single V_H6 family member and the J_H segments. An additional D segment, D_{11Q52} (homologous to the mouse D segment D_{Q52}), is located immediately upstream of the

six functional J_H segments, making a total of 25 functional D segments (Table 1). In addition to the functional V_H and D segments on chromosome 14, many V_H segments and more than one cluster of D segments are located on chromosomes 15 and 16. These so-called 'orphan' segments are not associated with J_H segments or constant region genes and therefore cannot encode functional heavy chains.

Human germline V_κ and J_κ segments

In humans, approximately 60% of light chains are of the κ type. A total of 40 functional V_κ and five J_κ segments are located on chromosome 2p11–12 upstream of the single C_κ gene (Table 1 and Figure 1B). The functional V_κ segments are divided into six families ($V_\kappa I$ to $V_\kappa VI$) based on nucleotide homology. These are arranged to two separate clusters: with 22 functional V_κ segments in the J_κ -proximal region spanning 450 kb (20 of these segments rearrange by deletional joining to the J_κ segments whereas two rearrange by invert joining) and 18 in the J_κ -distal region spanning 400 kb (all of these segments rearrange by invert joining). An additional 36 pseudogenes are interspersed amongst the functional segments. These two regions are separated by 800 kb of DNA that does not contain any V_κ segments. A comparison of the organization and V_κ segment content of the J_κ -proximal and J_κ -distal regions indicates that they are the result of a recent duplication event: on average, the sequences of the V_κ segments in the J_κ -proximal region differ by less than 1% from the corresponding segments in the J_κ -distal region. Studies of expressed κ chains have shown that V_κ segments in the J_κ -distal region are rarely used compared to those in the J_κ -proximal region. This is probably due to their distance from the J_κ segments and/or the fact that they must rearrange by inversion (see above). At least 24 additional V_κ segments are located outside the functional locus, some downstream of the functional locus on chromosome 2, some on the long arm of chromosome 2 and others on chromosomes 1 and 22. None of these 'orphan' segments are functional.

Human germline V_λ and J_λ segments

In humans, approximately 40% of light chains are of the λ type. The human immunoglobulin λ locus is located on chromosome 22q11.2 and contains 30 functional V_λ segments (Table 1) from ten V_λ families ($V_\lambda1$ to $V_\lambda10$) (Figure 1C). The transcriptional orientation of all segments in the locus (5' to 3' with respect to the constant region genes) is consistent with V_λ – J_λ joining occurring by a deletional mech-

anism. As with the human V_H and V_K segments, many pseudogenes are scattered throughout the locus. The J_λ segments in the λ locus are each paired with their own C_λ gene, such that there are four functional J_λ - C_λ pairs (Table 1 and Figure 1C). The choice of C_λ gene expression is therefore determined by V_λ - J_λ rearrangement. In contrast to the human heavy chain and κ light chain loci, where members of different V_H and V_K families are interspersed throughout the locus, the V_λ families are arranged in three distinct clusters. Cluster A (closest to the J_λ segments) contains the $V_{\lambda 2}$ and $V_{\lambda 3}$ families, cluster B (in the middle of the locus) contains the $V_{\lambda 1}$, $V_{\lambda 5}$, $V_{\lambda 7}$ and $V_{\lambda 9}$ families and cluster C (furthest from the J_λ segments) contains the $V_{\lambda 4}$, $V_{\lambda 6}$, $V_{\lambda 8}$ and $V_{\lambda 10}$ families. In addition to the functional genes on chromosome 22 at least one nonfunctional 'orphan' V_λ segment is located on chromosome 8.

For further details on the sequences and organization of the human V, D and J segments see <http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html> on the World Wide Web.

The creation of antibody diversity

Early studies on rearranged genes by Kabat and colleagues demonstrated that sequence diversity in antibodies is concentrated in six complementarity-determining regions (CDRs), three from the heavy chain variable domain (CDRH1, CDRH2 and CDRH3) and three from the light chain variable domain (CDRL1, CDRL2 and CDRL3). In the primary repertoire the CDRH1 and CDRH2 regions are encoded by the V_H segment, whereas CDRH3 is the product of V_H -D- J_H recombination. For the light chain, CDRL1, CDRL2 and the beginning of CDRL3 are encoded by the V_K or V_λ segment, whereas the end of CDRL3 is the product of V_K - J_K or V_λ - J_λ recombination. Subsequent crystallographic analyses have shown that these six CDRs correspond to the six antigen-binding loops that together form the antigen binding site of the antibody. As we have seen, this diversity is the result of germline diversity and junctional diversity in the primary repertoire and somatic hypermutation introduced during the process of affinity maturation. Since all human V_H , V_K and V_λ segments have now been sequenced and mapped it is possible to dissect their respective contributions in response to a wide range of antigens. A large database of rearranged genes was compiled and each was automatically assigned to its germline V_H , V_K and V_λ counterpart, thereby identifying the location of the somatic mutations in each rearranged gene. The percentage somatic hypermutation at each position in the heavy and light chain variable

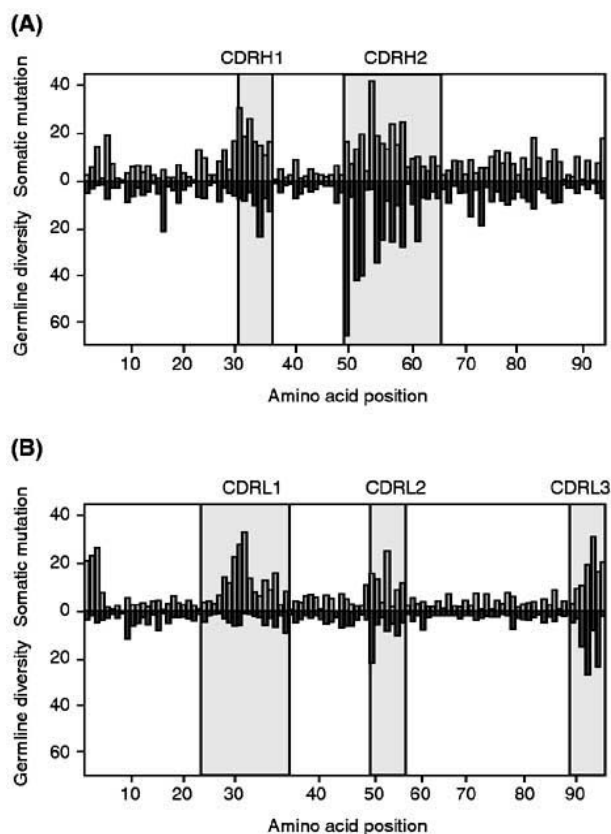


Figure 3 The patterns of germline diversity and somatic hypermutation. For each position, percentage somatic hypermutation (top of each panel, lighter tint) and germline variability (bottom of each panel, darker tint) are shown for (A) the human V_H domain and (B) the human V_K domain (corresponding plots for the V_λ domain are not shown here but can be found in Ignatovich *et al* (1997)). (Reproduced with permission from Tomlinson *et al* (1996).)

domains was then calculated (Figure 3). The CDRH3 and the end of the CDRL3 were not included in this analysis, as somatic hypermutation could not be distinguished from junctional diversity in this region. To compare the diversity introduced by somatic hypermutation with that present in the *same* repertoire of germline sequences, each of the rearranged genes was substituted with its corresponding germline V_H , V_K and V_λ segment. Germline diversity at each position was then calculated using the Kabat variability index (Figure 3).

Germline diversity is found mostly in CDRH2, at residue 50 in the CDRL2 and at residues 91, 92 and 94 in the CDRL3. In contrast, somatic hypermutation is most prevalent in the CDRH1 and CDRL1 regions. In order to illustrate these patterns of germline diversity and somatic hypermutation, a representative antibody structure was used to provide an 'antigen's eye' view of the binding site. Before

somatic hypermutation, germline diversity is concentrated at the centre of the antigen binding site. In contrast, somatic hypermutation spreads diversity to regions at the periphery of the binding site that are highly conserved in the primary repertoire. As a result, the final pattern of diversity (similar to that first represented by Kabat) is evenly distributed across the antigen binding site (see color plate 7).

The complementarity between germline diversity and somatic hypermutation seems to have evolved as an efficient strategy for producing high-affinity antibodies, given the limited number of B cells available for selection. Thus, antibodies are first selected from the primary repertoire based solely on the diversity at the center of the binding site. Somatic hypermutation is then left to optimize residues at the periphery without disrupting favorable interactions established during the primary response. The availability of a mechanism for somatic hypermutation has clearly led to the conservation of some sites in the germline V gene segments (for alteration by somatic hypermutation) and to the diversification of others. In this way, somatic hypermutation has left an evolutionary imprint on the repertoire of human germline V gene segments.

See also: Affinity maturation; Antibodies, specificity; Antigen-binding site; Immunoglobulin, evolution of; Immunoglobulin gene superfamily; Immunoglobulin structure; Antibody-antigen complexes, three-dimensional structures.

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IMMUNOGLOBULIN STRUCTURE

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An antibody molecule consists of four polypeptide chains or multiples thereof. There are two L ('light') chains and two H ('heavy') chains, with the H chains approximately twice as long as the L chains. Among human immunoglobulins there are two groups of L chains (κ and λ) and five classes of H chains (α , γ , δ , ϵ and μ), all with distinct amino acid sequences and antigenic properties. L Chains can associate with any of the H chain classes, producing immunoglobulin A (IgA) (with α -type H chains), IgG (γ), IgD (δ), IgE (ϵ), and IgM (μ) immunoglobulins.

Each type of antibody plays a different role in the body. IgA molecules are distributed both in the serum and in secretory fluids from the salivary glands and gastrointestinal tract. IgG proteins are the most abundant antibodies in serum, while IgD molecules are found predominantly on the surface of B lymphocytes. IgE antibodies are mediators of allergic responses and of immune reactions to parasitic infections. IgM proteins occur in two forms: 1) membrane-bound receptors on B cell surfaces and 2) secreted molecules assembled into pentamers or hexamers, with a molecular weight of approximately 900 kDa – hence the popular name of 'macroglobulin' for an IgM molecule in serum.

Components of an antibody and the genes that encode them

Both the L and H chains are composed of globular 'domains' connected by flexible segments with extended conformations, like cylindrical beads on a string (see **Figures 1–5**). An L chain has two domains – a V ('variable') unit with 108–111 amino acid residues, and a C ('constant') unit with about 105 residues (**Figure 1**). The variable domain is so named because its amino acid sequence differs from protein to protein, even within the same antigenic class and species. Within each isotype, the sequence of the constant domain changes very little (usually in four or fewer positions). Each V domain is encoded by two genes. The V_L gene specifies the first 95 residues and the J_L ('joining') gene dictates the remainder. Both the V_L and J_L gene products contribute to the antigen-binding site of the antibody (i.e. the CDRs, the 'complementarity-determining regions') and to the FRs ('framework regions') of the domain. The tail of the J_L segment joins the first few residues

of the C_L domain to form the flexible 'switch' region bridging the V and C domains.

In an H chain, the V domain is composed of the products of three genes: V_H , D ('diversity') and J_H . V_H encodes CDR1 and 2, as well as FR1, 2 and 3, and all three genes share responsibility for CDR3. J_H specifies FR4 and part of the switch region. Three C domains (four in IgM and IgE molecules), each encoded by a single gene (C_{H1} , C_{H2} , and C_{H3}), are connected to the V_H domain in human antibodies.

In IgG proteins the repeating unit is the combination of one L and one H chain (L–H heterodimer), normally linked covalently by an interchain disulfide bond. V_L and V_H domains are paired via noncovalent interactions to form an Fv ('fragment, variable') region, at the tip of which is the binding site for antigen. Pairing interactions of the C_L and C_{H1} domains involve a different set of surfaces than those of the V domains (see section on 'The immunoglobulin fold', below). Together, these V and C pairs constitute the Fab ('fragment, antigen-binding') (**Figure 2**).

To produce the normal four-chain IgG structure, the H chains in two L–H heterodimers are held together covalently by at least one interchain disulfide bond and noncovalently by very tight interactions between the C_{H3} domains. The combination of the C_{H2} and C_{H3} domains from the two heavy chains is called the Fc ('fragment, crystalline'), since this unit tends to crystallize when released from an IgG molecule by the enzyme papain (**Figure 3**).

The immunoglobulin fold

A single disulfide bond enclosing a loop of 65–70 residues is a common feature of every domain in L and H chains. The locations of the intrachain disulfide bonds in the V_L and C_L domains of a human λ -type light chain are shown in **Figure 1**. This illustrates the general repeated structural motif seen in antibodies: β sandwich or β barrel domains connected by flexible polypeptide segments. In an individual domain, the 'immunoglobulin fold' consists of two layers of antiparallel β pleated sheets linked by an intrachain disulfide bond. As exemplified by C domains, one sheet contains three strands of polypeptide chain and the second sheet has four strands. These sheets are not as clearly defined in all V domains. For instance, strand 1 of the four-chain layer may participate in few cross-strand hydrogen

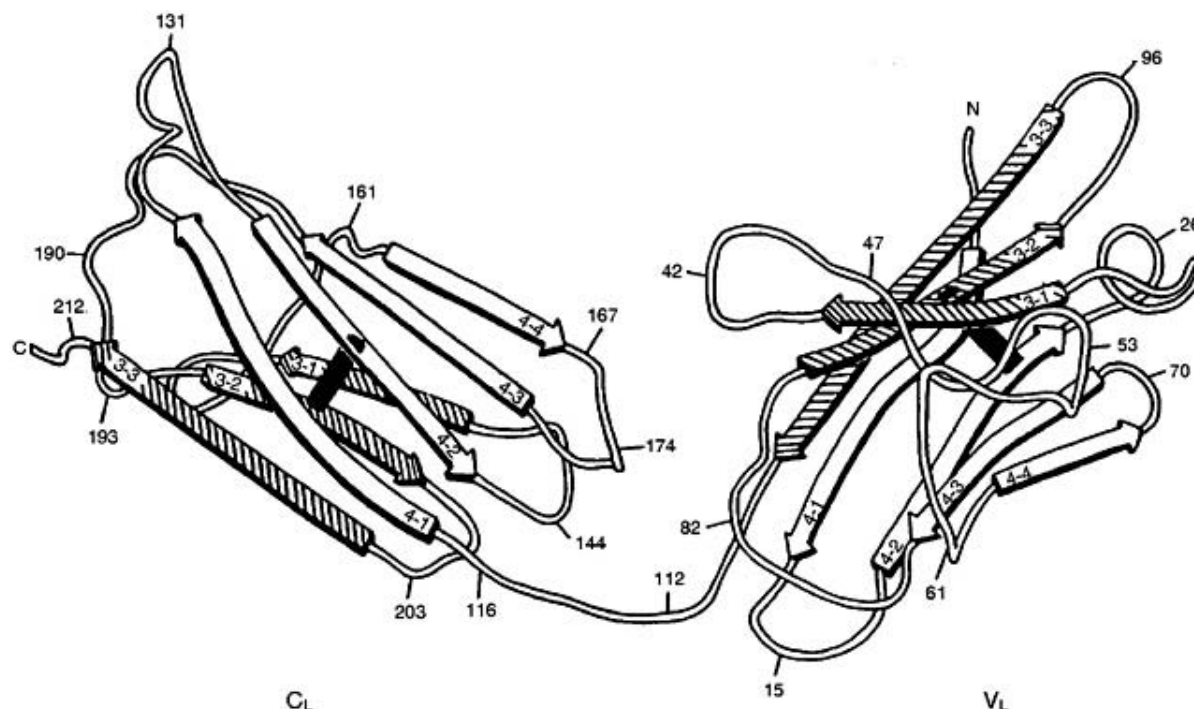


Figure 1 Tracing of the light chain from a human (Mcg) Bence Jones dimer with directional arrows superimposed (drawing by Kathryn R. Ely). The 3-stranded β -pleated sheets are represented by striated arrows and the 4-stranded layers by white arrows. The intrachain disulfide bonds are represented by the black bars between the β -pleated sheets. Positions of strategically located residues are numbered and the NH_2 - and COOH -termini are labeled N and C. The ascending and descending arms of CDR2 (marked by residue 53) form two additional β -strands in some molecules and expand the top layer to a 5-chain β -pleated sheet. The two domains can be approximately superimposed if the V_L domain is rotated 165° and translated $44 \text{ \AA}$. The V and C domains can be defined as rotational allomers, molecules related by rotation with similar 3-dimensional structures but different amino acid sequences.

bonds. The extra loops comprising CDR2 at the tip of the domain (but not present in C domains) may or may not exhibit the hydrogen bonding patterns that would expand the three-chain layer to a five-stranded sheet. These differences give the V domains more of a cylindrical outline and make them appear more like a barrel than a sandwich.

In antibodies the three-stranded sheets (or five-strand expanded sheets) provide the interface of the V_L - V_H pair, while the four-stranded sheets are exposed to solvent. The antigen-binding sites are composed mainly of three CDRs from each chain and are located at the outer edges of the three- or five-stranded sheets. Conserved FR residues bordering the CDR loops may also provide contact residues for binding an antigen, particularly in large active sites.

The modes of domain pairing are significantly different in the C domains. Relative to the V domains, the C_L and $\text{C}_\text{H}1$ domains rotate 160° - 180° . Their four-stranded β pleated sheets cross to form very close contacts which stabilize the dimer. The four-stranded layers of both the C_L - $\text{C}_\text{H}1$ and $\text{C}_\text{H}3$ - $\text{C}_\text{H}3$

dimers face each other to produce tight packing interactions and the three-stranded sheets are now relegated to the outer surfaces of each pair. Opposing faces of the $\text{C}_\text{H}2$ domains also involve the four-stranded sheets. However, covalently linked oligosaccharides partially cover the surfaces and effectively prevent close contacts of the protein moieties. Strict two-fold symmetry between half molecules of human and rabbit IgG antibodies may be broken because one of the oligosaccharide chains is shorter than the other (e.g. in 10-15% of patients with rheumatoid arthritis and in most if not all normal rabbits).

The λ light chain in **Figure 1** is covalently linked to the heavy chain by an interchain disulfide bond involving the penultimate residue. In a κ light chain, the C-terminal residue would serve this function. The interchain half-cystine residue contributed by the heavy chain is located in the 'hinge' region of a human IgG antibody (see 'Hinge region' below). However, the half-cystine residue in an IgM heavy chain is found in the sequence encoded by the $\text{C}_\text{H}1$ gene. In both cases, the strands containing the half-

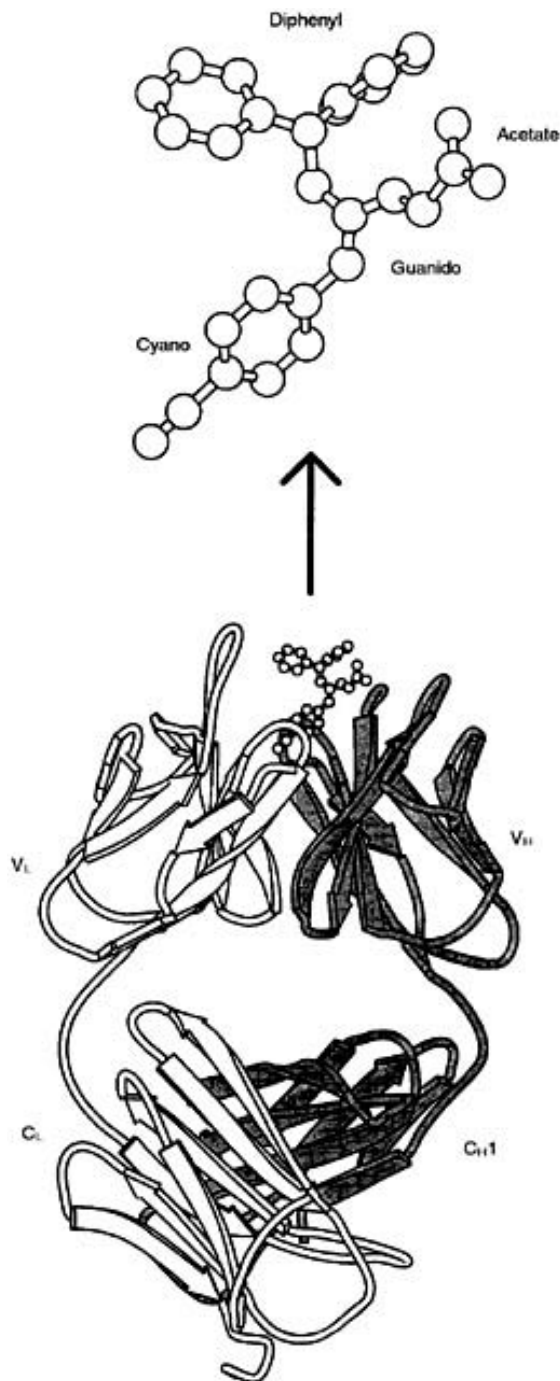


Figure 2 The model of the complex of the NC6.8 Fab with its haptent, N-(p-cyanophenyl)-N'-(diphenylmethyl)-N''-(carboxymethyl)guanidine. This haptent is shown as a ball-and-stick model between the V domains and the details of the constituent parts are enlarged above the arrow. When added to crystals of the native (unliganded) Fab, the haptent causes the crystals to dissolve. Immediately adjacent, new crystals of the haptent-Fab complex appear and these crystals have a different physical appearance, space group and unit cell dimensions. Relative to the native structure, the light chain of the complex is extended by 10 Å and the angle between V_L and C_L is increased by 20°. The heavy chain is shortened commensurately by flexing and the angle between V_H and C_H1 is decreased by 22°.

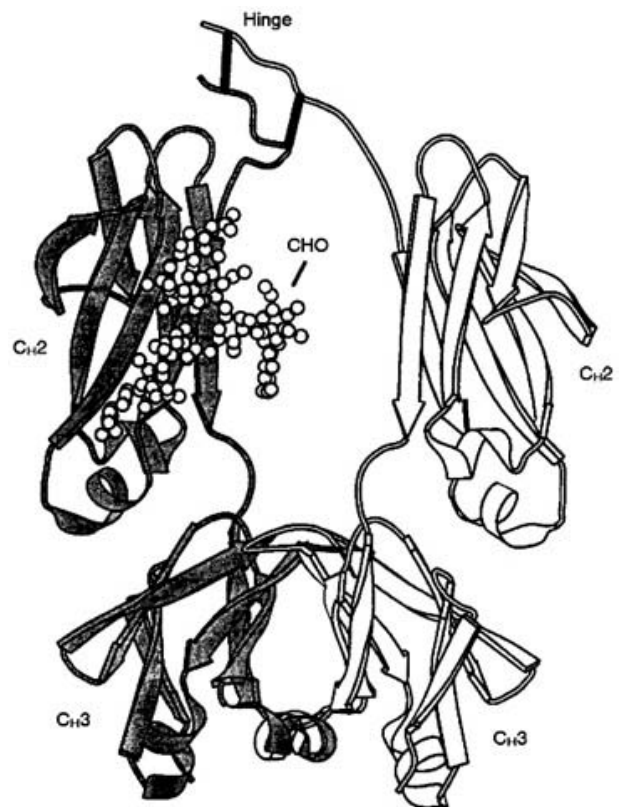


Figure 3 Model of an Fc from a human IgG1 myeloma protein. The carbohydrate (CHO) moiety is only shown for one C_H2 . The hinge region is asymmetrically placed above the C_H2 domains and the two polypeptide strands are linked by interchain disulfide bonds (black bars).

cystine-residues closely approach the C-terminal region of the light chain in the three-dimensional structures of the antibodies.

Structures of antigen-binding sites

The crystal structure of the NC6.8 Fab with its ligand bound in the active site is presented in **Figure 2**. Sufficient numbers of crystal structures of Fabs and Fvs have been determined by X-ray analyses to show that binding regions are widely diverse in size and shape. For protein antigens like lysozyme and neuraminidase, block end or shallow convex-concave types of binding seem to be favored. 'Linear' polymers like single-stranded DNA and some carbohydrates are most conveniently accommodated in large grooves in the V_L - V_H interface. Monosaccharides on branched carbohydrates and small haptent-like molecules fit into cavities or relatively small depressions on the surface of the antibody. A high-affinity site for fluorescein in the murine 4-4-20 antibody proved to be a very tight slot in the V_L - V_H interface.

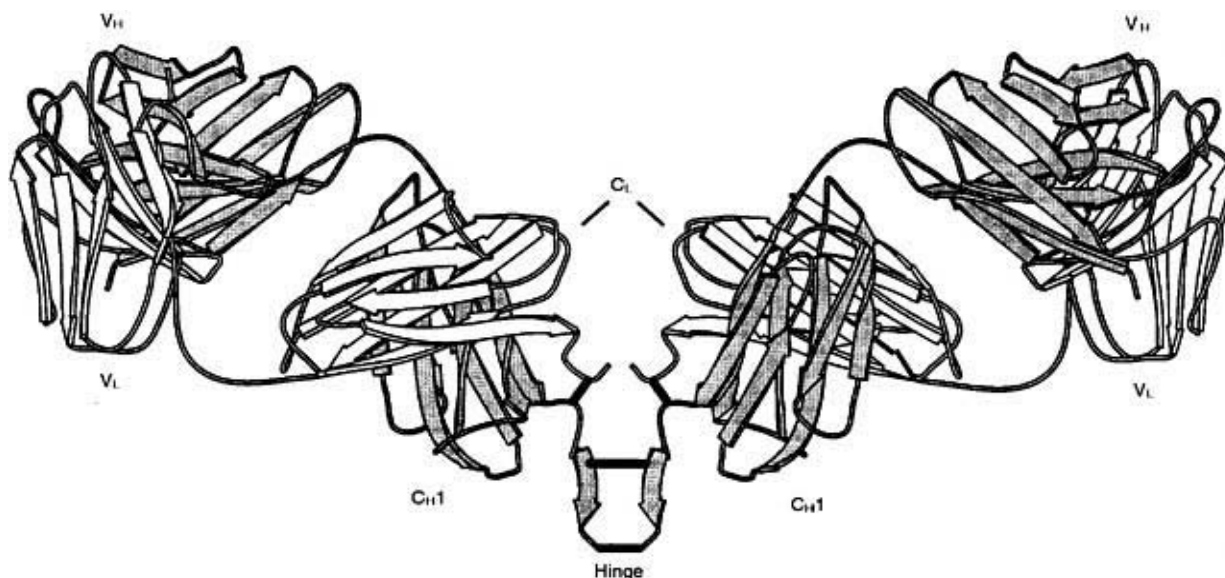


Figure 4 Model of the Kol IgG1 immunoglobulin, as adapted from Marquart *et al.* (1980). The Fab arms in the hinge region are shown in the model, but the Fc region is missing because it is disordered in the crystal. Interchain disulfide bonds in the hinge region are represented by black bars. Note that the Fabs have extended structures with close interactions between V_H and V_L .

One human L chain dimer (Mcg) has a two-compartment binding region, consisting of a large conical cavity and a deep pocket. More than 60 ligands, including fluorescent compounds, dinitrophenyl and other aromatic compounds, peptides, and oligonucleotides have been found to bind in a series of subsites along the entire interface of the two V_L domains. Moreover, human and insect hormones of eight or more residues have been shown to bypass the Mcg deep pocket, force passage between the domains through an unexpected route and span the space between the conventional binding cavity and the solvent pool between the V and C domain pairs.

In many but not all of the ligands tested for binding by Fab and Fv fragments, H chain substituents, particularly those in CDR3, seem to play more important roles than those in the L chains. CDR3 of the V_H domain often occupies an inordinate amount of space between the V_H and V_L domains and is therefore more likely to come into contact with the antigen. However, the L chain contributions may also be substantial or even dominant. For example, in the case of an anti fluoresceyl antibody (4-4-20), the light chain provides five of the primary contact residues for the hapten.

Generally, the interactions stabilizing the antibody–ligand complex are mediated through the amino acid side-chains rather than the atoms along the polypeptide backbone. However, there are examples in which the carbonyl oxygen and the proton on the amide nitrogen of a peptide bond participate in hydrogen bonding interactions with hydrogen

donors or acceptors on the ligand (e.g. as occurs in a complex of an anti-single-stranded DNA antibody with its oligodeoxynucleotide). In addition to hydrogen bonding, the interactions of antibodies with ligands include the usual types associated with proteins, namely the formation of ion pairs, hydrophobic interactions, ring stacking, and other aromatic–aromatic interactions.

The hinge region

A separately encoded ‘hinge’ region is inserted between the C_H1 and C_H2 domains. Portions of the hinge regions of two human IgG1 antibodies can be seen in **Figures 3 and 4**. In the human and murine IgG1 subclasses, the initial part of the hinge region supplies the half-cysteine residue which forms the interchain disulfide bond with the L chain (see **Figure 4**). Its half-cysteine counterpart in the L chain occupies the C-terminal location in a κ chain and the penultimate position in a λ chain. Disulfide bonds linking the two heavy chains are found in a relatively rigid ‘core’ segment (Cys-Pro-Pro-Cys-Pro) of the hinge region. Segments flanking this core section are responsible for the flexibility suggested by the name ‘hinge’. Papain hydrolyzes peptide bonds among residues 6–10 of the upper flexible segment (‘proximal hinge’) between the H–H and the H–L interchain disulfide bonds to produce Fabs. Pepsin cleaves the lower flexible segment (‘distal hinge’) after the disulfide bonds to release a $(Fab')_2$ fragment.

In the most prevalent subclass (IgG1) of human

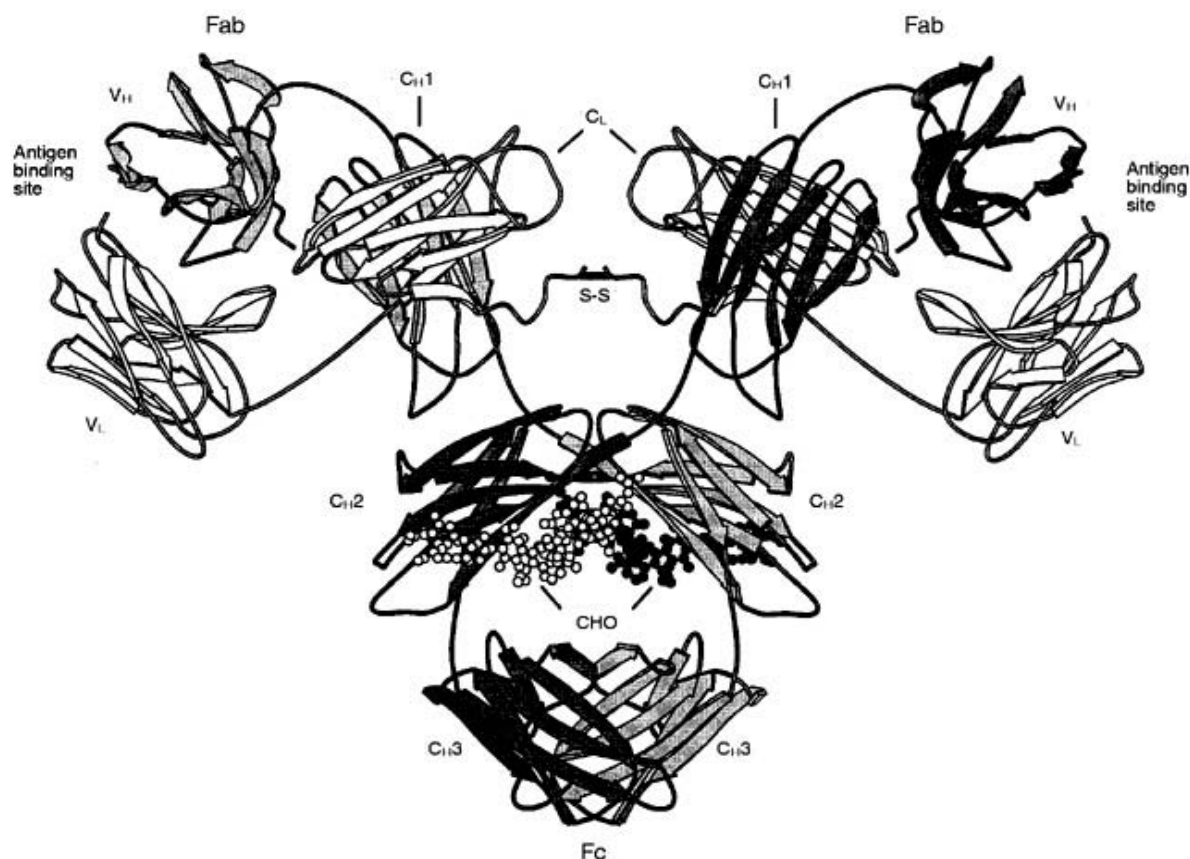


Figure 5 Model of the Mcg IgG1 myeloma protein, in which a discrete hinge region is deleted. The connecting segments between C_{H1} and C_{H2} are derived exclusively from the first seven residues of the C_{H2} gene product. Note that these connecting segments cross in the model. Because they cannot form a disulfide bond with the heavy chain, the appropriate half-cysteine residues in the two light chains form a disulfide bond across the crystallographic dyad relating the two halves of the IgG1 molecule. The shortening of the heavy chain, together with the inter-light chain disulfide bond, lead to different structural relationships between the light and heavy chains, including a very large, concave active site at the tips of the Fab arms. Note the large structural differences between the constrained Fc region in this intact IgG1 molecule and the Fc fragment released from a conventional IgG1 molecule by the action of papain (as seen in Figure 3).

IgG molecules, the hinge region typically consists of only 15 residues, while the corresponding segment in IgM and IgE molecules is sufficiently large to be considered an entire C domain. When hinge regions are present, as in the two human proteins Kol IgG1 and Zie IgG2, the Fab portions of these molecules adopt ordered structures in the crystals, while the Fc regions are 'disordered' and therefore cannot be analyzed by X-ray diffraction techniques. This causes the crystallographic structure of the intact Kol IgG1 immunoglobulin (Figure 4) to resemble a $(Fab')_2$ fragment.

In a small minority (1–2%) of IgG proteins, the hinge region is deleted from the heavy chain. These proteins (e.g. Dob, Lec and Mcg IgG1 immunoglobulins) are less flexible than molecules with hinge regions. However, the loss of flexibility appears to be advantageous for uniform packing in crystal lattices. Such proteins have therefore proved

to be favorable for X-ray crystallographic analyses. The crystal structure of the McG IgG1 immunoglobulin is shown in Figure 5.

When the hinge region is deleted, the interchain half-cysteine residue on the light chain can no longer pair with a heavy chain partner. Usually, alternative pairing is established by formation of an interchain disulfide bond with the second light chain in the antibody molecule (see Figure 5, center S–S bond). Although the effects are difficult to assess quantitatively, the formation of this disulfide bond partially restricts the freedom of the Fab arms to move independently and thus accentuates the loss of flexibility associated with a hinge deletion alone. Since the Fc region is pulled tightly up into the junction of the Fab arms, its rotational or translational mobility is also severely impaired.

Again we have an example of how the delineations of unusual features can lead to a better understanding

of the highly evolved and efficient structures of the dominant molecules in 'normal' sera. Just as the pathological effects of incomplete glycosylation focus attention on the role of carbohydrates in keeping IgG antibodies soluble, the deletion of the hinge and the formation of an L-L disulfide bond help us understand the tremendous advantages of a flexible hinge in the tracking and cross-linking of deleterious antigens.

See also: Antigen-binding site; Domains, immunoglobulin-type; IgA; IgD; IgE; IgG; IgM; Joining J chain; Antibody-antigen complexes, three-dimensional structures.

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IMMUNOLOGY, A BRIEF HISTORICAL PERSPECTIVE

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Immunology as a discipline has its roots in both applied and theoretical science. It has sought practical methods of immunizing and vaccinating to prevent specific infectious disease, and an understanding of the mechanisms involved in the induction of specific immunity. This entry is not an attempt to identify and catalog specific events in the history of immunology. Rather, it describes the large trends and sets them in a historical and intellectual context. In that sense, it is an intellectual history of immunology.

One philosophical approach to the history of science as intellectual history refers to the concept of a 'paradigm'; this approach is exemplified by Kuhn's book *The Structure of Scientific Revolutions*. According to Kuhn, a paradigm is a viewpoint held by a community of scientists. Paradigms form the foundation of experimental design and, most important, of interpretation of experimental results.

Kuhn has argued that a science becomes a 'mature' science only when it has a paradigm, and that it changes only when this paradigm changes, an event that happens only rarely. When a paradigm does change, there is a revolution in the way that the relevant science views itself. I refer here to the Kuhnian notion of a paradigm because it provides an instructive perspective from which to look at the history of immunology. I am fully aware that there are other ways to look at scientific progress and change (see Lakatos and Musgrave, *Criticism and the Growth of Knowledge*).

Preparadigmatic immunology

Protoimmunology: from the ancients to the Enlightenment

The scourges of infectious diseases have declined steadily and dramatically during the last century.

During this period, modern immunology had its beginnings and immunologists, not surprisingly, tend to see a causal relation between these events. But the discipline of public health was developed before immunology and has almost certainly played a larger role in the control of infectious disease and the elevation of the lot of humankind. It can be argued that the concepts and practices of public health raised the standard of living to the point at which individuals, now aware of the value of their own lives, wanted prevention of specific diseases (see Latour, *The Pasteurization of France*).

The fact that survivors of a plague were somehow spared from dire consequences of a second encounter with the disease was well known to the ancients. Thucydides, commenting on the plague of Athens of 430 BC: 'Yet still the ones who felt most pity for the sick and the dying were those who had had the plague themselves and had recovered from it. They knew what it was like and at the same time felt themselves to be safe, for no one caught the disease twice, or, if he did, the second attack was never fatal. Such people were congratulated on all sides, and they themselves were so elated at the time of their recovery that they fondly imagined that they could never die of any other disease in the future.' (Thucydides, *The Peloponnesian War*).

The realization that the survivors of a plague having once recovered were not going to succumb to it in the future came not from experiment but from observation, as did so much understanding of the world. So, too, must have come the eventual realization that these survivors were not to be spared from other diseases in the future. But the idea of specific immunity to a specific disease was probably not yet understood. Many examples from antiquity through medieval times suggest that people were aware that they could protect themselves from disease. This 'protoimmunology', the nonscientific awareness that there could be protection to a disease, does not however seem to have played an important part in magic or religious ritual in early societies. Perhaps life was so fraught with danger from diseases of all kinds that freedom from one offered only a small respite. Another disease could strike soon; women risked dying in childbirth, men in war, and both by famine. The souls represented in a Breughel painting were not likely to have been aware of the subtle differences between one form of suffering and another. A few diseases like the black plague, smallpox and erysipelas had sufficiently distinct symptoms to stand out, but the inhabitants of early modern Europe could not distinguish easily the various forms of fevers and pestilence. Since the reason for the existence of these fevers and pestilence, often tied to

magic and religion, was thought to be Divine retribution for collective sin, many people may have concluded that this suffering was not to be alleviated.

But if 'protoimmunology' was not part of ritual, it was a part of ordinary life in some societies. The Chinese inoculated healthy people with material removed from a pustule of a person with smallpox. They 'opened the pustules of one who has the Small Pox ripe upon them and drying up the Matter with a little Cotton . . . and afterwards put it up the nostrils of thowse they would infect . . .'. Smallpox was probably introduced into Europe by both the Crusaders and the Saracens in the sixth century. It is estimated that in sixteenth-century England, only five of 1000 persons escaped infection and over half of the population had obvious pockmarks. Smallpox was not as common by the eighteenth century, but it was still a real scourge for many people. Nevertheless, it was known in that century in some circles in England that people who had been infected with cowpox did not have the pockmarks of smallpox. Edward Jenner (1749–1823), who himself had had a traumatic experience when he had been treated with pustule material as a boy, turned this bit of folklore into what perhaps became the most famous clinical trial of all time. When he suggested to a Gloucestershire milkmaid patient that she had smallpox she replied, 'I cannot take the smallpox because I had had the cowpox.' Following her lead, in May of 1796, he inoculated 8-year-old James Phipps with cowpox and then, several months later, inoculated him with smallpox. James Phipps survived and the era of safe immunization began. Jenner's paper, which was rejected by the Royal Society ('lest he damage his reputation') and published privately, made him an international hero and opened the way for people to begin to think about prevention of specific diseases.

This advance coincided with the period we term the Enlightenment: public health had made life more 'civilized' and Europeans had come to believe in the goodness and perfectibility of human beings.

The first paradigm: specific etiology and immunization

Louis Pasteur and the 'wonderful century'

The middle of the nineteenth century (the 'wonderful century') saw political and scientific ferment and the career of Louis Pasteur (1822–1895) exemplifies that ethos. Trained as a chemist, he made important contributions to structural chemistry before he turned to the question of fermentation in 1857. Fermentation was understood loosely; it referred to the 'spontaneous' changes seen in the making of wine or beer (alcoholic fermentation), the souring of milk (lactic

fermentation), etc. Putrefaction of animal tissue was thought of as another form of fermentation, one that led to noxious products. All of these were thought to be caused by chemical agents that catalyzed chemical reactions. The catalysts were called 'ferments'; yeast was the ferment known to be responsible for the production of alcohol and wine and the rising of dough. Even though Schwann and others had showed in 1837 that yeasts were living entities more closely related to plants than animals, the great chemists and their followers did not see any value in microscopic evidence. The idea that living agents were involved was relegated to 'vitalism', which was anathema at that time to great chemists like Leibig, Berzelius, Helmholtz and Wohler. They considered the yeast seen in fermentation to be chemical precipitates. The great Leibig summed up the chemists' view of a vitalistic nature of ferments: 'As to the opinion which explains the putrefaction of animal substances by the presence of microscopic animicula, it may be compared to that of a child who would explain the rapidity of the Rhine current by attributing it to the violent movement of the many millwheels at Mainz' (quoted by Dubos, *Louis Pasteur: Free Lance of Science*). Pasteur was of the chemical tradition.

Between 1855, when he first addressed the problem of lactic fermentation, and 1857 when he presented his 'Mémoire sur la fermentation appelée lactique', Pasteur had moved from the view of the chemist to that of the biologist. By 1860, he had published the 'Mémoire sur la fermentation alcoolique', which formalized the theory that different kinds of fermentations are produced by different kinds of organisms. Pasteur worked on the theoretical questions of the nature of the ferments in the very practical context of 'diseases' of fermentation. He had become a national hero for saving first the wine and then the silk industries by curing their diseases and found that the 'diseases' were caused by specific microbes. Ultimately, this was to lead him to the germ theory of disease, namely the crucial idea that each infectious disease is caused by a specific microbe.

It is important to realize the tenor of the times. Darwin had published *Origin of Species* in 1859. The hold of church and the past on the European mind was changing rapidly. In France, a great (and final) debate on the subject of the spontaneous origin of life raged and Pasteur, who never avoided a conflict, was at the center of it. His now famous swan-necked flask experiment is still used as the final argument against the idea that microbial life arises spontaneously. By 1860, the idea that specific diseases are caused by specific microbial agents which do not

arise spontaneously had been formulated and accepted.

These ideas rank in the front line of advances in intellectual history. In 1879, he made a chance discovery that, while not of the intellectual scope of the first ones, began the field of modern immunology. (It is understandable that he is also remembered for the maxim that 'chance favors the prepared mind'.) In the spring of that year, he had begun experiments on chicken cholera. After the summer vacation, it was found that the cultures of chicken cholera bacillus that had been kept during the summer did not produce disease when inoculated into chickens. A new culture was obtained from a natural outbreak and was virulent when injected into new chickens. But when these virulent cultures were injected into the animals that had not succumbed to the old cultures, the animals remained free of disease. Pasteur immediately recognized the analogy between his result and that obtained when Jenner's cowpox was found to protect against smallpox. In homage to Jenner he called the phenomenon 'vaccination' and forever enlarged the meaning of the word. With this, Pasteur established the *general* principle that an organism can be altered so that it does not cause disease, but still retains the property of inducing immunity. What had been a brilliant but apparently anecdotal clinical insight for Jenner was given a scientific basis and raised to the level of a scientific principle by Pasteur. The science of immunology was born.

Louis Pasteur established that specific diseases are caused by specific organisms that do not arise spontaneously and that they can be used to induce specific immunity. Not surprisingly, Jenner and Pasteur were both revered in their lifetimes throughout the entire world because they had fulfilled the first aim of immunology, to protect humankind from infectious disease. In our time, only Jonas Salk has attained a similar status for the very same reason.

There followed two decades of competition between the French and German schools of immunologists, headed by Pasteur and Robert Koch in Berlin. They have important implications in both the history of immunology and international relations. In this time of extreme nationalism, both Pasteur and Koch saw themselves (and were seen by their countrymen) as defenders of national honor. It is rare in the history of nations that chauvinism bears socially useful results but this was one of them. In the attempt to be the first to identify the organism responsible for every disease, and to develop a procedure to immunize against them, the French and the Germans tried to outdo each other. While neither France nor Germany was ennobled, as their fervid

citizenry had hoped, the world acquired, along with war, hardship and hatred, the means of inducing specific immunity to many diseases and we began to understand the mechanism of this immunity.

One very important realization was that the mechanism of the immune response toward an innocuous substance, such as a foreign erythrocyte, was the same as that against a deadly bacillus. Thus it became possible to study immunology using surrogate pathogens. If this were not true, and if it had not been realized early in the history of the science, the enormous progress that has been made in understanding the mechanisms of immunity might not have been possible.

The second paradigm: clonal selection

Ehrlich, Haurowitz, Jerne and Burnet: the path to clonal selection

The path from Jenner to Pasteur established immunology as a mature science because it led to the first paradigm of immunology, namely that it is possible to inject altered forms of organisms to induce specific immunity. The next paradigm to be established, *clonal selection*, made it possible to study the mechanisms of immunity. The principle of clonal selection is that each of the cells involved in the immune response (the lymphocytes) are preprogrammed to express receptors and to respond to a single antigenic determinant. When antigen is introduced into the system it is processed and reacts with the receptor, inducing the cell to proliferate. Antigen thus acts as a selective agent in the Darwinian sense; the cell is induced to proliferate and undergoes clonal expansion, resulting in many more cells of the same specificity. The path to this idea began with Paul Ehrlich (1854–1915) in his Croonian Lecture to the Royal Society in 1901, which appears to be the first attempt to explain the observations of Jenner and Pasteur.

Ehrlich, in that lecture, postulated that the cells responsible for antibody production have *side-chains* on their surfaces, each of which have the ability to react with a single disease-causing entity. In Ehrlich's view, the reaction of a microbe or toxoid with the appropriate side-chain caused the cell to produce more of that side-chain so that soon the entire surface of the cell was covered with molecules of this specificity. In fact, the cell was thought to overproduce them and the excess appeared in the serum as antibodies. This first modern theory of antibody production is of a type we now call *selection* theories, because it postulates that the receptors (side-chains) are already present when the antigen is introduced and the role of the antigen is to select the side-chain.

The startling prescience of this idea that runs so counter to intuition cannot be overstated.

The side-chain theory stood until the 1930s when Karl Landsteiner (1868–1943) began his famous experiments on haptens and carriers. Landsteiner showed that when small molecules that by themselves were not able to induce an antibody response were conjugated to large immunogenic molecules, an animal was able to make an antibody response to them. The large molecule acted as a *carrier* and the small molecule that had become immunogenic by coupling to the carrier was called a *hapten*. Landsteiner showed that the antibodies produced to dinitrophenyl, for example, were specific for that hapten and did not react with antibodies raised to trinitrophenyl. In his influential book *The Specificity of Serological Reactions*, he attempted to develop the rules for immunogenicity. This attempt was not successful, but what did emerge was the fact that he could synthesize a seemingly endless array of substituted molecules, each of which gave rise to a specific antibody response. There could not be enough room on the surface of a cell for all of the side-chains that Ehrlich's theory had postulated. The scene was set for a change of paradigm and this was provided by Felix Haurowitz.

Haurowitz (1896–1988) was a German living in Prague. A physician, he was trained as a physical biochemist and had earned a reputation for his work on the structural forms of hemoglobin. A colleague, Breinl, who had spent a year at the Rockefeller Institute in New York, told him of Landsteiner's work and he and Breinl designed an experiment to determine the amino acid composition of gamma globulin before and after immunization. Using the methods available in 1930, they found that the amino acid composition was the same in both cases and concluded that since the quantity of each amino acid was the same, the *order* had to be different between non-immune and immune globulin. This conclusion led them to the theory that became known as the *template theory* of antibody formation. It postulates that a fragment of the antigen enters the antibody-producing cell and there acts as a template that directs the order of the placing of the amino acids into the protein. A positive-charged amino acid in the antigen fragment results in a negatively charged amino acid in the antibody molecule and vice versa. This theory is a fundamental departure from the selection type of theory of Ehrlich because instead of the antibody-forming cell being preprogrammed to produce antibody, in this theory the antigen is instructing the cell what antibody to make. It is an *instructive-type* theory of antibody formation.

Between 1931 when it appeared (Linus Pauling

expanded on the idea in an influential series of papers in the 1940s) and the 1950s when it was seen to be untenable, the direct template theory was appealing because it made sense and was testable. With the discoveries of the 1950s leading to the new genetics based on the 'trinity' of DNA–RNA–protein, the template theory of antibody formation was abandoned. It is one of history's small ironies that James Watson, the codiscoverer of the double helix and one of the founders of the new genetics, as a graduate student at Indiana University was a student in Haurowitz's class in biochemistry.

The template theory was replaced by another version of a selective theory by Neils Jerne (b. 1911). Jerne, a Danish polymath, was an immunologist at the Danish Serum Institute in Copenhagen but spent his summers as part of the famous 'phage group' of Max Delbrück at Caltech. These ideas were based largely on the 'Luria–Delbrück' experiment that showed that mutations are random events and not induced by the selective agent, i.e. the agent selects the mutant, it does not induce the mutation. In 1955, building on the theory of the phage group, Jerne proposed a new version of the original selective-type theory. This theory, called the *natural selection theory* of antibody formation, was based on his observations that all animals have detectable levels of antibody to a wide array of antigens in their serum even though they have never been intentionally immunized to them. His theory stated that, upon entering the system, antigen reacts with these 'natural antibody' molecules and the complex of antigen and antibody is transported to a cell and internalized, causing the cell to produce more antibody. F. M. Burnet (later Sir Macfarlane Burnet) suggested that the theory would be improved by having the antigen react at the surface of the cell rather than be internalized. The idea was thus changed to have the unit of selection become the receptor on a preprogrammed cell rather than the antibody in the serum. It was parsimonious to assume that the receptor was an antibody molecule of the specificity that the cell was preprogrammed to make. The theory stated that the reaction of antigen with cell surface antibody receptor induced the cells to proliferate, resulting in a clonal expansion of cells. Hence the theory was called the *clonal selection theory*.

The clonal selection theory was very quickly championed by a prominent immunologist, David Talmage, and by the Nobel prize-winning geneticist Joshua Lederberg. The theory appealed to both communities because it appeared at the time when genetics was establishing the basis of the modern era of biology and it served to bring immunology into the mainstream of biology and genetics. Within a short

time, both Haurowitz and Pauling conceded that the instructive-type theories were no longer tenable. Even though clonal selection was counter to the intuitive view that workers in the field had at the time because of decades of experimental evidence being interpreted through an instruction-type lens, it fulfilled Kuhn's description of scientific revolutions; when it was accepted, it was accepted totally.

Immunology now had its second major paradigm and the field changed dramatically from descriptive serology to one more closely related to cell biology because the intellectual groundwork had been laid to study the unit of selection, the preprogrammed cell. The questions that immunologists could now address were the cellular basis of the immune response and how the genes that encode the antibodies are organized. The stage was set for the cellular and molecular era.

The modern era: cellular and molecular immunology

With the establishment of clonal selection as the dominant paradigm, the framework was in place to attempt to explain the phenomena of the immune response. The problem was threefold:

1. What is the structure of the antibody molecule and how could the genes for molecules that react with an enormous number of antigens be organized?
2. What is the cellular basis for the immune response?
3. How is the system regulated (including the question of self/nonself discrimination)?

The great advances in the last three decades comprise the bulk of the entries in this encyclopedia and attest to the great progress that has been made in addressing the questions of mechanisms. The merging of molecular biology and immunology, along with giving us the tools to study the mechanisms, have allowed new approaches to vaccine development to occur and the promise of the next decade is the application of these great advances to the betterment of the human condition.

See also: Antibodies, specificity; *Bacillus*, infection and immunity; Carrier; Clonal selection; Diversity, generation of; Hapten; Lymphocytes; Natural antibodies; Poxvirus, infection and immunity; Vaccines.

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IMMUNOPATHOLOGY

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There are three ways in which the term 'immunopathology' has been adopted as a useful descriptive term:

1. Several immunological processes can be regarded as representative of normal 'immunophysiology'. In this framework, immunopathology is any process in which the physiological mechanism is damaged.
2. Rapid advances in immunology over the past 30 years have made a significant impact not only in our understanding of the immune response itself, but also in providing many of the modern technologies which are now applied to the study of pathological processes in general. Thus, in this framework, immunopathology is the discipline which is involved in studying disease processes using immunological methods; and the person who does this kind of investigation is the immunopathologist.
3. Although the immune response has evolved as part of the response to injury, it is now widely recognized that in several instances it is the response itself that is in fact what is recognised as characteristic of the disease. This form of immune injury is thus regarded as immunopathology.

Immunophysiology and immunopathology

The physiology of the immune system has to encompass the capacity of the cells that constitute it to respond to a wide range of exogenous stimuli, and

the capacity to operate under diverse conditions at the periphery as well as in central sites.

An attractive hypothesis is that the difference between immunophysiology and immunopathology should be regarded in evolutionary terms. In this hypothesis, a unicellular organism will respond to an exogenous stimulus (such as cell-cell contact) with a physiological reaction (such as repulsion and phagocytosis). As soon as multicellularity arises, there must be some mechanism to recognize the second cell as self, and hence not a potential target – but this mechanism must make use of the existing recognition pathways to generate such a 'nonresponse'. As soon as this nonresponse to self condition has been achieved, the organism has created for itself a new homeostatic status, which can be maintained and regulated as the molecules concerned turn over and are replaced, but which becomes the physiological immune system for that particular organism.

Subsequently, added on to this very basic recognition mechanism, as the immune system has evolved, we have acquired overlays, so that in mammals there is a complex pattern of genes which have the potential to rearrange and encode for proteins specific for target antigens. If the target is a self product, then these genes may rearrange to encode for the proteins that are involved in positive and negative selection in the thymus, as examples of immunophysiologic reactions. If, on the other hand, the target is extrinsic, then the rearrangement is not immunophysiologic.

In this context, the major type of immunopathology is clearly breach of tolerance to self. Although

this is the most obvious and well-established example, it is not difficult to envisage how other instances might arise. For example, most cytokines have been identified for their role in response to injury and in pathological processes. Presumably, these roles represent immunopathology, and the mediators do have a physiological homeostatic role which has thus far been much more difficult to define.

The use of immunological techniques in the study of disease

Traditionally, the basis for this 'investigative immunopathology' is serology. Detection of antibody against a specific pathogen, and of rising (or falling) titers of antibody, has long been the mainstay of the approach. The concept of autoimmunity is heavily based upon the identification of circulating antibodies directed against intrinsic antigens, and there is also reciprocal serology, based upon the use of antisera to detect a given antigen.

There are now many ways to detect and amplify the signal given in serology-based assays. The use of complement fixation to detect the formation of complexes was an early example; and there are now a host of other similar procedures, many of which rely on a second superimposed antigen-antibody reaction, or upon a combination of antibody with biotin, which can then be identified using an avidin-coupled detection system. Enzyme-linked antibody in ELISA assays has been used either alone or in conjunction with biotin-avidin and has proven a useful and efficient alternative to isotope. The most significant development in serology-based studies has undoubtedly been the advent of monoclonal antibodies, which have made a considerable impact upon the specificity that can be achieved.

In a sense, most of the other techniques that are used in investigative immunopathology are extensions of the serological approach. These methods have been used to purify proteins by affinity, and have also been used in more sensitive detection systems such as western blotting. Furthermore, serology is also the basis for studies that are orientated toward cells and tissues. Thus antibodies are used as the detection system for different cell types, both for the immune cells themselves (such as the lymphocyte subsets) and for other cells in epithelial and connective tissues.

Immunofluorescence, where an antibody is coupled to a fluorescent dye can be used in several different ways: for fluorocytometry (including preparative sorting), for detection of antibodies directed against cellular targets in autoimmunity, and for

both detection and isotyping of immune complex deposits. In histological examination of tissues, immunostaining methods, where the detection is via either peroxidase or alkaline phosphatase, have formed the basis for re-evaluation of previous tumor classification, and have made quantitative analysis possible. There are difficulties in this approach, in particular those related to the preservation of the antigen (in terms of accessibility, structure and configuration) under fixation conditions; but there are also unique advantages, since immunopathologic methods can be used in this context not only as cell detectors, but also to monitor cell function, where the antibody may be directed against cell cycle-related proteins, or against growth factors and their receptors.

Recently, immunopathology has also provided powerful adjunct techniques for molecular pathology, where the *in vitro* translation products of specific genes have been characterized using monoclonal antibodies; and, conversely, expression vector libraries have been generated which can be used to screen for identification of the gene which encodes for a particular protein.

However, investigative immunopathology is not confined to the study of antibody. Analysis of the complement components *per se* may also be used to monitor disease, either involving the complement system itself or as a marker of complex formation. Quantitative cellular studies are useful, and thus far have proven more reproducible than any of the functional immunopathological assays (such as phagocytosis, chemotaxis, natural killer activity, cytotoxicity, and alloresponses) that have been used as research tools to examine disease. Strictly, blood group serology and HLA typing are not classifiable as immunopathologic methods, since they document physiological markers rather than disease states, but both have been used to determine disease associations.

This armamentarium of procedures has provided the opportunity for investigating disease in many different ways, some of which have been mentioned already. It is probably true today that even if at a superficial level there is no obvious connection between a particular disease process and the immune system, in every instance there will be at least one investigative procedure which falls within the remit of immunopathological analysis.

Immunological mechanisms as pathologic processes

The assumption is generally made that the immune response is protective. To appreciate the concept of

immunologic mechanisms as pathologic processes, the paradigm of the immune system as a beneficial event has to be shifted completely.

There are some situations where the actual lesion is a relatively localized interaction between cell-cell and cell-organism. Perhaps the classic example is that of the most commonly recognized form of *Mycobacterium tuberculosis* infection, where a relatively minute area with only one or two bacilli is identifiable in the tissue lesions. However, it is the tissue response to these localized interactions that is so extensive and is what is in fact recognized as the disease. To continue the example: the extensive granuloma formation is itself what is destructive and spreading into surrounding tissues, and it is this reaction that is recognized as the immunopathology.

In these instances, where the disease itself can be regarded as immunopathologic, the initiating agent may no longer be identified, but nonetheless the ongoing processes are frequently destructive and may be life-threatening. Perhaps the hallmark of such processes is in fact that they readily become self-perpetuating, i.e. the immunologic cascade has been initiated and continues to generate further tissue damage way beyond the original insult.

Immunopathology in this context is thus very closely linked to the classic four patterns of tissue injury (hypersensitivity reactions) that have been described in the literature. Like these four reactions, there are often extrinsic antigens which cause the disease process, but autologous antigens often play either a primary or perpetuating role.

Although type I anaphylactic reactions have their own distinctive features, they are rapidly developing immunologic reactions which occur in minutes and long-standing changes such as tissue destruction are usually not a feature. These are the forms of tissue injury where IgE synthesis is the key component, and the immunopathologic process is recognized when there is perpetuation of the process with basophils and mast cells predominating.

In many cytotoxic reactions, such as transfusion and hemolysis, the clinical emphasis is usually placed upon the circulating components, but the immunopathologic event itself is the synthesis of antibody which destroys a self component present on the surface of the target cell. This component may be either autologous or extrinsic, such as when drugs are coupled to red cells. The immunologic events which follow include complement activation and target cell lysis.

Variants of the cytotoxic form of immunopathology include where the antibody is against a receptor, blocking ligand-receptor interaction. This may have the same consequences as lysis of target (i.e.

nonfunction), even though the receptor-bearing cell remains alive. Perhaps the best characterized example of this type of immunopathologic event is the synthesis of antibodies to the acetylcholine receptor, which is the cause of impaired neuromuscular transmission and hence muscle weakness in myasthenia gravis.

The other well-established variant is when antibodies are directed against connective tissue components such as basement membrane. The disease which is perhaps the best-known example of an immunopathologic process, where the primary injury is not known but the mechanism is immunologically mediated, is Goodpasture's syndrome. Antibasement membrane antibodies bind directly and block function to give linear glomerular deposits and also involve the alveolar wall interface with lung capillaries. That the lesion is immunopathologic has been confirmed by remission induction using plasmapheresis.

Immune complex formation leads to several different types of immunopathology. At a basic level, the question is really not why immune complexes constitute such an important component in immunopathologic processes, but rather what are the factors that make these particular immune complex patterns pathologic, whereas most immune complex formation is by definition a successful form of host defense.

Immune complex formation can be localized (as in an Arthus reaction) or systemic. The tissue hallmark is commonly a vasculitis, and this vasculitis may occur in many different sites: arthritis, endocarditis, glomerulonephritis and alveolitis are all possible. The nature of the complex varies, depending on whether the antigen is endogenous or exogenous, and on whether the immunopathologic event is the formation of a complex *per se*, as in lupus erythematosus, or of a complex of antigen, antibody and anti-antibody, as seen in rheumatoid arthritis.

The immunopathogenetic mechanism in immune complex disease starts as an acute local inflammatory reaction in response to the presence of the complex, but the key event is probably complement activation and fixation. Serum complement levels fall due to 'consumption' by complexes and neutrophils are attracted to the site. The natural history of the disease varies, but is again commonly persistent and chronic.

The granulomatous form of immunopathology has already been mentioned. These cell-mediated events are often a reflection of a classic delayed-type hypersensitivity and are the most obvious example of how a response can itself become the disease. Leprosy, with destructive paucibacillary disease, may be even more dramatic than tuberculosis, with nerve damage

due to the aggressive nature of the immunopathology. There is a cascade effect in which lymphocytes play a role. Necrosis occurs as a frequent concomitant of granulomas, and is a key immunopathologic change, since by definition it is a destructive rather than reparative event.

Recent developments in immunopathology

Many of the earlier descriptive reports about immunopathology anteceded the advent of recombinant DNA technology. As in most other areas of immunology, these methods now allow for a different approach to earlier questions in this field.

One change is that with the availability of recombinant cytokines, the role of these molecules has been recognized increasingly as mediators of immunopathologic events. For example, circulating recombinant tumor necrosis factor can cause widespread tissue damage; and local injection of recombinant interferon γ modifies the immunopathology of cutaneous leishmaniasis. Another is that the role of T cell immunopathology – cytotoxic events as a cause of damage rather than elimination of infected cells – has been increasingly recognized. Likewise, failure to induce a response (i.e. failure at the level of presentation) has been identified as a more significant form of immunopathology than was previously appreciated, based upon the experience with the acquired immune deficiency syndrome (AIDS).

Most recently, there has also been increasing awareness that the immunopathologic processes implicated in a particular disease may not involve a particular gene that encodes for a particular product, such as immunoglobulin. Rather, there may be dysregulation at the molecular level, via a controlling enhancer or suppressor sequence. It is from such concepts that the molecular immunopathology of the future is likely to develop.

See also: **Autoimmunity; Cytokines; Granuloma;**

Hypersensitivity reactions; Immune complexes; Mycobacteria, infection and immunity; Tolerance, peripheral.

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IMMUNOPOTENTIATION

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Immunopotential can be defined as a process that directly enhances one or more specific immune functions, or modifies one or more components of the complex immunoregulatory network to achieve its effects through indirect mechanisms. An enormous array of crude biological extracts, plant and animal

substances of varying degrees of purity, synthetic chemicals, and now cloned cytokines and highly purified immunoglobulins have been used as immunopotentiating agents. The subject includes both immunotherapy, directed at pathogenic microorganisms, malignant cells, immunodeficiency, and the immuno-

regulatory disturbances of autoimmune disease and the enhancement of immune responses during immunization, or the use of immunological adjuvants. There is a substantial overlap between the types of substances used for both these purposes and there may well be a parallel overlap in the mechanisms involved.

The idea of stimulating the immune system to tip the balance of the struggle between an invading pathogen or malignant cell and host is almost as old as ideas that have evolved this century about the components and function of the system itself. In 1902, a series of patients were vaccinated with a cancer cell extract by von Heyden and Blumenthal. The use of adjuvants has a long history too, with Pasteur and Joubert noting in 1877 that mixing additional substances with antigen intensified the immune response. Despite this long history, the mechanisms whereby the bulk of immunopotentiators mediate their action is not clear.

Unfortunately, it is pertinent to ask why there has been so little to show in real terms from the enormous effort put into examining the mechanisms and efficacy of immunopotentiating substances. In part, this is due to the complexity of the system that is being manipulated, but much of the lack of success may be due to an overoptimistic appraisal of the role the immune system plays in neoplasia and consequent unrealistic expectations about the potential for immunotherapy of cancer. Infectious diseases, many of them of profound importance in the developing world, may have much greater potential for immunorestorative therapy than cancer. In contrast to cancer, it is fairly clear that the immune system recognizes pathogenic organisms, even if in many cases it alone proves insufficient to rid the host of the invader. Autoimmune diseases at least represent one area where there has been some progress.

Classification of immunopotentialiation

It is possible to classify immunopotentialiation in a number of ways, and one such is shown in Table 1. There is some overlap between the different categories used and the classification should be viewed as a framework within which to base discussion. In addition, combinations of specific and nonspecific mechanisms are used, such as the addition of adjuvants to specific antigens in many vaccination protocols.

Specific mechanisms

Active specific immunopotentialiation

Vaccination Prophylactic vaccination against infectious diseases remains the single most clear-cut

Table 1 Classification of immunopotentiating procedures

<i>Specific mechanisms</i>	
Active	Vaccination
Passive	Xenogenic antibody therapy Hyperimmune globulin
Adoptive	Activated (sensitized) lymphocytes – allogeneic, xenogeneic or autologous Tumor-infiltrating lymphocytes (TIL) Lymphocyte extracts – transfer factor, immune RNA
<i>Nonspecific mechanisms</i>	
Active	Immunopotentiators of biological origin Thymic extracts and synthetic analogs Cytokines Synthetic chemicals
Passive	Immunoglobulin therapy (immune serum globulin, ISG) Plasmapheresis
Adoptive	Lymphokine-activated killer (LAK) cells

success of immunopotentialiation in the practical application of immunological principles to the improvement of human and animal health. Specific vaccination has also been used to treat a restricted number of chronic infectious diseases whose response to antimicrobial chemotherapy remains suboptimal. Two examples of this are the use of vaccination with killed *Leishmania* promastigotes and live BCG as an adjuvant in the therapy of localized cutaneous leishmaniasis. A randomized trial has shown that this regimen compares very favorably to standard antimonial chemotherapy in terms of efficacy, side-effects, and cost. A similar vaccination protocol in leprosy, involving the use of killed *Mycobacterium leprae* and live BCG as an adjuvant has resulted in conversion of 85% of borderline leprosy and 65% of polar lepromatous leprosy to the tuberculoid end of the disease spectrum. These clinical observations have been accompanied by histopathological and immunological changes and, it is hoped, the patients treated in this manner will become more responsive to antimicrobial agents. Such an approach may well be applicable to other chronic infectious diseases where cell-mediated immunity is critical for resistance.

These definitive and very encouraging results have not been accompanied by similar findings from a large number of therapeutic vaccination trials with alleged tumor antigens and a variety of adjuvants in malignant disease. Probably because there is little evidence for immunogenetic tumor-associated antigens in most human neoplasias, minimal practical success has been achieved with this approach. However, in veterinary practice it has proved possible to treat certain virus-induced tumors, such as bovine

fibropapilloma, by vaccination. Powerful effects of peptide immunization have been observed in tumor models, and pilot studies in human melanoma are underway. The presumed mechanism is the selective activation by vaccination of CD8 cytotoxic T lymphocytes (CTL).

Vaccination against recurrent miscarriage One specific form of active vaccination worthy of mention is the use of paternal or mixed allogeneic white blood cells or peripheral blood mononuclear leukocytes (PBMLs) in the treatment of what is termed 'allo-immune' pregnancy failure. It is believed that some women fail to make the normal immune recognition of paternal antigens to which bearing a fetus exposes them. This failure of recognition in turn results in a failure to develop specific protective mechanisms and the fetus is lost. The ill-defined deficiency can be overcome by prior immunization with paternal or mixed allogeneic cells, or some other source of alloantigens. The procedure is still one of some controversy, although efficacy has been demonstrated in a meta-analysis. The mechanism whereby a pregnancy survives what should be a hostile immune environment is still not understood.

Passive specific immunopotentialiation

Xenogeneic antibody therapy The administration of antibodies prepared by immunizing other species, initially large animals, but more recently rats and mice, combined with immortalization of the antibody-forming cells to produce monoclonal antibodies (mAbs), have proved/or promises to be an important part of immunopotentialiation therapy. Thus diphtheria and tetanus antitoxins prepared in horses or cattle were successful adjuncts to standard therapy in the early decades of this century. This approach has, of course, been replaced by widespread vaccination of the population and the preparation of human hyperimmune serum in the case of tetanus. Xenogeneic antibodies prepared in similar ways are widely used for the treatment of envenomation from a variety of poisonous species, especially snakes and spiders.

More recently, attempts have been made to use mAbs as therapeutic agents. Some success has been achieved with mAbs directed against lymphocyte determinants found on a variety of leukemias or lymphomas, although long lasting control has not been achieved. mAbs that react with certain infectious agents such as cytomegalovirus (CMV) have also been prepared, although their role remains to be defined. The major drawback to the use of xenoantisera is the development of anti-immunoglobulins in the recipient. Such antibodies may be associated with

serum sickness which precludes further therapy. In addition, although the side-effects with mAbs seem to be less severe, the antimouse/rat antibodies rapidly neutralize the potentially therapeutic mAb, curtailing the value of therapy. The inability to use repeated doses of antibody is thus the major limitation of this therapy. This is generally not a problem with antivenins but has so interfered with mAb therapy for tumors and graft rejection that it has led to attempts to make human mAbs and the successful 'humanization' of rodent mAbs by genetic engineering techniques. The latter process has given rise to a series of potentially useful therapeutic agents directed against important surface molecules such as CD4 and CDw52, and against cytokines such as TNF α . The ultimate aim of CD4 antibodies is eventually to induce tolerance, successful to date in rodent models, but not in humans. Anti-TNF α has proved a useful adjunct to the treatment of refractory rheumatoid arthritis (RA) and its use in combination with other approaches is being explored. The use of mAbs in this way is really a form of immunosuppression rather than immunopotentialiation but has been included here for completeness.

Hyperimmune globulin therapy For a restricted number of infectious diseases, we are fortunate enough to have high-titer antibodies prepared from pooled human blood collections. Such immune serum globulins (ISG) include those with specificity for hepatitis B, vaccinia, varicella, tetanus and rabies. The antibodies are logistically difficult to obtain, expensive to prepare and maintain, and may in part be replaced by intravenous immunoglobulins that are now more widely available with better characterized antibody specificity. Not all of these specific antibodies are readily available in all countries. Anti-D postexposure prophylaxis represents a special case of successful passive specific immunization. To prevent the occurrence of rhesus (Rh) antigen-associated hemolytic disease of the newborn, Rh-negative women who deliver an Rh-positive infant or who have a spontaneous or induced abortion receive anti-D immunoglobulin. The mechanism of inhibition of antibody synthesis is not clear, but rapid clearance and destruction of Rh-positive cells from the circulation seems to play a role.

Adoptive specific immunopotentialiation

Lymphocytes A number of experimental models exist that allow the transfer of 'sensitized' or activated lymphocytes which then have a therapeutic effect against tumors with shared antigens. Similar approaches have been tried in humans generally without consistent success. The development of

cloned interleukin 2 (IL-2) has allowed expansion and activation of cells from the peripheral blood into lymphocyte-activated killer (LAK) cells. There is no evidence for *antigenic* specificity and this mechanism will be considered below. However, there is a variant of this procedure involving the expansion of tumor infiltrating lymphocytes (TILs). It is possible to expand a small number of lymphocytes up to 10^{11} cells. Their specificity, if indeed they are specific, is unknown, and early clinical studies in both renal carcinoma and melanoma have not been as promising as was hoped. Lymphocytes from regional nodes are also being used as a source of 'specific' CTL for adoptive immunotherapy. This type of therapy has yet to be tried in situations other than neoplasia.

Transfer factor In the mid-1950s, Lawrence first reported that delayed-type hypersensitivity (DTH) could be passively transferred using dialyzable extracts of leukocytes obtained from skin test-positive, normal human donors to previously skin test-negative normal human recipients. Since then, transfer factor has been widely used, particularly in infectious diseases in which cell-mediated immune responses have been felt to be defective, such as chronic mucocutaneous candidiasis, vaccinia gangrenosum, and some mycobacterial infections. Despite this, it has not yet been completely characterized and the question of its efficacy and specificity remains. It is thought to be a mixture of purine- and pyrimidine-containing polynucleotides with some polypeptide moieties. How these proposed structures carry antigenic specificity is unclear. A very limited number of controlled trials support its efficacy, but these remain largely unconfirmed and its ongoing therapeutic role is questionable now that other low molecular weight potentiators like the cytokines are available in purified form.

Nonspecific mechanisms

Active nonspecific immunopotentialiation

Immunomodulators of biological origin A summary of the different categories of substances that have been used is shown in Table 2. This represents a small sample of a vast array, with an attempt made to select the more promising. In general terms, these agents have been used both as adjuvants in vaccination procedures and as immunotherapeutic tools for neoplasms and to a lesser extent infectious disease. Little is known about the specific mechanism(s) by which these compounds exert their effect.

Many studies tend to describe one or more effector cell read-outs which shed little light on the cell inter-

Table 2 Immunopotentiators of biological origin

<i>Mycobacteria and derivatives</i>
BCG
Complete/incomplete Freund's adjuvant
Methanol-extractable residue (MER) of BCG
Muramyl dipeptides
<i>Bacterial lipopolysaccharide (LPS) endotoxins</i>
Monophosphoryl lipid A
Lipid A analogs
<i>Fungal glycans</i>
Glucan
Lentinan
Krestin
<i>Polymeric microbially derived agents</i>
Polynucleotides
Maleic vinyl ether
<i>Miscellaneous</i>
<i>Corynebacterium parvum</i>
<i>Bordetella pertussis</i>
'Picibanil' – extract of <i>Streptococcus pyogenes</i>
'Biotin' – extract of <i>Klebsiella pneumoniae</i>
'Broncho-Vaxom' – extract of eight strains of bacteria

action involved. Some agents, such as those derived from lipopolysaccharide (LPS), seem to bind to CD14, an activation ligand on the surface of macrophages. Fungal glycans activate macrophages and induce the release of interleukin 1 (IL-1), tumor necrosis factor α (TNF α) and colony-stimulating factors. Mycobacterial-derived products probably have similar actions and the polymeric microbially-derived agents are interferon inducers. These agents almost all certainly have multiple effects and an attractive unifying concept is that their cellular effects may depend on their capacity to elicit the production and secretion of cytokines (e.g. IL-1, IL-2, TNF α and interferon γ) which are the direct cellular regulatory factors. The differences between the actions of various stimulatory biological products may therefore be related to minor variations in the production of different cytokines. Adjuvants including mineral oil or mineral salts have the additional effect of holding specific antigen within the vaccine in a depot to encourage slow release over a prolonged period resulting in a more sustained immune response.

These substances have primarily been used as immunotherapy for a wide variety of neoplasms in animals and in humans. Such therapy has been combined with proposed tumor-specific antigens or made part of a multimodality approach to cancer including surgery, chemotherapy and radiotherapy. Although there have been occasional reports that deserve the accompanying enthusiasm, in general the use of immunomodulators of biological origin in human cancer has proved disappointing. Currently local

BCG is used as adjunctive therapy in bladder cancer and a variety of products are used in gastric cancer, particularly in Japan. Biostin and Broncho-Vaxom are used in chronic bacterial infections. Indeed, although much of the emphasis to date has been on cancer therapy, the place of these agents may well be as safe adjuncts in chronic infectious diseases.

Following the observation that type 1 diabetes-prone NOD (non-obese diabetic) mice raised in non-specific pathogen-free (non-SPF) conditions had a lower incidence of diabetes, NOD mice were exposed to BCG and again the incidence of autoimmunity was diminished. Although this is not a general phenomenon in autoimmune disease, these findings have led to BCG trials in identified high-risk relatives of patients with diabetes with early results pointing to a modest prolongation of the 'honeymoon' (i.e. remission) period.

Thymosin and other hormone-like factors extracted from thymus glands There are a large number of compounds that come under this heading, many with quite a substantial body of literature describing their actions. Only those that have been recently used, largely in Europe, are listed below:

- Thyostimulin
- Thymomodulin
- T-activin
- Thym-uvoval.

As well as the extracted substances, there are synthetic 'hormones' such as thymosin α 1, thymulin and thymopentin (TP5). The latter pentapeptide corresponds to amino acid residues 32–36 of thymopoietin 2 and retains the biological activity of the parent compound.

The rationale behind the use of these compounds is to accelerate the maturation of T cells from precursor cells. Most work has aimed at increasing host resistance in cancer, particularly in overcoming the immunosuppressive effects of chemotherapy, radiotherapy, surgery or the tumor itself. Minor improvements in survival have been reported with some of the compounds listed, but no clearly defined role in antineoplastic therapy exists. Thymic hormones, particularly thymosin fraction 5, thymosin α 1 and TP5 have been used in children with primary immunodeficiency diseases such as the DiGeorge syndrome, chronic mucocutaneous candidiasis, the Wiskott–Aldrich syndrome, and other immunodeficiencies associated with thymic hypoplasia or dysplasia. In recent years these substances have been widely used in acquired immune deficiency syndrome (AIDS). There is little reproducible evidence of sustained benefit. These substances have also been used

to treat a number of autoimmune diseases, especially systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). The aim has been to reverse the immune imbalance believed to be associated with the disease process. The results, however, have been disappointing and none of these agents has secured a regular place in therapy. In a sense, this is hardly surprising as the physiological role of these compounds, extracted or synthetic, in normal T cell maturation in the thymus is far from clear.

Cytokines and other biological mediators The ability to produce a wide array of biological mediators by recombinant DNA techniques has allowed an enthusiastic expansion of biological response modifier (BRM) programs. Some of the substances discussed have been used therapeutically for several years, others at this time are little more than interesting compounds to investigate.

Cytokines that have been widely used are shown in Table 3, where they have been grouped into those with clearly defined functions and those in which early study looks promising. A fundamental problem with most cytokines as therapeutic agents stems from the fact that relatively large doses given systemically have many, generally proinflammatory, side-effects. Ultimately, agents derived from cytokines that act more exclusively on particular receptors may be of more use.

Table 3 Therapeutic use of cytokines

<i>Proven or widespread use</i>	
Interferon α	
	Hepatitis C, hepatitis B
	Papilloma virus (local)
	Hairy cell leukemia
	Multiple myeloma – a small response rate and maintenance of plateau phase
	Kaposi's sarcoma with HIV infection and a reasonably preserved CD4 count ($\geq 200/\mu\text{l}$)
	CML in chronic phase
Interferon β	
	Multiple sclerosis
Interferon γ	
	Chronic granulomatous disease
	Visceral leishmaniasis
<i>Early study looks promising</i>	
Interleukin 2	
	Rare cases of severe combined immunodeficiency (SCID) with IL-2 deficiency
	Metastatic renal carcinoma
	Melanoma ?
IL-2 with antivirals in HIV infection	
IFN γ to reduce a T $_H$ 2 response, e.g. severe atopy	
IL-10 – may be useful to enhance a T $_H$ 2 response	
IL-12 – may be useful to enhance a T $_H$ 1 response	

Synthetic chemicals (thymomimetic drugs) To date, the best characterized examples are levamisole, isoprinosine, ditiocarb, methyl inosine monophosphate (MIMP), azimexon and imexon. The mechanisms of action are not very clear but possibly include the induction of thymic hormones, possession of thymic hormone-like properties, and in most cases direct effects on phagocytic and perhaps natural killer (NK) cells.

Levamisole was first used as an immunopotentiating agent in 1971 and is considered to be capable of restoring defective cell-mediated immunity (CMI), but does not affect normal CMI. Lymphocytes, granulocytes and macrophages are all stimulated. Levamisole has been tried in a wide array of disease states, ranging from cancer and infections to autoimmune diseases. Overall, the effects have been disappointing and the side-effects considerable. It may find a substantive role in the treatment of large bowel cancer in conjunction with cytotoxic drugs.

Isoprinosine is thought to act through a receptor for an inosine-like compound on T cell precursors. *In vivo* it appears to induce the production of more mature T cells and there is some evidence that it can increase the CD4:CD8 ratio in a variety of conditions. It has been demonstrated to ameliorate a number of viral infections, particularly acute and recurrent herpes simplex virus, rhinovirus, and appears to be able to arrest the progress of measles-related subacute sclerosing panencephalitis. It has the advantage over levamisole of very few side-effects. Some data suggest an improvement in indices of CMI in patients with early HIV infection. Whether this is accompanied by any useful clinical effects is not clear. Overall, the effects are not very impressive, although it has achieved licensing for some of the above in many countries.

The other agents have largely been spawned from desperate attempts to find compounds that will impede the progressive immunodeficiency of HIV infection. To date, little success has been reported.

Passive nonspecific immunopotentialiation

Immunoglobulin therapy Although specific immune serum globulins (ISG) (as described above) and polyvalent ISG have been available for several decades, it was only with the purification of immunoglobulin G (IgG) in such a way that high-quality preparations suitable for larger-dose intravenous infusion were obtained that manipulation of the immune system with these agents became realistic. An important use remains antibody replacement in individuals with primary hypogammaglobulinemia. Intravenous immunoglobulins have also been used as an adjunct to standard antimicrobial therapy in severe bacterial

infections. At first sight, it would seem clear that this should be a useful maneuver, but in fact the data are far from clear-cut and will really only be settled by randomized controlled trials. High-dose polyvalent ISG has been used successfully for prophylaxis of cytomegalovirus (CMV) and to a lesser extent varicella zoster virus in immunocompromized patients.

The most interesting new role for high-dose polyvalent ISG has been in a variety of autoimmune diseases where perhaps there is an immunoregulatory disturbance corrected by the immunoglobulins. The mode of action is not well understood; if elucidated, it will undoubtedly expand our knowledge of the complex interactions of antibodies, antibody synthesis, immune complexes and the mononuclear phagocytic system.

Immune thrombocytopenic purpura (ITP) In children this disease tends to be acute and self-limiting, conventionally managed by expectant observation or if more severe by corticosteroids. High-dose ISG, $0.4 \text{ g kg}^{-1} \text{ day}^{-1}$ for 5 days has proved as efficacious as prednisone. The disease tends to be chronic and relapsing in adults. Again, the thrombocytopenia can be corrected with similar doses of ISG, although regular maintenance doses at weekly to monthly intervals are often required. ISG therapy is useful for preparing a patient for splenectomy, but its role in long-term management remains less convincing.

The success of ISG in ITP has prompted its use in several other immunologically mediated thrombocytopenias. Postinfectious, alloimmune and neonatal reactions have responded to therapy. Occasionally, the latter have been prevented by maternal infusion late in pregnancy, although this approach cannot be relied on, irrespective of the effect on the maternal platelet count.

Other diseases Intravenous ISG is standard therapy in Kawasaki's disease, has been trialled in autoimmune neutropenia and hemolytic anemia with varying success and has been successfully used to downregulate the production of factor VIII-specific antibodies in individuals with hemophilia A. Its applications are now being extended to other conditions such as myasthenia gravis, chronic relapsing polyneuropathy, recurrent pregnancy loss and bullous pemphigoid.

A number of mechanisms have been proposed for ISG in these different conditions. All may play a variable role in different circumstances, the effect varying with time and the diagnosis. Postulated mechanisms include:

- blockade of the Fc receptors on the mononuclear phagocyte system and platelets;
- downregulation of autoantibody production by anti-idiotypic antibodies present in ISG;
- removal of exogenous antigen that initiated the disease; and
- interference with the pathogenic role of antibody, immune complexes or both.

Plasmapheresis and staphylococcal protein A column treatment of plasma These two modalities have been used to remove putative blocking factors that inhibit an otherwise effective cellular immune system from dealing with malignant disease. In fact, the evidence for the existence of such factors is wanting in human neoplastic disease. Consequently, it is hardly surprising that plasmapheresis has no immunopotentiating role in malignant disease.

Removal of 'blocking antibodies' on protein A-coated columns has shown quite impressive results in some animal models, but these successes have not been transferable to the clinic.

Adoptive nonspecific immunopotentialiation

Most nonspecific immunopotentiating maneuvers have proved disappointing in the control of human neoplastic disease. With some exceptions, this also includes the *in vitro* expansion of autologous lymphocytes from cancer-bearing patients with IL-2 and their adoptive transfer back into the patient, usually with *in vivo* IL-2 as a way of further potentiating their activation and proliferation. This method was pioneered by Rosenberg and colleagues at the National Institutes of Health (NIH) in the 1980s. The protocols used are quite complex, with patients being infused with IL-2, rested, leukapheresed, and their cells returned to them over several days after *in vitro* expansion. The toxicity is considerable, due largely to a capillary leak syndrome induced by IL-2 and results in marked fluid retention, cardiac and renal failure, respiratory insufficiency, hypotension, and an increased rate of infections. Up to 4% of patients in these studies died due to these toxic effects. Immunosuppressive therapy can reduce this, although the latter then appears to interfere with the therapeutic effect of the procedure. Some workers have claimed that infusions of IL-2 alone are just as useful.

The nature of the cells involved as LAK is uncertain. The cells exhibit potent non-major histocompatibility complex (MHC)-restricted cytotoxicity against a wide range of targets including NK cell-resistant tumor lines. Normal parenchymal cells of the same type as the tumors tend to be resistant to LAK activity. A heterogeneous population of cytotoxic cells has been found, including an NK CD3⁻ cell,

which is probably the major cell phenotype, and also a CD3⁺ cell which in culture loses its MHC restriction and kills promiscuously.

Despite the attraction of LAK therapy, which is based on sound theoretical grounds, it remains disappointing. Results in other hands have not been as effective as those obtained originally at the NIH and the modest overall rate of complete (8%) and partial (14%) responses mainly against renal carcinoma and melanoma has to be balanced against the expense involved and the complication rate. More needs to be known about the duration of remissions, for if they are not sustained then the procedure represents an enormous potential diversion of health resources for limited overall ends.

See also: Adjuvants; Antibodies, bispecific; Bacterial cell walls; BCG; *Bordetella*, infection and immunity; Coryneform bacteria, infection and immunity; Cytokines; Gammaglobulin; Humanized antibodies; Immunotherapy of autoimmune diseases; Immunotherapy of tumors; Interferon α ; Interleukin 2; Lymphokine-activated killer (LAK) cells; Monoclonal antibodies (mAbs); Muramyl dipeptide; Plasmapheresis; Thymic hormones and peptides; Toxins; Transfer factors; Vaccines; Venoms.

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IMMUNOSUPPRESSION

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Regulation of the immune response can be influenced in two ways. The enhancement of the immune response is called *immunopotential* and its down-regulation *immunosuppression*. The early history of medical immunology is dominated by deliberate attempts to induce resistance to infectious agents by means of eliciting an immune response and by enhancing the immunological defense mechanisms. Vaccination or immunization with an antigen preparation can specifically protect the body against microorganisms such as smallpox, rabies or poliomyelitis. More recently, increasing awareness that several pathological conditions are caused by undesirable reactions of the immune system has led to attempts to reduce the immune response. Thus, immunosuppression is needed in transplantation to prevent the rejection of a transplanted and histoincompatible but life-saving organ as well as to induce remission and to prevent relapse in autoimmune diseases.

The advent, around 1900, of the feasibility of anastomosing vessels opened to the surgeons a wide vista into the field of transplantation surgery. The transplantation of organs such as kidneys was experimentally performed at that time by pioneers such as A. Carel in France and E. Ullman in Austria, but all attempts met with failure due to the invincible rejection phenomenon. It was Medawar who, in 1944, described the immunological basis for rejection of foreign tissues. Soon after, cell-mediated immunity and immunological tolerance were discovered by Billingham, Brent and Medawar. The investigation of the human leukocyte antigen (HLA) system since 1958, initially by Dausset and coworkers, has made a major contribution to improving organ transplantation. Finally, in the late 1950s, it was further realized that immunosuppressive methods were useful in controlling rejection.

If the immune response, as originally conceived, was to defend the individual against any foreign antigen, it should remain fully tolerant to self components. However, for various reasons, a breakdown in tolerance can occur and lead to more or less specific immune attacks directed against defined cells or organs of the host, thus inducing an autoimmune disease. The use of immunosuppressive agents has proven of therapeutic value in many such diseases, e.g. uveitis, myasthenia gravis, juvenile diabetes, psoriasis, primary biliary cirrhosis, ulcerative colitis,

systemic lupus erythematosus, nephrotic syndrome and rheumatoid arthritis.

The goal of selective immunosuppression

If any foreign material (antigen) makes its way into the body – virus, bacteria, fungi or parasites – a swift and complex immune response for destroying it is set into motion. The allelic products of the major histocompatibility complex (MHC) characterize the immunological identity of each individual. With the exception of identical twins, it is extremely unlikely that two individuals match exactly. Nature did not intend for organs to be transplanted from one individual to another. The speed and severity of rejection of a transplanted (foreign) organ is partly linked to the degree of incompatibility of MHC (HLA) antigens between donor and recipient.

Whether the antigenic trigger is a foreign microorganism, a histoincompatible transplant, or a self component to which self tolerance has been broken, the same humoral or cell-mediated immune response is set in motion. Besides the antigen-presenting cell, three main classes of lymphocytes exist to initiate (helper T cells), effect (cytotoxic T cells and antibody-producing B cells), and shut off (suppressor T cells) the immune response. In addition, natural killer cells recognize cell surface antigens in a non-MHC restricted fashion. Antibodies and killer or cytotoxic T cells are mobilized to destroy all target cells bearing the offending antigen. In the case of infection this would be a microorganism, but in the case of a transplant it is the vital life-saving organ which becomes the target of the immunological attack. In the case of *autoimmunity* it will be the cells or organs bearing the self antigen which has escaped self tolerance.

The goal of selective immunosuppression is to maintain the body's defense mechanism against viruses, bacteria, fungi, and parasites, to preserve its capacity to phagocytose necrotic cells and other debris, and finally to protect the mechanism of *immune surveillance* which eliminates malignant cells. This is outlined in **Figure 1**. On the other hand, one would like to induce antigen-specific tolerance for the transplanted organ such as kidney, liver and heart. In the case of autoimmune diseases, one would like to selectively inhibit the autoaggressive response against, for example, the retina in uveitis, the colon

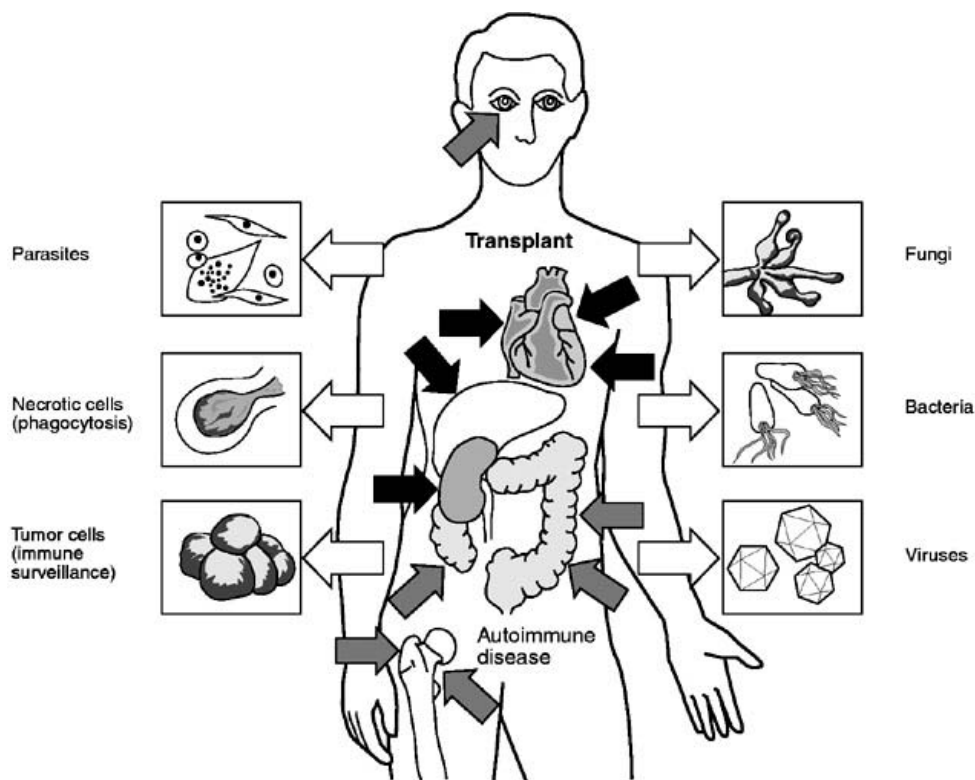


Figure 1 Goals of selective immunosuppression: 1) to maintain defense mechanism against foreign (viruses, bacteria, fungi, parasites) and internal (necrotic and tumor cells) invaders (white arrows); 2) to selectively suppress host reaction against its organ grafts (black arrows), i.e. in transplantation (kidney, heart, liver); and 3) to selectively suppress the pathological immune attack directed against self target organs (retina in uveitis, islet cells in diabetes, joints in rheumatoid arthritis, etc.), i.e. in autoimmune diseases (stippled arrows).

in Crohn's disease or the joints in rheumatoid arthritis.

Although a vast range of different compounds have been reported to be capable of depressing the immune response, it has been particularly disappointing that so few of these have shown potential as immunosuppressive agents in humans. The action of many has been to suppress lymphocyte functions *in vitro* but, of those that were capable of prolonging allograft survival or of suppressing autoimmune symptoms in animals, very few have been found to be safe and effective in humans. Therefore, for a drug to be of practical use in suppressing immunological responses, it should fulfill a number of basic requirements. These are tentatively summarized as follows:

1. Its major pharmacological impact should result in the depression or suppression of the immune response in relevant *in vitro* tests as well as in several animal models.
2. The drug should be able to prevent the onset of an immune response and also be effective against immune processes once they have already developed.
3. Its mechanism of action should selectively induce *clonal deletion* or inactivation of immunocompetent cells or preferably only certain subsets and inhibit some of their functions (anergy).
4. There should be a biologically meaningful therapeutic index, i.e. safety margin between the therapeutic and the toxic dose. This index has to be determined in several species.
5. The treatment protocol should not endanger the innate and already acquired immunity against opportunistic infections.
6. Thus, the drug should only be administered at full dose for a limited period. After this critical phase, dose reduction and eventually discontinuation of the drug should become feasible.
7. The ideal immunosuppressant should, therefore, help to induce tolerance, especially in transplantation. In other words, it should allow the immune system to adapt to the foreign antigens of the transplant by progressively recognizing them as part of self. (However, the induction of tolerance in man has not yet been fulfilled.)

Development of immunosuppressive therapy

With the advent of organ transplantation, immunosuppression became a pressing problem. Most of the surgical problems of organ allografting had been solved long before it was understood how to protect the transplant from its inevitable rejection. Donor-specific or unspecific blood transfusions before transplantation and tissue matching (HLA) have clearly been shown to be of importance in prolonging graft survival; however, these measures were of value only if integrated into a chemotherapeutic protocol.

The first stage in the development of immunosuppression introduced in the late 1950s and early 1960s consisted of using cytostatic drugs and antimetabolites which were used to control the proliferation of neoplastic cells. It seemed logical to observe their effect on the equally proliferative processes of the immune response. Those cytostatic or cytotoxic agents which proved of some value were alkylating agents such as cyclophosphamide, purine analogs (thiopurines) such as 6-mercaptopurine and azathioprine, folate analogs (antimetabolites) such as methotrexate (or amethopterin), and pyrimidine analogs such as cytosine arabinoside. It can be concluded that the concept of interference at various stages of the immune response, e.g. repression of the formation of precursor cells, destruction or blockade of immunocompetent cells, suppression of proliferation and differentiation of lymphocytes and monocytes by inhibition of the biosynthesis of nucleic acids and proteins, was basically correct. However, the use of nonspecific drugs – that is drugs whose action was not limited to the immunocompetent cells – remained hazardous. This was because they acted by indiscriminately blocking or damaging all cells that happened to be in mitosis, in particular normally functioning cells that are important to the organism's survival (e.g. hematopoiesis). The major drawback to the use of these cytostatic drugs is the high risk of overwhelming infections by organisms, many of which are not normally pathogenic (opportunistic infections). Although graft rejection could be held at bay by the use of these compounds, which interfered nonselectively with the induction or expression of the immune response, the toxic side-effects were usually so severe that the overall results were not considered satisfactory.

The next step was, therefore, the development of lymphocytotoxic drugs or procedures which were mostly restricted to the elimination of the immunocompetent cells, mainly the lymphocytes. This goal could be achieved by the use of very heterogeneous means, such as total lymphoid irradiation, thoracic

duct drainage, splenectomy, thymectomy, anti-lymphocyte serum or globulin, and steroids. Corticosteroids are naturally occurring hormones secreted by the adrenal cortex, of which cortisol (hydrocortisone) is by far the most powerful component known to possess lymphocytolytic activity, particularly with respect to T lymphocytes, to inhibit lymphokine production, and to exert a stabilizing effect on the lysosomal membranes, along with those of other cellular organelles. These effects are dose- or concentration-dependent. Corticosteroids not only intervene at many points of the immune response, such as preventing lymphocyte recirculation and generation of antibody-producing and cytotoxic effector cells, but they also possess a remarkable anti-inflammatory potency. They inhibit neutrophil adherence to the vascular endothelium at an inflammatory site and suppress monocytic functions such as microbicidal activity, monocyte response to lymphokines, and release of monokines. Antilymphocyte serum (ALS) or antithymocyte globulin (ATG) is prepared by the injection of lymphocytes or thymocytes into a xenogeneic species. Human thoracic duct cells or thymocytes can be used as antigens to immunize rabbits and horses; the purified immunoglobulin fraction containing the relevant antithymocyte antibodies is then used in the clinic for intravenous injection. Since regular administration of ATG leads to sensitization against xenogeneic proteins (antibody production directed against the antihuman ATG from the immunized species), this type of adjunctive treatment is usually given short term to overcome a graft rejection crisis or early to prevent sensitization to the allograft.

Immunosuppressive therapy during the 1960s and 1970s consisted of combinations of different agents intended to produce maximal suppression while keeping the side-effects as few as possible. The most common immunosuppressive protocol was the combination of azathioprine and corticosteroids, which significantly improved allograft survival but also created a variety of severe side-effects, especially long term, among which were overwhelming, sometimes fatal infections, direct organ toxicities, slow wound healing, anemia, leukopenia, diabetes, osteoporosis, stunted growth in children, and even malignancies. The average 1 year kidney survival rate from all transplantation centers reached about 50% using this protocol while in outstanding centers the figures reached 80% and higher. Under those conditions, liver transplantation remained an experimental procedure and heart transplantation, which had enjoyed a transient burst of activity in the late 1960s, was abandoned in all but three centers worldwide. In autoimmune diseases, steroids were most commonly

used, usually in increasing doses with time, and severe cases were sometimes treated with azathioprine, cyclophosphamide or methotrexate.

The current or third phase of immunosuppressive therapy is that of immunopharmacology, which is characterized by selective regulation of defined subpopulations of immunocompetent cells. This stage deals with new pathways and is aimed at the development of agents or procedures with selective action on the acquisition of immunologic responsiveness, recognition of immunogenic stimuli by receptor-bearing cells, induction of differentiation and maturation of lymphocytes, cellular interactions, and modulation of effector functions. Cyclosporin (WHO)/cyclosporine (US Adopted Name Council)/cyclosporin (British Approved Name) emerged as the first drug that fulfilled these requirements to some extent and which has proved of permanent clinical value. However, other promising and original attempts need to be mentioned here, such as the new technology of monoclonal antibodies directed toward lymphocyte subsets and their products, and other cytokines as well.

The final stage of immunosuppression will be the induction of antigen-specific depression of allograft reactivity. Classical transplantation tolerance has been induced in a developing immune system, but it is very difficult to achieve in a fully developed immune system. Several experimental approaches have been more or less successfully used in various models, but their practicability for clinical purposes appears still to be unproven.

Retrospectively, the 1980s can be viewed as the cyclosporine era. Although this novel immunosuppressant has triggered important developments in transplantation, autoimmunity and basic immunology, it is by no means the only factor responsible for the numerous advances recently achieved in these fields. Despite the astounding progress of experimental immunology in gaining more insight into the mechanisms controlling an immune response and, therefrom, in learning how to bypass an undesirable immune reaction, it still appears that clinical immunosuppression will continue to rely for this decade on a chemotherapeutic strategy using a subtle combination of more selective as well as better tolerated immunopharmacologically effective drugs. If not yet evident, a revolution is always possible.

Modern trends in immunosuppression

The 1990s have seen the emergence of several new drugs that exhibit marked immunosuppressive activity in clinical trials: tacrolimus (FK506), sirolimus (rapamycin), mizoribine and spergualin. Like

cyclosporine (cyclosporin A), they are all natural products originally isolated from cultures of microorganisms. Cyclosporine, a lipophilic, cyclic peptide, and tacrolimus, a lipophilic, macrocyclic lactone, have very similar mechanisms of action. They both act specifically on lymphocytes by inhibiting gene transcription induced by calcium-dependent pathways, i.e. they block the progression from G_0 to G_1 of the cell cycle. Sirolimus is a macrolide which inhibits the growth factor-dependent pathway of T cell proliferation by blocking the progression from late G_1 to the S phase. Sirolimus acts synergistically with cyclosporine, but not with tacrolimus, in the suppression of T and B cells. The antibiotic mizoribine, a nucleoside analog that inhibits guanosine (GMP) synthesis, and gusperimus (15-deoxyspergualin), a derivative of spergualin that suppresses polyamine biosynthesis, exhibit immunosuppressive activity without lymphocyte selectivity. Mizoribine, in contrast to gusperimus, has also been claimed to induce at least additive effects when used in combination with cyclosporine. Gusperimus has proved particularly effective in suppressing experimental xenograft rejection.

Moreover, mycophenolate mofetil (RS61443), a mycophenolic acid derivative that interferes with *de novo* purine synthesis, has yielded encouraging clinical results when used in combination with cyclosporine. In clinically attainable concentrations both sirolimus and mycophenolic mofetil are known to inhibit smooth muscle cell proliferation in the neointima, a major pathogenic mechanism that causes chronic rejection. In an appropriate combination with cyclosporine they might, therefore, help control chronic rejection in transplanted patients – currently the principal limitation of long-term graft survival.

While significant progress has been made with chemical immunosuppressants, other approaches have concentrated on using immunomodulating biological agents. Polyclonal antibodies present in anti-lymphocyte serum have been replaced with monoclonal antibodies (mAbs) that target selected cell surface antigens. OKT3, a T cell-depleting CD3-specific mAb, is the first mAb to have been introduced successfully in the clinic. Monoclonal antibodies directed against T helper cells (anti-CD4) and the interleukin 2 receptor (anti-CD25) have shown promising results in clinical trials. Another approach is to delete specific cell populations by using immunotoxins. These molecules consist of two fused parts, one being a recognition moiety and the other a toxic moiety (e.g. ricin or diphtheria toxin). In addition to T and B lymphocytes, antigen-presenting and regulatory cells also express characteristic target determinants. For instance, selected peptides have

been synthesized that bind to MHC antigens and thereby compete with antigen presentation.

A further option is to block costimulatory molecules, in particular CD28 and CD40, a strategy that leads to anergy due to loss of second signal activation. Since cytokines play a critical role in the process of an immune reaction, attempts have been made to neutralize them with soluble receptors, as an alternative to anticytokine mAb. Interference with adhesion molecules (selectins, integrins) on activated graft-invading leukocytes and on graft endothelial cells is a further means of intervening to prevent allograft rejection. Treatment with recombinant cytokines may also result in immune deviation. Finally, it is known that the modification of the graft by culture procedures or ultraviolet irradiation can induce prolonged survival, presumably by the removal of the passenger leukocytes (antigen-presenting cells) which play an important role in allograft rejection.

Although many of these widely differing approaches toward selective suppression or modulation of the immune response have yielded reasonably encouraging results under experimental conditions, they are not yet generally applicable to clinical practice. However, several of these procedures are now cautiously being tested in patients receiving organ transplants or suffering from autoimmune diseases. The appropriate combination of some of these chemical drugs and biological agents integrated into a well-synchronized regimen may eventually achieve graft tolerance – at least func-

tional unresponsiveness – or long-term remission in autoimmunity.

See also: Anti-inflammatory (nonsteroidal) drugs; Antilymphocyte serum; Autoimmune diseases; Autoimmunity; Hematopoietic stem cell transplantation; Cyclosporine; Glucocorticoids; Plasmapheresis; Radiation, effects on immune system; Tolerance, peripheral; Transplantation.

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IMMUNOTHERAPY OF ALLERGIC DISEASES

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Allergen immunotherapy represents the dominant immunologic, as opposed to environmental or pharmacologic, approach to management of allergic diseases such as allergic rhinitis, conjunctivitis and asthma. It was developed empirically, coincident with the modern-day discovery of allergy by Portier, Richet and von Pirquet. In essence, it involves identification of the allergen(s) to which a patient exhibits immunoglobulin E (IgE)-dependent sensitivity followed by subcutaneous administration of minute amounts of natural extracts containing these allergens. The goal is lasting modification of immune processes responsible for the maintenance of atopic symptoms. Other, infrequently used, forms of desensitization (i.e. IgE-mediated drug reactions)

operate by different mechanisms and are not reviewed here.

History

Given its beginnings at the turn of the century, the theory underlying allergen desensitization was closely associated with that of vaccination. Allergens, common environmental antigens that provoke allergic responses in some individuals, were seen as conceptually similar to bacterial toxins. It was thought that individuals with undesirable (hypersensitivity) responses to such substances would develop a protective immune response upon exposure to increased doses of allergen, resulting in

a qualitative shift from immediate hypersensitivity to clinical tolerance. Beginning in 1909–11, Noon and others established immunization programs for 'desensitization' that have continued largely unchanged to the present.

The question of efficacy

In insect venom immunotherapy, protection is achieved in well over 90% of subjects following a short (usually several day) course of injections. Efficacy is commonly tested by deliberate administration of an insect sting challenge in the presence of a physician, yielding clear-cut evidence of success or failure. In contrast, the efficacy of immunotherapy for inhalant allergens, used in therapy of allergic rhinitis or allergic asthma, remains controversial. Proponents argue that allergen immunotherapy is highly effective and that the equivocal results are attributable to use of insufficient or poorly prepared allergen extracts, use of inappropriate or insufficiently rigorous criteria for patient selection or unwillingness of patients to complete a sufficiently long course of treatment. They draw support from statistically significant differences that have been observed in a number of parameters measured in randomized, double-blind, placebo-controlled studies. On this basis, the formal position of medical associations in most developed nations is that desensitization with standardized allergenic material in high doses is effective in patients with an allergy to insect stings, allergic rhinitis, conjunctivitis and, in some cases, allergic asthma when patients have been selected based on meticulous history corroborated by positive results of skin tests and for whom avoidance of allergens and drug therapy is not sufficiently effective. The problem is that the degree of success achieved is highly variable and unpredictable on an individual basis. Generally, patients with multiple unrelated sensitivities have more severe or complex disease and do less well. Consequently, enthusiasm for allergen immunotherapy varies widely between different countries and groups of physicians. In most instances, the technique requires several months to years of therapy, hence substantial time and financial commitment with an uncertain probability of success and a very low, but real, risk of anaphylaxis resulting from treatment.

Current practice

Immunotherapy is easy to administer and in most patients is well tolerated, but to attain substantive efficacy strict criteria for patient selection are necessary. A variety of factors must be taken into account

but the first is to establish that the clinically apparent sensitivity is IgE-mediated. As no single objective test provides sufficient information, IgE-mediated disease is generally diagnosed by correlating history, skin tests and often specific IgE levels. Once the clinically relevant antigens have been identified, a course of treatment involving a series of subcutaneous allergen injections is begun. As most patients are polysensitized, this can require coadministration of multiple allergens. Initial injections contain very low amounts of allergen to minimize the intensity of local reactions and risk of systemic anaphylaxis. On each subsequent visit the amount of allergen, which may be aqueous or bound to adjuvants such as aluminum salts, is increased until a maintenance dose, often close to the maximum amount of antigen tolerated, is reached. The patient is then kept under medical supervision for 20–30 min in case of anaphylaxis or acute exacerbation of asthmatic symptoms. Contraindications include diseases of the immune system, conditions complicating management of adverse reactions to immunotherapy (i.e. coronary heart disease, hypertension or treatment with β -adrenergic antagonists, sometimes pregnancy) and patients developing severe reactions during therapy.

Allergic patients commonly vary by more than a thousand-fold range in their degree of allergic sensitivity. It is for this reason that the cumulative dose used in allergic immunotherapy is related to what can be tolerated by the patient. Doses that are too low are safe but have little or no effect relative to placebo. Doses that are too high may be effective but carry an increased risk of systemic reactions. Given the wide variability in the subject population and, until recently, poor standardization of the amount of biologically active allergen present in nominally identical commercial preparations, safe and effective desensitization is generally considered to require specialty training in allergology.

In contrast to other forms of desensitization therapy for common inhalant allergens (dust mite, cat, tree and grass pollens), which typically require 2 to many years of treatment, venom immunotherapy is commonly initiated via 'rush' methods in which rapidly increasing amounts of venom are injected over a period of several days. Regular booster treatments are usually required although recent studies have begun to address how long protection persists in the absence of continued therapy.

In the mid-1980s a cluster of immunotherapy-related fatalities in the UK and studies indicating that desensitization programs in the US had resulted in an average of approximately one fatality a year over 42 years resulted in an IUIS/WHO sponsored re-examination of desensitization practices. Serious

problems were primarily found to occur when therapy was administered by physicians inexperienced in its use and when standardized procedures for recognition and control of systemic reactions were not in place. A series of guidelines were developed that have been widely adopted.

Mechanisms of action

While immunotherapy is generally considered the final medical modality (after allergen avoidance or pharmacologic control of symptoms), and usually requires several years to make its effects seen, its appeal springs from a desire to modify the underlying disease state that is responsible for hypersensitivity. As such it has stimulated much interest by basic and clinical immunologists attempting to identify the mechanism by which it operates. No consensus is currently possible. Our inability to definitively identify a causative relationship linking changes in objectively measurable parameters (previously induction of 'blocking' IgG, currently inhibition or redirection of T_H2 -like cytokine responses to allergen) and clinical improvement continues to be the greatest hindrance impeding efforts to give immunotherapy the success rate of, for instance, immunization programs for tetanus or polio. Based on the status of desensitization following a century of empiricism, substantial progress will be difficult until a mechanistic cause-and-effect relationship is established.

Future developments

A major factor that has impeded accurate clinical diagnosis of allergy, development of immunotherapeutic protocols and basic research in hypersensitivity diseases has been the highly variable, poorly standardized quality of commercial allergen preparations available to clinicians and researchers for most of this century. Until recent years, most were characterized based on the total protein content rather than the amount of specific allergen present. International standardization based on units of biological activity rather than mass has partially dealt with this concern but such materials remain highly heterogeneous. The identification, cloning and expression of recombinant allergens over the last decade is having substantial impact on research, diagnosis and, perhaps in the future, therapy of allergic diseases.

Antigen-specific methods

A number of strategies that may result in substantial improvements in safety and efficacy are on the ho-

rizon (Table 1). Use of ultrapure (recombinant) allergens in classical immunotherapy protocols offers homogeneity, reproducibility and perhaps improved efficacy compared to the complex mixtures that currently dominate clinical practice. Several approaches (i.e. chemical modification of allergens, DNA vaccines) have been proposed that aim to reorient ongoing cytokine response *in vivo* from those of T_H2 -associated bias to T_H1 -dominated patterns. Alternatively, by identifying the immunodominant peptides driving allergen-specific T cell reactivity in humans, it may be possible to functionally delete them from the periphery upon inhalation, injection or oral administration. Each of these strategies focuses on altering the activity of allergen-specific regulatory populations (largely but not exclusively CD4 T cells) responsible for maintaining the allergic state.

A consistent finding from animal and human studies is that abrogating or redirecting established (memory) responses *in vivo* is much more difficult than directing the initial induction of naive cells to a desired response. Consequently, there has been discussion of the potential utility of prophylactic desensitization of children with a high (genetic) probability of developing asthma to common environmental allergens.

Antigen-nonspecific approaches

An independent series of strategies that may prove useful do not seek antigen specificity, circumventing a need for detailed identification of the specific allergens to which any particular patient is sensitive. Collectively, they differ from strategies seeking to inhibit or redirect T cell activation and antibody responses to semipermanent changes in clinical responsiveness

Table 1 Recent developments with potential for desensitization strategies

Use of recombinant allergens in classical desensitization protocols
Identification of immunodominant T cell epitopes and induction of T cell anergy with allergen-derived peptides
Redirection of established allergic responses to T_H1 -dominated patterns using modified native or recombinant allergens
DNA vaccines
Oral or intranasal induction of tolerance with intact antigens or immunodominant peptides
Antagonizing the action of T_H2 -associated cytokines via administration of mutant cytokine (i.e. IL-4) molecules or antibodies specific to cytokine receptors
Local administration of exogenous cytokines (i.e. IFN γ , IL-12)
Anti-IgE-based approaches

in that they assume a requirement for chronic treatment much as do current pharmacologic methods of symptomatic control. Candidates include: 1) rIL-4 engineered to bind but not trigger the IL-4 receptor, 2) antibodies specific to cytokines or cytokine receptors that aim to block the production or function of the IL-4 necessary to sustain ongoing IgE responses *in vivo* and 3) antibodies that bind ϵ -specific determinants on free but not mast cell/basophil-bound IgE molecules, thereby impeding their interaction with high-affinity IgE receptors without triggering already bound IgE. Substantial progress has been made in genetically engineering these antibodies to reduce their inherent immunogenicity in humans.

Each of these approaches has shown promise in preliminary animal studies and is at some stage of clinical trials. Each also faces substantial hurdles before it can be considered as a major improvement capable of raising the efficacy of desensitization programs. Detailed discussion of novel approaches is accessible via the citations listed under Further reading.

Summary

From strictly empirical beginnings, immunologists and clinical practitioners have developed immunization strategies that efficiently protect large populations from a host of infectious diseases. Increased understanding of how protective immune responses are initiated and maintained has played a substantial role. With allergic diseases currently affecting 20–25% of the population in developed nations at substantial social and financial cost, the need for consistently effective strategies is readily apparent. Better understanding of 1) the means by which exposure to ubiquitous environmental antigens leads to allergic sensitization versus clinical tolerance, 2) immunologic mechanisms responsible for maintenance of

hypersensitivity diseases and 3) the cause-and-effect relationship underlying successful immunotherapeutic regimens are key priorities in moving desensitization programs to high levels of efficacy and acceptance.

See also: Allergens; Asthma; Atopic allergy; Mast cells; IgE; Rhinitis, allergic; Venoms; Vaccines; Interleukin 4; Effector lymphocytes.

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IMMUNOTHERAPY OF AUTOIMMUNE DISEASES

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The term 'immunotherapy' is used in two somewhat different senses. One is 'therapy of the immune system', aimed at targets ranging from antigen-presenting cells to activated T cells, macrophages and B cells. Another context is the use of immunological reagents, such as antibodies, T cells or their modifi-

cations in therapy. Immunotherapy in both senses has been applied vigorously in animal models of autoimmune diseases, and increasingly attempts are being made to apply immunotherapy to a wide range of human diseases.

Types of immunotherapy

A major goal of immunotherapy is to selectively reduce the unwanted immune response, but retain other protective immune responses. Existing anti-immune drugs are not clearly selective. Selectivity could be obtained in a variety of ways. One is by the induction of immunological tolerance. This comes in various forms, all defined as antigen-induced, antigen-specific immune suppression. Other approaches, less specific, would include inhibiting a portion of the HLA class I and II antigens or the HLA-peptide complex involved in presenting peptides to T cells. Alternatively, as some T cell responses to antigen use a narrow range of T cell receptors for antigen, targeting a subset of T cell receptors (TCRs) by using monoclonal antibodies or superantigens is being attempted. Targeting lymphocytes bearing markers of activation, such as interleukin 2 receptor (IL-2R, CD25), also offers a degree of selectivity.

At the other end of the spectrum are drugs which are not selective, inhibiting all lymphocytes following activation (e.g. cyclosporine) or all lymphocytes responding to cytokines such as IL-2 (e.g. rapamycin).

Selective immunosuppression

Tolerance in its classical definition depends on knowing the antigenic epitopes to be recognized, and hence a major drawback of studies in human and experimental autoimmunity is our lack of understanding of the important epitopes. Nevertheless there are a variety of experimental approaches which are finding their way into human application, at least to the clinical trial stage. A major objective is to ensure that in established disease, with an ongoing immune response, the therapeutic strategy does not potentiate rather than suppress the immune response.

Oral tolerance

Oral tolerance has been described for many years, first by Merrill Chase in guinea pigs. Most recently it has been popularized by H. Wiener and colleagues, and clinical trials of bovine collagen type II in rheumatoid arthritis and myelin basic protein in multiple sclerosis are in progress, run by Autoimmune Inc. Multiple mechanisms of oral tolerance have been proposed, from inducing apoptosis at high doses to inducing regulatory T cells at low doses. An improved variant may be inhalation tolerance, via the nose, which is more potent, and has been popularized by David Wraith.

Peptide-induced tolerance

Peptides given systematically, without adjuvant, are of low immunogenicity and are being developed for therapy. Trials of myelin basic protein (MBP) peptides, based on the 89–102 immunodominant region, are in progress, with different variants being run by Neurocrine Inc. and Immunologic. Neurocrine's trial involves using peptide derivatives known as 'altered peptide ligands', which upon binding to TCR do not elicit a complete immune response, but instead favor induction of tolerance.

Peptide-MHC complexes

Peptides elicit their effects after binding to the major histocompatibility complex (MHC) class I or II binding groove. It was thus proposed that it may be more effective to induce tolerance by treatment with pre-formed MHC class II-peptide complexes. This is effective in animal models, and a clinical trial of an HLA-DR2-MBP peptide complex is being initiated by Anergis Inc.

In the past, it was considered that saturating MHC peptide-binding grooves with nonimmunogenic peptides may be an effective strategy, but this was found not to be the case. Nonpeptide MHC blockers are in development (Zeneca). There are also clinical trials using immunization with MHC-derived peptides, which are designed to induce anti-MHC autoantibody, for example to the DR4/DR1 conserved 'shared' epitope involved in rheumatoid arthritis (Anergis Inc.).

TCR peptides

Peptides from the TCR have been shown to induce a protective immune response in experimental allergic encephalomyelitis (EAE), and such an approach is being attempted in humans in phase I studies (Immune Response Inc.).

Bystander suppression

It was initially thought that a major problem of all the tolerance regimes designed to inhibit the dominant autoimmune response would be the fact that they would block only a fraction of the autoimmune responses, and with time, weaker residual responses would amplify to restore the pathologic status quo. However, there is increasing evidence that this may not be the case, and that 'bystander suppression' may develop.

The phenomenon of bystander suppression is that during 'tolerization' with a major epitope, responses to other epitopes found in the same site, either on the same or other proteins, are also diminished. This

has been reported to occur with oral tolerance, inhalation tolerance, and also with injected peptides. The mechanism is not well understood, hypotheses range from the production of protective T_H2 cells producing immunosuppressive cytokines such as IL-4 or IL-10, or ' T_H3 ' cells producing immunosuppressive cytokines such as transforming growth factor β (TGF β).

Nonspecific immune suppression

Even 'nonspecific' immune suppression comes in degrees of specificity. One popular target is activated T cells. It could be predicted that activated T cells would be enriched for cells involved in the autoimmune process. However this prediction may not be useful, as there is evidence that many activated T cells are involved in the endogenous immunoregulation which diminishes the immune responses to auto and other antigens.

Activation antigens that have been used as the target for immune suppression include CD25, the activatable component of the high-affinity IL-2 receptor. IL-2 toxin has been used by Seragen. Antibodies to CD25 by Sandoz and Hoffman La Roche appear to be effective. OX40 is another activation antigen which is being considered as a therapeutic target. Immunosuppressive drugs like cyclosporine and FK506 are widely used in transplantation and cyclosporine is being increasingly used in rheumatoid arthritis (in combination with other drugs) and in psoriasis. In autoimmunity, glucocorticoids and methotrexate, which are both immunosuppressive and anti-inflammatory are widely used (e.g. in systemic lupus erythematosus and rheumatoid arthritis).

Antibody-dependent immunotherapy

The capacity to use antibodies for immunotherapy has been reported by a number of groups, and provides a very flexible and specific approach. This approach has been used in a wide spectrum of diseases, in transplantation and cancer, as well as autoimmunity. One of the virtues of antibody-mediated therapy is its molecular specificity, which, unlike the case with organic chemical drugs, is precisely known. Thus, cross-reactions can be readily monitored and therapeutic trials offer insight into pathophysiology.

The murine monoclonal antibody OKT3 developed by Patrick Kung was the first antibody to reach the market for transplant rejection episodes, and is still in use. Because murine antibodies are highly immunogenic in humans, repeated administration was not likely to be practical in the treatment of chronic disease, unless the effect was permanent.

Subsequent developments have altered the structure of murine antibodies so that they more closely resemble human antibodies in order to reduce immunogenicity. Replacement of the Fc and hinge regions leads to 'chimeric' antibodies, retaining the murine Fv, and more recently only the murine hypervariable regions have been 'grafted' on to a human Ig backbone, forming 'humanized' antibodies. With the generation of transgenic mice with a considerable part of the functional human Ig gene locus in place, the goal of developing fully human antibodies is also close to becoming realized. Phage display technology has been used to construct recombinations of human heavy and light chains. These immunoglobulins can be selected to bind molecular targets, and can be used for antibody. Whether these strategies will prevent immune responses to neopeptides in the idiotypic (hypervariable) region is unclear.

One of the most studied antibodies is anti-CD4. It has been shown by H. Waldmann in animal models that this promotes the development of immunological tolerance. The mechanism of this is not fully understood, and probably several mechanisms exist. Both lytic (depleting) and nondepleting antibodies can be protolerogenic, the latter more so. The mechanism of tolerance involves the generation of regulatory T cells and takes several weeks to reach optimal effects. Since such tolerance is capable of being transferred by T cells, the induced tolerance is 'infectious'. In humans anti-CD4 has been used extensively. Initial trials with lytic anti-CD4 in rheumatoid arthritis were not effective, but current trials of nonlytic anti-CD performed by SmithKline Beecham and Glaxo-Wellcome are more encouraging, reflecting the animal model experience.

Immunotherapy with anti-CD7 and with anti-CD5 (the latter conjugated to ricin) have not been effective in rheumatoid arthritis. CAMPATH-1H reacting with CD52 present on T, B and monocytic cells is a very lytic antibody, and may be effective in rheumatoid arthritis, although its apparent toxicity has prevented its use in randomized placebo-controlled trials. Nevertheless, it has had dramatic success in vasculitis and in lymphomas and some success in multiple sclerosis.

Antibodies to cytokines have been used for immunotherapy. The most widely studied is anti-tumor necrosis factor α (TNF α), which was initially used in sepsis, without clearcut success. Subsequent open and placebo-controlled clinical trials in rheumatoid arthritis and Crohn's disease have been highly successful and reproducible using two different antibodies, first with cA2 from Centocor Inc. and subsequently CDP571 from Celltech. In addition, two immunoglobulin (Ig) Fc-TNF receptor fusion pro-

teins produced by Immunex (p75) and Roche (p53) have both been shown to be effective.

Antibodies to IL-6 and IL-6R have been reported to be beneficial in very small open studies in rheumatoid arthritis.

Fusion proteins, comprising the IgG Fc region coupled to a receptor, have been used for two reasons: in order to prolong half-life in the blood and to augment avidity due to the presence of two combining sites. In addition to the above-mentioned TNFR Ig proteins, another which has entered trials is CTLA4-Ig (Bristol Myers Squibb). This protein binds to the CD80 and CD86 costimulatory ligands on antigen-presenting cells for CD28, and is very effective in animal models.

Cytokines with immunoregulatory properties have been used for immunotherapy and clinical trials. These include IL-10 for Crohn's disease and rheumatoid arthritis (Schering Plough), IL-11 for Crohn's disease (Genetics Institute) and IL-4 for rheumatoid arthritis (Schering Plough). The clinical trials are based on the effectiveness of these cytokines in relevant animal models, but of these only IL-10 in Crohn's disease has yet reached the clinical trial stage where its effectiveness has been successfully assessed.

Interleukin 1 receptor antagonist has been used in sepsis without success, but recent trials in rheumatoid arthritis by Amgen Inc. have shown a trend to some benefit, not so much in reducing inflammation, but in reducing the rate of joint destruction as assessed by serial X-rays of the hands.

Conclusions

There is currently a large endeavor to assess which immunotherapeutic successes in animal models will translate to humans. The results are encouraging, and from little new activity in therapeutic trials in the 1980s, a wide spectrum of new immunotherapeutic approaches are under development in the clinic. Whilst not all will succeed, the preliminary data on a number of approaches are sufficiently encouraging to indicate that immunotherapy will revolutionize the treatment options in autoimmunity in the next few years.

See also: Autoimmune diseases; Autoimmunity; Campath-1 antigen (CD52); Cytokines; Humanized antibodies; Immunosuppression; Oral tolerance; Tolerance, peripheral; Tumor necrosis factor α ; Tolerance, central.

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IMMUNOTHERAPY OF TUMORS

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The concept of the immune system being involved in the development of cancer and of manipulating it as a part of cancer therapy dates back almost 100 years to the experiments of William Coley with bacterial toxins ('Coley's toxin') used in the treatment of advanced breast cancer, apparently with some success. Ehrlich developed the concept of immune surveillance against the development of cancer, the immune system acting to detect and eliminate cancerous or precancerous cells. This hypothesis has received new impetus with the recent development of monoclonal antibodies and recombinant cytokines,

particularly the interferons and more recently interleukin 2 (IL-2), thereby providing powerful tools for the manipulation of the immune response.

For immune surveillance against cancer to be plausible theory, cancer cells must express antigens recognizable as foreign by and accessible to the immune system, which must in turn be able to mount a response against cells bearing such antigens. Such a theory must also account for the somewhat bizarre tumors seen in patients with immunocompromised states such as post-transplant immunosuppression and the acquired immune deficiency syndrome

(AIDS), coupled with the failure to observe a large excess of the commoner epithelial malignancies in such disease states.

Tumor antigens

These may be examined under a variety of headings, not necessarily mutually exclusive: chemically induced antigens, virally induced antigens, major histocompatibility antigens, differentiation antigens, embryonic antigens, and inappropriate expression of adult proteins.

Chemically-induced antigens

Ideally, for an immune response to develop against a tumor, it should possess tumor-specific transplantation antigens (TSTAs). In animal models, chemically induced tumors in mice possess TSTAs of varying degrees of immunogenicity that are unique to each tumor and will be rejected by immunocompetent animals. Although many spontaneous human tumors are presumed to be chemically induced, for example lung and bladder cancer, such tumors are poorly immunogenic, suggesting the model is not of general applicability. The chemical nature of animal TSTAs remains unclear.

Virally-induced antigens

Tumors induced by viruses express antigens that are not found in uninfected cells. The tumor antigens expressed are consistent and are virally encoded. As with chemically induced tumors, the immunogenicity of the virally induced tumors varies with different models. As the virally induced antigens are the same, the difference in response must lie at the host level. Tumors induced by simian virus 40 (SV40) in mouse models are rejected by immunocompetent animals. It has been established that the response to such tumors is major histocompatibility complex (MHC)-restricted, providing a clue as to how human tumors may escape immune surveillance by downregulating MHC antigen expression. Tumors induced by SV40 have not been described in humans but another group of DNA viruses, the human papillomaviruses (HPVs), are implicated in the etiology of a variety of anogenital cancers, particularly carcinoma of the cervix. However, the HPV genes with transforming potential are the early genes E6 and E7, which probably act by binding to the antioncogenes p53 and Rb. There is currently much research examining targeting HPV oncoproteins as part of a vaccine strategy.

A slightly different pattern of findings apply to lymphomas induced by Epstein-Barr virus (EBV) in that the virus immortalizes the host cell (e.g. the B

lymphocyte) by means of early viral proteins. However, the cells do express viral antigens on their surface, allowing recognition and elimination of virus-infected cells by the immune system. In immunocompromised hosts, such elimination does not occur and the immortalized lymphocyte continues to divide and eventually may acquire further mutations resulting in malignant transformation. However, in established Burkitt's lymphoma, the only viral protein detectable is EBNA1, which may or may not be immunogenic and is the subject of current intensive investigation. It has recently been demonstrated that some immunosuppression-related lymphoproliferative disorders can be eliminated if the immunosuppression can be reversed.

The EBV/Burkitt's model fits the Ehrlich immune surveillance hypothesis in that the tumor precursors express TSTAs which can be identified by the immune system and the cells bearing the antigens eliminated before or in some cases even after a cancer develops, unless the host immune response is compromised, for example by post-transplant immunosuppression, AIDS or chronic malaria. In contrast, the HPV-induced tumors, although similar etiologically in some ways, do not appear to produce TSTAs accessible to the immune system in most cases. However, there is now evidence linking HLA types to risk of development of invasive carcinoma (see below) and thus ability to recognize viral proteins may be a factor in tumor progression. In addition, it is becoming apparent that anogenital squamous cell carcinomas, which share a likely HPV-related etiology, are increased in incidence in HIV infection and in the case of cervical carcinoma are now an AIDS-defining characteristic.

The association of Kaposi's sarcoma (KS) with sexually acquired human immunodeficiency virus (HIV) infection, and case reports of KS in HIV-negative homosexuals in San Francisco has led to the suggestion that induction of KS requires an ineffective cofactor, transmitted primarily by sexual contact and presumed to be viral. Precise figures for African patients with AIDS are more difficult to obtain but the pattern appears to be similar in that KS is relatively common and the principal mode of transmission of HIV among adults is sexual. Immunosuppression is an independent cofactor as KS also occurs among patients with other congenital or acquired immunodeficiency states such as post-transplant immunosuppression. Further KS lesions may regress in such patients if the immunosuppression is reversed. The prognosis of AIDS/KS correlates with the degree of immunosuppression of the patient.

Embryonic antigens

Carcinoembryonic antigen (CEA) is a fetal glycoprotein detectable on cells and in the serum of patients in a proportion of gastrointestinal malignancies. It has, however, proved disappointing as a tumor marker.

α -Fetoprotein, a fetal albumin, and human chorionic gonadotropin (hCG) β chain can also be detected on cells and in serum in a variety of malignancies including germ cell tumors and hepatocellular carcinoma and are useful as markers of established disease and in diagnosis. However, they are not sufficiently specific to be used for screening.

The importance of embryonic antigens lies in the light they shed on the underlying pathogenesis of the disease. The removal of controls on normally tightly regulated genes, in this case genes only expressed *in utero* (incidentally, a time of rapid growth – the human fetus grows faster than any malignant tumor), is likely to play an important role in the oncogenic process.

More recently, a series of probable embryonic antigens have been demonstrated to be present in a wide range of tumor types. These antigens have been identified using cytotoxic T lymphocytes (CTLs) to screen expression libraries derived from tumor cells. The initial family of antigens, the MAGE (melanoma antigen) family of melanoma tumor antigens have been further characterized to establish the precise peptide sequences necessary for presentation on defined HLA proteins. The MAGE proteins are of unknown function but are expressed during fetal development and in testis and brain in the adult. They are expressed in a wide range of common adult tumors in addition to melanoma. Despite their presumed fetal developmental functions, the method used to isolate the antigens, based on identification by CTLs, shows that these proteins are potential immune targets. Planning is underway to use the peptide epitopes described above in immunotherapy studies.

Inappropriate expression of proteins present in adult tissues

Cancers result from a series of mutations in normal cellular control mechanisms, resulting in either loss of inhibitory controls (so-called tumor suppressor genes) such as p53 and Rb, or gain of function mutations in positive regulators, usually involved in signal transduction pathways (so-called oncogenes). A number of these mutations lead to the expression of mutant proteins which therefore are potentially recognizable as foreign antigens. The malignant phenotype may also result in the inappropriate

expression of adult proteins, for example, ectopic ACTH production by small-cell lung carcinomas, which may be detected in biopsy specimens by immunostaining.

Major histocompatibility antigens

An effective immune response requires that a suitable target antigen exists. However, if the immune system is not presented with antigen by antigen-presenting cells, then no response will be possible. The MHC system is responsible for antigen presentation at the cell membrane and for interaction with T helper cells. Presentation of different epitopes is dependent on the MHC class I molecules present in the cell. Thus tumor antigens would be presented by some but not all HLA class I molecules and it should thus follow that either HLA type or decreased expression of HLA molecules on tumor cells may influence prognosis if escape from immune recognition is a factor in tumor progression. There is evidence from a variety of tumor types that downregulation of HLA expression occurs during disease progression. A variety of human tumors including lung, breast and skin tumors showed low levels of class I MHC expression. In contrast, benign epidermal lesions showed normal MHC expression, whereas a series of 15 basal cell carcinomas lacked both class I MHC and β_2 -microglobulin. Similar results have been obtained with squamous cell skin carcinomas. This phenomenon thus applies both to tumors that respond to immunotherapy such as melanoma as well as tumors not traditionally susceptible to immunotherapy. Bladder cancer is particularly intriguing as superficial disease can be effectively treated with intravesical BCG. Responses can be shown to be cell mediated and accompanied by markers of immune response such as release of cytokines including interleukins and tumor necrosis factor α . Muscle invasive tumors are not susceptible to BCG and have downregulated MHC expression, suggesting that this may be a factor in the development of invasive potential.

It has been known for some time that interferons modulate expression of MHC antigens, control being at the transcription level, providing one (though not the sole) rationale for their use in malignant disease. In contrast, N-myc expression induces downregulation of class I MHC in neuroblastoma cell lines and N-myc amplification is known to correlate with prognosis in neuroblastoma. Particularly intriguing in this context is the observation that patients with Evans stage 4S neuroblastoma, who have extensive disease, have a normal N-myc copy number and usually experience spontaneous tumor regression in con-

trast to the extremely poor prognosis of other patients with disseminated neuroblastoma.

It would be predicted that certain HLA types will be unable to present certain antigens and that this may allow more rapid tumor progression. There is evidence associating specific HLA types with risk of progression of cervical intraepithelial neoplasia to invasive carcinoma. As there is a well-defined role for human papillomaviruses in the etiology of this disease (see above), the possibility of targeting viral antigens for therapy is attractive and the converse, that the concept that the inability to 'see' viral antigens may be etiologically relevant, is persuasive.

Differentiation antigens

Tumors may express antigens normally expressed during the developing cells of the parent tissue, examples being the wide variety of lymphocyte antigens expressed on lymphomas, prostate-specific antigen in prostate carcinoma, and thyroglobulin in thyroid carcinomas. Such antigens are clearly not TSTAs but are nonetheless tumor associated and may thus be of use either diagnostically or therapeutically.

Diagnostic relevance of tumor immunology

The use of panels of monoclonal antibodies (mAbs) for immunophenotyping tumor material is now well established and of great value in subtyping lymphomas and for determining the histogenesis of undifferentiated tumors.

Antibodies directed against tumor-associated antigens have also been used for the immunolocalization of tumors by means of radiolabeled antibodies. So far, this conceptually appealing mode of imaging has failed to prove more effective than more conventional imaging techniques.

Therapeutic modification of the immune response

Immune response modifiers

A considerable literature now exists on the use of genetically engineered cytokines in the treatment of cancer. Initial enthusiasm has now abated with the realization that the immune system can not easily be turned on to tumor targets by the indiscriminate activation of the immune system by cytokines such as IL-2 and interferon α . However, interferons do have a defined role and proven efficacy in the management of certain conditions, particularly melanoma, renal cell carcinoma and hairy cell leukemia and as adjuvant therapy in certain hematological malignancies such as low-grade lymphoma and myeloma.

Interleukin 2 was first used in studies at the National Cancer Institute in the mid-1980s and attracted widespread attention as a potential breakthrough in the treatment of renal cell carcinoma and melanoma. Studies elsewhere have confirmed an effect in these tumors but only in a small number of patients and at high cost in toxicity. The *in vitro* expansion of cells from tumor samples, generating tumor-infiltrating lymphocytes (TILs), or peripheral blood to produce lymphokine-activated killer (LAK) cells, probably does not usefully increase the response rate. The combination of IL-2 with interferon and chemotherapy may increase the response rate although at present the high toxicity makes the routine use of IL-2 outside study settings in specialist units inadvisable.

Both IL-2 and interferon α , used as sole therapy for metastatic melanoma or renal cell carcinoma, produce response rates of 5–40% depending on patient selection criteria. In essence, fit patients with a low disease burden (and prior nephrectomy in the case of renal cell carcinoma) are most likely to respond to either agent. Some metastatic sites (e.g. lung) respond better than others (e.g. bone or liver). However, the median duration of response is generally less than a year and long-term survivors rare. Nonetheless, spectacular responses, and long-term survivors do occasionally occur with immunotherapy in a few lucky cases. Furthermore, these malignancies, whilst in some ways unusual, are not typically associated with immunosuppression, suggesting that they are able to evade an intact immune system but that this evasion can be surmounted. The search is thus continuing for ways of improving the response rate by more effective targeting of such therapies (see below).

Monoclonal antibodies

The development of monoclonal antibody technology also generated a wave of enthusiasm for these seemingly ideal anticancer agents – natural, nontoxic and exquisitely targeted. Unfortunately, problems of delivery to targets, reactions to murine antibodies and difficulties in the linking of suitable warheads to these biological 'guided missiles' prevented them from fulfilling their initial promise. There appear to be three problems preventing effective therapy: the first is dissociation of the antibody from its 'warhead'; the second is the therapeutic ratio between nonspecific binding (especially to lymphoid tissue) and target tumor tissue; the third is the development of a host response against the antibody, which is usually of murine origin and which limits the number of repeat treatments that may be given.

The problems are all amenable to at least partial

solution. For example, the use of F(ab)₂ fragments of 'humanized' mAbs may abrogate the nonspecific binding and host response problems. Improved chelation techniques may decrease the extent of complex dissociation. Other areas of research include the use of mAbs linked to enzymes which are then used to activate an inactive prodrug – antibody-directed enzyme prodrug therapy (ADEPT). This approach has the additional advantage that the half-life of specific binding is usually much greater than that of nonspecific binding, thereby improving the therapeutic ratio as administration of the active drug can be delayed to allow clearing of such nonspecifically bound antibody. By means of such approaches, it may become possible in the future to utilize mAbs for systemic therapy, probably as an adjunct to more conventional debulking treatment such as surgery, radiotherapy or chemotherapy although the clinical utility of ADEPT remains to be convincingly demonstrated.

A new generation of bispecific antibodies (BSABs) may circumvent some of these problems by aiming to cross-link targets to immune effector cells to initiate a cell-mediated antitumor response. This circumvents the problems of cross-linkage as stable covalently BSABs can be formed. Delivery is less of an issue than when the mAb is the primary effector molecule due to 'bystander' lysis of adjacent cells by activated immune cells. Promising results have been observed with BSABs given with recombinant cytokines in phase I/II trials using antibodies targeted to the HER2/NEU gene product and the macrophage Fcγ receptor.

Monoclonal antibodies have been used extensively to purge marrow for autologous bone marrow transplantation (ABMT), though with little direct evidence to support its use. Recent evidence from the Barts lymphoma ABMT program suggests that, at best, a 2 log cell kill can be obtained by treatment with a single monoclonal antibody, but that complete clearance of abnormal cells (measured by polymerase chain reaction amplification and detection of the t(14,18) translocation breakpoint region) only occurred in those with the lowest initial burdens. The significance of these results remains unclear.

Immune factors also play a role in the success of allogeneic transplantation for leukemia. Graft-versus-host disease (GVHD) is a major clinical problem, predicted by the classical adoptive transfer experiments of Billingham, Brent and Medawar. Unfortunately, attempts to reduce GVHD, for example by T cell depletion of donor marrow, resulted in increased leukemic relapse rates. Furthermore, patients receiving marrow from identical twins have a higher leukemic relapse rate and, conversely, patients with

severe GVHD a lower relapse rate, suggesting a significant graft-versus-leukemia effect. Thus, post-transplant immunosuppression protocols tread a wary balance between these two conflicting problems.

Cancer vaccines

The last 10 years have seen dramatic advances in our understanding of how human T lymphocytes recognize and in some situations destroy cancer cells. Major efforts are going into the development of various types of cancer vaccines using peptides, glycoproteins, antibody idiotypes as well as autologous tumor cell lines. Polynucleotides encoding for various tumor specific peptides have been claimed to raise a powerful immune response under certain situations.

Recently, the successful cloning of cytolytic T cells (CTLs) has led to the identification of a series of antigenic peptides degraded from intracellular proteins and ending up in the clefts of MHC molecules on the external surface of the cell. Three approaches have been used in their identification. Target cells transfected with cDNA libraries have been used to analyse specificity of CTL clones. This genetic approach was used to identify the MAGE series of melanoma antigens as well as MART, tyrosinase and melan-A. A biochemical strategy has been the separation and characterization of peptides from purified MHC molecules. A third approach has been the construction and analysis of the response to synthetic peptides that bind to MHC class I determinants.

Phase I clinical trials are now in progress using several vaccine strategies (Table 1). Most involve direct peptide injection with immunological adjuvant but enhanced responses may be obtained by using autologous dendritic cells pulsed with peptide antigens. Assays are available to measure the immunological effectiveness of such vaccines so that optimization can be achieved before moving to larger scale phase II trials aimed at determining efficacy.

Table 1 Current cancer vaccines

Autologous cell lines
Allogeneic cell lines
Genetically modified tumor cells
Glycoproteins
Stripped glycoproteins
Peptides
Antitumor antibody idiotypes
Polynucleotides encoding tumor antigens

Gene therapy

As outlined above, there is increasing evidence that tumor cells do express antigens not found on the parent normal tissue. Evidence from studies using cytokines such as interleukin 2 and interferon α demonstrate that in both experimental and clinical settings, the immune system can be stimulated to recognize and destroy tumor targets. Considerable recent research has thus focused on possible reasons why tumors escape elimination by the immune system. If the principal reason for the lack of an effective immune response is related to defects in immune processing rather than a lack of identifiable targets *per se*, then manipulation of the immune process may allow effective immunotherapy on a wider scale than hitherto has appeared possible.

There has been much recent interest in modifying tumors to make them more immunogenic. Experiments using nonimmunogenic animal tumors such as the B16F10 melanoma cell line have shown that modification of tumor cells to express the coimmunostimulatory molecule B7-1 will result in subsequent rejection of challenge with both modified and unmodified tumor cells. Similar results have been obtained with interleukin 12, which is also involved at a key initial stage of initiation of the cell-mediated immune response. However, although dramatic results have been obtained in certain well-defined models, there is also increasing evidence that single modifications to render tumors immunogenic are unlikely to be sufficient to overcome the profound inability of the immune system to recognize and eliminate most human tumors. Nonetheless, combination approaches utilizing genetic modification of targets, cytokines and linking molecules such as BSABs may in the future be able to deliver effective targeted anti-tumor immunotherapy.

Conclusions

There are many potential permutations for the application for immunologically based therapy for cancer. One of the most important developments has been the increased understanding of the molecular mechanisms of malignancy through which biological manipulation can be tailored to an individual tumor. Although current clinical studies are not demonstrating high response rates, there remain tantalizing pointers towards more effective therapies in the future.

See also: Antibodies, bispecific; Chromosome translocations of immune genes; Cytokines; Embryonic antigens; Epstein-Barr virus, infection and immunity; Immune surveillance; Immunopotential; Interleukin 2; Leukemia viruses; Lymphokine-activated killer (LAK) cells; Monoclonal antibodies (mAbs); Papillomavirus, infection and immunity; Targeting of immunological agents; Toxins; Tumor antigens; Tumor imaging; Tumors, immune response to; Tumors, immunological escape of.

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IMMUNOTOXICOLOGY

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Immunotoxicology has been defined as the study of adverse effects on the immune system resulting directly or indirectly from occupational, environmental or therapeutic exposure to chemicals (including drugs), biologic materials and, in certain instances, physiological factors, collectively referred to as agents. It encompasses studies of altered immunologic events associated with exposure of humans and wildlife species including immune regulation (suppression or enhancement), allergy and autoimmunity (**Figure 1**). In the former case, the systemic or local (e.g. skin, lung) immune system acts as a passive target for the agent, and the result may be an increased incidence or severity of infectious disease or neoplasia. In allergy, the immune system responds to chemical (hapten)–host protein conjugates or high molecular weight compounds. The most likely health consequences of the latter include respiratory tract allergies (e.g. asthma, rhinitis) or allergic contact dermatitis. Autoimmunity may occur either as a result of an agent-induced alteration in host tissue, endocrine function or immune regulation. More recently, studies in immunotoxicology have also included investigations into the role of inflammatory mediators, particularly cytokines and reactive oxygen species, in localized chemical toxicities as many of these mediators are produced by and/or act upon immune cells.

The potential for adverse health effects due to alterations in the immune system by toxic agents has been a matter of increasing scientific and public concern. As such, there have been marked efforts in both basic research undertaken within this area as well as development and incorporation of appropriate test methods to assess potential immunotoxicities in experimental animals, wildlife species and humans. Regarding testing methods, appropriate selection has been complicated by the need to employ distinct tests based upon the site examined (e.g. systemic, lung, skin) and the immunopathology of concern (i.e. hypersensitivity, immune regulation, autoimmunity, inflammation). However, our increased understanding of the molecular, cellular and genetic events responsible for mounting appropriate immune responses, as well as for the immune mediators

involved in organ-specific necrotic and apoptotic processes, has provided opportunities for the utilization of more streamlined and informative tests for risk assessment and the development of novel preventive or therapeutic measures.

Hypersensitivity

Pharmacologically mediated hypersensitivity responses, such as those elicited with β -lactam antibiotics, are well recognized. In addition, a number of industrial materials have known or presumed allergic activities in humans and laboratory animals (**Table 1**). This response can be mediated by T cells as well as antibodies (e.g. IgE) and can result in allergic contact dermatitis or allergic lung disease (asthma). A compound causing both these responses is toluene diisocyanate (TDI), a classic representative of the isocyanate compounds used in the production of plastics and resins. TDI, which acts as a hapten, is particularly reactive with amino groups on proteins. Chemical-induced allergic respiratory responses can also be induced by high molecular weight compounds from animal urine and saliva, enzymes in detergent, plant materials as well as therapeutic peptides. Clinical differentiation of allergic responses from nonimmune irritant responses, without detecting specific immune responses, are often difficult, but the former are usually more persistent and severe.

Tests to identify potential respiratory sensitizers are difficult to undertake and, as such, efforts to validate screening models are limited. Although the guinea pig has some significant immunological differences compared to humans (e.g. IgG1 versus IgE reagenic antibodies), it has proved a predictable animal model for humans given the limited comparative data available and has been used to test for both low and high molecular weight sensitizers. As with guinea pig skin tests, the method includes both an aerosol sensitization and challenge phase. There is also a need to measure both immediate and delayed-onset responses, although this does not distinguish between nonspecific pulmonary hyperreactivity and specific immune responses. The latter is commonly

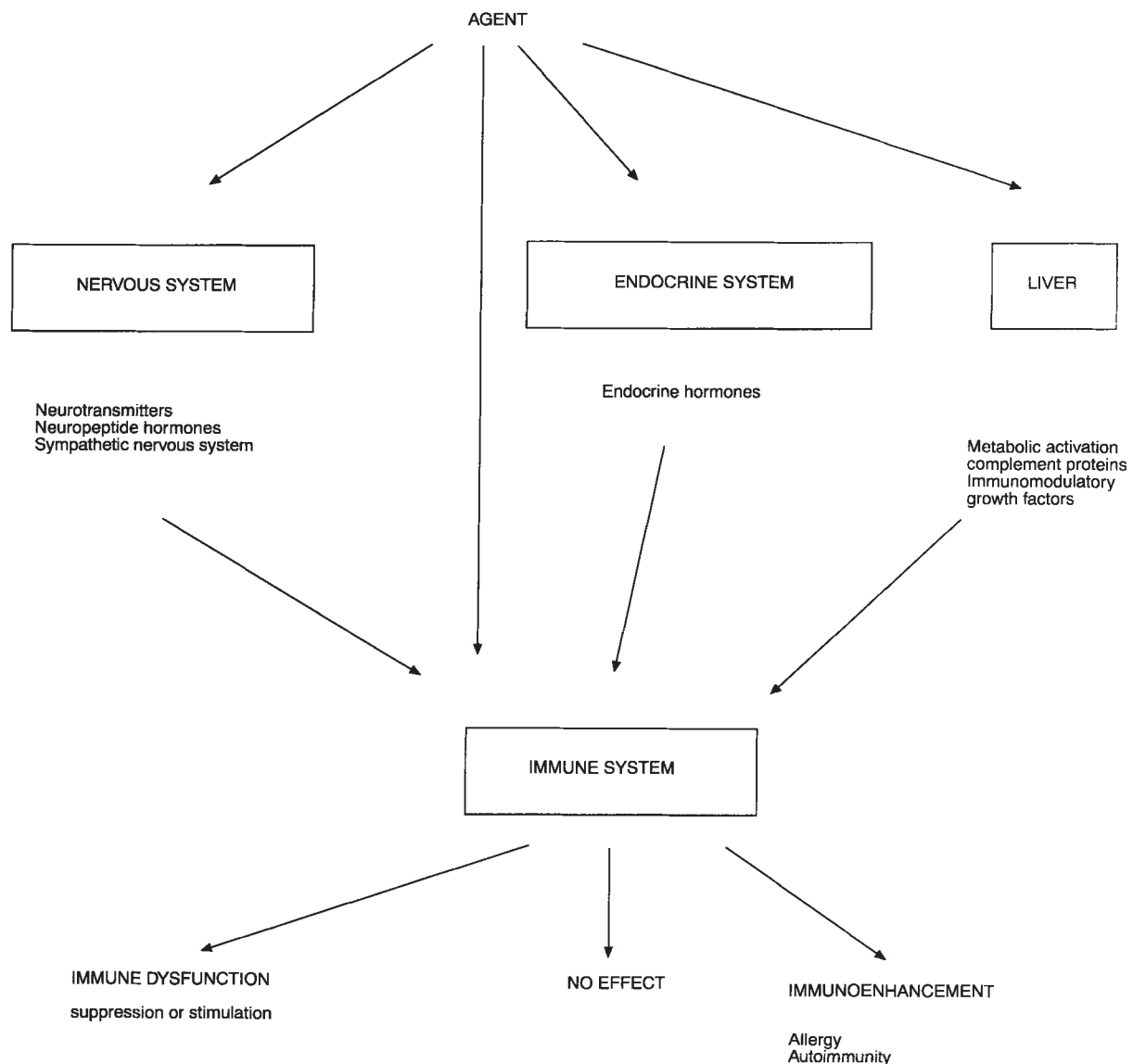


Figure 1 Scheme for direct or indirect effects of xenobiotic agents on the immune system and potential outcomes. (Reproduced with permission from Karrow JG and Holsapple MP (1995) Structure and function of the immune system. In: Smialowicz R and Holsapple M (eds) *Experimental Immunotoxicology*, pp. 3–12. Boca Raton, FL: CRC Press.

established by examining sera for the presence of reagenic antibodies.

Historically, the guinea pig has also been used to test for potential skin sensitizers and such tests have been incorporated into many regulatory guidelines. Many variations in guinea pig hypersensitivity assays exist, but the Buehler occluded and Magnusson–Kligman maximization tests have been most often employed. Because guinea pigs are large, several graded doses of antigen may be tested and an entire dose–response curve can be generated by comparing skin reactions in individual animals. However, it is expensive to purchase as well as maintain guinea

pigs, there are few inbred strains and immunological reagents are not widely available. As such, efforts are presently underway to replace guinea pig assays with mouse models. The most promising test is the local lymph node assay (LLNA). This procedure allows for the quantitation of lymphocyte proliferation in lymph nodes draining the site of chemical application, which is routinely the ear, and compared to guinea pig assays, is considerably less complex and time consuming. The LLNA may fail to detect some weak sensitizers and provide false positives for strong irritants, but modifications of the assay have been used, such as skin abrasion, to increase the

Table 1 Examples of agents reported to be immunotoxic

<i>Hypersensitivity</i>	<i>Immunosuppression</i>	<i>Autoimmune-like^a</i>
<i>Lung</i>	<i>Systemic</i>	<i>Drugs</i>
Toluene diisocyanate	Dioxins, PCBs	Procainamide
Anhydrides	Organotin	Hydrazine
Wood dust	Alcohol	Isoniazid
Antibiotic dust	Marijuana	Halothane
Detergent enzymes	Heavy metals	<i>Environmental</i>
<i>Skin</i>	<i>Lung</i>	Vinyl chloride
Nickel	Asbestos	Trichloroethylene
Chromium	Silica	Cadmium
Certain pesticides	Ozone	Mercury
Animal products	NO ₂	<i>Foods</i>
Citral	<i>Skin</i>	Alfalfa sprouts
Isoeugenol	Ultraviolet radiation	Tartrazene
Formaldehyde	Polycyclic aromatics	

^aAutoimmune like = the symptoms disappear when the offending agent is removed.

sensitivity. These procedures can also be modified to allow examination of potential photoallergens.

Autoimmunity

The well-documented examples of drug-induced autoimmune-like syndromes have led to studies to determine whether certain agents contribute to the incidence of autoimmune diseases. Some of the chemical and therapeutic agents suggested to be associated with autoimmune diseases or autoimmune phenomenon (i.e. increased levels of autoantibodies), in experimental animals or humans are listed in **Table 1** and include several heavy metals, pharmaceuticals, organic solvents and foods. As a number of factors participate in the development of autoimmune disease and it presents both diverse clinical symptomology and organ specificity, appropriate animal models have been difficult to develop. One method that has received attention is the popliteal lymph node assay (PLNA) which measures proliferative activity in the draining lymph nodes. Although this assay appears to detect low molecular weight compounds with immunomodulatory potential, it does not necessarily discriminate those that influence autoimmunity and may provide false positives with irritating agents. A number of animal models for autoimmune diseases exist, such as immunization (e.g. with myelin basic protein for multiple sclerosis) or animals that develop spontaneous autoimmunity. However, their evaluation as screening models in immunotoxicology has not been sufficiently investigated. A large database exists on animal strains with

genetic susceptibility to autoimmune disease from exposure to metals, such as cadmium or mercury, and include the Brown-Norway rat, ICR mouse and H2^s mouse. Silicone, used in surgical implants, has recently been implicated in autoimmune phenomena in humans, although epidemiological data clearly do not support such a relationship. This, however, does not preclude the need to have available predictable testing methods for detecting such agents and, in fact, may exemplify the need for such assays.

Immune regulation

Although immune regulation (i.e. suppression or stimulation of the local or systemic immune response) may represent a potentially more significant human health hazard than hypersensitivity and, as such, has been afforded considerable attention. Initially, efforts were made by immunologists working in this area of toxicology to identify agents which could potentially alter the immune system using various *in vitro* or rodent models (i.e. hazard identification). Subsequently, studies were conducted to develop more predictive test methods, determine mechanisms of action, or identify affected human populations. Based primarily upon animal studies, a number of agents have been shown to alter regulation of the immune system (**Table 1**). One of the most studied classes of chemicals are halogenated aromatic hydrocarbons, which include dioxins and polychlorinated biphenyls (PCBs). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a prototype of this class, is a potent immunotoxic chemical in mice with an ED₅₀ for antibody suppression of 1–2 µg kg⁻¹ body weight.

Although the exact mechanisms by which TCDD exerts its immunotoxicity has not been established, it is known to affect a number of immune cell types, to alter differentiation processes (possibly via altering tyrosine kinase activity) and to require binding to an intracellular receptor (referred to as the Ah-receptor for aromatic hydrocarbon). Once bound to the receptor, the complex is capable of translocating to the nucleus and interacting with 'dioxin responsive elements' on DNA that help regulate expression of numerous genes. Although immunological changes have been reported in human and wildlife populations exposed to dioxins and/or PCBs, the significance of these findings are hotly contested. One view states that the reported immune changes are not of sufficient magnitude to be considered relevant biologically. This conclusion is supported by the lack of clear evidence that the exposed populations show increased frequencies of immune-associated diseases such as infections. The opposing view states that

even small changes in the immune system can translate to increased frequency or severity of disease, but the ability to discern this is dependent upon the size of the population exposed and how closely they are monitored. Thus, while the immune system may be redundant, it does not possess significant reserve. The latter hypothesis is supported by mathematical models derived from experimental data bases. Data bases to conduct similar types of analysis do not exist in humans. A recent study by Weisglan-Kuperus and colleagues, exemplifies the debate. In this report, a dose-response relationship was observed between TCDD/PCB internal exposure and significant changes in cell surface markers, particularly, CD3⁺CD8⁺ T cells in infants exposed via breast milk. Similar observations were reported previously in mice and nonhuman primates receiving chronic, low-level dioxin exposure. The infants, however, showed no evidence of increased disease and the phenotype values fell within background levels which can show a coefficient of variation as high as 30%. The significance of such findings will probably be argued until improved disease monitoring and use of standardized methods are evoked.

Incorporating experimental animal data on toxicant-induced immune alterations as part of the evaluation of drugs and chemicals in human risk assessment has become increasingly common. Because of the complexity of the immune system, the initial strategies devised by immunologists working in toxicology and safety assessment have been to select and apply a tiered panel of assays to identify hazardous agents. These tests included both functional and non-functional (e.g. lymphoid organ histology) assays, as well as host resistance tests. More recently, it has been shown that only a minimal number of appropriate tests need be conducted for general immune screening. As such, both regulatory agencies and industry are now requiring or recommending (i.e. on a needed basis) immune system evaluation. A surprising exception to this is the Center for Biological Evaluation and Research of the Food and Drug Administration, which is responsible for testing biological materials including immunoregulatory cytokines and growth factors, and support the notion that in immunotoxicity issues should focus only on antigenicity. A general screening approach which is increasingly being required by many agencies and private industries involves the examination of lymphoid organs (e.g. histology, organ weight) and a functional assay requiring the quantitation of antibodies following immunization with sheep erythrocytes in rodents exposed to the test agent. The latter assay has undergone extensive validation studies and

appears to be a predictive and sensitive indicator for most immunotoxic agents.

Local immunity

As the portal of entry for many agents is the lung or skin, attention in immunotoxicology has also been directed to assessing local immunity at these sites. In the lung, for example, there is growing clinical evidence suggesting that nonspecific and specific immune mechanisms play a major role in pulmonary diseases associated with air pollutants including oxidant gases, such as ozone and nitrogen oxides, as well as particulates such as silica, asbestos and coal dust. For the most part, these appear to be associated with the synthesis and release of proinflammatory cytokines from alveolar macrophages and lung epithelial cells. Recent studies have also established immune-mediated mechanisms associated with the development of certain occupational pneumoconiosis (disorders caused by inhalation of particulates or aerosols) such as berylliosis (beryllium), byssinosis (cotton dust), and occupational asthmas (red cedar). Although validated methods to screen pulmonary immunotoxicants are not available, cellular and biochemical profiles of bronchoalveolar lavage constituents following inhalation exposure in experimental animals and humans can be useful for determining immune-mediated lung injury. These include, in addition to examining cellular infiltration and the release of cytokines, quantifying a variety of short-lived products which contribute to altering resistance to pulmonary infections and inflammation including arachidonic acid products and reactive oxygen species. Other immunological defenses operating in the lung include humoral- and cell-mediated immunity derived from bronchial-associated lymphoid tissue (BALT), as well as interstitial lymphocytes. The lung also possesses natural killer (NK) cells which are affected by a number of agents including phosgene. The overall alterations of such systems by these agents has been shown to play a role in respiratory infections, asthma severity, granulomatous lung disease and even interstitial pulmonary fibrosis.

Similar to the lung, mammalian skin is in contact with draining lymph nodes and contains immune components, including Langerhans cells, cytokine-producing keratinocytes and T lymphocytes. Skin-associated lymphoid tissue can respond to agents by the induction of allergic contact dermatitis or by induction of local or systemic (antigen-specific) immunosuppression. Immunosuppression can occur following dermal exposure to ultraviolet radiation and chemicals, such as polycyclic aromatic hydrocarbons. The mechanisms for this are complex but

appear to involve the release of soluble mediators from keratinocytes, including regulatory cytokines. Although *in vitro* tests are available for screening contact irritants, which often involve monitoring the release of inflammatory mediators, there are no standardized methods available for assessing altered skin immunity.

As a subdiscipline of toxicology, immunotoxicology studies are providing valuable information in risk assessment for humans and, to a lesser extent, wildlife. To improve risk assessment, however, it will be necessary to develop more sensitive human and wildlife epidemiological and immunological biomarkers, determine sensitive populations and better understand the quantitative relationships between changes in immune function and disease.

See also: Autoimmunity; Contact hypersensitivity; Cytokines; Drugs, allergy to; Hypersensitivity reactions; Immune surveillance; Immunosuppression; Monoclonal antibodies (mAbs); Toxins; Vaccines; Venoms.

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INBRED STRAINS

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According to Grunberg in 1952: 'The introduction of inbred strains into biology is comparable in importance with that of the analytical balance into chemistry.' While this is open to debate, there is little doubt that inbred strains have made a substantial contribution to the advance of biomedical sciences. Several Nobel Prizes in immunology (e.g. Medawar and Snell) have been for work which would have been difficult, or impossible, without using such strains.

Definition

For most practical purposes, an inbred strain can be regarded as an immortal clone of genetically identical individuals. According to the International Committee on the Genetic Nomenclature of the Mouse:

A strain shall be regarded as inbred when it has been brother $\times$ sister mated (hereafter called b $\times$ s) for 20 or more consecutive generations (F20), and can be traced back to a single ancestral breeding pair in the 20th or subsequent generations. Parent $\times$ offspring matings may be substituted for b $\times$ s

matings provided that, in the case of consecutive parent $\times$ offspring matings, the mating in each case is to the younger of the two parents.

Similar definitions apply to other species.

Brother $\times$ sister mating increases homozygosity at each gene locus at about 19.1% per generation. So a strain is never fully inbred, but homozygosity exceeds 98% after 20 generations. Eventually, an equilibrium is set up between the loss of heterozygosity due to continued inbreeding, and the generation of new genetic variation by mutation. A single breeding pair in the 20th or a subsequent generation is chosen to ensure that the strain is also isogenic, i.e. all individuals are genetically identical (see below). Where parent $\times$ offspring matings are necessary, the younger of the two parents is used to avoid several generations of backcrossing to a single individual, which is not genetically equivalent to brother $\times$ sister mating.

Most of the common inbred strains of mice and rats were developed between 1906 and about 1940, though a few strains continue to be developed each

year. For example, the most widely used strains of mice are C57BL, C3H, CBA, BALB/c, and AKR, which were all developed between 1920 and 1936. Several strains (including C3H and AKR) were selected for a high incidence of neoplasia. Currently there are over 400 inbred mouse and 200 inbred rat strains (many more if congenic and recombinant inbred strains are also counted), but only a handful of inbred strains of guinea pigs, hamsters, gerbils, chickens and rabbits.

Derivatives of inbred strains

A pair of strains are said to be coisogenic when they differ at a single genetic locus as a result of a mutation within one branch of an inbred strain. Congenic strains are inbred strains of mice and rats approximating the coisogenic status, developed by 12 or more backcrosses of a gene to an inbred strain. They may carry 'passenger' genes closely linked to the backcrossed gene. Sets of recombinant inbred strains (RIS) are developed by brother $\times$ sister inbreeding from the F1 hybrid between two standard inbred strains. If 7–20 or more such strains can be developed from a single cross, and each strain is phenotyped, the resulting strain distribution pattern can be compared with that already determined for other genes in the same RIS set. A close match would imply genetic linkage.

Recombinant congenic strains are produced by crossing two inbred strains, followed by 1–6 generations of backcrossing, then brother $\times$ sister mating. The resulting set of 20 or more strains is useful in the genetic analysis of polygenic characters and the identification of quantitative trait loci (QTLs).

Transgenic (including knockout) strains are developed by transferring DNA to an individual, typically at the preimplantation embryo stage. Inbred knockout strains are similar to coisogenic strains, with a single mutant locus. Inbred transgenic strains other than knockouts are also similar to coisogenic strains except that the DNA may have become integrated as tandem arrays of several copies of the foreign DNA in a 'random' location in the host genome.

The first-generation offspring of a cross between two inbred strains is known as an F1 hybrid. These are isogenic, though heterozygous at all loci at which the two strains differ (see below for the properties of inbred strains). They also tend to be more vigorous and robust than either parental strain as a result of hybrid vigor.

Nomenclature

Full details are given in *Genetic Variants and Strains of the Laboratory Mouse*, 3rd edn (see Further reading). Inbred strains are designated by a capital letter or letters (e.g. CBA, SJL), or less preferably by a combination of letters and numbers beginning with a letter (e.g. C3H, C57BL). Most designations have little or no meaning. The use of long strain names such as 'Brown Norway' instead of BN is not encouraged.

Where necessary, the number of generations of brother $\times$ sister mating can be indicated by appending an F followed by the number of inbred generations (e.g. F31). If, because of incomplete records, the number indicates only part of the inbreeding, this should be indicated with a question mark (e.g. F?+31).

Two colonies of the same strain would be regarded as substrains if there were known or probable genetic differences between them, as a result of residual heterozygosity at the time of separation (remember, no strain is ever fully inbred), or as a result of new mutations. Colonies separated between F20 and F40, and those separated from a common ancestor for more than 100 generations should be considered as substrains. Substrains are designated by a slash, followed by a substrain symbol, which can be a number or letter(s) indicating the name of an investigator or laboratory, or a combination of the two (e.g. DBA/1 and DBA/2, C3H/He and C3H/Bi).

Environmental, maternal or cytoplasmic factors may also alter strain characteristics. Cross-fostering, embryo transfer or ovarian grafting may alter the complement of vertically transmitted retroviruses. Hence, in addition to any substrain symbol, a laboratory code should be appended to the strain name following an @ symbol (e.g. C3H/He@N, mice of the C3H/He strain and substrain bred at the National Institutes of Health) in order to indicate the laboratory of origin and to emphasize the fact that even genetically identical animals may differ as a result of nongenetic factors.

Maintenance of inbred strains

The aim is usually to keep the strain genetically constant with an easy and efficient breeding protocol. A self-supporting stem line colony of 10–40 breeding pairs, which are mated brother $\times$ sister, can provide breeding stock for one or more expansion colonies of several hundred breeding pairs. These may be mated at random for 2–3 generations without any deleterious consequences, provided the strain is fully inbred to F >20.

Genetic quality control or genetic monitoring is essential as a single nonstrain mating can destroy an inbred strain. The following methods have proved useful, though the last method is likely to be most widely used in future.

Skin grafting

Skin grafts between members of the same strain should not be rejected. This provides a way of testing for isogenicity, and genetic contamination by a non-strain mating. However, it cannot easily be used to confirm that the strain is AKR rather than BALB/c (both of which are albino), and it takes 5–6 weeks to ensure that grafts are not rejected due to a weak incompatibility.

Protein polymorphism

Polymorphic loci, in which different alleles can be detected electrophoretically, can be used as genetic markers. For example, strain C57BL carries the Hbb^s allele, and DBA/2 carries the Hbb^d allele coding for the hemoglobin β chain. These give a single and a double band on the gel, respectively, following electrophoresis of a red blood cell lysate. The disadvantage is that these biochemical methods require considerable skill, and will not normally distinguish between congenic strains which differ, say, only at the major histocompatibility complex (MHC).

Serological methods

A strain-specific alloantiserum can be produced by injecting lymphocytes from 5–10 different strains with various lymphocyte alloantigens into animals of the strain to be monitored in the future. Such an antiserum will not react with lymphocytes from the host strain, but will usually react with lymphocytes from other strains. Similar serological methods can be used with monoclonal antibodies to detect the presence of polymorphic alloantigens (e.g. on lymphocytes), which can be compared with published data for the strain.

DNA polymorphisms

DNA fingerprinting, DNA probes for minisatellite loci and restriction fragment polymorphisms offer powerful methods for genetic quality control. However, 'microsatellite' markers appear to be even more convenient for most purposes. More than 6000 microsatellite markers identifying specific locations on all chromosomes have been described and mapped in the mouse, and a similar number will be available soon in the rat. These are highly polymorphic in the number of repeats, and can be detected

rapidly using the appropriate primers and the polymerase chain reaction followed by gel electrophoresis. These provide a powerful tool for genetic quality control, requiring only very small quantities of DNA. Data on microsatellites are available by e-mail (see Further reading).

Properties of inbred strains

The properties of these strains can be briefly described as follows.

Isogenicity All individuals of an inbred strain are genetically identical at all but a handful of loci. The consequence is that a single individual of the strain can be typed, say at the MHC or a microsatellite locus, and this will serve to type all members of the strain. In this way, a genetic profile of the genes present in the strain can be built up and used in the planning and interpretation of experiments. Isogenicity also ensures that cells, tumors and organs can normally be exchanged within a strain without immunological rejection. It also ensures that a new colony established from one or more pairs will be an exact genetic representation of the parent colony. This is not the case with a noninbred colony, where an individual only has a sample of the genes present in the colony.

Homozygosity Each inbred individual is homozygous at almost all loci. There are no 'hidden' recessive genes which are not expressed because of the presence of their normal allele. Within-strain matings should breed true, with all offspring genetically identical to the parents, apart from any new mutations that might occur. Note that F1 hybrids are heterozygous at all loci at which the parental strains differed, hence F1 hybrids will not breed true.

Phenotypic uniformity Isogenicity leads to phenotypic uniformity, in the same way that pairs of identical twin humans are more similar than pairs of random individuals. One consequence is that, usually, fewer animals are needed to achieve a given level of statistical precision in an experiment than if outbred animals are used.

Long-term stability In inbred strains, the frequency of all alleles is fixed at zero or one. Genetic change can only occur as a result of residual heterozygosity, or the occurrence of new mutations. Thus inbred strains stay constant for long periods of time, allowing the accumulation of background information which can be used in the planning and interpretation of experiments.

Identifiability Once the genetic profile at a number of polymorphic (e.g. microsatellite) loci is known, this information may be used to identify the strain. In contrast, there is no way of checking the genetic authenticity of outbred stocks such as Wistar rats or Swiss mice.

Sensitivity Inbred strains are more sensitive to adverse environmental conditions than are outbred stocks, so must be maintained under good conditions.

International distribution Because of their long-term stability, isogenicity, identifiability and homozygosity, colonies distributed throughout the world will stay very similar to the parental colony for several years, so research done on such strains is comparable. In contrast although outbred 'Wistar' rats or 'Swiss' mice (for example) have an international distribution, each colony is in fact genetically unique.

Individuality Each inbred strain represents a unique combination of genes at about 30 000 different genetic loci, resulting in a unique phenotype. For example, there are strains with a high and others with a low level of spontaneous tumors, immune responses to particular antigens, spontaneous activity or relative brain weight, to give just a few examples. Care must be taken to ensure that the chosen strain is suitable for the particular experiment.

Fitness

During the process of inbreeding, some deleterious genes become fixed in a strain. As a result, inbred strains tend to be less vigorous, and have a poorer breeding performance than outbred stocks. F1 hybrids show hybrid vigor and are generally fitter as a result of deleterious recessive genes being 'covered up' by more advantageous genes present in one or other of the parental strains.

Research uses

Some experiments, such as those involving the transfer of cells or tissues between individuals without immunological rejection, can usually only be done with inbred strains or immunodeficient strains (which are not always easy to maintain). In other

cases, comparison of the responses of two or more strains, particularly if the strains differ at a relevant genetic locus, can be highly informative.

There are a few experiments for which inbred strains are unsuitable. In some cases, their use is rejected because they are more expensive than outbred animals, though this may be a false economy. They may be unsuitable for experiments which require a very good breeding performance, and for certain genetic experiments such as those aimed at changing strain characteristics by selection. Generally, the advantages of inbred strains are so overwhelming that, where available, they should be used for all research unless a case is specifically made to use an alternative type of stock.

See also: Congenic mice; Genetic analysis at the molecular level; Genetic analysis at the phenotypic level; Guinea pig, inbred strains of; Immune response (Irr) genes; Mouse inbred strains; Mouse inbred strains, origins of; Rat, inbred strains of.

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- genome_database@genome.wi.mit.edu An e-mail message with the single word *help* sent to this address should provide access to an extensive list of microsatellite markers of the mouse with full details of the required primer sequences and the map position of the marker. These can be used for genetic quality control and genetic mapping.

INFERTILITY, IMMUNOLOGICAL CAUSES OF

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According to the common definition of infertility, a couple is regarded as being infertile if conception has been pursued unsuccessfully for 2 years or more. The incidence of infertility varies in different parts of the world. In Western countries it has been estimated that 12–15% of all couples eventually experience infertility, either primary or secondary in nature. Besides a general physical examination, investigations of infertile couples include semen analysis and tests for ovulation and tubal function. However, in 12–25% of cases, these basic investigations reveal no significant abnormalities, and such cases are commonly referred to as ‘unexplained’ infertility.

Since the 1950s, autoimmune responses to organ-specific reproductive tract antigens in both males and females and so-called isoimmune reactions of females towards components in semen have been extensively studied as possible causes of ‘unexplained’ infertility, leading to the conclusion that immune responses may cause or contribute to the infertility in about 10% of these couples.

Immunity to semen

Semen contains a multitude of antigens, in seminal plasma as well as in sperm. Some overlap between the two groups of antigens exists because some seminal plasma antigens adhere so firmly to the sperm membrane that they cannot be removed by washing (sperm-coating antigens). However, not all of these antigens are semen specific and only some are able to induce auto- or isoimmunity. A glycoprotein in seminal plasma may rarely induce immunoglobulin E (IgE) antibodies in women, causing severe anaphylactic reactions immediately after exposure to semen. More important, sperm-immobilizing antibodies to a sperm-coating carbohydrate antigen (N-acetylglucosamine) have been found in about 15% of women with unexplained infertility in Japan, but apparently not in other parts of the world. The possibility that the immune response might be caused by a cross-reacting microorganism has therefore been discussed.

The sperm-specific autoantigens are listed in **Table 1** together with some of the techniques used to detect the corresponding autoantibodies. More recently, by means of monoclonal antibodies a number of sperm-specific antigens implicated in gamete interactions have been identified and characterized, e.g. PH-20,

Table 1 Autoantigens in human sperm

<i>Antigen</i>	<i>Localization</i>	<i>Detection of antibodies</i>
Glycoproteins (3 or more)	Membrane in intact sperm	Immunobead test, MAR, agglutination and immobilization tests
LDH-C4, acrosin, hyaluronidase	Enzymes in the acrosome	Radioimmunoassay (for LDH-C4)
Protamine 1 and 2	Sperm nucleus	Immunofluorescence on swollen sperm heads
Various poorly characterized subsurface antigens	Acrosome, equatorial segment, postnuclear cap, main tail piece, tail end-piece	Immunofluorescence on fixed spermatozoa

FA-1, SP-10, but is not yet clear to what extent they are true autoantigens.

For many years, sperm antigens were considered sequestered, i.e. normally secluded from contact with immunocompetent cells and thus unable to induce immunological tolerance. This theory has now been questioned, and local immunoregulatory factors have been assumed to play a role in preventing immune responses. Nevertheless, sperm-reactive antibodies are quite often present in human sera. Using the immunoblotting technique, nearly 50% of male and female sera have been found to react with at least one band, but since reactions are observed as frequently with sera from fertile as from infertile persons, most of these antibodies are obviously of no interest in relation to fertility. So far, only immune responses to the membrane glycoproteins (i.e. the antigens which are accessible for antibody binding in intact sperm) have been closely linked with subfertility or infertility.

Also lactate dehydrogenase C4 isoenzyme (LDH-C4) is expressed in the sperm membrane, and in immunization experiments with mice, rats and baboons, high levels of LDH-C4-specific antibodies have been found to interfere with female fertility. However, the significance of such responses in humans has not been clarified. By means of a sensitive radioimmunoassay, modestly elevated levels of antibodies reacting with LDH-C4 have been detected

in about 10% of males and females with unexplained infertility.

Antibodies to sperm membrane antigens

The occurrence of sperm-specific antibodies in 60–70% of vasectomized men shows that occlusion of the efferent ducts can induce autoimmunity, and infections may also stimulate immune responses, possibly through an adjuvant effect. However in most cases no explanation for the immune response can be found.

Male laboratory animals immunized with homologous sperm in complete Freund's adjuvant develop orchitis with destruction of the germinal tissue, but in men the autoimmunity apparently does not affect spermatogenesis. Apart from modest deposits of immunoglobulin around seminiferous tubules, testicular histology has been normal in most men with sperm-specific antibodies who (for other reasons) have had testis biopsies performed, and semen samples often show normal sperm counts and normal motility. However, the presence of antibodies on motile sperm can be demonstrated by simple techniques such as the immunobead-binding test, in which particles covered with anti-immunoglobulin attach to the sperm, or the rather similar mixed antiglobulin reaction (MAR). The use of class-specific anti-immunoglobulins in these tests has led to the observation that IgA antibodies (but not IgG antibodies) impair or inhibit the migration of sperm through cervical mucus. Sperm covered with IgA are seen to shake vigorously in the mucus without forward progression. This explains the strong fertility reducing effect of locally produced IgA antibodies in semen or cervical mucus.

The significance of IgG antibodies, which predominate in serum and are transudated to semen (1% of serum concentration), is less clear, because in men from infertile couples IgG antibodies are rarely found without IgA antibodies also being present. The percentage of eggs being fertilized by *in vitro* fertilization (IVF) is usually reduced when the sperm reveal strong immunobead or MAR reactivity, but also here IgA may be playing the major role. *In vitro* experiments with human IgG antibodies have indicated that in certain cases they could block fertilization, but on the other hand vasovasostomized men with only IgG anti-sperm responses have been found to have the same high fertility rate as vasovasostomized men without sperm-specific antibodies. The explanation why responses with only IgG antibodies are rarely seen among men from infertile couples might therefore be that such men usually do not end up in infertility clinics!

Evidence of immunological subfertility or infertility, i.e. strong IgG and IgA reactivity in immunobead test or MAR combined with detection of free antibodies in seminal plasma or high titers in serum, is found in approximately 10% of the men from couples with unexplained infertility.

In women, most laboratories have found sperm-specific antibodies in serum to be rare and generally present only in low titers. The explanation seems to be that women are exposed only to sperm mixed up with seminal plasma, which contains potent immunosuppressive factors, some of which adhere to the sperm membrane as sperm-coating substances. A multilaboratory study revealed significant immune responses in only 2% of women from couples with unexplained infertility. Detection of antibodies in liquefied cervical mucus (e.g. by indirect immunobead test) seems more informative, particularly since antibodies can apparently occur in cervical mucus without being detectable in serum.

High-dose corticosteroid therapy – with a 30–40% success rate – is now rarely used due to occasional severe side-effects (aseptic necrosis of femoral head). To overcome the impaired migration of IgA-covered sperm through cervical mucus, intrauterine insemination has been used with some success. In cases with strong immunization, IVF may offer the best choice. Although the fertilization rate is usually reduced, once fertilization has occurred the pregnancy rate seems to be the same as for patients without sperm-specific antibodies.

Immunity to zona pellucida

In the mid-1970s, animal studies revealed that the zona pellucida (zp) contains several organ-specific glycoproteins (now known as ZP1, ZP2 and ZP3) and that antibodies to these antigens were capable of blocking fertilization *in vitro*. Human and porcine zp antigens showed cross-reactivity, and interest in this antigen–antibody system was therefore stimulated in 1977 when a high percentage of sera from infertile women by immunofluorescence was found to stain porcine zonae. However, when similar reactivities were subsequently observed, also with sera from fertile females and even with male sera, the specificity of the reactions had to be questioned, and when absorption with pig and human erythrocytes was found to remove the reactivity of most of the sera, serious doubt was raised concerning the presence and clinical significance of zona-specific antibodies. After 20 years and numerous studies there is still no convincing evidence for the occurrence of true zona-specific autoantibodies as the cause of human infertility.

In one of the best controlled studies, with a passive hemagglutination technique using purified porcine zona material for coating of the erythrocytes, weakly positive reactions were recorded in 5.6% of 320 women with unexplained infertility, in 1.7% of 1552 women with infertility of known cause, in 1.5% of 193 fertile women, but in none of 292 men.

In several animal species (dog, rabbit, horse and monkey) active immunization with porcine zona has, on the other hand, produced long-term infertility. The immunization usually also leads to abnormalities in ovarian structure and function with disruption of folliculogenesis and changes in menstrual cycle, and in veterinary medicine immunization with pig zona material is now used to sterilize bitches.

Immunity to trophoblast

Trophoblast-specific antigens have been identified in the syncytiotrophoblast plasma membranes. Therefore, since the surface of the trophoblast is exposed to maternal immunocompetent cells, strong immunoregulatory mechanisms must have been developed during evolution. At the same time as recognition by the maternal immune system of the semi-allogeneic trophoblast seems necessary for the maintenance and growth of the fetus, immune responses to the organ-specific antigens in the trophoblast are apparently effectively prevented. Even though low levels of antibodies reactive with these antigens have occasionally been detected in female sera, there is no

evidence that such responses play a role in infertility. Antibodies to human chorionic gonadotropin (hCG) which, as shown in recent trials of hCG vaccines for immunological contraception, can cause infertility in women, do apparently not occur as 'natural' antibodies.

See also: Autoimmunity; Contraception, immunological; Fetus as allograft; Hypersensitivity reactions; Immunosuppression; Privileged sites; Tolerance, peripheral.

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INFLAMMATORY BOWEL DISEASE

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Inflammatory bowel disease (IBD) consists of two major illnesses, ulcerative colitis and Crohn's disease, which are chronic disorders of the intestine of unknown origin. Both diseases exhibit chronic inflammatory changes consisting of an intense infiltration of macrophages and lymphocytes with large numbers of plasma cells. In ulcerative colitis, the pathological changes include diffuse inflammation with mucosal ulcers. Involvement is restricted primarily to the mucosa, and not to deeper layers, such as muscle. In Crohn's disease, the inflammatory infiltrate frequently forms granulomas and may extend through all layers of the bowel wall. In both diseases, chronic inflammatory changes dominate the

histological picture. However, Crohn's disease and ulcerative colitis can also exhibit acute inflammatory components. In active inflammatory bowel disease, there is a constant movement of acute inflammatory cells, including neutrophils and monocytes, from the circulation into the inflamed mucosa.

Research into the immunological abnormalities of IBD originally focused on describing alterations in various inflammatory and immune mechanisms. A large body of information has been accumulated over the last 50 years from studying many such factors. More recently, researchers have tried to understand how inflammatory processes are initiated and why the normal mechanisms controlling resolution of

inflammation may be ineffective. While most investigators agree about the inflammatory changes established in the intestine in these conditions, there is still little consensus as to how they are initiated in diseased subjects. Possibilities include an enhanced permeability of the mucosal barrier (Figure 1); inappropriate T cell activation; bacterial and viral etiologies; and vasculitis in the microcirculation of the intestine.

As a host defense mechanism to infectious agents, acute intestinal inflammation is usually self-limiting. However, in ulcerative colitis and Crohn's disease, the normally protective T and B cell immune responses are not appropriately regulated resulting in prolonged activation of mucosal effector cells which may cause damage to the intestine. The immune and inflammatory processes within the intestine may, therefore, exacerbate and perpetuate intestinal injury in inflammatory bowel disease.

Lymphocyte activation

Peripheral blood lymphocytes

Recent studies utilizing monoclonal antibodies against T lymphocytes and their subsets have not

demonstrated any differences between IBD patients and untreated controls. Patients with long-term, steroid-treated Crohn's disease may have lymphocytopenia (particularly T cells), depending on disease duration. However, patients with Crohn's disease exhibit activation markers of peripheral blood T cells in IBD. In one study, the early lymphocyte activation marker, T9 antigen, was expressed by 24% of active Crohn's disease peripheral blood T lymphocytes and by only 10% of inactive Crohn's disease peripheral T lymphocytes. Autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, were also associated with an increased state of activation of T cells. In contrast, T cell activation was not increased in bacterial or viral colitis. In IBD, a high percentage of activated T9 antigen-positive T cells coexpressed Fc receptors for polymeric immunoglobulin A (IgA), a finding not present in the autoimmune disorders that also exhibited increased peripheral blood T cell activation.

During lymphocyte activation, soluble interleukin 2 (IL-2) receptor molecules may be shed from the surface of T cells or macrophages and serum soluble IL-2 receptor levels are increased in Crohn's disease

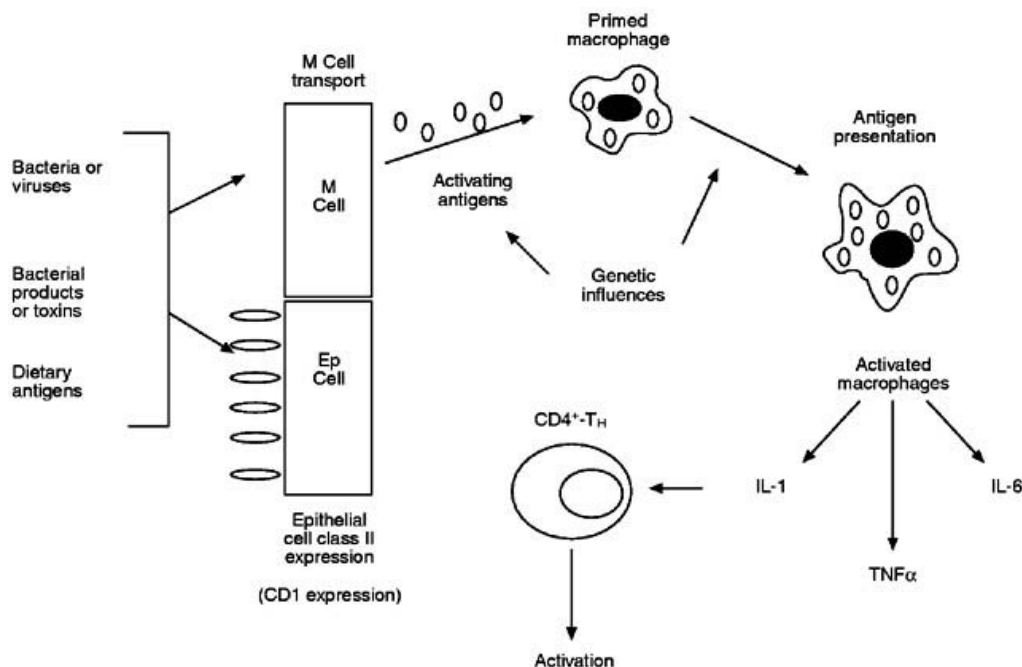


Figure 1 Possible activating events in IBD. Bacterial cell wall products, infectious agents and/or toxins can be transported across M cells or presented by class II antigens on epithelial cells to prime and activate macrophages. After antigen presentation, the primed and activated macrophages synthesize and secrete a variety of molecules including proinflammatory cytokines, such as IL-1, IL-6 and TNF α . The proinflammatory cytokines in turn activate a number of cell-mediated events and processes, including activating CD4 $^{+}$ T helper cells in the intestine. In IBD, macrophages and lymphocytes are in a highly activated and upregulated state with the resultant secretion of proinflammatory and immunoregulatory molecules. (Adapted with permission from Reinecker H-C, Schreiber S, Stenson WF and MacDermott RP (1994) The role of the mucosal immune system in ulcerative colitis and Crohn's disease. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee JR and Bienenstock J (eds) *Handbook of Mucosal Immunology*, p 440. London: Academic Press.)

patients. Crohn's disease peripheral blood mononuclear cells have an enhanced capacity to secrete soluble IL-2 receptor, both spontaneously and after stimulation with phytohemagglutinin (PHA) *in vitro*. The increased capacity of mononuclear cells isolated from intestinal lamina propria to release soluble IL-2 receptor is not specific to IBD because similar findings have been observed in colonic diverticulitis. Nevertheless, increased serum levels of soluble IL-2 receptor in Crohn's disease patients correlate with intestinal inflammatory activity, and the receptor is detected in the portal venous system from inflamed intestine of Crohn's disease patients. These studies, therefore, demonstrate that activated lymphocytes in IBD may be a central feature of the immunological changes that result in inflammation (Figure 2). Measurement of such activation molecules provides a useful marker to correlate the level of tissue inflammation with ongoing disease activity.

Intestinal lymphocytes

Immunohistological studies of the inflammatory cells in IBD mucosa have yielded conflicting results regarding intestinal lymphocyte activation in

Crohn's disease and ulcerative colitis. A number of workers have demonstrated the increased expression of lymphocyte activation antigens, such as 4F2, transferrin receptor, IL-2 receptor, and HLA-DR in Crohn's disease and ulcerative colitis, whereas other groups have failed to do so. Studies by James and colleagues found that IL-2 mRNA levels were elevated in the mucosa of patients with Crohn's disease but not ulcerative colitis. In contrast, Allison and colleagues demonstrated the appearance of the CD7 activation marker but no increase in IL-2 receptor expression. Studies examining flow cytometric analysis of isolated colonic lamina propria mononuclear cells revealed increased expression of activation markers, including IL-2 receptor, transferrin receptor, and the 4F2 antigen on intestinal B and T cells as well as CD4⁺ and CD8⁺ T lymphocyte subpopulations in both active ulcerative colitis and Crohn's disease. These studies provide evidence that activation of B and T cell lymphocyte populations occur in the intestine as well as in the peripheral blood of patients with IBD.

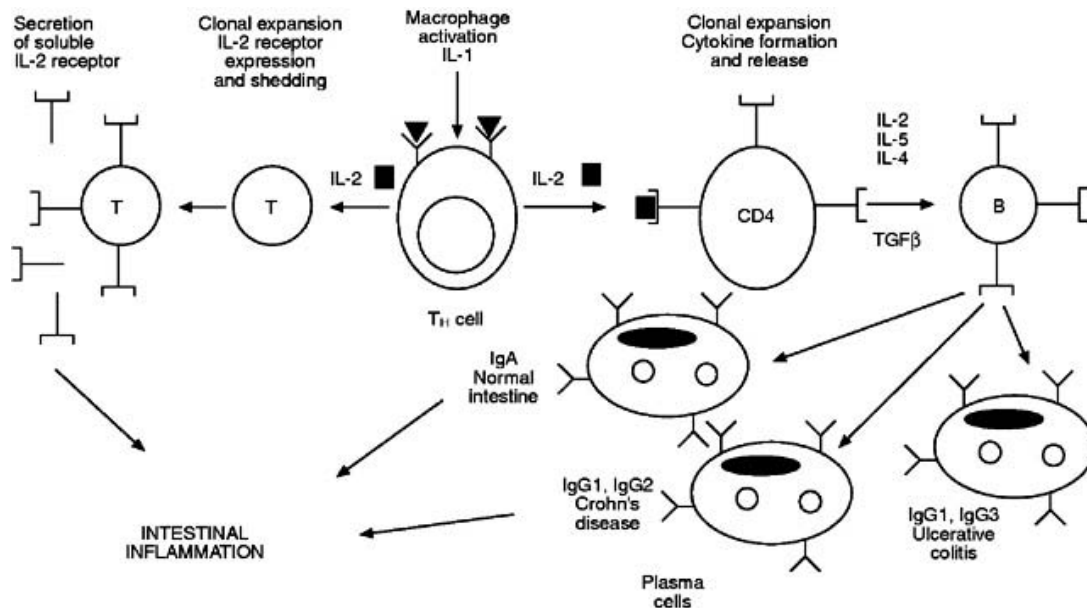


Figure 2 Cell-mediated immunity in IBD. After luminal bacterial cell wall products lead to macrophage activation, specific antigen presentation occurs, resulting in the activation and expansion of CD4⁺ T helper lymphocytes. The activation of T helper cells leads to enhanced B cell proliferation and maturation, resulting in increased numbers of IgG-producing plasma cells. Resultant secretion of soluble IL-2 receptor by activated mucosal lymphocytes involved in the inflammatory process leads to increased levels of circulating soluble IL-2 receptor, which can be found to correlate with disease activity. In the normal intestine, the predominant immunoglobulin isotype secreted by B cells is IgA, which protects against pathogens by antigen exclusion. IgA overall increase in IgG-secreting lamina propria B cells has been demonstrated. In marked contrast to IgA, IgG is capable of fixing complement, arming NK cells and activating phagocytes. The increase in IgG subclasses is composed primarily of IgG1 and IgG3 in ulcerative colitis, whereas in Crohn's disease the increase is predominantly in IgG1 and IgG2. (Adapted with permission from Screiber S, Raedler A, Stenson WF and MacDermott RP (1992) The role of the mucosal immune system in inflammatory bowel disease. In: MacDermott RP and Elson CO (eds) *Gastroenterology Clinics of North America*, 21: 453. Philadelphia: WB Saunders.)

Immunoglobulin synthesis and secretion

Intestinal mononuclear cells from healthy mucosa normally secrete large amounts of IgA. In IBD, IgG cells are increased relative to IgA cells and are present in deeper tissue layers. The mucosal immune system exhibits a markedly heightened IgG immune response because of defective or altered immunoregulation. In IBD, intestinal and peripheral blood mononuclear cells secrete increased immunoglobulin, particularly of the IgG isotype. The greatest increase in spontaneous IgG secretion is seen in ulcerative colitis where intestinal mononuclear cells secrete predominantly IgG1 and IgG3. Crohn's disease intestinal mononuclear cells also exhibit increased IgG1, but secrete IgG2 rather than IgG3 (Figure 2).

The increased secretion of IgG by isolated intestinal mononuclear cells *in vitro* is most likely due to the increased numbers and skewed ratios of intestinal plasma cell populations in IBD. Compared with control specimens, the number of IgG-containing cells is 30 times greater than normal, while the number of IgA-containing cells is double that of normal tissue. There are significantly fewer IgG-containing cells in biopsy specimens of patients with acute infectious colitis when compared to IBD.

IgG1 and IgG3 antibodies, which are increased in ulcerative colitis, are more potent activators of complement and opsonins than are IgG2 and IgG4. They are the major antibodies produced in response to protein antigens. IgG2, on the other hand, is the predominant antibody that responds to carbohydrates and many bacterial antigens. The increased production of IgG2 in Crohn's disease may represent an inappropriate immune response that could partially contain, but not completely eradicate, a chronic infectious process. The delineation and analysis of immunoglobulin isotype regulation may provide important insights into the pathophysiological mechanisms underlying ulcerative colitis and Crohn's disease.

Complement pathway activation

Activation of complement results in the generation of proinflammatory products which may ultimately lead to tissue destruction. Various studies have demonstrated the possible role of complement in tissue damage in ulcerative colitis. Monoclonal antibodies against a neoepitope exclusively expressed by activated C3b and the cytolytically active terminal complement complex have been used to identify potential complement-induced damage in tissue sections from inflamed IBD intestine. Eighty per cent (9 of 11) of patients with ulcerative colitis showed

activated C3b deposited apically on the surface epithelium of involved mucosa. No deposits were seen in 31 matched noninflamed specimens or in 94% (16 of 17) of healthy controls. Strong colocalization of IgG1, activated C3b, and terminal complement complex was shown in 36% (4 of 11) of ulcerative colitis patients. Moreover, increased vascular deposition of terminal complement complexes occurred in both ulcerative colitis and Crohn's disease. Both C3 and C4 levels are elevated in jejunal perfusates of Crohn's disease patients when compared to healthy controls. Complement activation may initiate acute as well as chronic destruction of intestinal tissues and may result in the enhanced activation of granulocytes and macrophages leading to the increased release of potent chemotactic molecules by neutrophils (Figure 3).

Granulocyte and macrophage function

Peripheral blood granulocytes and monocytes isolated and radiolabeled with indium-111 *in vitro* and reinjected into patients, migrate to inflamed areas of the intestine. In one study, more than 90% of radiolabeled phagocytes accumulated rapidly in the inflamed intestine of 91% (20 of 22) of Crohn's disease patients. A further study on 15 patients with ulcerative colitis showed enhanced migration of similar cells into areas of inflamed bowel. The migration of leukocytes from the vascular lumen into the inflamed bowel is mediated through adhesion molecules, particularly LFA-1 (lymphocyte function-associated antigen 1, CD11a), which binds to ICAM-1 (intercellular adhesion molecule 1, CD54). In IBD, the expression of ICAM-1 on endothelial cells is increased and is accompanied by an increase of CD11a on mononuclear phagocytes (Figure 4).

Cytokine production

Inflammatory events are mediated partly through the effects of various cytokines, including IL-1 α , IL-1 β , IL-6, or tumor necrosis factor α (TNF α). Both IL-1 α and IL-1 β induce fever, stimulate acute phase protein synthesis, and activate the immune system. TNF α causes hemodynamic shock and cachexia in a number of disease states. Both TNF α and IL-6 enhance cellular catabolism, induce acute phase proteins, and stimulate pyrogenic activity. TNF α activates endothelial cells and can induce IL-1 and IL-6. Both TNF α and IL-2 stimulate prostaglandin I₂ (PGI₂), PGE₂ and platelet-activating factor (PAF) secretion by cultured endothelial cells.

Levels of IL-1 are increased in inflamed mucosa from patients with both Crohn's disease and ulcer-

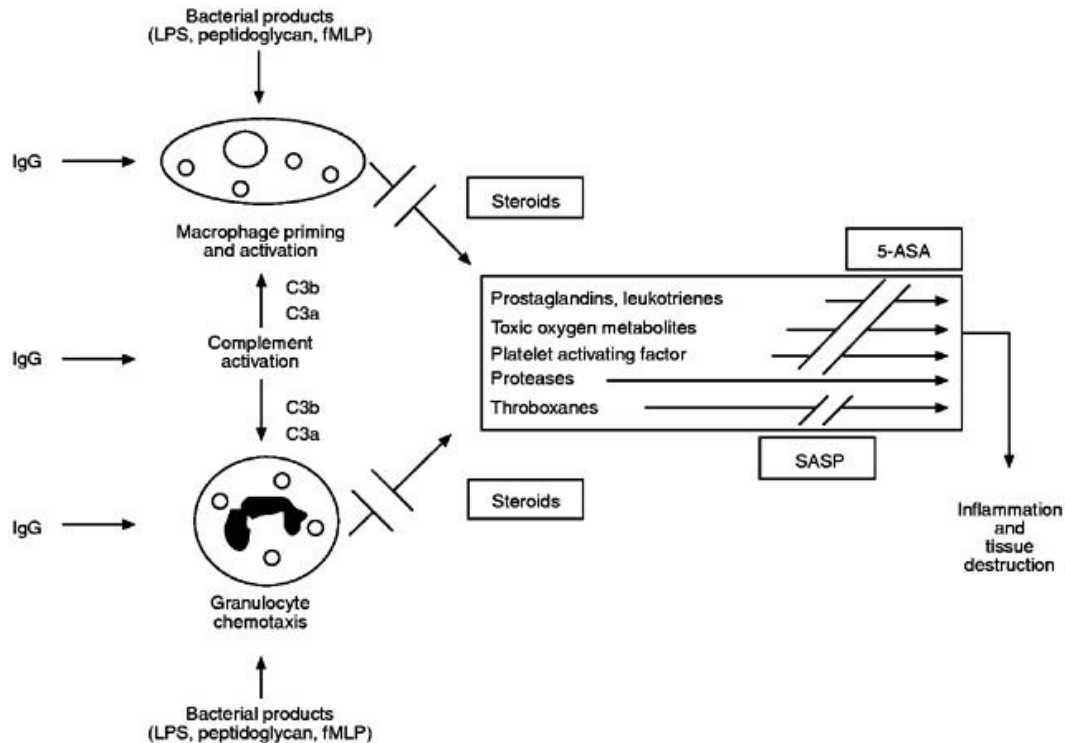


Figure 3 Mechanism of tissue injury in IBD. Inflammatory processes can be dramatically increased by a variety of mechanisms, including: 1) direct activation of granulocytes or neutrophils by bacterial products, 2) priming of phagocytes directly by IgG, or 3) specific immunological effector events mediated by IgG activation of the complement cascade. After priming or activation of granulocytes and macrophages, a host of destructive inflammatory mediators and toxic oxygen radicals can lead to direct tissue damage by a variety of destructive mechanisms. The two primary agents used to treat IBD patients (steroids and 5-ASA products) are effective because of their ability to inhibit many of the pathways that lead to inflammation-induced injury. (Adapted with permission from Scriber S, Raedler A, Stenson WF and MacDermott RP (1992) The role of the mucosal immune system in inflammatory bowel disease. In: MacDermott RP and Elson CO (eds) *Gastroenterology Clinics of North America*, 21: 453. Philadelphia: WB Saunders.)

ative colitis. The spontaneous secretion of IL-1 β is increased in intestinal lamina propria mononuclear cells isolated from IBD patients when compared with normal controls.

Lipopolysaccharide further enhanced IL-1 β production by IBD lamina propria mononuclear cells, but not by those from normal controls. Increased IL-1 concentrations play a key role in the pathogenesis of rabbit immune complex colitis, and tissue levels of IL-1 correlate with the severity of inflammation. Lamina propria mononuclear cells isolated from endoscopic biopsy specimens from patients with active IBD spontaneously secrete high amounts of IL-1 β .

Using polymerase chain reaction, higher levels of IL-1, IL-6, and TNF α mRNA have been demonstrated in Crohn's disease and ulcerative colitis patients than in normal subjects. MacDonald and colleagues using a spot ELISA technique, demonstrated that TNF α -secreting intestinal mononuclear cells are increased in frequency in both Crohn's disease and a subgroup of ulcerative colitis patients in comparison with normal controls. These obser-

vations have therapeutic implications because antibodies against TNF α are now undergoing trials as a form of treatment.

The spontaneous secretion of IL-6 is increased by lamina propria mononuclear cells from ulcerative colitis patients. This secretion appears to be less marked in Crohn's disease than in ulcerative colitis. Many of the biologic activities of IL-1, IL-6, and TNF α that amplify inflammatory and immunological processes overlap. Therefore, sustained inflammation leading to tissue destruction in IBD could potentially be mediated by the potent proinflammatory activities of IL-1 α , IL-1 β , IL-6, and TNF α (Figure 4).

The role of chemotactic cytokines in the pathophysiology of ulcerative colitis and Crohn's disease has just begun to be examined. Chemokines may play an important role in the perpetuation of inflammation in IBD because, in contrast to other chemoattractant molecules, such as leukotriene B₄ (LTB₄), formyl-Met-Leu-Phe (fMLP) and PAF, they are relatively resistant to inactivation *in vivo*. Furthermore, chemokines are rapidly produced by a number of different cell types after stimulation by proinflammatory

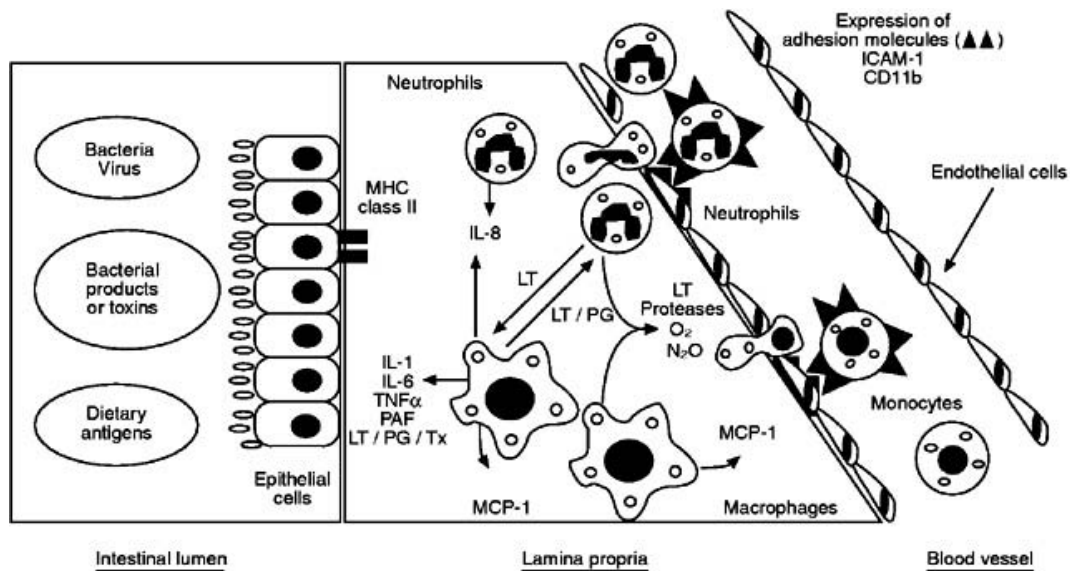


Figure 4 Phagocytic activation and inflammatory events in IBD. Bacterial cell wall products from the intestine prime monocytes and granulocytes to increase the expression of adhesion molecules with increased recognition of endothelial cell receptors. Macrophages and granulocytes migrate from the capillaries and venules into the intestinal lamina propria, from which they migrate into the intestinal lumen through the intestinal wall. The primed macrophages and granulocytes within the intestinal lamina propria are activated by bacterial cell wall products, such as LPS, as well as by antigenic luminal factors to release a variety of proinflammatory cytokines and mediators including IL-1, TNF α , IL-6, PAF, leukotrienes and prostaglandins (PG/Tx). In addition, destructive molecules, such as oxygen radicals and proteases, are produced. The involvement of granulocytes and macrophages in IBD adds an acute inflammatory component to the chronic cell-mediated intestinal injurious processes involved in IBD.

cytokines. Chemokines are relatively resistant to inactivation and have comparatively long half-lives *in vivo*. They are secreted by many different cell types and have specific chemotactic activities with regard to defined subtypes of leukocytes. Therefore, chemokines may play pivotal roles in the perpetuation and upregulation of the chronic inflammatory processes in ulcerative colitis or Crohn's disease patients (Figure 4). IL-8, an α -chemokine, is a potent neutrophil chemoattractant and activator. Increased synthesis and production of IL-8 is present in ulcerative colitis. Very high levels of IL-8 mRNA have been observed in involved ulcerative colitis mucosa and moderately increased IL-8 mRNA levels in involved Crohn's disease mucosa. Involved intestinal mucosal sections exhibited higher levels of IL-8 mRNA than noninvolved mucosal sections obtained from the same patients. Monocyte chemoattractant protein 1 (MCP-1), a β -chemokine and a potent monocyte chemoattractant and activator, is also increased in inflammatory bowel disease.

Prostaglandin production

Prostaglandins are synthesized from arachidonic acid catalyzed by the enzyme cyclooxygenase. Prostaglandins, particularly the E series, have biologic properties that can enhance microvascular permeability with subsequent edema, vasodilatation and

induction of pain. In IBD, there is an increased level of prostaglandin production. Elevated levels of prostaglandins are found in the stool, venous blood and rectal mucosa in IBD. Prostaglandin levels in IBD in mucosa, serum or rectal dialysates correlate with disease activity. Successful medical management results in reduction in prostaglandin levels.

There is some evidence, however, that contradicts the role of prostaglandins as proinflammatory mediators in IBD. The use of potent cyclooxygenase inhibitors, indomethacin and other nonsteroidal anti-inflammatory drugs (NSAIDs), results in both a decrease in prostaglandin synthesis and in clinical improvement in rheumatoid arthritis and other inflammatory diseases. However, NSAIDs have no role in the medical management of IBD. Small trials of indomethacin administered orally or rectally revealed no improvement in ulcerative colitis. Some reports have shown that both indomethacin and flurbiprofen may cause clinical deterioration despite causing a decrease in prostaglandin production. The failure of NSAIDs to induce clinical improvement in IBD suggests that the secretion of prostaglandin and other inflammatory mediators such as PAF, leukotrienes and proinflammatory cytokines including chemokines, must be inhibited concurrently to reduce tissue injury.

Leukotriene synthesis

Leukotrienes are derived from arachidonic acid via the 5-lipoxygenase pathway found primarily in cells of bone marrow origin involved in the inflammatory process, including mast cells, neutrophils, monocytes and macrophages. The major products of this pathway are 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and leukotrienes B₄ (LTB₄), C₄ (LTC₄), D₄ (LTD₄), and E₄ (LTE₄). LTB₄ and 5-HETE are potent neutrophil chemoattractants. LTB₄ also increases vascular permeability. The sulfidoleukotrienes (LTC₄, LTD₄, LTE₄) induce smooth muscle contraction in the lung, blood vessels and gastrointestinal tract.

IBD mucosa produces larger quantities of lipoxygenase and cyclooxygenase products than normal mucosa. Lipid extracts of IBD mucosa contain large amounts of LTB₄, up to 50-fold as much as normal mucosa. Levels of LTB₄ are markedly higher in rectal dialysates from ulcerative colitis patients and decrease to normal levels after treatment with prednisolone. Crohn's colitis mucosa produces a three-fold increase in sulfidoleukotrienes (LTC₄, LTD₄, LTE₄) compared to normal mucosa.

Conclusions

In IBD, the state of activation of the intestinal immune system and mucosal inflammatory pathways are markedly upregulated. The mechanisms that normally downregulate the immunologic and inflammatory protective processes, once the inflammatory stimulus has been cleared, appear to be deficient or are suboptimal. Knockout mice that do not express IL-10, a cytokine that normally downregulates immune responses, have a number of features of inflammatory bowel disease. It is possible that

changes in immunological responsiveness in IBD are genetically controlled. A critical area of investigation is the elucidation of the genetic factors that predispose certain individuals either to having a heightened immune and inflammatory response or an inability to downregulate ongoing chronic immunologic and inflammatory events.

See also: Acute inflammatory reaction; Adhesion molecules; Antigen, entry into the body; Chemokines; Chemotaxis; Cytokines; Endotoxin (lipopolysaccharide (LPS)); Macrophage activation; Monocyte chemotactic protein 1 (MCP-1); Mucosal immunity; Prostaglandins.

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INFLAMMATORY BOWEL DISEASE, ANIMAL MODELS

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Environmental, immune and genetic susceptibilities have all been implicated in the pathogenesis of inflammatory bowel disease (IBD). The development of various experimental models (Table 1) has enabled researchers to study the development of inflammation in the intestine, under conditions where the underlying lesion is better understood.

Using recombinant DNA technology to target gene insertions and deletions, mutant animals with known genetic abnormalities are produced. Exogenous agents such as chemicals, bacterial products and immune complexes have also been used in animal models of disease. These models also serve as tools to study therapeutic strategies in IBD.

Table 1 Models of IBD*Genetically engineered*

Dominant negative N-cadherin in intestinal epithelia
 IL-2 deficient
 IL-10 deficient
 T cell receptor mutant
 HLA-B27 transgenic rats
 G α_{12} Knockout
 CD45RB $\rightarrow$ SCID transfer

Spontaneously

Cotton top tamarin
 C3H/HeJ Bir mice

Exogenously induced

Acetic acid
 Immune complexes/formalin
 Indomethacin
 Trinitrobenzene sulfonic acid
 Carrageenan
 Cyclosporine
 Dextran sulfate
 Peptidoglycan polysaccharide

Animal models of inflammation induced by gene disruption

Dominant negative N-cadherin mutants

Cadherins are transmembranous glycoproteins that mediate cell adhesion. The cytoplasmic domain of N-cadherin includes a β -catenin which, in turn, binds an α -catenin. The catenin-cadherin complexes regulate a variety of cell functions, including proliferation, polarity and migration. Disruptions of these interactions result in the development of IBD that closely resembles Crohn's disease. Chimeric mice, generated from normal blastocysts and genetically manipulated embryonic stem (ES) cells, have been used to study N-cadherin function. These B6 $\leftrightarrow$ 129 SV chimeric mice have patches of ES cell-derived, as well as the normal B6-derived, crypt villous units. The ES cell lineage is distinguished from the B6 cells by their ability to bind to *Ulex europaeus* agglutinin type 1. Promoters are available that specifically express genes localized to the intestinal epithelium. ES cells transfected with such a promoter, the fatty acid binding promoter (Fabpi), linked to the negative cadherin (NCAD) was used to generate B6 $\leftrightarrow$ 129 SV NCAD chimeras. Expression of the dominant mutant resulted in loss of endogenous E-cadherin, cell-to-cell adhesion and promoted early entry of epithelial cells into programmed cell death. E-cadherin is normally localized to the basolateral surface of intestinal epithelial cells and apical junctional complexes. Changes, confined to the intestine covered by the ES cell-derived epithelium (which expresses

NCAD) include lamina propria infiltration with lymphocytes and histiocytes, immunoglobulin A (IgA)- and IgG-secreting plasma cells and increased expression of class II major histocompatibility complex (MHC). By 3 months of age, crypt distortion and crypt abscesses appear. Chimeras expressing NCAD along the entire crypt villous axis have greater disease than those with NCAD expression confined to the villous epithelium. In these animals, intestinal dysplasia and neoplasms can also be seen. This animal model exemplifies the importance of the epithelial barrier in the prevention of IBD.

IL-2 knockout mice

Interleukin-2 (IL-2) activates macrophages, lymphokine-activated killer (LAK) cells and natural killer (NK) cells and induces differentiation of B cells, in addition to promoting growth and expansion of T cells. In a study of the function of IL-2 in mice by gene disruption, it was noted that these mice develop a lesion that is similar in many ways to ulcerative colitis. IL-2 knockout mice develop normally until 4 weeks of age. Approximately half die after the first month of age, mainly from anemia, while those surviving more than 10 weeks develop a pancolitis characterized by bloody diarrhea and rectal prolapse. Colonic wall thickening, ulceration, crypt distortion and abscesses are seen on histological examination. Immune abnormalities in the lamina propria include an increase in activated T cells (as demonstrated by markers for CD44 and CD69), in activated B cells and in MHC class II expression, suggesting both T and B cells are stimulated in the colonic immune response. No bacterial pathogen has been identified but germ-free animals do not develop disease, suggesting that an abnormal immune response to enteric flora contributes to the pathogenesis of IBD.

IL-10 knockout mice

Derived from T cells, macrophages and certain B cells, IL-10 is a potent inhibitor of macrophages and T_H1 lymphocytes. IL-10-deficient mice, generated by gene disruption in ES cells, develop intestinal disease resembling IBD, with both small and large intestinal involvement. The enterocolitis is segmental and associated with a chronic inflammatory infiltrate. Loss of epithelial integrity is also present, and there is an increase in class II MHC expression. The disease is ameliorated, but not eradicated by housing mice in a pathogen-free environment, as focal proximal colitis is still evident. Resolution, however, occurs on administration of IL-10 antagonists, suggesting that an aberrant T_H1 cell response, normally inhibited by IL-10, may be responsible.

T cell receptor and MHC class II mutants

Chronic colitis resembling human ulcerative colitis is observed in mice that do not express normal T cell receptors on the T cell surface resulting from an introduced mutation in the β chain. In contrast, disruption in the recombinant activating gene RAG1, which results in animals lacking both B and T cells, does not result in bowel inflammation. Increased B cell activation is observed in the T cell receptor mutant mice with an increase in IgA, IgM and IgG secretion, perhaps due to a lack of T cell-mediated suppression of B cells. MHC class II^{-/-} mice which lack class II-restricted CD4⁺ T cells also develop bowel inflammation, again implicating a loss of immunoregulatory T cells in the development of the disease. The mice with these lesions predominantly exhibit colonic disease. Histological sections show mucin depletion, crypt distortion and a mixed inflammatory histology. Ulceration, granulomas and fibrosis are not seen. The incidence and severity of the disease vary with the genetic background of the mice examined, implying that other genes modify disease susceptibility.

G α_{12} knockout

G protein heterodimers of α , β and γ chains serve as mediators of signal transduction for numerous surface receptors. G α_{12} is widely distributed in intestinal epithelial cells and in lymphocytes. Its functions include activation of MAP kinase, stimulation of tumor necrosis factor α (TNF α) and production of colony-stimulating factor 1. Mice with an introduced deletion of G α_{12} develop pancolitis by 8–12 weeks of age. Approximately a third of the animals develop adenocarcinoma by 15–33 weeks of age. There is an increase in CD4⁺ T cells, and B cells. Unlike the knockouts of IL-2, IL-10 and the T cell receptor, pathogen-free mice continue to develop a pancolitis; however, variation in genetic background affects disease susceptibility.

CD45RB

The T cell and B cell deficiency in SCID mice can be reconstituted by B cell and T cell transfer. If the transferred CD4⁺ T cells express high levels of CD45RB (CD45RB^{hi}), the recipient mice exhibit a diarrhea which is chronic but does not contain blood. Transfer of CD4⁺ cells with low levels of CD45RB (CD45RB^{lo}) does not induce the disease, indeed CD45RB^{lo}, when cotransferred with CD45RB^{hi}, abrogates the disorder. Murine IL-10, interferon γ (IFN γ) and TNF α administration prevents the disease, while antibiotics abrogate the colitis, suggesting a role for bacterial pathogens in the development of the disease.

HLA-B27 transgenic rats

Expression of the human MHC class I haplotype HLA-B27 in rats results in an animal that develops enterocolitis resembling IBD. These rats also exhibit arthropathies in addition to the gastrointestinal lesions. The disease is abrogated in germ-free animals, again suggesting a role for normal colonic bacteria in the pathogenesis of inflammation. The colitis can be transferred to healthy controls by bone marrow cells derived from B27 transgenic rats. Transgenic mice expressing HLA-B27 do not show any abnormal phenotype, suggesting that the genetic background is important. This model, which was the first of the transgenic IBD models to be developed, demonstrates the interrelationship between a specific gene, the environment (bacterial flora) and the genetic background of the animal.

Models of spontaneous colitis

C3H/HeJ Bir mice

A spontaneous colitis occurs in a selective strain of C3H/HeJ mice noted at the Jackson laboratories to have diarrhea and rectal ulceration. The disease coincides with the onset of bacterial colonization and is focal in distribution. It spontaneously resolves as the mice age. The severity of the disease is variable and depends on the animal facility in which the mice are housed.

Cotton top tamarin

A remitting and relapsing colitis arises spontaneously in the cotton top tamarin by the second year of life. This disease, which appears to be found only in animals in captivity, shows considerable similarities to ulcerative colitis. The entire colon is involved. Serum antibodies to gut epithelial cells have been detected but no pathogen has been isolated. The intestinal epithelium exhibits an increased dysplasia with age. Again this animal shows the importance of the interaction between genetics and environmental factors in the development of disease. Some researchers have suggested that it may be a model in which the effects on the severity of colitis of neurogenic and stress factors can be studied.

Intestinal inflammation induced by exogenous agents

Trinitrobenzene sulfonic acid-induced colitis

Administration of an enema containing trinitrobenzene sulfonic acid (TNBS) results in the development of colitis in animals studied. The colitis has the

features of a delayed-type hypersensitivity response to TNBS. Variations are seen in the response amongst different strains of mice, and mice could potentially be used to define genes that control susceptibility of disease. These mice have responded well to drugs used in the treatment of IBD and provide a useful model with which to study newer modalities of treatment.

Acetic acid-induced colitis

Rectal irrigation of Sprague Dawley rats with 1 ml of 4% acetic acid results in epithelial injury and necrosis. The model, although lacking in chronicity, is useful for studying anti-inflammatory drugs. Mice given butyric acid also exhibit colitis, but it is not clear whether this is due to a different mechanism from acetic acid because butyric acid is a much weaker acid; butyrate itself has specific effects on intestinal epithelial cells.

Immune complex/formalin colitis

A model of colitis is seen when 0.45% unbuffered formaldehyde is inserted rectally with 0.85 ml of immune complexes injected into the ear vein of New Zealand white rabbits. The distal colitis is associated with crypt distortion, abscess formation, increased production of IL-1 and leukotriene B₄. Attenuation of the disease is seen when IL-1 antagonists are given, suggesting a central role for this cytokine in the disease. In addition sulfasalazine, mesalamine and azathioprine (all agents effective in treating IBD) decrease the severity of the colitis.

Indomethacin

Dose-dependent ulceration is seen in rodents given subcutaneous injections of indomethacin. Chronic intestinal inflammation results and is associated with extra intestinal manifestations of hepatic inflammation, anemia and leukocytosis. Prostaglandins appear to be protective, while bacteria may promote the inflammatory response. Sulfasalazine, metronidazole and IL-1 antagonists attenuate the disease.

Complex carbohydrate molecules

Guinea pigs given drinking water with 5% degraded carrageenan develop cecal inflammation in 5–7 days. Germ-free animals do not develop disease. The inflammation resolves with sulfasalazine and mesalamine. The model provides insights into possible interactions between endogenous bacterial flora and dietary constituents in the gut.

Cyclical administration of 5% dextran sulfate results in a chronic intestinal inflammation in Swiss Webster mice. Again IL-1 receptor antagonists will

ameliorate the disease. Luminal cyclosporine is also beneficial, while metronidazole prevents colonic inflammation and dysplasia.

Lewis rats given intramural injections of peptidoglycan-polysaccharide polymers develop an enterocolitis associated with granulomatous hepatitis, arthritis and anemia. Cecal mRNA for IL-1, IL-6 and cytokine-induced neutrophil chemoattractant (CINC/GRO) are increased. Cyclosporine and steroids aid in resolution of the disease.

Conclusions

Many factors are important in the pathogenesis of inflammatory bowel disease in humans and these include genetic susceptibility and environmental factors. A recurring characteristic of the animal models used to study inflammatory bowel disease is that, here too, both genetic background and environmental effects, particularly bacterial flora, appear to be important. Animal models with intestinal disease that have similarities to IBD thus provide one avenue for the study of the factors and their interaction. The use of these models provides information that not only illuminates our understanding of IBD but also aids in the development of therapeutic strategies.

See also: Adhesion molecules; CD45 (the leukocyte common antigen); Inflammatory bowel disease; Interleukin 2; Interleukin 10 and its receptor; Knock-out, genetic; Transgenic animals.

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INFLUENZA VIRUS (ORTHOMYXOVIRUS), INFECTION AND IMMUNITY

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The orthomyxovirus family of segmented RNA viruses is classified into three types (A, B and C), based on differences between the internal matrix (M) and nucleoproteins (NP). Influenza A and B viruses have eight RNA segments while influenza C has seven, each coding for at least one protein. The following deals solely with the A viruses, which have received the most attention because changes in their surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins lead to repeated epidemics and pandemics. The B and C viruses do not vary in this way.

The influenza A viruses comprise at least 15 HA and nine NA subtypes, all of which are found in birds. There are three HA subtypes in humans, two in pigs, two in horses, and occasional isolations from seals, mink and whales. Severe outbreaks of influenza have occurred in many of these species, with the 1918 epidemic killing more people than the preceding European war.

Infection and humoral immunity

Influenza in mammals, including humans, is essentially an aerosol infection confined to epithelial cells in the upper and lower respiratory tract. The disease

in birds is different, being largely gastrointestinal in nature and spread by fecal contamination. Some of the avian viruses (H5 and H7) are so virulent that death may result from generalized infection with central nervous system involvement.

Mucosal (IgA) and serum (IgG) antibodies to the HA molecule neutralize virus infectivity and are primarily responsible for resistance to reinfection. This is the basis of vaccination against current epidemic strains with killed virus. The immunoglobulin (Ig) response to the HA is subtype specific, but accumulation of point mutations (antigenic drift) permits infectious virus to escape antibody-mediated destruction. Five antigenic sites, all of which are associated with protection, have been identified on the globular head of the HA molecule. 'Antigenic drift' results from the accumulation of amino acid substitutions at these sites, reproduced in the laboratory by selection of escape mutants with mouse monoclonal antibodies (mAbs). In addition, crystallographic studies with the mutants have shown that the antibodies bind to the regions of the molecule where the amino acid substitutions occur.

Antibodies to the neuraminidase do not prevent infection but reduce the spread of virus. The enzyme-

active centers on each of the four subunits of the NA lie in a central cavity surrounded by two antigenic sites. Escape mutants can again be selected with mAbs to these sites, which inhibit virus release by blocking enzyme activity. Complexes of NA–Fab have been crystallized and their structure established. Each defines an epitope comprising five polypeptide loops on the NA, involving about 22 amino acids in direct contact.

Variability is the key feature of the influenza A viruses. This depends on two, separate mechanisms. Antigenic drift (see above) gives rise to variants of existing epidemic strains which are sufficiently different from their predecessors to escape neutralization by the Ig pool available in the population at risk. The more profound change associated with ‘antigenic shift’ may result from a chance dual infection with a human and an animal influenza A virus. Reassortment of the segmented genomes of the two viruses can then lead to the emergence of a novel pandemic strain, for which there is no pre-existing antibody in humans. Such reassortants are readily generated experimentally.

Cell-mediated immunity (CMI)

The current paradigm is that influenza A viruses are normally cleared from the infected lung by CD8⁺, virus-immune cytotoxic T lymphocytes (CTLs). Lymphocytes of this subset are driven to recognize virally infected cells by antigen-presenting cells expressing viral peptides in the context of major histocompatibility complex (MHC) class I glycoproteins to the T cell receptor (TCR) and by subsequent interaction of the CD8 molecule with the constant region of the MHC glycoprotein which is believed to increase the overall avidity of the interaction. Adoptive transfer experiments with T cell clones specific for one, but not another influenza A virus used to doubly infect mice, indicate that immune elimination depends on direct contact between the effector lymphocyte and the virus-infected target cell. This is generally thought to operate via a Ca²⁺-dependent lytic degranulation pathway mediated by perforin and granzymes. There is no ‘bystander’ destruction of adjacent cells infected with the virus that is not recognized. Most cytolytic T cells are also capable of lysis by a largely Ca²⁺ and pore-forming independent mechanism utilizing as the Fas–Fas ligand interactions. Recent experiments have shown that CD8⁺ T cells can clear influenza virus via perforin/granzyme or Fas-dependent processes.

Immune CD4⁺ T cells contribute to the lung consolidation characteristic of influenza pneumonia, and also play a significant role in virus clearance. Cloned,

CD4⁺ T cell lines can eliminate virus from the lung in the apparent absence of primed CD8⁺ lymphocytes. Cytolytic CD4⁺ T cells can be generated *in vivo*, though such generation is dependent on depletion of CD8 T cells. The Fas–Fas ligand pathway of cytotoxicity is believed to have a more prominent role in CD4⁺ versus CD8⁺ T cell-mediated cytotoxicity.

The one task that can be definitely attributed to the CD4⁺ population is help for antibody production, which is now known to be the mechanism underlying the clearance of virus from the lung by the CD4⁺ clones. A major role of CD4⁺ T cells specific for viral peptides associated with MHC class II glycoproteins on B cells is primarily the generation of ‘helper’ cytokines such as interleukin 4 (IL-4) and IL-5. These cytokines promote the clonal expansion and differentiation of B cells to antibody-secreting plasma cells.

The inflammatory exudate that can be lavaged from the respiratory tract of mice with influenza is dominated by macrophages, natural killer (NK) cells and T lymphocytes, with the CD8⁺ subset being 2–3 times more prevalent than the CD4⁺ cells. Macrophage recruitment and activation occurs in the absence of the CD4⁺ population and is dependent on nonspecific chemokine/cytokine recruitment probably produced from infected respiratory epithelia. The number of T cells in the exudate declines rapidly from the time that it is no longer possible to recover infectious virus. A role for NK cells in mediation of virus clearance has not been well established. Major questions concerning the nature of influenza pneumonia include the relative roles of different lymphokines, cytokines and chemokines in the disease process.

T cell specificity and memory

Infection with any influenza A virus primes for a secondary CD8⁺ T cell response to any other influenza A virus. This reflects that many of the CD8⁺ T cells are specific for peptides of conserved, internal virus proteins presented on the cell surface in the binding site of self MHC class I molecules. The NP is particularly prominent in this regard, though the M protein, nonstructural protein (NS1), and even the viral polymerases (PA, PB1-2) may contribute to antigenicity. The significance of a particular viral peptide depends on whether or not it binds to the MHC class I glycoproteins expressed in the individual at risk. Presence of these cross-reactive memory T cells has been correlated with less severe disease and their absence is probably the reason why influenza tends to be more lethal in very young children and in the elderly. Mouse experiments using

limiting dilution analysis (LDA) suggest that following infection, increased numbers of CD4⁺ or CD8⁺ T cells persist in lymphoid tissue for life. CD8⁺ T cell clonotypes that survive in the long term have virus-specific frequencies at least 30-times those found in naive animals, and express characteristic profiles of adhesion and activation antigens such as CD44 and CD62L. These cell surface molecules are believed to influence lymphocyte recirculation and homing.

Influenza-immune CD4⁺ helper T cells are specific for viral peptides presented in the context of self MHC class II glycoproteins. Following infection, the normal sequence of events is that antigenic determinants on the viral coat glycoproteins are bound by Ig receptors on appropriate B lymphocytes. This leads to the interiorization of the virus-Ig complex, degradation in lysosomes, and expression of constituent peptides + MHC class II molecules on the surface of the B cell. Lymphokines, such as IL-2 and IL-4, that are needed to promote the growth and differentiation of B lymphocytes specific for the influenza hemagglutinin (HA) molecule, can thus be provided by T helper cells reactive to epitopes derived from any of the viral proteins. This may be one reason why whole, inactivated influenza virus vaccines are more effective than, for instance, those consisting solely of HA subunits.

See also: **Antigen-binding site; Antigen presentation**

via MHC class I molecules; Antigen presentation via MHC class II molecules; Antigenic variation; B lymphocytes; MHC, functions of; T lymphocytes; Vaccines; Vaccines, adverse reactions to; Viruses, immunity to.

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INNATE IMMUNITY

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Innate, or natural, immunity includes all non-specific resistance or immune mechanisms, in contrast to *specific or acquired immunity* which exhibits exquisite antigen specificity and immunological memory (Figure 1).

History and nomenclature

Metchnikoff, toward the end of the nineteenth century, fully realized the capabilities of mononuclear phagocytes, and in a series of brilliant comparative and descriptive studies, he related the phagocytic function of metazoan amoebocytes to vertebrate macrophages. The applications of his work concerned host-defense reactions to infectious agents

and other materials that cause inflammation. Later, during the first quarter of the twentieth century, cytochemical procedures facilitated the recognition that phagocytic cells such as monocytes and macrophages were of widespread occurrence throughout the body. Three categories of mononuclear phagocytes have been defined: 1) those found circulating in the blood (monocytes); 2) those that are fixed in tissues and organs; and 3) those that move about or wander around in loose connective tissues. Much confusion has existed in terminology because of variations in organ localization and methods of study, multiplying synonyms for functionally related types. Simply defined, mononuclear phagocytes found in the peripheral blood derived from bone marrow are

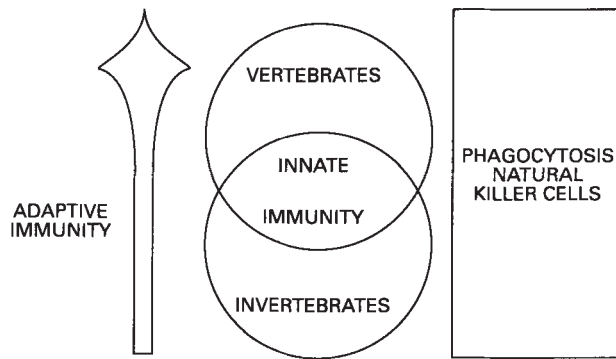


Figure 1 Nonspecific innate immunity is a characteristic of all living animals, a universal defense system found in invertebrates and vertebrates. Phagocytosis and the activities of natural killer cells are inherent in this form of immunity. In contrast, specified adaptive immunity evolved and reached its most complex development in vertebrates including fish, amphibians, reptiles, birds and mammals.

monocytes. Once they leave the peripheral blood, we will refer to the more mature cells of the tissues as *macrophages*.

Nongranular leukocytes

Vertebrates, and many invertebrates, have evolved a system of internal transport for communicating between components of the immune system. This system comprises the blood and lymph within the circulatory and lymphatic systems. Blood contains two major types of cells: erythrocytes, or red blood cells, and leukocytes, or white blood cells. There are two basic types of leukocytes, nongranular and granular, which play important roles in the immune system. Nongranular (agranular) leukocytes are further divided into two types: *lymphocytes* and *monocytes*. Lymphocytes possess receptors for antigens on their surfaces and are thus vital to specific immune responses throughout the body. Like other blood cells, monocytes are produced in the bone marrow and then enter the blood system. They frequently exhibit ameboid movement, and become voracious phagocytes when they leave capillaries and enter connective tissues as macrophages. In adaptive immune responses, macrophages are involved in the presentation of antigens to T cells.

Granular leukocytes

Granular leukocytes include *neutrophils*, *eosinophils* and *basophils*. The number of neutrophils increases during infections to provide one of the first lines of defense against invading foreign material and microorganisms. In allergies and parasitic infestations, the number of eosinophils increases. Basophils are

important for the production of histamine, the primary cause of skin reactions in allergic responses. The most actively phagocytic of leukocytes are neutrophils and macrophages, a functional characteristic carried out mainly in connective tissue. Both cell types ingest foreign particles, bacteria and degenerating cells and fragments, and are thus crucial to the body's nonspecific immune reactions. Such responses involve rapid elimination of foreign material, often without the intervention of antibody.

Phagocytosis

Phagocytosis is the process of ingesting foreign material. Macrophages and neutrophils will engulf almost anything. To verify the presence of phagocytic cells one need only inject an animal with carbon particles; shortly after the phagocytes can be seen to be blackened and heavily engorged with the ingested particles. Phagocytosis contributes significantly to an animal's resistance to infectious organisms. When the macrophage engulfs antigen (e.g. foreign particles or infectious organisms) it is processed and then presented to lymphocytes, setting in motion a chain of events that begin a specific immune response.

Enzyme synthesis

Since the original description of macrophages in the nineteenth century, it has been known that the cytoplasmic granules of activated macrophages take up more of the dye neutral red than do those of nonactivated macrophages. Neutral red is a basic dye that is specific for the enzyme components of macrophage granules. Procedures in histochemistry, coupled with the more sophisticated techniques of electron microscopy, have revealed that acid phosphatase is abundant in the lysosomes of macrophages. These protein enzymes in lysosomes are actively synthesized by macrophages. After the fusion of a phagosome (which forms immediately after phagocytosis) with a lysosome, enzymes are discharged into the resulting phagolysosome. A residual body consisting of digested material is formed and is then extruded by exocytosis.

Synthesis of lipids

Macrophages play an important role in the synthesis of lipids through their involvement in the uptake, hydrolysis and esterification of cholesterol. Reduced phagocytic function may contribute to the lowered rate of cholesterol metabolism during aging. Macrophages are probably involved in the onset of atherosclerosis during the degradation and synthetic utiliz-

ation of cholesterol and other lipids. By using either ^3H , ^{14}C , or ^{32}P to label different lipids, it has been shown that when cholesterol, triglyceride and phospholipid are ingested simultaneously by macrophages of lymph nodes, cholesterol is removed more slowly than either of the other two lipid components. During lipid metabolism, peritoneal macrophages from rabbits can incorporate labeled acetate into cholesterol, and they can degrade cholesterol. Alveolar macrophages contain cholesterol esterase and lipase. Fatty acids and triglycerides are taken up and oxidized by rabbit peritoneal macrophages, which also show increased phospholipid synthesis during phagocytic activity. Since lipids constitute an important component of all cell membranes, the universal presence of lipids is crucial to effective cell functions such as phagocytosis.

Interferon production

Macrophages are an important source of interferon, especially in the lungs, a place vulnerable to countless viral infections. When tissues which are rich in macrophages (e.g. spleen and liver) are cultured *in vitro*, they synthesize interferon within 24 h, whereas tissues which are poor in macrophages (e.g. kidney and brain) produce negligible quantities of interferon within the same time period.

Natural killer cells

Natural killer (NK) cells are not clonally distributed, nor do they exhibit memory responses. They patrol the body looking for cells which have lost expression of major histocompatibility complex (MHC) class I molecules, e.g. cells infected with certain viruses and some types of tumor cells. If such cells are encountered, they are then killed by the NK cells.

See also: Acute phase proteins; Basophils; Complement, alternative pathway; Complement, classical

pathway; Eosinophils; Interferon α ; Interferon β ; Interferon γ ; Monocytes; Neutrophils; Phagocytosis; Phylogeny of the immune response; Skin, contribution to immunity; Mast cells.

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INSULIN-DEPENDENT DIABETES MELLITUS, EXPERIMENTAL MODELS

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Type I diabetes, also known as insulin-dependent diabetes mellitus (IDDM), results from the destruction of insulin-producing pancreatic β cells, culminating in hypoinsulinemia and hyperglycemia. IDDM appears to be a disease of autoimmunity. Genetic susceptibility is believed to be a prerequisite for the development of IDDM and environmental factors, such as viruses, diet and toxins are also considered to

be involved in the clinical expression of this disease (Figure 1).

Animal models of human autoimmune IDDM, such as the BioBreeding (BB) rat and the nonobese diabetic (NOD) mouse, have enhanced our understanding of the pathogenic mechanisms of this disease. The spontaneously diabetic BB rat was discovered in 1974 in a colony of outbred Wistar rats

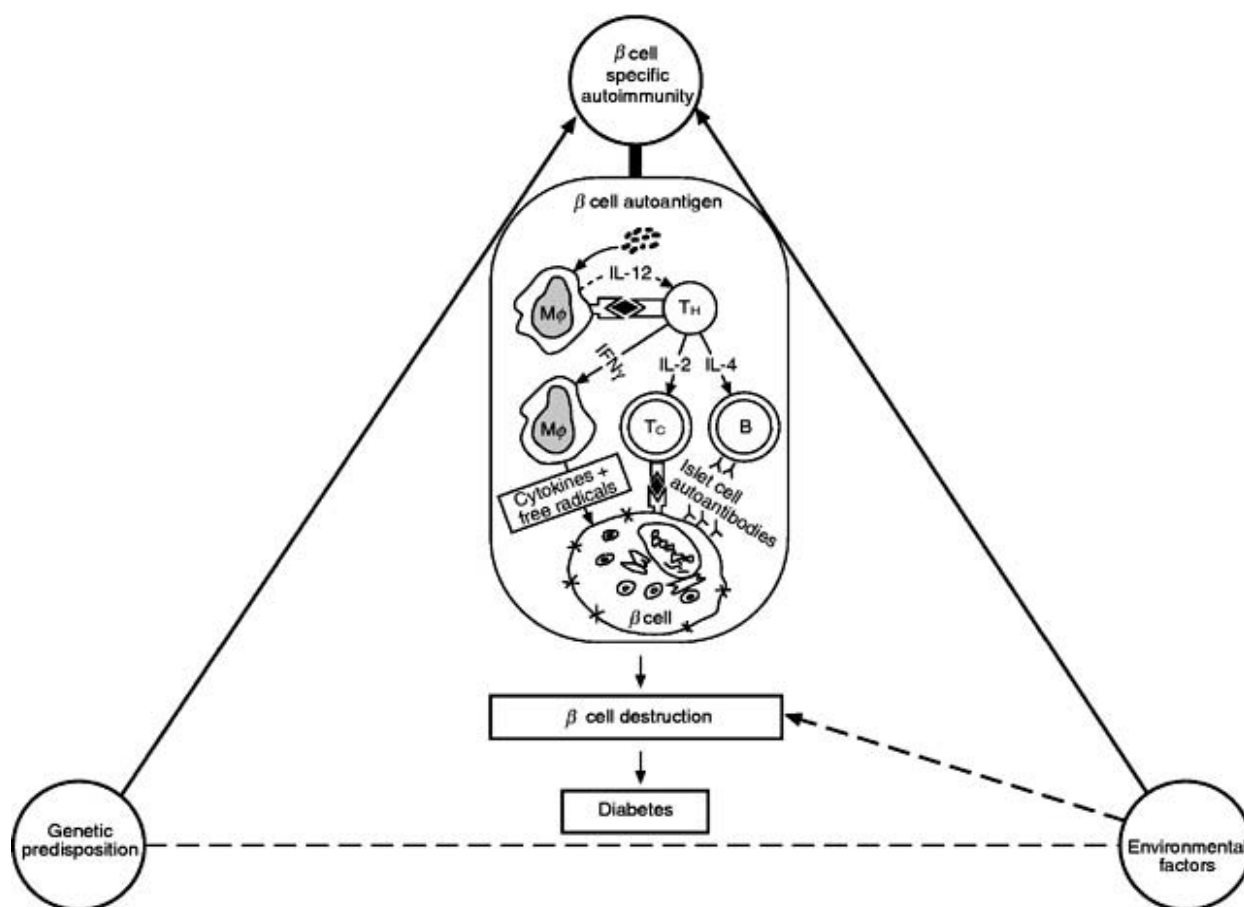


Figure 1 Etiology of IDDM. Genetic susceptibility appears to be prerequisite for most, if not all, cases of IDDM. Environmental factors influence the clinical expression of genetic susceptibility. Certain environmental factors, such as viruses and toxins, may act as primary injurious agents to pancreatic β cells, or as triggering agents for autoimmunity in humans and animals. Once β cell-specific autoantigens, macrophages, helper T cells and autoantigen-specific cytotoxic T lymphocytes (CTLs) are involved in the autoimmune process. In addition, free radicals and cytokines released from macrophages and T cells, and autoantigen-specific T effector cells synergistically destroy β cells.

at the BioBreeding Laboratories in Ottawa, Canada. The cumulative incidence of diabetes in DP-BB/Wor rats is greater than 80%. The pattern of disease in the DP-BB rat is, in many ways, similar to human IDDM, with the exception of the association of T cell lymphopenia with the DP-BB rat (Table 1). The NOD mouse was developed in the course of a breeding program to establish a cataract-prone subline (CTS) from a subline outbred ICR mouse at Shionogi Research Laboratories in Osaka, Japan in 1974. The diabetic syndrome observed in NOD mice is similar to that of humans and BB rats with the exception of the incidence of disease between the sexes (Table 1). In the NOD mouse there is a greater incidence of diabetes in females (70–80%) when compared to males (10–20%).

Cumulative evidence indicates that β cell autoantigens, macrophages, $CD4^+$ T cells, and $CD8^+$ T cells are involved in the pathogenesis of β cell-specific autoimmune IDDM in the BB rat and in the NOD mouse. Cytokines produced from immunocytes and oxygen free radicals also contribute to the destruction of β cells. In addition, chemical toxins and viruses induce a diabetes-like syndrome in animals. Various strategies have been employed to prevent insulinitis and diabetes in animal models. Most strategies are based on immune modulation or immune intervention in order to block the process of autoimmune β cell destruction at different stages.

Genetics

The development of IDDM is controlled by multiple susceptibility genes including a locus in the major histocompatibility complex (MHC). In the BB rat, diabetes susceptibility is strongly linked to genetic markers for the MHC and the inheritance of phenotypic markers, such as T lymphopenia. The class II region of the rat MHC is comprised of the RT1B and RT1D loci, homologous to mouse I-A and I-E regions, and flanked by RT1A, RT1E and RT1C class I loci. The RT1D locus is homologous to the human HLA-DR. The BB rat expresses the RT1A^u, RT1B^u and RT1D^u haplotypes. MHC-associated diabetes susceptibility genes map to the class II region on chromosome 20 and the MHC class II RT1^u haplotype is required for the expression of diabetes, but MHC class I molecules are not associated with the expression of diabetes. The sequences of the first external domains of RT1B and RT1D β chains in BB rats are different from the sequence of other rat or mouse class II genes. It is noteworthy that Lewis rats, which are not diabetes-prone, and BB rats both lack aspartic acid at position 57 of the class II β chain. This suggests that the allelic amino acid at this position in the BB rat may not serve as a disease marker and that there may be other unidentified class II allelic differences involved in the susceptibility of BB rats to IDDM. T cell receptor polymorphisms have

Table 1 Characteristics of human, BB rat and NOD mouse IDDM

Characteristic	Human	BB rat	NOD mouse
Genetics			
MHC association	+	+	+
Non-MHC association	+	+	+
Clinical features			
Insulin dependence	+	+	+
Equal sex distribution	+	+	—
Obesity	—	—	—
Insulinitis	+	+	+
Humoral immunity			
ICA	+	—	+
ICSA	+	+	+
Insulin autoantibody	+	+	+
Cellular immunity			
Lymphopenia	—	+	—
T lymphocyte dysfunction	—	+	+
Passive transfer	Not determined	+	+
Prevention by immunosuppression	Not determined	+	+
Association with other autoimmune diseases	+	+	+
Environmental factors	+	+	+

not been linked to diabetes in the BB rat. The development of diabetes in the BB rat also correlates with the inheritance of severe T cell lymphopenia and is attributed to a lack of T cells expressing the RT6 cell surface alloantigen. Lymphopenia segregates independently of the RT1 haplotype. While the development of diabetes in nonlymphopenic BB-derived rats has occurred in rare instances, no permanently nonlymphopenic strain of rat that develops spontaneous diabetes has been created. Therefore, it is likely that the severe lymphopenic phenotype of the BB rat, inherited in an autosomal recessive fashion, is not essential for the development of diabetes but increases the probability of its development. Recently, a lymphopenic gene (*lyp*) was mapped close to the neuropeptide Y (*Npy*) gene on chromosome 4.

Early genetic studies in mice revealed that the susceptibility of NOD mice to IDDM is also linked to the H2 complex and is inherited as a recessive trait. Later studies with H2 congenic strains of NOD mice revealed that susceptibility to IDDM by H2 (Idd-1) is not recessive, but rather a dominant trait with varying degrees of penetrance for the phenotypes of insulinitis and diabetes. The NOD mouse is unique in that its H2 complex encodes for novel I-A molecules ($A\alpha^d$ and unique $A\beta$) in which aspartic acid at position 57 of the β chain is replaced by serine. Also the NOD mouse lacks I-E expression. However, the mutated I-A^{g7} $A\beta$ gene, which encodes aspartate at position 57, reduces the incidence of diabetes but does not prevent insulinitis. Studies with nondiabetic

NOD transgenic mice expressing normal I-E or other I-A molecules revealed that the unique H2 complex of the NOD mouse plays a direct role in the mouse's susceptibility to IDDM, but by itself the H2 complex is not sufficient for the onset of diabetes. The CTS strain, which expresses the same MHC class II allele as NOD mice but a different MHC class I allele, does not develop diabetes, indicating that H2 linked genes (at class I or class II loci) in addition to class II genes may also contribute to disease development. Although 50% or more of the genetic risk is transmitted by Idd-1, susceptibility of the NOD mouse to IDDM is also determined by a large number of non-MHC-linked genes (Table 2). The Idd-2 gene is weakly linked to the *Thy1/Apoa1* cluster on chromosome 9. The Idd-3 diabetes susceptibility locus mapped near the interleukin 2 (IL-2) gene on chromosome 3 and is associated with both insulinitis and diabetes. Idd-10 on chromosome 3 is associated with the expression of a defective Fc receptor (*Fcgr1*) of IgG. Idd-4, which maps to chromosome 11, is associated with the early onset of IDDM and may influence the frequency of insulinitis and may control the progression of severe insulinitis to overt diabetes. The mouse inducible nitric oxide synthase (iNOS) gene *Nos 2* is a candidate for Idd-4. Idd-5 is linked to the IL-1 receptor and the *Lsh/Ity/Bcg* genes which control resistance to intracellular pathogens on chromosome 1. Also, Idd-5 is the most important gene affecting insulinitis after the H2 gene. Idd-6, which maps to chromosome 6, affects the incidence of diabetes, but not the frequency of insulinitis. Idd-7 and

Table 2 IDDM susceptibility loci identified in the NOD mouse

IDDM locus	Chromosome	NOD allele	Linkage	Candidate gene
Idd-1	17	Susceptible	H-2	<i>Ab, Ea</i>
Idd-2	9	Susceptible	<i>Thy 1</i>	<i>T3g, T3d, T3e</i>
Idd-3	3	Susceptible	<i>IL-2, D3Nds1</i>	<i>IL-2</i>
Idd-4	11	Susceptible	<i>Acrb</i>	<i>Nos-2</i>
Idd-5	1	Susceptible	<i>D1Nds6, Bcg, D1Nds7</i>	<i>Bcg, ILr1, Bcl2</i>
Idd-6	6	Susceptible	<i>Kras-2</i>	<i>Bphs, Ins-1</i>
		Susceptible	<i>D6Mit14</i>	
		Resistant	<i>D6Nds1</i>	
Idd-7	7	Resistant	<i>D7Nds6</i>	
Idd-8	14	Resistant	<i>D14Nds1</i>	
Idd-9	4	Susceptible	<i>D4Nds16</i>	
Idd-10	3	Susceptible	<i>D3Nds11, D3Nds7</i>	<i>Fcgr1</i>
Idd-11	4	Susceptible	<i>Nhe 1</i>	
Idd-12	14	Susceptible	<i>Tcra</i>	
Idd-13	2	Susceptible	<i>IL1, B2m</i>	<i>Rec, IL1</i>
Idd-14	13	Susceptible		
Idd-15	5	Susceptible		
Idd-16	17	Susceptible	H-2	<i>Tnfa</i>

Idd-8 are located on chromosome 7 and chromosome 14, respectively. Another diabetes susceptibility gene on chromosome 14 near *Tcra* (T cell receptor α chain gene) is Idd-12. Idd-9 and Idd-11 are both located on chromosome 4 and the locus of the Na^+/H^+ exchanger 1 (*Nhe1*) is a candidate gene for Idd-11. Idd-13 was identified as a resistance allele on chromosome 2, linked to the *Il1a* gene. Idd-14 and Idd-15 are located on chromosome 13 and chromosome 5, respectively. Recently Idd-16 was located adjacent to the Idd-1 region. The *Tnfa* gene is a candidate gene for Idd-16. Thus, a series of alleles at different loci, each necessary but not sufficient for the development of diabetes, underlie the susceptibility to diabetes (Table 2).

Islet pathology

The DP-BB rat and the NOD mouse have many features that make them advantageous models of human autoimmune diabetes (Table 1). These models develop diabetes spontaneously; their symptoms characterized by hyperglycemia, glucosuria, ketonuria, polyuria, polydipsia, weight loss and death if insulin is not administered. In the BB rat, mononuclear cell infiltration, known as insulitis, of the pancreatic islets usually occurs at an age of 60–120 days in both males and females and lymphocytes are scattered throughout the islets. In contrast, lymphocytes are detected in the peri-islets at 4 weeks of age in the NOD mouse and these mononuclear cells appear to migrate toward the islets as the NOD mouse matures. Diabetes develops at 120–200 days of age in the NOD mouse. Insulitis often precedes or accompanies the onset of hyperglycemia in these animal models.

The progressive and selective loss of pancreatic β cells is an important characteristic of IDDM. This destructive process excludes other islet cell types, such as alpha (α), delta (δ), and polypeptide-producing (pp) cells, leaving them intact. After the onset of hyperglycemia, the degree of mononuclear cell infiltration decreases and end-stage islets have few or no inflammatory cells. The islet infiltrates consist of macrophages, dendritic cells, CD4^+ T cells, CD8^+ T cells, natural killer (NK) cells, and, to a lesser extent, B cells. Among them, macrophages and dendritic cells are the primary infiltrates of the pancreatic islets.

Immunopathology

Humoral immunity

One of the most commonly reported immunological

abnormalities of humans and animals with autoimmune diabetes is the presence of autoantibodies in their sera, directed against pancreatic islet cell antigens. Anti-islet cell autoantibodies have been divided into two types: islet cell cytoplasmic antibodies (ICA) and islet cell surface antibodies (ICSA). Both ICAs and ICSAs have been detected in the NOD mouse, but ICAs have not been detected in the BB rat. Autoantibodies against bovine serum albumin (BSA) and insulin have been detected in the BB rat and the NOD mouse. The presence of anti-64 kDa glutamic acid decarboxylase (GAD) autoantibodies and anti-38 kDa autoantibodies have been detected in the sera of BB rats. Although autoantibodies directed against a 64 kDa autoantigen have been detected in the NOD mouse, the detection of anti-GAD autoantibodies in NOD sera is controversial. Also, autoantibodies directed against a 52 kDa protein, peripherin and heat shock protein have been detected in the NOD mouse. However, anti-37/40 kDa (tyrosine phosphatase/IA2) autoantibodies detected in human diabetic sera have not been detected in either the BB rat or the NOD mouse. It is believed that these islet cell autoantibodies are predictive markers rather than pathogens.

Cell-mediated immunity

The role of cell-mediated autoimmunity in the pathogenesis of IDDM has been extensively studied in animal models. Evidence exists that supports the hypothesis that macrophages and T cells are clearly involved in the destruction of β cells.

Macrophages The major populations of infiltrating cells during the early stage of insulitis appear to be macrophages and dendritic cells. The infiltration of these cells is followed by the invasion of T cells, B cells and NK cells. Treatment of BB rats and NOD mice with silica, known to be toxic to macrophages, results in the near complete prevention of the development of insulitis and diabetes, suggesting that macrophages play an important role in the initiation of insulitis. Inactivation of macrophages in NOD mice at an early age with a long-term treatment of silica prevents the development of β cell-specific effectors. However, late, short-term treatment of NOD mice with silica does not affect the destruction of β cells by pre-existing effectors. Concanavalin A (con A)-activated spleen cells prepared from long-term silica-treated BB rats do not induce insulitis and diabetes in BB neonates, whereas con A-activated spleen cells from untreated BB rats induce both insulitis and diabetes, indicating that the inactivation of macrophages results in the loss of ability of the splenocytes to adoptively transfer insulitis and diabetes.

Late, short-term silica administration to BB rats does not, however, alter the ability of the splenocytes to adoptively transfer insulinitis and diabetes after con A activation. Thus, macrophages appear to be essential for the development of β cell-specific cytotoxic effectors in the initial phase of insulinitis in NOD mice and BB rats.

T cells There is much evidence to indicate that T cell-mediated autoimmunity is involved in the destruction of β cells. Neonatal thymectomy prevents diabetes in the BB rat. The lymphocytes from diabetic BB rats transfer disease to young diabetes-prone BB rats. BB rats treated with monoclonal antibodies (OX19) directed against antigens expressed on the surface of all T cells do not develop diabetes. T cell lymphopenia, characterized by a reduced level of CD4⁺ T cells and a substantial decrease in the number of CD8⁺ T cells, is observed in the BB rat. In contrast, the BB rat exhibits a relative increase in the number of NK cells (OX19⁻/OX8⁻). The proliferative response of T cells to mitogens, such as con A, phytohemagglutinin (PHA), and the production of con A-induced IL-2, is greatly reduced in DP-BB rats. The expression of the RT6⁺ antigen, which is a marker of mature lymphocytes, is defective in the DP-BB rat. However, the mechanisms involved in T cell lymphopenia and the defective development of mature T cells are not known.

It is known that autoimmune β cell destruction is mediated by both CD4⁺ and CD8⁺ T cells in the NOD mouse. Neonatal thymectomy prevents IDDM in the NOD mouse and adoptive transfer studies using cloned and uncloned T cells show that both CD8⁺ and CD4⁺ T cell subsets are required for the transfer of diabetes in NOD mice, NOD athymic nude mice, and NOD severe combined immunodeficiency (SCID) mice. Islet cell-specific T cell clones have been isolated from insulinitic lesions and the splenocytes of both prediabetic and diabetic NOD mice. CD4⁺ islet-specific T cell clones accelerate the development of diabetes in young NOD mice and destroy islet grafts in CD8⁺ T cell-depleted diabetes-resistant mice. The BDC2.5/NOD transgenic mouse, which expresses the rearranged T cell receptor (TCR) α and β chain genes of a diabetogenic islet-specific CD4⁺ T cell clone (BDC2.5), exhibits an increased incidence of diabetes. In contrast, some reports have shown that the CD4⁺ T cell subset alone is not sufficient for the transfer of diabetes in NOD mice. As well as CD4⁺ T cell involvement in β cell destruction, experimental evidence reveals that CD8⁺ T cells play a role as effector cells in the destruction of β cells in the NOD mouse. CD8⁺ cytotoxic T cell clones (CTLs), isolated from the islets of

diabetic NOD mice, can destroy β cells *in vitro* and transfer diabetes *in vivo* with the help of CD4⁺ T cells. Transgenic mice (TCR β /NOD) with a TCR β rearrangement from a diabetogenic CD8⁺ T cell clone exhibited an accelerated onset of diabetes. In addition, a β cell-specific CD8⁺ CTL clone, which expresses CD28 and CD44, effects β cell damage without the assistance of CD4⁺ T cells. Furthermore, β cell-specific CD8⁺ cytotoxic T cells directly destroy β cells in association with MHC class I-restricted antigens in islet transplanted murine models. In conclusion, both CD4⁺ and CD8⁺ T cells are required for the development of spontaneous diabetes in NOD mice (not transgenic models). However, the specific roles that CD4⁺ and CD8⁺ cells play in diabetogenesis remain unclear.

T cell receptor Most CD4⁺CD8⁺ and CD4⁺CD8⁺ T cells express $\alpha\beta$ T cell receptors and recognize antigenic epitopes presented by MHC class II and class I molecules, respectively. The great majority of CD4⁺CD8⁺ T cells express a TCR composed of γ and δ chains. Studies with T cells from NOD mice exhibiting insulinitis and islet-reactive T cell clones suggest a diverse TCR repertoire. Thus, the TCR repertoire of islet-infiltrating T cells in NOD mice is known to be polyclonal. However, the TCR-V β repertoire of islet-infiltrating T cells in very young NOD mice (2 weeks old) exhibits a monoclonal TCR-V β 8.2, suggesting that IDDM in NOD mice may be initiated by the recognition of a single autoantigen.

Collaboration of macrophages with CD4⁺ and CD8⁺ T cells in the destruction of β cells Macrophages, CD4⁺ T cells, and CD8⁺ T cells are involved in the destruction of β cells in NOD mice and BB rats. The interactions among these cells, however, are not well understood. Many studies indicate that macrophages are one of the first cell types to infiltrate the pancreatic islets. These cells must be involved in the pathogenesis of IDDM early on, since inactivation of macrophages results in the near complete prevention of insulinitis and diabetes in both NOD mice and BB rats. One of the key functions of the macrophage is the MHC class II-restricted presentation of processed antigen to helper T cells. Thus, it is reasonable to speculate that prior to the development of insulinitis, there may be antigenic changes on the β cell which are recognized as 'nonself'. Also, it is speculated that β cell autoantigens are released by the spontaneous turnover of β cells, or damage to the β cells by environmental factors (such as viral infection) resulting in their processing and presentation by macrophages. Thus, the presentation of β cell-specific autoantigens by macrophages to CD4⁺

helper T cells might be the initial step in the development of autoimmune diabetes (Figure 2). During this process, the activated macrophages secrete interleukin (IL)-12, IL-1 β , and other cytokines. IL-12 activates T_H1-type CD4⁺ T cells which, in turn, secrete interferon γ (IFN γ) and IL-2 (Figure 2). The secretion of IFN γ generates cytotoxic macrophages from resting macrophages. These cytotoxic macrophages secrete IL-1 β , tumor necrosis factor α (TNF α) and free radicals; substances known to be toxic to β cells. Simultaneously, IL-2 and other cytokines released from T_H1-type CD4⁺ T cells activate precytotoxic CD8⁺ T cells. These substances may also induce the migration of peripheral precytotoxic CD8⁺ T cells to the inflamed islets, perhaps by the induction and expression of specific homing recep-

tors on the endothelial cells. Precytotoxic CD8⁺ T cells bearing β cell antigen-specific receptors may differentiate into CD8⁺ cytotoxic effector T cells upon recognition of β cell-specific peptides bound to MHC class I molecules. Cytotoxic CD8⁺ T cells then effect β cell damage by the release of perforin and granzyme after recognition of MHC class I/peptide complexes on the surface of β cells. The Fas-FasL interaction appears also to be involved in the destruction of β cells. β cell-reactive CD8⁺ T cells may not have the ability to migrate to the pancreatic islets or to effect β cell damage in the absence of T_H1-type CD4⁺ T cells and macrophages. In this way, activated macrophages, T_H1-type CD4⁺ T cells, and β cell-specific CD8⁺ T cells synergistically destroy β cells in association with β cell-specific autoantigens

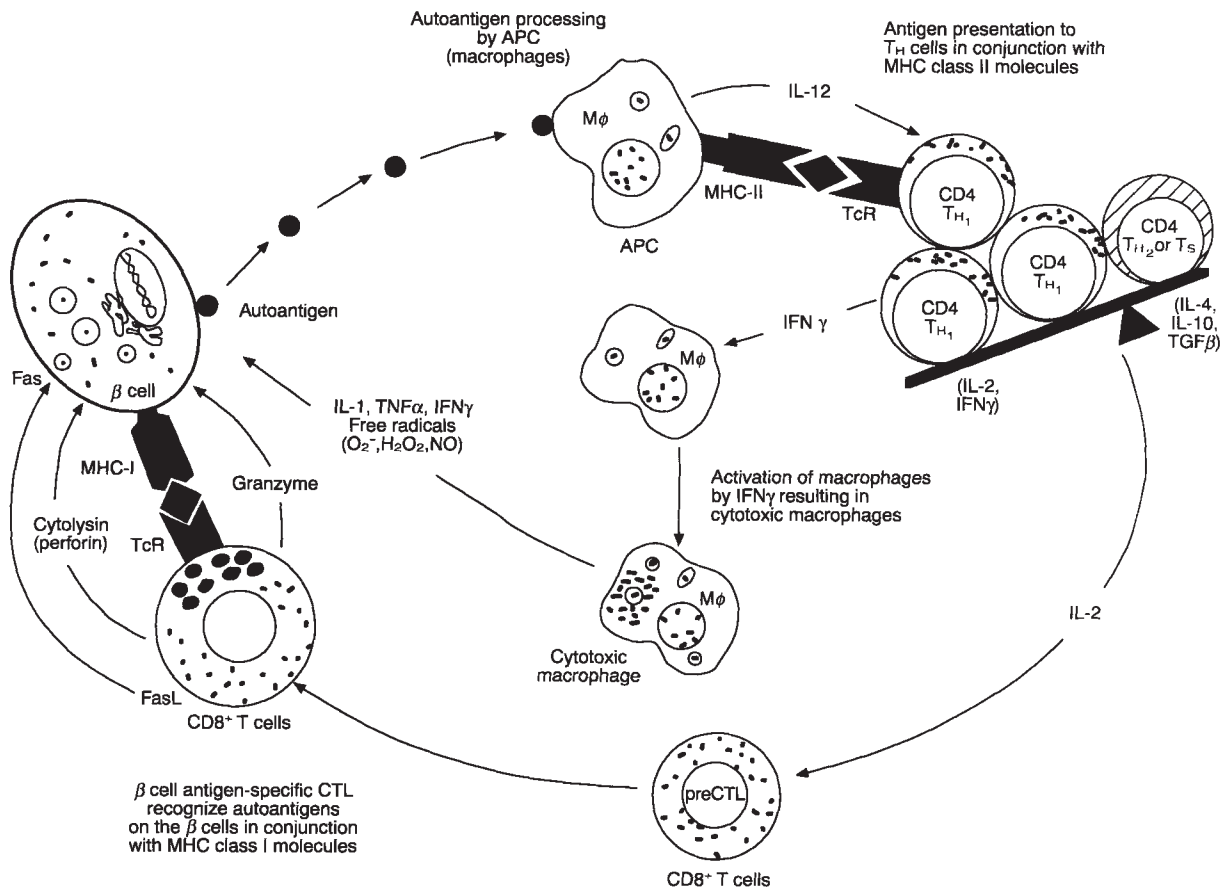


Figure 2 Schematic representation of the development of β cell-specific autoimmune IDDM. β cell autoantigens released from the β cells during spontaneous turnover or insult by viral infection are processed by macrophages and presented to helper T cells in association with MHC class II molecules. Macrophages release IL-12, which activates T_H1-type CD4⁺ T cells. While this process is taking place, β cell-specific precytotoxic T cells may be recruited to the islets. These precytotoxic T cells may be induced by IL-2 and other cytokines released by CD4⁺ helper T cells to differentiate into effector T cells. IFN γ released by helper T cells may cause macrophages to become cytotoxic. These cytotoxic macrophages release substantial amounts of β cell-toxic cytokines (including IL-1 β , TNF α , and IFN γ) and free radicals. In addition, the helper T cells secrete interleukins that activate other helper T cells, B lymphocytes, and cytotoxic T cells. The autoantigen-specific CD8⁺ cytotoxic T cells, as final effectors, may recognize the autoantigens expressed on many unaffected β cells, in association with MHC class I molecules. These CTLs release granzyme and cytolysin (perforin), which are toxic to β cells. In addition, Fas-mediated apoptosis is involved in β cell destruction. In this way, macrophages, T cells, and cytokines synergistically destroy β cells, resulting in the development of autoimmune IDDM.

and MHC class I and class II molecules, resulting in the development of autoimmune IDDM (Figure 2).

Cytokines

Biochemical mediators of cytotoxicity, such as cytokines, perforins and proteases, may destroy β cells. Macrophage-mediated β cell damage is caused by cytokines, such as IL-1 β , TNF α , and IFN γ released from activated macrophages. IL-1 is selectively cytotoxic to pancreatic β cells *in vitro*. IL-1 β , TNF α , lymphotoxin and IFN γ act alone or synergistically to damage isolated islets or cultured islet cells. IL-1 β -stimulated and TNF α -stimulated endogenous iNOS activity is revealed as a major source of β cell injury. These cytokines are expressed in the insulinitic lesions of NOD mice and BB rats. Monoclonal antibody directed against IFN γ prevents the development of diabetes in these animal models. Transgenic expression of IFN γ in the β cells of normal mice results in the autoimmune destruction of β cells and the development of IDDM.

The genetic absence of IFN γ in NOD mice, inactivated by a mutation in the IFN γ gene, results in a significant delay of the development of diabetes. Transgenic expression of IFN α in the β cells also exhibits an immune-mediated destruction of islet β cells, and antibody against IFN α prevents β cell damage and IDDM in these transgenic mice. In general, T_H1-like cytokines, such as IL-2 and IFN γ , are β cell destructive, while T_H2-like cytokines, such as IL-4 and IL-10, are preventive although the local expression of IL-10 accelerates the development of diabetes. The administration of IL-12, a cytokine that is thought to play a key role in the promotion of T_H1 immune responses to NOD mice, accelerates the onset of diabetes. It was reported that IL-12 mRNA expression was elevated in prediabetic DP-BB rat islets and that this cytokine is associated with the rapid development of diabetes in NOD mice. Thus, it is likely that cytokines play an important role in the destruction of β cells. However, the precise mechanisms whereby cytokines effect the destruction of β cells remain to be determined.

Free radicals

The generation of oxygen free radicals in target cells has been implicated as one of the various mechanisms involved in the destruction of β cells. The β cell is very sensitive to the production of free radicals because islet cells exhibit very low free radical scavenging activities. The cytokines IL-1 β , TNF α , and IFN γ are known to induce iNOS, which in turn generates the free radical nitric oxide (NO) from L-arginine. NO exerts a toxic effect on β cells by inhibiting iron-dependent enzymes, thereby impairing cellular

mitochondrial function and DNA synthesis. NO is also capable of generating cytotoxic hydroxyl radicals. Islets with advanced infiltration highly express iNOS, suggesting that expression of iNOS may be associated with the development of diabetes in BB rats and NOD mice. Thus it appears that free radicals, such as NO (generated by iNOS), may contribute to β cell destruction.

Environmental factors

Susceptibility to IDDM is determined by genetic factors; however, the concordance rate for IDDM between monozygotic twins approaches only about 35%, suggesting that as well as genetic factors, non-genetic environmental factors influence the clinical expression of this disease. Viruses, toxins, diet and stress have all been implicated as possible environmental determinants of IDDM.

Viruses

There are at least two different mechanisms by which viruses cause IDDM. One mechanism involves the direct infection of β cells and virus replication within them, resulting in their lysis. The best-studied model of virus-induced IDDM is the encephalomyocarditis (EMC) virus-induced animal model in which the EMC virus acts as the primary injurious agent to β cells. Susceptibility to EMC virus-induced diabetes is controlled by genetically determined host factors. The involvement of T cells in the destruction of β cells in EMC-M-infected mice was suggested in early studies; however, later studies using EMC-D virus-infected animals did not support T cell-mediated involvement. Recent studies reveal that macrophages play a critical role in the process of β cell destruction soon after EMC-D virus infection. The development of EMC-D virus-induced diabetes is controlled by a single gene of the host and a single amino acid of the polyprotein of the virus.

The other mechanism for virus-induced diabetes involves the triggering of β cell-specific autoimmunity, leading to the autoimmune destruction of β cells. Kilham rat virus (KRV) causes autoimmune diabetes in DR-BB rats without the distinct infection of β cells. Since KRV widely infects peripancreatic and other lymphoid tissue, but not pancreatic β cells, it has been suggested that KRV triggers autoimmune diabetes by perturbing the immune system of genetically predisposed DR-BB rats, although the exact role KRV plays in the induction of diabetes in DR-BB rats is not known.

In addition, coxsackievirus B4 induces diabetes in mice and produces transient diabetes in nonhuman primates. It is suspected that bovine viral diarrhea

mucosal disease virus causes IDDM in cattle. When neonatal golden Syrian hamsters are infected with β cell-passaged rubella virus they develop diabetes. β cell-specific expression of endogenous retrovirus is associated with the development of insulinitis and diabetes in NOD mice. It was also demonstrated that reovirus causes autoimmune IDDM in mice. Transgenic mice expressing lymphocytic choriomeningitis virus (LCMV) glycoprotein (gp), or influenza virus hemagglutinin in the β cells have been developed in order to investigate the potential association between viruses and IDDM. Young hosts that express LCMV glycoprotein in their β cells develop diabetes when they are exposed to the same virus later in life. Transgenic mice that express influenza virus hemagglutinin in their β cells develop autoimmune diabetes. In contrast to the induction of diabetes, LCMV and mouse hepatitis virus (MHV) prevents the development of autoimmune IDDM in the BB rat and the NOD mouse.

Toxins

Diabetogenic chemicals such as alloxan, streptozocin (STZ), chlorozotocin, vacor and cyproheptadine may destroy β cells by several different mechanisms. These chemicals may generate oxygen free radicals and alter endogenous scavengers of these reactive species. By breaking DNA, these chemicals may also increase the activity of the enzyme that depletes nicotinamide adenine dinucleotide poly-ADP ribose synthetase in β cells and they may inhibit active calcium transport and calmodulin-activated protein kinase activity. One high dose of STZ causes complete β cell necrosis and the onset of diabetes within 24 h in dogs, hamsters, monkeys, mice, guinea pigs, and many species of rats. Multiple subdiabetogenic doses of STZ produce pancreatic insulinitis, progressing to overt diabetes in certain inbred strains of mice. An autoimmune mechanism may be involved in the onset of diabetes after multiple low doses of STZ since antilymphocyte serum plus 3-O-methylglucose, prior to STZ treatment, prevented the onset of diabetes. Also, athymic nude mice were resistant to STZ-induced diabetes. It has been suggested that IFN γ plays a role in the induction of diabetes in this model. However, it was reported recently that STZ induces diabetes in NOD-SCID mice, suggesting another mechanism is involved. In addition, type C retrovirus particles have been found in the β cells of animals with STZ-induced diabetes.

Diet

Diet may alter metabolism, cellular structure and function, as well as the immune system. It has been shown that food diabetogens affect the expression of

the diabetic syndrome in BB rats and NOD mice. The source of dietary protein seems to be the most important factor and intact protein may be required for expression of genetic susceptibility to diabetes in BB rats. Wheat gluten, soybean meal, alfalfa and skim milk powder have been identified as the most likely sources of dietary diabetogens in rodents.

Prevention

There are a number of immunotherapeutic interventions that can delay or prevent autoimmune destruction of pancreatic β cells. Neonatal thymectomy, which prevents the generation of mature T lymphocyte populations, prevents IDDM in BB rats and NOD mice. Treatment of animals with antibodies directed against T lymphocytes or T lymphocyte subsets (CD4⁺ T cells or CD8⁺ T cells) has been reported to prevent diabetes in BB rats, NOD mice and the STZ-induced diabetes animal model. Depletion of macrophages by the administration of silica also prevents diabetes in BB rats and NOD mice.

Immunosuppressive agents, such as cyclosporine, FK506 and rapamycin prevent insulinitis and diabetes in NOD mice and BB rats. Paradoxically, it has been reported that immune stimulation prevents IDDM. In NOD mice, OK-432 (streptococcal product), the fungal polypeptide IZ-8, BCG, complete Freund's adjuvant (CFA), and certain staphylococcal enterotoxins prevent diabetes. In addition, the systemic administration of cytokines, such as IL-4 and IL-10, protects β cells from autoimmune destruction in NOD mice. TGF β , secreted from suppressor T cells, prevents the development of diabetes in NOD mice. Some T cell clones isolated from NOD mice also prevent diabetes.

Blocking β cell-specific cytotoxic activity produced by free radicals with antioxidants also prevents diabetes. Activated macrophages and cytokines produced by immunocytes that have infiltrated the pancreatic islets contribute to β cell destruction by a process that generates free radicals, such as NO. The administration of antioxidants such as nicotinamide, vitamin E, probucol, MDL 29311, and U78518F has been reported to prevent or delay the development of diabetes. The induction of immune tolerance by immunization with islet antigens, such as GAD, heat shock protein and insulin is another useful method for the prevention of diabetes. Intrathymic or intravenous injection or intranasal administration of GAD protein to young NOD mice prevents insulinitis and diabetes. The administration of insulin reduces the incidence of insulinitis and diabetes in both NOD mice and BB rats.

Conclusion

The mechanisms responsible for the development of IDDM are multifactorial; thus, the simplification of these pathogenic mechanisms is not easy. Both genetic and environmental factors are involved in the development of autoimmune IDDM. β cell autoantigens, macrophages, T cells and immunocyte-derived soluble mediators, such as cytokines and oxygen free radicals, synergistically destroy β cells, resulting in the development of clinical IDDM. The control of pathogenic processes may depend upon the delicate balance of immunoregulatory T cells and/or their secreted cytokines. Animal models of IDDM, such as the BB rat and the NOD mouse, have greatly enhanced our understanding of the role that macrophages and T cells, as well as their soluble mediators, play in the pathogenesis of autoimmune IDDM. Although these animal models are not identical to human autoimmune IDDM, the knowledge gained from their use is invaluable for understanding the pathogenic mechanisms of human autoimmune IDDM and for the development of immunological methods for the prevention of this disease in humans, since there are extraordinary similarities between animal and human systems at the clinical, pathological and immunopathological levels.

See also: Autoimmune disease, spontaneous experimental models; Autoimmune diseases; Autoimmunity; Cell-mediated immunity; Cytokines; Cytotoxic T lymphocytes; Insulin-dependent diabetes mellitus, human.

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INSULIN-DEPENDENT DIABETES MELLITUS, HUMAN

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Contemporary management of insulin-dependent diabetes mellitus (IDDM) is intrusive and cannot guarantee complication-free survival. Improved understanding of the pathogenesis of IDDM has, however, provided optimism that this autoimmune disease will soon become either preventable or curable. While determined by multiple genes, the predisposition to destruction of the insulin-producing β cells in the pancreatic islets of Langerhans is transmitted predominantly through the class II human leukocyte antigen (HLA) DR and DQ loci. These predispositions involve the failure of multiple regulatory mechanisms and breakdown of immunological tolerance to self, probably induced by exposure to one or more incompletely identified environmental triggers. Particular attention has focused on the identification of specific islet autoantigens, soluble mediators of cytotoxicity (e.g. cytokines, superoxides), the balance between antigen-specific CD4⁺ T_{H1} and T_{H2} cells, and the cytotoxic effects of macrophages and CD8⁺ T lymphocytes. Future treatments will focus on preservation of pancreatic β cells and/or provision of permanent and physiological insulin replacement.

Genetic predisposition

The risk for IDDM in a first-degree relative of a diagnosed patient exceeds by some 15-fold the 0.3% disease prevalence observed in the general population, documenting the complex genetic predisposition (Table 1).

A hierarchy of autoantigen epitope affinities to the antigen binding cleft has been proposed to explain HLA-mediated IDDM susceptibility. Whether more tightly bound autoantigens aberrantly stimulate autoreactive lymphocytes or else fail to induce tolerance normally remains speculative. Two amino acids within the dimeric DQ antigen binding site, one on the α chain and the other on the β chain, have particularly important influences. A neutral amino acid (alanine, valine, serine) at amino acid 57 of the β chain is associated with susceptibility, whereas aspartic acid at this residue is protective. Arginine at position 52 of the DQ α chain occurs frequently in IDDM patients. If all four possible *cis*- and *trans*-encoded HLA-DQ α/β heterodimers comprise such

Table 1 Genetic influences of IDDM susceptibility

Relationship to proband	Risk for IDDM (%)
Parent	6
Offspring	
Diabetic mother	2
Diabetic father	6
Sibling	5
No HLA identity	1
HLA haploidentical	5
HLA-identical	15
Identical – DRB1*03/04;DQB1*0201/0302	25
Monozygotic twin	33
HLA genotypes	Risk in general population
DRB1*03/03;DQB1*0201/0201	1–2
DRB1*04/04;DQB1*0302/0302	1–5
DRB1*03/04;DQB1*0201/0302	6–10
DRB1*X/X;DQB1*0602/X ^a	0.02–0.1

^aX indicates any allele.

HLA data kindly provided by J-X She, PhD, University of Florida. Adapted from Muir A and Schatz D (1995) Prevention of insulin-dependent diabetes – 1995. *Trends in Endocrinology and Metabolism* 6: 312–317.

high-risk alleles, the patient is maximally susceptible. Transracial studies of IDDM susceptibility appeared to present data that conflicted with the Asp57 hypothesis. Blacks but not Caucasians with a DRB1*07;DQB1*0201 haplotype are susceptible. This may occur since the linked DQA chain (0501) in Blacks is the same as that found in Caucasians with the high-risk DRB1*03;DQB1*0201 haplotype. The DRB1*07 haplotype typically encodes a low risk DQA*0201 allele in Caucasians. Further, DQB1*0302;DQA*0501 is a susceptibility haplotype in Caucasians and Blacks, but not in Chinese or Japanese. This difference may arise from interactions between DR and DQ molecules. Indeed, DR β chains with aspartic acid at position 57 (DRB1*0403 or *0406) are protective even when present in HLA-haplotypes containing DQB1*0302;DQA*0301. Thus, susceptibility appears to arise mainly from DQ alleles, but it is influenced by HLA-DR and even LMP7, a locus within the haplotype that encodes part of the intracellular antigen processing complex.

Eleven other genetic intervals outside the HLA

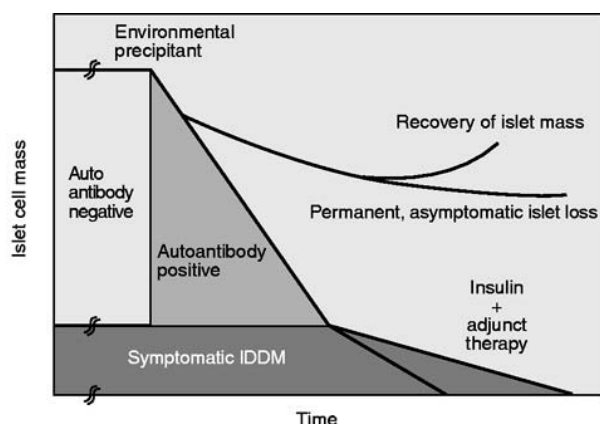


Figure 1 Natural history of autoimmune IDDM. Islet autoimmunity is a multistage process that becomes symptomatic only in its very late stages. An environmental stimulus in people with an inherited predisposition to disease leads to an initial insult to β cells that is associated with the appearance of circulating autoantibodies. Some islet cell mass may be lost, but the process may be transient and perhaps even partially reversible. Small amounts of β cell loss are clinically insignificant, but some individuals suffer inexorable depletion of islet cell mass, causing symptomatic hyperglycemia (i.e. IDDM). The rate of islet loss may be slowed by the initiation of insulin therapy and this process may be decelerated further by adjunct therapies, such as immunosuppression. The goal of preventive therapies is to arrest the loss of islet cell mass. It is anticipated that the maximal success of such treatments will depend on their initiation early in the preclinical stages.

locus have been reported to affect IDDM susceptibility. While the discovery of more intervals is expected, only four of those found so far have been confirmed (Table 2). IDDM2 has been mapped to the variable number of tandem repeats (VNTR) locus, upstream of the insulin gene (INS). The region is thought to enhance insulin transcription, the high-risk allele being associated with higher levels of INS mRNA. Higher rates of preproinsulin transcription may result in enhanced autoantigen production in β cells. Interestingly, the 2–5-fold relative risk for IDDM conferred at IDDM2 is preferentially or solely transmitted through paternal alleles. This could be best explained by a maternal imprinting, an effect strongly observed for the adjacent IGF2 gene. Diabetic fathers are at least three times more likely to

have affected children than are diabetic mothers, consistent with a parentally-biased transmission of the INS alleles.

Environmental factors

Autoantibody evidence that the disease process has started begin finally during infancy or early childhood (Figure 1). The role of viral or dietary triggers of β cell autoimmunity have long been postulated, but not clearly identified. Proposed pathogenic mechanisms have included direct islet cell viral infection, resulting in either altered self molecules or release of previously sequestered antigens from damaged β -cells, and/or a superantigenic event. Alternatively, molecular mimicry between a viral or dietary protein antigen and an islet autoantigen may provide the inductive event.

Congenital rubella, the first proven viral mediator of IDDM, is associated with an onset of hyperglycemia during the first 5–20 years of life in some 12% of such patients. More recently, enteroviruses, particularly Coxsackie B4, have been proposed as disease precipitants. SJL/J mice became hyperglycemic after their infection with a Coxsackie virus that had been isolated from the pancreas of a child who died at IDDM onset. Conflicting retrospective analyses of enteroviral serology in children with new-onset IDDM have appeared both before and after this. Acute enteroviral infection during pregnancy, documented by IgM titers, was prospectively associated with a higher risk of IDDM in the offspring before age 3 years. A putative molecular mimic of the Coxsackie P2C protein has been suggested to exist on the β cell autoantigen, glutamic acid decarboxylase₆₅ (GAD₆₅). A superantigen effect was implicated in IDDM pathogenesis after a disproportionate representation of V β 7 T lymphocyte clones was found in the pancreas of two children who died at their onset of IDDM. However, the polymerase chain reaction methodology used to detect this is fraught with the potential for quantitative artifacts.

Epidemiologic studies have suggested a direct association between cow's milk ingestion during infancy and diabetes, whereas an inverse relationship has been observed between the disease and duration of breast feeding. Antibodies to both the albumin and the globulin fractions of milk have been variably reported at higher rates in new-onset IDDM patients than in healthy controls. Further support for the hypothesis has arisen in experimental animal models of IDDM, in which dietary replacement of bovine protein has reduced diabetes incidence. It has been suggested that the ICA-69 protein of islets is mimicked by a 17mer (ABBOSS) contained in bovine

Table 2 Confirmed genetic intervals mapping IDDM susceptibility

Interval designation	Chromosome map	Identified genes
IDDM1	6p21	HLA-DQ, HLA-DR
IDDM2	11p15	VNTR 5' of insulin
IDDM4	11q13	
IDDM5	6q24–25	
IDDM8	6q25–27	

serum albumin (BSA). ICA-69 however is a ubiquitous antigen and in some studies antibodies to the protein occur at equal frequencies among diabetic and healthy control subjects. Antibodies against ABBOSS, as well as peripheral blood T cell proliferation in response to it, have been reported but not independently confirmed. T cell responses to β -lactoglobulin have also been reported in 55% of new-onset IDDM patients compared with 22% of controls. Multicenter prospective trials of cow's milk withdrawal are planned to address definitively this important public health issue.

Islet cell autoantigens

Pancreatic β cell components that are recognized by autoantibodies include insulin, proinsulin, GAD₆₅, and two protein tyrosine phosphatases, insulinoma antigen-2 and -2 β (IA-2 and IA-2 β). Other islet antigens suggested include carboxypeptidase H, ICA-69 and GM gangliosides. Humoral reactivities against these antigens are not believed to be directly harmful; however, they have provided a valuable means of identifying patients whose pancreatic β cells are under autoimmune attack. Islet cell autoantibodies (ICAs) in serum of healthy individuals are identified by indirect immunofluorescence of frozen human pancreatic sections. They are the most sensitive single predictor of future IDDM because they represent composite antibody reactions to multiple antigens. Interpretation of ICA positivity requires knowledge of the titer, the presence of other autoantibodies, the age and genetic risk of the patient, and the ability of pancreatic β cells to produce insulin after an intravenous glucose stimulus. Importantly though, the predictability of a positive ICA result is the same for relatives of patients as it is for the general population. However, the ICA procedure is technically demanding and simpler procedures for detecting autoantibodies against defined autoantigens are more reproducible. In our experience, detection of autoantibodies to GAD₆₅ plus those to insulin and/or to IA-2 could substitute for ICAs without loss of sensitivity in predicting IDDM.

Insulin was the first defined autoantigen to be identified in IDDM. In radiobinding assays, IAA are highly predictive of IDDM when they coexist with ICAs in subjects under 10 years of age. In a 1991 report, autoantibodies to proinsulin were detected by radiobinding assay 50% more often than IAA in 97 new-onset diabetic subjects. A 13 amino acid T lymphocyte epitope in IDDM patients that spans the C peptide-B chain junction of the prohormone has been reported, the sequence of which is 70% homologous to a T cell epitope of GAD₆₅.

Absorption of ICA activity from the sera of some IDDM patients by preincubation with the recombinant GAD₆₅ has been demonstrated and autoantibodies against GAD₆₅ (GADAb) can now be detected by immunoassays that are sensitive, accurate and reproducible. GADAb are found in 75% of new-onset IDDM patients, one-third of whom test negative for ICAs. Most longstanding ICAs after disease onset are, in reality, GADAb. As predictors of future IDDM however, their value is reduced by their moderately high frequency in normal adults. When GADAb coexist with additional ICAs, however, they indicate a high risk of IDDM.

The membrane-associated protein tyrosine phosphatase, IA-2, is expressed in islets, brain and pituitary gland. Its sequence confirms that its truncated form is the target of the previously identified autoantibody called ICA-512. Some 60–75% of new-onset patients have autoantibodies against the protein, compared with only 2% of healthy controls. In addition, among IDDM patients who were prospectively identified before disease onset (true prediabetics), seroconversion to IA-2 positivity increased from 60% to a maximum frequency of 75% in the year or so prior to IDDM diagnosis. Intra-islet expression of a homologous protein, IA-2 β , is restricted to β cells and displays an autoreactivity that is generally a subset of IA-2-positive persons. Autoantibodies against 40 kDa and 37 kDa fragments of trypsin-treated islet preparations that are closely related to IDDM susceptibility recognize portions of the intracellular domains of IA-2 and IA-2 β respectively, whereas those of 64 kDa and 50 kDa are directed against GAD₆₅ (Table 3). It is important to note that risk of IDDM is confined to those with more than one type of autoantibody.

Cellular immunity

Cellular autoimmune cytotoxic T cell effectors deliver the lethal hit to the pancreatic β cells. Aberrant peripheral blood T lymphocyte responses

Table 3 Islet autoantibodies in children

Autoantibody	Approximate frequency (%)			
	General population	Relatives	Pre-IDDM	New-onset IDDM
ICA	0.6	3.6	75	80
IAA	0.7	2.4	30	50
GAD ₆₅	1.0	2.5	75	75
IA-2	2.0	3.0	60	75
IA-2 β	2.0	3.0	35	40

to specific islet autoantigens have however been notoriously difficult to demonstrate in humans. Thus, insights into possible defects of cellular immunity in IDDM have arisen from a handful of human studies and a large volume of work performed in experimental animal models. NOD mice and BB rats have been the most frequently used comparative models of spontaneous anti β cell autoimmunity. Islet inflammation or insulinitis is the hallmark lesion of IDDM. Mononuclear cells – initially macrophages and dendritic cells – accumulate around the islets. Subsequently, B and T lymphocytes (both $CD4^+/CD8^-$ and $CD4^-/CD8^+$) arrive and form a periinsulitis before the cells invade the islets. Lymphocyte invasion, unaccompanied by macrophages, has been suggested as resulting in a nondestructive insulitis. Limited studies of human islets obtained through autopsy material and pancreatic biopsies performed at the time of IDDM onset have demonstrated similar, but less dense mixed-cell infiltrates. The $CD8^+$ T lymphocytes are more prominent in human insulitis than in the lesions of the rodents. There is also a class I HLA hyperexpression that usually accompanies insulitis that may potentiate cell-mediated β cell destruction.

There is an inability to stimulate a syngeneic/autologous mixed lymphocyte reaction against self MHC in IDDM patients and in NOD mice, suggesting that antigen-presenting cell (APC)-dependent regulatory function may be defective. Intrathymic deletion of autoreactive T lymphocytes in NOD mice has also been shown to be defective. Whereas studies in the NOD mouse have demonstrated incomplete maturation of macrophages after exposure to colony-stimulating factor 1 (MSF-1) and interferon γ ($IFN\gamma$), similar effects have not yet been reported in humans.

$CD4^+$ T cells cloned from NOD mice can transfer diabetes to immunodeficient (SCID) recipients, but the process is greatly accelerated by co-infusion of $CD8^+$ cells. Recently, however, cloned $CD8^+$ cells have also been reported to transfer diabetes to naive recipients. The role of B lymphocytes in insulitis is undefined. Since neither insulitis nor diabetes can be transferred by B lymphocytes, they have been considered to have a nondestructive function in the insulitis lesion. However, a NOD μ heavy chain knockout mouse, which has normal T lymphocytes but no functioning B lymphocytes, did not develop insulitis. This suggests a possible regulatory role for these cells.

T lymphocyte clones, some recognizing either insulin or GAD₆₅, have been isolated from NOD mice. Whereas diabetes is transferred to recipients by injecting some of these clones, other clones actually

confer protection from the disease. Cytokine production by invading cells provides part of the explanation for these contrasting results. $CD4^+$ T_H1 -like cells that produce $IFN\gamma$ and interleukin 2 (IL-2) support the destructive processes of insulitis. In contrast, T_H2 -like cells that secrete IL-4 and IL-10 are more likely to inhibit the harmful actions of the T_H1 cells. Host genetics, the nature of the antigen, its route of entry, its affinity for the MHC-binding cleft, and accessory molecule interactions (e.g. B-7 on the APCs and CD28/CTLA4 on T lymphocytes) are among the variables determining whether immune reactions will be predominantly T_H1 or T_H2 . The pathogenesis of IDDM is, however, more complex than a simple T_H1 - T_H2 dichotomy. Production of soluble mediators of inflammation, including prostanooids, interleukins, chemoattractants and chemokines, exert both destructive effects on pancreatic β cells and regulatory actions on immune cells. Transient molecules, such as reactive oxygen species and nitric oxide, have also been implicated in β cell destruction.

Treatment

The mainstay of treatment for diabetes since the discovery of insulin in the 1920s has been hormone replacement and glucose level monitoring. Intensive insulin replacement has been made possible by the advent of home blood glucose monitoring (to replace urine testing) and the introduction of purified, recombinant insulins with variable kinetics (short, intermediate and long acting). Until β cells can be regenerated or replaced without triggering renewed autoimmunity, or until the autoimmune process can be arrested before critical depletion of β cells has occurred, a cure is unlikely.

Even with modern modes of insulin replacement, such as open-loop continuous subcutaneous insulin infusions, chronic complications of the disease persist at high rates. Closed-loop delivery systems, which include a glucose sensor and automated insulin delivery system (i.e. artificial pancreas), are not yet ready for routine clinical use. Engraftment of autologous, non- β cells, containing recombinant insulin-producing constructs, is unlikely to become a reality in the coming decade. Islet and pancreas transplantation has found a niche in clinical medicine for some long-term IDDM patients who are undergoing immunosuppression for coincident renal transplantation. Prolonged survival of grafts in the absence of immunosuppression has not, however, been consistently demonstrated, even in transplantation between identical twins. Encasement of isolated islets in inert capsules that are permeable to

soluble insulin and glucose (e.g. alginate) have attempted to subvert recrudescant autoimmunity. Unfortunately, these devices have been prone to structural breakdown and graft destruction.

Disease prevention has recently become the subject of clinical trials. Generalized immunosuppression with azathioprine/steroids or cyclosporine retards the progression of islet destruction but does not ultimately prevent it. Nonspecific immunostimulation with tuberculin antigen (PPD) or BCG and anti-inflammatory therapy with nicotinamide have not yet afforded the levels of protection in humans that were observed with much larger doses in NOD mice. Trials of daily subcutaneous injections of insulin in mice result in marked delays of onset and profound reductions of insulinitis. In a recent trial of the treatment in patients with late-onset autoimmune diabetes, improved C-peptide production and glycemic control compared with five ICA-positive controls receiving an oral hypoglycemic agent was seen. An ongoing multicenter US trial of daily subcutaneous insulin, the DPT-1, should have the statistical power to determine definitively whether such prophylactic therapy is efficacious. Our own NOD mouse studies have suggested that intentional immunization to autoantigens, such as insulin B-chains but not insulin A-chains, and the peptide AA 9-23 of the B-chains especially, may redirect immune responses towards those unassociated with harmful anti-islet cytotoxicity. Such an observation could be the starting point for a diabetes vaccine in man in the future. Similarly, the induction of immunological tolerance to autoantigens through oral administration has proven effective in experimental animal models of a number of autoimmune conditions, including IDDM. A trial to induce oral tolerance to insulin has therefore commenced as part of the DPT-1, and a companion study in newly diagnosed patients is also underway. A number of immunotherapies that have demonstrated efficacy in rodents await further study before human trials can be launched. These include long-acting cytokine therapy (e.g. IL-4 or IL-10) and infusion of soluble MHC-antigen complexes to anergize autoreactive T lymphocytes.

Conclusion

The autoimmune pathogenesis of IDDM has been established beyond any reasonable doubt. Specific emphasis must now be placed on unravelling the web of genetic influences, environmental triggers and immunoregulatory failures that conspire to cause pancreatic β cell depletion.

See also: **Autoimmune diseases; Autoimmunity;**

Cytokines; Insulin-dependent diabetes mellitus, animal models; MHC disease associations; Polyendocrine autoimmunity.

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INTEGRINS

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In 1986 the term 'integrin' was proposed by Richard Hynes to describe a family of integral membrane receptors which promoted cell adhesion and communication through linking the intracellular activities of one cell with that of another cell or with the extracellular matrix. The integrins are now a superfamily of 22 receptors composed of noncovalently associated heterodimers of α and β subunits: 16 α and eight β subunits have been identified to date (Table 1). Leukocytes collectively express 13 of these 22 integrins but exclusively express only the six members of the β_2 - and β_7 -integrin families. The superfamily was previously subdivided into three main groups based on the sharing of a common β subunit (β_1 , β_2 or β_3) by α subunits of the very late activation (VLA) proteins, the leukocyte integrins (CD11/CD18 family) and the cytoadhesins, respectively. With the discovery of new integrins, it has become apparent that greater complexity exists. For example, the same α_4 subunit which binds to β_1 in VLA-4 can associate with a second β subunit, β_7 , and the α_v subunit of the vitronectin receptor (VNR) may unite with as many as five different β subunits. The cell-cell contacts formed by integrins are essential for many aspects of immune responsiveness, such as antigen presentation, leukocyte-mediated cytotoxicity and myeloid cell phagocytosis. Integrins are an essential part of an adhesion receptor cascade which guides leukocytes from the bloodstream across endothelium and into injured tissue in response to the chemotactic signals. The integrins expressed by leukocytes do not function constitutively but undergo a process of activation which is thought to involve changes both in integrin clustering and conformation, having the net effect of increasing the overall avidity of binding to ligand.

Structural relationships

α subunits

There is an overall 25% amino acid identity between the α subunits that increases to 45–63% between those which are from the same group. At the N-terminal end of the α subunits are seven homologous tandem repeated sequences (I–VII) of approximately 65 amino acids, of which three (V–VII) or four (IV–VII) contain putative divalent cation binding sites (Figure 1). Seven leukocyte integrins, including the four β_2 -integrins, VLA-1 ($\alpha_1\beta_1$), VLA-2 ($\alpha_2\beta_1$) and $\alpha_E\beta_7$ contain an extra 'inserted/interactive' or 'I' domain of ~200 amino acids located between repeated domains II and III. This domain belongs to the von Willebrand factor (vWF) protein family which is represented many times in nature in proteins involved in adhesion interactions. The crystallization of the Mac-1 and LFA-1 I domain in 1995 represented the first part of an integrin to yield to structural analysis. The I domain adopts a classic $\alpha\beta$ dinucleotide binding fold like the gp21 ras protein. Recently integrin domains I–VII have been modeled as a β -propeller domain resembling the β subunit of the same trimeric G protein complex.

β subunits

The eight β subunits share an overall 37–45% amino acid identity, with high homology in the cytoplasmic and transmembrane domains. The β_4 subunit, partnered by α_6 and found in hemidesmosomes, is distinctive in that it has a long cytoplasmic 'tail' of ~100 residues compared with the more typical ~45 residues found in the other β subunits. At the N-terminus of the β subunit extracellular domain there is

Table 1 The integrin α and β subunits and their characteristics

Family	β Sub-unit	α Sub-unit	CD group	$\alpha\beta$ Sub-unit	Receptor (R) name	Other name	Chromosome localization in humans	I region	Mol. wt (kDa) reduced	Ligand	RGD role	Distribution
VLA proteins	β_1		CD29				10 (pter-q22)		130			
		α_1	CD49a	$\alpha_1\beta_1$	VLA-1		5	+	210	C, L	?	Widespread
		α_2	CD49b	$\alpha_2\beta_1$	VLA-2	Collagen R, gpla	5	+	160	C(L)	-/?	Widespread
		α_3	CD49c	$\alpha_3\beta_1$	VLA-3		17 (q21-23)	-	125/25	C, L5, FN, entactin	-?	Widespread
		α_4	CD49d	$\alpha_4\beta_1$	VLA-4	LPAM-2	2 (q31-32)	-	150/80/70	FN (III-CS), VCAM-1	-	Widespread
		α_5	CD49e	$\alpha_5\beta_1$	VLA-5	Fibronectin R	12 (q11-13)	-	135/25	FN (RGD site)	+	Widespread
		α_6	CD49f	$\alpha_6\beta_1$	VLA-6	Laminin R	2	-	125/30	L (E8 fragment)	-	Widespread
		α_7		$\alpha_7\beta_1$						FN, VN, TN		
		α_8		$\alpha_8\beta_1$						TN, osteopontin		
		α_9		$\alpha_9\beta_1$			2		125/24	FN (or VN?)	+	Epithelium, Muscle, Hepatic Cell lines
Leukocyte integrins CD11/CD18	β_2		CD18				21 (q22.3)		95			
		α_L	CD11a	$\alpha_L\beta_2$	LFA-1		16 (p11-13.1)	+	180	ICAM-1, -2, -3	-	Leukocytes
		α_M	CD11b	$\alpha_M\beta_2$	Mac-1	CR3	16 (p11-13.1)	+	165	iC3b, Fb, factor X, ICAM-1	-	Neutrophil, monocytes, LGL
		α_X	CD11c	$\alpha_X\beta_2$	p150,95	CR4	16 (p11-13.1)	+	150	iC3b LPS	-	Mph, monocytes (neut, some T cells)
		α_D	CD11d	$\alpha_D\beta_2$			?	+	155	ICAM-3		Mph subsets (some T cells)
		α_4	CD49d	$\alpha_4\beta_7$	LPAM-1		12 (q13.13)		105			Lymphocytes
		α_E	CD103	$\alpha_E\beta_7$				+	150/25	VCAM-1, FN, MAdCAM-1		Intraepithelial, lymphocytes
			CD61			gpIIa	17 (q21-23)		105			
		α_{IIb}	CD41	$\alpha_{IIb}\beta_3$	gpIIb/IIIa		17 (q21-23)	-	120/25	Fb, FN, vWF, VN, Thr	+	Megakaryocytes, platelets
		α_V	CD51	$\alpha_V\beta_3$	Vitronectin R		2	-	125/24	VN, Fb, FN, vWF, Thr, bone sialoprotein, TN	+	Widespread
Other inter-actions	β_3	β_5		$\alpha_V\beta_5$					110 = β_5	VN (FN)	+	Widespread
		β_6		$\alpha_V\beta_6$					106 = β_6	FN, TN	?	Lung epithelium
		β_8		$\alpha_V\beta_8$	$\alpha_V\beta_8$				<114 = β_8	VN	+	Some cell lines
		β_4	CD104	$\alpha_6\beta_4$			17 (q11-ter)		205 = β_4	Lam 5 (kalinin), epiligrin	+	Epithelium hemidesmosomes

C, collagen; Fb, fibrinogen; FN, fibronectin; L, laminin; LGL, large granular lymphocyte; Mph, macrophage; Neut, neutrophil; Thr, thrombospondin; TN, tenascin; VN, vitronectin; vWF, von Willebrand factor.

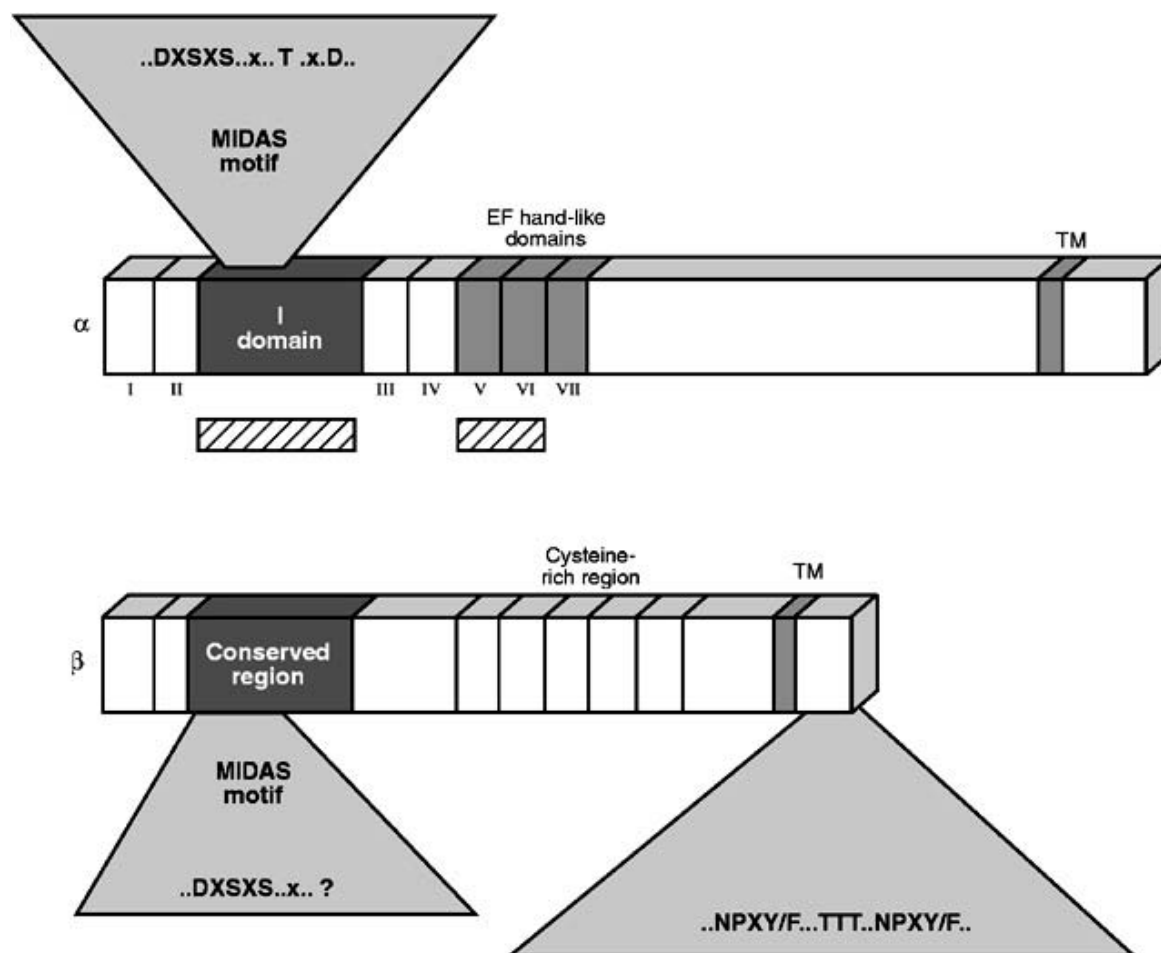


Figure 1 The domain structure of integrin heterodimers. The α and β subunits both contain the highlighted MIDAS (metal ion-dependent adhesion site) motif. Domains V–VII in I domain containing integrins (or IV–VII in others) have EF hand-like cation-binding sequences with a conserved motif DxDxDxxxDxxx (X is any amino acid). The positions of ligand-binding domains for ICAM-1 within $\alpha_4\beta_2$ are indicated as $////\Delta$. The β -subunit cytoplasmic domain insert shows the two conserved NPXY/F motifs present in all β subunits except β_4 and β_8 . The first NPXY motif has been shown to be important for affinity changes in β_1 and β_3 . The TTT motif in β_2 regulates adhesion through postreceptor events. TM, transmembrane sequence. (Reproduced with permission from Stewart *et al.* (1995).)

an area of high homology which is thought to participate in $\alpha\beta$ heterodimer formation and in ligand binding. Secondary structure prediction provides convincing evidence that this domain is homologous to the I domain of the α subunit, suggesting that seven integrins have two I domains, whereas the remainder have only the single I domain of the β subunit. More C terminal to this region, the β subunits have another distinctive feature in the positioning of 37 cysteines within a restricted area of 190 amino acids, giving rise to extensive disulfide bonding. Electron microscopic analysis of $\alpha_5\beta_1$ reveals a mushroom-shaped globular 'head', containing the ligand-binding area, supported on the membrane by 'stalks' formed by the C-terminal regions of the α subunit and the rigidly cross-linked β subunit.

Molecular aspects of ligand-binding metal ions and ligands

Integrins are metalloproteins and require divalent cations Mg^{2+} and Ca^{2+} for activity. In general the Mg^{2+} -bound integrin is functionally active, with the full role of Ca^{2+} still not completely understood. When agents such as phorbol esters are used to activate integrins, Ca^{2+} synergises with Mg^{2+} to increase integrin-mediated adhesion, whereas when integrins are directly activated with Mg^{2+} , adhesive activity is negatively regulated by Ca^{2+} . Potential metal-binding sites are located in the I domains of α and β subunits and the EF hand-like domains IV/V–VII of the α subunit. The I domains have an unusual Mg^{2+} -binding

site (Figure 1) termed the MIDAS (metal ion-dependent adhesion site) motif (DxSxS..T...D..). There is no definite information about which of these 4–5 sites are actually used during integrin activation.

The finding that the I domain of the α subunit contains a key ligand-binding site has stimulated much interest in ligand-binding areas of the α subunit. Elsewhere in the α subunit, ligand-binding sites are associated with the short repeating domains: V of GPIIb and domains V/VI of LFA-1 (Figure 1). Early experiments, in which RGD-containing peptides were cross-linked to the β_3 -integrins, indicated that approximately 80% of 'cross-linked' RGD bound to the common β_3 subunit within the highly conserved β 'I domain-like' sequence close to the MIDAS motif. Whether ligand can independently bind to these different sites or whether they come together to form a single 'maxi' binding pocket has still to be resolved. The concordance between ligand binding and cation binding suggests that cations might be directly part of the ligand-binding site or involved in conformational changes in association with the binding of ligand.

Ruoslahti and Pierschbacher showed that many but not all of the receptors which bind to matrix molecules recognise the tripeptide Arg-Gly-Asp (RGD). Another unusual feature of some integrins is their ability to bind to more than one ligand or counter-receptor. The VNR has the widest specificity, with binding activity for vitronectin, fibrinogen, vWF, fibronectin, thrombospondin and bone sialoprotein. In contrast, VLA-5 the classical fibronectin receptor, seems to bind only to fibronectin via recognition of the RGD sequence. VLA-3 and VLA-4 also recognize fibronectin, with the specificity of VLA-4 now known to be for another region of the fibronectin molecule, called the CS-1 domain. Further complexity arises from evidence that the same receptor isolated from two different cell types may have different binding specificities. For example, VLA-2 from platelets binds only collagen, but VLA-2 from endothelial cells binds both collagen and laminin. The ICAM-1, -2 and -3 recognised by the β_2 -integrin LFA-1 may serve as ligands in different situations: ICAM-3 is highly expressed by naive T cells and on antigen-presenting cells; ICAM-2 on endothelium; and ICAM-1 is widely induced by inflammatory stimuli and provides the most robust interaction with LFA-1.

Integrin function

β_2 -integrins

Integrins participate in cell interactions in many tissues, including those of the immune system. Of the

integrins specific to leukocytes, Mac-1 and p150,95 are largely confined to the cells of the myeloid lineage. These β_2 -integrins are present in cytoplasmic granules but are rapidly translocated to the membrane following exposure of myeloid cells to inflammatory stimuli such as cytokines and chemokines. Mac-1 has an impressive array of ligands, including iC3b, fibrinogen, factor X as well as ICAM-1, and the binding activities of p150,95 largely reflect that of Mac-1. Mac-1 is also found in *cis* associations with a number of surface receptors, including Fc γ RIIA, Fc γ RIIIB, urokinase and LPS/LBP-bound CD14, and it is proposed that Mac-1 may function as a transducer of signals resulting from the binding of these associated receptors to their ligands. Interestingly p150,95 also binds Fc γ RIIIB and LPS/LBP-bound CD14 and Petty and Todd have suggested that ligands may 'shuffle' between these two integrins during cell locomotion. The recently described $\alpha_D\beta_2$ is highly expressed by tissue macrophages and so far is the only β_2 -integrin to bind exclusively to ICAM-3.

In contrast, LFA-1 is found on all leukocytes. It functions in antigen presentation between T cells and different types of antigen-presenting cell where ICAM-3 may serve as an initial ligand; inhibition of T cell-mediated killing (but see next section); natural killer cell activity; neutrophil antibody-dependent cellular cytotoxicity; binding of tumor cells by activated macrophages and homotypic aggregation of proliferating leukocytes. The rapid response of memory T cells to antigen is facilitated by the presence of increased levels of LFA-1 and ligand ICAM-1, permitting enhanced cell-cell interactions. It is important to note that this increase in expression of adhesion receptors does not automatically cause elevated integrin activity. Triggering of T cells through specific receptors or with phorbol esters is necessary to bring about increased LFA-1 adhesion to ICAM-1. This control of receptor function through 'inside-out' signaling has the advantage that high avidity intercellular adhesion will occur only when the involved cells are activated. The increase in avidity is thought to depend on either redistribution of receptor in the membrane and/or conformational changes in LFA-1.

α_4 -integrins

The VLA antigens were first identified by Martin Hemler on T cells growing in long-term culture but are known to be more widely distributed. *In vivo* VLA-4 ($\alpha_4\beta_1$) is expressed on early thymic precursor cells and is retained until the 'double-positive' CD4⁺8⁺ blast stage prior to the exiting of functionally mature 'single-positive' CD4 or CD8 T cells

from the thymus into the circulation. Memory T cells and monocytes have enhanced levels of VLA-4, VLA-5 ($\alpha_5\beta_1$) and VLA-6 ($\alpha_6\beta_1$) and these receptors become activated like LFA-1 when these cells are triggered through the T cell receptor and are thought to function as the cells move within tissue compartments. The $\alpha_4\beta_7$ -integrin binds to fibronectin and VCAM-1 but is known as the mucosal homing receptor because it recognizes the mucosal addressin MADCAM-1. The second β_7 -integrin, $\alpha_E\beta_7$, is expressed by intraepithelial T cells and is the only integrin to recognize a member of the cadherin adhesion family, E-cadherin.

β_3 -integrins

The cytoadhesins (β_3 family) are also represented on leukocytes. The integrin gpIIb/IIIa is exclusively found on megakaryocytes and platelets and, by its binding to fibrinogen, functions as the key receptor on platelets in the clotting cascade. Fibrinogen binding by gpIIb/IIIa is tightly controlled; it occurs only after activation with agonists such as thrombin and involves a conformational change in gpIIb/IIIa (deduced from expression of ligand-induced binding site (LIBS) epitopes). Similar activation events permit gpIIb/IIIa to bind fibronectin or vWF, mediating the adhesion of platelets to endothelium or the subendothelial matrix. The other classical cytoadhesin, the VNR, is found on tissue culture-matured monocytes and is implicated in recognition of senescent or apoptotic leukocytes. This specialized form of phagocytosis ensures that dying cells are removed from tissues, limiting the damage caused by their potentially harmful contents.

Adhesion cascade

The cooperative action of integrins with the selectin adhesion receptors in successfully guiding leukocytes from the bloodstream across the endothelium and into injured tissue has been termed the adhesion cascade (Figure 2). It was initially thought that selectins alone were responsible for tethering leukocytes to stimulated endothelium but it is now recognized that the α_4 -integrins can also perform this role. In fact α_4 -integrins appear to be capable of participating in all stages of leukocyte transmigration, including leukocyte arrest on stimulated endothelium and movement across endothelium into the tissue space. LFA-1 and Mac-1 dominate as the integrins causing firm binding and cell flattening of cells on endothelium. Neutrophils rely only on LFA-1 and Mac-1 as they lack α_4 -integrins. Although chemokines are attractive candidates as integrin activators, more proof is needed that they are major activators of α_4 - or β_2 -integrins in this situation.

Integrin 'knockouts' in mouse and human

Many integrins have now been the target of gene deletion or 'knockout' (KO) experiments in mice. The majority of KOs are either embryonic or perinatal lethal, which indicates the importance of integrins in development. For example, the lethal α_4 KO characterized by the Hynes laboratory has confirmed a role for α_4 -integrins in muscle and neural crest development. Other approaches, such as inducible KOs or 'integrin null' chimeric mice, will allow exploration of the role of this integrin, which is of

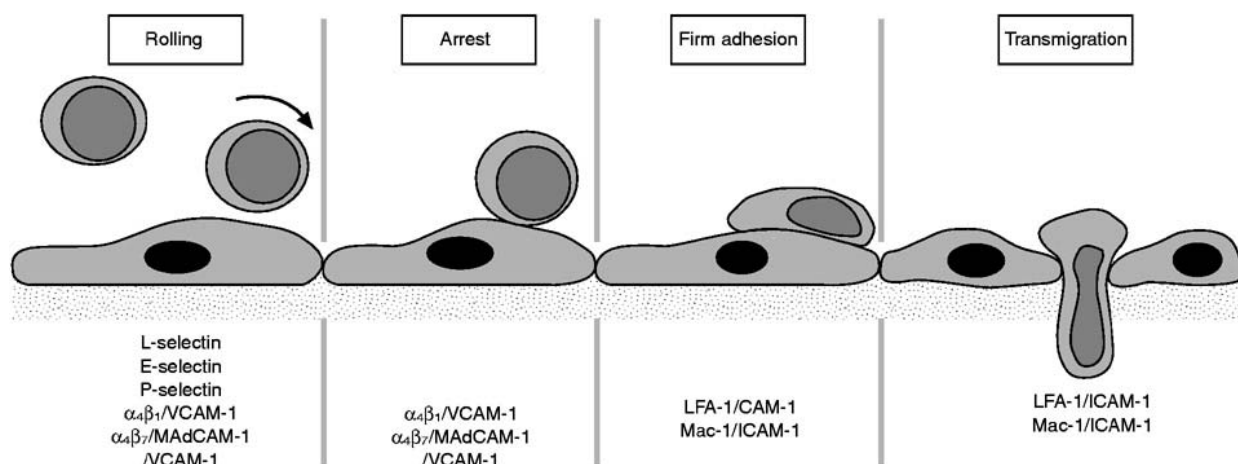


Figure 2 Sequential interactions of a leukocyte with endothelium under the guidance of the 'adhesion cascade'. ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1. (Reproduced with permission from Hogg N and Berlin C (1990) *Immunology Today* 16: 327–330.)

such importance in the adhesion cascade. On the other hand β_2 (CD18) KO mice have a normal phenotype but have severely depressed immune responses, e.g. reduced response to chemically induced peritonitis and also delay in transplant rejection. This analysis has become more specific with the characterization of the LFA-1 KO. These mice show the expected deficiencies when challenged in *in vitro* adhesion assays and also fail to mount an antitumor response *in vivo*, suggesting cell trafficking problems. Surprisingly, cytotoxic T lymphocyte responses toward common viruses are normal, indicating that LFA-1 may not always be necessary in the interaction between leukocytes and their targets.

The human 'KO' of the β_2 subunit or CD18, termed leukocyte adhesion deficiency (LAD), is a rare condition recognized in some 60 patients worldwide. Patients suffer from severe bacterial and fungal infections which arise from the inability of myeloid cells to perform phagocytosis and chemotaxis. In LAD, expression of three leukocyte integrins LFA-1, Mac-1 and p150,95, is reduced or absent owing to five types of genetic defect in the β_2 subunit, which prevents membrane expression of the $\alpha\beta$ complex. Another recognized integrin defect occurs in the equally rare Glanzmann's thrombasthenia, in which the expression of gpIIb/IIIa on platelets is reduced or absent due to mutations in the α subunit gpIIb.

See also: Adhesion molecules; CD2; Complement receptors; Fibronectin; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Lymphocyte function-associated antigen 1 (LFA-1); Lymphocyte function-associated antigen 3 (LFA-3).

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INTERCELLULAR ADHESION MOLECULES: ICAM-1, ICAM-2 AND ICAM-3

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The intercellular adhesion molecules ICAM-1 (CD54), ICAM-2 (CD102) and ICAM-3 (CD50) mediate a variety of critical intercellular adhesion events in the immune system through interactions with their counter-receptors, the β_2 -integrins LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), p150,95 (CD11c/CD18), and speculatively, CD11d/CD18. The binding of members of the ICAM family with the β_2 -integrins physically stabilizes interactions between collaborating pairs of T and B cells, T cells

and antigen-presenting cells (APCs), and brings effector cells such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells into close apposition with their target cells. In addition to promoting these intercellular collaborations, the binding of the ICAMs with the β_2 -integrins also plays a key role in leukocyte transendothelial migration during normal circulation and trafficking, and in the specialized localization of leukocytes in the vasculature and subsequent transendothelial migration into inflamed tissue.

All three ICAMs share a common polypeptide homology and structural motif, and the ability to bind LFA-1. The distinct functional role of each ICAM is dictated by their relative affinities for LFA-1 (ICAM-1 > ICAM-2 > ICAM-3), by differential binding to other β_2 -integrins (such as Mac-1 (CD11b/CD18)), their cellular distribution and regulation of expression, and differences in the ability to transduce costimulatory signals to lymphocytes (Table 1).

Expression and regulation

Overall, ICAM-1, ICAM-2 and ICAM-3 have complimentary patterns of expression and regulation which probably reflect different temporal and cellular roles in mediating leukocyte adhesion. ICAM-1 can be expressed on all leukocytes and a wide variety of nonhematopoietic cells, including endothelial and

epithelial cells. The expression of ICAM-1 is highly inducible and this appears to be a major mechanism of regulating ICAM-1 activity. Basal expression levels are typically low, but can be induced to much higher levels by inflammatory mediators such as interleukin 1 (IL-1), tumor necrosis factor (TNF), and interferon γ (IFN γ). Different mediators induce expression in different cells, and in combination may act additively or synergistically. On T cells, ICAM-1 expression is induced by activation. Typically, cell surface expression on activated or cytokine-induced cells can be detected within 4 h after induction, becomes maximal with 16–24 h, and remains high for some time thereafter. This inducible expression is usually the result of *de novo* transcription and translation, but mobilizable intracellular pools have been identified in monocytes. In addition to regulation through expression levels, some cells such as T lymphocytes may also regulate the net adhesive

Table 1 The intercellular adhesion molecules

	ICAM-1 (CD54)	ICAM-2 (CD102)	ICAM-3 (CD50)
Ligand for:			
LFA-1 (CD11a/CD18)	+	+	–
Mac-1 (CD11b/CD18)	+	+	–
p150,95 (CD11c/CD18)	+	?	
CD11d/CD18	–	?	+
Expression	Inducible	Constitutive	Constitutive
Distribution	Broad, at sites of inflammation Not limited to hematopoietic cells	Lymphocytes Endothelial cells Platelets	Resting leukocytes
PMN	Weak	–	+
Monocytes	Activated	+	+
Lymphocytes	Activated	+	+
Endothelial cells	Activated	+	+/-
Platelets	–	+	–
Soluble forms	Yes	No	Yes
Costimulatory signal through LFA-1	Yes	Yes	Yes
Direct signal transduction	Yes	No	Yes
Major role in leukocyte adhesion	Expansion of immune response effector Target interactions Transendothelial migration at sites of inflammation	Transendothelial migration during normal leukocyte trafficking? Initiation of immune responses	Initiation of immune responses
Receptor/ligand for pathogens	Receptor for rhinoviruses, malaria- infected erythrocytes		

activity of ICAM-1 by rapidly and transiently clustering the molecule at sites of cell-cell interaction.

In contrast to the inducible expression of ICAM-1 on activated cells, ICAM-2 and ICAM-3 are constitutively expressed on unactivated cells, and their cellular distribution is much more restricted. ICAM-2 is expressed on lymphocytes, monocytes, platelets and endothelial cells, but not on granulocytes. ICAM-3 is expressed on all leukocytes, but is absent from endothelial cells. The expression of ICAM-3 is most pronounced on cells capable of presenting antigen, including monocytes, resting lymphocytes, and dendritic cells.

Role in leukocyte adhesion

The role of the ICAMs in promoting adhesion between cells in the immune system has been demonstrated by a number of *in vitro* and *in vivo* approaches including functional inhibition with monoclonal antibodies, cell-binding experiments with purified proteins or transfected cells, and by mutagenesis. Experimental evidence suggests that together, ICAM-1, -2 and -3 can completely account for LFA-1-dependent adhesion. However, the distinct cellular distribution and expression patterns of the three molecules indicates that they are not just redundant systems, but rather serve different roles.

ICAM-1

ICAM-1 facilitates a variety of both afferent and efferent immune responses that require intercellular contact and collaboration, including T helper responses, T-dependent B cell responses, antigen-induced T cell proliferation, cell-mediated cytotoxicity, and the binding of leukocytes to vascular endothelium prior to extravasation into inflamed tissue. In these events, the binding of ICAM-1 to LFA-1 acts as an antigen-independent, generalized 'accessory' adhesion mechanism that holds cells in close proximity long enough for other receptor/ligand interactions (MHC/Ag receptor, cytokines, etc.) and cellular functions to take place.

The phenotypes of ICAM-1-deficient mice show that ICAM-1 is not essential for leukocyte development and maturation. However, these mice display impaired contact hypersensitivity, defective neutrophil migration, and their cells are defective stimulators of mixed lymphocyte responses. In addition, ICAM-1-deficient mice are resistant to septic shock induced by either superantigen or endotoxin, and this resistance appears to reflect defects in T cell activation by superantigen and decreased neutrophil infiltration into the liver stimulated by endotoxin.

In addition to its role in the immune system,

ICAM-1 has been subverted by some human pathogens for their own use; the major group of human rhinoviruses (causative agents of the common cold) use ICAM-1 as an obligate receptor for cell entry and infection, and red blood cells infected with the malarial parasite *Plasmodium falciparum* bind to ICAM-1 expressed on endothelial cells.

ICAM-2

In contrast to ICAM-1 and ICAM-3, the functional role of ICAM-2 is not clear. Although it is constitutively expressed on most leukocytes and platelets, it is most strongly expressed on normal endothelial cells, and its expression is not modulated by inflammatory cytokines. ICAM-2 may thus serve a role in mediating the normal recirculation of LFA-1⁺ lymphocytes in the absence of an inflammatory or immune response. Acting in concert with ICAM-1, ICAM-2 may also help to trigger further adhesion strengthening and transendothelial migration by inducing an increase in the avidity of LFA-1 for all of the ICAMs. Combinations of anti-ICAM-2 and anti-ICAM-3 antibodies inhibit the mixed lymphocyte reaction (MLR), suggesting that ICAM-2, like ICAM-3, may help to initiate immune responses. Finally, ICAM-2 is the only known β_2 -integrin ligand on platelets, which suggests that it might play a role in leukocyte-platelet interactions.

ICAM-3

The constitutive expression of ICAM-3 and its restricted distribution on resting lymphocytes and antigen-presenting cells indicate that its major role is in facilitating intercellular adhesion in the initiation of immune responses, and indeed, ICAM-3 accounts for most of the functional LFA-1-dependent adhesion of resting T cells. Furthermore, the binding of purified ICAM-3 to LFA-1 delivers costimulatory signals to both resting and activated T cells, and as noted below, ICAM-3 may also transduce signals itself after receptor binding and cross-linking. The combined adhesion and signal transduction properties of ICAM-3 indicates that it plays an essential role in the initial stages of T cell-dependent immune responses.

Signal transduction

Optimal activation of lymphocytes through specific receptors such as the CD3/TCR complex requires additional receptor-ligand interactions that generate 'costimulatory' signals. The binding of each of the ICAMs to LFA-1 has been shown to provide some form of costimulatory signal to T cells. In addition,

ICAM-1 and ICAM-3 appear to be capable of direct signal transduction; cross-linking with certain anti-ICAM-1 or ICAM-3 antibodies can provide costimulation for T cell proliferation, and can induce intracellular Ca^{2+} mobilization and the phosphorylation of cellular proteins. Cross-linking of ICAM-1 costimulates the formyl-Met-Leu-Phe-induced oxidative burst in mononuclear cells, and cross-linking of specific epitopes on ICAM-3 induces enhanced LFA-1-dependent intercellular adhesion. Overall, the ability of ICAMs to provide costimulatory or accessory signals directly, or via LFA-1, is consistent with their role as adhesion and accessory molecules.

Structure and relationship to function

All three ICAMs are typical integral membrane glycoproteins and are members of the immunoglobulin gene superfamily base on sequence homology. The extracellular domains are composed of discrete immunoglobulin (Ig) domains of the C2 type comprised of seven antiparallel β strands which form two β sheets held together by at least one intradomain disulfide bond. The extracellular domains of both ICAM-1 and ICAM-3 contain five Ig domains, while ICAM-2 contains only two. The Ig domains are predicted to provide a stable, rigid structural framework, and electron microscopy of purified molecules shows that ICAM-1 and ICAM-3 are rod-shape molecules approximately 19 and 15 nm long, respectively. These dimensions are consistent with an end-to-end arrangement of the Ig domains. ICAM-1 has a characteristic bend between domains D3 and D4 that may represent an area of molecular flexibility that provides increased receptor accessibility to key binding sites located at the N-terminus. In both the native membrane-bound and shed forms, ICAM-1 can exist as a homodimer which has significantly greater binding to LFA-1 than the monomeric form.

Site-specific mutagenesis and domain deletion analyses have localized the receptor-binding sites on each ICAM. For binding to LFA-1, the primary site of contact appears to be the N-terminal domain (D1) on each molecule, with some contribution from the penultimate domain D2. The binding site on ICAM-1 for human rhinoviruses is also located in domain D1, perhaps reflecting the overall molecular accessibility of this domain as it is held above the cell mem-

brane by the rigid framework of the other Ig domains. In contrast, the binding site for Mac-1 on ICAM-1 is localized on domain D3, raising the possibility that LFA-1 and Mac-1 might bind simultaneously, or independently in a regulated manner.

See also: Adhesion molecules; Immunoglobulin gene superfamily; Integrins; Lymphocyte function-associated antigen 1 (LFA-1).

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INTERFERON α

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In 1957 Isaacs and Lindenmann discovered that supernatants from virus-infected cell cultures contained a substance that could react with other cells to render them resistant to infection by the same or unrelated viruses. They named it interferon (IFN), which turned out to be a family of molecules all of which to some degree are able to interfere with viral infection. Moreover, IFNs are part of a superfamily of small proteins known as cytokines, that carry signals locally from one cell to another to play an important role in the body's natural defense system. In this respect, IFNs are considered to be the first line of defense against viral infection.

Although IFNs were first recognized for their potent antiviral properties, they were also shown to inhibit cell growth and promote differentiation. In addition, IFNs modulate the activity of virtually every component of the immune system including the antibody response, stimulation of cytotoxic lymphocytes, expression of major histocompatibility complex (MHC) and other surface antigens, recruitment of natural killer (NK) cells and activation of macrophages. Recently, one form of IFN has been implicated as playing an essential role in early pregnancy in several species of animals.

The IFNs have been classified into two types on the basis of their biological and physical properties. IFN α and IFN β are type I IFNs; IFN γ is a type II IFN. Type I IFNs can be produced by many cell types and induced by many substances, but the amount produced and the subspecies induced can vary per cell type and per inducer. Viruses and synthetic double-stranded RNAs are amongst the best inducers. Type II IFN is largely produced by T lymphocytes stimulated by foreign antigens or mitogens and by NK cells in response to viral infection. In general, type I IFNs are more potent inducers of viral resistance in cells while type II IFN is a better modulator of the immune system.

To elicit their biological properties, it is necessary that the IFNs bind to specific receptors on the target cells. The interaction of IFNs with their cognate receptors activates signaling pathways in which cytoplasmic activated transcription factors translocate to the nucleus and stimulate the induction of a set of primary genes which are central in mediating the biological response. This transcriptional activation is rapid and does not require protein synthesis. Similar

genetic activation of cells by IFNs appears to be required for most of its biological actions (e.g. antiviral, cell growth inhibitory and immunomodulatory activities).

The IFN α / β family

In all mammalian species studied, the α IFNs are encoded by a family of closely related genes. In the human genome at least 14 active IFN α genes and 4 pseudogenes can be discriminated. In addition, in the human genome a subfamily of IFN α -related genes, called IFN ω genes, have been identified. The IFN ω genes exhibit approximately 70% sequence homology with the other human IFN α genes and are mainly pseudogenes. In the mouse genome, 12 different complete IFN α genes have been isolated and characterized. Eleven of these encode biologically active proteins, the other is a pseudogene. The IFN ω genes appear to be absent in mice. Multiple functional IFN ω genes have been described in cattle, horse and sheep. Recently, it was shown that the IFN α genes are also present in birds; the chicken genome as well as the duck genome contain at least 20 IFN α genes. Human IFN β is encoded by a single copy gene, which exhibits approximately 40% homology at the nucleotide level to the IFN α genes, and which is physically closely associated with the IFN α gene family. The mouse genome, in common with human, possesses a single IFN β gene, whereas cattle, sheep and pigs possess multiple IFN β genes.

In humans, type I IFN genes are located within the band p22 on the short arm of chromosome 9. Recently, the first complete physical map of the type I IFN gene cluster has been established. In mouse, type I IFN genes are clustered on chromosome 4; in hamster on chromosome 2q.

In all mammalian species studied, type I IFN genes are devoid of introns and encode proteins of 186–190 amino acids, including a signal peptide of 23 amino acids. The IFN ω proteins, which have approximately 60% sequence similarity to the IFN α s, contain a C-terminal extension of 6 amino acids. All IFN α s contain four cysteine residues (positions 1, 29, 99 and 139) which are involved in disulfide bridges. An additional cysteine at position 86 is present in all murine, hamster and rat, and in

some human subspecies. Most murine and a few human IFN α subspecies contain an N-glycosylation site at position 78, and were shown to be glycosylated. The function of this glycosylation site is not understood, but it is not necessary for the biological activity of IFNs.

It is assumed that type I IFN genes share a common ancestor and that the IFN α gene family arose by repeated duplications of the ancestral α gene. The IFN α genes are relatively well conserved. Within a species the homology between the proteins is 70% or more. Between human and murine IFN α proteins the homology is 50–60%. Despite these structural similarities, IFN α s are relatively species-specific: most human IFNs have only a low activity on mouse cells. However, certain mouse IFN α s reveal high antiviral activity on hamster cells. Similarly, some hamster IFN α s show antiviral activity on mouse cells.

In response to developmental stimuli, trophoblasts of ruminants and humans secrete a distinct class of IFNs, IFN τ or trophoblast IFNs, which are involved in regulating maternal recognition of pregnancy. Several IFN τ genes have been identified in sheep and cattle and more recently in humans. Structurally, the amino acid sequence of IFN τ shows 30–70% homology to the type I IFNs from various species. Like the IFN ω proteins, the IFN τ proteins contain a C-terminal extension of 6 amino acids. In addition, the τ IFNs exhibit antiviral activity in common with other IFNs, and are able to bind the same receptor as IFN α , β and ω .

Regulation of IFN α expression

IFN α and IFN β gene expression is induced upon viral infections. The induction is transient and involves transcriptional activation by several transcription factors, which bind to upstream regulatory *cis*-elements. The region involved in the virus inducibility of the human IFN α genes is known as the virus-inducible element or VRE. Similarly, it was reported that the IE (inducible element), which overlaps with the VRE, was able to confer inducibility to murine IFN α genes. These elements contain repeats of the AGTGAA motif, or the variants of GAAANN and form cooperative binding sites for the positive factors IRF1 (IFN regulatory factor 1), TG-binding factor and α F1 (α factor 1). A fourth factor, IRF2, binds to the same element as IRF1 and can suppress the function of this factor. However, the precise role of these factors in the regulation of the IFN α genes remains to be established. Differences in the VRE sequences of individual IFN α genes in both men and mice have been suggested to account for differences

in the activity of their respective promoters. The IFN β gene is regulated in a different way, which is described in the entry on 'Interferon β '.

In general, no IFN activity is found in normal sera or organs, however some evidence is provided that small amounts of IFNs are constitutively produced by peritoneal macrophages *in vivo*.

IFN α / β signal transduction

All cellular responses to IFNs require the interaction of the ligand with a low number of high-affinity, species-specific cell surface receptors. There are two types of IFN receptors: type I and type II. The functional type I IFN receptor is composed of at least two transmembrane subunits and permits the productive binding of IFN β and the many related subtypes of IFN α . IFN α / β subspecies differ considerably in their affinity for the receptor, which correlates with the level and type of biological activity. The type II receptor also consists of two subunits and specifically binds IFN γ . The pathways activated by type I IFNs are slightly different from those activated by type II IFN, which will be discussed in the article on 'Interferon γ receptor'. Interestingly, the signal transduction pathway used by type I and type II IFNs has become a prototype for signaling from many cytokine receptors.

In response to type I IFN, within minutes the IFN α / β receptor activates two members of the JAK or Janus family of cytoplasmic tyrosine kinases, TYK2 and JAK1, that constitutively associate with the receptor cytoplasmic tail. Activated kinases become autophosphorylated and phosphorylate the receptor on specific tyrosine residues, creating docking sites for the transcription factors STAT1 and STAT2, that are members of a family known as STAT (signal transducers and activators of transcription). The STAT1 and STAT2 proteins subsequently become rapidly activated by tyrosine phosphorylation at a single site catalyzed by JAK1 and TYK2. This activation allows them to stably assemble into a STAT1–STAT2 heterodimer complex through specific src homology 2 (SH2) phosphotyrosyl interactions, and together with the DNA-binding subunit p48 (a member of the IRF family) join in the multiprotein complex ISGF3 (IFN-stimulated gene factor 3). ISGF3 translocates to the nucleus where it binds to the IFN-stimulated response element (ISRE; consensus sequence AGTTTCNNTTTCT/C, where N is any nucleotide) sequence in the promoters of IFN α / β -inducible genes (ISG), to initiate transcription. In addition to ISGF3, several other protein factors have been identified which recognize the ISRE: IRF1, IRF2, IRF3, ICSBP

(IFN consensus binding protein), Pip (Pu.1 interaction partner) (all members of the IRF family) and IBP1 (ISRE-binding protein 1). Although these factors all bind specifically to the ISRE, ISGF3 has been shown to be the primary factor involved in the early transcriptional activation of ISGs. ISGF3 is induced very rapidly by IFN α/β without novel protein synthesis. The kinetics of appearance and decline of ISGF3 and its lack of dependence on protein synthesis correlate with transcriptional activation and deactivation of ISGs. The precise role of the other ISRE-binding factors in the regulation of the ISGs, however, remains to be established.

Lately, a second class of early response ISG1 genes has been identified, that are activated by both type I and type II IFNs and contain an enhancer element known as gamma activated site (GAS; TTNNNNNAA). In response to both types of IFN, a transcription factor known as GAF (composed of a homodimer of phosphorylated STAT1) binds the GAS and activates transcription. GAF, like ISGF3, is resident in the cytoplasm of untreated cells, is activated in response to type I and type II IFNs, and then quickly migrates to the nucleus and binds DNA. Activation of GAF correlates with transcriptional activation of GAS-containing genes.

Recently, disruption of the gene for STAT1 in embryonic stem cells and in mice revealed that the STAT1 protein is required for all transcriptional responses to IFN. STAT1-deficient mice grow and

develop similar to wild-type animals, but show compromised innate immunity to viral disease (Figure 1).

IFN α/β -inducible genes

Type I and type II IFNs induce the expression of overlapping sets of genes. Some of these genes are induced in common by the type I and type II IFNs, whereas others seem to be preferentially induced by one of the IFN types. The similarities and differences in the biological properties of the type I and type II IFNs may be a reflection of partially overlapping and differential regulation of cellular genes by the two types of IFNs.

ISG mRNAs are detected in cells within about 1 hour of treatment with type I IFN. In some cases, the mRNAs accumulate to a steady-state level which is maintained for over 24 hours. In other cases, the mRNAs are induced to a peak level which then declines, despite the continued presence of IFN. The induction of gene expression by IFNs is often of great magnitude, reaching high rates of transcription from nearly undetectable levels. Although the induction is primarily at the transcriptional level, additional regulation at the post-transcriptional level has been proposed in certain cases. Induction of transcription will occur even in the absence of cellular protein synthesis. The transcriptional response of ISGs correlates with receptor occupancy. Some ISGs were also found to be directly inducible by virus or double-

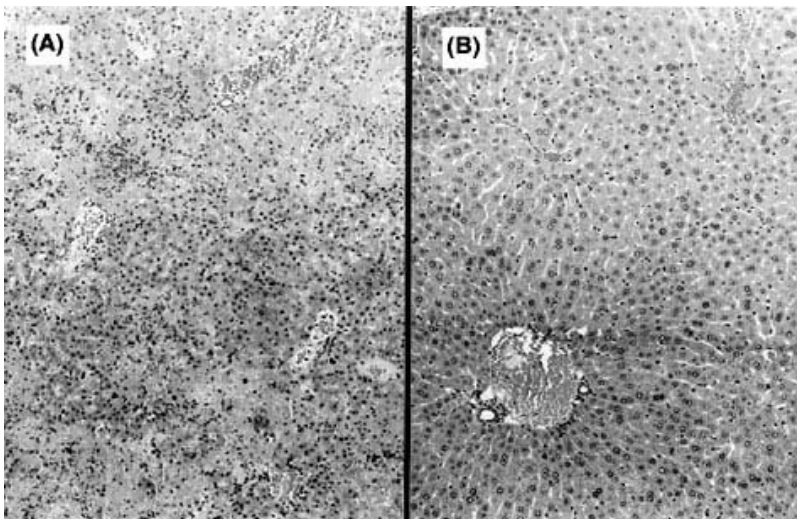


Figure 1 STAT1-deficient mice are extremely susceptible to viral infections. Transcriptional responses to interferons (IFNs) are mediated by tyrosine phosphorylation and nuclear translocation of transcription factors of the signal transducer and activator of transcription (STAT) family. Disruption of the gene for STAT1 in embryonic stem cells and in mice revealed that the STAT1 protein is required for all transcriptional responses to IFN (both type I and type II). STAT1-deficient mice grow and develop similar to wild-type animals, but show compromised innate immunity to viral disease. These animals fail to survive in the presence of otherwise innocuous pathogens, including mouse hepatitis virus. Histological examination of liver tissue from STAT1-deficient animals dying of disease showed multifocal hepatic necrosis with syncytial cell formation (A) and scant inflammatory response, consistent with viral hepatitis (caused by infection with mouse hepatitis virus). Analysis of hepatic tissue taken from wild-type siblings demonstrated no pathology (B). (Courtesy of Dr JE Durbin and Dr DE Levy.) (See also color **Plate 8**.)

stranded RNA, in the absence of IFNs. The mechanism of this induction, which does not seem to involve ISGF3 or GAS, is still unclear.

A search for IFN-induced proteins responsible for mediating the antiviral and other effects of IFNs led to the identification of novel enzymes (e.g. two double-stranded RNA-activated enzymes: a protein kinase (PKR) and a 2',5'-oligoadenylate synthetase (2,5OAS)), cell surface molecules (MHC class I and class II antigens, Fc receptors) and a number of new proteins, like the murine Mx protein.

A direct role in the antiviral actions has been demonstrated for the Mx protein and PKR and the 2,5OAS systems. In Mx⁺ mouse strains, for example, the Mx1 gene is induced by IFN and confers resistance selectively to influenzavirus. The IFN-induced PKR protein phosphorylates translation initiation factor eIF-2 α which results in inactivation of this factor and consequently to inhibition of protein synthesis. PKR activation is dependent on double-stranded RNA, which is present in a cell only when a virus replicates its genetic material. Thus, protein synthesis is blocked by PKR in infected cells but not in healthy ones. The 2,5OAS family of enzymes which are induced by both type I and type II IFNs, also interfere with the production of viral proteins, but they do so by activating an enzyme called ribonuclease RNAase L, which cleaves RNA without any specificity before it can be translated into protein.

Some of the proteins that inhibit protein synthesis may also have a function in the growth-inhibiting effects that IFNs display on certain tumors in culture and in the body. For example, the IFN-inducible 9-27 gene encodes for an RNA-binding protein that inhibits Rev-mediated HIV-1 expression and vesicular stomatitis virus. On the other hand, this protein has also been implicated in the IFN-induced inhibition of cell proliferation. Recently, the IFN-inducible cyclin-dependent kinase inhibitor p21 has also been implicated in the growth-inhibiting effect of IFNs, by negatively regulating the cell cycle.

Despite the fact that over 30 IFN-inducible proteins have now been identified, unfortunately it remains largely unclear how exactly they mediate the biological effects of IFN.

Therapeutic use of IFN α

With all their impressive properties, IFNs have come under close attention as potential therapeutic agents. In particular, the antitumor effects of IFNs led to high expectations in the battle against cancer. Unfortunately, IFN did not turn out to be a magic bullet. An additional limitation that IFNs display in clinical use, especially IFN α , is that they can produce side-

effects, including fever, headache, chills, fatigue, anorexia, leukopenia and thrombocytopenia. Despite their current limitations, IFNs are now well established as useful drugs. IFN α is the most widely used and has received approval in many countries, including Food and Drug Administration (FDA) approval in the US, for several clinical indications. Since 1986 it has been used as a therapy against hairy-cell leukemia. Significant regression of the cancer is observed in more than 90% of patients. Since 1988, IFN α has been successfully used as a therapy against diseases associated with human papillomavirus infection such as juvenile laryngeal papillomatosis and condyloma acuminatum. Since that same year it has also been used in the treatment of Kaposi's sarcoma in patients infected with HIV. Around 30% of the patients who receive IFN α , however, withdraw from treatment because of side-effects associated with the high IFN α doses. In 1991, IFN α was licensed for the treatment of chronic hepatitis C (non-A, non-B hepatitis) and in 1992 for chronic hepatitis B. IFNs also have demonstrated efficacy in basal cell carcinoma, cutaneous squamous cell carcinoma, chronic myelogenous leukemia, early HIV infection, carcinoid, multiple myeloma and some low-grade lymphomas.

The IFN τ proteins, like the other IFNs, show potent antiviral properties as well as antitumor effects and are currently also considered as therapeutic agents. The most promising quality of the τ IFNs, from a clinical point of view, may be their low toxicity as compared to IFN α . This low toxicity suggests that human IFN τ might prove to be a safer alternative to the other IFNs in the near future.

IFN α dosages per treatment range from 1 million units in hairy cell leukemia to 18–36 million units in Kaposi's sarcoma. Therapy duration ranges from a few weeks in condyloma acuminatum patients to several months in chronic hepatitis C cases. The severity of side-effects of IFNs are dose related and observed in most patients receiving more than 1 million units of IFN per treatment.

Whereas IFNs have so far clearly shown their value in medicine as a monotherapy, they may turn out to be most helpful when combined with standard treatments such as surgery, chemotherapy or radiotherapy or in combination with other drugs. For example, IFN α in combination with tamoxifen is more effective against breast cancer than either drug alone. Likewise, in combatting HIV, IFN α in combination with zidovudine (AZT) enhances the drug's effects. Alternatively, strategies for gene therapy involving IFN genes are being developed to treat infectious diseases such as the acquired immune deficiency syndrome (AIDS). Although this kind of

therapy is still in a very early stage, it may be a promising tool in the near future.

See also: Hepatitis B virus, infection and immunity; Cytokine genes, regulation of; Cytokines; Hepatitis C virus, infection and immunity; Interferon β ; Interferon γ ; Leukemia; Viruses, immunity to.

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INTERFERON β

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Interferon β (IFN β) and the multiple species of interferon α constitute the type 1 IFNs. The historical term 'fibroblast interferon' is misleading, since clearly more tissues than fibroblasts produce this type of IFN. Interferon β and α are related in a number of ways:

1. Their primary structure suggests that they originate from a common ancestor gene.
2. They may be produced by the same type of cell following the same inducing agent.
3. They bind to the same cell surface receptor and show an almost 100% overlap in their biological activities.

Thus, most of the antiviral and antiproliferative activities discussed below apply to IFN α also. Nevertheless, there are some observations that clearly point to a different functional role for IFN β *in vitro*. These observations include:

- the molecular mechanisms of IFN β induction;
- the concept of autocrine IFN β production during differentiation or physiological growth control; and
- different pharmacokinetics for IFN β and IFN α .

This entry on IFN β structure, induction and production will focus on human IFN β and include some

data on its murine counterpart. It is, however, anticipated that similar conclusions apply to other organisms, since in all mammalian species studied so far an equivalent structure of the IFN system has been found.

Structures of the IFN β gene and protein

The mature human IFN β is a glycoprotein of ~23 kDa apparent molecular weight. The entire primary sequence as deduced from complementary DNA consists of 166 amino acids which are preceded by a signal peptide of 21 amino acids. An N-glycosylation site has been identified at position 80 of the mature protein (Asn80-Glu81-Thr82). A disulfide bond between cysteines 31 and 141 is essential for biological activity. The more hydrophobic character of the IFN β protein is in agreement with a high content of leucine, tyrosine and isoleucine. The physiological significance of the carbohydrate moiety is still unclear, since unglycosylated recombinant IFN β retains its biological activity, although it has been suggested that glycosylation may contribute to its stability.

The IFN β gene has been mapped to human chromosome 9 in the vicinity of the IFN α gene cluster. Like the IFN α genes, the IFN β gene lacks any intron. Only one IFN β gene has been identified in human and mouse, whereas at least three closely related IFN β genes have been found in cattle.

Native murine IFN β has an apparent molecular weight of 35–40 kDa. The primary sequence of the mature protein consists of 161 amino acids preceded by 21 amino acids of signal sequence. There are three potential sites for N-glycosylation (at positions 29, 69 and 76) and one single cysteine residue at position 17. Thus, unlike human IFN β , the murine species does not require intramolecular disulfide linkage for biological activity. Like the murine IFN α genes, the murine IFN β gene is devoid of introns and has been localized to chromosome 4.

Induction and production of IFN β

Most cell types seem to be capable of producing IFNs under the appropriate conditions of induction. The type and amount of IFN produced, however, is extremely variable and depends on the given combination of cell type and inducer. Efficient inducers of IFNs include not only double-stranded RNAs (dsRNA) and viruses, but also bacteria or bacteria-derived lipopolysaccharide and protozoa.

There is no clear distinction between IFN α and IFN β inducers. Whether either or both types are produced depends to a large extent on the cell type and on the species. While dsRNA treatment of human fibroblasts will produce exclusively IFN β , treatment of murine fibroblasts will produce IFN β and IFN α . Following exposure to dsRNA, human blood monocytes will make IFN α , whereas murine macrophages make a mixture of IFN α/β . Thus, there seem to be cellular factors which convert the same inducing signal to a tissue specific pattern of expression. The following discussion will focus on the induction of IFN β by dsRNA and virus.

Regulation of IFN β gene expression

In general, the IFN β gene is only transiently activated following induction by virus or dsRNA. Maximum levels of IFN β mRNA are reached at around 6–8 h after induction and then gradually decrease to undetectable amounts by 18–24 h. In virus-infected cells, transcriptional activation is not dependent on *de novo* protein synthesis, in contrast to postinduction repression that is sensitive to protein synthesis inhibitor drugs. Expression of the IFN β is controlled by a transcriptional enhancer located between nucleotides –104 and –37 upstream of the transcriptional start site. Dissection of this DNA region has revealed the presence of four positive regulatory domains called PRDI to IV and one negative regulatory domain, NRDI, that partially overlaps with PRDII. When analyzed individually, the PRDs are unable to stimulate transcription unless they are present in multiple copies. However, such

multimerized subunits of the IFN β gene promoter show only moderate inducibility and, in addition to virus, are also responsive to tumor necrosis factor α (TNF α), IFN γ or cAMP. In contrast, the intact promoter is only inducible by virus and its activity exceeds by far that of any synthetic construct.

The PRDI region comprises a sequence between nucleotides –77 and –64 and was the first functional element identified within the IFN β enhancer. There is a strong sequence similarity between PRDI and an adjacent element named PRDIII, giving rise to the assumption that these sequences arose by duplication of a common ancestor. Two nuclear proteins, interferon regulatory factor 1 and 2 (IRF1, IRF2), have been shown to compete for a hexanucleotide sequence within PRDI. Both factors share extensive sequence homologies in their N-terminal parts which are involved in DNA binding; however, whereas IRF1 is a transcriptional enhancer, IRF2 represses IFN β expression. Following infection by virus, both mRNAs are induced and it is assumed that the finely tuned balance between these two factors is important for the shut-off of IFN β expression.

Nevertheless, the importance of IRF1 for the activation of PRDI is still a matter of debate. On the one hand, mutations within the PRDI element that reduce the affinity for IRF1 decrease the levels of virus-induced IFN β gene synthesis. In addition, IRF1 is not inducible in undifferentiated cells, which also lack the capacity to produce IFN β in response to virus. Upon retinoic acid-induced differentiation, however, these cells gain the capacity to express both IRF1 and IFN β . On the other hand, elimination of IRF1 by targeted gene disruption has been shown to result in deficient IFN β production only in response to dsRNA but not in response to virus. It has therefore been suggested that an alternative, IRF1-independent, pathway of IFN β induction also exists.

The PRDII element of the IFN β enhancer extends from nucleotides –64 to –55 and specifically binds transcription factors of the NF κ B/rel family. Among the four PRD elements, PRDII displays the strongest activity and it has been shown that oligomers of PRDII confer inducibility by virus but can also act as constitutive transcriptional activators. In the non-induced state the intact IFN β promoter is silent due to the presence of NRDI that partially overlaps with PRDII and further extends downstream to nucleotide –37. NRDI also contains a conserved 11 bp element at the 5' end with a strong silencing effect on constitutive NF κ B/rel-containing promoter constructs. NF κ B is necessary for induction of the IFN β gene by virus; it is, however, not sufficient since a variety of other activators of NF κ B fail to stimulate the gene promoter. In addition to NF κ B, the high mobility

group (HMG) protein I(Y) has been shown to bind to PRDII and to enhance DNA binding activity of the p50 and p65 subunits of NF κ B.

Like PRDII, the PRDIV element alone can also function as a virus-inducible enhancer when present in multiple copies. In the intact IFN β enhancer PRDIV is located between nucleotides -104 and -86. The activating transcription factor 2 (ATF2) specifically binds to a nonamer region within PRDIV, either as a homodimer or as a heterodimer with the cellular proto-oncogene *c-jun*. Similarly to NF κ B and PRDII, HMG I(Y) has also been found to specifically recognize DNA sequences adjacent to the ATF2/*c-jun* binding site at PRDIV. In addition, HMG I(Y) interacts with the basic/leucine zipper domains of ATF2/*c-jun* and facilitates the binding of the heterodimer to PRDIV.

Recently, it has been proposed that HMG I(Y) facilitates the assembly of an 'enhanceosome' which constitutes the regulatory unit for the control of IFN β gene expression. According to this model the enhanceosome of the IFN β promoter comprises a DNA complex including two or more HMG I(Y) molecules and at least three transcription factors that bind to PRDI-IV.

IFN β mediated biological activity

At least five complex types of biological activity are mediated by IFNs: 1) receptor binding, 2) antiviral, 3) cell growth inhibitory, 4) immunomodulatory, and 5) antitumor activity. This summary will focus on the first three activities.

The IFN α / β receptor

Any IFN-mediated activity requires binding to a specific receptor on the cell surface. The number of receptors varies from some hundred to a few thousand per cell. All human IFN α species and IFN β share the same type I IFN receptor to which they bind with similar affinity (K_d ranging from 1 to 2×10^{-10} M). Receptor-bound IFN is internalized via receptor-mediated endocytosis. The receptor is composed of two chains with molecular weights of 115 kDa and 102 kDa. The gene for the type I IFN receptor α chain encodes the 557 amino acids of the 115 kDa protein. A second, recently identified cDNA encodes for a 51 kDa protein of 331 amino acids, but it is not yet clear whether the 102 kDa chain consists of two disulfide-linked 51 kDa subunits. When expressed in mouse cells, only the 51 kDa chain displays high-affinity binding for all type I IFN species including IFN β , but is biologically inactive. The 115 kDa chain only binds one IFN α subspecies and not IFN β ; however, a tenfold increase in ligand

affinity is observed when the 51 kDa and 115 kDa chains are coexpressed in mouse cells, indicating that both receptor chains associate upon binding of IFN to form a high-affinity ternary complex. In addition, they also seem to be involved in signal transduction in human cells via two cytoplasmic tyrosine kinases. Jak1 and tyk2, that are constitutively associated with the native 102 kDa and 115 kDa chains, respectively.

On the basis of nucleotide sequence homology, a murine homolog of the human 115 kDa receptor chain has been identified and expressed in human cells. Similar to the corresponding human counterpart, this chain also displays selective binding characteristics for some but not all type I mouse IFN species, suggesting that the natural mouse IFN receptor also consists of two chains. Studies using mutant mice lacking this receptor chain proved the important role of the IFN system *in vivo* since these animals showed high susceptibility to infection by several viruses including vesicular stomatitis, Semliki Forest, lymphocytic choriomeningitis and vaccinia virus. These findings demonstrate that mouse cells probably also have an additional receptor chain and that the loss of one functional subunit results in failure to respond to IFN.

Antiviral activity

Exposure of cells to IFNs renders them less permissive to viral replication. Current evidence suggests that this antiviral state is composed of a number of independent antiviral mechanisms which may interfere at various stages of the viral growth cycle. Thus, it has been demonstrated that IFN treatment inhibits 1) virus uptake (vesicular stomatitis virus (VSV)), 2) events during late virus uncoating or primary transcription (e.g. SV-40, herpes simplex virus type 1 (HSV-1), influenza virus, VSV), 3) viral protein synthesis (e.g. reovirus, picornavirus, VSV, SV-40), and 4) virus maturation and release (retrovirus, HSV-1). Further targets for antiviral activity may exist.

The mechanisms by which these inhibitions are brought about are not completely understood. Crucial to the translational inhibition are two pathways which require dsRNA for activation. One pathway leads to increased degradation of RNAs through stimulation of the IFN-induced 2',5'-oligoadenylate synthetase and activation of an endoribonuclease by its product, 2',5'-oligoadenylate. This RNAase is involved in IFN-mediated resistance to encephalomyocarditis virus but not to VSV, as shown by overexpression of a dominant negative mutant in cell culture. Central to the second pathway is an IFN-induced protein kinase (PKR), which upon activation by dsRNA phosphorylates the translation factor eIF-2 α and thus blocks protein synthesis at the level of

binding of mRNAs to ribosomes. In the intact cell, these translational control mechanisms are fairly specific for viral gene expression, leaving cellular protein synthesis unchanged.

The Mx proteins are a family of related, IFN-induced proteins which mediate resistance to specific virus groups. The murine Mx1 is a nuclear protein which blocks primary transcription of influenza A virus. Its human homolog is a cytoplasmic protein which in addition to influenza A virus inhibits the replication of VSV. Both proteins have a GTPase activity, however its importance for the antiviral activity is currently unknown.

Some proteins involved in IFN signaling such as STAT1 and IRF1 are also required for establishing an antiviral state. IRF1 has been shown to exhibit antiviral properties in the absence of IFN whereas STAT1, the common subunit of the signal transduction and activator of transcription protein family, requires phosphorylation on a tyrosine residue following IFN treatment since a mutation in this position prevents its activation, nuclear translocation and IFN-induced virus resistance.

In addition to these intracellular antiviral activities whose target is the replicating virus, IFN-mediated antiviral protection in the organism may involve effector cells of the immune system, e.g. natural killer (NK) cells, macrophages or cytotoxic T cells, whose target is the infected cell itself.

Cell growth inhibiting activity

The antiproliferative activity of IFN type 1 has been observed on cultured cells from various tissues, e.g. fibroblasts, epithelial cells, endothelial cells, and cells of the hematopoietic system. Furthermore, normal, transformed and tumorigenic cells have been shown to be sensitive to this activity, although the degree of sensitivity varies widely. It is now clear that multiple parallel pathways are involved in IFN-mediated growth inhibition and some of the molecular targets have been identified during the past few years. In IFN-sensitive cell lines, strongly reduced DNA-binding activity of the transcription factor E2F was observed and found to be associated with growth arrest in the G₀/G₁ phase of the cell cycle. E2F activates several genes involved in cell cycle regulation and recent reports have documented an IFN-mediated inhibition of cyclins and cyclin-dependent kinases (cdk), indicating that IFN acts, at least in part, by functional modulation of E2F. Most likely, inhibition of cdk also results in reduced phosphorylation of the retinoblastoma protein (pRB), a tumor suppressor gene known to form a complex with E2F, thus preventing its interaction with DNA target

sequences. Upon phosphorylation, pRB detaches from E2F and the released factor becomes active. In addition, IFN also induces expression of pRB which predominantly exists as the underphosphorylated form able to interact with E2F. Since E2F also drives the expression of *c-myc*, there is also a concomitant inhibition of this cellular proto-oncogene in IFN-treated cells. The transcription factor IRF1 has also been shown to cause inhibition of cell proliferation when overexpressed in cell culture. IRF1 is not only involved in the regulation of IFN gene expression but is itself inducible by IFN and transactivates IFN-stimulated genes through binding to the IFN-stimulated response element (ISRE). The dsRNA-dependent kinase PKR is induced by IRF1 and there is substantial evidence that PKR represents a downstream target of IRF1-mediated growth inhibition since cells engineered to overexpress a dominant negative mutant of PKR fail to respond to the antiproliferative action of IRF1.

'Endogenous' IFN β induction

Circumstantial evidence points to a low level of IFN β production under various conditions in cell culture and *in vivo* in the absence of known IFN inducers, a situation which has been described as physiological, spontaneous or autocrine IFN β production. This endogenous IFN β has been implicated in control of cell growth and differentiation. Primary mouse macrophages proliferate in response to macrophage colony-stimulating factor (M-CSF). Inclusion of an antimouse IFN α/β serum further stimulates the mitogenic response and increases the susceptibility of the culture to virus infection. In mouse myeloid M1 cells, IFN β mRNA has been detected following differentiation by cytokines (e.g. M-CSF, TNF α , interleukin 6 (IL-6)) and differentiation was inhibited by antiIFN β serum. Activities similar to those of IFN β have also been demonstrated in dimethyl sulfoxide (DMSO)-differentiated Friend erythroleukemia cells and in phorbol-12-myristate-13-acetate (PMA) differentiated U937 cells.

Endogenous IFN β production is not restricted to cells of hematopoietic origin; IFN β RNA has been found in IL-1- and TNF-treated Hep-2 cells and is of crucial importance to the antiviral activity of TNF, whereas in FS-4 fibroblasts the mitogenic stimulus provided by TNF is enhanced by antibodies against IFN β .

Why IFN β ?

The division of the IFNs into one (or a few) IFN β and a multispecies group of IFN α which has so far

been found in all mammalian species, suggests a unique role for these different IFNs. This assumption, however, finds little support when one compares their biological activities, since the overlap seems to be almost complete and synergism has not been found when IFN α and IFN β are combined in treatment. Thus, the biological 'justification' for IFN β may be the tissue specificity of its expression and its pharmacokinetic behavior. From clinical experience, it is known that IFN β is more 'sticky' than IFN α , i.e. whereas the latter is easily found in the serum following intramuscular injection, IFN β administered by the same route is usually not found in the circulation. It has been assumed that, due to a faster uptake in tissues and in the lymphatic system, IFN β exerts its biological activity in close proximity to its site of production and is thus especially suited as an autocrine/paracrine effector in control of growth and differentiation.

See also: Cytokine genes, regulation of; Cytokines; Interferon α ; Interferon γ ; Interleukin 6; Viruses, immunity to.

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INTERFERON γ

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The cytokine now known as interferon γ (IFN γ) was first described in 1965 by Wheelock as an interferon-like antiviral substance produced by leukocyte cultures stimulated with phytohemagglutinin (PHA). Although in the virus inhibition assay the factor was indistinguishable from IFN, Wheelock noted that, unlike typical IFN, the PHA-induced leukocyte-derived factor was inactivated by exposure to pH 2. Subsequently, other investigators showed that various mitogenic agents can induce the production of a factor with IFN-like antiviral activity in cultures of lymphocytes, especially T cells. In 1973, Youngner and Salvin showed that IFN derived from lymphocyte cultures by mitogenic stimulation is antigenically distinct from 'classical' IFN and they pro-

posed to term the former 'type II IFN'. Other investigators used the term 'immune IFN' to describe this IFN-like protein. In 1980, these earlier names were replaced with the term IFN γ . At the same time, the names IFN α and IFN β were introduced for the two antigenically distinct varieties of 'classical' or 'type I' IFN. The human IFN γ protein was first isolated by Yip and colleagues in 1981.

Cloning of the human IFN γ gene by Goeddel and coworkers in 1981 revealed that IFN γ is structurally distinct from the IFN α/β family. (There is a large family of mutually interrelated IFN α/β proteins, each encoded by a separate structural gene.) Likewise, the cellular receptor for IFN γ is distinct from the receptor for IFN α/β .

The IFN γ gene and protein

Only a single IFN γ gene has been identified in all animal species examined. The human IFN γ gene has been mapped to chromosome 12, the structural gene for murine IFN γ is on chromosome 10 (Table 1). The overall organization of the IFN γ gene is very similar in different animal species, comprising three introns and four exons.

The human IFN γ gene encodes a mature protein 143 amino acids long, in addition to a 23 amino acid signal peptide sequence removed before the secretion of the molecule. The protein is rich in basic amino acids. Native IFN γ was found to lack between 9 and 16 residues from the C-terminus, apparently due to exopeptidase digestion during or after secretion. There are two potential N-glycosylation sites at positions 28 and 100. Under denaturing conditions, the fully glycosylated molecule has an apparent molecular weight of 25 kDa, but in some naturally produced molecules only one of the two sites is glycosylated (resulting in the generation of a molecule with an apparent molecular weight of 20 kDa under denaturing conditions) and a small portion of the secreted IFN γ molecules apparently lacks carbohydrate altogether (having an apparent molecular weight of 15 kDa). There is no evidence that the presence or absence of carbohydrate has a significant influence on the functional activity of IFN γ . Nonglycosylated *Escherichia coli*-derived recombinant human IFN γ shows the same spectrum of biological activities as natural, glycosylated human IFN γ . Native human IFN γ is a homodimer, with an apparent molecular weight of approximately 40–50 kDa on gel filtration columns. X-Ray crystallographic analysis of recom-

binant IFN γ indicates that the sites contacting the cell surface receptor contain segments from both the N- and C-termini of the IFN γ dimer.

The murine IFN γ gene encodes a 134 amino acid-long mature peptide (i.e. nine residues shorter than human IFN γ), with a cysteine at the C-terminus. However, the actual C-terminal sequence of native murine IFN γ is not known. Human and murine IFN γ are only about 40% homologous at the amino acid level. Therefore, human IFN γ is virtually inactive in mouse cells, and so is murine IFN γ in human cells.

Generation of IFN γ

Although production of trace amounts of IFN γ by monocytic and some other cells has been reported, lymphoid cells are the only significant source of this cytokine. Natural killer (NK) cells, helper T (T_H) cells, cytotoxic T (T_C) cells and some B cell lines all generate IFN γ upon appropriate stimulation. IFN γ production in these cells can be induced by various activating treatments; in T cells IFN γ can be induced by polyclonal activators, e.g. PHA, concanavalin A or antibodies to CD3, or by antigen-specific, clonally restricted activation. In helper T cells, IFN γ production is abundant by T_H1 cells and is characteristically absent in T_H2 cells. The cytokine interleukin 12 (IL-12) is now recognized to play an important role in the regulation of IFN γ production by both NK and T cells. In the intact organism, during early stages of an infection, lipopolysaccharide (LPS) and other products of bacteria, as well as components of protozoan parasites and other infectious agents, induce IL-12 production in monocytes and macro-

Table 1 Major characteristics of IFN γ

Location of structural gene	Chromosome 12 (human) Chromosome 10 (mouse)
Location of receptor genes	Chromosome 6 and 21 (human) Chromosome 10 and 16 (mouse)
Main cellular sources	T cells (mainly T_H1 and T_C), NK cells
Major biological actions	Activation of monocytes/macrophages Induction of class I and II MHC molecules Promotion of antigen processing and presentation by accessory cells Stimulation of high-output nitric oxide production Modulation of immunoglobulin synthesis Promotion of T_H1 response, inhibition of T_H2 response Modulation of cytokine synthesis Inhibition or stimulation of cell growth Inhibition of the multiplication of viruses and some other intracellular infectious agents Inhibition of tumor growth
Roles in host resistance and disease	Protection against infectious agents (mainly through innate immune mechanisms) Increased production during infections can contribute to pathology (together with TNF α , lymphotoxin (TNF β) and some other cytokines) Increased production at a local site may promote autoimmune disease

phages. IL-12, together with IL-1, tumor necrosis factor α (TNF α) and some other cytokines produced by activated macrophages, then stimulates IFN γ generation in NK cells and subsequently in T cells. IFN γ , in turn, promotes its own production through complex regulatory circuits. In contrast, cytokines associated with the T_H2-type response, notably IL-10 and IL-4, inhibit IFN γ generation. Glucocorticoids and transforming growth factor β (TGF β) also downregulate IFN γ production.

The induction of IFN γ appears to be regulated mainly at the transcriptional level. A combination of several positive and negative regulatory elements located in the 5'-flanking region and in the introns of the IFN γ gene is thought to govern the expression of IFN γ , but the details of this process have not yet been fully elucidated. In addition, IFN γ synthesis can be regulated by the level of DNA methylation and post-transcriptionally by changes in mRNA stability or translation efficiency.

The IFN γ receptor and molecular mechanisms of action

The biological actions of IFN γ can be attributed to its ability to activate or inhibit the expression of specific target genes. All biological actions of IFN γ require the binding of IFN γ to specific cell surface receptors, expressed on virtually all types of cells. The receptor is composed of two chains, the ligand-binding α chain and the accessory β chain, which are encoded by genes located on two different chromosomes (Table 1). Although binding of IFN γ to the receptor is the function of the α chain, IFN γ binds with higher affinity in the presence of the β chain. IFN γ signaling is initiated by the formation of a multisubunit complex that contains both IFN γ receptor chains and IFN γ .

IFN γ signaling leading to activation of gene expression proceeds via the JAK-STAT pathway, so named because it involves the activation of receptor-bound JAK (Janus) tyrosine kinases, followed by the tyrosine phosphorylation and resulting activation of the signal transducer and activator of transcription 1 (STAT1) protein. IFN γ binding first brings about dimerization of the receptor α chain, thereby creating a binding site for the receptor β chain and leading to the activation of the JAK1 and JAK2 kinases. The activated JAK1 kinase phosphorylates a specific tyrosine residue on the cytoplasmic domain of the IFN γ receptor α chain, thus creating a docking site for the SH2 domain-containing STAT1 protein. Docking of STAT1 is followed by its tyrosine phosphorylation and subsequent release from the receptor. Activated STAT1 protein forms homodimers that translocate

to the nucleus and bind to DNA recognition sequences (termed interferon γ -activated sites, GAS) present in some IFN γ -inducible genes, thereby stimulating their transcription. In addition, activated STAT1 protein can form a heterocomplex with a protein termed p48, and this heterocomplex binds to other DNA recognition sequences (termed interferon-stimulated response elements, ISRE) present in some IFN γ -inducible genes, and thus activate their transcription. Some genes thus activated by IFN γ encode transcription factors, which then can activate additional genes. One transcription factor whose synthesis is induced by IFN γ is IRF1 (interferon regulatory factor 1). This is required for the IFN γ -induced activation of several genes, including the murine gene encoding the inducible nitric oxide synthase (iNOS), an enzyme responsible for the high-output nitric oxide production that mediates the killing of intracellular infectious agents and tumor cells. Another transcription factor induced by IFN γ is CIITA (class II transcriptional activator) responsible for the activation of class II major histocompatibility complex (MHC) genes.

Recent progress in the understanding of the pathways of intracellular signaling revealed why there are many similarities in the actions of IFN α/β and IFN γ , despite the fact that they are structurally dissimilar and bind to different receptors. It is now known that the IFN α/β receptor activates a similar pattern of JAK-STAT signaling as the IFN γ receptor, with some signaling proteins being shared (e.g. the JAK1 kinase and STAT1 protein) and some being utilized only in IFN α/β signaling (Tyk2 kinase and STAT2 protein) or only in IFN γ signaling (JAK2 kinase). Among the genes known to be activated by both IFN γ and IFN α/β are some genes encoding proteins responsible for the antiviral action (2'-5'-oligoadenylate synthetase and a double-stranded RNA-dependent kinase termed PKR) and genes encoding class I MHC molecules. In contrast, class II MHC genes are inducible by IFN γ but generally not by IFN α/β .

IFN γ action can also result in the inhibition of expression of some genes, such as genes encoding some other cytokines (chemokines). The inhibitory effects on gene expression can occur either at the level of transcription or post-transcriptionally, but the mechanisms of these inhibitory actions have not been fully explained.

Immunoregulatory actions of IFN γ

IFN γ plays a major role in the activation of monocytes or macrophages, resulting in their enhanced tumoricidal and microbicidal activities. Bacterial lipopolysaccharide (LPS) or TNF α and IL-1 can act

synergistically with IFN γ in this process. IFN γ stimulates monocytes/macrophages to synthesize TNF α , which mediates resistance to intracellular bacteria and the killing of some types of tumor cells. Monocyte/macrophage activation by IFN γ is accompanied by the release of the toxic gaseous compound nitric oxide (NO) which, in conjunction with reactive oxygen intermediates, mediates the killing of intracellular infectious agents and tumor cells. IFN γ is also known to enhance the expression of high-affinity Fc receptors for immunoglobulin G (IgG) on monocytes/macrophages, and this action may play a role in the stimulation of antibody-dependent cellular cytotoxicity (ADCC). Many examples of a differentiation-inducing activity of IFN γ in myelomonocytic cell lines have been described. In addition to their actions on monocytes/macrophages, IFNs (both IFN α/β and IFN γ) can activate other cytotoxic effector cells, most notably NK cells and cytotoxic T cells. In neutrophils IFN γ induces high-affinity IgG Fc receptors, leading to increased phagocytosis and cytotoxicity.

IFN γ can enhance antigen presentation and immune recognition in T cell-mediated immunity. IFN γ stimulates the expression of both class I and class II MHC molecules on various cell types by activating transcription of the respective structural genes. IFN γ enhances the expression of class II MHC molecules on accessory cells that constitutively express these proteins (monocytes, macrophages and dendritic cells) and induces the *de novo* synthesis of class II MHC genes in other cells, such as skin fibroblasts, vascular endothelial cells, thyroid epithelial cells and astrocytes. As a result of class II MHC molecule induction, these cells acquire an ability to function as antigen-presenting cells, and can thus participate in the process of specific immune recognition. To date, IFN γ is known as the most potent inducer of class II MHC expression, although some other cytokines (e.g. IL-4, IL-10, TNF α and granulocyte-macrophage colony-stimulating factor (GM-CSF)) also enhance the expression of these proteins in different types of cells. IFN γ also induces the expression of other MHC-encoded genes involved in antigen presentation such as TAP-1, TAP-2, and proteasome subunits. By inducing the peptide transporters TAP-1 and TAP-2, IFN γ promotes the translocation of antigenic peptides from the cytosol into the endoplasmic reticulum where the peptides bind to MHC molecules. By inducing the proteasome subunits LMP2 and LMP7, IFN γ alters the proteolytic specificity of proteasomes, facilitating the generation of peptides for antigen presentation.

IFN γ also has a regulatory role in the control of immunoglobulin isotypes produced by activated B

cells. IFN γ enhances the secretion of IgG2a by LPS-stimulated purified B cells *in vitro*, and concomitantly inhibits the production of IgG1, IgG2b, IgG3 and IgE while sparing IgM synthesis. There is evidence that IFN γ exerts its unique IgG2a-inducing effect directly on B cells, by both increasing the frequency of precursors of IgG2a⁺ cells and enhancing the number of IgG2a⁺ daughter cells emerging from each precursor. Also, IFN γ was found to enhance serum IgG2a and reduce serum IgG1 levels in mice immunized with *Brucella abortus*. Similar effects of IFN γ on IgG levels were observed in mice injected with anti-IgD antibodies; in addition, treatment with IFN γ also suppressed serum IgE levels in these mice. In contrast to IFN γ , IL-4 can stimulate the secretion of IgG1 and IgE, and suppress IgG2a secretion. Therefore, the relative quantities of IFN γ and IL-4 produced during an immune response are thought to regulate the selective production of immunoglobulin isotypes.

As mentioned, IFN γ production is a hallmark of the T_H1 response. Not only is IFN γ produced by T_H1 cells and not by T_H2 cells, IFN γ can also selectively inhibit the growth of T_H2 cells while sparing T_H1 cells. IFN γ can exert immunomodulatory actions indirectly, by affecting the generation of other cytokines. In monocytes/macrophages, IFN γ enhances IL-12 and TNF α production. IFN γ also promotes the T_H1 response by augmenting IL-12-induced T_H1 cell development.

IFN γ alone, or in combination with TNF α , induces chemokine secretion by endothelial cells and monocytes. (However, in other situations IFN γ can selectively inhibit chemokine production.) Furthermore, IFN γ can modulate cytokine receptors, e.g. the expression of TNF receptors in a variety of cells is enhanced by IFN γ action. It is also apparent that the actions of IFN γ are modified by the presence of other cytokines; many examples of synergistic and antagonistic interactions involving IFN γ and other cytokines (e.g. TNF α , lymphotoxin (TNF β), IL-1, IFN α/β) are known.

Another important action is the ability of IFN γ to augment the expression of the adhesion receptor ICAM-1 on vascular endothelial cells, leading to an increased adhesion of LFA-1-expressing cells. In this activity too, IFN γ acts cooperatively with TNF α or IL-1. This action is thought to contribute to the recruitment of lymphocytes to a local inflammatory site.

IFN γ was shown to be involved in the regulation of apoptosis in lymphocytes. Thus, pre-B cells can undergo apoptosis when exposed to IFN γ ; T cell receptor cross-linking-induced T_H1 cell apoptosis also requires IFN γ . The level of IFN γ receptor

expression on lymphocytes appears to be critical in the outcome of IFN γ actions; high-level expression tends to be associated with apoptosis, whereas low-level expression often leads to IFN γ -induced proliferation.

Roles in disease and therapeutic applications

A better understanding of the roles of IFN γ in the intact organism is emerging from studies with animals in which either the gene encoding IFN γ or the gene encoding the α chain of the IFN γ receptor has been inactivated by gene targeting. The major defect in animals that lack functional IFN γ (but retain the functions of IFN α/β) is a decreased ability to resist infection with some bacteria (*Listeria*, *Mycobacteria*), parasites (*Leishmania*) and viruses (vaccinia, Theiler's virus). Other changes seen in mice lacking functional IFN γ include an increased resistance to the toxic action of bacterial LPS and a decreased production of some other cytokines (TNF α , IL-1, IL-6 and IL-12). Such mice also showed a decreased capacity to develop protective immunity against *Plasmodium yoelli* sporozoites and against pseudorabies virus. There was also an altered pattern of generation of immunoglobulin isotypes, most notably a decrease in IgG2a production.

Several studies investigated the consequences of an aberrant expression of IFN γ in transgenic mice. Overexpression of murine IFN γ led to increased class I and II MHC expression, localized inflammation and tissue destruction. In a recent study aberrant expression of IFN γ in the neuromuscular junctions produced myasthenia gravis-like symptoms.

In view of its stimulatory action on MHC expression and antigen presentation IFN γ has long been suspected to play a role as a mediator of autoimmunity. However, it appears that the role of IFN γ in autoimmune disease may vary depending on the stage of the disease process or site of action. McDevitt and coworkers showed that in complete Freund's adjuvant (CFA)-induced arthritis in rats, administration of IFN γ 24 h before CFA caused an enhancement of arthritis, whereas injection of IFN γ 24–48 h after CFA suppressed the disease. In collagen-induced arthritis in mice, locally injected IFN γ promoted arthritis whereas systemically administered IFN γ was protective. There are other diseases in which IFN γ apparently plays a dual role. While there is evidence that IFN γ is an important factor in host defenses against some infections, recent data from animal models suggest that IFN γ may contribute to the pathology in septic shock, cerebral malaria and cachexia associated with tumor growth. These

adverse effects may be due at least partly to the ability of IFN γ to promote the generation of toxic levels of TNF α and to synergize with TNF α .

Many recent studies have investigated the effects of artificial overexpression of IFN γ (by introduction of cDNA expression vectors) on the ability of tumor cell lines to generate tumors in mice. In several models IFN γ -producing tumor cells were rejected due to the efficient generation of tumor-specific cytotoxic CD8⁺ T cells. However, in one study tumor cells transfected with IFN γ cDNA showed an increased metastatic potential.

In view of its many immunoregulatory actions, recombinant IFN γ has been tested in clinical trials in humans. Many of the trials were in patients with malignancies. Although some clinical responses have been seen (e.g. in renal cell cancer), the value of IFN γ in neoplastic diseases has not been confirmed in controlled clinical trials. One approved therapeutic application of IFN γ is in patients with chronic granulomatous disease, a rare genetic disorder in which defective phagocyte function represents the major clinical problem. Administration of IFN γ in these patients resulted in a significant improvement in the ability of their phagocytes to kill bacteria and reduced the incidence of life-threatening infections. The use of IFN γ for the treatment of some chronic infections affecting macrophages (e.g. leishmaniasis, toxoplasmosis, tuberculosis) is under consideration. Low doses of IFN γ were reported to have a beneficial effect in some patients with rheumatoid arthritis and in Germany IFN γ has been licensed for the treatment of this disease.

See also: Autoimmune diseases; Autoimmunity; Cytokine assays; Cytokine genes, regulation of; Cytokines; Cytokine receptors; Interferon α ; Interferon β ; Interferon γ ; Macrophage activation; T lymphocyte activation.

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INTERFERON γ RECEPTOR

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The interferon γ receptor (*ifngr*; IFN γ R) is a cell surface glycoprotein complex that uniquely binds interferon γ (IFN γ) initiating cellular responses (antiviral, macrophage activation, immunomodulatory). Recent progress includes the identification of critical molecular components, determination of the structures of the ligand and receptor, elucidation of a major intracellular signaling path-

way and investigation of the physiological roles of the IFN γ system (Table 1).

The *ifngr* consists of at least two glycoproteins (Table 1). *ifngr-1* (IFN γ R1 or α chain) is necessary and sufficient for high-affinity IFN γ binding (K_d of about 0.85 nM); however, biological activity and intracellular signaling require a second cell surface transmembrane protein, *ifngr-2* (IFN γ R2; accessory

Table 1 Properties of IFN γ receptor subunits

	<i>ifngr-1</i>		<i>ifngr-2</i>	
	Human	Murine	Human	Murine
Protein size (amino acids)				
Precursor	489	477	337	332
Mature	472	450/451 ^a	310/316 ^a	314
Extracellular domain	228	227/228 ^a	220/226 ^a	224
Transmembrane domain	23	23	26/24 ^a	24
Intracellular domain	221	200	64/66 ^a	66
Mature protein size (kDa)				
Predicted	50.5	49.6	34.8–35.0 ^a	34.5
Observed	80–95	90	~45	?
Potential N-linked glycosylation sites	5	5	6	6
Chromosomal gene location	6(q23–q24)	10	21q22.1	16
Major transcript size (kb)	2.3	2.2–2.3	1.8	2

^aPredictions of domain boundaries and signal sequence cleavage sites may differ between laboratories.

factor 1 or β chain), which does not independently bind IFN γ , although the *ifngr-1/ifngr-2* complex binds IFN γ with about 2–5-fold higher affinity than does *ifngr-1* alone. The gene for *ifngr-1* is on human chromosome 6q, while that for *ifngr-2* is on human chromosome 21q, in a cluster with several known cytokine receptor genes, including both known components of the type I IFN (IFN α/β) receptor. *ifngr-1* and *ifngr-2* proteins have a tripartite structural motif: an N-terminal extracellular ligand-binding domain is connected to the C-terminal intracellular domain by a short hydrophobic membrane-spanning sequence which is probably α -helical. N- and O-linked glycosylation contribute to the apparent molecular size of the mature proteins. *ifngr-1* and *ifngr-2* are members of the cytokine receptor 2 structural family, as are the known subunits of the type I IFN receptor and the interleukin 10 (IL-10) receptor. Their extracellular portions are formed by two fibronectin III (FBN III)-like domains (Figure 1). The coexpression of human *ifngr-1* and *ifngr-2* in heterologous cells is sufficient for high-affinity ligand binding and for one or more biological activities, the latter depending on the experimental system employed. Additional receptor components or associated proteins may yet be discovered.

The three-dimensional structure of homodimeric IFN γ complexed with two molecules of the ligand-binding extracellular domain of *ifngr-1* (Figure 1) shows extensive contact between the ligand and receptor, including residues from several loops of each FBN III domain, in a manner reminiscent of the human growth hormone complex with its receptor. However, the membrane-proximal FBN III subdomains of the two bound *ifngr-1* receptors do not

make close contact with each other, leading to the suggestion that *ifngr-2*, not included in this crystalline complex, might interact in this region. It is unclear whether direct interactions with IFN γ help recruit *ifngr-2* into the active ligand–receptor complex.

Binding of IFN γ to the receptor activates the cell via a JAK/STAT (Janus Kinase/Signal Transduction and Activator of Transcription) pathway. While the order of events has not totally been established, IFN γ binding likely induces dimerization of *ifngr-1*, with the recruitment of *ifngr-2*, producing an activated complex of one IFN γ dimer, two *ifngr-1* chains and two *ifngr-2* chains. Jak1 and Jak2 tyrosine kinases are rapidly recruited, activated and phosphorylated. The subsequent phosphorylation of *ifngr-1* on its cytoplasmic domain creates a binding site for the cytoplasmic latent transcription factor STAT1 α through its SH2 (Src homology 2) domain. STAT1 α is consequently phosphorylated, leading to its dissociation from the receptor and homodimerization. The phosphorylated STAT1 α homodimer enters the nucleus through an undefined mechanism and serves as a transcription factor for genes having GAS (IFN Gamma Activated Sequences) elements in their 5' regulatory regions. The critical role of STAT1 α for IFN γ activity (and type I IFN activity) has been demonstrated in mice having a homozygous disruption of this gene. Molecular biological approaches have permitted the mapping of other important sites on the cytoplasmic segments of *ifngr-1* and *ifngr-2*. Whether other signaling or catalytic molecules are recruited to the active IFN γ –*ifngr* complex and whether the JAK/STAT pathway intersects with other known signaling pathways (e.g. mitogen-



Figure 1 Three-dimensional structure of the IFN γ homodimer complexed with two ligand-binding domains of the *ifngr-1* protein. Each extracellular ligand-binding domain, composed of tandem FBN III-type domains with seven β strands (represented as flat arrows), flanks the IFN γ homodimer (darker structure in the center, with α helices represented as coils). The receptor proteins would enter and traverse the plasma membrane (not shown) at the bottom of the figure. (Used by permission of Dr MR Walter.)

activated protein (MAP) kinase) is not known, nor have any phosphatases involved in the system yet been identified.

The distribution of the *ifngr* is undoubtedly related to its physiological roles. Most human and murine tissue culture cells bind IFN γ , with 100–10 000 receptors per cell. However, immunohistochemical studies of *ifngr* in human surgical specimens, while not as sensitive as radioligand-binding studies, suggest tissue-specific and developmental regulation of receptor expression. Thus, B cell areas of lymphoid tissues were strongly positive for *ifngr*, whereas lymphocytes in T cell areas did not have detectable expression. Moreover, evidence was presented for upregulation of *ifngr-1* during activation of T cells, and downregulation during B cell activation. Endothelial and epithelial tissues also varied considerably. *ifngr-2* appears absent in T_H1 (helper) cells, but not T_H2 cells, apparently underlying selective inhibition by IFN γ of T_H2 proliferation; *ifngr-2* may emerge as a marker for the T_H2 subset of helper T cells.

The physiological contribution of the IFN γ system is apparent in mice homozygous for targeted disruptions of the *ifngr-1* locus ('knockout' or *ifngr-1*^{0/0}). The IFN γ response is not critical for the *in vivo* control of several viruses (most examined were RNA viruses), but had an important role in some, such as vaccinia and Theiler's virus. On the other hand, a functioning IFN γ system was critical for defense against several intracellular bacteria (bacille Calmette–Guérin (BCG) and *Listeria*) and parasites (*Leishmania*). Developmental defects were not obvious in the immune system of *ifngr-1*^{0/0} mice, although effects possibly related to T_H1/T_H2 balance, such as immunoglobulin G (IgG) isotype ratios, were noted, as were effects on macrophage activation.

The inappropriate or prolonged activation of IFN γ has been related to various autoimmune and inflammatory reactions, and delayed-type hypersensitivity. The understanding and control of such responses would be aided by the development of IFN γ antagonists. No small-molecule peptidic or nonpeptidic antagonists have evolved, but experimentally useful macromolecular antagonists include the soluble ligand-binding domain of *ifngr-1* and antibodies to IFN γ or to *ifngr*. The soluble *ifngr-1* fragment has also been produced as a fusion protein with the immunoglobulin (Ig) heavy chain (an 'immunoadhesin'), enhancing its stability and serum half-life. These antagonists have demonstrated their efficacy in several mouse disease models. This receptor-based strategy for blocking IFN γ and other cytokines is seen in poxviruses: myxoma, which is immunosuppressive, encodes an *ifngr-1* homolog which is secreted from infected cells; however, a

definitive demonstration that this protein is a virulence factor is awaited.

Continuing basic and applied issues include the physiological regulation of the expression of *ifngr*, the interaction of its signaling pathways with others, and the development and application of receptor antagonists or mimics.

See also: Cytokine receptors; Interferon γ .

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INTERLEUKIN 1 AND ITS RECEPTORS

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Interleukin 1 (IL-1) was originally described by Gery and Waksman in 1971 as a macrophage-derived, 'lymphocyte-activating factor' (LAF) with comitogenic effects on thymocytes and T cells. Subsequent studies revealed structural and biochemical similarities of LAF to other partially characterized mediators such as endogenous pyrogen, leukocytic endogenous mediator, B cell-activating factor, mononuclear cell factor, catabolin and osteoclast-activating factor. In 1979, the term IL-1 was proposed for the putative protein with all these pleiotropic activities.

The bioassay for LAF could be performed in multiwell assay plates and was sufficiently sensitive to effectively monitor purification and molecular cloning studies aimed at identifying IL-1. Before the advent of recombinant DNA technology, efforts to biochemically purify human IL-1 detected the existence of high (31 kDa) and low (17 kDa) molecular forms of LAF/IL-1, representing aggregated and mature forms, respectively. In 1984–1985, by using a combination of hydrophobic chromatography, gel filtration, isoelectrofocusing and high performance liquid chromatography, the low molecular weight protein was shown to consist of two molecules with different isoelectric points, pI 5.4 and pI 6.8. Cloning of cDNA for human IL-1 by Auron and colleagues in 1984 and March and coworkers in 1985 identified two distinct proteins with IL-1 activities, IL-1 α with a pI of 5.4 and IL-1 β with a pI of 6.8. All the aforementioned biological activities of IL-1 were manifested by recombinant IL-1. A third member of the IL-1 family, IL-1 receptor antagonist (IL-1ra), was cloned by Eisenberg and colleagues in 1990. IL-1ra was initially detected as an IL-1 inhibitor found naturally in the urine of patients with fever or myelomonocytic leukemia. IL-1ra acts as a specific antagonist of IL-1 α and IL-1 β by binding to IL-1 receptors without transducing a signal.

An endogenous mediator of host inflammatory responses, IL-1 also participates in regulating specific immune responses by enhancing the growth and differentiation of both B and T lymphocytes. It is active over low concentrations of 10^{-9} to 10^{-15} M. When locally produced by stimulated cells, IL-1 acts in an autocrine or paracrine manner to augment early non-specific inflammatory and later specific immune responses. When released in larger quantities into the circulation, IL-1 acts systemically to exert its endocrine effects such as activation of the fever center, the hypothalamus-pituitary-adrenal (HPA) axis and in inducing acute phase proteins.

IL-1 genes, mRNA and proteins

The three members of the IL-1 family are products of different genes located close to one another on human chromosome 2. The IL-1 α gene is 11 kb and the IL-1 β gene is 7.5 kb. Each gene contains seven exons, and transcription gives rise to a 2.1 kb mRNA for IL-1 α and a 1.6 kb mRNA for IL-1 β . The human IL-1ra gene spans 15 kb, has four exons, and the mRNA is 1.8 kb. At the nucleotide level, the homology between human IL-1 α and human IL-1 β is 45%. Sequence comparisons and analysis of mutational rates for IL-1 α , IL-1 β and IL-1ra by Eisenberg and colleagues suggests that all three molecules arose from a common ancestral gene through the process of gene duplication about 350 million years ago.

The regulatory sequences in IL-1 α and IL-1 β genes differ. The typical transcriptional promoter motifs, TATA box and CAAT box, are found immediately upstream from the transcription initiation site in IL-1 β , but are absent in IL-1 α . An NF-IL-6 site and a novel lipopolysaccharide (LPS)-inducible site have been identified in the IL-1 β promoter. Glucocorticoid regulatory elements are present in both genes. Both genes for IL-1 are rapidly transcribed upon

stimulation. IL-1 production is regulated at both the transcriptional and post-transcriptional level.

Unlike conventionally secreted proteins, IL-1 α and IL-1 β contain no hydrophobic signal peptide sequence and are not secreted via the classical secretory pathway. IL-1 is found in the cytoplasm but not in the endoplasmic reticulum or Golgi apparatus, and neither IL-1 α nor IL-1 β is *N*-glycosylated. Both IL-1 α and IL-1 β are synthesized as 31 kDa precursors (271 amino acids for IL-1 α and 269 amino acids for IL-1 β) which are proteolytically cleaved to generate the mature, 17 kDa proteins (159 amino acids for IL-1 α and 153 amino acids for IL-1 β). Most IL-1 α remains as pro-IL-1 α in the cytosol, while some is transported to the cell surface, where it becomes membrane-bound by a lectin interaction involving mannose residues on IL-1 α . Pro-IL-1 α as well as membrane-associated IL-1 α is biologically active, but IL-1 β is inactive and must be processed to the 17 kDa form before it can exert any biological effects. Several serine proteases are capable of cleaving pro-IL-1 β to the mature, biologically active form. However, an intracellular cysteine protease, named IL-1 β -converting enzyme (ICE) is required to cleave pro-IL-1 β specifically between Asp116 and Ala117 to generate the mature 17 kDa IL-1 β . ICE consists of both a 19.8 kDa and a 10.2 kDa subunit, which are derived from a 45.2 kDa proenzyme. ICE is found in most cells but requires two proteolytic cleavages for activation. The precise mechanism by which IL-1 is cleaved by ICE and is enabled to exit from the cell remains to be established. Both LPS and interferon γ (IFN γ) upregulate the expression of ICE. ICE does not cleave pro-IL-1 α , but knockout mice lacking the ICE gene fail to secrete IL-1 β as well as IL-1 α . IL-1 α is cleaved between Arg112 and Ser113 by calpain and other extracellular nonspecific proteases. Depending on cell type and the stimulant used, release or processing of IL-1 may not occur and pro-IL-1 may accumulate and be stored intracellularly. IL-1 release *in vitro* may also occur as a consequence of cell injury and cell death.

The precursor protein of IL-1ra is a single, non-glycosylated peptide of 177 amino acids with a 25 amino acid leader sequence. IL-1ra is secreted extracellularly in adherent immunoglobulin G (IgG)-stimulated monocytes as three structural variants; the two larger forms of approximately 22 kDa are glycosylation derivatives of the 18 kDa form (152 amino acids). All three forms of IL-1ra exhibit identical receptor binding, indicating that the *N*-linked sugar is not essential for this inhibitory activity (Figure 1).

IL-1 structure

Crystallography by Priestle and colleagues indicated that IL-1 β is a tetrahedron with the triangular faces formed by 12 β strands held together by hydrogen bonds. There are no intramolecular disulfide bonds. The hydrophobic core consists of side-chains arising from multiple positions along its primary sequence. The three-dimensional structure of IL-1 α is probably very similar to that of IL-1 β , as they have a similar frictional ratio and Stokes radius. Both N- and C-termini are exposed and residues involved in receptor binding and activation are located in a number of sites over the surface of IL-1. At the amino acid sequence level, IL-1ra is 26% homologous with IL-1 β and 19% homologous with IL-1 α ; the sequence of IL-1 β is 26% identical to that of IL-1 α . The hydrophilicity plot of IL-1ra is similar to that of IL-1 β , indicating similarities in tertiary structure.

Deletion mutation studies by Mosley and coworkers suggested that the minimum polypeptide size for retaining biological activities for IL-1 α is 140 amino acids, extending from 128 through 267. The minimum size for IL-1 β is 147 amino acids, representing amino acids 120 through 266. Both minimal IL-1 forms require an arginine at the N-terminus to retain biological activity. Most attempts to identify smaller fragments of IL-1 have failed but a fragment of nine residues of human IL-1 β (fragment 163–171) when fused to an antigen has been reported to have reproducible significant immunoadjuvant capacity. This fragment is absent from IL-1ra.

Cell source and inducers

Mononuclear phagocytes are the major cell source of IL-1. However every mature nucleated cell type examined has been shown to be capable of producing IL-1, including keratinocytes, dendritic cells, astrocytes, microglial cells, normal B lymphocytes, cultured T cell clones, natural killer cells, other leukocytes, fibroblasts, neutrophils, epithelial cells, endothelial cells and smooth muscle cells. Normal cells must be stimulated to produce IL-1. IL-1 production by macrophages can be triggered by a variety of proinflammatory stimuli, including bacterial cell wall products, LPS, microbial superantigens, zymosan, phorbol esters, muramyl dipeptide, antigens, mitogens, leukotrienes, activated complement components (C5a), immune complexes, ultraviolet irradiation, silica particles, viruses, parasites and other microorganisms. Agents that activate lymphocytes can stimulate macrophages to produce IL-1 either in response to direct cell interactions, or by

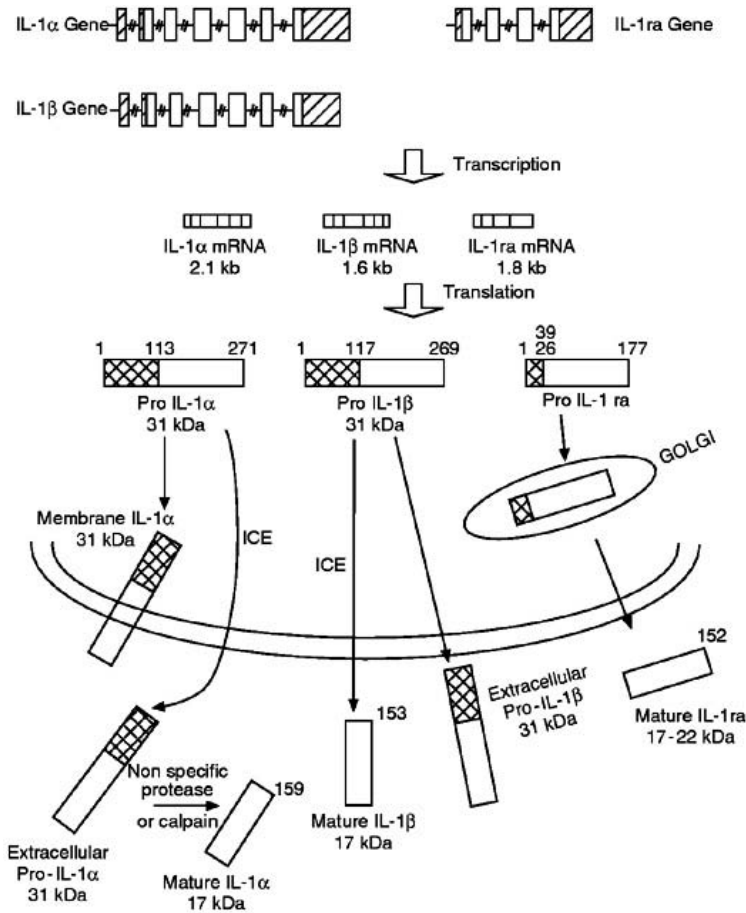


Figure 1 Interleukin 1: from genes to proteins.

producing cytokines such as lymphotoxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-1 itself, which can stimulate macrophages and other cells to produce more IL-1.

The production of IL-1 β and IL-1ra in human monocytes is reciprocally regulated. *In vitro* studies by Arend and colleagues showed the production of both IL-1 β and IL-1ra by LPS-stimulated monocytes. Monocytes cultured adherent to IgG-coated surfaces exhibited no IL-1 β gene expression, but high levels of IL-1ra due to enhanced transcription and prolonged mRNA stability. The mechanism of this differential regulation of IL-1 and IL-1ra may involve cross-linking of different carbohydrate groups on the cell surface, resulting in differential signal transduction pathways. Other studies also suggest that production of IL-1 β and IL-1ra are differentially regulated by cytokines. For example, IL-4, IL-10 and transforming growth factor β (TGF β) decrease production of IL-1 β , but can concomitantly increase synthesis of IL-1ra in monocytes. Corticosteroids suppress the production of IL-1 α and IL-1 β but have no effect on IL-1ra. Studies of the kinetics of production of IL-1 generally reveal that IL-1 β is made

before IL-1 α , and IL-1ra is made last. In addition, patients treated with IL-1 β develop elevated plasma IL-1ra levels indicating that even IL-1 induces IL-1ra production.

Many cell types express all three IL-1 genes, but levels of IL-1 α , IL-1 β and IL-1ra vary widely. Keratinocytes express mostly IL-1 α and IL-1ra. Human monocytes produce predominantly IL-1 β while mouse macrophages produce mostly IL-1 α . During differentiation of monocytes into macrophages, production of IL-1ra is upregulated while production of IL-1 β is decreased. Thus IL-1ra is a major product of tissue macrophages, particularly during disease. Most of the IL-1 activity found in the circulation is IL-1 β whereas IL-1 α is more often detected on cell surfaces. Plasma concentrations of IL-1 β in normal subjects are usually undetectable (<40 pg ml $^{-1}$), except in women after ovulation and in subjects after strenuous exercise. Normal human subjects have circulating levels of IL-1ra in the range of 200–300 pg ml $^{-1}$. Studies by Dinarello and coworkers showed that during experimental endotoxemia in humans, peak levels of 150–200 pg ml $^{-1}$ of IL-1 β occurred at 3–4 h. In contrast, peak levels of 6000–8000 pg ml $^{-1}$

of IL-1ra typically occurred after 4 h and remained high for another 8 h.

Bioassay and other detection methods

Various laborious biological assays can be used to detect IL-1 α and IL-1 β activity. These include costimulation of murine thymocyte proliferation with phytohemagglutinin A, IL-2 production by IL-1-dependent T cell lines, stimulation of IL-1-dependent T cell line proliferation, stimulation of fibroblast proliferation, production of collagenase and prostaglandin E₂ (PGE₂) by synovial cells. The bioassay for IL-1ra is based on the inhibition of mitogenic activity of IL-1 in mouse thymocyte proliferation. Although bioassays are actually more sensitive than immunoassays, they are not generally preferred because they are more laborious, less reproducible and respond to other cytokines and therefore lack specificity. Moreover, other naturally occurring substances or inhibitors can interfere with the biological activity of IL-1 in bioassays. ELISA and RIA using monoclonal and polyclonal antibodies specific for each species of IL-1 are most commonly used. These assays have some limitations in that they cannot distinguish functional IL-1 from inactive material and can be affected by interfering molecules, such as soluble IL-1 receptors, which can prevent IL-1 binding to antibodies. Nevertheless, ELISAs are rapid, with sensitivity in the picogram per ml range, and are highly specific, as they can differentiate the three different forms of IL-1, which are often present together in biological fluids.

IL-1 receptors

Two distinct IL-1 receptors have been identified as shown by the cloning studies of Sims and coworkers. Both receptors are members of the immunoglobulin superfamily. The type I receptor is an 80 kDa transmembrane protein, 552 amino acids long, with a single 22 amino acid transmembrane region and a long cytoplasmic tail of 213 amino acids. Its extracellular ligand-binding region consists of three immunoglobulin-like domains. The type II receptor is a 60 kDa protein and is similar to the type I receptor in its extracellular and transmembrane regions. The type II receptor has a short cytoplasmic tail of 29 amino acids and is incapable of signal transduction. The type II receptor actually competitively inhibits IL-1 activity by acting as a 'decoy' receptor for IL-1, and regulates the level of extracellular IL-1.

IL-1 acts on target cells by binding with high affinity (K_d of 10^{-10} M) to IL-1 type I receptors. Type I receptors are found on T cells, endothelial cells,

hepatocytes, fibroblasts and keratinocytes, whereas type II receptors appear to have a more restricted distribution. Type II IL-1 receptors are the predominant receptor on B cells, monocytes and neutrophils. IL-1 α , IL-1 β and IL-1ra show detectable affinity for both types of IL-1 receptors. However, IL-1 α binds preferentially with somewhat higher affinity to type I receptor and IL-1 β binds preferentially to type II receptor. IL-1ra binds type I receptor with considerably higher affinity than it binds to type II 'decoy' receptor, and it can block the binding to type I receptor and consequent biological activities of both IL-1 α and IL-1 β . However, a 10- to 500-fold excess of IL-1ra may be necessary to achieve a 50% inhibition of the biological effects of IL-1 *in vitro*. Pro-IL-1 β does not bind to type I receptor and therefore has no detectable biological activity. The receptor binding affinity and biological activities of the pro and mature forms of IL-1 α appear indistinguishable. Despite the low number of type I receptors (200 per cell), all biological responses of IL-1 are mediated by type I receptors. A receptor occupancy of less than 5% per cell is sufficient to activate cells. These data suggest that a major amplification of IL-1 signal must occur after IL-1 ligand/receptor interaction. Recently, Greenfeder and colleagues identified an IL-1 receptor accessory protein which is the putative signal transducing subunit of the IL-1 receptor complex. IL-1 receptor accessory protein does not bind IL-1 directly but forms a complex with type I IL-1 receptor after it is bound by either IL-1 α or IL-1 β but not IL-1ra.

Expression of both type I and type II IL-1 receptors is regulated to control inflammation and immune responses. Dexamethasone, PGE₂ and cytokines with known anti-inflammatory properties, such as IL-4, IL-10, IL-13 and TGF β , can increase receptor expression and shedding from cell membrane. The extracellular domain of both type I and type II receptor is shed by activated neutrophils and monocytes and the solubilized shed receptor binds and inhibits IL-1 β functions. Recently, it was shown that vaccinia and cowpox viruses encode a protein with 30% sequence homology to the soluble type II IL-1 receptor. The presence of this protein counteracts the host inflammatory response to these viruses because viruses genetically engineered to lack these genes become less pathogenic.

Signal transduction by IL-1

The role of internalization in signal transduction remains unclear. After IL-1 binding, IL-1 type I receptor is rapidly internalized. This ligand/receptor complex is relatively resistant to biochemical degra-

dation and can subsequently be found associated with the nucleus. The identification of the intracellular signal transduction pathways and second messenger systems for the biological effects of IL-1 remain to be completely determined. Different types of responding cells may utilize diverse signal transduction pathways for IL-1. IL-1 induces the phosphorylation of a number of cellular proteins such as epidermal growth factor receptor and heat shock proteins. Various kinases including protein kinase C (PKC), tyrosine kinase, mitogen-activated protein kinase (MAP kinase), and ceramide-activated kinase are activated by IL-1. Phosphorylation of phospholipase A₂ (PLA₂)-activating protein activates PLA₂, resulting in the release of arachidonic acid from membrane phospholipids. This leads to enhanced cyclooxygenase activity and the production of prostanooids. In T cells, IL-1 stimulates phosphatidylcholine-specific phospholipase C to generate 1,2-diacylglycerol, which is a potent activator of protein kinase C. IL-1 also activates the transcription factors AP-1 and NFκB which bind to the promoter regions of certain genes (IL-6, IL-8, IL-2 receptor, endothelial cell adhesion molecule). IL-1 enhances the transcription of *c-jun* by activating two novel nuclear factors. Recent studies by Mathias and colleagues suggest that IL-1 binding to receptors activates a neutral sphingomyelinase, hydrolyzing sphingomyelin to ceramide. Ceramide then stimulates a ceramide-activated protein kinase that phosphorylates a specific subset of cellular proteins, thereby altering their function.

Inflammatory and immunological effects of IL-1 (Tables 1 and 2)

Biological activities of IL-1 are mostly indirect and the result of induction of a cascade of other mediators. IL-1 is a pleiotropic mediator of the host response to infection and injury. Its biological activities largely overlap with those of tumor necrosis factor α (TNFα) and IL-6. Many of the effects of IL-1 are mediated through its capacity to increase the production of other cytokines, such as granulocyte colony-stimulating factor (G-CSF), GM-CSF, TNFα, IL-6, IL-8 and homologous members of the chemokine family, platelet-derived growth factor (PDGF), and IL-11. In addition, the effects of IL-1 are amplified through the ability of IL-1 to upregulate receptor expression for itself, for the IL-2 receptor α chain, and for the receptors for IFNγ, IL-3 and GM-CSF.

IL-1 stimulates a variety of cells that function as effectors of immune and inflammatory responses. IL-1 promotes the proliferation of T cells by enhancing the production of IL-2 and the expression of IL-2

Table 1 IL-1 effects *in vivo*

Fever and anorexia
Somnolence and slow-wave sleep
Vasodilation, hypotension and shock
Increased acute phase proteins
Decreased hepatocyte cytochrome P450 activity
Cytotoxicity for β islet cell
Neutrophilia
Hypozincemia
Hypoferremia
Hypercupremia
Hypercalcemia
Increased hormone levels: corticosteroids, ACTH, CRF
Increased insulin and glucagon
Increased heart rate
Endothelial cell activation
Neutrophil accumulation at inflammatory sites
Bone resorption
Cartilage proteoglycan release
Increased Na excretion
Protection against hyperoxic lung injury
Radioprotection
Bone marrow cell cycling
Induction of circulating CSFs
Adjuvant effect
Increased NK activity
Resistance to bacterial infection
Antitumor immunity
Increased tumor metastasis

receptors on T cells. IL-1 augments antigen-induced T cell proliferative and functional responses partly because IL-1 coinduces major histocompatibility complex (MHC) class II expression on antigen-presenting cells. IL-1 also induces the production of IL-6 and TNFα. Recently, Lenardo and colleagues showed that IL-1 production within the thymic microenvironment induces IL-2 receptor α chain expression on early immature thymocytes. Thus, IL-1 is required for thymocyte maturation and CD4⁺CD8⁺ differentiation. The normal thymic development seen in knockout mice that lack the IL-1 type I receptor therefore may be based on compensatory pathways.

IL-1 also promotes B cell development as evidenced by the induction of κ light chain synthesis in pre-B cells. IL-1 augments B cell proliferation, surface IgM expression and antibody production. However, knockout mice that lack the IL-1 type I receptor have normal serum immunoglobulin levels and a normal immune response. IL-1 acts on monocytes and neutrophils, inducing secretion of several cytokines, including IL-8 and IL-1 itself. IL-1 is reported to prolong the survival of neutrophils *in vitro*.

IL-1 participates in the recruitment of leukocytes to inflammatory sites by inducing adhesion molecules (ICAM, ELAM and VCAM) on vascular endothelium and a number of chemokines that attract

Table 2 IL-1 effects *in vitro*

Target cell	Activity
T cell	Induction of IL-2, IL-4, IL-5, IFN γ , IL-2R Potentiation of T cell activation Contributes to thymic development Induction of chemokines
B cell	Induction of IL-6 Enhanced B cell proliferation Potentiation of antibody production
Monocytes/ macrophages	Induction of IL-1, IL-6, IL-8, TNF, GM-CSF, PGE $_2$ and IFN γ receptor Potentiation of cytotoxicity Induction of chemokines
NK cells	Augmentation of cytotoxicity
Neutrophils	Priming
Eosinophils	Degranulation
Dendritic cells	Increased T cell activation
Epithelial cells	Comitogenic Induction of collagen IV
Endothelial cells	Induction of IL-1, IL-6, IL-8, TNF and GM-CSF Induction of procoagulant activity Upregulation of adhesion molecules
Fibroblasts	Induction of IL-1, IL-6, IL-8, TNF and GM-CSF Stimulation of proliferation Induction of PGE $_2$ and collagenase
Synoviocytes	Synthesis of PGE $_2$ and collagenase Induction of hematopoietic growth factors
Chondrocytes	Degradation of proteoglycan Induction of PGE $_2$, collagenase and plasminogen activator
Osteoclasts	Increased proliferation and bone resorption
Osteoblasts	Decreased alkaline phosphate levels
Hepatocytes	Decreased cytochrome P450 and C3
Adipocytes	Decreased synthesis of lipoprotein lipase
Tumor cells	Cytostasis

leukocytes. Other mediators such as IL-1, IL-6, prostanooids, platelet-activating factor and procoagulant factor (tissue factor) are also produced by endothelial cells in response to IL-1.

Other biological activities of IL-1

IL-1 is an important regulator of hematopoiesis, although the phenotype of IL-1 β -deficient mice is remarkably normal. IL-1 induces the production of CSF by bone marrow stromal cell and receptors for CSF. Thus IL-1 synergizes with CSF to stimulate growth of early hematopoietic bone marrow progenitor cells and can counteract the myelosuppressive effects of radiotherapy and chemotherapy. The latter effect is mediated partly through the induction of mitochondrial manganese superoxide dismutase

activity that scavenges potentially toxic superoxide radicals.

IL-1, an endogenous pyrogen, acts on the fever center and also stimulates the hypothalamus to produce corticotropin-releasing hormone (CRH), resulting in the release of adrenocorticotrophic hormone (ACTH) from the pituitary. IL-1 can also directly stimulate pituitary ACTH release. This activation of the HPA axis culminates in induction of the production of glucocorticoids by the adrenals. Glucocorticoids act as a negative feedback regulator by suppressing the production of IL-1 and upregulating both type I and type II IL-1 receptors. IL-1 also inhibits the release of both gonadotropin-releasing hormone and luteinizing hormone (LH), as well as thyroid-stimulating hormone (TSH) and decreases plasma thyroid hormone.

IL-1 is postulated to play a major role in destructive joint disease such as rheumatoid arthritis. It regulates bone resorption by inducing metalloprotease secretion by chondrocytes, resulting in cartilage breakdown and proteoglycan release. IL-1 stimulates fibroblast proliferation and secretion of collagenase, IL-6, IL-8 and G-CSF. It induces cyclooxygenase synthesis and hence prostaglandin release from fibroblasts, but suppresses expression of mRNA for matrix proteins.

IL-1 can have antitumor as well as tumor growth promoting effects. IL-1 has been shown by Onozaki and coworkers to act both as an *in vitro* cytostatic and a cytotoxic agent for a number of tumor cell lines. This activity is associated with inhibition of ornithine decarboxylase activity by IL-1. IL-1 increases natural killer (NK) cell activity and T cell-dependent tumor immunity *in vivo*. Fibrosarcoma tumor cell lines transfected to overexpress IL-1 α have been shown by Apte and colleagues to be more readily rejected and to induce the development of immune resistance upon rechallenge. However, systemic administration of IL-1 has been reported to promote the development of tumor metastases in some animal models. Thus under some circumstances IL-1 appears to promote tumorigenesis.

Therapeutic effects of IL-1 inhibitors

Many of the biological effects of IL-1 are similar to those observed after a severe infection (see Table 1). As a mediator produced in response to microbial invasion, IL-1 will induce fever, myalgia, somnolence, vasodilation and shock, cause bone resorption, induce muscle proteolysis, and induce synthesis of acute phase proteins such as C-reactive protein, serum amyloid A, and fibrinogen by hepatocytes. IL-1 causes a decrease in plasma zinc and iron, which

in turn inhibits growth of virulent bacteria. IL-1 also induces chemokines that are responsible for neutrophil and macrophage emigration to sites of injury, all to arm the host against pathogens. Recent reports of the acquisition of soluble IL-1 type II receptors and inhibitors of ICE by certain viruses underscores the importance of IL-1 in host defense.

IL-1 has been implicated *in vivo* in the pathogenesis of a variety of acute and chronic inflammatory diseases such as septic shock, rheumatoid arthritis and atherosclerosis. Synovial fluids contain IL-1, which acts as a potent stimulator of cartilage resorption. Synovial tissue macrophages also produce high levels of IL-1 β followed by IL-1ra. Endogenously produced IL-1ra levels may be a normal response to limit the proinflammatory, pathogenic effects of IL-1.

A number of naturally occurring inhibitors of IL-1 are present in serum. PGE₂ suppresses the synthesis and release of IL-1. CRH inhibits IL-1-induced anorexia, sleep and prostaglandin synthesis. Corticosteroids suppress expression of IL-1 mRNA. TGF β , IL-4, IL-10 and IL-13 inhibit IL-1 production. β -melanocyte-stimulating hormone inhibits the synthesis and activities of IL-1.

The role of the natural antagonist, IL-1ra, in normal physiology remains to be understood. IL-1ra can block IL-1-induced T cell proliferation, although 50% inhibition of IL-1 biological effects *in vitro* requires a 10- to 500-fold excess amount of IL-1ra. IL-1ra does not alter *in vitro* T cell proliferative responses to mitogens, antigens or alloantigens. IL-1ra blocks LPS-induced IL-8 production in mononuclear cells. IL-1ra also decreases IL-2 receptors and secretion of IL-1 α , IL-1 β , IL-2 and TNF in these cells *in vitro*. Several studies have shown that inhibition of IL-1ra by neutralizing antibodies results in considerable exacerbation of inflammatory diseases. Thus, IL-1ra plays a role in downregulating *in vivo* inflammatory reactions. In animal models, IL-1-induced hypotension can be inhibited by pretreating the animals with large doses of IL-1ra. However, the large amounts of IL-1ra (10 000-fold molar excess, 2.5 g day⁻¹ estimated for human use) required to counteract IL-1-mediated effects reduces the clinical usefulness of IL-1ra. Thus, IL-1ra as yet has no significant therapeutic potential in human phase III clinical trials for sepsis. However, the demonstrated proinflammatory effect of suppressing IL-1ra *in vivo* suggests that improved delivery systems may make IL-1ra more effective.

Other approaches to block IL-1-mediated responses include the use of IL-1-specific antibodies, IL-1 receptor-specific antibodies, soluble IL-1 receptors and inhibitors of ICE.

Gene deletion of IL-1

Despite the plethora of IL-1 activities, knockout mice that are defective in the production of IL-1 α or IL-1 β or in the expression of IL-1 type I receptor are viable and show only relatively limited phenotypic deficiencies. Kluger and coworkers showed resistance to fever induction in IL-1 β -deficient mice. Studies by Dinarello and colleagues indicated that IL-1 β -deficient mice have an impaired acute-phase response to a local injection of turpentine although systemic administration of LPS gave a normal inflammatory response in these mice. This normal response to LPS is probably due to *in vivo* compensatory mechanisms that correct for the lack of IL-1 signals in IL-1 knockout mice and to the redundancy in the activities of IL-1, TNF α and IL-6.

In summary, IL-1 plays a central role in regulating immune responses, often through the induction of other mediators and cytokines. IL-1 is the only cytokine for which a natural endogenous inhibitor with specificity exists, IL-1ra, to counteract its biological effects. An additional natural mechanism to moderate the effects of IL-1 is suggested by adrenalectomy in animals, which results in a more pronounced inflammatory response, indicating that normal adrenal activity buffers IL-1 activity. Thus, multiple mechanisms such as the shedding of IL-1 receptors, corticosteroids and IL-1ra exist naturally to carefully regulate the levels and activities of IL-1.

See also: Cytokine receptors; Interleukin 1 and its receptors.

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INTERLEUKIN 2

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Interleukin 2 (IL-2) was one of the first interleukins to be characterized. Initially called T cell growth factor (TCGF), its activity was detected in the supernatant of mitogen-stimulated peripheral blood lymphocytes. It was immediately used for the culture of T lymphocytes *in vitro*. After complete molecular characterization (purification and cloning) and identification of its receptor, IL-2 was quickly recognized as a central factor in controlling the immune response. Large quantities of IL-2 have been produced and used in clinical trials aimed at stimulating the immune system, particularly against tumors.

Molecular characterization of IL-2

IL-2 belongs to the hematopoietin family which includes growth hormone, prolactin, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-15, GM-CSF, etc. The IL-2 gene, located on chromosome 4 (q26-q27), contains four exons coding for a 153 amino acid precursor including a 20 amino acid leader sequence. Human IL-2 contains only one O-glycosylation site (Thr3); glycosylation at this site is heterogeneous but does not affect biological activity. The three-dimensional structure of IL-2 is essentially composed of four α helices (A, BB', C and D) connected by three loops (Figure 1). A disulfide bridge between Cys58 and Cys105 is necessary for the bioactivity of the molecule. The regions of the molecule involved in binding to the three chains of the

IL-2 receptor (α , β and γ) have been identified. IL-2/IL-2R β interactions mainly involve helix A and IL-2/IL-2R γ interactions involve helix D, whereas IL-2/IL-2R α interactions mainly involve the loop between helix A and helix BB'.

IL-2 production

IL-2 is produced by CD4 T lymphocytes. During the immune response CD4 lymphocytes differentiate into T_H1 and T_H2 functional subsets. This pattern of differentiation is very clear in the mouse, where T_H1 lymphocytes produce IL-2 and interferon γ , and T_H2 lymphocytes produce IL-4, IL-5 and IL-6. In humans, however, this functional dichotomy of CD4 lymphocytes is less distinct.

Resting CD4 lymphocytes do not produce IL-2. Two types of signals (signal 1 and signal 2) induce the production of IL-2. Signal 1 is dependent on the binding of the T cell receptor (TCR) to the antigen/MHC (major histocompatibility complex) complex. It can be mimicked by antibody against the TCR or by some lectins. Signal 2 follows the binding of T cell surface molecules such as CD28 or CTLA-4 to B7-2 and B7-1 present on the surface of the antigen-presenting cells. Maximum production of IL-2 occurs when both signals are present. Other costimulating molecules (cell surface antigens, cytokines) are also involved in the optimal production of IL-2. Alone, signal 1 induces an anergy of the T_H lymphocytes. This anergic state is characterized by the inability to react to a second antigenic signal. IL-2 can replace signal 2 and prevent the induction of an anergic state.

Some CD8 clones can produce small amounts of IL-2. Under certain conditions B lymphocytes are also able to produce IL-2: Epstein-Barr virus (EBV)-infected lines produce IL-2 as well as normal B lymphocytes cultured in the presence of anti-CD40 monoclonal antibodies (mAb) and IL-4 and subsequently stimulated by phorbol esters and calcium ionophore.

The transcription of the IL-2 gene is highly regulated by a 300 bp promoter. This promoter region can bind many transcription factors. Among them two NFAT factors (p and c) and AP-1 (heterodimer of fos and jun) are essential.

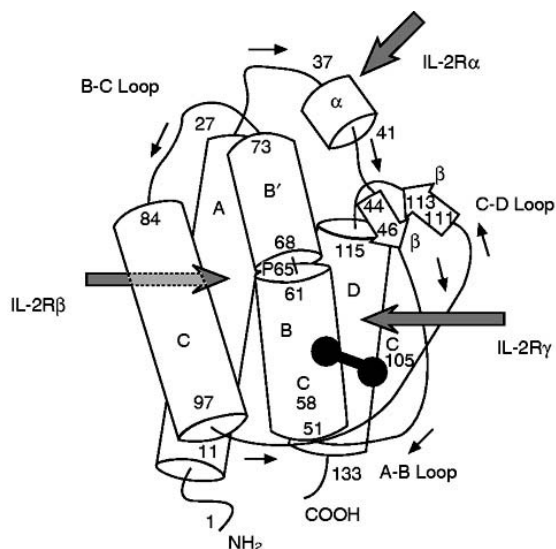


Figure 1 Structure of interleukin 2.

Targets of the stimulatory activity of IL-2

Most cells of the immune system can express the high-affinity (IL-2R α , β , γ) or the intermediate-affinity (IL-2R $\beta\gamma$) IL-2 receptors, either spontaneously or after stimulation. Consequently IL-2 has many targets (Figure 2).

T lymphocytes

The primary role of IL-2 is to expand activated CD4 and CD8 lymphocytes. On T_H1 cells IL-2 acts in an autocrine fashion. IL-2 also induces the growth of T_H2 and CD8 lymphocytes as a paracrine factor. IL-2 has no influence on the T_H1/T_H2 balance. On CD8 lymphocytes IL-2 stimulates cytotoxic activity. After stimulation T lymphocytes produce many cytokines and express the corresponding receptors. However IL-2 remains the main cytokine controlling the proliferative response of CD4 lymphocytes. The proliferation of purified CD4 lymphocytes stimulated by anti-CD3 is completely neutralized by anti-IL-2 monoclonal antibodies.

B lymphocytes

Activated B lymphocytes express the high-affinity IL-2 receptor and respond to IL-2. Under these conditions they proliferate intensively and produce immunoglobulins of the IgM, IgG and IgA isotypes. Different stimulations can induce the susceptibility of B lymphocytes to IL-2. Experimentally anti-Ig, or lipopolysaccharides (LPS) are good stimulators. Physiologically, in the context of T/B collaboration T_H1 lymphocytes (or T_H1-like in humans) may induce an intense proliferation and differentiation of the B lymphocyte population. Unlike IL-4, IL-5 and transforming growth factor β (TGF β), IL-2 does not exert an influence on isotype selection.

Natural killer (NK) cells

All NK cells are spontaneously sensitive to IL-2. In the majority of these cells IL-2 can stimulate cytotoxic activity without stimulating NK cell proliferation. In rare NK cells, expressing the high-affinity IL-2R, IL-2 stimulates both cytotoxic and proliferative responses. NK cells play a dominant role as the first defense barrier against viral infections and tumor growth.

Monocytes

IL-2 spontaneously stimulates the killing of tumor cells and bacteria by monocytes. Interferon γ (IFN γ) and LPS stimulate the appearance of high-affinity IL-2R on the surface of monocytes and therefore increase sensitivity to IL-2. During IL-2 stimulation monocytes produce a large array of mediators such as H₂O₂, prostaglandin E₂ (PGE₂), thromboxane B₂, and tumor necrosis factor α (TNF α). These products may be responsible for the side-effects observed during IL-2 therapy.

Granulocytes

Neutrophils are also sensitive to IL-2. Their anti-fungal activity is greatly enhanced by IL-2, perhaps because of the production of lactoferrin and TNF α by these cells.

IL-2 is a pleiotropic cytokine with proinflammatory activity both on the nonspecific arm of the immune system (NK cells, monocytes) and on the specific response generated by T and B cells.

IL-2 in the negative control of lymphocyte proliferation

Autoimmunity in IL-2-deficient animals

The precise role of IL-2 *in vivo* is not well understood. An important tool for studying this question

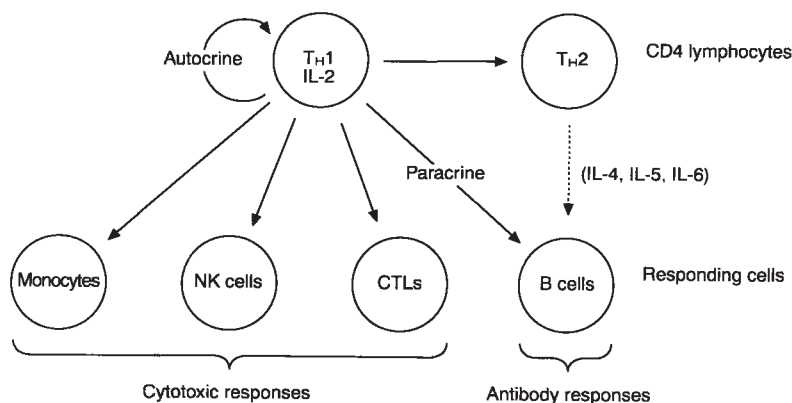


Figure 2 Interleukin 2 immune activity.

is provided by gene knockout mice produced after homologous recombination. In IL-2^{-/-} animals the ontogeny of the immune system is not dramatically affected, as demonstrated by the normal development of lymphoid organs and the presence of the major lymphocyte subsets. However IL-2^{-/-} animals have an overproduction of lymphocytes, which is morphologically reflected by lymphoid organ hyperplasia. This observation is surprising since IL-2 was first described as a T cell growth factor.

As expected, the immune response of IL-2^{-/-} mice is abnormal at the T and B cell level. The *in vivo* cytotoxic response against viruses is reduced, although *in vitro* cytotoxic responses can be restored by addition of IL-2.

The most striking observation made with IL-2^{-/-} animals is the development of an autoimmune syndrome. The intensity of the disease is dependent both on environmental factors and the genetic background of the animals. Fifty per cent of IL-2^{-/-} mice in a 129/01a × C57B16 genetic background die of autoimmune hemolytic anemia before 9 weeks of age. All animals that survive past 10 weeks of age develop an inflammatory bowel disease comparable to ulcerative colitis in humans. Mice kept in germ-free conditions do not develop the disease. The data obtained with IL-2^{-/-} mice as well as with IL-2Rα^{-/-} and IL-2Rβ^{-/-} animals indicate that there may be a defect in cell apoptosis in these animals.

IL-2 in the control of apoptosis

Shortly after antigen recognition an autocrine loop is established based on the coexpression of IL-2 and the IL-2R by T cells, which functions to drive responding cells through the cell cycle. IL-2 also promotes the survival of T cells in culture, presumably by inducing the expression of Bcl-2, a protein that protects cells from passive apoptotic cell death. *In vivo* this mechanism may be related to the persistence of some memory cells.

On the other hand, several recent studies have demonstrated that clonal expansion of activated T cells can be controlled by the induction of programmed cell death of some activated lymphocytes. The major pathway of this activation-induced cell death (AICD) involves the interaction of Fas expressed by activated T and B cells and Fas ligand produced by a subset of activated T cells. IL-2 is a critical determinant of AICD: addition of IL-2 to stimulated T cell clones increases AICD whereas anti-IL-2 antibodies reverse the deletion of these cells. The effect of IL-2 on AICD may be explained by the acquisition of susceptibility to Fas-mediated

apoptosis after treatment with IL-2, but not by increased Fas expression.

IL-2-dependent immune responses are transient since IL-2-induced activation and clonal expansion is followed by AICD. Furthermore IL-2 appears to be critical for triggering a mechanism that regulates self-tolerance and prevents pathological autoimmunity.

Clinical aspects

The major clinical use of IL-2 to date has involved tumor immunology. The potential for IL-2 as a cancer treatment is based on activation of cells which are cytotoxic for the tumor, and some success has been obtained with renal cell carcinoma and metastatic melanoma. However the use of IL-2 is limited by various side-effects. To circumvent these difficulties lymphokine-activated killer (LAK) cells as well as tumor-infiltrating lymphocytes (TILs) have been tested after *in vitro* activation by IL-2. Local delivery of IL-2 to the tumor site and genetic modification of the tumor cells by the IL-2 gene are currently under clinical trial.

Coupled to toxin, IL-2 can be used as an immunosuppressive agent. IL-2 fragments coupled to *Pseudomonas* exotoxin (IL-2-PE40) or diphtheria toxin (IL-2-DAB486) have been studied. They inhibit graft rejection in animals and were found to have beneficial effects on rheumatoid arthritis. Drugs which limit the production of IL-2 (cyclosporine and FK506) are classically used during graft transplantation in humans.

IL-2 has been used in AIDS (acquired immune deficiency syndrome) patients as an immunostimulatory agent able to limit the effects of CD4 depletion. After IL-2 injection and under some clinical conditions, IL-2 treatment is followed by an increase of the CD4 count without affecting the viral load.

See also: Interleukin 2 receptor; Interleukin 4; Interleukin 7 and its receptor; Interleukin 9 and its receptor; Interleukin 15 and its receptor; Tumors, immune response to.

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INTERLEUKIN 2 RECEPTOR

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Interleukin 2 (IL-2) is a growth factor for T cells and natural killer (NK) cells, where it induces G_1 - to S-phase progression, and a differentiation factor for B cells, where it induces immunoglobulin secretion. The high-affinity IL-2 receptor (IL-2R) is composed of three distinct chains, designated α , β and γ . Intracellular signaling is initiated by ligand-induced heterodimerization of the IL-2R β and γ chains, which results in the activation of multiple intracellular kinases, including members of the Janus kinase (JAK) family. These biochemical events induce expression of a variety of target genes with mitogenic and differentiative functions.

Structure and expression of the IL-2R

Subunit composition of the three IL-2R complexes

The IL-2 molecule has a four α helix bundle structure characteristic of many cytokines and, from molecular modeling and mutational studies, is thought to contain distinct binding sites for each of the three IL-2R chains. Depending on which subunits are expressed by a cell, three distinct forms of the IL-2R can be detected. The high-affinity IL-2R (K_d 5–10 pM) is found on activated lymphocytes and consists of an $\alpha:\beta:\gamma$ trimeric complex (Figure 1). As this receptor complex generates a proliferative response at low concentrations of IL-2, it is thought to represent the physiologically relevant IL-2R for *in vivo* responses. IL-2R α alone can form a low-affinity receptor for IL-2 (K_d 5–10 nM), however this complex fails to signal. Finally, the human IL-2R β and γ chains together form an intermediate affinity receptor (K_d 0.5–1 nM) which is found on NK cells, macrophages and resting T cells and can signal in the presence of high concentrations of IL-2. In contrast to the human receptor, however, it remains controversial whether the murine IL-2R β and γ chains form a functional intermediate-affinity IL-2R.

On activated lymphocytes coexpressing all three IL-2R chains, IL-2R α and β appear to preassociate as a stable heterodimer in the absence of ligand. IL-2 is thought to bind first to this $\alpha:\beta$ heterodimer and

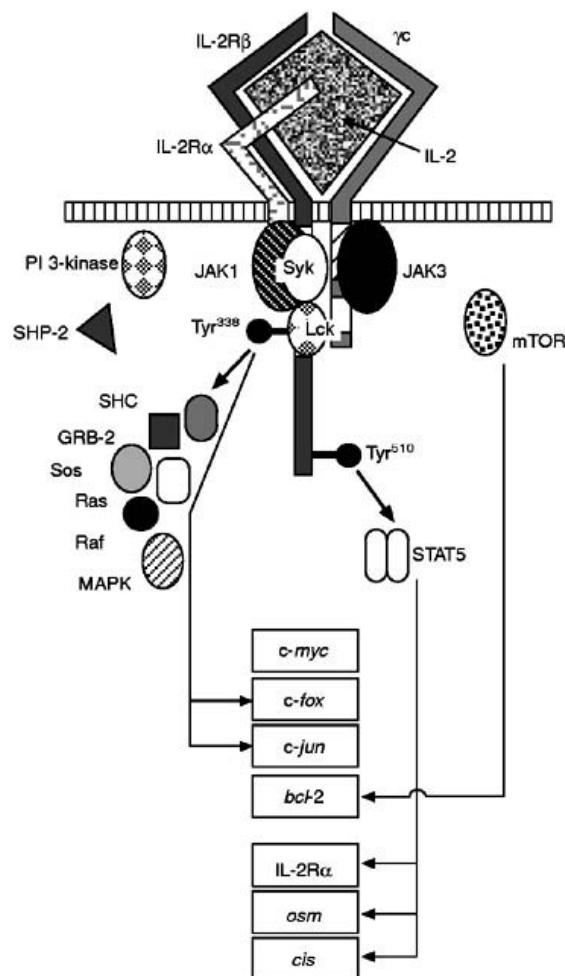


Figure 1 Schematic overview of the high-affinity interleukin-2 receptor complex, including the receptor chains, downstream signaling components and target genes (boxes). Also shown are two tyrosine residues (Tyr³³⁸ and Tyr⁵¹⁰) on IL-2R β that are implicated in mitogenic signaling. Arrows indicate established connections between signaling proteins and target genes. Tyr³³⁸ is required for SHC-mediated activation of the Ras/MAP kinase pathway, which is thought to regulate expression of *c-fos* and *c-jun*. Tyr⁵¹⁰ mediates activation of the transcription factor STAT5, which is implicated in the regulation of the IL-2R α , oncostatin M (*osm*) and *cis* genes.

to subsequently recruit the γ chain. IL-2 binds the high-affinity receptor complex with a rapid on-rate ($t_1 \sim 35$ s), contributed by IL-2R α , and remains bound due to a slow off-rate (t_2 250–300 min), con-

tributed primarily by IL-2R β . Intracellular signaling is initiated by IL-2-induced heterodimerization of the cytoplasmic domains of the β and γ c chains (see below), which is followed by rapid internalization of the ligated receptor complex.

The IL-2R α chain

The α chain of the IL-2R was the first to be defined at a molecular level due to its unique ability to independently bind IL-2. IL-2R α (also known as Tac antigen or CD25) is a glycoprotein of approximately 55 kDa and contains an extracellular domain of 219 amino acids, a transmembrane domain of 19 residues and a cytoplasmic domain of only 13 residues. The chain shares sequence homology with the α chain of the IL-15 receptor, particularly within an extracellular region known as the sushi domain, which contains residues required for ligand binding. Resting T cells do not express detectable amounts of IL-2R α and consequently are unable to bind IL-2 with high affinity. However, antigen stimulation rapidly induces expression of the IL-2R α gene by activating a variety of transcription factors, including NF κ B, c-Rel, serum response factor, Elf-1 and HMG-I(Y), that bind to proximal sites in the IL-2R α promoter. IL-2 itself can also further upregulate the expression of IL-2R α by activating STAT5 and other transcription factors that bind sites 1–4 kb upstream of the transcriptional start site.

Antibodies to IL-2R α (anti-Tac) are being evaluated clinically as T cell-specific immunosuppressants for the treatment of graft-versus-host disease. In addition, anti-Tac conjugated to yttrium-90 is being investigated for the treatment of T cell lymphomas.

The IL-2R β chain

The mature β chain of the IL-2R is a glycoprotein of approximately 75 kDa and contains an extracellular domain of 214 amino acids, a transmembrane domain of 25 residues and a long cytoplasmic domain of 286 residues. IL-2R β belongs to the hematopoietic receptor superfamily by virtue of 1) conserved cysteine residues and a Trp-Ser-Pro-Trp-Ser motif in the extracellular domain, residues that are required for ligand binding, and 2) Box 1 and Box 2 motifs in the membrane-proximal cytoplasmic region that are essential for mitogenic signaling and for association with Janus tyrosine kinases (JAKs).

IL-2R β is generally expressed at low levels on resting T cells and is modestly upregulated by antigen stimulation. It is constitutively expressed at moderate to high levels on NK cells and macrophages. As the IL-2R β chain is also a component of one

form of the IL-15 receptor, it is sometimes referred to as IL-2R β c, for common IL-2R β chain.

The γ c chain

The γ c chain was originally identified as the third subunit of the IL-2R but shortly thereafter was found to also be a component of the receptors for IL-4, IL-7, IL-9 and IL-15, hence the designation γ c (for common γ chain). The mature γ c chain is a glycoprotein of approximately 64 kDa with an extracellular domain of 232 residues, a transmembrane domain of 29 residues, and a cytoplasmic domain of 86 residues. The extracellular domain contains conserved cysteine residues and a Trp-Ser-Glu-Trp-Ser motif, qualifying γ c also as a member of the hematopoietic receptor superfamily. The membrane-proximal cytoplasmic region of γ c shares homology with the α chains of the IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors, which are additional members of this superfamily. This region of γ c also shows partial resemblance to an SH2 domain, but is unlikely to function as such since it lacks most of the residues necessary for binding phosphotyrosine.

Consistent with the involvement of γ c in diverse cytokine receptor complexes, the chain is expressed constitutively by multiple hematopoietic cell types, including macrophages and T, B and NK cells. The γ c gene is located on the X chromosome and constitutes the genetic locus of X-linked severe combined immune deficiency (X-SCID; see below).

IL-2R signal transduction

Although all three chains of the IL-2R are required for high-affinity binding of IL-2, experiments with chimeric receptor chains containing alternative extracellular domains have demonstrated that ligand-induced heterodimerization of the cytoplasmic domains of the β and γ c chains is necessary and sufficient for intracellular signaling. Thus, the role of the α chain appears to be limited to conferring high-affinity binding of IL-2 to the receptor complex. The membrane-proximal cytoplasmic regions of IL-2R β and γ c associate noncovalently with the tyrosine kinases JAK1 and JAK3, respectively (Figure 1). IL-2R β has been shown to also bind and activate the tyrosine kinases Lck, Fyn and Syk. In T cells, heterodimerization of IL-2R β and γ c induces rapid catalytic activation of JAK1 and JAK3 and rapid tyrosine phosphorylation of multiple substrates including JAK1, JAK3, IL-2R β , γ c, the transcription factor STAT5, the adapter molecules SHC and GRB-2, the tyrosine phosphatase SHP-2, the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3K), and the MAP

kinase ERK-2 (Figure 1). Signaling results in the induction of multiple target genes associated with proliferation, including *c-myc*, *c-fos*, *c-jun*, and *bcl-2*, as well as a number of nonmitogenic genes, including IL-2R α and oncostatin M (osm).

Activation of the tyrosine kinase JAK3 is a critical early event in IL-2 signaling, as disruption of JAK3 catalytic activity abrogates almost all of the above tyrosine phosphorylation events and virtually eliminates the mitogenic response. The few JAK3 kinase-independent events described to date include low-level tyrosine phosphorylation of IL-2R β and SHP-2, induction of the proto-oncogene *bcl-2* and prolongation of cell viability. By contrast, disruption of JAK1 activity is reported to have little effect on mitogenic signaling. The critical role of JAK3 catalytic activity indicates that tyrosine phosphorylation of one or more intracellular substrates is essential for mitogenesis. Indeed, mutation of all cytoplasmic tyrosine residues of IL-2R β to phenylalanine eliminates the mitogenic response in the face of normal activation of JAK1 and JAK3. Restoration of either Tyr338, which is required for SHC phosphorylation, or Tyr392 or Tyr510, which can mediate activation of STAT5, rescues the proliferative response in lymphocytes. This suggests that the mitogenic component of the IL-2R signal involves JAK3-dependent tyrosine phosphorylation of IL-2R β , followed by the recruitment and phosphorylation of SHC, STAT5 or possibly other downstream signaling molecules. Consistent with this model, T cells transformed by the human retrovirus HTLV-I (human T lymphotropic virus type I) demonstrate a correlation between IL-2-independent growth and constitutive activation of JAK3 and STAT5.

Aside from tyrosine kinases, several other enzymatic activities are associated with IL-2R signaling. Components of the Ras/MAP kinase pathway are activated in response to IL-2, and are thought to regulate the expression of *c-fos* and *c-jun*. The lipid kinase PI3K is also activated by IL-2, although the role of this molecule in signaling remains undefined. The IL-2R also induces catalysis of glycosylphosphatidylinositol through activation of an unidentified enzyme. Finally, the novel protein serine kinase mTOR (mammalian target of rapamycin) has been implicated in IL-2R signaling, as rapamycin, a pharmacological inhibitor of mTOR, greatly reduces IL-2R-mediated proliferative responses. Rapamycin acts in part by delaying the onset of *bcl-2* expression induced by IL-2 and by preventing the downregulation of p27^{kip1}, an inhibitor of G₁- to S-phase progression. Rapamycin is currently being evaluated clinically as an immunosuppressant.

Genetic disruption of IL-2R subunits

As IL-2 is required to maintain the growth and viability of T cells *in vitro*, one might expect disruption of the genes encoding IL-2 or any of the IL-2R subunits to significantly impair T cell development or expansion. However, mice lacking the IL-2, IL-2R α or IL-2R β genes demonstrate apparently normal development of T, B and NK cells and, rather than showing reduced lymphocyte expansion, frequently demonstrate hyperproliferation of lymphocytes and development of T cell-dependent autoimmune disorders such as hemolytic anemia and inflammatory bowel disease. In contrast, $\gamma c^{-/-}$ mice show severely impaired lymphocyte development, consistent with the involvement of γc in other cytokine receptor complexes. Indeed, the IL-7 receptor utilizes γc and has been shown to be essential for B cell and thymocyte development. Collectively, these results suggest that in the absence of IL-2 or a functional IL-2R, other γc -dependent cytokine receptors can mediate lymphocyte development and expansion *in vivo*. However, an intact IL-2R signaling pathway appears to be required to limit the magnitude of immune responses and prevent the development of certain forms of autoimmunity, perhaps reflecting a role for IL-2 in the regulation of antigen-induced cell death.

In humans, the γc gene represents the locus of X-SCID, a syndrome characterized by a lack of functional T and B cells. Interestingly, patients with mutations in the JAK3 locus suffer from a syndrome very similar to X-SCID, underscoring the critical role of JAK3 in γc -mediated signaling. Genetic therapies are being developed for these immunodeficiencies based on retrovirus-mediated transduction of the wild-type γc or JAK3 genes into the hematopoietic stem cells of patients.

See also: Cytokines; Cytokine receptors; Immunosuppression; Interleukin 2; Interleukin 4 receptor; Interleukin 7 and its receptor; Interleukin 9 and its receptor; Interleukin 15 and its receptor.

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INTERLEUKIN 3

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Tissue culture systems for generating *in vitro* cells of hematopoietic origin were established in the early 1960s by Ginsberg and Sachs. The cells were mast cells and they appeared in cultures of murine thymocytes. In hindsight, it is likely that the generation of these mast cells resulted from the release of interleukin 3 (IL-3), now known to be a potent mast cell growth factor, from activated T cells. Activation of the T lymphocytes probably occurred as a result of a mixed lymphocyte reaction, as the mice used as sources of the thymocytes were not inbred. Sachs and Pluznick, and Metcalf and Bradley in Australia, went on to develop the colony assays that allowed single bone marrow progenitor cells to grow into small colonies of differentiated cells under the influence of soluble factors termed colony-stimulating factors (CSFs).

At the end of the 1960s, Metcalf and his colleagues noticed, using the colony assay, that a murine myelocytic leukemia, WEHI-3B, released activities which stimulated the growth from bone marrow progenitors of colonies with a distinctive morphology, now known to result from the action of IL-3. However, the recognition of IL-3 as a distinct hematopoietic factor, affecting multiple hematopoietic cell lineages, had to wait until the late 1970s and came simultaneously from laboratories studying quite different

phenomena. Dexter and colleagues observed that long-term cultures of mouse bone marrow occasionally gave rise to lines of cells which were dependent for their growth on a factor that was released by the myelomonocytic tumor WEHI-3B, but was distinct from granulocyte-macrophage colony-stimulating factor (GM-CSF). At the same time, a number of groups (Aaronson, Hasthorpe, Nabel, Moore, Razin, and Schrader) observed that cells from mouse spleen, fetal liver or bone marrow that had been cultured in the presence of medium that had been conditioned by activated T cells, or by WEHI-3B, gave rise to homogeneous populations of cells with the characteristics of mast cells. In another distinct line of research, Ihle observed that activated T cells and WEHI-3B released a factor that stimulated increased levels of an enzyme 20 α -hydroxysteroid dehydrogenase, thought at that time to be an exclusive marker of T lymphocytes.

Pleiotropic effects of IL-3 on hematopoietic cells

Biochemical purification demonstrated that the activities studied by these groups all resided in a single molecule that had gone under many names reflecting its multiple activities: namely, IL-3, mast

cell growth factor, P cell stimulating factor, panspecific hematopoietin, multicolony-stimulating factor, CSF2a, CSF2b, hematopoietic growth factor, burst-promoting activity, histamine-producing cell-stimulating activity, Thy-1 inducing factor, and colony-forming units (CFUs) stimulating activity. This molecule stimulated the growth of colonies of mast cells, neutrophils, macrophages, eosinophils, megakaryocytes and erythroid progenitors, and colonies containing a mixture of these cell types. Its presence in bone marrow cultures also permitted the survival of CFUs and cells that could repopulate the lymphoid compartment of irradiated mice, the latter observation pointing to a direct or indirect effect on pluripotential hematopoietic stem cells.

The role of IL-3 in the generation of lymphocytes continues to be the source of some controversy. IL-3 appears to be capable of stimulating the growth of early B cell progenitors, but only in the absence of myeloid cells, which appear to inhibit the growth of B cell progenitors. At present, it would seem reasonable to conclude that IL-3 is active on all of the cell lineages derived from pluripotential hematopoietic stem cells, with the exception of those giving rise to T lymphocytes.

Structure

Structurally, IL-3 is a member of a family of cytokines that includes the CSFs, many interleukins, and other cytokines. These share a characteristic three-dimensional structure and have been termed '4-helix-bundle cytokines'. IL-3 is a polypeptide of 140 amino acids in the mouse and 133 in the human. Although there is little amino acid homology between IL-3 and other peptide regulatory factors, IL-3 does share with GM-CSF, IL-2, erythropoietin, and certain other cytokines, a common motif of Ala-Pro at the N terminus. The functional significance of this motif is unclear; it can be deleted without affecting the ability of IL-3 to activate its receptor.

The genes for IL-3 and GM-CSF have broad structural similarities and are closely linked, consistent with their origin from a common ancestor. The genes for IL-3 in the human and mouse have diverged very considerably, the amino acid sequences being only 29% homologous. Rat IL-3 is only about 70% homologous with mouse IL-3, and rat, mouse and human IL-3 do not act across these species barriers. Similarities between human and mouse genes are in fact greater in the noncoding sequences.

Structure-function studies of the murine IL-3 molecule have shown that significant portions of the N- and C-termini can be deleted without affecting bioactivity *in vitro*. IL-3 became the first protein of

its size to be successfully chemically synthesized in biologically active form using automated peptide synthesis.

IL-3 is heavily glycosylated and runs on SDS-PAGE gels as a series of glycoforms with M_r ranging from 22 000 to 34 000, compared with 14 000 for the nonglycosylated peptide. The function of this carbohydrate is completely unknown. It does not seem to be related to the stability of the molecule in the blood and only modestly prolongs its half-life in the plasma. It may play a more subtle role, for example in localization of the molecule in specific sites *in vivo*.

Receptor

IL-3, like most other 4-helix-bundle cytokines, binds to an oligomeric receptor made up of subunits that are members of a family of proteins termed the 'cytokine or hemopoietin receptor superfamily'. IL-3 binds with relatively low affinity to a specific α -chain receptor subunit (IL-3R α). High-affinity binding and signal transduction requires interaction of the IL-3-IL-3R α complex with a larger β -chain receptor subunit. This β chain is shared with the GM-CSF and IL-5 receptors (each of which have a GM-CSF- or IL-5-specific α chain) and is thus termed ' β common' or β_c . In the mouse, the situation is more complex in that the complex of IL-3 and IL-3R α can also interact with an IL-3-specific β chain (AIC-2A or β_{IL-3}), which has resulted from duplication of the β_c gene and itself has low affinity for IL-3.

Binding of IL-3 to the IL-3R α occurs with relatively low affinity and is followed by interaction with a β_c , or, in the mouse, β_{IL-3} , and the formation of a high-affinity complex. The resultant approximation of the cytoplasmic domains of the α and β chains is probably sufficient to activate cytoplasmic tyrosine kinases such as JAK2 kinase, which is known to be activated following binding of IL-3 to its receptor. More complex mechanisms that require the assembly of a larger complex, resulting from recruitment of an additional β chain or of other IL-3-IL-3R α / β chain complexes, however, have not been completely excluded. Interaction of IL-3 with its receptor results in activation of many of the intracellular signaling paths also stimulated by other growth factors. These include activation of Ras and three branches of the MAP kinase family, the ERK-1,2 Map kinases, the JNK/SAPK kinases, and the p38MAP kinases. These in turn activate other kinases, in the case of Erk-1/2, p90RSK and in the case of p38MAP kinase, MAPKAP-2 kinase. The Map family kinases also directly phosphorylate transcription factors such as c-Jun, ATF-2, CREB, E1k-1 and Sap, upregulating

their activity. IL-3 induces upregulation of the expression of genes such as c-myc, c-fos, c-jun, bcl-2 and bclx_L. Phosphatidylinositol 3 kinase is also activated. Activation of the transcription factor STAT5 is a relatively specific consequence of IL-3 stimulation, although it also occurs in response to GM-CSF, and IL-5, and IL-2 and prolactin. When activated by tyrosine phosphorylation and dimerization, STAT5 upregulates expression of a series of genes including those encoding oncostatin-M and Cis, as well as contributing to upregulation of expression of c-fos.

Sources of IL-3

The major physiological sources of IL-3 appear to be T lymphocytes and, in the human, natural killer (NK) cells. IL-3-dependent mucosal mast cells, connective tissue mast cells and eosinophils also produce small amounts of IL-3 when they are stimulated, for example, by crosslinking of antibodies bound to Fc receptors on their surface. IL-3 released during allergic reactions may affect the functions of mast cells and eosinophils as well as those of other cells such as macrophages. There is no evidence that any of the stromal cells of the bone marrow normally produce IL-3.

Physiology of IL-3

An extremely potent molecule, IL-3 is detectable in *in vitro* bioassays in concentrations of 0.1 pM or less. The sensitivity of various target cells to the action of IL-3 varies considerably, probably due to differences in receptor levels. Thus, whereas certain IL-3-dependent mast cell lines, particularly those that have been in culture for many years, are sensitive to subpicomolar concentrations of IL-3 progenitor cells and stem cells may require 10–50-fold higher concentrations for maximal effects and some mature cells, such as macrophages, even higher concentrations.

As is the case with many peptide regulatory factors, it is likely that in many physiological circumstances IL-3 acts in the vicinity of the cell that has produced it. However, when there is relatively massive activation of T lymphocytes, for example in graft-versus-host disease, IL-3 can appear in the bloodstream and act systemically. IL-3 has a relatively short half-life in the blood, of the order of 40 minutes, and is destroyed at sites such as the renal tubule and liver.

Physiologically, IL-3 links activation of T lymphocytes and mast cells to stimulation of the generation of the accessory cells – granulocytes, phagocytes and platelets – which carry out defense and repair

responses. It is thus an important participant in the response of the hematopoietic system to stress.

There is no good evidence that IL-3 is normally present in the blood or indeed is produced in a normal animal that is not undergoing some immunological stimulation. In particular, there is no evidence that bone marrow stromal cells produce IL-3. Thus there are no data to support the view that IL-3 is involved in regulating normal, steady-state hematopoiesis. Mice lacking functional IL-3 genes have no overt phenotype and the hematopoietic system appears to develop normally.

Clinical significance of IL-3

The *in vivo* administration of IL-3 results in all of the phenomena that might be predicted from its activity in tissue culture systems. Thus there is a rapid and dramatic increase in the number of mast cells in tissues such as spleen and also the skin. In the mouse spleen, there are increases in the number of progenitor cells of various lineages and of pluripotent hematopoietic stem cells. In the bone marrow, there is an increase in the frequency of pluripotent hematopoietic stem cells.

IL-3 has synergistic effects with other hematopoietic growth factors such as GM-CSF and there is evidence that the sequential administration of first IL-3, stimulating primitive stem and progenitor cells, and then a factor, such as GM-CSF, that affects specific lineages, will provide maximal stimulation of myelopoiesis. Administration of IL-3 also results in mobilization of hematopoietic stem cells into the blood. The clinical utility of IL-3 has yet to be established. Studies are in progress to determine whether, particularly when used in conjunction with other growth factors such as GM-CSF, IL-3 will enhance the recovery of the bone marrow after procedures such as bone marrow transplantation or chemotherapy.

In the long term, as is the case with many peptide regulatory factors, antagonists may have more clinical significance than the factors themselves or agonists. Animal studies are revealing a variety of disease states – ranging from allergic and infectious diseases to leukemia – where antagonists may have a role to play in therapeutic procedures. For example, antibodies to IL-3 given together with antibodies to IL-4 block the mastocytosis seen in parasitized rats. Administration of IL-3 results in accelerated IgE responses and expulsion of *Trichinella spiralis*, and appears to promote the production of IL-10 and IL-13, cytokines which promote T_H2 responses. Thus IL-3 antagonists may find a useful role in allergic diseases or asthma. Antibodies to IL-3 and GM-CSF

have also been shown to block the pathogenic accumulation of macrophages in the brain of mice suffering from cerebral malaria. In leishmaniasis in mice, there is evidence that IL-3 exacerbates the disease process and antibodies to IL-3 favor disease regression. These findings may reflect an antagonistic activity of IL-3 on the action of interferon γ , which is known to activate mechanisms that kill intracellular leishmania.

The investigation of IL-3 has provided insights into the role of autostimulatory production of growth factors in tumorigenesis. It is now clear that WEHI-3B, the monocytic tumor from which IL-3 was originally purified, produces IL-3 as a result of pathological activation of one IL-3 gene by insertion of viral DNA. Moreover, early passages of WEHI-3B appeared to be dependent on this IL-3. In other experiments, a spontaneous variant of an IL-3-dependent cell line was isolated through its ability to survive and grow in the absence of IL-3. This property of the cell was shown to be due to autogenous IL-3. Significantly, unlike the parental line, when injected into a mouse this variant cell gave rise to a disseminated leukemia. These leukemic animals had detectable amounts of IL-3 in their serum. A number of other such examples in which the growth of leukemic cells depends on the production of autostimulatory growth factor, such as IL-3 or GM-CSF, have now been uncovered. Antagonism of the action of autostimulatory growth factors can result in death of the leukemic cells. However, the autostimulatory production of IL-3 does not appear to be a significant cause of human malignancy.

Conclusion

Structural analysis of IL-3 and its receptor and the IL-3-specific components of IL-3 signal transduction pathways should ultimately lead to the design of small molecules that can mimic or antagonize IL-3. The capacity of IL-3 to stimulate primitive cells in the hematopoietic system is likely to be an advantage in the design of strategies to stimulate recovery of bone marrow, especially after chemotherapy for cancer or other aplastic diseases of the marrow. In inflammatory diseases, particularly where mast cells

play a key role, such as asthma or allergy, IL-3 antagonists may find a useful role.

See also: Mast cells; Bone marrow and hematopoiesis; Hematopoietic stem cell transplantation; Colony stimulating factors; Cytokine assays; Cytokine genes, regulation of; Cytokines; Cytokine receptors; Granulocyte-macrophage colony stimulating factor (GM-CSF).

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INTERLEUKIN 3 (IL-3) RECEPTOR

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Interleukin 3 (IL-3) is a T cell-derived pluripotent hematopoietic colony-stimulating factor required for the survival and proliferation of primitive hematopoietic progenitor cells. It signals target cells through a high-affinity cell surface receptor. The IL-3 receptor was first described on murine bone marrow cells. These cells appeared to express a single high-affinity binding site with a K_d of around 100 pM, and only a few hundred sites per cell. In several murine hematopoietic cell lines however, a low-affinity site ($K_d = 10$ nM) has also been described. Chemical cross-linking studies indicated multiple cross-linked proteins with molecular weights of 140, 120 and 70 kDa. Studies with human hematopoietic cells also described an IL-3 receptor with a K_d of 70–100 pM and about 100–200 sites per cell. Chemical cross-linking revealed two binding components: a larger, 120 kDa species, and a smaller one with a molecular weight of 70 kDa. Interestingly, in human hematopoietic cells, granulocyte-macrophage colony-stimulating factor (GM-CSF) competes with IL-3 for binding to the IL-3 receptor. This cross-competition was not observed in the murine hematopoietic cells.

Structure of the IL-3 receptor

Molecular biology of the murine IL-3 receptor

Using an antibody (Aic2) that partially blocked IL-3 binding to murine cells, a cDNA termed AIC2A was cloned that encoded an 878 amino acid protein including a signal sequence. The mature protein is a glycoprotein of about 120 kDa. The AIC2A protein is a member of the type I cytokine receptor superfamily that includes the β and γ subunits of the IL-2 receptor, the receptors for IL-4 through IL-7, IL-9, GM-CSF, G-CSF, erythropoietin, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor. They have a common structural motif in their extracellular domains. This motif contains approximately 200 amino acids with fibronectin type III modules. Each module contains seven antiparallel β strands. There are four conserved cysteines in the N-terminal module and a unique tryptophan-serine-variable amino acid-tryptophan-serine (WSXWS) motif in the C-terminal module.

The AIC2A protein binds murine IL-3 with very

low affinity ($K_d = 10$ –20 nM). These binding characteristics are consistent with the low-affinity binding site on murine cells. A second cDNA was subsequently cloned that conferred high-affinity IL-3 binding in cooperation with AIC2A. The cDNA was termed SUT-1 and coded for a protein of 396 amino acids that is also a member of the type I cytokine receptor superfamily. The mature protein is about 70 kDa and binds IL-3 with a K_d of 40 nM. Coexpression of SUT-1 and AIC2A reconstitutes a receptor that binds murine IL-3 with a K_d of 300 pM. Thus, SUT-1 is now referred to as the α or ligand-binding subunit, and AIC2A as the β subunit of the murine IL-3 receptor. Further expression cloning using the Aic2 antibody led to the molecular cloning of a second AIC2 cDNA termed AIC2B. This cDNA encodes a protein of 896 amino acids with 91% homology to AIC2A, and its structure is basically the same as the IL-3 receptor β subunit; however, AIC2B binds neither IL-3 nor any other cytokine. Further studies have revealed that AIC2A is a subunit specific to the IL-3 receptor binding complex, and AIC2B is a β subunit shared with the GM-CSF receptor (Figure 1).

Molecular biology of the human IL-3 receptor

Using the AIC2A cDNA as a probe, the human homolog of the murine IL-3 receptor β subunit was cloned. The cDNA was termed KH97 and its overall structure was remarkably similar to the AIC2A/AIC2B proteins. It is about 56% homologous to the amino acid sequence of the murine AIC2 proteins. KH97 has two WSXWS motifs, but does not bind any IL-3 or other cytokines. Expression cloning using a cotransfection strategy with KH97 cDNA allowed the isolation of a cDNA that bound IL-3 with extremely low affinity. This cDNA, termed DUK-1, encodes a protein of 378 amino acids, and the extracellular domain contains a single WSXWS motif. The mature protein is about 70 kDa, and its structure is similar to the murine IL-3 receptor α subunit. The murine and human proteins however, show only about 70% homology at the amino acid level.

Transfection of COS-7 cells with the human IL-3 receptor α cDNA (DUK-1) yielded cells that bound IL-3 with very low affinity ($K_d = 100$ nM); however,

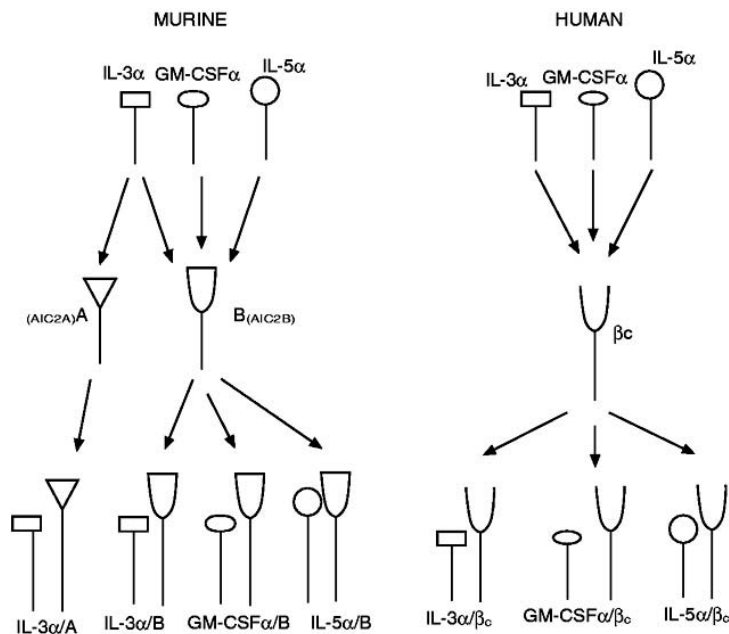


Figure 1 Structure of the murine and human IL-3 receptors and shared subunits. The murine system has two β subunits – AIC2A and AIC2B. AIC2A associates specifically with the α subunit of the IL-3 receptor, while AIC2B is shared by the IL-3/GM-CSF, and the IL-5 receptor α subunits. The human system possesses only one type of β subunit (β common or β_c), which forms high-affinity receptors with the α subunits of the IL-3/GM-CSF and IL-5 receptors.

cotransfection with KH97 reconstituted a high-affinity human receptor ($K_d = 100$ pM). Further studies of the GM-CSF receptor revealed that the GM-CSF and IL-3 ligand-binding subunits share the protein encoded by KH97 to form their respective high-affinity receptor complexes. Therefore the IL-3 receptor β subunit is termed β common or β_c , and the cross-competition between human IL-3 and GM-CSF is probably caused by competition for a limiting number of β_c by different ligand binding (α) subunits (Figure 2).

Signal transduction through the IL-3 receptor

Subunit requirements for signaling specificity and optimal cytokine response

Neither subunit of the IL-3R contains any known consensus sequences for intrinsic tyrosine kinase motifs or SH2/SH3 domains. Functional assays, however, have defined well-conserved domains in both the α and β_c subunits which are important for signal transduction. While dimerization of the β subunit appears to be essential for receptor activation, the α subunit is proposed to augment interactions of β_c with downstream signaling proteins. The ability of IL-3/GM-CSF/IL-5 to induce various effects depending on the target cells, may also be derived from the distinct cytoplasmic domains of their respective ligand-specific α subunits. Ligand-stimulated increases in tyrosine phosphorylation of the β_c subunit triggers IL-3-mediated signaling pathways. The observed lack of a correlation between cell pro-

liferation and β_c tyrosine phosphorylation has led some researchers to suggest that the IL-3R complex may contain additional components besides the α and β subunits.

Activation of non-receptor tyrosine kinases, serine/threonine kinases and recruitment of effector proteins into IL-3R-associated signaling complexes

Ligand stimulation of the IL-3 receptor rapidly induces tyrosine phosphorylation of effector proteins which subsequently trigger a cascade of intracellular responses. The activated receptor recruits nonreceptor tyrosine kinases and proteins containing SH2/SH3 structural motifs that participate in protein:protein interactions with β_c . Abrogation of IL-3 dependence in murine myeloid cells by retroviral tyrosine kinase oncogenes suggests that tyrosine phosphorylation events are critical to signaling pathways regulating cell proliferation and survival. Several members of the Src family of tyrosine kinases – p53/p56^{lyn}, p62^{yes} and p93^{fes} – have been implicated in IL-3R action. The β_c subunit appears to be constitutively complexed to p53/p56^{lyn}, phosphatidylinositol-3-kinase (PI3K) and JAK2. Engagement of the IL-3R leads to enhanced tyrosine phosphorylation and enzyme activity of JAK2 and PI3K, which are thought to play a role in signaling cell proliferation. Activation of JAK2, a non-Src type tyrosine kinase, is known to be necessary for IL-3-initiated mitogenesis and phosphorylation/nuclear translocation of the transcription factor STAT5 (signal transducer and activator of transcription). Stimulation with IL-3

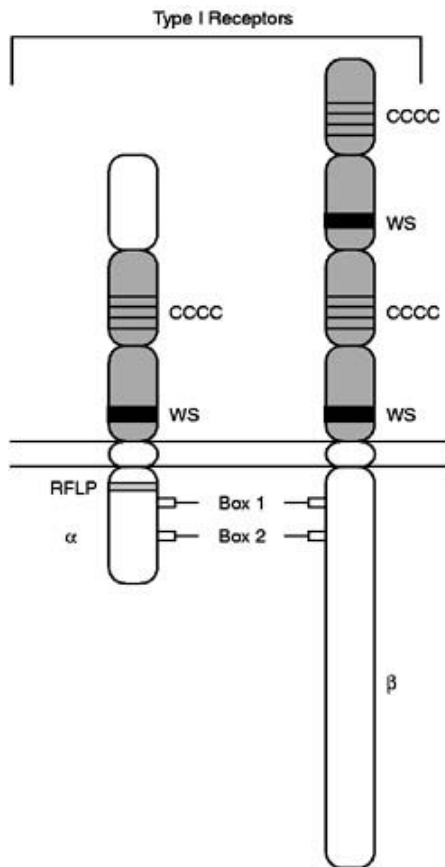


Figure 2 Structural organization of the IL-3 receptor α and β subunits, both of which are members of the type I cytokine receptor family. The extracellular portion of the IL-3 receptor comprises a conserved motif (stippled boxes), which bears four conserved cysteine residues (CCCC) in the N-terminal side, and the sequence WSXWS in the C-terminal side. A short stretch of conserved amino acid sequence (RFLP) is found in the cytoplasmic domain of the α subunits as well. Box 1 and 2 motifs are part of a serine-rich region in the membrane-proximal domain, which is critical for IL-3-mediated cell proliferation.

also induces transient increases in the tyrosine phosphorylation status of the SH2-containing proteins Vav and p52^{shc}, and facilitates association of the latter with the docking protein GRB-2. These proteins are regulators of the interaction between IL-3R and the Ras pathway (Figure 3).

IL-3 stimulation of the serine/threonine kinases Raf-1 and members of the MAPK cascade serves to link tyrosine kinase-initiated signals to nuclear events involved in mitogenesis. *In vitro* experiments with glutathione-S-transferase/human IL-3R β c fusion proteins reveal that the membrane-proximal domain (amino acids 455–517) of the β c subunit is also capable of directly binding, and being phosphorylated by cytosolic serine/threonine kinases. Interestingly, the IL-3R activates a phosphatidylcholine-specific

phospholipase C, in conjunction with membrane translocation and activation of protein kinase C (PKC). IL-3R-activated PKC phosphorylates Raf-1 and the myristoylated alanine-rich C kinase substrate (MARCKS) protein. Phosphorylation of MARCKS is proposed to release the calmodulin bound to it, and lead to activation of calmodulin kinase. Calmodulin appears to be required for IL-3-induced DNA synthesis and primitive hematopoietic progenitor colony formation. Activation of PKC lies downstream of receptor-mediated increases in tyrosine phosphorylation, and seems to play a significant role in both IL-3R-regulated expression of the antiapoptotic gene *bcl-2*, and the suppression of apoptosis in factor-dependent cells.

Receptor subdomains mediating IL-3-dependent proliferative responses and suppression of apoptosis

Structure–function analyses using cytoplasmic domain deletion mutants of the β c subunit have revealed two regions which appear to transduce distinct signals, both of which are required for long-term survival and proliferation (Figure 4). The membrane-proximal region of the human β c subunit (amino acids 455–562), containing a Pro-X-Pro amino acid motif and an acidic residue-rich region, is required for activation of JAK2 tyrosine kinase and PI3K induction of the proto-oncogenes *pim-1*, *c-myc*, and mitogenesis. The amino acid sequence of the membrane-proximal region is highly conserved among β c, AIC2A and AIC2B (Figure 5). The membrane-distal region (amino acids 626–763) is essential for activation of Ras, Raf-1, MAPK and p70S6K and is related to induction of the *c-fos* and *c-jun* genes. A C-terminal-deleted β c, which lacks the membrane-distal cytoplasmic sequences required for activation of the Ras-Raf-1-MAPK cascade, induces DNA synthesis, but is incapable of supporting cell survival. Thus, despite a transient mitogenic response, cells expressing this mutant die by apoptosis in the presence of growth factor. Interestingly, the expression of a constitutively activated Ras protein appears to complement defective signaling through this mutant and restores the long-term proliferation response to cytokine stimulation. These data suggest that the IL-3R prevents apoptosis by stimulating a signaling pathway distinct from the induction of DNA synthesis.

See also: Cytokines; Cytokine receptors; Granulocyte-macrophage colony stimulating factor (GM-CSF); Interleukin 3; Interleukin 5 and its receptor.

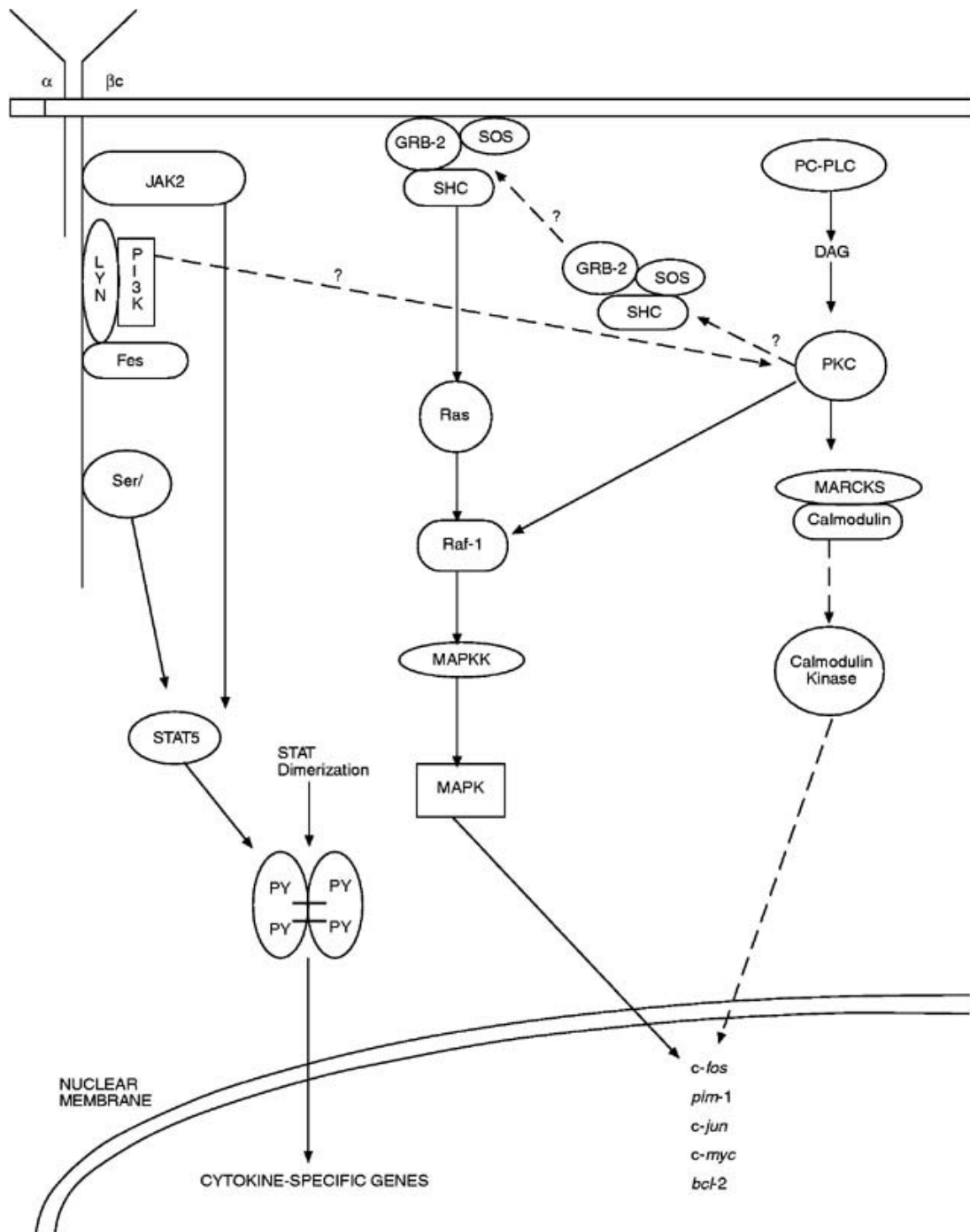


Figure 3 A generalized schematic illustration of nonreceptor kinases, serine/threonine kinases, downstream effector molecules and target genes involved in IL-3 receptor signaling (proposed pathways indicated by dashed lines). The tyrosine kinases depicted seem to be capable of directly associating with human βc . Ligand stimulation leads to increases in βc tyrosine phosphorylation. activation of tyrosine kinases and PI3K, the latter catalyzes formation of the second messenger PI3,4,5- P_3 (phosphatidylinositol 3,4,5-triphosphate). PKC ζ is known to be activated by PI3,4,5- P_3 , suggestive of a role for this isotype of PKC in signaling through the IL-3 receptor. Evidence indicates that a 110 kDa serine-threonine kinase is also constitutively associated with the βc subunit. the substrates of this enzyme are not yet known. Serine/threonine phosphorylation of STATs is known to enhance their transactivation potential, the pathway/enzyme(s) that mediate this are unknown. IL-3 stimulates the tyrosine phosphorylation and complex formation between adapter proteins regulating activation of the Ras pathway (SHC, SOS-1, GRB-2). However, these events apparently do not promote the mass membrane translocation of the SH2 adapter protein complex. PY, phosphorylated tyrosine; DAG, diacylglycerol; Ser/ThrK, serine/threonine kinase.

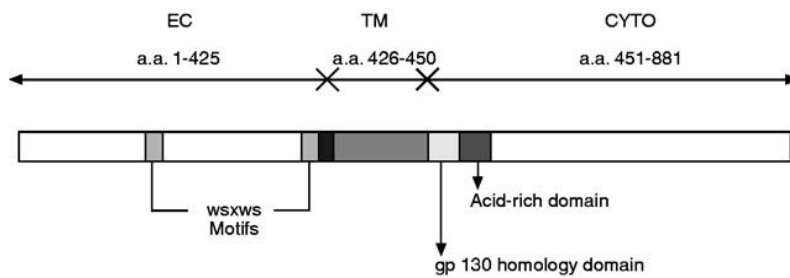


Figure 4 Structure of the human IL-3 β c subunit: EC, extracellular domain; TM, transmembrane domain; gp130 homology domain, region containing the i) Pro-Asn-Pro motif that is important for tyrosine phosphorylation and cell proliferation, and ii) acid-rich domain containing 11 acidic and 1 basic amino acids which comprise a motif shared with the p75 signaling subunit of the IL-2 receptor. The membrane-proximal domain, extending between Arg456 and Asp544 is involved in mediating the proliferative signal through the IL-3 receptor. A region encompassing amino acids Val518 to Leu626 is responsible for major tyrosine phosphorylation, and the membrane-distal region (Leu626-Ser763) is required for activation of Ras, Raf-1, MAPK, and p70 S6K, as well as induction of *c-fos* and *c-jun*.

(A)

h β_c	(454-487)	LPRKWEKIPNPSKSHLFQNGSAELWPPGSM	SAF
AIC2A	(450-483)	TYRKWEKIPNPSKSLLFQGGKGLWPPGSM	AAAF
AIC2B	(451-484)	TYRKWEKIPNPSKSLLFQGGKGLWPPGSM	AAAF

(B)

h β_c	(517-544)	EVSPLTIEDPKHVC	DPPSGPDTTPAAS	D
AIC2A	(513-540)	NVSPLTIEDPNIIRD	PPSRPDTTPAAS	S
AIC2B	(514-541)	NVSPLTIEDPNIIRV	PPSGPDTTPAAS	S

(C)h β_c (450-562)

YGYLRRKWEEKIPNPSKSHLFQNGSAELWPPGSM^SSAFTS
 GSPPHQGPWGS^RFPELEGVFPVGFGDSEV^SPLTIEDPKH
 VCDPPSGPD^TTPAASDLTEQPPSQPGPAASHTPE

Figure 5 Primary amino acid sequences of regions in the human β_c , which are involved in IL-3-mediated cell proliferation. Sequences from corresponding regions of mouse AIC2A and AIC2B are also aligned to highlight the extensive homology between human β_c and mouse β subunits. Region (A), between Arg456 and Phe487 appears to be essential for proliferation, while region (B) enhances the growth signal. (C) Underlined residues are acidic amino acids comprising the acid-rich region of human β_c ; residues in bold identify a motif homologous to that present in the IL-6 receptor gp130.

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INTERLEUKIN 4

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The cytokine interleukin 4 (IL-4) is produced by several subsets of lymphoid cells and has pleiotropic actions on a variety of effector cells of the immune system (Table 1). Its major roles include promoting type 2 T helper (T_H2) cell differentiation, immunoglobulin E (IgE) secretion and eosinophil recruitment.

The human and murine IL-4 genes map to human chromosome 5 and mouse chromosome 11 and encode proteins that are comprised of 153 and 140 amino acid residues, respectively. Despite sharing 50% amino acid identity, human IL-4 is inactive on mouse cells and murine IL-4 is inactive on human cells. While natural IL-4 is an 18–20 kDa glycoprotein, recombinant IL-4 lacking glycosylation is fully active. As for most secreted polypeptides, IL-4 contains an N-terminal signal sequence that is post-translationally cleaved.

IL-4 is produced by several subsets of activated lymphoid cells. T_H2 cells are particularly important sources of this cytokine, as they are critical for initiating humoral immunity against extracellular pathogens. In addition to secreting IL-4, T_H2 cells also require IL-4 for their own development. It has been suggested that specialized T cells that express natural killer (NK) cell markers and secrete large amounts of IL-4 immediately after T cell receptor stimulation produce the IL-4 that promotes T_H2 cell differentiation. However, T_H2 cell differentiation can proceed normally in strains of genetically altered mice that lack these NK-like T cells. Activated mast cells, basophils and γ/δ T cells can also produce IL-4 and thus may be other sources of the IL-4 that is required for T_H2 cell differentiation.

Secretion of IL-4 is transcriptionally regulated and, as such, the IL-4 promoter has been extensively studied. Functionally important binding sites for AP-1 and NF-AT transcription factors are present in the IL-4 promoter. However, AP-1 and NF-AT also bind to the promoters of other cytokine genes expressed independently of IL-4, suggesting that cell-specific expression of IL-4 is dictated by additional factors. Indeed, recent studies indicate that activity of the proto-oncogene *c-maf* may be responsible for the specific transcription of the IL-4 gene in T_H2 cells.

As is the case with other cytokines, IL-4 elicits biological responses by interacting with a specific cell surface receptor. The IL-4 receptor is expressed on a wide variety of cell types including T and B lymphocytes, macrophages, fibroblasts, keratinocytes and endothelial cells. The receptor is composed of at least two chains: a 140 kDa ligand-specific α chain and a 75 kDa γ_c chain. The α chain is also a component of the IL-13 receptor, while the γ_c chain is shared by several other cytokine receptors, including those for IL-2, IL-7, IL-9 and IL-15.

Table 1 Pleiotropic functions of IL-4

- Acts as a costimulant for growth in B cells, T cells and mast cells.
- Increases the survival of cultured B cells, T cells and other hematopoietic cell types
- Induces the expression of MHC class II on B cells and monocytes and CD23 on B cells
- Directs immunoglobulin class switching to the IgG1 and IgE isotypes
- Directs T helper cell differentiation to the T_H2 type
- Diminishes the inflammatory functions of monocytes and macrophages while enhancing their antigen presenting functions
- Can inhibit the growth of transplanted tumors *in vivo* and can diminish the growth of certain cancer cells *in vitro*
- Induces growth and chemotaxis in human fibroblasts and the production of IL-6, extracellular matrix proteins and ICAM-1
- Regulates expression of VCAM-1 on human endothelial cells and VLA-4 on T cells and eosinophils
- Reduces Cl^- secretion by intestinal epithelial cells

Activities of IL-4

IL-4 was first identified as a helper T cell product with the capacity to costimulate B cell growth *in vitro*. Early studies also revealed that IL-4 augments expression of major histocompatibility complex (MHC) class II and Fcε receptor molecules on murine B cells and promotes their secretion of IgE and IgG1. Consequently, IL-4 was initially named B cell stimulatory factor. Both transgenic IL-4-overexpressing and IL-4-deficient mice have been generated. *In vivo*, transgenic overexpression of IL-4 dramatically increases basal levels of serum IgE, and IL-4-deficient mice fail to produce antibodies of the IgE-isotype and produce diminished amounts of IgG1. Thus, IL-4 is essential for B cell secretion of IgE and augments production of IgG1 *in vivo*.

In addition to regulating B cell growth and immunoglobulin secretion, IL-4 also affects T cells. *In vitro*, IL-4 promotes T cell growth and can potently induce cytolytic T cell activity. IL-4 also induces the differentiation of naive CD4⁺ T cells into T_H2 cells, which are characterized by their capacity to secrete the cytokines IL-4, IL-5 and IL-10 upon activation, while simultaneously inhibiting the generation of T_H1 cells, which secrete IL-2 and interferon γ (IFNγ). Indeed, *in vivo* the development of T_H2 cells is critically dependent upon IL-4, as it is completely abrogated in transgenic IL-4 deficient mice. Accordingly, IL-4-deficient mice are hypersensitive to infections that are normally eradicated by T_H2 cells, such as the nematode *Nippostrongylus brasiliensis*.

IL-4 may also influence T cell maturation in the thymus. A burst of IL-4 expression occurs at day 15 of gestation in the fetal thymus of normal mice, suggesting that thymic IL-4 expression plays a role in normal T cell development. *In vitro*, IL-4 affects thymocyte maturation, as CD8⁺ T cells selectively develop from IL-4-treated thymocyte cultures. Likewise, the total number of immature thymocytes is reduced, while the number of mature CD8⁺ thymocytes is increased when IL-4 is overexpressed *in vivo*. However, all thymocyte subsets develop normally in IL-4-deficient mice. Thus, *in vivo* IL-4 expression is not critical for T cell maturation in the thymus.

In vivo, IL-4 regulates eosinophil recruitment. Transgenic mice with T cells overexpressing IL-4 develop an ocular inflammatory lesion characterized by a striking number of eosinophils and mast cells. IL-4-mediated eosinophilia is also observed when nonimmunogenic tumor cells are transfected with an IL-4 cDNA and implanted into mice. The IL-4-expressing tumor cells become immunogenic, and their clearance is associated with an infiltrate of tumoricidal eosinophils. The IL-4-promoted eosino-

philia has been shown to be dependent upon IL-5. Thus, the depressed eosinophil responses in IL-4-deficient mice are probably a consequence of their failure to generate IL-5-producing T_H2 cells.

Molecular mechanisms of IL-4 activity

At least two distinct IL-4 receptor-associated signaling pathways are activated when IL-4 binds to the membrane-bound form of its receptor. One pathway leads to the phosphorylation of an insulin receptor substrate (IRS), named IRS-2. Phosphorylated IRS-2 then associates with phosphatidylinositol 3-kinase. A mutant cell line that cannot phosphorylate IRS-2 exhibits diminished growth in response to IL-4, suggesting that the IRS-2 pathway may regulate IL-4-associated proliferative responses.

The second pathway activated by binding of IL-4 to its receptor is initiated by the Janus kinase family members, JAK1 and JAK3. These JAKs are constitutively associated with the IL-4 receptor and are activated by phosphorylation when IL-4 binds to the receptor. Once activated, the JAKs phosphorylate a member of the signal transducing and activation of transcription (STAT) family, STAT6. This protein exists in a latent form in the cytoplasm and it dimerizes and migrates to the nucleus when activated by phosphorylation. There it promotes the transcription of genes containing promoters with STAT6 binding sites. Lymphocytes from transgenic STAT6-deficient mice do not differentiate into T_H2 cells in the presence of IL-4. In addition, IL-4-induced increases in B cell expression of MHC class II antigens are abrogated in the absence of STAT6, as are IL-4-induced B and T cell proliferative responses. Thus, STAT6 appears to mediate most responses to IL-4, including T_H2 cell differentiation.

As the transcription factor STAT6 is a critical component of signaling through the IL-4 receptor, the expression of a specific set of genes presumably mediates many of the biologic effects of IL-4. As mentioned, IL-4 is critical for B cell class switching to IgE and promotes the switching of murine cells to IgG1. Class switching is initiated by IL-4-induced transcription of immunoglobulin genes, and functional STAT6 binding sites have been identified in the promoters of the IgE and IgG1 genes. In addition, B cell expression of MHC class II antigens, CD23, CD40, Thy-1 and receptors for IL-4 and Fcε are each augmented by exposure to IL-4. Thus, transcription of STAT6-dependent genes can account for many of the activities of IL-4 on B cells.

In contrast, the mechanisms by which IL-4 regulates T cells are less well understood. IL-4 and IL-12 exert antagonizing effects on T cell differentiation.

While IL-4 promotes the differentiation of naive T cells into T_H2 cells, IL-12 promotes their differentiation into T_H1 cells. When both cytokines are present, IL-4 dominates and T_H2 cells develop. In part, this is because IL-4-exposure causes a downregulation of the β chain of the IL-12 receptor, thus abrogating signaling via IL-12 receptors. Thus, the production of IL-4 during early phases of an immune response will critically affect the types of effector cells that develop.

However, the mechanism by which IL-4 directly promotes the generation of T_H2 cells is currently unknown. Few IL-4-regulated T cell genes have been identified. The proto-oncogene *c-maf* is a potent inducer of IL-4 transcription, and *c-maf* is expressed in differentiated T_H2 cells, but not in T_H1 cells. Thus, if IL-4 induces *c-maf* expression, *c-maf* could in turn promote IL-4 expression and perhaps induce stable T_H2 cell differentiation. However, it has not yet been demonstrated that either IL-4 or STAT6 directly promote *c-maf* expression. Thus, despite its critical requirement for T_H2 cell differentiation *in vivo*, the mechanisms by which IL-4 promotes the development of T_H2 cells from naive precursors remain to be elucidated.

See also: Mast cells; Helper T lymphocytes; Cytokines; Effector lymphocytes; Eosinophils; IgE; Immunoglobulin class switching; Interleukin 4 receptor.

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INTERLEUKIN 4 RECEPTOR

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Interleukin-4 (IL-4) is a cytokine, produced by T cells, mast cells and basophils, that elicits a diverse array of biological responses. These functions range from the regulation of helper T cell phenotype and the production of immunoglobulin E (IgE) by B lymphocytes to the regulation of the adhesive properties of endothelial cells via VCAM-1 and the regulation of ion secretion by intestinal epithelial cells. In keeping with this diverse array of biological effects, high-affinity binding sites for IL-4 (K_d 20–300 pM) have

been detected on a variety of hematopoietic and non-hematopoietic cell types at levels ranging from 100 to 5000 sites per cell.

Cell surface IL-4 receptor complex

The structure of the IL-4 receptor complex was initially characterized using chemical cross-linking of [¹²⁵I]IL-4 to molecules on the surface of IL-4-responsive cells. These early studies found two major com-

plexes. One complex contained [^{125}I]IL-4 bound to a protein of ~140 kDa. The second complex often contained 80 and 70 kDa molecules cross-linked to [^{125}I]IL-4. These molecules have since been cloned and sequenced.

The molecular cloning of cDNA encoding IL-4 binding and its expression in COS-7 cells indicated that a single chain of ~140 kDa constituted a high-affinity IL-4 binding site (IL-4R α). The IL-4R α bears the characteristic pairs of cysteine residues and the WSXWS motif found in other members of the hematopoietin receptor superfamily, a classic transmembrane domain, and one of the largest cytoplasmic domains of this family with over 500 amino acids. During the cloning process, a second naturally occurring form of mRNA was observed in murine cells. This form gave rise to a soluble high-affinity IL-4-binding protein of 40 kDa. The nucleotide sequence of the cDNA encoding these molecules indicated that both shared the extracellular, ligand-binding domain, but that the 40 kDa form lacked the transmembrane and cytoplasmic domains. Examination of the structure of the murine gene (Figure 1) revealed an exon (exon 8) that specified a soluble version of the IL-4 receptor. Thus the two species of receptors are likely to arise from alternative splicing.

The IL-4 receptor complex also contains other polypeptide chains, consistent with the early cross-linking studies. The γ chain of the IL-2 receptor (common γ or γc) associates with the IL-4R α chain following the binding of IL-4. This complex has been called the type I IL-4 receptor (Figure 2). In addition, the low-affinity binding chain for IL-13 (IL-13R α), a cytokine that elicits biological responses similar to IL-4, can also function as an IL-4R α -associating chain. The IL-4 receptor containing the IL-13R α has been termed type II. Interestingly, a chimeric receptor consisting of the extracellular domain of the erythropoietin receptor and the cytoplasmic domain of the IL-4R α is competent to signal cellular proliferation, suggesting that association of two IL-4R α chains is sufficient to induce signaling. However, it is not yet

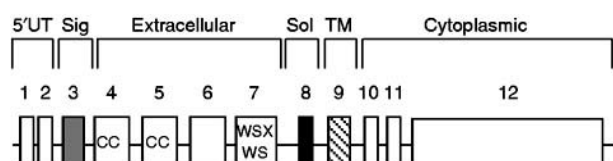


Figure 1 Exon structure of the gene encoding the murine IL-4R α . Exons 1–12 are depicted. Introns are not to scale. The two pairs of cysteine residues (CC) and the WSXWS motif are shown in the appropriate location. Exon 8 codes for the carboxy-terminus of the soluble form of the receptor. In the cell surface form, exon 8 is spliced out leaving exons 7 and 9 adjoining.

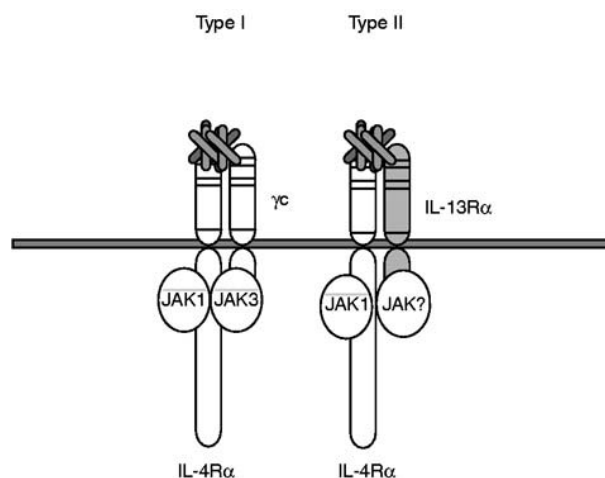


Figure 2 Models of IL-4 receptor complexes. Models of the IL-4 receptor complexes best supported by experimental data are shown. The type I receptor consists of the IL-4R α and the γc . JAK1 binds the IL-4R α and JAK3 binds the γc . The type II IL-4 receptor consists of the IL-4R α and the low-affinity IL-13 binding chain called IL-13R α .

clear whether this type of IL-4 receptor is functional *in vivo*.

Signal transduction by the IL-4 receptor complex

Treatment of cells with IL-4 activates several signaling pathways that involve the activation of cytoplasmic tyrosine kinases (Figure 3). The Janus family of tyrosine kinases (JAKs) are of critical importance in initiating these pathways. JAK1 is associated with IL-4R α , while JAK3 is coupled to the γc . The identity of the kinase(s) associated with IL-13R α is not yet clear, but it is not JAK3. Heterodimerization of the receptor subunits is thought to activate the kinases.

One of the signaling pathways initiated by IL-4 includes the activation of a latent, cytoplasmic transcription factor that is a member of the signal transducers and activators of transcription (STAT) family, termed STAT6. The JAK-dependent tyrosine phosphorylation of STAT6 results in its dimerization and translocation to the nucleus. By virtue of its ability to bind to DNA sequences found in the promoter elements of IL-4 responsive genes, STAT6 participates in the IL-4-induced regulation of gene expression.

A second signaling pathway activated via the IL-4 receptor involves the insulin receptor substrate (IRS) family (IRS-1 and IRS-2). Tyrosine phosphorylated sites within IRS-1 and IRS-2 associate with high

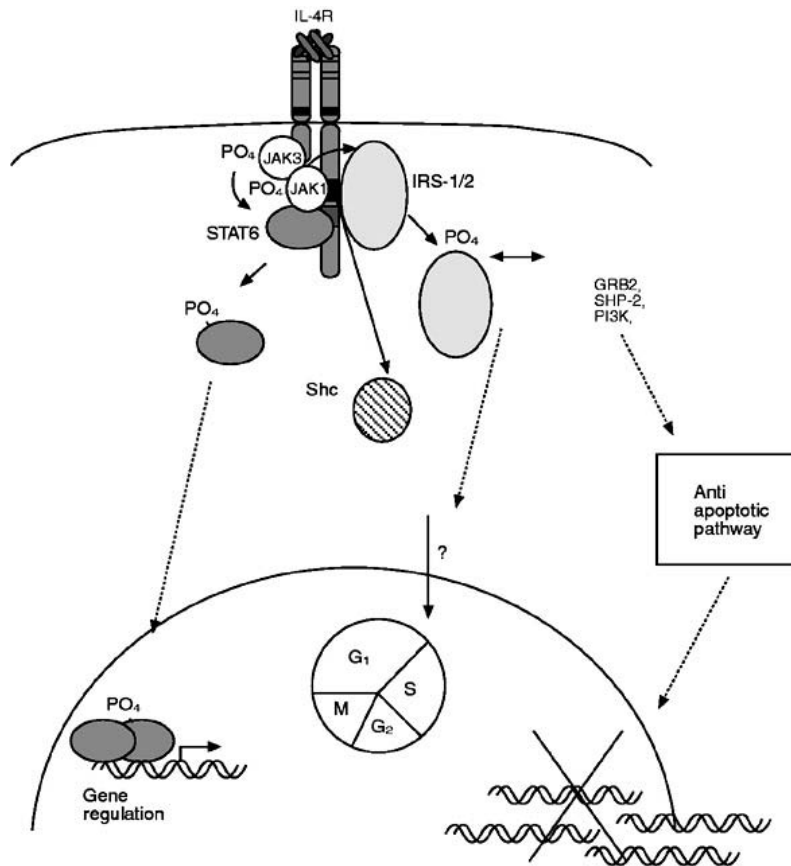


Figure 3 Signaling pathways emerging from the IL-4 receptor complex. The binding of IL-4 to the IL-4R α induces its association with γ c (or with the IL-13R α). The Janus family tyrosine kinases JAK1 and JAK3 bind to the IL-4R α and the γ c cytoplasmic domains, respectively, and become activated when they are brought in close proximity. Activation of the kinases leads to the tyrosine phosphorylation of the IL-4R α and several signaling molecules including STAT6, IRS and Shc. The phosphorylation of STAT6 results in its dimerization and translocation to the nucleus where it can bind to promoter elements and regulate gene expression. The phosphorylation of IRS results in its association with and activation of other signaling molecules. IRS has been shown to be important in regulating cell survival and entry into cell cycle in response to IL-4. The function of phosphorylated Shc is unclear.

affinity to cellular proteins that contain SH2 domains, including the p85 subunit of phosphatidylinositol 3-kinase (PI3K), growth factor receptor-bound protein 2 (GRB2), the SH2 and SH3 domain containing adaptor protein nck, the src-family kinase fyn, and the src-homology protein tyrosine phosphatase 2. This pathway contributes to the IL-4-induced protection from cell death and entry into cell cycle.

The importance of the transforming protein Shc in IL-4 signaling is not yet known. However, IL-4 clearly induces the tyrosine phosphorylation of Shc in several cell types. The precise roles of the STAT6, IRS and Shc pathways (Figure 3) in regulating biological responses transmitted by the IL-4 receptor will require further detailed analyses.

See also: Cytokine receptors, soluble; Cytokine receptors; Interleukin 2 receptor; Interleukin 4; Interleukin 13 and its receptor.

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INTERLEUKIN 5 AND ITS RECEPTOR

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Interleukin 5 (IL-5) controls the production of eosinophils. It has remarkable biological specificity *in vivo*. Overexpression leads to eosinophilia, and gene ablation or treatment with anti-IL-5 antibody abolishes eosinophilia. While IL-5 has activity on B cells in the mouse *in vitro*, the biological role of this activity remains doubtful. Furthermore, human IL-5 has little if any activity on human B cells *in vitro*.

Production of eosinophils *in vivo*

Transgenic mice overproducing IL-5 showed high-level eosinophil production which was proportional to the transgene copy number. These animals have detectable levels of IL-5 in the serum, and show a profound and lifelong eosinophilia, with large numbers of eosinophils in the blood, spleen, bone marrow, lung and gut wall. Interestingly, despite the massive long-lasting eosinophilia, the mice remained apparently normal. This illustrates that increased numbers of eosinophils are not themselves harmful. The tissue damage seen in allergic reactions and other diseases must be due to other factors.

Mice infected with *Nippostrongylus brasiliensis* develop eosinophilia and increased levels of immunoglobulin E (IgE). However, when treated with anti-IL-5 antibody, no eosinophils were observed and the number of eosinophil precursors present in the bone marrow was also depressed. As the anti-IL-5 antibody does not block the effect of IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF), these experiments demonstrate the essential role that IL-5 performs in the control of eosinophilia in such parasite infections. In addition they show that the apparent redundancy seen *in vitro*, where both IL-3 and GM-CSF are also able to induce eosinophil production, does not operate in these infections.

Targeted disruption of the IL-5 gene by homologous recombination results in animals which do not develop eosinophilia when infected with *Mesocostoides corti*. Normal mice infected with *N. brasiliensis* exhibit peripheral eosinophilia, eosinophilic infiltration of the lung and lung damage. The IL-5-deficient mice show no peripheral eosinophilia, a 10-fold decrease in lung cellularity and no lung damage. There was no effect on baseline levels of immunoglobulin isotypes in these mice. These experiments confirm the link between IL-5 and eosinophilia.

To examine the role of eosinophils in lung dam-

age, IL-5-deficient and normal mice were sensitized to ovalbumin and then exposed to ovalbumin aerosol. Normal mice developed massive lung eosinophilia accompanied by considerable lung damage, the IL-5-deficient mice however, showed no increase in eosinophils and no lung damage. When these mice were infected with recombinant vaccinia virus engineered to express IL-5, the eosinophilic infiltration and lung damage was completely restored. In contrast when the same experiment was carried out with mice in which the IL-4 gene had been disrupted and which did not produce IgE, aeroallergen-induced recruitment of eosinophils and lung damage was similar to that seen in the normal mice.

Structure

In its native form IL-5 is a heterogeneous glycoprotein of mean M_r of 45 kDa. The cDNA encodes a protein of 135 residues which includes a leader sequence of 20 residues. Thus the mature polypeptide consists of 115 residues with a calculated mass of 12 000. The active molecule is a homodimer of calculated mass 24 000, indicating that the native molecule is heavily glycosylated. Human IL-5 has N-linked carbohydrate at position Asn28, and O-linked at Thr3, and murine IL-5 is glycosylated at analogous sites, with an additional N-linked oligosaccharide at Asn55. A possible N-linked site at Asn71 is not glycosylated in either species.

The two polypeptide chains form helical bundles each containing three helices (A-C) from one chain and one from the other (D'). The dimeric protein is held together by two disulfide bridges between residues 42 and 84 of opposing chains. Mutation of one of the cysteines prevents dimerization and the resulting monomer is inactive, probably because it is unstable.

Contact points between IL-5 and its receptor chains have been investigated by mutation analysis, constructing species hybrid molecules and the mapping of neutralizing antibodies. These studies indicate that residues in the D helix, Asn108 and Glu110 and in loop 3 (between the C and D helices), Lys84 and Arg91, are important for binding to the receptor α chain. The receptor β chain interacts with Glu13 (of helix A). Despite the dimeric nature of IL-5 suggesting two potential receptor binding sites, only one α chain binds to IL-5, although the number of β chains in the complex is not known.

Gene structure and expression

In humans, the IL-5 gene is located on chromosome 5q31 in a cluster with IL-13, IL-4, IL-3 and GM-CSF. The coding sequence of the IL-5 gene forms four exons. The 5' flanking region of the IL-5 RNA initiation site contains several motifs involved in the transcription of the gene. There is a short sequence named the conserved lymphoid element 0 (CLE0), just upstream of the TATA box. This element is essential for promoter activity. It binds factors of the AP-1 family (*c-fos* and *junB*) together with NF-AT regulatory proteins and has been shown to mediate the response to T cell activation signals generated by phorbol myristate acetate (PMA) in combination with either anti-CD28 or cAMP. The participation of OCT factors and GATA4 proteins at two sites immediately upstream of the CLE0 element are also critical for the regulation of the gene. Two related palindromic elements which resemble the NF- κ B consensus sequence, have been identified. These have counterparts in the mouse gene and appear to be specific for IL-5. Electrophoretic mobility shift assay (EMSA) suggests that similar proteins bind to both elements and an interaction between the elements has an important regulatory effect.

A series of positive and negative regulatory elements involved in the induction of the mIL-5 gene has been identified using the mouse T cell clone D10.G4.1. A sequence located between positions -130 and -176 contains at least two protein-binding sites which participate in the activation of the IL-5 promoter and two negatively acting elements were mapped between positions -261 and -300 and positions -392 and -431. A novel repressor element (mDRE1) consisting of four repeats of an 8 nucleotide element have been identified in the 3' region of the mouse gene. This is active in both the EL4 lymphoma cell line and primary mouse T cells. No obvious human counterpart has yet been identified.

IL-5 has a remarkable translational regulatory mechanism identified by a deletion in the 3' region of the RNA. This deletion results in a 10-fold increase in expression in monkey COS cells and in reticulocyte lysates. Both this region and nucleotides forming a loop structure in the 5' region are necessary for the downregulation of translation. The proteins binding to the RNA are present in reticulocytes, COS cells as well as T cells, and so this may be a general control mechanism in the regulation of many proteins.

IL-5 receptor complex

The receptor complex consists of an α chain and a β_c chain. Both chains have structures characteristic of the hematopoietin receptor superfamily. The α chain is a glycoprotein of 415 amino acids and binds with low affinity to IL-5. The β chain itself does not bind to IL-5, but associates with the α chain to generate the high-affinity receptor. The β chain is common to the receptors for IL-3 and GM-CSF.

Because of the difficulty in obtaining sufficient numbers of eosinophils, progress in characterizing the eosinophil IL-5R has been slow. Murine eosinophils have been calculated to express approximately 50 high-affinity receptors (K_d in the range $4-6 \times 10^{-11}$ M), and approximately 5000 low-affinity receptors (K_d in the range $0.3-2.0 \times 10^{-9}$ M). Initial work on the human receptor used a clone of the human promyelocytic leukemia cell line HL-60.

These cells express only a single population of high-affinity IL-5-binding sites and cross-linking studies show IL-5 associating with two membrane proteins of M_r 60 000 and 13 000. Similarly, only a single high-affinity receptor population has been identified on human eosinophils. This was found to be a high-affinity receptor in the range K_d 170-330 pM ($3-6 \times 10^{-11}$ M).

Human eosinophils express three different transcripts from the same IL-5R α locus. In addition to the membrane-anchored receptor, two soluble isoforms can be produced. Intriguingly, one of these soluble variants is the predominant (>90%) transcript detected in mature, circulating eosinophils or in eosinophils obtained from cord blood cultures. This major soluble isoform arises from splicing to a soluble-specific exon, which precedes the exons encoding the transmembrane anchor.

See also: Cytokines; Cytokine receptors; Eosinophils.

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INTERLEUKIN 6

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Interleukin 6 (IL-6) is a multifunctional cytokine produced by various cells. The molecular cloning of the cDNAs encoding B cell stimulatory factor 2 (BSF-2), interferon β 2, and 26 kDa protein showed that all these molecules are identical. Furthermore, hybridoma/plasmacytoma growth factor (HPGF) and hepatocyte-stimulating factor (HSF) were also found to be identical to this molecule and, therefore, this molecule has been called IL-6. Subsequent studies demonstrated that IL-6 acts not only on B cells but also on hematopoietic stem cells and hepatocytes and induces hematopoiesis as well as acute phase reactions. As summarized in **Figure 1**, it was also shown to act on T cells, nerve cells, keratinocytes, renal mesangial cells, megakaryocytes and myeloma/plasmacytoma cells. Since antibody production, hematopoiesis, and acute phase reactions are three major responses against infection, inflammation and tissue injury, IL-6 may have a central role in host defense mechanisms. On the other hand, the deregulation of IL-6 gene expression was shown to be involved in the pathogenesis of polyclonal and monoclonal B cell abnormalities, such as rheumatoid arthritis and multiple myeloma.

Structure of IL-6

The characterization of the IL-6 molecule is summarized in **Table 1**. Only granulocyte colony-stimulating factor (G-CSF) showed a significant homology with IL-6, especially as the position of four cysteine residues of both proteins matches completely. However, these cysteine residues have no effect on the biological activities of IL-6. Furthermore, the organization of the IL-6 gene shows a distinct similarity with G-CSF. Both genes have the same number of exons and introns and their exon sizes are strikingly similar. Taken together, these findings suggest that both genes might be derived from a common ancestor gene.

Functions of IL-6

Immune response

Interleukin 6 was originally identified as a T cell-derived lymphokine that induces final maturation of B cells into antibody-producing cells. Recombinant human IL-6 acts on B cells activated with *Staphylococcus aureus* Cowan I or pokeweed mitogen (PWM) to induce immunoglobulin M (IgM), IgG and IgA production, but not on resting B cells. Anti-IL-6 antibody was found to inhibit PWM-induced Ig production, indicating that IL-6 is one of the essential factors in PWM-induced Ig production. Furthermore, IL-6 was shown to augment the primary and secondary anti-SRBC (sheep red blood cell) antibody production in mice *in vivo*. Also IL-6 could enhance IgA synthesis in murine Peyer's patch B cells which were already committed to IgA production. Murine IL-6 was also shown to act on murine B cells activated with anti-Ig or dextran sulfate; IL-6 and IL-1 synergistically stimulate the growth and differentiation of these murine B cells. Interleukin 6 could also induce the growth and differentiation of T cells and promoted the growth of mitogen-stimulated thymocytes and peripheral T cells. It was also shown to induce the differentiation of cytotoxic T cells in the

Table 1 Characteristics of IL-6

	Mouse	Human
Apparent mol. wt (kDa)	22–29	21
Number of amino acids		
Total	211	212
Mature protein	187	184
N-glycosylation sites	0	2
Sequence homology		G-CSF
Genomic structure		5 exons
Chromosomal location	Ch 5	Ch 7

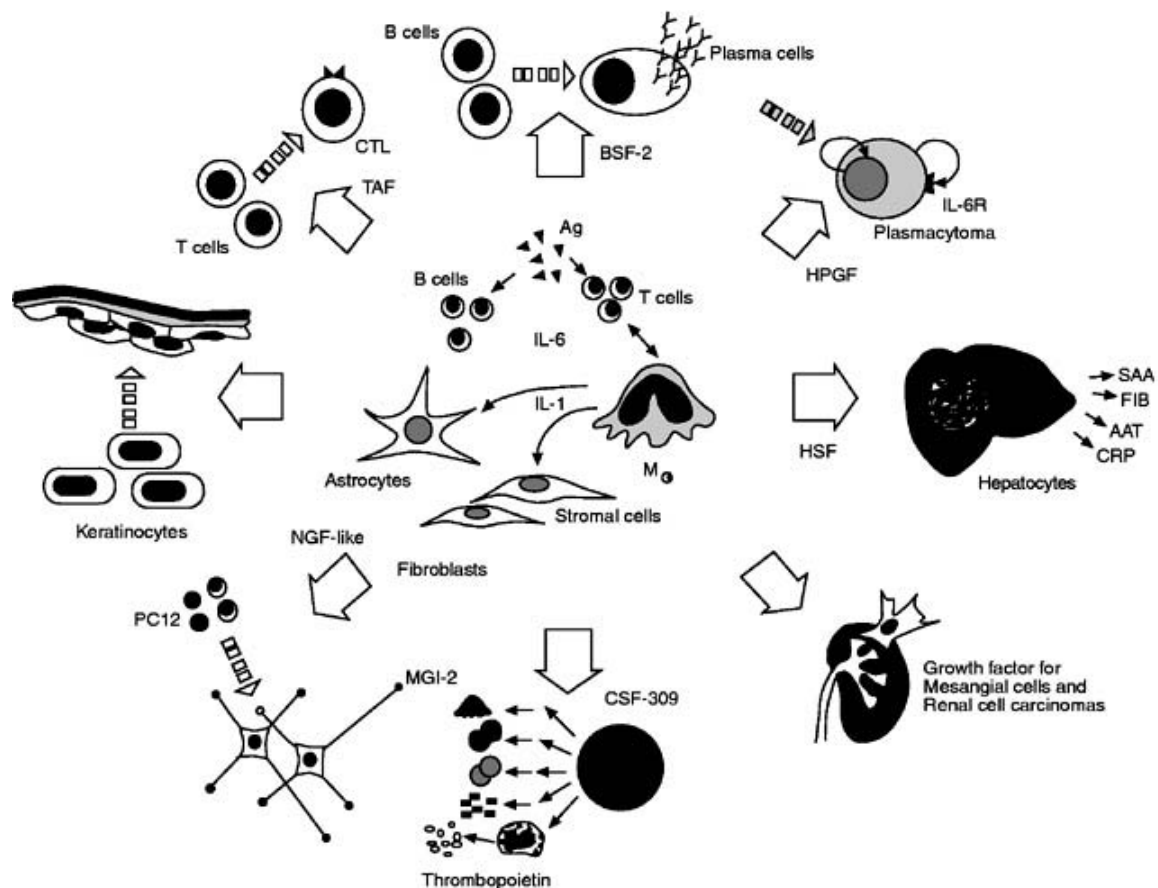


Figure 1 Schematic representation of pleiotropic functions of IL-6. BSF-2, B cell stimulatory factor 2; HPGF, hybridoma/plasmacytoma growth factor; HSF, hepatocyte-stimulating factor; SAA, serum amyloid A; FIB, fibrinogen; AAT, α_1 -antitrypsin; CRP, C reactive protein; CSF-309, colony-stimulating factor 309; MGI-2, macrophage/granulocyte differentiation-inducing protein 2; PC12, rat pheochromocytoma cell line; NGF, nerve growth factor; TAF, T cell-activating factor.

presence of IL-2 from murine as well as human thymocytes and splenic T cells.

Hematopoiesis

By shortening the G_0 period of the stem cells, IL-6 and IL-3 synergistically induce the colony formation of multipotent hematopoietic progenitors. This synergic effect of IL-3 and IL-6 was also observed in serum-free culture conditions, suggesting that IL-6 may enhance the sensitivity of multipotent stem cells to IL-3. When bone marrow cells were transplanted to lethally irradiated recipients, the survival rate at day 30 was only 20%. However, when these cells were precultured with IL-6 and IL-3 before transplantation, the survival rate was raised to 90%.

Furthermore, IL-6 induced the maturation of megakaryocytes *in vitro* and *in vivo*. IL-6 promoted marked increments in size and acetylcholinesterase activity, a marker enzyme of this lineage. IL-6 also induced a significant shift toward higher ploidy classes.

Acute phase response

The acute phase response is a systemic reaction against inflammation, infection, or tissue injury, which is characterized by leukocytosis, fever, increased vascular permeability, alteration in plasma metal and steroid concentration, along with increased levels of acute phase proteins. The production of acute phase proteins by hepatocytes is regulated by several soluble factors, such as IL-1, tumor necrosis factor (TNF) and HSF. Among these factors, only HSF could induce the full acute phase proteins. It was demonstrated that recombinant IL-6 can function as HSF. It can induce various acute phase proteins, such as fibrinogen, α_1 -antichymotrypsin, α_1 -acid glycoprotein, and haptoglobin, in a human hepatoma cell line. In addition, IL-6 induces serum amyloid A, C reactive protein, and α_1 -antitrypsin in human primary hepatocytes. In the rat, IL-6 induces fibrinogen, cysteine proteinase inhibitor, and α_2 -macroglobulin. Serum albumin was negatively regulated by IL-6.

Nerve system

Interleukin 6 mRNA was induced by IL-1-stimulated glioblastoma cells or astrocytoma cells, suggesting that IL-6 may have certain effects on neural cells. The rat pheochromocytoma cell line PC12 is a typical neural differentiation model. Nerve growth factor (NGF) induces chemical, ultrastructural, and morphological changes in the PC12 cell. Also IL-6 was found to induce the typical differentiation of this cell into neural cells and found to be produced by virus-infected microglial cells and astrocytes. It also induced the secretion of NGF by astrocytes. In CNS IL-6 production may be involved in repair mechanisms in the course of viral infection.

IL-6 and disease

The involvement of IL-6 in disease was first suggested in cardiac myxoma. The patients frequently show symptoms related to polyclonal plasmacytosis, such as hypergammaglobulinemia and the presence of various autoantibodies and an increase in acute phase proteins. These symptoms disappear upon the resection of the tumor, suggesting that myxoma-derived factors may induce these phenomena. It was found that myxoma cells express a large amount of IL-6. Abnormal IL-6 production was also observed in patients with Castleman's disease. In these patients, activated B cells in the germinal centers of hyperplastic lymph nodes were found to produce IL-6. After the resection of these lymph nodes, clinical improvement and a decrease in serum IL-6 levels were observed. This evidence suggests that deregulated IL-6 production may induce polyclonal B cell activation and increases in acute phase proteins. This possibility was also suggested in rheumatoid arthritis (RA). High levels of IL-6 were detected in synovial fluid from the joints of patients with active RA. Interleukin 6 is a potent growth factor for murine hybridomas/plasmacytomas, suggesting a possible involvement of IL-6 in the generation of plasmacytomas/myelomas. Furthermore, a study with human myeloma cells isolated from patients with multiple myelomas demonstrated that IL-6 is an autocrine growth factor for human myeloma cells. All the evidence suggests that deregulated gene expression of IL-6 may be involved in the polyclonal B cell activation and generation of plasma cell neoplasia.

Mesangial proliferative glomerulonephritis (PGN) is histologically characterized by proliferation of mesangial cells (MC), suggesting the involvement of the growth factor for MC in the pathogenesis of this disease. It was shown that IL-6 is an autocrine growth factor for MC. It could be detected in urine

Table 2 Diseases related to deregulated expression of IL-6

<i>Polyclonal B cell activation or autoimmune diseases</i>
Cardiac myxoma
Rheumatoid arthritis
Castleman's disease
AIDS
Uterine cervical carcinoma
Alcoholic liver cirrhosis
<i>Neoplasias</i>
Plasmacytoma and myeloma
Lennert's T cell lymphoma
Renal cell carcinoma
<i>Others</i>
Mesangial proliferative glomerulonephritis

samples from patients with PGN. Furthermore, a close relationship was observed between the level of urine IL-6 and the progression of PGN. These results suggest that deregulated IL-6 production in MC is involved in the pathogenesis of PGN. Other diseases related to the deregulated expression of IL-6 are summarized in Table 2.

IL-6 transgenic mice

Transgenic mice with the human IL-6 gene conjugated with the immunoglobulin enhancer have been produced. The E μ -IL-6 gene was expected to express IL-6 constitutively in B lineage cells. In all founder mice, a marked increase in serum IgG1 and enlargement of spleen, thymus and lymph nodes were observed. All of the mice died within several months and the histology of lymph nodes showed a massive infiltration of plasma cells. However, Southern blot analysis of DNAs from lymph nodes or spleen did not show any monoclonal or oligoclonal rearrangements of the Ig heavy chain genes. Furthermore, plasma cells were not transplantable to syngeneic recipients. The other changes consistently observed in E μ -IL-6 transgenic mice were an increase in mature megakaryocytes in bone marrow and mesangial proliferative glomerulonephritis.

In IL-6 knockout mice approximately 80–90% reductions of antiviral antibody response and acute phase reaction have been observed, confirming that IL-6 is one of the essential factors for antibody production and the acute phase reaction.

See also: Acute phase proteins; B lymphocyte differentiation; Bone marrow and hematopoiesis; Cytokine assays; Cytokine genes, regulation of; Cytokines; Cytokine receptors; Granulocyte colony stimulating factor (G-CSF); Interleukin 3; Interleukin 6 receptor; T lymphocyte differentiation.

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INTERLEUKIN 6 RECEPTOR

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The specific receptor for interleukin 6 (IL-6) was found to be expressed on lymphoid as well as non-lymphoid cells in accordance with the multifunctional properties of IL-6. Among human cell lines, IL-6 receptor is expressed on Epstein-Barr virus-transformed B cell lines (CESS, SKW-CL4), a histiocytic leukemia cell line (U937), a promyelocytic leukemia cell line (HL-60), myeloma cell lines (U266, ARH77), hepatoma cell lines (HepG2, Hep3B), and a glioblastoma cell line (SK-MG-4). In normal tissues, IL-6 receptor is expressed on hepatocytes and monocytes, not on resting B cells but on activated large B cells with IgD negative phenotype, and on CD4⁺/CD8[−] as well as CD4[−]/CD8⁺ T cells (in the case of T cells, no significant change after phytohemagglutinin (PHA) stimulation). The number of receptors on each cell is relatively small; i.e. 100–20 000. Scatchard plot analysis demonstrated the presence of high- and low-affinity IL-6 binding sites – $K_d = 10^{-11}$ M and 10^{-9} M respectively. The number of high-affinity sites is usually 5–10% of the total binding sites.

Complementary DNA encoding human IL-6 receptor capable of expressing both high- and low-affinity binding sites has been cloned. Human IL-6 receptor consists of 468 amino acids (a.a.), ~19 a.a. signal peptide, ~339 a.a. extracellular portion, ~28 a.a. transmembrane domain, ~82 a.a. intracytoplasmic portion. There are six potential N-linked glycosylation sites (Asn-X-Ser/Thr) and 11 cysteine residues. The mature receptor has O- and N-glycosylation and its molecular weight is about 80 kDa.

Inspection of the IL-6 receptor sequence shows that the region between position ~20 and ~110 fulfills the criteria for the constant 2 (C2) set of the immunoglobulin (Ig) superfamily. The extracellular

region following the C2 set domain has homology with the extracellular portion of IL-2 receptor β chain, IL-3 receptor, IL-4 receptor, IL-7 receptor, growth hormone receptor, and erythropoietin receptor (a cytokine receptor family having four cysteines and the motif Trp-Ser-X-Trp-Ser). The intracytoplasmic portion of IL-6 receptor is short and has no tyrosine kinase domain, unlike certain other growth factor receptors, and shows no homology with other known functional proteins. Thus it indicates that transduction of the IL-6 signal could be mediated through another molecule associated with the IL-6 receptor.

The mouse IL-6 receptor has 460 amino acid residues and has homology to the human receptor over its whole sequence.

When the IL-6 receptor was precipitated with monoclonal anti-IL-6 receptor antibody under mild lysis (1% digitonin), only the 80 kDa IL-6 receptor molecule was observed. Another polypeptide chain of 130 kDa was coprecipitated with IL-6 receptor, when the cells were incubated with IL-6 at 37°C for 30 min before digitonin lysis. This observation suggested that IL-6 triggered the association of the 80 kDa IL-6 receptor with a 130 kDa molecule. This 130 kDa molecule was a membrane-anchored glycoprotein (gp130), since it was labeled when labeling the cell surface and its molecular weight was decreased by glycanase treatment.

The murine gp130 homolog could be associated with the human IL-6 receptor by IL-6 stimulation, using a mouse transfectant with human IL-6 receptor cDNA. gp130 alone cannot bind IL-6 if the parental cell line has no IL-6-binding sites.

The murine myeloid cell line M1 transfected with either normal human IL-6 receptor cDNA or deletion

mutant IL-6 receptor cDNA lacking the intracytoplasmic portion was growth inhibited and induced to differentiate to macrophages by IL-6 stimulation. This result indicates that the intracytoplasmic portion of IL-6 receptor is not required for the signal transduction.

Soluble IL-6 receptor lacking the transmembrane and cytoplasmic domains could associate with gp130 in the presence of IL-6 and provide the IL-6 signal.

These results show that the IL-6 receptor system is composed of two functional chains, the ligand-binding 80 kDa IL-6 receptor and the nonligand binding gp130. Interleukin 6 triggers the association of IL-6 receptor and gp130 on the cell membrane and gp130 provides the IL-6 signal. gp130 functions as a signal transducer not only for IL-6 but also for various cytokines belonging to the IL-6 family, such as LIF, CNTF, oncostatin M, IL-11 and cardiotrophin. Homodimerization of gp130 or heterodimerization of gp130 and LIF receptor activates the JAK-STAT3 signaling pathway.

See also: Cytokine receptors; Immunoglobulin gene superfamily; Interleukin 6.

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INTERLEUKIN 7 AND ITS RECEPTOR

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Millions of lymphocytes are produced each day and recruited to the periphery. In human and mouse, the major sites of their production are bone marrow and thymus for B and T lymphocytes, respectively. Although *in vitro* propagation of T cell progenitors and human B cell progenitors is yet to be attained, it has been possible to maintain the growth of murine B cell progenitors in culture since Whitlock and Witte developed culture conditions modified from those of Dexter's long-term bone marrow culture. Whitlock–Witte type long-term culture consists of two components: a hematopoietic cell lineage including B cell progenitors and adherent stromal cells which support their growth. Cloned stromal cell lines were established which were able to support the proliferation of single purified B cell progenitors.

Interleukin 7 (IL-7) was identified and molecularly cloned from one such stromal cell line as a soluble factor able to stimulate the proliferation of stromal cell-dependent pre-B cells. Subsequently, cDNA encoding the receptor for IL-7, which is now known as IL-7R α , was cloned from both human and mouse. Recent evidence demonstrates that IL-7 is an essential molecule for the production of murine B and T cells. Although its role in human B lymphopoiesis is still unclear, it would appear to play a role in T cell production in the human thymus.

Structure of IL-7 and the IL-7 receptor

The cDNA of IL-7 (initially designated lymphopoietin 1) was first cloned from the murine stromal

cell line IXN/A5. Subsequently, human IL-7 cDNA was cloned from the human hepatoma cell line SK-HEP-1. Murine IL-7 consists of 154 amino acid (aa) residues including a 25 aa signal peptide, whereas human IL-7 is 19 aa longer. This difference in size is due to the absence of an exon in the mouse gene, which corresponds to the 5th exon of the human IL-7 gene. Because of this deletion, murine IL-7 has two N-linked glycosylation sites in contrast to three in human IL-7. However, as deletion of these 19 aa from the human IL-7 molecule has no effect on its biological activity, this region is not essential. Except for this 19 aa region, human and mouse IL-7 are homologous and their overall homology in nucleotide sequence is 81% in the coding region. Both contain three intramolecular disulfide bridges. Although no significant sequence homology has been found with other cytokines, a structural model predicted from the amino acid sequence suggests that its tertiary structure is similar to that of IL-2, IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in that it has four α helices connected by long crossing loops.

Human IL-7R α was cDNA cloned from a lung cancer cell line which bound recombinant IL-7, and the murine homolog was cloned from a pre-B leukemia cell line by using human IL-7 cDNA. Both encode 439 aa peptides and consist of extracellular, transmembrane and cytoplasmic regions. The presence of two fibronectin type III domains and a WSXWS motif indicates that this is a member of the cytokine receptor family. Although no domains with apparent enzymatic activity have been found in the cytoplasmic region, it shares a number of common motifs such as a box, acidic and serine-rich regions with other cytokine receptor family molecules. Evidence indicates that these regions play an important role for signaling through association with various other molecules. The homology between human and mouse IL-7R α is about 60% at the amino acid sequence level, and human recombinant IL-7 is able to bind and stimulate the murine IL-7R α .

While IL-7R α alone can bind recombinant IL-7, it functions only in association with another cytokine receptor molecule, IL-2R γ (γ c). Indeed, a monoclonal antibody against γ c inhibited, though not completely, IL-7 binding to the receptor complex, suggesting a close association of the two molecules upon IL-7 stimulation.

Tissue expression of IL-7 and IL-7R α

Varying levels of IL-7 mRNA expression are detected in spleen, thymus, kidney, gut epithelium and liver. Unexpectedly, its expression level in the bone mar-

row is low. As no *in situ* hybridization result is available in these tissues, its expression at the cellular level is still unclear. *In vitro* IL-7 expression in stromal cell lines correlates well with their ability to support B lymphopoiesis. Addition of recombinant IL-7 restores the B lymphopoiesis supporting ability of a stromal cell line that does not express IL-7.

The stage-specific expression of the receptor during both B and T cell differentiation has been characterized in the mouse using an IL-7R α -specific monoclonal antibody (mAb) (Figure 1). During B cell differentiation, IL-7R α is expressed through pro-B and pre-B cell stages until surface μ chain is produced. Exact timing of its expression is not clear, but evidence suggests that the earliest B220⁺ progenitors do not express it, indicating that its expression may take place after commitment to the B cell lineage. IL-7R α expression has never been detected in mature surface μ ⁺ B cells in bone marrow nor in peripheral lymphoid organs. On the other hand, it is expressed in both mature and immature T cells. In thymus, IL-7R α expression is found in CD4⁺CD8⁻ (double negative), CD4⁺CD8⁺, CD4⁻CD8⁻, but not in CD4⁺CD8⁺ (double positive) populations. Among double negative immature T cells, IL-7R α expression is believed to correlate well with *c-kit* expression in the CD44⁺CD25⁻ population. Indeed, in the fetal thymus containing only nascent T lineage cells, most of them are IL-7R α ⁺ and γ c⁺. Although IL-7R α expression has also been detected in human T and B lymphocytes, it remains to be determined if the pattern of expression, particularly during early differentiation, is the same as that in the mouse.

Function and signal transduction

In complete agreement with the expression pattern of IL-7R α , blocking of IL-7 or IL-7R α functions results in defective production of lymphocytes but the animals otherwise remain normal. In IL-7 or IL-7R α null mutant mice, B cell genesis is blocked at the pro-B and pre-B stage, though some leakage into mature B cells does exist. Interestingly, the pro-B cell stage is reduced more in IL-7R α (-) than IL-7(-) mice, suggesting the presence of another molecule that can substitute IL-7 at the pro-B stage. Identification of this molecule in a thymic stromal cell clone was recently reported. Likewise, thymic cellularity is markedly reduced in IL-7 or IL-7R α null mutant mice. In the thymus of IL-7R α null mice, CD44⁺CD25⁻ double negative cells increase, while later stages including CD44⁺CD25⁺ double negative cells are reduced, suggesting a requirement for IL-7 signaling during the shift from double negative to double positive T cell populations. In contrast, the

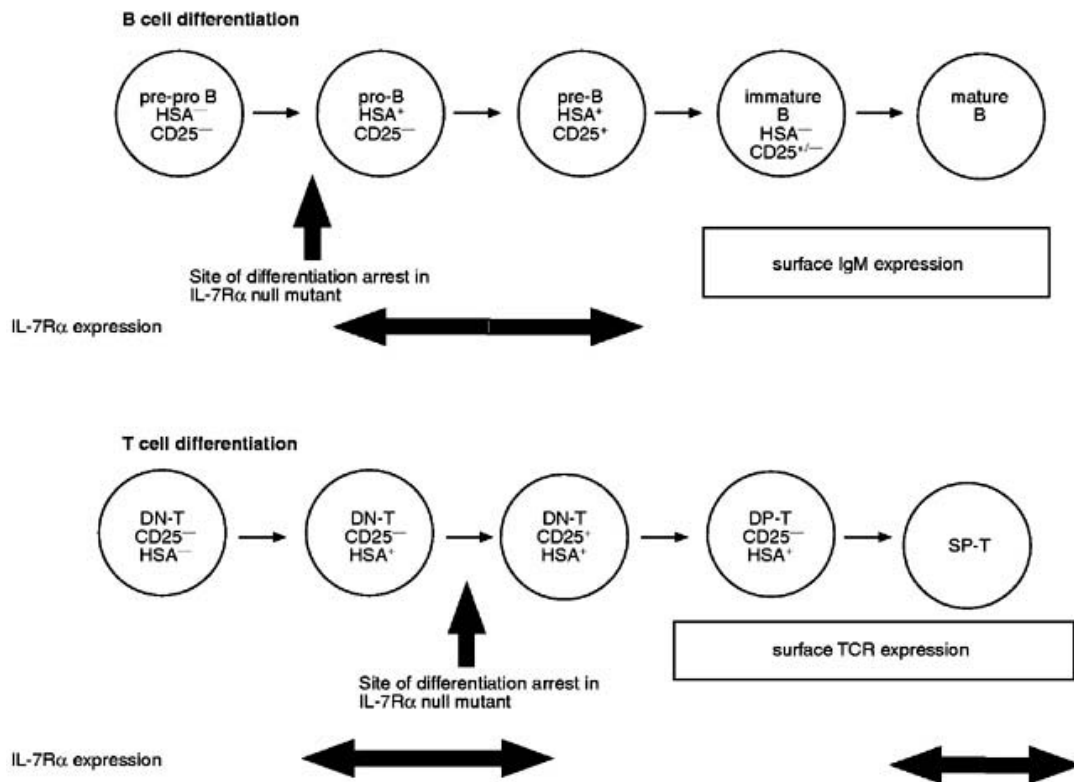


Figure 1 IL-7Rα expression during lymphocyte differentiation in mouse. HSA and CD25 expression of each stage of B and T cell differentiation is indicated. DN, DP and SP represent double negative, double positive and single positive in terms of CD4 and CD8 expression.

proportion of each T cell subset remains relatively unaffected in the IL-7 null mouse, though thymic cellularity is only 5% of normal. This discrepancy in the phenotype of IL-7 and IL-7Rα null mutant mice again suggests the presence of another molecule bound to IL-7Rα, which is now known as TSLP (thymic stromal-derived lymphopoietin). The only lymphoid cell lineage that is not affected in these mice is natural killer (NK) cells.

Although IL-7 can induce the proliferation of human T and B cells *in vitro*, its *in vivo* role in humans is not clear. Two distinct severe combined immunodeficiency (SCID) mutations that have relevance to IL-7 signaling have been reported; one is a mutation in the γ_c gene and the other a mutation in the JAK3 gene. As mentioned previously, an association of IL-7Rα with γ_c is essential for the IL-7 signal. Moreover, as summarized in Figure 2, signal transduction by the IL-7Rα/ γ_c complex requires JAK3 tyrosine kinase. Hence, it is conceivable that a null mutation in either of these genes resulted in defective lymphocyte production. Indeed, mice bearing null mutations of either JAK3 or γ_c genes have severe defects in lymphocyte production. NK cells are also deficient in the γ_c null mouse. As γ_c is also involved in the IL-2, IL-4, IL-9, IL-13, and IL-15

receptors, the difference between the IL-7Rα and γ_c null mice indicates the involvement of one or more of these signals in NK cell production. However, in human, complete loss of JAK3 or γ_c causes a phenotype in which T but not B cell lineages are absent. This indirect evidence indicates that the role of IL-7 signaling in human B lymphopoiesis is redundant.

What is the role of the IL-7 signal in lymphocyte differentiation? Studies on normal pro-B cells indicate that it controls cell cycle progression, particularly entry and maintenance of the S phase. Moreover, evidence suggests that it is involved in stage-specific gene expression such as the VDJ recombinase machinery. As multiple molecules are activated upon stimulation by IL-7, it is conceivable that the IL-7 signal may have multiple functions. Molecules directly associated with the IL-7Rα/ γ_c complex include JAK1, JAK3 and fyn. The phenotype of the JAK3 null mutant mouse strongly suggests that the JAK3 pathway is essential for the induction of cell proliferation. In fact, the activation of JAK3 upon IL-7 stimulation subsequently activates STAT1, STAT5 and phosphatidylinositol 3-kinase (PI3K) through tyrosine phosphorylation. Evidence indicates that PI3K is essential for cell proliferation. However, if and how STAT1 and 5 are involved in

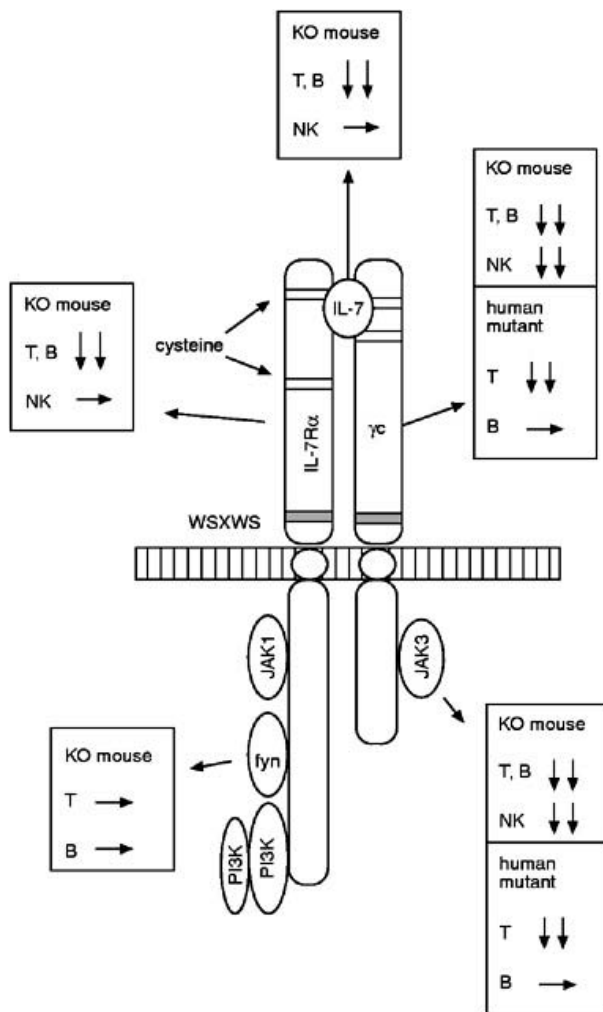


Figure 2 IL-7 signal transduction pathway. Molecules involved in IL-7 signal are depicted schematically. With respect to genes for which mice or human that bear null mutation are available, the phenotypes are given in the corresponding box.

cell proliferation is as yet unclear. Recent studies to reconstitute the IL-7R α signal of IL-7R α (-) pro-B cells by transfecting the receptor genes bearing various mutations indicates that STAT recruitment is not sufficient for the induction of pro-B to pre-B differentiation.

Future questions

While the role of IL-7 in the production of both T and B cells in mouse, particularly in their progenitor stage, is now well established and the biochemical analysis of its signal pathway is in progress, two major questions remain for future study. One is the role of IL-7 in human B lymphopoiesis. If the signal transduction pathways of human IL-7 use the same intracellular machinery as in mouse, the fact that B cell genesis continues in γc or JAK3-deficient patients

suggests the IL-7 signal is not required for the generation of human B cells. This, in turn, raises the question of which molecule(s) are required in humans.

The other major question is the role of IL-7 signaling in mature T cells. Because half of the T cells in peripheral lymphoid organs express IL-7R α , it is reasonable to assume that IL-7 may be involved in the stimulation of mature T cells. Because of the severe lymphopenia in the IL-7 or IL-7R α null mutant mice, however, this question has not yet been addressed in an appropriate way.

See also: B lymphocyte differentiation; B lymphocytes; Bone marrow and hematopoiesis; Colony stimulating factors; Cytokines; Cytokine receptors; Immunodeficiency, animal models; Immunodeficiency, primary; Interleukin 2; Proliferation, lymphocyte; Stromal cells; T lymphocyte differentiation.

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INTERLEUKIN 8 AND ITS RECEPTOR

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Partially purified interleukin 1 (IL-1) and tumor necrosis factor (TNF) were claimed to be directly chemotactic for neutrophils in the early 1980s. However, recombinant IL-1 and TNF lacked leukocyte chemotactic activities *in vitro* although they induce leukocyte infiltration upon intracutaneous injection. This led to the discovery of a neutrophil chemotactic factor distinct from IL-1 and TNF. The cytokine was purified from lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMC)-conditioned media and was initially termed monocyte-derived neutrophil chemotactic factor (MDNCF). The cDNA cloning of MDNCF revealed that it was identical to 3-10C, which was reported by Schmidt and Weissmann as a gene inducible in enterotoxin-stimulated human PBMC. Subsequently, a biochemically identical cytokine was reported by several groups and termed granulocyte chemotactic peptide (GCP), neutrophil-activating factor (NAF), and neutrophil-activating peptide 1 (NAP-1). Moreover, Larsen and colleagues detected a T lymphocyte chemotactic factor present in phytohemagglutinin (PHA)-stimulated human PBMC-conditioned media that was identical to MDNCF. Since MDNCF has multiple target cells and is produced by various types of cells, it was renamed interleukin 8 (IL-8).

Biochemical properties

Interleukin 8 is synthesized as a precursor (99 amino acids) and processed to a mature secreted form (72 amino acids). In addition to a truncated form (69 amino acids) which is as potent as the mature IL-8, intermediately processed forms (79 and 77 amino acids) with weaker activities have been purified from culture supernatants. The mature IL-8 is not glycosylated and contains four cysteine residues. Due to the existence of many basic amino acids (lysines and arginines), the isoelectric point of mature IL-8 is

>8.5. The sequence Lys–Phe–Leu–Lys–Arg at the C-terminal region is the most probable heparin-binding site. Two intermolecular disulfide bonds are formed between Cys7–Cys34 and Cys9–Cys50. A triple-stranded β sheet is formed between residues 7 and 54, while an α helix is formed in the C-terminal region. The sequence of Glu4–Leu5–Arg6 and the residues near the turn at His33 are presumed to be important for binding to the receptor. At high concentrations, IL-8 dimerizes through formation of hydrogen bonds between the first β strand (residues 23–29) in each molecule. However, a mutant IL-8 protein, which cannot dimerize, still possesses neutrophil chemotactic activity, suggesting that dimerization is dispensable for biological activities.

The IL-8 gene family and related molecules

Since the discovery of IL-8, more than 20 similar cytokines have been identified (Figure 1). These cytokines contain four cysteine residues at the well-conserved positions with a molecular mass of around 8 kDa and possess the ability to attract and activate various types of leukocytes. Hence, the term 'chemokines' was proposed for this novel class of cytokines.

Chemokines can be subdivided into two subclasses, depending on whether the first pair of cysteine residues are separated by one amino acid (CXC) or adjacent (CC). The genes for CXC and CC chemokines reside on human chromosome 4q12–13 and 17q11–12, respectively. CC chemokine, platelet factor 4 and platelet basic protein genes consist of three exons and two introns, whereas the other CXC chemokine genes have an additional exon and intron. However, the similarity of the splice junction and of the sizes of the introns and exons suggest that they all stem from a common ancestral gene.

Amino Acid Sequences of Chemokines

CXC Chemokines

IL8 AVLP^{RS}SAKELRCQ^{CI}KTY^SKPF^{HK}FIKEL^{RV}IESGPH^{CA}TEI^{IV}KLSD-GR^{IC}LD^{DP}KEN^{WV}Q^{RV}VEK^{FL}KRA^{ENS}
 GRO α ASVATEL^{RC}Q^{CI}LQ^{TI}-LQGI^{HL}KN^{IQ}SV^{VK}SPG^{PH}CA^QTE^{VI}AT^{LK}N-GR^{KA}CL^{NP}AS^{PI}V^{KK}II^{EK}ML^{NS}DK^{SN}
 GRO β APLATE^{LR}CQ^{CI}LQ^{TI}-LQGI^{HL}KN^{IQ}SV^{VK}SPG^{PH}CA^QTE^{VI}AT^{LK}N-GQ^{KA}CL^{NP}AS^{PM}V^{KK}II^{EK}ML^{KN}GK^{SN}
 GRO γ ASV^{VT}EL^{RC}Q^{CI}LQ^{TI}-LQGI^{HL}KN^{IQ}SV^{VN}SPG^{PH}CA^QTE^{VI}AT^{LK}N-GK^{KA}CL^{NP}AS^{PM}V^{KK}II^{EK}IL^{KN}GS^{TN}
 NAP2 AEL^{RC}MC^{IK}TS^{GI}HP^{KN}IQ^{SV}LE^{VG}IK^{GT}HC^{NQ}VE^{VI}AT^{LK}-GR^{IC}LD^{DP}AD^{PA}IP^{AK}IV^{QK}KL^{AG}ES^{AD}
 AEA-78 AGPAA^{AV}LR^{EL}RC^{VI}LQ^{CI}-TQ^{GH}VP^{HK}MIS^{NL}QV^{FA}IG^{QC}SK^{VE}VV^{AS}L^{KN}-GK^{IC}LD^{PE}AP^{LP}KK^{VI}Q^{IKL}DDG^{GN}KN^{EN}
 PF4 EA^{ED}GD^{GL}Q^{CI}LV^{CK}T^{SQ}VR^{PR}ETS^{LV}EA^{IG}AP^{HC}PT^{AQ}LI^{AT}L^{KN}-GR^{IC}LD^{LP}Q^{LA}PI^{KK}II^{KL}KL^{ES}
 IP-10 VPLSTVR^{CT}IS^{IS}NQ^{VP}NP^{RS}LE^{KI}PA^{SO}Q^{FCR}VE^{IA}IT^{AK}ML^{KK}GK^{ER}CL^{NP}ES^{KA}IK^{NL}LA^{VSK}EM^{SK}SR^{SP}

CC Chemokines

	CC	C	C
MCAF/MCP-1	QPDAINAPVITCCYNFTNRKISVQRLASRYRRTSSKCPKEAVIFKTTVAKELCADPKQKWVQDSMDDELDKQTQTPKT		
MCP2	QPDVSISPTCCNVINKRIPQRLSEYTRITNIQCPKEAVIFKTKRGEVCADPKERWVRDSMKELDQIFQNLKP		
MCP3	QPVGINTSTTCCYRFINKKIPQRLSEYRRTTSSHCPEAVIFKTKLDKELCADPTQKWVQDFMKELDKKTQTPKL		
I-309	SKSMQVPFS-RCFCSFAEQEIPRLAILCY-RTSSICNSGELIFLKGKGEACALDVTGWVQREKRMLRHCPSKRK		
MIP-1α, LD78	ASLAADPTACCFSTSRQIPNFVIADYFE-TSSCSKPGVIFLTKRSRQVCADPSEEWQKYVSDLESLA		
MIPβ, Act-2	APMGSDPPTACCFSYTARKLKNFVVDYYE-TSSSCSPAVVVFQTKRSKQVCADPSESWEQYVYDLELN		
RANTES	SPYSSDT-TPCCFAIYARLPLRAEIKYFY-TSGCKCNPAVVFVTRKNQVCANPEKKWVREYINSLEMS		
human Eotaxin	GPASV-PTTCCFNLRNKIPLQRLSEYRRTISGKCPQKCAVIFKTKLDKELCADPKKKWQDSMKYLDQCSPTPKP		
gp Eotaxin	HPG-I-PSACCFRVTNKISKISFORLSYKIITSSSKCPQTAIVFEIKPKDMICADPKKXXWQDQAKKYLDQISQXTKP		

Figure 1 Comparison of amino acid sequences of chemokines. Amino acid sequences are aligned according to the positions of cysteines.

Production and gene expression of IL-8

Various stimuli, including LPS, proinflammatory cytokines and viruses, induce IL-8 mRNA very rapidly in a variety of cells, including PBMCs, fibroblasts, endothelial cells and keratinocytes. The activation of gene transcription as well as stabilization of its mRNA are responsible for the accumulation of IL-8 mRNA.

IL-8 genomic DNA consists of four exons and three introns. The transcription of IL-8 gene requires, in a cell type-specific manner, the activation of three *cis* elements in the 5'-flanking region of the IL-8 gene: AP-1 (-126 to -120 bp), NF-IL6 (-94 to -81 bp), and NF κ B binding sites (-80 to -70 bp). NF κ B is the most crucial transcription factor and activates IL-8 gene transcription in cooperation with NF-IL6 or AP-1. In addition, several agents such as glucocorticoids, interferon α/β , and FK506, inhibit IL-8 gene transcription by interfering with the activation of NF κ B.

The IL-8 receptor and signaling pathway

Two types of human IL-8 receptor, type A and B, have been identified molecularly. Types A and B consist of 351 and 360 amino acids, respectively (Figure 2). Both belong to a family of G protein-coupled receptors with seven transmembrane domains and possess high sequence homology with each other at the amino acid level, reaching more than 80% homology except for their N-terminal portion. Both genes map to chromosome 2p35 and are accompanied by one pseudogene.

Cells transfected with type A IL-8 receptor cDNA bind only IL-8 with a high affinity, whereas cells transfected with type B bind gro as well as IL-8 with similar high affinity. The difference in their N-terminal portions accounts for the differences in their specificity. Scanning mutagenesis on type A receptor demonstrates that besides the four cysteine residues, Arg199, Arg203 and Asp265 are important for the overall folding of the receptor. Immunofluorescence analysis reveals a selective and coordinate expression of both types of IL-8 receptors (Table 1) although the functional significance of each receptor has not been fully determined yet.

In addition to these specific receptors on leukocytes, Duffy antigen on erythrocytes, which is the entry site of *Plasmodium vivax*, can bind IL-8 as well as several other chemokines including gro, RANTES and MCAF/MCP-1 with a high affinity.

IL-8 activates pertussis toxin-sensitive and receptor-coupled G proteins including $G\alpha_{14}$, $G\alpha_{16}$, $G\alpha_{12}$ and $G\alpha_{i3}$. G proteins, upon conversion to the GTP-bound form, activate phospholipase C β_2 (PLC β_2), RhoA and phosphatidylinositol 3-kinase (PI3K) (Figure 3). Activation of PLC β_2 results in the generation of inositol 1,4,5-triphosphate (IP $_3$). IL-8 also stimulates phosphatidylinositol 4-phosphate kinase (PIP-K), which synthesizes phosphatidylinositol 4,5-bisphosphate (PIP $_2$), the source of IP $_3$. The IP $_3$, in turn, induces the release of Ca $^{2+}$ from intracellular stores, which is sufficient to induce exocytosis and respiratory burst. RhoA, upon conversion to the GTP-bound form, activates a serine-threonine kinase, protein kinase N (PKN) and enhances leukocyte

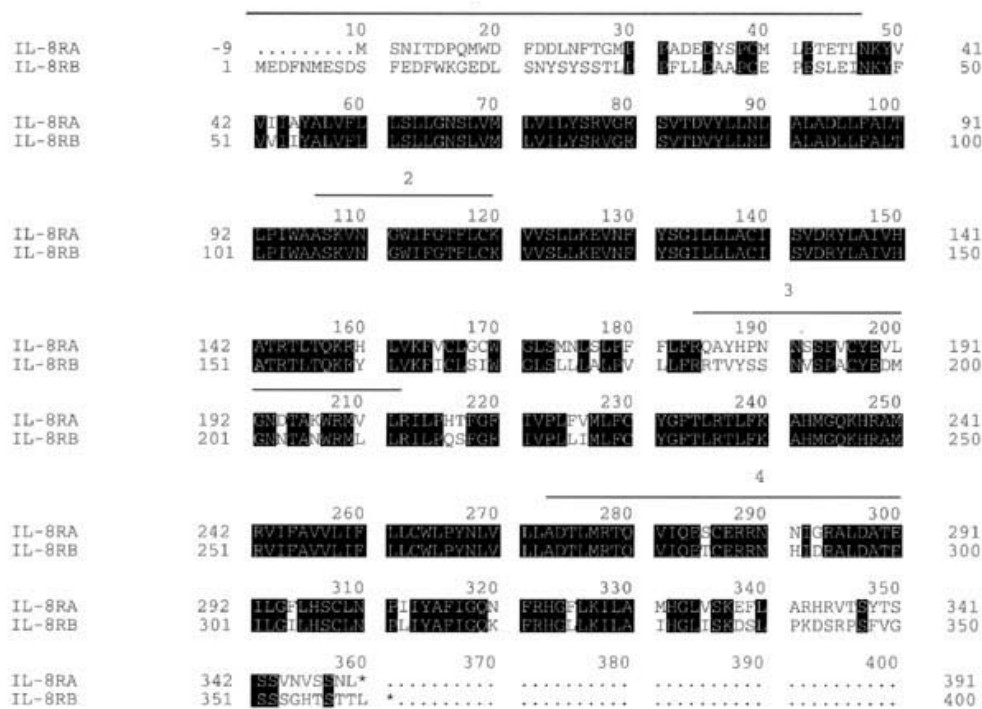


Figure 2 Alignment of the amino acid sequences of type A (upper) and type B (lower) human IL-8 receptors. Extracellular domains are indicated by bars and numbered from the N-terminal end. The positions occupied by conserved sequences are indicated by filled boxes.

Table 1 Distribution of each type of IL-8 receptor on various types of leukocytes as revealed by immunofluorescence analysis

Cell type	Type A	Type B
Neutrophils	++	++
Eosinophils	-	-
CD16 ⁺ lymphocytes	+	+
CD8 ⁺ lymphocytes	+	+
CD4 ⁺ lymphocytes	-	-
CD20 ⁺ lymphocytes	-	-
Monocytes	+	+
CD34 ⁺ progenitor cells	-	-

adherence through integrins. Activation of PI3K eventually activates ras/raf/mitogen-activated protein kinases (MAPK), resulting in granule release and neutrophil adherence. Moreover, IL-8-induced MAPK activation is negatively regulated by RGS (regulator of G protein signaling).

***In vitro* biological activities**

The actions of IL-8 on neutrophils are diverse (Figure 4): chemotaxis, transendothelial migration with concomitant shedding of leukocyte adhesion molecule 1,

induction of lysosomal enzyme release, respiratory burst, shape change and generation of superoxide anions. Moreover, IL-8 induces chemotaxis of T and B lymphocytes, IL-2-activated natural killer cells, basophils and eosinophils if primed with either IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-8 inhibits IL-4-induced immunoglobulin E (IgE) production by normal B lymphocytes and inhibits *in vitro* colony formation from human bone marrow progenitor cells.

IL-8 has profound effects on nonleukocytic cells. IL-8 is haptotactic (induces cell migration to cell surface- or matrix-bound gradient), and promotes adhesion and proliferation of melanoma cells. It inhibits collagen expression in synovial fibroblasts and enhances cytomegalovirus replication in lung fibroblasts. Furthermore, IL-8 induces the migration of human umbilical vein endothelial cells *in vitro* and angiogenesis in rat cornea.

The roles of IL-8 in experimental animal models

Systemic injection of IL-8 induces a transient granulocytopenia followed by granulocytosis in several animals, including humans. Moreover, it also

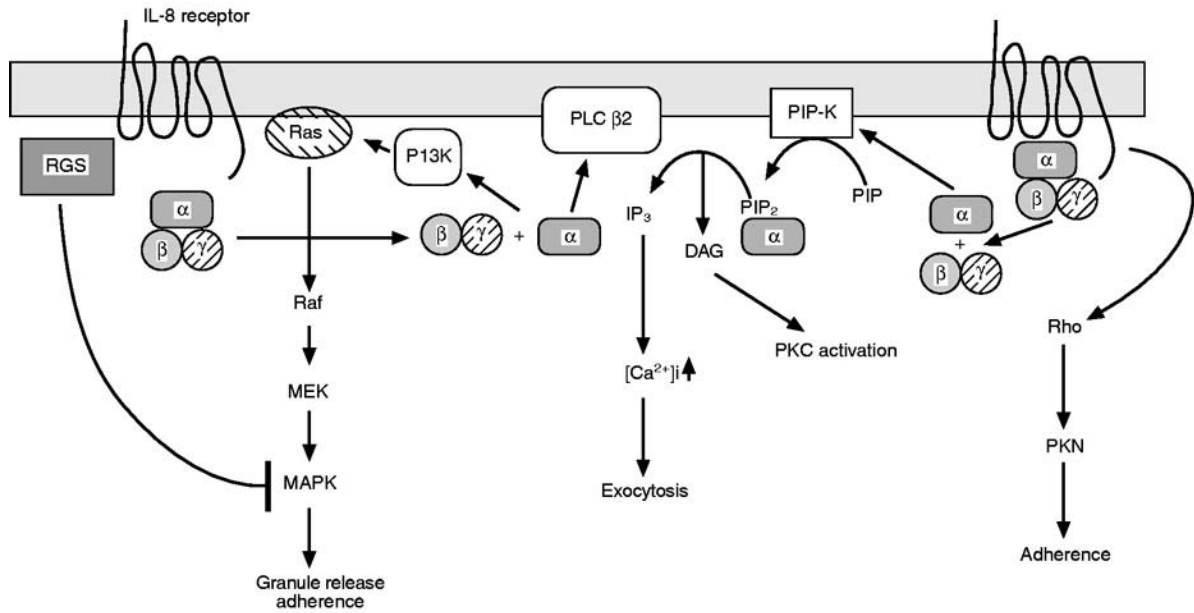


Figure 3 Proposed IL-8 receptor-mediated signaling mechanisms.

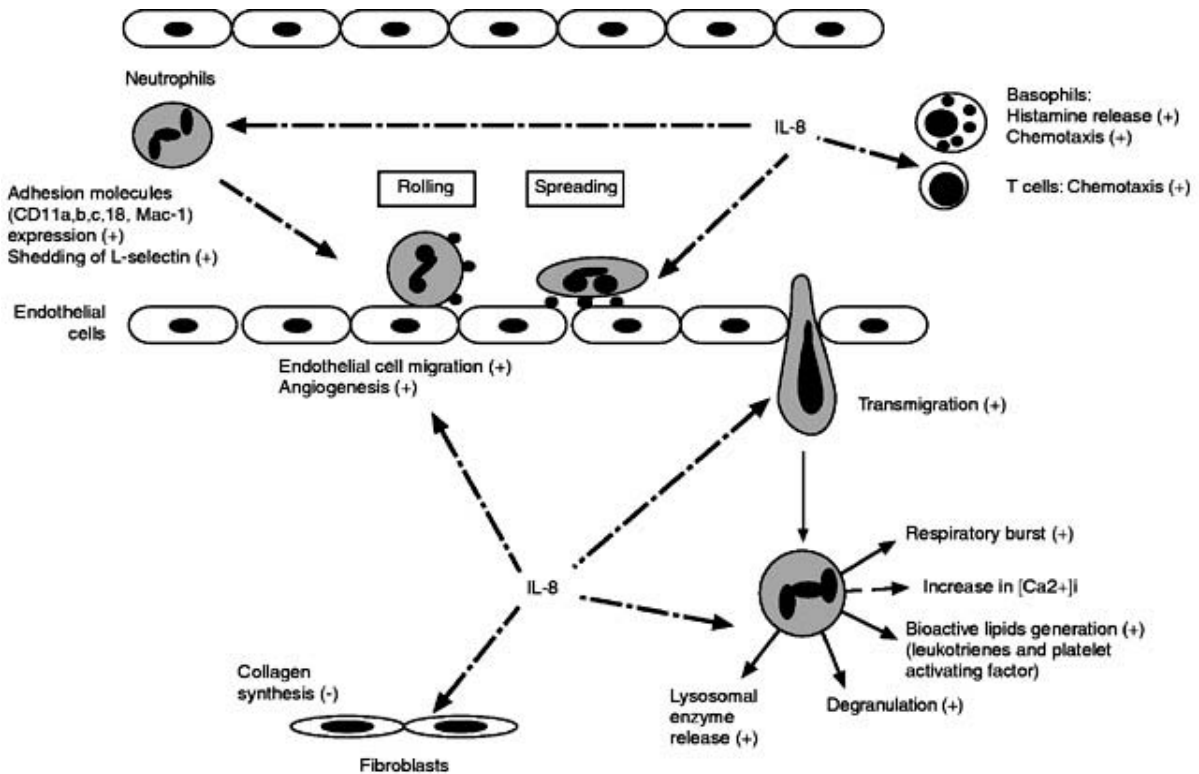


Figure 4 *In vitro* biological activities of IL-8.

causes a rapid (<30 min) mobilization of bone marrow stem cells into the peripheral blood in mice and primates. Intradermal injection of IL-8 induces a rapid and concentration-dependent neutrophil infil-

tration and neutrophil-dependent plasma leakage at the injected sites. In addition to neutrophils, local injection of IL-8 at a lower dose induces lymphocyte migration to the injected sites. In guinea pigs,

intravenous and intraperitoneal injections of IL-8 causes eosinophil accumulation in skin and lung, respectively.

Administration of neutralizing antibodies to IL-8 prevented neutrophil-dependent tissue injuries observed in several experimental models including lung perfusion injury, LPS-induced acute dermatitis, LPS- or IL-1-induced acute arthritis, immune complex-mediated acute glomerulonephritis, immune complex-mediated pneumonia, and aspiration pneumonia. These observations may implicate IL-8 as one of the major mediators of acute inflammation.

IL-8 transgenic mice (with expression of the transgene directed to either the liver or the gastrointestinal tract) exhibit increases in serum IL-8 levels and circulating neutrophil number, and decreases in L-selectin expression on the surface of neutrophils. They show an accumulation of neutrophils in the microcirculation of the lungs, liver and spleen without neutrophil extravasation, plasma exudate and tissue damage. No apparent change is observed on the bone marrow of the transgenic mice compared with the control littermates. However, neutrophil migration into the inflamed peritoneum is severely impaired in the transgenic mice.

Targeted disruption of the murine IL-8 receptor homolog gene (which has a high of homology with both the type A and type B human IL-8 receptors) results in lymphadenopathy due to an increase in B cells. Serum IL-6 level is also increased in IL-8 receptor-deficient mice. There is an increase in bone marrow cellularity composed of the normal myeloid cells in addition to splenomegaly resulting from an increase in myeloid cells in splenic white pulp. Neutrophil migration into the inflamed peritoneum is severely reduced although neutrophils retain their normal ability to kill bacteria. It should be noted that IL-8 receptor deficient mice, if kept in germ-free environments, do not develop increased myelopoiesis, splenomegaly or lymphadenopathy.

Clinical relevance and possible clinical application of IL-8

Increases in IL-8 protein levels in body fluids has been reported in various types of diseases which are accompanied by neutrophilia and/or neutrophil infiltration into lesions (Table 2). Urinary IL-8 levels increase in several types of glomerulonephritis where neutrophil infiltration is observed, and reflect the disease activity of these types of glomerulonephritis. IL-8 levels in bronchoalveolar fluids are reported to predict the complication of the adult respiratory distress syndrome (ARDS) in patients at risk. Thus, the

Table 2 Diseases associated with elevated levels of IL-8 in body fluids

Conditions	Body fluids with elevated IL-8 levels
Endotoxemia	Blood
HIV infection	Blood
Bacterial meningitis	Spinal fluid
Urinary tract infections	Urine
Relapsing fever	Blood
Acute peritonitis in CAPD patients	Peritoneal fluids
Pleural empyema	Pleural fluids
Gouty arthritis	Joint fluids
Rheumatoid arthritis	Joint fluids
Psoriasis	Skin lesions
Contact dermatitis	Skin lesions
ARDS	BALF
Idiopathic pulmonary fibrosis	BALF
Summer type hypersensitivity pneumonitis	BALF
Acute asthmatic attack	Sputum
Acute myocardial infarction	Blood
Rejection after transplantation	Blood, urine
Kawasaki disease	Blood
Inflammatory bowel diseases	Blood
Glomerulonephritis	Urine
<i>Helicobacter pylori</i> -related gastric diseases	Gastric juice
Parturition	Amniotic fluids

ARDS, adult respiratory distress syndrome; BALF, bronchoalveolar lavage fluids; CAPD, continuous ambulatory peritoneal dialysis; HIV, human immunodeficiency virus.

determination of IL-8 levels may be a useful tool to monitor some disease activities.

The ability of IL-8 to rapidly mobilize bone marrow stem cells into the peripheral blood may prove helpful for peripheral blood stem cell transplantation. Since neutralizing antibodies to IL-8 prevent tissue injuries observed in several models of inflammation, the development of antagonists at the receptor level, inhibitors at the postreceptor level, or inhibitors of IL-8 production will be important for the treatment of neutrophil-mediated inflammation.

See also: Acute inflammatory reaction; Chemokines; Chemotaxis; Cytokine assays; Cytokine receptors, soluble; Cytokines; Interleukin 1 and its receptors; Neutrophils; Recruitment; Tumor necrosis factor α .

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INTERLEUKIN 9 AND ITS RECEPTOR

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Interleukin 9 (IL-9) was originally described in the mouse system as a T cell growth factor (TCGF P40) which could support the antigen-independent proliferation of certain murine T helper cell clones. Human IL-9 cDNA, on the other hand, was initially isolated based on its ability to stimulate the proliferation of a human megakaryocytic leukemic cell line. Subsequent studies have indicated that IL-9 has a broad spectrum of biological activities within the hematopoietic, lymphopoietic and neuronal systems. The sharing of the common signal transducer among IL-9, -2, -4, -7, and -15 receptors has provided, at least in part, the explanation for the biological redundancy and pleiotropy of these cytokines.

Molecular biology of IL-9 and IL-9R

Both mouse and human IL-9 cDNAs contain a single long open reading frame of 432 nucleotides, which encodes a 144 amino acid polypeptide with an 18 amino acid signal sequence. They also contain four N-linked glycosylation sites, which is consistent with

the extensive size heterogeneity of IL-9 protein observed with IL-9-expressing cells. The overall homology between mouse and human IL-9 cDNAs is 67% at the nucleotide level and 56% at the amino acid level. The human IL-9 gene has been mapped to the long arm of human chromosome 5 while the mouse IL-9 gene has been localized to mouse chromosome 13. Both genes are 4 kb in length and consist of five exons and four introns. The 5'-flanking region of the IL-9 gene contains several potential transcriptional control elements. It has been shown recently that the cooperation of different transcriptional factors including NFκB, *c-jun* and a 35 kDa protein is essential for the constitutive and induced human IL-9 gene expression in human T lymphotropic virus type I (HTLV-I) transformed T cells. Although no IL-9 message can be detected in freshly isolated human peripheral blood mononuclear cells, T cell mitogens such as phytohemagglutinin (PHA) or anti-CD3 induced substantial IL-9 expression that can be further enhanced by phorbol myristate acetate (PMA) or CD28. In the mouse system, IL-9 is mainly

produced by T_H2 clones *in vitro*, and *in vivo* IL-9 production is also associated with T_H2-like T cell responses.

The mouse IL-9R encodes a 468 amino acid polypeptide with two potential N-linked glycosylation sites and six cysteine residues in the extracellular domain. The position of these cysteine residues, the presence of a WSEWE motif in the extracellular domain and the lack of consensus sequences for tyrosine or serine/threonine kinases in the intracellular domain suggest that IL-9R is a member of the hematopoietin receptor superfamily. The human IL-9R encodes a 522 amino acid polypeptide with 53% homology with the mIL-9R. The extracellular region of human and mouse IL-9R is well conserved while the intracellular domain is quite different between the two. Despite the high sequence homology of ligand and receptor between the two species, mouse IL-9 interacts with mouse and human IL-9R while human IL-9 only targets human but not mouse cells. Mouse IL-9R gene is a single-copy gene composed of nine exons and eight introns, on the other hand, the human genome contains at least four IL-9R pseudogenes with 90% homology with the IL-9R gene. Some of the molecular characteristics of IL-9 and its receptor are summarized in Table 1.

Signal transduction mechanisms of IL-9

Because of the overlapping biological activities and the sharing of IL-2R γ chain as common signal transducer of IL-9 and other cytokines such as IL-2, -4, -7 and -15, many investigators have compared the signal transduction pathways mediated by this group of cytokines. These studies indicated that there are convergent and divergent points along the signaling pathways mediated by these cytokines. Figure 1 summarizes some of the signaling events mediated by IL-9 in hematopoietic cells.

Although most of hematopoietin receptors do not possess tyrosine kinase activity, it appears that protein tyrosine phosphorylation plays an important role in growth factor-mediated signal transduction. It has been shown that IL-9 stimulation of different IL-9 responding cells results in enhanced tyrosine phosphorylation of proteins of different molecular weights. Some of the tyrosine kinases involved in the process are believed to be members of the JAK (Janus kinase) family of nonreceptor tyrosine kinases, which have been shown to play important roles in signaling pathways mediated by different cytokines. It was found that JAK family kinases, such as JAK1 and JAK3, can physically associate with receptor complexes and are tyrosine phosphorylated and activated following stimulation with IL-9 and other related

Table 1 Molecular characteristics of IL-9 and IL-9R

Human/mouse IL-9		
Amino acids		
Precursor	144	
Signal sequence	18	
Mature	126	
Molecular mass	30–40 kDa	
N-Linked glycosylation sites	4	
Cysteine residues	10	
pI	10	
mRNA size	0.8 kb	
Overall homology between mouse and human IL-9:		
Amino acid level:	56%	
Nucleotide level:	67%	

IL-9R	Mouse	Human
Amino acids		
Precursor	468	522
Mature		
Extracellular	233	233
Transmembrane	21	21
Cytoplasmic	177	231
Molecular mass		
Core	52 kDa	60 kDa
Mature	64 kDa	?
N-Glycosylation sites		
Extracellular	2	2
Intracellular	0	0
mRNA size	4.5, 2.5 and 2 kb	?
Amino acid homology between the extracellular domains of mouse and human IL-9R: 67%		

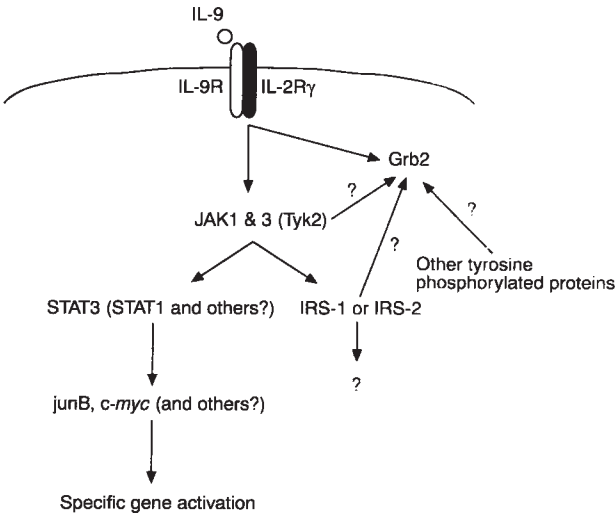


Figure 1 Possible control mechanisms of IL-9-mediated signal transduction pathways in hematopoietic cells.

cytokines. Although common JAKs are activated by IL-9 and related cytokines, the cytokine specificity may in part be determined by downstream signaling molecules such as IRS proteins (insulin receptor substrate 1 or 2) and STATs (signal transducers and acti-

vators of transcription). IRS proteins contain multiple tyrosine residues which may serve as substrates for upstream kinases and as docking sites for the SH2 domains of Grb2, SHP-2, Nck and PI3K. It is believed that the ability of IRS proteins to interact with different downstream molecules following cytokine stimulation may determine part of cytokine specificity. IL-9 and related cytokines have also been shown to activate different members of the STAT family transcriptional factors. In addition, differences have also been observed among primary response genes activated by IL-9 and related cytokines. These studies strongly suggest that the specificity for IL-9 and related cytokines could be achieved through the unique tyrosine phosphorylated proteins, their associated molecules, specific transcriptional factors and primary response genes triggered by individual cytokines.

Biological activities of IL-9

IL-9 is a T cell-derived cytokine with a variety of *in vitro* biological activities within the hematopoietic, lymphopoietic and neuronal systems. Some of the biological activities known to be associated with IL-9 are summarized in Table 2.

T cell growth factor activity of IL-9

IL-9 was originally discovered based on its ability to support the growth of certain mouse helper T cell clones. Subsequent studies have shown that this cytokine does not appear to function as a general growth factor for T cells. In fact, freshly isolated T cells do not respond to IL-9 even in the presence of costimuli such as lectins or anti-CD3 antibodies. Fetal thymocytes, on the other hand, can proliferate in response to the combination of IL-2 and IL-9, implicating the role of IL-9 in T cell ontogeny in the thymus. It appears that IL-9 responsiveness correlates with the

activation status but not the phenotype of T helper clones.

Mast cell-enhancing activity of IL-9

Several studies have shown that mouse IL-9 possesses mast cell-enhancing activity (MEA) and can enhance the mast cell growth elicited by IL-3 and IL-4. It not only provides a proliferative signal, but also leads to a marked functional activation of responsive mast cells as evidenced by the production of IL-6 and the expression of mast cell proteases and the α chain of the high-affinity IgE receptor. Interestingly, IL-9 overexpression in transgenic mice results in a massive intestinal mastocytosis and increased circulating levels of mast cell specific proteases.

Erythroid burst-promoting activity of IL-9

Human IL-9 can support erythroid colony formation (BFU-E) in cultures supplemented with erythropoietin using CD34⁺ progenitors for human bone marrow, peripheral and cord blood. The colony-enhancing effect elicited by human IL-9 is erythropoietin-dependent although IL-9 alone sustains the survival of erythroid progenitor cells *in vitro* as assessed by the delayed addition of erythropoietin to the cultures. It has also been shown that IL-9 does not accelerate cycling of adult or fetal BFU-E, CFU-Mix or CFU-GM to the extent observed after incubation with IL-6. In contrast to its maturation activity on adult erythroid progenitors, IL-9 has a wider spectrum of action on fetal progenitors and, when combined with IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), results in clonogenic maturation of progenitors that do not undergo maturation after stimulation with IL-3 and GM-CSF. The erythroid burst promoting activity has also been ascribed to mouse IL-9.

Biological effects of IL-9 on B cells

IL-4 has been shown to induce immunoglobulin E (IgE) production by normal B lymphocytes from human peripheral blood without preactivation of these cells. In the absence of IL-4, IL-9 does not induce IgE and IgG production by human B lymphocytes. However, in the presence of a suboptimal dose of IL-4, IL-9 markedly enhances IgE and IgG production in a dose-dependent manner. The ability of IL-4 to induce IgG and IgM by purified B lymphocytes preactivated with *Staphylococcus aureus* Cowan strain I was also enhanced in the presence of IL-9. These results implicate a role for IL-9 in the regulation of immunoglobulin production by B lymphocytes.

Table 2 *In vitro* biological activities of human and mouse IL-9

Human IL-9

- supports erythroid colony formation
- synergizes with IL-4 in the production of IgE and IgG by B cells
- supports the growth of certain H-RS cells

Mouse IL-9

- supports the growth of certain helper T cell clones
- stimulates erythroid colony formation
- induces fetal thymocyte proliferation in combination with IL-2
- enhances mast cell growth elicited by IL-3 or IL-4
- promotes maturation of mouse embryonic hippocampal progenitor cell lines in combination with TGF α and bFGF

Biological effects of IL-9 on neuronal cells

In combination with transforming growth factor α (TGF α) and basic fibroblast growth factor (bFGF), IL-9 has been shown to promote neuronal morphological maturation of mouse embryonic hippocampal progenitor cell lines, as evidenced by enhanced neurite outgrowth and nuclear enlargement, induced expression of high molecular weight mature neurofilament protein and reduced expression of nestin and NF66. In addition, neuronal cells thus treated can also give action potentials sensitive to tetrodotoxin, which is indicative of sodium channels with more mature physiological properties.

Potential role of IL-9 in leukemia and lymphoma

Since T cell-stimulating activity of IL-9 depends on the activation status but not the phenotype of T cells and the fact that transfection of IL-9-dependent cells with IL-9 cDNA resulted in autonomous growth and tumorigenicity of these cells, Renauld and coworkers have hypothesized that T cell transformation may in part be due to the dysregulated expression of IL-9. To test this hypothesis, transgenic mice expressing high levels of IL-9 constitutively were generated. Although no major morphological changes were noted in the lymphoid organs of most mice, the animals showed a high incidence of thymic lymphomas, were highly susceptible to chemical mutagens, and exhibited a high sensitivity to the tumorigenic effect of gamma irradiation. These results support the notion that dysregulated expression of IL-9 may 'prime' T cells to a transformed state that is more susceptible to secondary transforming agents such as mutagens or irradiation.

It is known that certain mouse helper T cell clones can proliferate in response to cytokines such as IL-2, -4 or -9 in the absence of antigen-presenting cells and antigen stimulation. Although dexamethasone, a glucocorticoid hormone, was able to induce apoptosis in these cells, the inhibition of IL-2-induced proliferation was found not to be the result of apoptosis, because this process can be antagonized by IL-2 as well as IL-4 and IL-9. These results indicate that different signaling events may be involved in growth stimulation and inhibition of apoptosis in the presence of glucocorticoids. IL-9 has also been shown to be more potent in protecting mouse thymic lymphomas against apoptosis than inducing cell proliferation. The antiapoptotic effects of IL-9 may explain certain aspects of its role in leukemogenesis but may also have potential therapeutic usage in certain clinical settings.

To examine whether IL-9 may be involved in the pathogenesis of human lymphomas, Mertz and colleagues have analyzed the expression of IL-9 in a variety of tumors and found that IL-9 message was detected in several cases of large cell anaplastic lymphoma (LCAL) and Hodgkin's disease (HD). It has been shown that primary and cultured Hodgkin's Reed-Sternberg (H-RS) cells can coexpress IL-9 and IL-9R. The possible role of IL-9 in the pathogenesis of HD was also suggested based on the inhibition of cultured H-RS cell growth in the presence of antisense oligonucleotides and antibodies to IL-9. Recent studies have also shown a cooperative effect of IL-9 and CD40 ligand in promoting the clonogenic growth of certain HD cell lines. The possible involvement of IL-9 as an autocrine growth factor or in concert with surface antigens for some human lymphomas requires further investigation.

See also: Apoptosis; Mast cells; Bone marrow and hematopoiesis; Cytokine receptors, soluble; Cytokines; Cytokine receptors; Leukemia; Lymphoma; Proliferation, lymphocyte.

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INTERLEUKIN 10 AND ITS RECEPTOR

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Interleukin 10 (IL-10) is an important immunoregulatory cytokine which was initially known as the cytokine synthesis inhibiting factor (CSIF) and its discovery was based upon characterization of its biological activity. Fiorentino described a type 2 T helper (T_H2) cell-derived factor that could selectively inhibit the secretion of interferon γ (IFN γ) by type 1 T helper (T_H1) cells. Murine Ly-1⁺ B cell lymphomas were shown to have a 'B cell-derived growth factor' activity that strongly enhanced IL-2- or IL-4-dependent thymocyte proliferation. O'Garra demonstrated that the same murine lymphoma supernatant also had mast cell growth factor activity. Based on these biological activities, cDNA encoding mouse IL-10 (mIL-10) was isolated from an concavalin A-activated T_H2 clone. Subsequently, the human IL-10 (hIL-10) was identified by screening a tetanus toxin-specific human T cell cDNA library using probes based on the mouse cDNA sequence.

18.5 kDa acid-sensitive protein that belongs to the four α -helical bundle cytokine superfamily. Unlike murine IL-10, which is glycosylated at the N-terminus, hIL-10 lacks detectable carbohydrate moieties. This N-linked glycosylation is, however, not necessary for its biological activity. Both mIL-10 and hIL-10 protein contain intrachain disulfide bonds that are essential for the biological activity of this cytokine. hIL-10 has been shown to be active on both murine and human cells, but mIL-10 is not active on human cells. Interestingly, both the mIL-10 and hIL-10 exhibit a strong DNA (71%) and amino acid sequence (84%) homology to the open reading frame in the Epstein-Barr virus (EBV) genome called BCRF-1. Since hIL-10 and BCRF-1 (viral IL-10) are closely related in the amino acid sequence, it is postulated that EBV may have captured this mammalian gene during evolution to confer survival advantage.

Physical properties of IL-10

The mIL-10 and hIL-10 cDNAs clones exhibit a high degree of nucleotide sequence homology (>80%) and also encodes very similar (>73% homology) open reading frames of 178 amino acids. Murine IL-10 is a homodimeric cytokine with an apparent molecular weight of 35 kDa due to the mixture of 17, 19 and 21 kDa polypeptides. Human IL-10 is a

IL-10 gene structure

The gene for both the murine and human IL-10 has been located to chromosome 1 and is present as single copies in the genome. The mIL-10 gene contains five exons spanning over 5.1 kb of the genome with a similar structure determined for human IL-10. IL-10 gene transcription produces a single mRNA of 1.4 kb in mouse and 2 kb in humans. Transcriptional

regulation of IL-10 is still unclear though its upstream regulatory sequence elements closely resemble that of IL-6. Transcriptional control is the predominant feature in regulating IL-10 levels in T lymphocytes and appears to involve cyclosporine-resistant pathways. Furthermore, regulation of post-transcriptional mRNA stability/degradation also seems to be an important feature which in part may be mediated via the multiple copies of the AUUUA motifs present within the 3'-untranslated region (3'-UTR). Similar regulatory mechanisms may also be operative in monocytes and B lymphocytes.

Cellular source of IL-10

IL-10 is produced by several types of cells, including activated T lymphocytes, monocytes, B cells as well as nonhematopoietic sources such as keratinocytes, colon carcinoma and melanoma cells. In humans though T_H2 T cell clones are the main source of IL-10, many T_H1 clones will also secrete IL-10 following antigen-specific stimulation. In contrast, mIL-10 is produced by the T_H2 subset of $CD4^+$ T cells but not by T_H1 or $CD8^+$ T cells. In humans both $CD4^+$ and $CD8^+$ T cells secrete IL-10 following stimulation with anti-CD3, although significantly higher levels are produced by $CD4^+$ T cells. Among the $CD4^+$ T cells, $CD45RO^+$ memory cells produce 10-fold higher level of IL-10 than naive T-cells ($CD45RA^+$). Murine Ly-1 B cells as well as Epstein-Barr virus transformed human B cells produce IL-10. Human monocytes are also a major source of IL-10 in response to activation with lipopolysaccharide. Interestingly, kinetics studies reveal that IL-10 is synthesized later than other immunoregulatory cytokines by activated T cells or monocytes. This suggests that IL-10 may play a regulatory role for the later phases of the immune response.

Effect of IL-10 on the immune system

Like most other cytokines, IL-10 exerts multiple effects upon the function of various cell lineages including lymphocytes, monocytes, natural killer (NK) cells, and dendritic cells.

T lymphocytes

In the mouse, IL-10 specifically inhibits T_H1 cell cytokine synthesis ($IFN\gamma$ and IL-3) particularly when macrophages were used as antigen-presenting cells (APCs). IL-10 has been demonstrated to have inhibitory effects on the proliferation, survival and cytokine production of human T cells. In humans, direct interaction of IL-10 with the IL-10 receptor (IL-10R) on T cells results in specific suppression of IL-2 gene

transcription thus inhibiting T cell proliferation. Since presence of IL-10 also reduces the expression of IL-2R on T cell clones, exogenous IL-2 cannot completely overcome the inhibition produced by IL-10. T Cells are also indirectly inhibited by IL-10 through alteration of the antigen-presenting function of monocytes. IL-10 accomplishes this effect by downregulating the expression of major histocompatibility complex (MHC) class II antigen on monocytes thus decreasing the peptide/MHC complexes available for interaction and adequate stimulation of T cells. Besides the effects on $CD4^+$ T_H1 cells, IL-10 also inhibits $IFN\gamma$ synthesis by $CD8^+$ T cells without affecting their cytotoxic function. In contrast to these inhibitory effects, IL-10 also has positive effects where it can promote survival of IL-2-starved T cells which otherwise are destined to undergo apoptosis. hIL-10 has also been shown to stimulate chemotaxis, proliferation, differentiation and cytolytic activity of human $CD8^+$ T cells.

Macrophage/monocytes

Experimental evidence indicates that monocytes/macrophages are the prime targets of IL-10 and has prominent effects on their morphology, phenotype and cytokine production. Besides causing de-adherence and rounding up of monocytes, IL-10 also strongly inhibits their constitutive and inducible expression of MHC class II antigen. IL-10 also has the ability to block B7 and ICAM-1 expression in monocytes in response to $IFN\gamma$, thereby disrupting monocyte-T cell interaction. Production of IL-1 α , IL-1 β , IL-6, IL-8, tumor necrosis factor α (TNF α), granulocyte-macrophage (GM-) and granulocyte colony-stimulating factor (G-CSF) by human monocytes following activation is inhibited by IL-10 at the mRNA and protein level. IL-10 also prevents IL-12 expression from monocytes, thereby inhibiting T_H1 cell development. Furthermore, IL-10 inhibits its own production by monocytes indicating the presence of a self-regulatory negative feedback loop.

Natural killer (NK) cells

IL-10 can inhibit monocyte-induced NK cells production of $IFN\gamma$. IL-10 has this effect on NK cells indirectly by suppressing monocyte production of IL-12. However production of $IFN\gamma$, TNF α and GM-CSF by IL-2-activated NK cells is significantly enhanced by IL-10. IL-10 has also been shown to induce significant NK cytotoxic activity against NK-resistant tumor cell targets.

B lymphocytes

Unlike the effects on monocytes, IL-10 induces the

expression of MHC class II antigen on resting B cells. IL-10 leads to enhanced viability of B cells *in vitro* and augments their differentiation into antibody-secreting cells particularly after activation by anti-CD40 antibody. IL-10 synergizes with IL-2 to induce B cell differentiation by upregulating their high-affinity IL-2 receptor. Upon stimulation with polyclonal activators or transformation by EBV, purified B cells will produce IL-10. The ability of B lymphocytes to produce and to respond simultaneously to IL-10 has suggested that this cytokine may act as an autocrine growth factor in humans.

IL-10 receptor

The various biological effects of IL-10 are mediated by engaging with its cell surface receptor. Recently both the murine and human receptors for IL-10 were identified, characterized and cloned. There is 75% DNA sequence homology between the mIL-10R and the hIL-10R, with 60% identity between the predicted amino acid sequence. The hIL-10R gene is located on chromosome 11 and its mRNA expression has been detected in all IL-10-responsive cells (mainly hematopoietic cells). Binding studies of radioiodinated hIL-10 to various human cell lines characterized the hIL-10 receptor to be a 110 kDa glycoprotein. As mentioned earlier, hIL-10 will bind to both murine and human IL-10 receptors, whereas mIL-10 binds only to mIL-10R. Major differences in the N-terminal third of mIL-10 and hIL-10 protein may be in part responsible for the apparent species specificity.

Structural analysis reveals homology to the class II cytokine receptor gene family which includes the receptors for IFN α/β and γ . This is interesting considering the fact that IL-10 and IFN γ antagonize each other's function. Receptors belonging to the type I and II cytokine receptor families are now known to utilize the JAK/STAT family of proteins for signal transduction. Characterization of mIL-10's intracellular signaling demonstrates that it activates STAT1, STAT3 and STAT5 that subsequently form multiple STAT heterocomplexes and bind to the IFN γ response region (GRR) of the Fc γ R1 gene. STAT3, but not STAT1 or STAT5, is directly recruited to the ligand-activated mIL-10R by binding to a specific tyrosine-containing docking site in the receptor intracellular domain. In human T cells and monocytes, IL-10/IL-10R complex induces phosphorylation of tyk2 and JAK1 with subsequent activation of STAT1 α and STAT3. STAT3 is predominantly activated over STAT1 particularly in monocytes and the subsequent distinct homodimer/heterodimers complex of the two STAT proteins may

explain why T cells and monocytes individualize their response to IL-10. IL-10 has also been shown to mediate monocyte proliferation through selective activation of the phosphatidylinositol 3-kinase and p70 S6 kinase. Thus recent data suggest that multiple and distinct signaling pathways may mediate the various pleiotropic activities of IL-10.

See also: Cytokine genes, regulation of; Cytokine inhibitors; Cytokines; Cytokine receptors; Effector lymphocytes; Epstein-Barr virus, infection and immunity.

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INTERLEUKIN 11 AND ITS RECEPTOR

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The cDNA encoding interleukin 11 (IL-11) was originally isolated from a primate bone marrow-derived stromal fibroblast cell line based on its mitogenic activity on an IL-6-dependent mouse plasmacytoma cell line. The same cDNA was also isolated independently by its ability to inhibit adipogenesis. Subsequent *in vitro*, *in vivo* and clinical studies have shown that IL-11 is a multifunctional cytokine important in hematopoiesis. Mouse and human IL-11 receptor (IL-11R) cDNAs have been isolated by hybridization using degenerate oligonucleotides for the WSXWS motifs of hematopoietin receptors and an enhancer-trap method. Sharing of the common gp130 signal transducer among IL-11, IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and possibly cardiotropin 1 (CT-1), at least in part, the biological redundancy and pleiotropy of these cytokines. *In vitro* and *in vivo* studies have implicated IL-11 as a multifunctional growth factor with therapeutic potential in enhancing thrombopoiesis, protecting gastrointestinal injury and modulating immune response. Like many other hematopoietic growth factors its clinical efficacy should be interpreted with caution and may be further improved by combination with other hematopoietic growth factors.

Molecular biology of IL-11 and IL-11R

IL-11 cDNA and genomic sequences are well conserved among different species. IL-11 cDNAs contain a single open reading frame of 199 amino acids with a 21 amino acid signal peptide and a calculated molecular weight of 23 kDa. The cDNA encodes no cysteine residues and no N-linked glycosylation sites. The overall homology between mouse and human IL-11 cDNAs is 88% at the amino acid level and

86% at the nucleotide level. The human IL-11 gene has been mapped to the long arm of human chromosome 19 at 19q13.3–13.4. The gene is 7 kb in length and consists of five exons and four introns. The 5'-flanking region contains several potential transcriptional control elements including AP-1, SP-1 and NF- κ B. There are at least nine copies of ATTTA sequence in the 3'-flanking region. The IL-11 transcripts (2.5 and 1.5 kb) have been detected in primate and human bone marrow-derived stromal fibroblast cell lines, human lung fibroblast, trophoblast, pulmonary and tracheal epithelial, cervical carcinoma, osteogenic sarcoma, megakaryoblastic and other leukemic cell lines. In addition, IL-11 transcripts have also been detected in human chondrocytes, synoviocytes and umbilical vein or adult aortic endothelial cells. The expression of the IL-11 transcripts in different cell types is constitutive but can be attenuated by different positive regulators including IL-1, transforming growth factor β (TGF β), tissue plasminogen activator (t-PA), calcium ionophore, respiratory syncytial virus (RSV), prostaglandin E₁ and E₂ and forskolin, and negative regulators such as retinoic acid, IL-4, dexamethasone and heparin. The mechanisms of IL-11 gene expression mediated by different modulators have been shown to be at either the transcriptional or post-transcriptional level. In stromal fibroblasts, a 10 bp region (5'GGTGAGTCAG3') containing a consensus AP-1 site has been found to be responsible for the basal level transcription of the IL-11 gene and JunD is one of the major components of the DNA-protein transcriptional complexes. The 3'-UTR of IL-11 mRNA can function as a destabilizing component in IL-11 mRNA metabolism, possibly through the AUUUA motifs. In addition, the IL-11 5'-UTR and the IL-11 coding sequence, in conjunction with certain protein

tyrosine kinases, are required for the stabilization of the IL-11 mRNA by IL-1 in stromal cells.

IL-11R consists of at least one ligand-binding subunit (IL-11R α) and a signal transduction subunit (gp130). The mouse and human IL-11R cDNAs encode a 432 and a 422 amino acid polypeptide, respectively, with two potential *N*-linked glycosylation sites and four cysteine residues in the extracellular domain. The position of these cysteine residues, the presence of a WSTWS motif in the extracellular domain and the lack of consensus sequences for tyrosine or serine/threonine kinases in the intracellular domain suggest that IL-11R α is a member of the hematopoietin receptor superfamily. The overall homology between mouse and human IL-11R α is 84% at the amino acid level and 85% at the nucleotide level. In the mouse genome, there are at least two IL-11R α genes, IL11R α and IL11R α 2, that can potentially encode the IL-11R α . The IL-11R α gene is ubiquitously expressed while the IL11R α 2 gene is only expressed in certain strains of mice in specific organs such as testis, lymph node and thymus. It appears that the molecular composition of IL-11R is more complicated than initially anticipated. Further biochemical molecular and *in vivo* knockout studies of IL-11R are underway to dissect the essential components for transducing IL-11 signals. Some of the known biochemical properties of IL-11 and IL-11R α are summarized in Table 1.

Signal transduction mechanisms of IL-11

Because of overlapping biological activities and sharing of the gp130 signal transducer by IL-11R and the receptors for other cytokines such as IL-6, LIF, OSM, CNTF and possibly CT-1, many investigators have compared the signal transduction pathways mediated by this group of cytokines. These studies indicated that there are convergent and divergent points along the signaling pathways mediated by these cytokines. Some of the signaling events mediated by IL-11 in mouse preadipocytes are shown in Figure 1 and summarized below.

Involvement of kinases

Kinases involved in IL-11 signaling include JAK kinases, MAPKs, pp90^{rsk}, Src family kinases and PI3K. JAK (Janus kinase) family nonreceptor tyrosine kinases play important roles in signaling pathways mediated by different cytokines. It was found that JAK family kinases physically associate with gp130 and are tyrosine phosphorylated and activated following stimulation with IL-11 and other IL-6-type cytokines. MAPKs and pp90^{rsk} have been shown to

Table 1 Molecular characteristics of IL-11 and IL-11R

Human/primate/mouse IL-11		
Amino acids		
Precursor	199	
Signal sequence	21	
Mature	178	
Molecular mass	23 kDa	
<i>N</i> -linked glycosylation sites	0	
Cysteine residues	0	
pI	11.5	
<i>IL-11 receptor α chain (IL-Rα)</i>		
		Mouse Human
Amino acids		
Precursor	432	422
Mature	409	399
Extracellular	344	342
Transmembrane	26	26
Cytoplasmic	39	31
Molecular mass		
Core	50 kDa	50 kDa
Mature	151 kDa	?
<i>N</i> -glycosylation sites		
Extracellular	2	2
Intracellular	0	0
Immunoglobulin-like domain	1	1
mRNA size	2.0 kb	2.0 kb
Amino acid homology between mouse IL-11R and:		
Human IL-11R	84%	
IL-6 receptor α chain	24%	
CNTF receptor α chain	22%	
IL-12 receptor p40 subunit	16%	

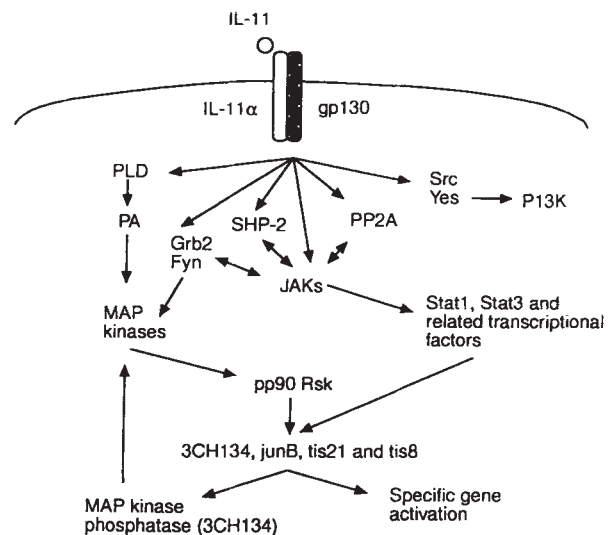


Figure 1 Possible control mechanisms of IL-11-mediated signal transduction pathways in preadipocytes.

be activated by a variety of growth factors and mitogens. MAPKs and pp90^{rsk} can be activated by IL-11 and related cytokines. pp90^{rsk} is one of the H7-sensitive protein kinases involved in the expression of primary response genes induced by gp130 family cytokines. In addition to pp90^{rsk}, various H7-sensitive kinases may control the expression of primary response genes at either the transcriptional or post-transcriptional level. Src family tyrosine kinases represent an expanding class of closely related intracellular enzymes that participate in the signal transduction pathways of a variety of surface receptors. It has been shown that Src family kinases, such as p60^{src} and p62^{yes} can be activated by IL-11 in mouse preadipocytes. PI3K, which phosphorylates the D3 position of phosphatidylinositol or its derivatives, has been associated with many cellular processes. IL-11-stimulated cells revealed increased PI3K activity and the increased PI3K was found to be associated with tyrosine phosphorylated proteins, including p62^{yes}. The amount of p62^{yes} associated with PI3K transiently increased in response to IL-11. Recent studies have shown that IL-11 induces Grb2 to bind to p59^{fyn} and JAK2.

Involvement of phosphatases

Phosphatases involved in IL-11 signaling include MAPK phosphatase, SHP-2 and PP2A. IL-11 significantly induces expression of the 3CH134 gene, which encodes a phosphatase with dual specificity that can specifically dephosphorylate and inactivate MAP kinases both *in vitro* and *in vivo*. IL-11 has also been shown to stimulate tyrosine phosphorylation of SHP-2, a SH2-containing protein tyrosine phosphatase shown to be important for signaling of different cytokines. The C-terminus of SHP-2 has been shown to associate with several tyrosine phosphorylated proteins including gp130 and JAK2. The association of SHP-2 and gp130 or JAK2 can be further induced by IL-11 stimulation. Peptide competition studies suggest different interactions between SHP-2/gp130 and SHP-2/JAK2. Recent studies have also shown that IL-11 can induce the association of PP2A, PI3K and p62^{yes} to JAK2.

Transcriptional factors involved in IL-11 signaling

STAT (signal transducers and activators of transcription) family transcriptional factors are cytoplasmic proteins which become tyrosine phosphorylated and activated following ligand-receptor interactions and activation of JAK family kinases. STAT1 and associated proteins are tyrosine phosphorylated and translocated to the nucleus following IL-11 treatment in 3T3-L1 mouse preadipocytes. The activation of STAT3 has been demonstrated for other cytokine

receptors within the gp130 family. The activation of primary response genes such as junB and tis11 by IL-11 may require transcriptional factors other than STATs.

Biological activities of IL-11

IL-11 is a bone marrow fibroblast-derived cytokine with a variety of *in vitro* biological activities within the hematopoietic, lymphopoietic, hepatic, adipose, bone and neuronal systems. IL-11 has been tested in various animal models including mice, rats, hamsters, rabbits and nonhuman primates. Some of these *in vitro* and *in vivo* studies are summarized in Tables 2–4. It appears that IL-11 may be a potential therapeutic agent for thrombocytopenia and neutropenia following myeloablative therapy and bone marrow transplantation.

In addition, based on its immunomodulatory activity and ability to maintain the integrity and pro-

Table 2 *In vitro* biological activities of IL-11

Supports the growth of certain plasmacytoma, hybridoma and myeloma cell lines
Enhances the growth of myeloid leukemic cells in combination with IL-3, GM-CSF or SCF
Stimulates increases in numbers, sizes and ploidy values of megakaryocyte colonies in human and murine systems in combination with IL-3 or SCF
Stimulates primitive multilineage hematopoietic progenitors as well as precursors at various stages of erythroid differentiation in combination with IL-3 or SCF
Supports the survival and expansion of primitive progenitor cells in combination with other cytokines
Increases the tritiated thymidine suicide rate of human fetal but not adult CFU-MIX, CFU-GM and BFU-E
Enhances the generation of antigen-specific plaque-forming cells (PFCs) in the <i>in vitro</i> mouse spleen cell culture system
Acts directly on human T (not B) cells, particularly the CD4 ⁺ CD45RA ⁺ T cell subset, to enhance immunoglobulin secretion
Enhances the effects of IL-7 and SCF to differentiate AA4.1 ⁺ B220 ⁺ Mac-1 ⁺ fetal liver progenitor cells
Induces the synthesis of several major human acute phase plasma proteins
Inhibits adipogenesis and the associated lipoprotein lipase (LPL) activity
Induces neuronal maturation of immortalized mouse embryonic hippocampal cell lines
Induces the formation of osteoclasts in mouse bone marrow and calvaria cultures
Stimulates the production of the tissue inhibitor of metalloproteinases in chondrocytes and synoviocytes
Increases the histamine responsiveness of airway smooth muscle cells
reduces the production of proinflammatory cytokines including TNF- α , IL-1 β , IL-6 and nitric oxide (NO).

Table 3 Biological effects of IL-11 in normal animals*Mice*

IL-11 enhances primary and secondary immune responses; increases the absolute numbers of CFU-GM and stimulates CFU-GM, BFU-E and CFU-GEMM progenitor cells to a higher cell cycle rate

IL-11 stimulates megakaryocyte maturation and an increase in peripheral platelet number in normal and splenic mice

IL-11 in combination with G-CSF results in a synergistic increase in splenic cellularity and elevated numbers of CFU-MK, BFU-E, CFU-GM and CFU-S_{day12} and the increased numbers of MK progenitors in the spleen are highly responsive to IL-11 and Epo

Rats

IL-11 alone significantly induces thrombocytosis, and in combination with G-CSF, induces an increased myelopoiesis. Combinations of two early-lineage CSFs (IL-11 + CSF) and a later-lineage CSF (G-CSF) significantly induces new-born rat myelopoiesis and IL-11 alone significantly induces new-born rat thrombopoiesis

Primates

IL-11 has thrombopoietic effects in nonhuman primates when administered by constant intravenous infusion or subcutaneous administration. Megakaryocytes from IL-11-treated monkeys have many ultrastructural characteristics indicative of increased maturation.

IL-11 either alone or in combination with GM-CSF or IL-3 decreases red blood cell counts, hemoglobin and hematocrit; the thrombopoietic effects of the combination of IL-11 and GM-CSF or IL-11 and IL-3 are greater than each cytokine administered alone; IL-11 alone has no effect on leukocyte counts but enhances leukocytosis in response to IL-3 or GM-CSF

mote the healing of the gastrointestinal mucosa, IL-11 can also be used to reduce the severity of chemotherapy-induced mucositis and associated inflammatory complications.

Potential clinical significance of IL-11

Two clinical trials of IL-11 in cancer patients have been performed. In a phase I trial in patients with breast cancer, IL-11 was administered daily for 14 days during a 28-day prechemotherapy "cycle 0". Patients subsequently received up to four 28-day cycles of cyclophosphamide and doxorubicin chemotherapy (day 1) followed by IL-11 (days 3 through 14). The results indicated that IL-11 has thrombopoietic activity at all doses studied (10, 25, 50, 75 and 100 $\mu\text{g kg}^{-1} \text{ day}^{-1}$), is well tolerated at doses lower than 50 $\mu\text{g kg}^{-1}$ and at doses greater than 25 $\mu\text{g kg}^{-1} \text{ day}^{-1}$ has the potential to reduce chemotherapy-induced thrombocytopenia in these patients. The effects of IL-11 were subsequently evaluated in a ran-

domized phase II trial in cancer patients who had previously received platelet transfusions for severe chemotherapy-induced thrombocytopenia. Ninety three patients who had received platelet transfusion for nadir platelet counts less than 20 000 μl during chemotherapy cycle immediately prior to entry into the study were randomized to receive placebo or IL-11 at doses of 25 or 50 $\mu\text{g kg}^{-1}$ subcutaneously once daily for 14 to 21 days beginning one day after chemotherapy. Chemotherapy was continued during the study without dose reduction. The results demonstrated that IL-11 treatment at a dose of 50 $\mu\text{g kg}^{-1}$ significantly reduces the need for platelet transfusion in subsequent chemotherapy cycles for patients who had required platelet transfusions for severe thrombocytopenia in a previous cycle. The thrombopoietic effect of IL-11 is being further evaluated in other phase II and phase III trials.

Summary

IL-11 was originally identified based on its biological similarities to IL-6; these two cytokines, however, are not functionally identical. Identification and characterization of receptor components of IL-11R and the associated signaling molecules will be required to dissect the mechanisms responsible for cytokine specificity. IL-11 is a cytokine with a more restricted spectrum of target cells than IL-6 and this unique property may explain the minimal or no toxicity associated with IL-11 in the *in vivo* studies. *In vivo* administration of IL-11 in normal and myelosuppressed animals has demonstrated the thrombopoietic effect of this cytokine. Phase I and II clinical trials provide further support that IL-11 is an important thrombopoietic agent that can be used safely in patients with severe chemotherapy-induced thrombocytopenia. Because of its immunomodulatory activity and ability to promote healing of the gastrointestinal mucosa, IL-11 may also reduce the severity of chemotherapy-induced mucositis and associated inflammatory complications. These biological activities have led to clinical trials for treatment of chemotherapy-induced mucositis and Crohn's disease. Based on its ability to enhance thrombopoiesis, protect gastrointestinal epithelial injury and reduce inflammatory complications, IL-11 therefore represents a multifunctional cytokine that may potentially offer several benefits in cancer therapy. In addition, IL-11 is being evaluated further in the treatment of various inflammatory disorders such as inflammatory bowel disease, rheumatoid arthritis and sepsis. Future clinical trials with IL-11 alone and in combination with other biologic, radiation or

Table 4 Biological effects of IL-11 in experimentally induced animal models**HEMATOPOIETIC EFFECT***(a) Mice immunosuppressed by cyclophosphamide*

IL-11 augments the number of spleen antigen-specific PFC as well as serum antigen-specific antibody titers, and decreases the time required to regain normal levels of leukocyte and platelet counts

(b) Mice myelosuppressed by nitrosourea

IL-11 diminishes nitrosourea-induced pancytopenia and leads to a significant reduction in chemotherapy-related mortality. The increase in multilineage peripheral blood counts is associated with a higher number of committed progenitor cells and HPP-CFC in the bone marrow

(c) Mice myelosuppressed by combined sublethal irradiation and carboplatin

IL-11 accelerates the recovery of peripheral platelet, red blood cell counts and hematocrit

(d) Mice receiving syngeneic bone marrow transplants or sublethal irradiation

IL-11 increases peripheral platelet and leukocyte (acceleration of neutrophil recovery) counts

(e) Mice receiving syngeneic bone marrow infected with IL-11-expressing retrovirus and lethal irradiation

The IL-11 expressing mice have an accelerated recovery of circulating platelets and red blood cells and the chronic IL-11 exposure results in the loss of body fat, thymus atrophy and alterations in plasma protein levels

(f) Primates myelosuppressed by carboplatin

IL-11 treatment following chemotherapy significantly improves platelet nadirs and accelerates platelet recovery.

NON-HEMATOPOIETIC EFFECT**1. Gastrointestinal damage***(a) Mice myelosuppressed by 5-FU and radiation*

IL-11 decreases morbidity and mortality caused by endogenous infection from gut organisms; stimulates the proliferation of small intestine crypt progenitor cells and induces recovery of small intestine villus structure; results in a mild increase in peripheral platelet counts.

(b) Hamsters with oral mucositis resulting from 5-FU treatment
IL-11 decreases the frequency, severity and duration of mucositis in a dose-dependent manner and increases animal survival.

2. Inflammatory bowel diseases*(a) HLA-B27 transgenic Fisher 344 rats*

IL-11 decreases the clinical signs and histologic lesion scores of inflammatory bowel disease in these rats.

(b) Acetic acid induced colitis in rats

IL-11 increase platelet counts and decreases the severity of acetic acid-induced colonic injury.

(c) TNBS (trinitrobenzene sulfonic acid) induced colitis in rats

IL-11 decreases the inflammatory lesions in acute colitis induced by TNBS.

3. Systemic inflammatory conditions*(a) Radiation induced thoracic injury in mice*

IL-11 reduces the levels of radiation induced TNF α mRNA in the lung which has been implicated in acute lung injury and interstitial lung disorders.

(b) Toxic shock in mice given D-galactosamine and Staphylococcus aureus enterotoxin B

IL-11 increases survival of these animals.

(c) Pseudomonas sepsis in rats

IL-11 reduces the level of lung infection, pulmonary edema and injury to gastrointestinal epithelium.

(d) Antibiotic-treated newborn rats during experimental group B streptococcal sepsis

IL-11 enhances platelet recovery as well as survival rate of these animals.

(e) Endotoxemia in rats

IL-11 lowers serum levels of inflammatory cytokines such as TNF α , IFN α and IL-1B.

(f) Endotoxemia in rabbits

IL-11 reduces serum levels of proinflammatory cytokines and prevents the hypotension that occurs as a consequence to endotoxemia and is associated with decreased plasma nitric oxide levels.

chemotherapeutic agents will be necessary to evaluate the efficacy of IL-11 in various clinical settings.

See also: Bone marrow and hematopoiesis; Cytokines; Cytokine receptors; Interleukin 6; Interleukin 6 receptor.

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INTERLEUKIN 12 AND ITS RECEPTOR

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IL-12 was discovered between 1986 and 1990 independently by four groups by its costimulatory activity for T and natural killer (NK) cells. This is illustrated by the original functional designations of CLMF (cytotoxic lymphocyte maturation factor, M.

Gately *et al*) and NKSF (natural killer cell stimulatory factor, G. Trinchieri *et al*) for human interleukin 12 (IL-12) as well as TSF (T cell stimulating factor, T. Germann, E. Rüde) and IL-2RIF (IL-2 receptor inducing factor, H. Nariuchi *et al*) for murine IL-12.

Whereas TSF and IL-2RIF were obtained from normal splenic accessory cells or bone marrow-derived macrophages upon interaction with activated T_{H1} cells, NKSF and CLMF were both secreted by Epstein-Barr virus (EBV)-transformed human B cell lines activated by phorbol ester. The human factors could be produced and purified in amounts sufficient for N-terminal sequence analysis. It became apparent that NKSF and CLMF comprised two polypeptide chains, IL-12p40 and IL-12p35. Cloning of both proved the identity of CLMF and NKSF. The designation IL-12 was agreed upon. The mouse IL-12 chains were then cloned by cross-hybridization to the human cDNA probes.

Structure of IL-12 and its receptor (IL-12R)

Structural characteristics of IL-12 and its receptor are summarized in Table 1. The homologies to other cytokines, mainly to IL-6 and its receptor, suggest that IL-12 has evolved from a primordial cytokine and its soluble receptor. On activated T cells (human and mouse) IL-12 binding sites of high (K_d 5–50 pM), intermediate (K_d 50–200 pM) and low (K_d 2–6 nM) affinities have been identified. In bioassays IL-12 (mouse and human) is active at concentrations of about 0.1 pM. This indicates that signaling can be induced even at low receptor occupancy. Similarly, a cell line cotransfected with the two cloned hIL-12R β_1 and β_2 chains exhibited binding sites of K_d 50 pM and K_d 5–10 nM and was responsive to IL-12 at 1 pM. Signal transduction induced by IL-12 in T cells was found to include activation of tyrosine kinases JAK2 and TYK2 and phosphorylation of STAT1 (mouse), STAT3 and STAT4 (mouse and human).

Competitive inhibition of IL-12 function by murine IL-12(p40) $_2$

The p40 chain of IL-12 (human and mouse), when synthesized in the absence of p35 (e.g. in cells transfected with p40 cDNA) or when produced in excess over p35, can be secreted as a monomer, IL-12p40, and as a disulfide linked homodimer, IL-12(p40) $_2$. Murine IL-12(p40) $_2$ because of its high affinity binding to the murine IL-12R (Table 1) is an efficient inhibitor of IL-12 activities both *in vitro* and *in vivo*. Synergistic inhibition was observed in combination with certain IL-12-specific antibodies. There are indications that murine IL-12(p40) $_2$ may be synthesized as a physiological inhibitor of IL-12. Human IL-12(p40) $_2$ is a much poorer competitor for human IL-12 (Table 1); possibly it could be used in combination with suitable antibodies for more efficient inhibition of IL-12 functions.

Production of IL-12 and its regulation

Although human IL-12 was originally discovered in the supernatants of transformed human B cell lines, normal B cells (human or mouse) were not found to synthesize significant amounts of IL-12 (murine B cells stimulated by bacterial DNA may be an exception). The main producers of IL-12 belong to the macrophage/dendritic cell (DC) lineage. It is important to note that the capacity of macrophages to synthesize IL-12 depends on their functional stage (Table 2). Neutrophils, upon stimulation by lipopolysaccharide (LPS), were also found to produce IL-12, although less efficiently than monocytes. Furthermore, Langerhans cells, microglia cells, astrocytes, connective tissue type mast cells and keratinocytes could be identified as IL-12 producers.

Regulation of IL-12 synthesis is complex because it is controlled by two independent genes which have to be coexpressed in the same cell. There are indications that mRNA expression for the two chains does not always correlate. The p40 chain is generally produced in excess over the IL-12 heterodimer. The ratio of p40 to IL-12 can vary from 5:1 to 100:1, depending on the cell type and the stimulus. This may not be trivial because in the case of murine macrophages the IL-12 antagonist, IL-12(p40) $_2$, could be detected among the excess p40 produced. It is obvious that this complicates the determination of IL-12 considerably (see below).

The stimuli leading to IL-12 production have been mainly studied with macrophages/DCs (Figure 1). With respect to the function of IL-12 as a mediator of innate resistance and a link to specific immunity, it is essential that this cytokine (together with other cytokines such as tumor necrosis factor α (TNF α), IL-1, etc.) is produced upon infection/interaction of macrophages with gram-negative as well as gram-positive bacteria on their products (e.g. LPS and others). Furthermore, infection with certain parasites and viruses can induce IL-12 synthesis. A second pathway leading to IL-12 production in macrophages/DCs is initiated by ligation of CD40, which occurs under physiological conditions by interaction with activated, CD40L-expressing T cells of the T_{H1} subtype. No IL-12 is produced upon interaction of macrophages/DCs with T_{H2} cells. This is probably due to the release of IL-10 which inhibits IL-12 production (see below). This pathway of IL-12 production is important to maintain and perhaps further expand an already existing T_{H1} -type response.

Some of the modulators of IL-12 production and their effects are listed in Table 2. It should be noted that IL-10 and transforming growth factor β (TGF β) not only inhibit IL-12 synthesis but also its biological

Table 1 Some structural characteristics of IL-12 and its receptor*Interleukin 12 (IL-12)*

Human (h) and murine (m) IL-12 are both 70–75 kDa proteins composed of two polypeptide chains, IL-12p40 and IL-12p35, linked by one disulfide bridge. Murine IL-12 can function on human cells but human IL-12 does not function on mouse cells

hIL-12p40:	~40 kDa, 306 amino acids calculated mol. wt 34 699, 10 Cys: 8 in intrachain disulfide linkages. 1 Cys forms a bridge to IL-12p35, 1 Cys modified by thioglycolic acid or by cysteine, 4 potential <i>N</i> -glycosylation sites, 10% carbohydrate Amino acid sequence shows homology to IL-6R α chain Encoded on chromosome 5q31–q33
hIL-12p35:	~35 kDa, 197 amino acids, calculated mol. wt 22 500, 7 Cys: 6 in intrachain disulfide linkages, 1 Cys forms bridge to IL-12p40, 3 potential <i>N</i> -glycosylation sites, 20% carbohydrate Amino acid sequence shows homology to IL-6 and G-CSF Encoded on chromosome 3p12–q13.2
mIL-12-p40:	~40 kDa, 70% cDNA sequence and 70% amino acids sequence identity with hIL-12p40 Encoded on chromosome 11
mIL-12p35:	~35 kDa, 67% cDNA sequence, 60% amino acids sequence identity with hIL-12p35 Encoded on chromosome 6(3)

Interleukin 12 receptor (IL-12R)

Two receptor chains IL-12R β_1 and IL-12R β_2 have been identified and cloned

hIL-12R β_1 :	~100 kDa, type I membrane protein, 638 amino acids calculated mol. wt 70 400, extracellular portion 516 amino acids, transmembrane region 31 amino acids, intracellular portion 91 amino acids.
hIL-12R β_2 :	~130 kDa, type I membrane protein, 835 amino acids, calculated mol. wt 94 059, extracellular portion 595 amino acids, transmembrane portion 24 amino acids, intracellular portion 216 amino acids Both β_1 and β_2 chains are members of the cytokine receptor superfamily and are related to gp130 of the IL-6R and to receptors of LIF and G-CSF. Coexpression of IL-12R β_1 and β_2 confers IL-12 responsiveness and results in binding sites of K_d 50 pM and of K_d 5–10 nM; expression of either chain alone results in binding sites of K_d 2 to >50 nM.
mIL-12R β_1 :	54% amino acid sequence identity with hIL-12R β_1 The cDNA of mIL-12R β_1 was reported to occur in 3 additional splice variants (β_{1a} , β_{1b} and β_{1c})
mIL-12R β_2 :	68% amino acid sequence identity with hIL-12R β_2
h + mIL-12R:	Expressed on 'resting' NK cells but not on resting T and B cells. Upregulation upon activation on T cells and NK cells. Expression on activated B cells is not yet firmly established

IL-12 (pP40) $_2$: a competitive inhibitor of IL-12

IL-12(p40) $_2$ is a disulfide-linked homodimer that can be secreted in addition to the IL-12p40 monomer by cells producing IL-12p40

hIL-12(p40) $_2$:	Binds to the high affinity hIL-12R with 5- to 10-fold lower affinity than hIL-12 and is therefore not a potent inhibitor of hIL-12
mIL-12(p40) $_2$:	Binds to the high affinity mIL-12R with similar affinity as mIL-12 itself but without stimulation of the cells. mIL-12(p40) $_2$ is therefore a potent competitive inhibitor of mIL-12
mIL-12p40: (monomer)	Binds with 25- to 50-fold lower affinity to the IL-12R than mIL-12(p40) $_2$

activities on T cells. Furthermore, the role of CD46, which is a receptor for measles virus and a binding protein for C3b/C4b, is remarkable because it selectively inhibits the synthesis of IL-12 but not that of TNF α and IL-6 by macrophages.

Cells responsive to IL-12

The major cell types that respond to IL-12 are NK cells and T cells, the latter including CD4 $^+$ and CD8 $^+$ T cells as well as $\gamma\delta$ T cells (Figure 1). Furthermore, hematopoietic precursor cells were found to respond

to IL-12 *in vitro* with increased proliferation and differentiation, whereas application of IL-12 *in vivo* leads to anemia, neutropenia and lymphopenia due to indirect effects of interferon γ (IFN γ) induced by IL-12. IL-12 has also been reported to act on activated B cells to increase immunoglobulin (Ig) secretion. Recently, murine B cells activated by crosslinking of CD40 were found to produce relatively high concentrations of IFN γ in response to a combination of IL-12 and IL-18 and this cytokine combination led to a shift in the production of IgG $_1$ /IgE to IgG $_{2a}$. The main effects of IL-12 on NK

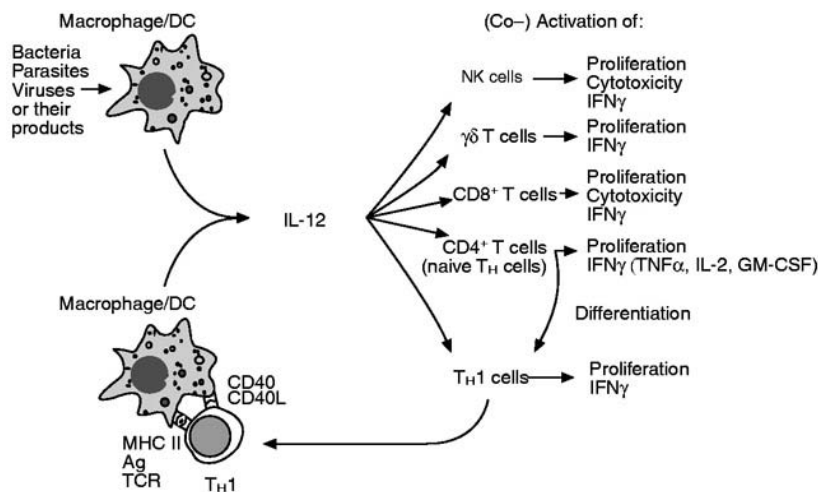


Figure 1 Stimuli that induce IL-12 production in macrophages/dendritic cells (DCs) and some of the biological effects of IL-12 on NK and T cells.

Table 2 Modulators of IL-12 production^a

Positive modulators

IFN γ :	Preincubation of macrophages or neutrophils
GM-CSF:	Preincubation of macrophages (DCs are generally cultivated with GM-CSF)
IL-4, IL-13:	Monocytes (human); depending on conditions the synthesis of IL-12 can also be inhibited (see below)

Negative modulators

IL-10:	Potent inhibitor for macrophages and DCs, also inhibits synthesis of other cytokines (e.g. TNF α , etc.)
TGF β :	Strong inhibitor, also for other cytokines (e.g. TNF α , etc.)
IL-4, IL-13:	Partial inhibition in monocytes (human) (see above)
Prostaglandin E ₂ :	Leads to elevation of cAMP, inhibition of IL-12 and TNF α production
Phosphodiesterase inhibitor:	Leads to elevation of cAMP, inhibition of IL-12 and TNF α production
CD46 cross-linking	Induced by measles virus, dimerized C3b or antibodies to CD46 Selective inhibition of IL-12 but not of TNF α or IL-6 synthesis in human macrophages

^aThe results summarized in this table were mainly obtained with monocytes/macrophages as the IL-12-producing cells.

and T cells are the enhancement of cytokine production, costimulation of proliferation, and the enhancement of cytotoxic activity. Possibly most important is the induction of IFN γ synthesis at different levels. In addition, the production of TNF α , granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 by T cells is promoted. The importance of IL-12 for optimal IFN γ production in the course of immune responses *in vivo* was demonstrated by the use of mice with targeted disruption of the IL-12p40 or p35 genes.

Innate resistance to infections is mediated by IL-12 that synergizes with IL-1 β and/or TNF α in inducing IFN γ production by NK cells. This proinflammatory effect of IL-12 which via IFN γ leads to activation of macrophages can not only mediate protection but also pathological septic shock-like effects. The fact that IL-12 is a potent costimulator for T cells constitutes an important link between innate and acquired immunity (Figure 1).

IL-12 synergizes with T cell receptor (TCR)-stimu-

lation and with other costimuli (IL-2, CD28-ligation) in inducing IFN γ production by T cells. Another important aspect of IL-12 function is the induction of differentiation of naive CD4⁺ T helper cells to T_H1 cells. These are characterized by their high capacity to produce IFN γ . Furthermore, IL-12 functions as a costimulator that synergizes with signaling via CD28 inducing IFN γ production and proliferation of T_H1 cells. Thus, IL-12 is generally known as the major inducer of T_H1-type immune responses *in vitro* and *in vivo*. However, one should notice that in the presence of small amounts of IL-4, which by itself will lead to T_H2 development of naive T_H cells, the addition of IL-12 does not suppress but rather enhances this T_H2 development. Such effects of IL-12 have been also observed *in vivo*. Injection of IL-12 into mice led to expression of IL-10 (in addition to IFN γ) in not yet identified cell types. It was shown that *in vitro* priming of human T cells in the presence of IL-12 not only resulted in the development of cells producing IFN γ but also IL-10. This would represent a feedback mech-

anism since IL-10 can inhibit further IL-12 synthesis by macrophages and IL-12R expression on T cells (see below).

Regulation of responsiveness to IL-12

Responsiveness of T cells to IL-12 is controlled by IL-12R expression. Resting T cells do not express the receptor, which is transiently upregulated relatively late (day 3–4) after activation of T cells (Table 1). In addition to this basic regulation, the responsiveness of activated IL-12R expressing human T cells is strongly enhanced by interaction of CD2 with CD58 on accessory cells. No such effect has yet been observed with mouse T cells. Negative regulation of IL-12 responsiveness occurs in naive T_H cells developing toward T_H2 cells under the influence of IL-4. This loss of IL-12 reactivity is due to the downregulation of IL-12R β_2 and is counteracted by the presence of IFN γ . An important modulator that strongly inhibits the effects of IL-12 is TGF β . Indeed it was reported that TGF β and IL-10 strongly inhibit upregulation of IL-12R β_2 , and moderately inhibit upregulation of IL-12R β_1 , on human T cells.

Immune modulation by IL-12

The importance of early IL-12 production for stimulation of innate immunity and the development of protective T_H1 -type immune responses in infectious disease models has already been mentioned. Resistance to a malaria infection could be achieved by a prophylactic treatment of monkeys or mice with IL-12. In cases of an insufficient T_H1 response (e.g. *Leishmania* infection of susceptible BALB/c mice) the early administration of IL-12 could correct this defect. However, an already established disease-promoting T_H2 response could not be reverted.

IL-12 could be detected in the early phases of some but not all virus infections and was found to assist in the development of immunity. While administration of IL-12 depending on its dosage had beneficial effects with some virus infections it led to toxic, septic shock-like effects in others (e.g. lymphocytic choriomeningitis virus; LCMV). Since macrophages of patients with the acquired immune deficiency syndrome (AIDS) are deficient in producing IL-12, and addition of IL-12 to peripheral blood mononuclear cells (PBMC) of AIDS patients restored antigen-induced IFN γ production and proliferation of T cells, treatment with IL-12 *in vivo* in analogy to the mouse model of MAIDS might contribute to restoration of cellular immune functions.

When administered together with protein antigens, IL-12 showed a strong adjuvant effect. It promoted the development of T_H1 -type responses and, after a moder-

ate reduction of early IgG1 production, it led to weakly enhanced late IgG1 and strongly enhanced IgG2a, IgG2b and IgG3 responses. An established IgE response could not be permanently suppressed. Under certain conditions, the induction of an IgE response was even enhanced. This adjuvant effect of IL-12 has been successfully employed to immunize mice with defined bacterial protein antigens. While poor *in vivo* protection was achieved with Al(OH) $_3$ as adjuvant, IL-12 supported the development of protective immunity comparable to whole bacterial vaccines. The involvement of endogenously produced IL-12 was also demonstrated for the induction of contact sensitivity that represents a classical T_H1 -type response. When administered together with a contact allergen, IL-12 also functioned as an adjuvant.

T_H1 -type responses to autoantigens are considered to be characteristic for several organ-specific autoimmune diseases. It could be demonstrated that in certain mouse models of antigen-induced or spontaneous autoimmune diseases administration of IL-12 can accelerate the development of disease. But IL-12 could also inhibit or delay disease development if higher doses or a different application regimen were employed. Furthermore, in some of the models IL-12-specific antibodies, when applied therapeutically, led to a reduced incidence and severity of the disease.

Finally, IL-12 has been extensively tested as an adjuvant for inducing tumor specific immunity or as a therapeutic agent against established tumors in a variety of mouse models. Protective immunity could be induced upon preimmunization in some models and striking inhibitory effects on tumor growth and metastasis formation were observed in others. In some models CD8 $^+$ T cells were found to be required, yet generally the underlying mechanisms appear to be complex.

Determination of IL-12

The determination of IL-12 can be carried out by biological assays or by ELISA. Biological assays for IL-12 are very sensitive and are mostly based on the 'capture' technique. The assay wells are precoated with an antibody against IL-12 that should be specific for the heterodimer, but not neutralizing and not binding to IL-12p40 or IL-12(p40) $_2$. After capturing IL-12 the responding cells (e.g. activated T cells) are added and the resulting IFN γ production or proliferation can be measured. This avoids, especially in the mouse system, inhibitory effects of IL-12(p40) $_2$, which may be also present in the test fluids. A sandwich ELISA uses the same primary antibody to bind IL-12 but not the p40 chain that is usually produced in excess over IL-12. A

secondary anti-IL-12p40 or anti-IL-12p35 antibody can then be used for detection of the IL-12 bound.

See also: Adjuvants; Autoimmunity; CD40 and its ligand; Helper T lymphocytes; Cytokine receptors; Dendritic cells; Immunotherapy of tumors; Innate immunity; Interferon γ receptor; Interleukin 6 receptor; Macrophage activation; Natural killer (NK) cells; Septic shock.

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INTERLEUKIN 13 AND ITS RECEPTOR

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Interleukin 13 (IL-13) has biological activities on B cells, monocytes/macrophages and endothelial cells. IL-13 is primarily produced by T_H2 cells, but it is also secreted by other T helper cell subsets $CD8^+$ T cells, mast cells, eosinophils and basophils following activation. IL-13 induces proliferation and immunoglobulin E (IgE) synthesis by human B cells, suggesting that it may play a role in the pathogenesis of IgE-mediated allergic diseases. Moreover, IL-13 inhibits production of proinflammatory cytokines and chemokines by monocytes/macrophages both *in vitro* and *in vivo*, indicating that IL-13 also has important anti-inflammatory properties.

Molecular characteristics of IL-13

Human IL-13 is a 12 kDa nonglycosylated four-helix, α -helical protein. Alternative splicing results in two functionally similar isoforms of hIL-13 consisting of 131 or 132 amino acids, the latter having an additional glutamine residue at position 98. IL-13 protein is approximately 25% homologous to IL-4, and all residues that contribute to the hydrophobic structural core of IL-4 are conserved, or have conservative replacements in IL-13, suggesting that the three-dimensional structures of IL-4 and IL-13 are comparable. The gene encoding human IL-13 is located on chromosome 5q31, in the same cluster of genes encoding the cytokines IL-3, IL-4, IL-5, IL-9 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The IL-13 gene is only 12 kb upstream from the gene encoding IL-4, indicative for gene duplication during the evolution of these two genes. IL-13 and IL-4 share many, but not all, biological properties, which is partially due to differential expression of IL-4 and IL-13 receptors.

IL-13R complex

The high-affinity IL-13 receptor complex comprises the IL-4R α chain and an IL-13 binding protein, termed the IL-13R α chain. The IL-13R α chain is a ~70 kDa glycosylated molecule, which is homologous with the IL-5R α chain (51% similarity and 27% identity). The IL-13 receptor is expressed on B cells, monocytes/macrophages, basophils, mast cells and endothelial cells, but not on T cells. The IL-13R complex also functions as a second receptor for

IL-4, whereas the other IL-4R complex, consisting of the IL-4R α chain and the common γ chain, is specific for IL-4 (Figure 1). The IL-4R α chain alone binds IL-4 with a relatively high affinity ($K_d \sim 10^{-10}M$), whereas the IL-13R α chain is a specific binding protein for IL-13 ($K_d 2.5 \times 10^{-10}M$).

Data obtained with cells from patients with X-linked severe combined immunodeficiency (SCID) indicate that IL-4 can signal through both IL-13R and IL-4R complexes. SCID patients have mutations in the gene encoding the common γ chain, a shared component of receptors for IL-2, IL-4, IL-7, IL-9 and IL-15. Therefore, their T cells are unable to respond to these cytokines, causing severe abnormalities in both T and B cell functions. However, B cells derived from SCID patients proliferate and produce IgE fol-

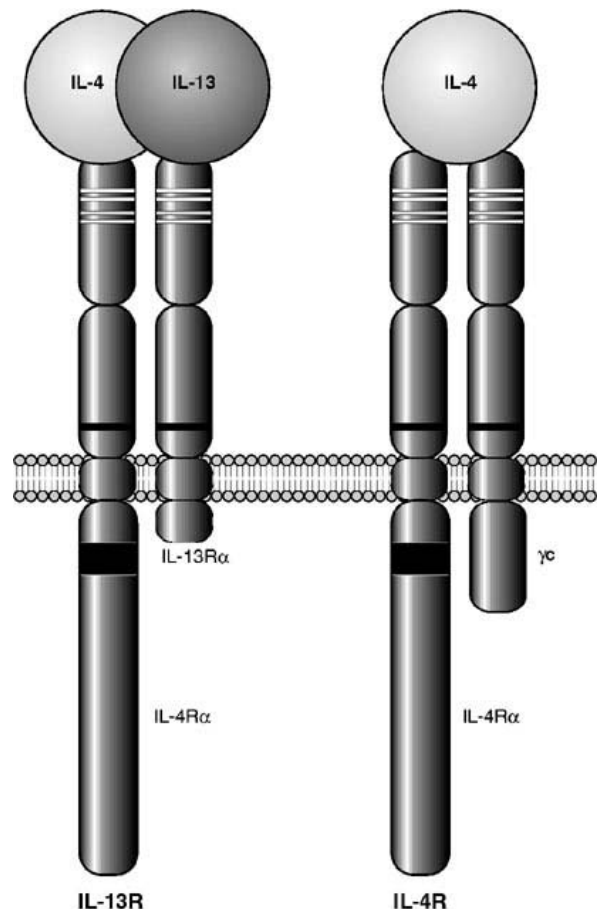


Figure 1 IL-13 receptor and IL-4 receptor complex.

lowing stimulation through CD40 in the presence of IL-4 or IL-13, whereas IL-2 and IL-15 were ineffective, demonstrating that both IL-4 and IL-13 can mediate their biological effects through the IL-13R complex in the absence of a functional common γ chain. Binding of IL-13 to the IL-13R α chain, leads to its dimerization with the IL-4R α chain and initiation of the signal transduction cascade.

Stable expression of the murine IL-13R α chain in CTLL-2 cells, which normally respond to IL-4 but not to IL-13, results in the generation of a high-affinity IL-13 binding complex, which transduces a proliferative signal in response to IL-13.

Effects of IL-13 on B lymphocytes

IL-13 induces IgG4 and IgE synthesis by human B cells cultured in the presence of activated CD4⁺ T cells, anti-CD40 monoclonal antibodies (mAbs) or CD40 ligand (L) transfectants. In contrast to human B cells, IL-13 does not induce IgE by mouse B cells under conditions that induce IgE switching in response to IL-4, suggesting that mouse B cells lack functional IL-13R complexes. However, IL-4-deficient mice produce low levels of IgE *in vivo*, following viral or parasite infections, indicating that IL-4-independent IgE synthesis occurs in these mice. However, it is not known whether this IgE production is mediated by IL-13.

The effects of IL-13 on human B cells are largely similar to those of IL-4, but IL-13 is generally less potent than IL-4 in inducing IgE synthesis.

IL-13 modulates the phenotype of normal human B cells. It upregulates the expression of CD23, CD71, CD72, surface IgM (sIgM) and major histocompatibility complex (MHC) class II molecules on purified human B cells. IL-13 has growth-promoting activity on normal B cells stimulated by anti-IgM or anti-CD40 mAbs, but it inhibits IL-2-induced proliferation of chronic lymphocytic leukemia B cells, suggesting that IL-13 has antiproliferative effects on malignant B cells.

IL-13 does not have additive or synergistic effects with IL-4 when both cytokines are added at optimal concentrations, which is consistent with the observation that the receptors for IL-4 and IL-13 share a common component, which is important for signal transduction.

Role of IL-13 in atopic diseases

Although both IL-4 and IL-13 induce IgE synthesis by human B cells *in vitro*, the relative contribution of the two cytokines to IgE synthesis under physiological situations remains to be determined. This is largely due to lack of suitable animal models.

Several lines of indirect evidence suggest that both IL-4 and IL-13 are required for optimal induction of human IgE synthesis. Both IL-4 and IL-13 are upregulated in the lungs of asthmatic patients after allergen challenge, indicating that both cytokines play a role in the regulation of allergic inflammatory responses. Atopy has been linked to genes in chromosome 5q31.1 in the area where the genes encoding IL-4 and IL-13 are located, suggesting a genetic predisposition for enhanced IL-4 and IL-13 production in atopic individuals. However, neutralization of either IL-4 or IL-13 activities in supernatants of activated allergen-specific T_H2 cells with anti-IL-4 or anti-IL-13 mAbs indicated that IL-4 is the dominant cytokine in inducing IgE synthesis. On the other hand, the fact that IL-13 is also produced by naive T cells and T cell subsets not producing IL-4 suggests that IL-13 may be the dominant cytokine inducing IgE synthesis in situations where IL-4 is absent or present at low levels, e.g. during the initiation of allergic responses.

Effects of IL-13 on monocytes and endothelial cells

The effects of IL-13 on monocytes/macrophages and endothelial cells are similar to those of IL-4. IL-13 modulates the phenotype of both human and murine monocytes/macrophages and it inhibits production of proinflammatory cytokines, chemokines and nitric oxide by these cells. IL-13 inhibits synthesis of IL-1 α , IL-1 β , IL-6, IL-12, tumor necrosis factor α (TNF- α) and the chemokines IL-8, macrophage inflammatory protein (MIP-1 α) and MIP-1 β by monocytes. In addition, it enhances production of the IL-1 receptor antagonist, which is a secreted protein with anti-inflammatory properties. IL-13 also inhibits IL-1 β and TNF α production by synovial fluid macrophages from patients with rheumatoid arthritis, suggesting that IL-13 may have therapeutic potential in the treatment of inflammatory arthritis. This notion is supported by the observation that IL-13-producing cells attenuate collagen-induced arthritis in mice. IL-13 was also shown to suppress experimental autoimmune encephalomyelitis in rats, further demonstrating its anti-inflammatory properties *in vivo*. In addition, IL-13 enhances expression of CD11b, CD11c, CD18, CD23 and MHC class II molecules, whereas it decreases expression of CD14, CD16, CD32 and CD64 on monocytes and macrophages.

IL-13 induces VCAM-1 expression on human umbilical vein endothelial cells, which results in adhesion of eosinophils to these cells. Eosinophils accumulate at sites of allergic inflammation, and they are thought to play a crucial role in the pathogenesis

of the lung inflammation and in lung epithelial cell destruction in asthmatic patients. Consequently, the ability of IL-13, like that of IL-4, to induce VCAM-1 expression on endothelial cells further emphasizes the importance of these cytokines in the pathogenesis of allergic and asthmatic responses.

Comparison of the functional properties of IL-4 and IL-13

Despite the structural and functional similarities between IL-4 and IL-13, there are also important differences (Table 1), which implies that IL-4 and IL-13 are likely to have distinct roles in the regulation of immune responses *in vivo*.

In contrast to IL-4, IL-13 does not have growth-promoting effects on activated human peripheral blood CD4⁺ or CD8⁺ T cells or T cell clones, and does not direct T_H2 cell differentiation. However, IL-13 may indirectly affect T cell functions and T cell differentiation through its downregulatory effects on the production of IL-12 and interferon α (IFN α), which direct T_H1 development. These differential effects of IL-4 and IL-13 on activated T cells appear to be due to absence of high affinity IL-13R complexes on these cells.

In addition to T cells, B cell precursors differ in their responses to IL-4 and IL-13. Both IL-4 and IL-13 induce proliferation and differentiation of immature CD19⁺, s μ ⁺ fetal B cells, but in contrast to

IL-4, IL-13 does not induce CD23, CD40 or HLA-DR expression or germline ϵ RNA synthesis in CD19⁺, s μ ⁺ human pre-B cells. These distinct effects of IL-4 and IL-13 on pre-B cells suggest that functional IL-13R is expressed at a later stage of B cell ontogeny than IL-4R and that IL-13, in contrast to IL-4, does not regulate early B cell maturation.

The different roles of IL-4 and IL-13 in the regulation of inflammatory responses *in vivo* are not only determined because they act on partially different target cells but also because both cytokines can be produced by different cells. IL-13 is not only produced by T_H2 cells, but also by T_H0 cells, and in contrast to IL-4 by naive CD45RA⁺ T cells and T_H1 cells. However, all T cells that produce IL-4 produce IL-13 when studied at the single cell level. The kinetics of IL-13 production by activated T cells also differs significantly from that of IL-4. IL-4 mRNA appears 2–4 hours after activation, and it is almost undetectable after 12 hours, whereas IL-13 mRNA can still be observed 48 hours after T cell activation, suggesting that IL-13 is produced for significantly longer time periods after antigen-specific T cell activation.

There are also similarities in IL-4 and IL-13R signal transduction pathways. Both IL-4 and IL-13 induce phosphorylation of STAT6 (STAT = signal transducer and activator of transcription), which is also necessary for the biological activities of both cytokines. STAT6-deficient mice are unable to produce IgE and their T_H2 cell differentiation is impaired. However IL-4, but not IL-13, induces phosphorylation of JAK3, which is probably due to the fact that JAK3 associates with the common γ chain, which is not an essential component of IL-13R complex.

Table 1 Functional properties of human IL-13 in comparison with IL-4

	IL-13	IL-4
Production by		
T _H 2 cells	†††	†††
T _H 1 cells	†	–
T _H 0 cells	††	†
CD45RA ⁺ naive T cells	†	–
CD45RO ⁺ memory T cells	††	††
Mast cells/basophils	††	††
Effects on B cells		
Proliferation	††	†††
IgG4/IgE synthesis	††	†††
CD23 and HLA-DR expression	††	†††
Activation of immature B cells	†	††
Activation of pre-B cells	–	††
Effects on T cells		
Proliferation	–	††
T _H 2 cell differentiation	–	†††
Effects on monocytes		
CD23 and HLA-DR expression	†††	†††
Synthesis of proinflammatory cytokines	‡‡‡	‡‡‡
Synthesis of nitric oxide	‡‡‡	‡‡‡
Effects on endothelial cells		
VCAM-1 expression	†††	†††

See also: Atopic allergy; CD40 and its ligand; Cytokines; IgE; Immunoglobulin class switching; Interleukin 4; Interleukin 4 receptor; T lymphocyte differentiation.

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INTERLEUKIN 15 AND ITS RECEPTOR

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Interleukin 15 (IL-15) is a cytokine that shares many *in vitro* activities with IL-2. The IL-2-like activity of this cytokine was initially described by two groups. The activity detected in concentrated supernatants of a monkey kidney epithelial cell line was molecularly cloned and given the name IL-15, whereas the activity in supernatants from a human T lymphotropic virus type I (HTLV-I)-transformed, human adult T cell leukemia line was designated IL-T. The IL-T protein has recently been shown to be identical to human IL-15.

The shared activities of IL-15 and IL-2 stem from their use of common receptor subunits. IL-15 interacts with a receptor complex that consists of both the β and γ_c chains of the IL-2R as well as a specific, high-affinity IL-15-binding subunit, designated IL-15R α . Although IL-2 and IL-15 have overlapping activities on cells of the immune system, the differential expression of these cytokines within tissues and by various cell types suggests that these cytokines perform some distinct physiological functions. The production of IL-15 by macrophages and other cell types in response to environmental stimuli and infec-

tious agents suggests that IL-15 plays a role in protective immune responses and immune disorders.

Expression of IL-15 mRNA and protein

In contrast to the expression of IL-2 mRNA, which is limited to activated T cells and to tissues that contain T cells, IL-15 mRNA is not expressed by activated normal T cells but is expressed constitutively (but often at low levels) in a wide variety of cell types and in many different tissues (Table 1). There is no evidence that normal human or mouse T or B cells express IL-15 mRNA. The predominant IL-15 message produced by the HTLV-I transformed human T cell line HuT-102 is smaller than that produced by nontransformed human cells and represents a chimeric mRNA that joins a segment of the HTLV-I long terminal repeat with the open reading frame of IL-15.

The expression of IL-15 mRNA by macrophages is enhanced in response to microbial products (Table 1). Although IL-15 mRNA expression is easily detected in lipopolysaccharide (LPS)-stimulated

Table 1 Characteristics of IL-15

Chromosomal location	4q31 (human); central region of 8 (mouse)
Polypeptide length	Mature protein: 114 amino acids; signal sequence: 48 amino acids
Predicted disulfide bonds	Cys35-Cys85, Cys42-Cys88, conserved in simian, human and mouse
Carbohydrate	Potential <i>N</i> -linked sites: Asn71, Asn79, Asn112 (human); Asn56, Asn60, Asn71, Asn112 (mouse)
Sequence homology of full-length proteins	Human and simian IL-15: 96% amino acid identity human and mouse IL-15: 73% amino acid identity
Heterotrimeric receptor complex	IL-15R α /IL-2R β / γ_c
Cellular sources of IL-15 mRNA	Monocytes/macrophages, epithelial cells, endothelial cells, fibroblasts, keratinocytes
Stimuli that increase IL-15 expression	IFN γ , LPS, infectious agents, UVB radiation
Biological effects of IL-15	Growth factor for activated T, B, and NK cells; chemoattractant and chemokinetic factor for T cells; induces cytotoxic activity and cytokine production by NK and T cells (synergizes with IL-12)

monocytes/macrophages, it has been difficult to detect IL-15 in unconcentrated supernatants from such cells. It has been suggested that this disparity is due in part to 10 upstream AUGs in the 5' untranslated region of IL-15 mRNA, which act to attenuate translation. However, recent studies have shown that IL-15 is produced *in vitro* as well as *in vivo*. For example, IL-15 is secreted by human keratinocytes upon exposure to UVB radiation. In addition, human peripheral blood mononuclear cells (PBMCs) or monocytes and mouse macrophages produce IL-15 in response to bacterial products, such as LPS, or in response to bacterial or viral infection. The optimal production of IL-15 by mouse macrophages has been shown to require both priming (by interferon γ , IFN γ) and triggering (by bacterial products).

The IL-15R complex

IL-15 interacts with a heterotrimeric receptor that contains both the β and the γ_c chains of the IL-2R as well as a unique IL-15-binding subunit. This subunit has been designated IL-15 α (Table 2) by analogy

Table 3 IL-15 binding affinities and proliferative response of cells expressing various forms of the IL-15R

Receptor subunits	$K_a(m^{-1})^a$	Response to IL-15 ^b
IL-15R α^c	10^{11}	—
IL-15R $\alpha\beta^{c,d}$	10^{11}	—
IL-15R $\alpha\beta\gamma^{c,d,e}$	10^{11}	+++
IL-15R $\alpha\Delta\text{cyt}\beta\gamma^{d,e,f}$	10^{11}	+++
human $\beta\gamma$	10^9	+
mouse $\beta\gamma$	Not detectable	+ ^g
IL-2R α^c	Not detectable	—

^aBinding affinities were determined using ^{125}I -simian IL-15. Limits of accurate determination of binding affinities are between 1×10^7 and $1 \times 10^{11} \text{ m}^{-1}$.

^bMeasured by incorporation of [^3H]thymidine by various cell lines.

^cRefers to either the mouse or human IL-15/IL-2R subunits.

^dThe β chain of the IL-15R is also a component of the IL-2R.

^eThe γ chain of the IL-15R is also a component of the receptors for IL-2, IL-4, IL-7, IL-9.

^fCells were transfected with IL-15R $\alpha\Delta\text{cyt}$, a deletion mutant of mouse IL-15R α that lacks all but five amino acids of the predicted cytoplasmic domain.

^gProliferation was detected in response to high concentrations (500 ng ml^{-1}) of human or simian IL-15. Such cells are 10–100 fold more responsive to mouse IL-15.

Table 2 Characteristics of the IL-15R α chain

Chromosomal location	10, bands p14–p15 (human); proximal region of 2 (mouse)
Polypeptide length	Mouse IL-15R α : mature protein: 231 amino acids; signal peptide: 32 amino acids; extracellular domain: 175 amino acids; cytoplasmic domain: 37 amino acids
Predicted disulfide bonds	Mouse IL-15R α : Cys4-Cys46, Cys30-Cys64
Carbohydrate	Mouse IL-15R α : potential <i>N</i> -linked site at Asn19, many potential sites for <i>O</i> -linked glycosylation
Alternatively spliced forms	Mouse: none detected Human: one is homologous to mouse IL-15R α cDNA, a second has a deleted exon in the extracellular domain, a third has an alternative cytoplasmic domain. No alternatively spliced mRNAs encoding soluble forms of mouse or human IL-15R α have been detected.
Cellular sources of IL-15R α mRNA and cell surface protein	Epithelial and bone marrow stromal cell lines, fibroblasts, T cells, NK cells, B cells, endothelial cells, monocytes/macrophages
Stimuli that increase or decrease IL-15R α expression	Increase: Anti-CD3, PMA (T cells); IFN γ (macrophages) Decrease: IL-15 (human T and B cells)

to the IL-2R α to which it is structurally related. As compared to IL-2R α , IL-15R α has a broader distribution of expression in different cell types and shows a much higher affinity for its ligand in the absence of the IL-2R β and γ_c chains. Whereas IL-2R α exhibits a low affinity ($K_a = 10^8 \text{ M}^{-1}$) for IL-2, IL-15R α binds IL-15 with a high affinity ($K_a = 10^{11} \text{ M}^{-1}$). The expression of IL-15R α can be upregulated in response to various stimuli (Table 2). Interestingly, although both IL-2 and IL-15 upregulate IL-2R α expression on human B and T cells, IL-15 downregulates the expression of IL-15R α on these cells. In addition, although a soluble, shed form of IL-2R α can be detected in biological fluids, it is not known whether a shed form of IL-15R α exists.

In cells of hematopoietic origin, the IL-2R β and γ_c chains are essential for signaling by either IL-2 or IL-15. Similar to what is observed in the IL-2/IL-2R system, the IL-15R α chain is required for high-affinity binding, but not signaling, by IL-15 (Table 3). It is therefore not surprising that the signaling pathways thus far described for IL-2 and IL-15 are identical. However, the IL-2R and IL-15R α chains control a cell's sensitivity to these two cytokines since specific, high-affinity binding of IL-2 or IL-15 requires the presence of the appropriate α chain of the receptor. Human cells that express IL-2R β and γ_c , but not IL-15R α , can bind and respond to human IL-15 *in vitro*, although relatively high concentrations of the cytokine are required to induce a response. Mouse cells expressing IL-2R β and γ_c , but not IL-15R α , are unable to bind IL-15 with a measurable affinity but are responsive to human or mouse IL-15. However, such cells are 10–100 fold more responsive to mouse IL-15. It is not clear whether human or mouse cells expressing IL-2R β and γ_c in the absence of IL-15R α will respond to physiological concentrations of IL-15 *in vivo*.

The significance of cells that express IL-15R α , but lack IL-2R β and/or γ_c , is also not clear. However, such cells would probably bind IL-15 with high affinity but would not be capable of responding to it. These cells might serve either to sequester IL-15 or to create high local concentrations of IL-15 that could be 'presented' to cells capable of responding to it. It is also possible that IL-15R α associates with as yet unidentified receptor subunits to signal in cells that express IL-15R α but lack IL-2R β and/or γ_c .

Biological effects of IL-15

IL-15, like IL-2, is a growth factor for activated mouse and human T cells, activated, purified human B cells, and a subpopulation of human natural killer (NK) cells. In addition, IL-15 synergizes with IL-12

to induce proliferation of resting mouse T_H1 clones and CD4⁺ T cell blasts. IL-15 induces locomotion and migration of human T cells, but appears to have no chemokinetic or chemotactic activity on human monocytes, neutrophils or B cells. IL-15 stimulates cytokine production by NK cells, especially when used in combination with IL-12, and will also induce cytokine production by T cells. Human B cells stimulated with the combination of soluble CD40L and IL-15 secrete polyclonal immunoglobulin M (IgM), IgG1, and IgA, but not IgG4 or IgE.

IL-15 enhances cytotoxic activity of both T cells and NK cells. It induces alloantigen-specific cytotoxic T lymphocytes (CTLs) from human mixed leukocyte cultures and lymphokine-activated killer (LAK) activity from cultures of human PBMCs, large granular lymphocytes, or NK cells. IL-15 treatment induces protective antitumor responses in mouse tumor models that have been shown to be responsive to IL-2 therapy and in which antitumor activity involves either the induction of LAK activity or tumor-specific CTLs.

Potential physiological roles of IL-15

The presence of IL-15 mRNA in many normal tissues and the increased production of IL-15 by macrophages/monocytes in response to bacterial or viral products suggests a role for IL-15 in protective immune responses, allograft rejection, and the pathogenesis of autoimmune diseases. Recently, a possible role for IL-15 in the pathogenesis of rheumatoid arthritis has been suggested. IL-15 is present in the synovial fluid of rheumatoid arthritis patients where it might contribute to recruitment of T cells and provide a means of stimulating T cells to produce macrophage-activating cytokines such as IFN γ , tumor necrosis factor α (TNF α) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Studies of IL-2 and IL-2R α knockout mice have revealed that IL-2 provides an immunoregulatory role in the immune system that cannot be compensated for by IL-15 or other cytokines. Comparative studies using the IL-2, IL-2R α , and IL-2R β knockout mice are likely to reveal defects in the IL-2R β knockout mice that can be attributed to the lack of both IL-2- and IL-15-mediated responses. The use of IL-15 antagonists *in vivo* and the generation of IL-15 knockout mice will provide further insight regarding the physiological roles of IL-15.

See also: Cytokines; Interleukin 2; Interleukin 2 receptor; Interleukin 12 and its receptor.

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INVARIANT CHAIN (Ii)

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Since its discovery as the third polypeptide chain coprecipitated with polymorphic class II products of the major histocompatibility complex (MHC), molecular immunologists have attempted to understand the physiological significance of the so-called invariant (Ii) chain (CD74) and its possible contributions to the structure and function of MHC-encoded class II membrane glycoproteins. Early observations made in transfection assays suggested that the Ii chain was not required for class II surface expression, but instead played a role during antigen presentation. Recent analysis of mutant mouse strains created using ES cell technology have confirmed the essential nature of Ii chain activities with respect to class II maturation and antigen presen-

tation and also demonstrated its role during thymic selection of the CD4⁺ T cell subset.

Structure

The application of molecular cloning techniques in the mid to late 1980s led to the isolation of Ii chain cDNAs and characterization of the corresponding genomic locus. The Ii chain was found to be encoded by a highly conserved single copy gene unlinked to the MHC, located on human chromosome 5 and mouse chromosome 18. An interesting aspect of the overall structure of Ii chain as shown in **Figure 1**, is that this type II transmembrane protein is oppositely oriented in comparison to MHC class II α and β

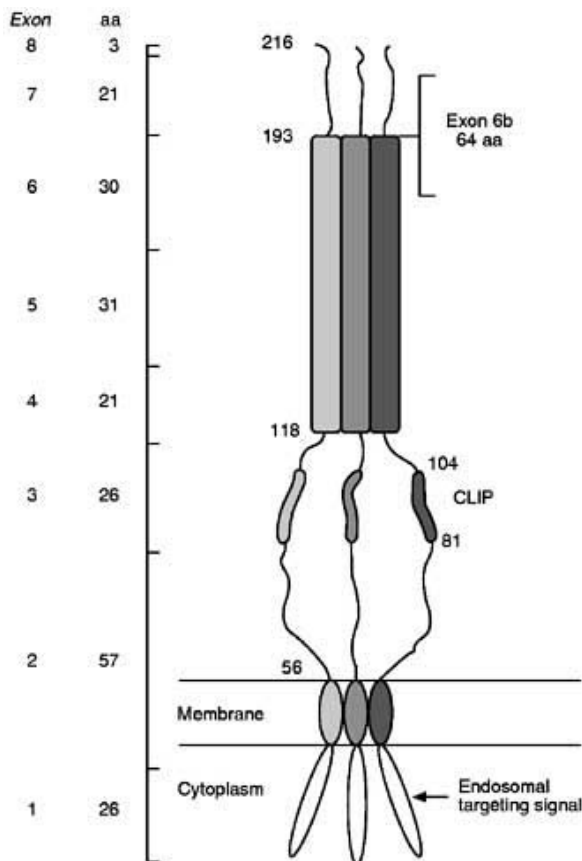


Figure 1 The overall structural organization of Ii chain, showing the important functional domains of this type II transmembrane protein.

chains, having its cytoplasmic tail at the N-terminus, followed by the transmembrane domain. The luminal portion of Ii chain responsible for its assembly into oligomeric complexes is located further towards the C-terminus.

Expression

The conserved Ii chain and polymorphic MHC class II molecules are selectively coexpressed in a tissue-specific fashion in conventional antigen-presenting cells such as B cells, macrophages and dendritic cells known to be responsible for initiating CD4⁺ T cell responses. Ii chain and MHC class II gene expression are coordinately upregulated in response to cytokines such as interferon (IFN) and interleukin 4 (IL-4). Many of the transcriptional regulatory elements mapping to their promoter regions appear largely conserved, but control of gene expression differs in at least one important aspect because Ii chain transcripts are far more abundant than MHC class II, probably reflecting in part the

actions of a transcriptional enhancer located in the Ii chain first intron.

Function

The intracellular transport route taken by newly synthesized class II molecules to the cell surface diverges from the constitutive secretory pathway used for class I export. Thus, class II molecules selectively capture antigenic peptides inside endocytic vesicles. The Ii chain contains endosomal targeting signal(s) located near the N-terminus, and has been shown to direct transport of immature class II dimers to peripheral acidic compartments. Selective Ii chain degradation subsequently results in class II peptide occupancy. As far as we know, under physiological conditions the vast majority of mature class II molecules expressed at the cell surface are devoid of Ii chain and are instead tightly occupied by an appropriate peptide ligand.

The Ii chain associates with MHC class II heterodimers to form nine subunit complexes shortly after chain synthesis in the endoplasmic reticulum (ER). As a general rule, assembly of allelically matched $\alpha\beta$ pairs does not require Ii chain coexpression, but in the exceptional case of A α^b A β^b molecules, Ii chain expression has been shown to facilitate production or maintenance of $\alpha\beta$ dimers. In the absence of Ii chain, empty class II molecules tend to aggregate, a property reversed upon occupancy of the peptide-binding site. Thus, the Ii chain serves as the functional equivalent of peptide for class II during early folding of the nascent chains, and protects the otherwise exposed, empty groove from being recognized by ER resident proteins. As for other incompletely or partially assembled oligomeric surface receptors, class II in the absence of Ii chain associates with molecular chaperones such as BiP and calnexin, thought to be responsible for quality control in the ER.

The Ii chain facilitates transport of correctly folded class II $\alpha\beta$ dimers through the Golgi apparatus *en route* to the cell surface. Under physiological conditions, association with Ii chain insures that newly formed $\alpha\beta$ heterodimers are quantitatively exported to endocytic vesicles, thus promoting selective interactions with peptides derived from internalized antigens.

Several closely related Ii chain polypeptides arise from a single Ii chain gene. Comparisons of the Ii chain gene products expressed in humans, mice and rats demonstrate a high degree of evolutionary conservation of structure and function. In humans, the 16 amino acid extension encoded by an alternative in-frame AUG start codon, has been found to contain an ER retention signal. The cytoplasmic domain

of Ii chain contains its endosomal targeting signal(s) including a di-Leu motif thought to be responsible for directing transport to acidic peripheral compartment(s).

The CLIP region

Evidence for class II association with the so-called CLIP region originally came from peptide elution studies. Transfection assays similarly demonstrate this portion of Ii chain is essential for class II chaperone activity. Recent X-ray crystal studies have given us a clear picture of CLIP peptide bound to the human DR3 class II molecule. Interestingly, the overall structure of this ternary complex shows a striking similarity to the class II groove occupied by a conventional antigenic peptide. Thus, CLIP peptide is bound in an extended conformation and has extensive atomic contacts with class II residues contributed by both α and β chains. The CLIP region present on intact Ii chain is part of an unfolded flexible domain and is thus accessible for binding to the MHC class II peptide groove.

CLIP peptide release is mediated by DM, the non-conventional class II product originally described as a facilitator of antigen presentation in mutant human cell lines. CLIP/class II complexes have also been found to accumulate on the surface of mutant mouse spleen cells lacking DM expression. Interestingly, CLIP/class II complexes in these DM mutant mice are not immunogenic and fail to stimulate CD4⁺ T cells. This demonstration of immunological tolerance provides strong evidence that under normal physiological conditions, class II maturation including CLIP removal, is an extremely efficient process.

Other functional domains

Further towards the C-terminus, the highly ordered segment of Ii chain encoded by exons 4–6 comprises a compact α -helical region necessary for trimer formation. There is some evidence that nine subunit complexes are generated via the progressive addition of three $\alpha\beta$ dimers to a core Ii chain trimer. However, experiments analyzing transfected cell lines also suggest that the Ii chain assembles with free α and β subunits.

The cysteine-rich, thyroglobulin-like domain encoded by the alternatively spliced exon 6b represents the most highly conserved portion of Ii chain. Amino acid sequence comparisons between humans, mice and rats show 90% identity. Such a degree of conservation is suggestive of an important function for this region. Two striking features of this portion of Ii chain are especially noteworthy, namely the presence

of two extra conserved sites for attachment of N-linked glycans, and the six luminal cysteines probably involved in disulfide bond formation in the strongly oxidative ER environment. Recent evidence suggests that this domain associates selectively with the lysosomal cysteine protease cathepsin L, and inhibits its activity. On the other hand, numerous experiments seem to suggest a great deal of functional redundancy shared by Ii chain p31 and p41 isoforms. Mutant mouse strains selectively expressing individual Ii chain isoforms exhibit no noticeable differences with respect to class II maturation or antigen presentation to CD4⁺ T cells. Thus the functional significance of this highly conserved region of Ii chain for the most part remains a mystery.

Consequences of invariant chain knockout

The activities of the Ii chain as a specific class II chaperone have been extensively analyzed in transfection assays and mutant mouse strains produced using gene targeting techniques. Under physiological conditions, the loss of Ii chain expression has been shown to cause a dramatic reduction in surface class II expression. As a general rule, mutant spleen cells are defective in their ability to present intact protein antigens, and have greatly reduced numbers of thymic and peripheral CD4⁺ T cells. However, exceptional antigens such as RNAase are presented in association with class II molecules via an Ii chain-independent pathway. Moreover, the extent of Ii chain influence on positive selection of individual CD4⁺ T cells is dependent on their receptor specificities. As yet, only limited information exists concerning the various functional contributions made by Ii chain to CD4⁺ T cell responses *in vivo*. It will be interesting over future years to learn more about the structure and intracellular transport of MHC class II/Ii chain complexes formed in various types of accessory cells such as those responsible for T_H1/T_H2 development, antigen-driven immunoglobulin class switching, somatic diversification, induction of autoimmune diseases, and tissue graft rejection.

See also: Antigen-presenting cells; Antigen presentation via MHC class II molecules; MHC peptide-binding specificity; B lymphocyte, antigen processing and presentation; Dendritic cells; Germinal center; H2 class II; HLA class II; Immune response (Ir) genes; Knockout, genetic; Langerhans cells; MHC, functions of; MHC restriction.

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INVERTEBRATE IMMUNE SYSTEMS

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Invertebrates, despite their lack of lymphoid immune systems, recognize and respond to nonself substances at least as efficiently as do vertebrates. Invertebrates rely on a diversity of mechanisms, some of which are inducible; however, the responses are in most cases short-lived and do not discriminate between individual pathogens. Therefore, responses mounted by invertebrates to potentially infectious agents are mediated by immune systems only in the sense that they resemble qualitatively the 'innate' or 'natural' immune responses of vertebrate myeloid cells and nonimmunoglobulin, humoral components. As invertebrates include about 95% of the extant animal species, and represent a vast diversity of organisms, from unicellular protozoans to the more complex echinoderms and protochordates, it is not surprising that a considerable range of strategies for recognition and defense against potential pathogens and parasites are to be found in these taxa.

Through his studies with starfish and water fleas in the nineteenth century, Metchnikoff established comparative immunology. His observation that foreign objects entering the body became covered with white blood cells (leukocytes) led him to consider phagocytosis and encapsulation as fundamental

processes in immune defense. In the 1970s, Hildebrand contributed substantively to the development of a global perspective on the evolution of cellular and humoral immune competence across the spectrum of animal diversity. More recent studies by Boman established the usefulness of invertebrate models for the understanding of antibacterial strategies, some of which have merited conservation throughout the evolution of the chordate lineages.

Invertebrate internal defense responses exhibit common themes such as phagocytosis and encapsulation, but the underlying molecular recognition and effector mechanisms are diverse. Some of the factors involved, such as α_2 -macroglobulins (α_2 -M), C reactive proteins (CRP), antibacterial peptides, serine proteinases and proteinase inhibitors, have been substantially conserved through the evolutionary lineages leading to the chordates, whereas others, such as C-type lectins and complement-related factors, retained only those regions of the molecule that are relevant to recognition/effector functions. Finally, for other factors such as glucan-binding proteins (crustaceans) and some antibacterial peptides, no homologs have been identified in vertebrates so far, and they appear to be restricted to invertebrates. The

potential for finding novel molecules or mechanisms in invertebrates has focused attention on a growing number of species as model organisms, and progress has been substantial during recent years.

Cellular and humoral immune responses

While some leukocytes in protochordate invertebrates (close relatives of the vertebrates) have been termed 'lymphocyte-like cells', evidence that these are homologs of vertebrate lymphocytes remains tenuous: these animals lack the rearranging genes and clonal expansion that characterize the vertebrate lymphoid receptors for antigen.

Among both primitive (acoelomate) taxa, such as the sponges and the corals, and more derived (coelomate, with blood circulatory systems) taxa such as the tunicates (sea squirts, phylum Chordata), many species live permanently fixed to solid substrata where competition for living space is severe, and the risk of being overgrown by neighbors is constant. Here, the ability to preserve one's genome (and pass it on through reproduction) is dependent on aggression toward neighbours. Such responses are effected by cellular and molecular components of the interacting individuals. The kinetics of rejection (establishment of a necrotic zone where contact has occurred) is reduced on second or later encounter. This heightened reactivity is directed specifically at the genotype of the individual that was previously encountered; rejection responses to third party encounters follow kinetics similar to those of first encounters. These responses thus resemble vertebrate graft rejection responses in that they involve leukocyte-mediated alloaggression with specific memory! The fine discrimination of cell surface markers implied by these phenomena is also essential for correct tissue differentiation in all Metazoa. This ability to discern specific markers within a species should not be surprising, since even the earliest free-living eukaryotic amoebae must have been able to distinguish nutritive particles worthy of ingestion (food) from inorganic detritus, and potential mates from potential competitors/aggressors. Mating types, known for modern unicellular eukaryotes (e.g. yeast, *Paramecium*), are identified by means of specific carbohydrate markers (epitopes) on cell surfaces. Also, a diversity of cell surface markers and receptors able to distinguish these is essential for the maintenance of self-sterility and nonself-fertility in plants and animal gametes.

So-called alloaggressive responses occur also in nonsedentary (free-moving), noncolonial invertebrates. Leukocytes from different individual sipunculid worms and echinoderms kill one another on

encounter *in vitro*. And body wall allotransplants made between earthworms, between ribbon worms, between echinoderms, and between solitary tunicates meet fates like those described above for sponges, corals and colonial tunicates. It is surprising to find complex invertebrates in which alloaggression appears to be lacking. This is the case in molluscs (snails) and in insects, where nonself-recognition mechanisms remain poorly understood. Nevertheless, a fascinating variety of effector mechanisms in these animals is responsible for defense against microbial and parasitic infection.

The great variety of blood cells in invertebrates precludes meaningful treatment here. Suffice to say that cells containing respiratory pigments (usually hemoglobin) for the transport of oxygen occur in a small percentage of species. Other pigments, thought not to transport oxygen, occur in echinoderm (sea urchins) granular cells; their functions are unclear. In the majority of taxa, leukocytes (nonpigmented, free cells) are diverse. In primitive species lacking circulatory systems, amoebocytes wander throughout the body, scavenging foreign and damaged material and, in some cases, contributing significantly to the nutrition of the organism. Most invertebrates have not only discrete nervous, excretory and musculoskeletal systems, but circulatory systems as well. In annelids (earthworms and their relatives) and echinoderms, more than one coelomic compartment contains free cells, and the role of scavenger may be met by distinct cell types in distinct locations. Among the more intriguing of the scavenging cells are the motile free cells of sipunculids and the vibratile cells of echinoderms. Common to all taxa, however, are phagocytic cells resembling vertebrate macrophages.

The fate of most foreign particles entering the body of a metazoan invertebrate is to be phagocytosed or, if too large, encapsulated by leukocytes. Fixed phagocytes, analogous to the reticulo-endothelial system, occur in crustacean gills and in the midgut glands of crustaceans and molluscs. All invertebrates, even those lacking body cavities – like sponges, anemones and flatworms – contain phagocytes, and in some species there are more than one phagocytic cell type. Like vertebrate macrophages, these cells orchestrate inflammatory responses, synthesize and use lysosomal enzymes, and possess cytotoxic mechanisms dependent on superoxide and its metabolites.

In addition to phagocytosis, both encapsulation and nodule formation occur in invertebrates. Encapsulation is the net result of frustrated phagocytosis by many leukocytes. Metazoan endoparasites, unless they enter rare susceptible host individuals, elicit encapsulation, often with consequent death to the

invader through toxic intermediates from an enzymatic cascade resulting in melanization. One recognition/effector mechanism, characterized in crustaceans, is the prophenoloxidase activating system that is present in many invertebrates and yields cytokines. A plasma protein binds microbial polysaccharides (LPS or β 1–3 glucans) and induces activation of a prophenoloxidase-activating enzyme that cleaves the proenzyme prophenoloxidase yielding phenoloxidase. This oxidizes phenols to quinones that polymerize and form melanin, all exhibiting antimicrobial properties. Encapsulation may also involve sclerotization, an essential reaction in cuticle development. Nodule formation occurs in insects when large numbers of bacteria enter. In addition to phagocytosis, encapsulation and nodulation, these cells can exhibit cytotoxic responses against foreign pro- and eukaryotic cells.

The interaction of hemocytes with foreign materials can trigger clotting of plasma, limiting the spread of microbes in the body. In horseshoe crabs, hemolymph contains abundant proteins (hemocyanin, limulin/CRP and α_2 -M) and two types of hemocytes. The first – granulocytes – contain two types of secretory cytosolic granules that selectively store proteins participating in defense mechanisms. L-granules contain clotting factors, protease inhibitors (serpins and cystatin), anti-LPS factor and lectins with LPS-binding or bacterial-agglutinating activity. S-granules contain basic proteins with antimicrobial or agglutinating activity. Except coagulogen, all the clotting factors are serine proteases activated by limited proteolysis. LPS from gram-negative bacteria and (1–3)- β -D-glucans from fungi activate factors C and G respectively, triggering the clotting cascade. In synergy with other aforementioned factors, this comprises an effective host defense against pathogens. Hemocytes of the American horseshoe crab (*Limulus polyphemus*) have provided commerce with a sensitive probe for endotoxin.

Recognition molecules

While lacking the rearranging receptor genes of vertebrate lymphocytes, invertebrates do produce members of the immunoglobulin superfamily. In *Drosophila*, these include cell adhesion molecules (fasciclins, neuroglian and lachesin), secreted molecules (amalgam and IMP-L2), receptor-linked protein tyrosine phosphatases (DLAR and DPT), a growth factor (DFGF) and a hybrid protein (peroxidasin). Receptors for tyrosine kinases occur in the sponge *Geodia* and the nematode *Caenorhabditis*. However, within the Ig superfamily, only hemolin, a protein isolated from lepidopterans, can be

induced upon bacterial challenge. A function in non-self recognition has been proposed. Although hemolin exhibits four immunoglobulin-like domains, their primary structure is closer to cell adhesion molecules than to immunoglobulins, and hemolin actually may modulate hemocyte adhesion.

Lectins (carbohydrate-binding proteins) are widespread components of invertebrate fluids and tissues. Commonly multivalent, these molecules, either constitutive or inducible, can aggregate microbes with appropriate saccharide moieties on their surfaces, thereby limiting the spread of potential pathogens. Furthermore, conformational changes of the lectin upon binding to ligand can facilitate phagocytosis. In vertebrates, some calcium-dependent lectins including mannose-binding proteins and the pentraxins CRP and SAP are acute phase proteins, and are involved in innate defenses by way of opsonization and activation of complement. Based on primary structures, *bona fide* homologs of vertebrate pentraxins such as CRP occur in horseshoe crabs, snails and tunicates. The *Limulus polyphemus* CRP is a hexameric structure with a certain degree of polymorphism and not only recognizes carbohydrate structures but also is hemolytic. Together with antibacterial peptides, lysozyme, protease inhibitors and other effector factors, lectins constitute an ancient but efficient defense system. As in vertebrates, lectins in invertebrates function both in recognition and as effector molecules, by direct antibacterial activity or by promoting opsonization/melanization.

Phagocyte surface receptors for nonself are evident when phagocytosis of foreign particles like yeast, bacteria, and erythrocytes occurs in physiological media lacking host molecules – a common observation.

Effector molecules

The most fully understood system of invertebrate defensive molecules is a group of substances synthesized by moths and flies in response to gram-negative or gram-positive bacterial peptidoglycans. In some insects, lysozyme titers also rise in response to this stimulus. Not only have many of these peptides been isolated and sequenced, but their genes and the regulation of their expression have been characterized. Antibacterial peptides are classified into several families: lysozymes, cecropins, sapecins or insect defensins, attacin-like, and proline-rich antibacterial peptides. Some occur even in vertebrates. There may be considerable sequence diversity within each family. Some, such as the cecropins, are targeted broadly and are bactericidal whereas others, such as the attacins, have restricted antibacterial spectra and are

bacteriostatic. Defensive effector molecules must efficiently target putative pathogens without affecting host cells. It appears that, because fatty acids are distributed differently in the prokaryotic and eukaryotic membrane lipid bilayers, antibacterial peptides may selectively target microbial membranes and not those from the invertebrate host. Multiple antibacterial peptides can be synthesized in insect fat bodies and secreted into the blood at relatively high concentrations following immune challenge.

Like some lectins, antimicrobial peptides may have roles in both defense and development. Sapecin, a potent bactericidal peptide from *Sarcophaga*, can be synthesized in larval hemocytes and the fat body in response to body wall injury. However, sapecin is expressed in the embryonic and in the early pupal stages in the absence of exogenous stimuli, and it may be a growth factor for embryonic cells. Some of these defensive molecules have been produced *in vitro* in insect cell, tissue and organ cultures.

The ability to lyse vertebrate erythrocytes (models for more biologically relevant targets), either alone or in combination with cobra venom factor and/or vertebrate serum, occurs in some invertebrate body fluids. Although in some the hemolytic activity is due to phospholipase C-like factors, other lytic factors resemble complement in being heat labile and/or divalent metal ion dependent. In vertebrates, several members of the complement system are mosaic or hybrid molecules constituted by several modules and domains. Complement-related proteins, their modules or domains have been identified in invertebrate species: these include CCP, TSRLDL, C1r/2, EGF, vWF-A, CD5 and the C-type lectin. In echinoderms, there is evidence of a complement-like system which can opsonize erythrocytes for mammalian phagocytes. Echinoderm phagocytes appear to possess complement receptors CR3 and possibly CR1.

Cascades of endogenous serine proteinases (e.g. coagulation and prophenoloxidase activation), important defense responses of invertebrates, require precise modulation in order to avoid damage to host tissues. In vertebrates, complement activation and plasma coagulation are regulated by proteinase inhibitors such as serpins, and equivalent mechanisms are present in invertebrates. Amino acid sequences of invertebrate proteinase inhibitors allow classification into families defined for those from vertebrate origin. For example, α_2 -M, a proteinase inhibitor containing an intrachain thioester bond and structurally related to the vertebrate complement system, has been purified as a 180 kDa dimer in *Limulus*, lobster, crayfish, crab and octopus, and as a tetramer in snails. However, other proteinase inhibitors isolated from some arthropods and one echino-

derm species do not appear to be related to any previously characterized families. In addition to the regulation of endogenous enzymes, proteinase inhibitors may protect invertebrates from exogenous proteinases produced by, for example, entomopathogenic fungi. Finally, other inducible effector factors elicited by toxins represent interesting avenues for future research in invertebrate immune responses. Cockroaches injected with toxoids from bee or snake venom acquire resistance to the otherwise lethal toxins. This response is accompanied by the appearance in the hemolymph of a 102 kDa molecule which precipitates the toxins. Responses to the two toxoids are purported to be specific, and an anamnestic response has also been postulated.

Regulation of recognition/effector responses

Recruitment and activation of effector cells that initiate the acute phase response are mediated in vertebrates by cytokines such as interleukins 1 and 6, and tumor necrosis factor α (TNF α). In starfish and tunicates, cytokine-like activity (presumably mediated by factors equivalent to IL-1 and IL-6) has been identified. So too have specific receptors on phagocytic cells. Invertebrate putative cytokine-like factors may participate in the regulation of non-specific responses to tissue damage and infection. If these factors are homologous to the vertebrate equivalents, additional roots of the vertebrate immune system may be detected within invertebrate phyla of the deuterostome lineage, from which vertebrates are considered to have arisen.

Chemotaxis and cell adhesion may be modulated in invertebrates by opioid peptides, opiate alkaloids and other neuropeptides. There is evidence of these factors and their receptors in the mussel *Mytilus edulis*. At the genetic level, however, *Drosophila* has resulted in the best model system for analyzing the regulation of immune responses: at least ten genes coding for antibacterial peptides have been cloned and structurally characterized. Their transcriptional profiles and the control of their expression during the immune response have been defined. The *cis*-regulatory elements appear to be homologs of promoter regions of mammalian acute phase response genes, such as NF-IL6, IL6-RE BP, κ B-related, Oct 1, NF-ELAM1 and HNF5. It appears that several inducible transactivating factors are involved in the coordinated expression of insect immune genes and some share characteristics with the *rel*-related systems, namely the factor NF κ B and the *dorsal* morphogen.

Conclusions

Beyond the intellectual interest in function and evolution of invertebrate immune systems, from the practical standpoint there is a clear need to address a variety of problems related to disease prevention and remediation for economically and ecologically important invertebrate species, as well as those that serve as vectors of disease to humans. Marine and freshwater fisheries and aquacultured crustaceans (shrimp, prawn and lobster) and molluscs (oysters, mussels and clams) now constitute significant sources of food, employment and commerce. Despite considerable improvements in the design and implementation of production techniques, the ability to prevent mass mortalities due to viral, bacterial and protozoan epizootics has not made comparable progress; catastrophic losses in productivity are becoming increasingly frequent. There is a continuing need for intense research in this area.

See also: Autoimmune disease, pathogenesis; Cytokines; Endotoxin (lipopolysaccharide (LPS)); Innate immunity; Lectins; Opsonization; Phagocytosis; Phylogeny of the immune response; Prokaryotes, immunity in.

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IRIDOVIRUS, INFECTION AND IMMUNITY

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The Iridoviridae family of viruses are large, icosahedral, double-stranded DNA viruses that infect a wide range of both vertebrate and invertebrate hosts. This family currently consists of five recognized genera (Table 1) with the iridoviruses being those that infect invertebrates (mostly insects). To date 33 iridoviruses have been classified with major studies being carried out on *Tipula* iridescent virus (TIV), *Chilo* iridescent virus (CIV) and *Wiseana* iridescent virus (WIV). The term 'iridescence' stems from the blue hue initially observed in the TIV-infected larvae of the crane fly *Tipula paludosa*; however, this phenomenon is not consistent with all infections.

Characteristics of iridoviruses

Virion structure

Virions are comprised of an icosahedral outer capsid, an internal lipid membrane and an electron-dense central core which contains the viral DNA. The capsid is made up of a single polypeptide of about 50 kDa in size, which is approximately 40% of the total mass of the virus. This protein has been shown to be highly conserved throughout the Iridoviridae family and therefore is of use in comparison studies. The tight packing of these icosahedral capsids can form a crystalline array (Figure 1) which diffracts

Table 1 Classification of the Iridoviridae family

Genus	Vernacular name	Host species	Type species
<i>Iridovirus</i>	Small iridescent insect virus	Diverse invertebrates (mostly insects)	<i>Chilo</i> iridescent virus (CIV)
<i>Chloriridovirus</i>	Large iridescent insect virus	Diptera (mosquitoes)	IV Type 3 from <i>Aedes taeniorhynchus</i>
<i>Ranavirus</i>	Frog virus	Amphibia	Frog virus 3 (FV3)
<i>Lymphocystivirus</i>	Lymphocystis disease virus	Fish	Lymphocystivirus type 1 (LCDV-1)
Goldfish virus-like	Goldfish virus	Goldfish	Goldfish virus (GFV-1)

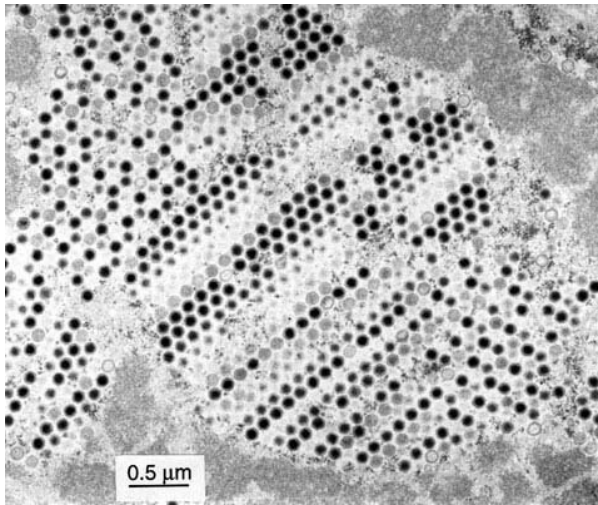


Figure 1 Electron micrograph of *Wiseana* iridescent virus. Cross-section of an infected insect showing virions in a crystal-line array.

light, causing a distinctive iridescence; however, this phenomenon has no biological significance.

The number of proteins detected for each iridovirus varies depending on the method and conditions of analysis. CIV has been shown to consist of at least 20 polypeptides, ranging from 10 to 213 kDa, with reports of up to 50 polypeptides by two-dimensional gel electrophoresis. Structural polypeptides of TIV have been shown to be nonglycosylated. Closely associated with the capsid is the internal lipid membrane, through which proteins pass, connecting the outer capsid to the inner core polypeptides. The lipid composition of the iridovirus membrane differs significantly to the lipids of the host cell membrane, indicating that their synthesis is virally encoded. The inner core is highly hydrated and comprises DNA packed with DNA-binding proteins. The DNA is linear, double stranded and nonmethylated and exhibits cyclically permuted terminal redundancy. CIV has a terminal redundancy in which 12% of the genomic DNA is repeated at the termini of the genome. This

feature is unique for eukaryotic viruses and it is more commonly observed in prokaryotic viruses such as the bacteriophages T4 and P22.

Host range and classification

Iridoviruses vary in their host range: some exhibit a broad range of host species while others are restricted to one or two specific hosts. Both TIV and CIV have a wide host range, infecting dipteran, coleopteran and lepidopteran hosts. CIV has been shown to infect over 100 insect species, woodlice, centipedes and two species of Crustacea. The greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) is highly permissive to most iridoviruses and is used for general virus propagation. Iridoviruses infecting insects have not been found to infect any vertebrates. The current classification scheme for these viruses uses names according to the host species of initial isolation and a type number relating to the chronological order of discovery. A system using PCR sequencing and DNA–DNA hybridization methods is currently being investigated for classification of iridoviruses.

Replication

These viruses have a low pathogenicity and the frequency of subclinical infections (those not exhibiting iridescent coloration) is probably high. Cannibalism and wounding are the likely routes of infection, although entry via spiracles, transovarial transmission and ingestion have also been proposed. The iridescence of infected individuals is not a reliable diagnostic criterion and has in the past lead to many false, negative results. The primary site of infection is thought to be the hemocytes and fat bodies of the larvae, with eventual spread to the entire body via the hemolymph. Virus particles enter the insect cell via phagocytosis and are incorporated into lysosomes where the virus is uncoated and replication initiated. Frog virus 3, isolated from the leopard frog *Rana pipiens*, has been shown to synthesize viral

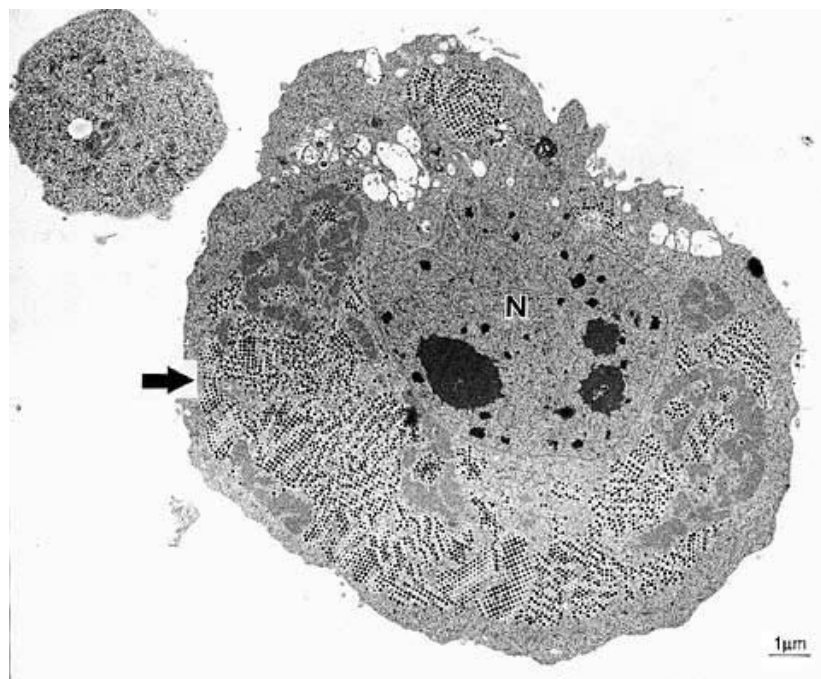


Figure 2 Electron micrograph of a *Wiseana* iridescent virus-infected *Spodoptera frugiperda* cell. Arrow indicates region of virus assembly in the cell cytoplasm. N, cell nucleus.

DNA in two stages. DNA is first synthesized in the cell nucleus in genome lengths, then transported to cytoplasm where it is replicated as a large concatemer of DNA. This two-step replication of DNA is a feature unique to the Iridoviridae family and it enables the linear genome to be replicated completely, resulting in the headful packaging of DNA and the terminal redundancy. Capsids are preformed in the cytoplasm where packaging occurs (Figure 2) and complete virions can be released via budding from the cell membrane or by cell lysis. Iridoviruses induce a shutdown of host macromolecular synthesis while utilizing some machinery for replication. This shutdown eventually leads to host death, although not until the individual is highly infected: in some cases up to one-third of the insect body weight can be virus.

Virus–host responses

The immune system of the insect is simple in comparison to that of the vertebrate. Primarily there is a cellular response comprising phagocytosis, encapsulation, nodule formation or coagulation to recognize 'foreignness'. Inducible substances such as attacin, cecropins, lysozyme and phenol oxidase have been isolated from a variety of dipteran and lepidopteran species and may play a toxic role in the defense mechanism. There is no evidence of specific antibody–antigen reactions, a complement system or interferon and any immunity is short lived and of a nonspecific nature. As infection predominantly

occurs in the insect larval stage, which exists for about 3 weeks, there is little reason for the evolution of an acquired immune system. CIV has been shown to encode a putative apoptosis-inhibiting factor, thus enabling the virus to prevent the programmed death of a cell in response to infection. In general, iridovirus infection of insect populations is a welcome occurrence as they are seen as a form of biological control. However, infection of a honey bee species is of concern to the honey industry. Within the Iridoviridae family there are also examples of viruses that infect fish, lymphocystis disease virus (LCDV-1), and frogs (FV3) which are important to both the fisheries industry and environmental agencies. However, the immune response to infection with these viruses remains to be investigated in both fish and amphibians.

See also: **Amphibian immune system; Apoptosis; Fish immune system; Invertebrate immune systems; Iridovirus, infection and immunity; Vaccines; Viruses, immunity to.**

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IRON AND THE IMMUNE SYSTEM

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Iron deficiency is the most common nutritional deficiency in populations around the world, especially in women, infants and children. In addition to the role of iron as an integral component of many proteins and as a cofactor for enzyme systems, it appears to be active in the immune response. Both iron deficiency and iron excess can alter immunocompetence (Table 1).

Effect of iron status on immunocompetence

Iron has a dual role in its association with immune function. First, micronutrients, such as iron, are redistributed in the body during infection. Second,

dietary deficiency of iron causes iron-deficiency anemia and decreased immunocompetence. The effects of iron excess depend on the route of entry of iron.

Iron is required for growth and proliferation of microorganisms. Upon invasion of a host by bacterial microorganisms, iron is rapidly redistributed in the host to stunt proliferation of bacteria by removing iron from circulation and storing it in organs, such as the liver and spleen. Redistribution of iron during infection can be misinterpreted as iron deficiency anemia by hemoglobin and hematocrit measurements. Upon recovery from infection, the iron status of the host will return to normal, assuming that iron status was normal before the onset of infection. This redistribution of iron during

Table 1 Effects of iron status on immune response components

Immunity	Effect of iron deficiency	Effect of iron overload
Immunocompetence		
Response to infection	↓ (humans)	↑ (humans, rats)
Growth of <i>E. coli</i> and <i>S. aureus</i>		↑ (humans)
Humoral Immunity		
Antibody production	↑ (humans), ↓ (rats)	
IgM response to sheep red blood cells		↓ (mice)
Opsonization		↓ (humans)
Cell-mediated Immunity		
Cellularity of spleen/thymus	↓ (rats)	
Circulating T cells	↓ (humans, mice)	
B and T cell response to mitogens	↓ (humans, rats, mice)	↓ (humans, mice)
Hypersensitivity response	↓ (humans, mice, guinea pigs)	
NK cell cytotoxicity	↓ (rats, mice)	↓ (humans)
CD8 T cells		↑ (humans)
Granulocyte phagocytosis		↓ (humans)
Cytokine production		
Macrophage inhibitory factor	↓ (humans)	
Interferon	↓ (rats)	
Interleukin 1	↓ (rats)	
Interleukin 2	↓ (humans)	

infection has a protective role for the host. Repletion of iron in malnourished children of poor populations by injection of iron dextran or oral iron supplements has been found to increase the incidence of infection. The excess iron was most likely increasing bacterial proliferation rather than altering immunocompetence.

Effect of iron deficiency on susceptibility to infection

Decreases in response to infection have been demonstrated in iron-deficient humans and animal models. The first study of the effects of iron deficiency on susceptibility to infection in infants was conducted in the late 1920s by Mackay, who recommended the use of iron fortified cow's milk. Iron fortification resulted in increased hemoglobin concentration and a 50% reduction in respiratory and gastrointestinal infections. In the multitude of studies conducted over the past 70 years, children with iron deficiency have demonstrated either increased or unchanged morbidity. Some studies support the hypothesis that iron deficiency may protect against certain infections. Increased infections after high-dose iron supplementation and/or a hypoferremic response in the host after microbial invasion have been documented in both humans and rats. Conflicting results are probably due to differences in experimental design, methodology and data collection. The presence of health and nutritional problems, as well as differences in socioeconomic conditions, contributes to variations in results.

In a number of clinical studies using different population groups, impairments in chemotactic activity and bactericidal capacity, and in the generation of an oxidative burst by the hexose monophosphate shunt have been reported in neutrophils of noninfected iron-deficient children compared with age-matched controls. No defects in opsonization and phagocytosis were found in iron-deficient subjects. Generation of an oxidative burst and bacterial capacity could be restored with 2 weeks of oral or parenteral iron treatment. In peripheral blood of iron-deficient rat pups, generation of an oxidative burst in phagocytes was decreased even though the percentage of phagocytes was increased. Myeloperoxidase, an iron-containing oxidative enzyme, has been reported to be either normal or decreased in iron deficiency. Defective destruction of organisms by oxidative substances normally produced in the myeloperoxidase pathway may contribute to decreased bactericidal capacity of phagocytes during iron deficiency.

The iron chelator desferrioxamine has been shown

to have antiproliferative effects on tumor growth and infections such as malaria. These observations are likely to be due to the direct action of the desferrioxamine, and not to its iron-chelating ability.

Effect of iron deficiency on humoral immunity

Iron-deficient children demonstrate either normal or elevated basal or stimulated antibody production to diphtheria and tetanus toxoid antigen, suggesting that humoral immunity is not impaired by iron deficiency. In contrast, use of a Jerne plaque assay in iron-deficient rat pups revealed decreased antibody formation to sheep red blood cell antigen. Dietary repletion of pups improved iron status, but did not correct immunoglobulin M (IgM) and IgG antibody production. Maternal iron deficiency resulted in irreversible impairment of antibody production which was not corrected by short-term iron repletion. Human implications of these findings in rat studies are important because many pregnant women develop iron deficiency.

Effect of iron deficiency on cell-mediated immunity

Cellular immunity is impaired in animals and humans with iron deficiency. Cellularity of immune tissues, such as spleen and thymus, is decreased in iron-deficient neonatal rats, and not easily repaired by iron repletion. A decreased percentage of circulating T cells has been found in iron-deficient children and mice. A decreased blastogenic response of T cells to concanavalin A and phytohemagglutinin and/or B cells to lipopolysaccharide has been found in iron-deficient humans and rodents. Since the iron-dependent enzyme ribonucleotide reductase is required for DNA synthesis, decreased cell proliferation may be due to decreased DNA synthesis. A depressed delayed hypersensitivity response has also been found in adult humans, mice and guinea pigs with iron-deficiency anemia.

Natural immunity against malignancy may be impaired in iron deficiency. Rat pups from iron-deficient dams have impaired peritoneal macrophage cytotoxicity. In iron-deficient rats and mice, decreased capacity of stimulated natural killer (NK) cells to kill tumor cells has been reported. Iron deficiency may decrease the responsiveness of effector cells toward the target tumor cells, decrease the percentage of effector cells capable of killing, or decrease the functional capacity of effector cells to kill once activated.

Effect of iron deficiency on cytokine production

Interferon is a cytokine that is produced by macrophages and activates NK cell cytotoxicity against virus-infected cells or tumor cells. Interferon added *in vitro* does not restore the cytotoxic capacity of iron-deficient NK cells to control levels. It appears that the functional capacity of interferon as a mediator of cellular immunity is altered by iron deficiency. Interleukin 1 (IL-1) is an important regulator of the acute phase response to an infection. Production of IL-1 by peritoneal cells is decreased in iron-deficient rats. IL-1 stimulates T cells to produce IL-2. Iron deficiency in children, and in rats, causes a decrease in IL-2 production. Decreased production of macrophage inhibitory factor has been demonstrated in adults with iron deficiency anemia.

Effect of iron excess on immunity

Iron excess has been associated with increased susceptibility to infections and abnormal neutrophil and lymphocyte function. Patients with iron overload undergoing dialysis showed decreased phagocytosis and bactericidal capacity of blood neutrophils. Hemochromatosis patients with elevated levels of storage iron were reported to have elevated numbers of CD8 T cells and impaired ability of peripheral blood mononuclear cells to respond to mitogens. Increased transferrin saturation in cancer patients receiving chemotherapy has been associated with increased *in vitro* growth of *Escherichia coli* and *Staphylococcus aureus*. NK cell cytotoxicity against K562 target cells was decreased in patients with β -thalassemia major who are iron overloaded as a consequence of chronic transfusion therapy. In patients with β -thalassemia intermedia, decreased opsonization and granulocyte phagocytosis, decreased serum immunoglobulin levels, and changes in B and T cell numbers and function have

been reported. Iron-loaded mice were reported to have reduced IgM antibody response against sheep red blood cells, but no changes in IgG antibody response were seen. Iron-loaded mice exhibit impaired mononuclear cell responses to various mitogens.

Conclusions

A number of studies have demonstrated that iron deficiencies and states of iron excess compromise the immune system. Further research needs to concentrate on biochemical, metabolic and genetic aberrations caused by altered iron status to discover the mechanisms causing compromised immunocompetence associated with altered iron status.

See also: Immunodeficiency, secondary; Microbicidal mechanisms, oxygen-independent; Transferrin receptor (CD71).

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ISCOM (IMMUNO-STIMULATING COMPLEX)

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ISCOM is a delivery system for antigen and adjuvant together in the same particle. The unique component of ISCOM is a mixture of *Quillaja* saponins extracted from the bark of the tree *Quillaja saponaria* Molina. Three groups of *Quillaja* components

with a triterpenoid structure (QH-A, QH-B and QH-C) have been characterized by reverse-phase high-pressure liquid chromatography (HPLC) and have been functionally identified to be of particular interest for ISCOMs. They differ in terms of adjuvant

activity, ISCOM-forming capacity and toxicity. A defined composition of QH-A and QH-C called Iscoprep 703™ is now used in ISCOM vaccines in human clinical trials.

The ISCOM system was first created to make antigens optimally immunogenic by:

1. presenting the antigens in a physically immunogenic form, i.e. several copies in a submicroscopic particle to resemble that of an infectious agent;
2. optimizing the targeting of antigen and adjuvant to lymphatic organs and lymphoid cells by enclosure of adjuvant and antigen in a stable, uniform particle;
3. optimizing the immunomodulatory capacity by presenting the adjuvant and antigen components in the same particle, thus reducing the amount of antigen and adjuvant required to enhance the immune response.

Preparation of ISCOM–antigen complexes

ISCOM is composed of a matrix (Figure 1) contain-

ing *Quillaja* saponins, cholesterol and phospholipid at a molar ratio of about 1:1:1. The assembly of ISCOM is facilitated by hydrophobic interaction between these components and the antigen. The ISCOM antigen may be an envelope protein of a native virus, a cellular membrane protein, or peptides containing hydrophobic domains or any antigen with a hydrophobic domain. In the case of virus, the envelope is disintegrated by a detergent, preferably nonionic, to release a protein–detergent complex (Figure 1). Upon the removal of the detergent (e.g. by dialysis or density gradient centrifugation) in the presence of *Quillaja* saponins, cholesterol and additional lipid, ISCOM assembly takes place. Primarily nonamphipathic proteins (e.g. gp120 of human immunodeficiency virus (HIV)) may also be integrated into ISCOM by refolding the protein to expose certain hydrophobic sequences, which are then available to form hydrophobic interactions. By efficient coupling methods, peptides may be conjugated to the external protein moiety of an ISCOM, which will then act as a carrier providing T cell help for a peptide antigen.

Electron microscopic analysis of ISCOMs shows

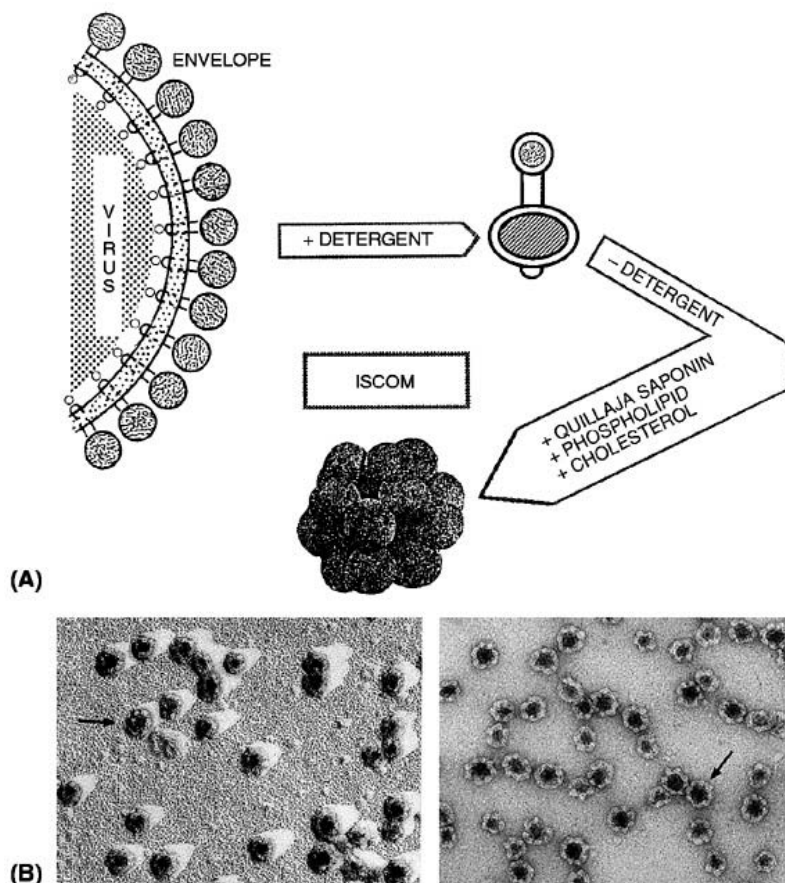


Figure 1 (A) Schematic representation of the disintegration of a viral envelope by a detergent, thus releasing a protein–detergent complex. By removal of detergent, in the presence of cholesterol, phospholipid and *Quillaja* saponin, the amphipathic proteins are assembled into a symmetrical shell of globular subunits. (B) Electron micrographs of ISCOM particles containing HIV proteins (arrows indicate hexagonal outline of subunits).

Table 1 Protective immunity induced by ISCOMs containing proteins derived from various microorganisms

Protein	Animal	Disease
Hemagglutinin (H) and neuraminidase (N) of influenza virus	Mouse Horse	Pneumonia, fatal Pneumonia, general infection
Glycoprotein of rabies virus	Mouse	Fatal
Measles virus hemagglutinin (H) protein	Mouse	Encephalitis, fatal
Measles virus fusion (F) protein	Mouse	Encephalitis, fatal
Bovine virus diarrhea virus	Sheep	Abortion
Feline leukemia virus	Cat	Viremia
Epstein-Barr virus	Tamarin monkey	Tumor
Equine herpes 1 virus	Hamster	Lethal
Equine herpes 2 virus	Horse	Pneumonia
Bovine herpes 1 virus	Cattle	Pneumonia
Pseudorabies virus	Lamb	Lethal
Pseudorabies virus	Swine	Weight loss, illness
Herpes simplex 1 virus	Mouse	Lethal
Herpes simplex 2 virus	Mouse	Lethal
gp120 of SIV	Monkey	Lethal infection
gp125 of HIV-2	Monkey	Viremia
gp120 of HIV-1	Monkey	Viremia, challenge with a chimeric HIV-1/SIV

40 nm cage-like structures exhibiting icosahedral symmetry with a number of morphological subunits. Multiple copies of antigens are exposed on the ISCOM particle. The construct is physically remarkably stable; it can be frozen and freeze-dried and dissolved in various buffers. Lyophilization may thus be useful for storage of ISCOM vaccines.

Immunogenicity of antigens borne by ISCOMs

Antibody-mediated immunity is efficiently induced by parenteral administration of antigens included in ISCOMs. In mice, the immune response was found to be a classical one, with an initial immunoglobulin M (IgM) response followed by an IgG response. The IgG response is distributed in all subclasses but the IgG2a response is dominant. In general, the antibody response to an antigen in an ISCOM is about 10 times higher than that induced by the same dose of the killed virus particles in saline or in micellar form.

After local intranasal administration of the hemag-

glutinin or neuroaminidase envelope proteins of influenza virus in ISCOMs, a high serum antibody response of the same order of magnitude as that following parenteral immunization was observed. A secretory IgA response is also efficiently evoked after intranasal immunization both at local respiratory mucosa and distant, e.g. genital tract, mucosa. In general, antigens derived from virus envelopes or cell membranes incorporated into ISCOMs retains biological activity and conformation provided their isolation is carried out under mild conditions. Thus, the biological activities of influenza virus surface glycoproteins borne by ISCOMs are conserved, facilitating the penetration of mucus, which explains their efficiency in evoking mucosal immune responses.

ISCOMs efficiently stimulate antigen-presenting cells (APCs) to produce interleukin 1 (IL-1) and IL-12. Other cytokines such as IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNF α) are also induced. The ISCOM induces strong T cell responses of the T_H1 type characterized by delayed-type hypersensitivity (DTH) and enhanced production of interferon γ (IFN γ) and IL-2, while IL-5 is enhanced IL-4 is not generally enhanced and IL-10 is downregulated. In general, ISCOMs are excellent inducers of IL-2 and IFN γ .

ISCOMs efficiently induce antigen-specific cytotoxic T lymphocytes (CTLs) following either parenteral or mucosal routes of administration. It appears that the protein antigens contained in ISCOM can be delivered not only to endosomes but also to the cytosol of APCs, leading to cytosolic processing, transportation to the endoplasmic reticulum, presentation by major histocompatibility complex (MHC) class I molecules to T cells, and a subsequent CD8⁺ T cell response. For example, ISCOMs containing the gp160 of HIV-1 have been shown to induce MHC class I restricted CD8⁺ CTLs. The induction of relevant cytokines such as IL-12, IL-2 and IFN γ is an additional benefit.

Protective immunity has been induced against a variety of microorganisms including viruses, bacteria, mycoplasma and parasites. Of particular interest is that protective immunity was induced by ISCOM-borne antigens to a number of retroviruses, e.g. feline leukemia virus infection in cats, to SIV, HIV-1 and HIV-2 in primates, to a tumor-inducing herpesvirus (e.g. Epstein-Barr virus) in cotton top tamarin monkeys, and against lethal infection of mice with *Trypanosoma cruzi* causing Chagas' disease in humans. Table 1 lists examples of ISCOM-borne antigens having induced protective immunity.

See also: Adjuvants; Immunogen; Immunopotent-

ation; Liposomes; Vaccination, methods of administration; Vaccines.

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ISOELECTRIC FOCUSING

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Isoelectric focusing (IEF) is an electrophoretic procedure capable of extremely high resolving power. During IEF, zwitterionic molecules are separated according to their isoelectric point (pI; defined as the 'pH at which a zwitterion possesses no overall charge'), and the technique can therefore be used to analyze the composition of a mixture and/or to determine the precise pI of a component under investigation. Although usually applied to proteins or protein-containing extracts, the method can, at least theoretically, be used for analysis of any zwitterionic species. Methodology has been developed to allow IEF to be carried out on analytical and preparative scales.

Principles

The underlying basis of IEF can be simplified to the following principles. If a zwitterion is not at its pI it will, by definition, possess an overall charge that will depend upon the pH of its environment and the number of ionizable chemical groups (especially carboxyl and amino residues) contained within its structure. When subjected to an electric field, it will move toward one or other of the electrodes depending upon the polarity of the charge on the molecule, which is in turn dependent upon the surrounding pH. If a pH gradient is established between the electrodes, then the zwitterionic molecule will migrate toward the electrode of opposite charge to that on

the molecule. As it moves along the pH gradient, the charged chemical groups responsible for the overall charge will be neutralized by the increasing concentration of oppositely charged ions derived from the surrounding environment. Eventually, all of the charged groups on the zwitterion will be neutralized and, as it will then possess no overall charge, it will no longer migrate in the electrical field, but will 'focus' or 'condense' into a tight band. The precise pH at which the molecule is focused is, by definition, the pI of the zwitterion as it is the pH at which it is electrically neutral, i.e. possesses no overall charge. The zwitterion will remain at this focused position as long as the pH gradient and electric field are maintained; IEF is, therefore, unlike most electrophoretic techniques, a 'steady-state' procedure.

Although a pH gradient will automatically form as the result of the application of an electric field to an H⁺ containing ionizable solution, such gradients are insufficiently robust or stable to be used experimentally for IEF. The problem of producing suitable pH gradients was the major technical difficulty experienced by pioneers of IEF such as Svensson, and delayed its introduction as a routine laboratory procedure for many years after its theoretical potential was realized (see Righetti, 1983, for a more detailed discussion of the principles of IEF and its early development).

In the mid-1960s, this problem was overcome by Vesterberg, who chemically synthesized a complex mixture of poly amino carboxylic acids which, when subjected to an electrical field, formed an array which produced a pH gradient. He was able to form fairly linear pH gradients over the range 3–10 and these were found to be suitable for IEF.

Methodology

Although it is possible to chemically synthesize poly amino acids suitable for IEF in the laboratory, most biologists now rely on commercial suppliers for these reagents. Such compounds are usually referred to as 'carrier ampholytes' and their function is to produce a stable, linear pH gradient when subjected to an electrical field. They are almost always poly amino acids, but several different chemical backbones to support the charged groups have been developed. The precise pH gradient obtained depends on the spectrum of ampholytes used. The commercial supply of carrier ampholytes was monopolized for many years by LKB, who almost certainly reproduced Vesterberg's synthesis of polyprotic amino carboxylic acids on a commercial scale. The LKB ampholytes are marketed under the trade name 'Ampholines' and are available for wide range gradients (pH 3.5–10.0)

and are also subfractionated using IEF itself to produce various narrower range gradients.

In the late 1970s, other commercial sources of chemically distinct carrier ampholytes became available, e.g. 'Pharmalytes' (Pharmacia) and 'Servalytes' (Serva), which are also available in wide and narrow pH ranges ('Ampholines', 'Pharmalytes' and other more recently developed ampholytes are now marketed by Pharmacia Biotech). Although pH gradients produced using such commercial ampholytes are in general linear and stable, 'weak spots' may occur at some pI values, particularly near the anode and cathode. For this reason, some workers prefer to add acids, bases or additional ampholytes such as arginine, lysine, glutamic and aspartic acids to strengthen these parts of the gradient. For most purposes, ampholytes are used at a final concentration of 1–2% w/v.

For practical purposes, it is necessary to provide some way of protecting the pH gradient (and focused proteins) from physical disruption during focusing and particularly during analysis. This is achieved by different means according to the procedure used. Isoelectric focusing was originally carried out on both analytical and preparative scales in the liquid phase using vertical glass columns. This apparatus usually consists of a central cylindrical compartment for focusing and electrode chambers at either end; these chambers contain platinum electrodes which are connected to a power supply. The focusing chamber is cooled using a water jacket connected to a chiller. Commercially available columns are equipped with a system which allows careful filling of the electrode chambers and ampholytes and a density gradient of an inert, nonionic substance (e.g. sucrose, glycerol, ethylene glycol) is used to prevent convection and mixing.

Prior to focusing, the lower chamber is filled with an electrode solution suitable for the required polarity (the anode is usually chosen for the lower electrode); this contains the highest concentration of anticonvection substance. The ampholyte solution is then prepared in low and high concentrations of anticonvection agent and this is pumped into the focusing chamber using a gradient former to produce a linear density gradient which decreases from the bottom to the top of the chamber. As IEF is a steady-state process, which 'condenses' the focused components of the preparation under study, it is not necessary to apply the sample in a small volume or as a thin band; the sample can be added to a portion of the ampholyte/anticonvection agent solution or even included in the whole of the focusing solution. When the focusing chamber is full, it is overlaid with electrode solution compatible with the upper

electrode. Electrode solutions are usually dilute acids or bases such as NaOH (cathode) and H_2SO_4 or H_3PO_4 (anode). For narrow range IEF weaker acids, aliphatic amines or ampholytes are more appropriate as electrode solutions.

When the column filling procedure is completed, IEF is carried out by applying a potential difference between the electrodes. At first the current is fairly high as the ampholytes arrange themselves to form the pH gradient; after this the current falls so that when the steady state is reached only a very small current flows. As the applied voltage concentrates the focused bands, it is usual practice to increase the voltage during focusing so that resolution of components is maximized. Although this can be done manually, it is easier and probably better to use a constant-power pack which automatically increases the voltage during IEF. The voltages required for high-resolution IEF are usually higher than for other types of electrophoresis. Column IEF is normally carried out overnight or for even longer. When focusing is complete, the contents of the focusing chamber are carefully removed either under gravity or using a pump, and fractions are collected for analysis. This usually involves the detection of biological material (e.g. protein) and/or assay for biological activity (e.g. enzyme or immunological activity).

Although column IEF has some advantages – for example, fractions can be assayed for several different parameters and it can be carried out on a fairly large scale (commercial columns of 110 and 440 ml are available) – it also suffers from several disadvantages. These are that it is laborious, expensive (considerable quantities of ampholytes are required), it is time-consuming, only one sample can be analyzed per experiment, and fractions may become mixed during collection. For this reason, alternative anticonvection devices have been developed, particularly for analytical IEF.

A particularly useful analytical approach is to use polyacrylamide gel as a solid support for IEF. For this, a gel is prepared in water containing ampholytes. Either slab or rod gels can be used: the former is normally carried out horizontally, whereas for the latter a conventional disk electrophoresis apparatus is used. Electrodes for slabs consist of carbon rods soaked in acid or base or platinum wire in contact with acid- or base-soaked filter paper. For rod gels, dilute acid or base is used to fill the electrode chambers. Samples are applied directly to the gel surface or absorbed on to filter paper strips; other details are as for column IEF. After focusing, proteins are identified by staining with dye (e.g. Coomassie Blue) or by detection of biological activity (e.g. enzyme activity or immunoreactivity).

Other useful anticonvection devices are agarose gel (low electroendosmosis agarose is required as 'ordinary' agarose or agar is charged and therefore unsuitable for IEF) and Sephadex slurries. Both of these (especially the latter) can be used for preparative IEF as focused protein can be eluted from the gel and recovered in the supernatant after low-speed centrifugation. The use of agarose for IEF allows higher molecular weight proteins (e.g. IgM) to readily enter the gel, which does not occur if polyacrylamide gel is used. For reasons still not entirely clear, a phenomenon known as 'cathodic drift' occurs with gel IEF in which the pH gradient slowly collapses into the cathode; for this reason, focusing time for gel IEF is fairly short.

Modifications to the basic procedure have been devised to allow IEF analysis of proteins which are insoluble under physiological conditions, e.g. membrane and cytoskeletal proteins. These involve the use of denaturing agents such as deionized urea and/or nonionic detergents (e.g. Nonidet P-40).

Uses of IEF

Analytical IEF can be used to assess the complexity of protein extracts and to identify components of interest. However, the great resolving power of IEF makes it ideal for detection of microheterogeneity in purified proteins. Enzymologists have used IEF to detect isoforms of purified enzymes and protein biochemists have demonstrated 'multiple forms' of many proteins which appear homogeneous using other biochemical techniques (including other electrophoretic methods). Focusing is used for the diagnosis and study of some genetically transmitted disorders. The basis of the slight differences in the pI of isoforms as revealed by IEF is often post-translational modification of a protein, i.e. a single primary structure which is modified after synthesis. This is frequently due to variation in the glycosylation of a protein and, in particular, the number of sialic acid residues present. Immunochemists often use IEF for the analysis of a variety of antigens and preparations. The technique is very useful when combined with immunoblotting; this enables the precise antigen-binding profile of an antiserum or monoclonal antibody to be established.

Isoelectric focusing has been used to demonstrate the microheterogeneity of immunoglobulins. Thus focusing of immunoglobulin G (IgG) derived from whole serum produces a 'smear' of protein over a fairly wide pH range due to the very large number of different immunoglobulin molecules present; IEF of affinity purified IgG usually shows a complex multiband pattern. The focusing of murine mono-

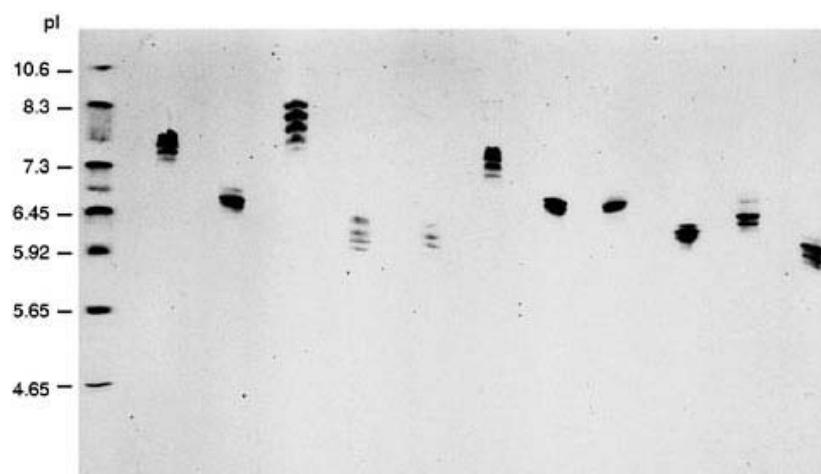


Figure 1 Isoelectric focusing in polyacrylamide gel of different purified IgG mouse monoclonal antibodies.

clonal antibodies shows different IgG species to possess very different pI values and that the mAbs can show variable microheterogeneity (see **Figure 1**). The microheterogeneity is very often due to differences in glycosylation, but other post-translational modifications of the monoclonal IgG molecule also produce multiple bands on focusing. The IEF profile of a mAb can be useful for identification purposes and is sometimes referred to as the 'spectrotype' of the antibody. Focusing can be used to demonstrate the presence of oligoclonal immunoglobulin in biological fluids which may be significant in certain pathological conditions, e.g. multiple sclerosis. Isoelectric focusing is also used for analysis of the polymorphism of the major histocompatibility complex.

Isoelectric focusing is often used as part of the quality control testing of therapeutic biological products to demonstrate batch consistency. Its high resolving power and ability to detect subtle changes in charge of proteins is particularly valuable for this (see **Figure 2**).

Preparative IEF is used for isolation of proteins and is usually included at a later stage of a purification procedure, to separate a specific component from contaminants of slightly different pI. It is usually the method of choice for the preparation of isoforms of proteins.

An important development of the basic polyacrylamide gel IEF procedure described by O'Farrell in 1975 is the concept of two-dimensional electrophoresis. For this, proteins are initially separated using IEF in rod gels and then the rods are overlaid at a right angle on an SDS-PAGE gel and subjected to secondary separation. This technique is particularly powerful as first-dimensional separation is on the basis of fine differences in charge, whereas second-dimensional resolution is size dependent. It is possible to resolve more than 1000 individual proteins in an extract using this method.

A preparative procedure related to but distinct from IEF known as 'chromatofocusing' was introduced in the early 1980s. The principle of the tech-

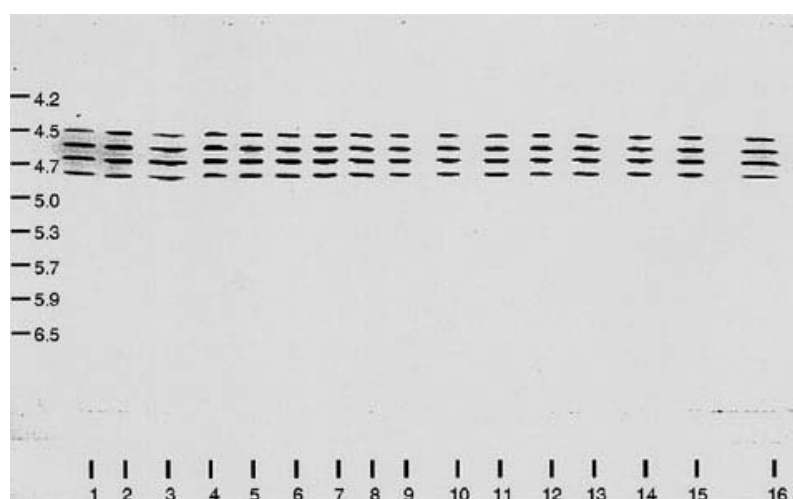


Figure 2 Isoelectric focusing in polyacrylamide gel of a number of batches (lanes 2-15) of clinical grade erythropoietin, showing consistency of production. All batches show an identical spectrotype and the IEF pattern matches that produced by the reference standard (lanes 1 and 16). The pH values at points measured on the gradient are shown on the vertical axis.

nique is that proteins will be focused on an ion-exchange column if there is a pH gradient formed along the length of the column, and they will be eluted from the column at a pH value near to their individual pIs. A mixture of acids, bases and ampholytes are used to form the gradient and equipment and reagents for chromatofocusing are available commercially. The procedure has the advantages that it uses a robust solid column support and has high sample capacity.

A caution

Although IEF is a very valuable analytical and preparative technique, it can produce artifactual results. One problem is that proteins are very poorly soluble at or near their pI and so precipitation of components may occur during focusing. Also, apparently different isoforms of a protein (as revealed by focusing) can be due to conformational isomers, oxidation or reduction, or formation of complexes with other proteins or ampholytes. A good check for this is to apply the sample at different positions in the pH gradient and check for an identical pattern and to ensure IEF-isolated components retain their identity on repeated focusing.

See also: Antibodies, detection of; Monoclonal antibodies (mAbs); Protein separation techniques; SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

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ISOTYPE

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Immunoglobulin (Ig) isotypes are responsible for the biological effector functions of antibody molecules. In humans, there are nine Ig heavy (H) chain and two light (L) chain isotypes which differ in the structure of their carboxy-terminal portion, or constant (C) region. The L chain isotypes, K and λ , are not known to be associated with biological functional differences. There are five H chain isotypes, or classes: IgA, IgD, IgE, IgG and IgM. Based on 95% or greater amino acid homology, IgA and IgG have two and four subclasses, respectively. C region genes are located on chromosome 14, 3' to the JH genes of the human Ig locus: C μ for IgM, C δ for IgD, C γ 1

for IgG1, C γ 2 for IgG2, C γ 3 for IgG3, C γ 4 for IgG4, C α 1 for IgA1, C α 2 for IgA2, and C ϵ for IgE. Each one of these genes encodes glycoprotein molecules with four structural components: C μ 1, hinge, C μ 2 and C μ 3. The C μ 1 interaction with the L chain C region is of importance for the structural integrity of antibody molecules. The C μ 2 regions interact through branched chain N-linked carbohydrate moieties which facilitate antibody solubility and secretion. The hinge region enables bivalent variable region antigen-binding sites to engage two epitopes: it enhances antigen binding and cross-linking, influences steric relationships between Fab sites and Fc

regions, and is responsible for antibody molecule flexibility. IgM and IgE have a C_H4 region and lack a genetic hinge, although the pentameric structure of IgM facilitates multivalent antigen binding. IgG3 has an extended hinge region encoded by several exons rather than one. IgG4 has a short, inflexible hinge that creates steric hindrance for fixation of complement. Allotypic variants, designated as Km, Am, Mm, and Gm, may exist at any of the H chain C region loci. Such allelic differences may also influence individual isotype levels and produce differences in biological activity. There are two pseudogenes (ψ) in the H chain C region locus and multiple λ light chain pseudogenes.

Complement fixation is a critical isotype function in host defense. The classical pathway is activated by C1q binding to the C₁2 region of cross-linked IgG (IgG3>IgG1>IgG2) and to the C_H3 region of IgM. C1q binds immune complexes and IgM pentamers with higher avidity than single molecules. Antibody is not required for the activation of the alternative pathway. The C_H2 and C_H3 regions of antibody molecules are responsible for Fc receptor binding to a large spectrum of host effector cells. Fc receptor binding results in aggregation, phagocytosis and lysis of antibody-coated targets. Unique Fc receptors demonstrate different specificities and affinities for individual isotypes.

Ig class switching, regulated by T and B cell differentiation factors, determines isotype commitment during B cell maturation. Developmental maturation of IgG subclasses results in a delay in the appearance of IgG2 antibodies until age 2 years. In adults, T-independent antigens such as polysaccharides often elicit isotype restricted responses which are predominantly IgM and IgG2. T-dependent antigens such as proteins primarily elicit IgG1 and IgG3 antibodies. IgG subclass deficiency, such as the physiologic IgG2 deficiency of infancy and early childhood, is frequently compensated by increased production of alternative IgG subclasses. Isolated isotype or IgG subclass deficiencies usually do not result in increased disease susceptibility, although combined deficiencies may be a risk factor for given infections.

The five major human isotypes are variably distributed. The IgG subclasses constitute 70–75% of the total Ig pool and 50% of these antibodies are located in the intravascular space. IgG antibodies are the predominant isotype of the secondary antibody response and through a transcytosis-mediated process are the only Igs to cross the human placenta. IgM is the predominant isotype in primary antibody responses and is typically secreted as a pentamer. It represents 10% of the Ig pool and is largely (75%) intravascular. IgA constitutes 15–20% of the Ig pool. It provides

mucosal immunity and is distributed in saliva, tears and breast milk, and respiratory, genitourinary and intestinal secretions. Secretory IgA, which is predominantly IgA2, is secreted from the extracellular matrix to the lumen by transcytosis in association with a non-Ig protein, the secretory component, which is synthesized by epithelial cells. Dimeric IgA and pentameric IgM are each associated with another non-Ig protein called the J chain. Serum IgA is mainly monomeric and of the IgA1 subclass. IgD accounts for less than 1% of human Ig and appears mainly on the surface membrane of circulating B lymphocytes. The long hinge region of IgD may play a role in B cell activation by cross-linking membrane IgD to antigen. Finally, significant levels of serum IgE are rarely found in nonallergic individuals, although it is present on the surface of basophils and mast cells. It is homocytotropic because of its presence on the surface of mast cells and basophils serving as an antigen-binding molecule. IgE appears in response to helminthic infections and in hypersensitivity reactions, allergy and asthma.

See also: Allotypes, immunoglobulin; Idiotype; IgA; IgE; IgG; IgM; Immunoglobulin class switching; Immunoglobulin genes; Immunoglobulin structure.

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J

JOINING (J) CHAIN

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Although J (joining) chain was detected earlier as a polypeptide with high electrophoretic mobility in the light (L)-chain fraction from human colostrum IgA, its existence as a third polypeptide typical of polymeric immunoglobulins (Igs), distinct from H and L chains, was established in the early seventies. Subsequent studies revealed that J chain, a glycosylated polypeptide with a molecular mass of 15–16 kDa, displays a fast electrophoretic mobility and is linked by disulfide bridges to the Fc fragment of polymeric IgM or IgA of secretory and serum (myeloma) origin. Based on the given criteria (molecular mass, fast electrophoretic mobility, characteristic amino acid composition and sequence, immunochemical cross-reactivity, and sequence of DNA) the presence of J chain has been established, with variable degrees of confidence, in polymeric Ig from many vertebrate species, including mammals (human, monkey, rabbit, mouse, pig, dog, goat, cat, cow, horse, rat, guinea pig and sheep), birds (chicken and pheasant), reptiles (turtle), amphibians (frog and toad) and fishes (catfish, sardine and shark). Recently, mRNA for J chain has been detected in various species of invertebrates, including earthworm, silkworm, spider, shrimp, crab, oyster, clam, sea squirt and sea cucumber.

Structure

Human, rabbit, cow and mouse J chains consist of 136–137 amino acid residues. The sequence analyses of J chain amino acids and/or cDNA of human, mouse, cow, pig, rabbit, bull frog and earthworm revealed a high degree of interspecies homology.

These findings, together with remarkable similarity in physicochemical properties and interspecies cross-reactivities, suggest that many of the J chain features are conserved through evolution. J chains are rich in acidic (aspartic, glutamic) amino acid residues and low in the content of glycine, serine and phenylalanine; tryptophan is absent. Six of the eight cysteine residues form three intrachain disulfide bridges; two residues close to the N-terminus are involved in disulfide bonds that link J chain to the penultimate cysteine residues of α and μ chains. These residues are present in the C-terminal segments of α and μ chains which extend by 18–20 residues beyond the C-termini of γ , δ and ϵ chains. Approximately 8% of the molecular mass is contributed by a single N-linked carbohydrate side-chain that consists of fucose, mannose, galactose, glucosamine and sialic acid. Despite the low degree of sequence homology with H and L chains, studies of the secondary structure reveal that J chain folds into an eight-stranded antiparallel β barrel (with 35% β sheet and the remainder in random coil). Thus, J chain displays a secondary structure consistent with a single Ig domain. In native molecules of polymeric Ig, the antigenic determinants of J chain are poorly exposed and, furthermore, J chain, even in native Ig molecules, is highly susceptible to proteolysis; these findings may explain the failure to detect J chain in some polymeric Ig. Stoichiometric studies reveal that a single chain is present in one molecule of polymeric Ig, irrespective of the number of constituent monomers. Cleavage of interchain disulfide bonds is usually suf-

ficient for its release from Ig, suggesting that only weak noncovalent interactions exist between J chain and the Fc region of polymeric Ig.

Cellular origin

Immunohistochemical studies reveal that J chain is present in the cytoplasm, but not on the surface membrane of lymphocytes of B cell lineage at various stages of their differentiation. In tissues from healthy individuals, almost all subepithelial IgA- or IgM-producing cells of the gastrointestinal and respiratory tracts, and also the interstitium of mammary, salivary and lacrimal glands, prominently display the presence of intracellular J chain. Surprisingly, a few IgG- as well as IgD-producing cells from these tissues also contain J chain. In contrast, IgA- or IgG-containing plasma cells from the normal bone marrow are uniformly J chain negative, while cells from lymph nodes and the spleen express a variable proportion of J chain-positive cells. In multiple myeloma, IgG, IgA or IgD plasma cells in the bone marrow frequently display intense immunofluorescence, indicating the presence of this polypeptide. Similarly, J chain has been detected in IgG plasma cells from inflammatory sites and mucosal tissues, and in mitogen-stimulated peripheral blood lymphocytes. The expression of J chain in cells that are not engaged in the synthesis of polymers has been taken as a sign of their clonal immaturity. Extensive studies of human cells from leukemic patients, established lymphoid cell lines and Epstein-Barr virus (EBV)-transformed fetal bone marrow cells (depleted of surface Ig-positive cells) reveal that J chain may be expressed in the cytoplasm of lymphocytes from the earliest stages of their differentiation along the B cell axis. Thus, cells phenotypically characterized as null, pro-B or pre-B cells contain J chain, in the absence of μ chain. EBV-transformed fetal bone marrow cells or cells from a few patients with clinically established diagnosis of multiple myeloma display the morphological features of plasma cells, but contain no intracellular Ig, and in the former case, exhibit a germ-line configuration or abortive VDJ rearrangement of H chain gene segments. Recently, mRNA for J chain has been detected in T cells from human fetal thymus; peripheral T cells were negative. J chain is detectable in free form only in trace amounts in various human body fluids (urine, serum and milk) or in the supernatants of *in vitro*-cultured cells, even though these contain cytoplasmic J chain; it is not secreted unless disulfide-linked to polymeric IgA or IgM. J chain expression in B cells at early stages of differentiation is apparently different in mice, in

which J chain is detectable only in Ig-secreting cells but not in pre-B or B cells.

Sequence analyses of mouse genomic DNA reveal that J chain-encoding information is contained in four exons of a single gene located on chromosome 5; in humans the J chain gene is on chromosome 4. The expression of J chain may be upregulated by lipopolysaccharide and by interleukins, including IL-2, IL-5 and possibly IL-6.

Function

Initial findings indicated that J chain participates, directly or indirectly, in disulfide bond-exchange reactions which ultimately result in the intracellular polymerization of IgA or IgM monomers. The detection of J chain in polymeric Ig-secreting cells, the presence of cysteine residues involved in the covalent linkage to J chain in secreted but not membrane forms of the α and μ chains, correct *in vitro* assembly of IgM and IgA in the presence of J chain (which becomes incorporated) in some but not all experiments, reinforce this view. Furthermore, by fusion of IgG-secreting, J chain-positive myeloma cells with surface IgM-positive B lymphoma cells, hybrids that secrete pentameric IgM have been obtained. Contrary to these findings, site-directed mutagenesis of μ chain genes leading to the replacement of penultimate cysteine residues involved in the formation of intersubunit and J-chain disulfide bonds did not abolish the assembly of IgM polymers. Analogous results were obtained when genes encoding μ and λ chains were expressed in J chain-negative tumor cells: polymeric IgM was synthesized in the absence of J chain. The presence of J chain in cells that produce monomeric IgG or IgD remains an enigma.

Studies of the selective transport of polymeric Ig into external secretions provide convincing evidence for the essential role of J chain in the binding of polymeric Ig to secretory component (SC). The binding of polymeric IgA or IgM preparations to free or cell membrane-associated SC is proportional to their J chain content, and proteins devoid of J chain do not bind to SC. Although J chain-deficient mice produce both polymeric and monomeric IgA, the transport of polymeric forms into external secretions is greatly impaired. Thus, the SC-binding site on polymeric Ig includes J chain, in addition to constant domains of α and μ chains. Because SC also displays Ig domain secondary structure, it appears that the interactions of the component polypeptides of secretory IgA and IgM are based on complementarities of their Ig domain-like structures.

See also: Antibodies, synthesis; Domains, immuno-

globulin-type; IgA; IgM; Immunoglobulin structure; Mucosa-associated lymphoid tissue (MALT); Secretory component (the polymeric Ig receptor).

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K

KALLIKREIN-KININ SYSTEM

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The kallikrein-kinin system was first recognized as a plasma and tissue proteolytic system responsible for the liberation of the vasoactive proinflammatory mediator bradykinin (BK). However, examination of the molecular, biochemical, biological and physiological properties of the kallikrein-kinin system has shown that this system initiates or interacts with a number of physiologic and pathophysiologic systems in plasma as well as blood and vascular cells. New activities of this plasma system include protease inhibition, antithrombin function and antiadhesive properties. Tissue kallikreins are ubiquitously distributed and regulate local blood flow and glandular secretion.

Biological effects of bradykinin

The first and perhaps most important function of plasma kininogens is the delivery of BK, a potent biologically active peptide. BK itself is a potent stimulator of endothelial cell synthesis of prostacyclin and nitric oxide formation (both vasodilators and inhibitors of platelet function), phagocyte superoxide formation, tissue plasminogen activator release and

endothelial cell-dependent smooth muscle hyperpolarization factor formation. When vessels are injured, BK stimulates protein kinase C and subsequently MAP kinase, which can result in vascular smooth muscle growth and proliferation. However, when vessels are intact, BK appears to prevent vascular muscle growth and proliferation, thus keeping blood flow and vessels patent. These actions of BK are mediated through a constitutive hepta-spanning G protein-coupled receptor known as the B2 receptor. A receptor called the B1 receptor is induced by inflammatory cytokines and binds des Arg⁹-BK but its function is not yet clear.

BK plays a role in blood pressure regulation although little is known about what regulates its liberation from kininogens by tissue kallikreins. Transgenic mice overexpressing tissue kallikrein are hypotensive and lose the diurnal fluctuation of blood pressure. Intramuscular delivery of rat kallistatin, a naturally occurring serine proteinase inhibitor, reverses hypotension in transgenic mice overexpressing human tissue kallikrein. Gene delivery of tissue kallikrein reduces mean blood pressure of spontaneous hypertensive rats and this inhibition is

blocked by kallistatin. These molecular genetic studies directly indicate that presumed tissue kallikrein-induced bradykinin liberation directly modifies local and potentially systemic blood pressure regulation.

The contact system of blood coagulation

In 1975, three individuals were described with deficiency of high molecular weight kininogen (HK), a precursor of BK. All had a prolonged activated partial thromboplastin time (APTT), a surface-activated clinical coagulation screening test. Studies on the plasma kallikrein-kinin system first focused on defining the procoagulant property of HK, despite the fact that 1) none of these individuals had a hemorrhagic state and 2) it was already known that factor XII and prekallikrein deficiencies were not associated with bleeding. Factor XII and prekallikrein are required for enzymatic cleavage of HK. These plasma proteins together were grouped as the 'contact system' because of the requirement for an artificial surface for their efficient activation (Table 1).

Factor XII is produced by a single gene on chromosome 5. Activation of factor XII by plasma kallikrein, trypsin or plasmin results in an enzyme with a decreasing size, a decrease in its artificial surface-binding properties, and a decrease in its coagulant activity. There are two major forms of activated factor XII: an 80 kDa protein consisting of two disulfide-linked polypeptide chains (factor XIIa), and factor XII fragment (factor XII_f) a 28–30 kDa protein fragment derived from factor XIIa by removal of its heavy chain. Factor XII_f has no artificial surface-binding properties but retains its enzymatic ability to activate prekallikrein and the first component of the complement C1.

Prekallikrein (PK) is produced by a single gene on chromosome 4. When prekallikrein is cleaved and activated to kallikrein by either factor XIIa or factor XII_f, the active enzyme is composed of two disulfide-linked subunits: a heavy chain of approximately

52 kDa and a light chain which occurs as one of two variants of approximately 36 and 33 kDa. At least 75% of PK circulates bound noncovalently to HK. In the absence of factor XII, prekallikrein *in vitro* will not become activated on an artificial surface.

The human kininogens, HK and low molecular weight kininogen (LK) are proteins with multiple associated activities. HK and LK are the products of a single gene on chromosome 3 as a result of alternative mRNA splicing. HK has been divided into six domains. Domain 1 (which binds calcium) and D2 and D3 (which inhibit cysteine proteases including cathepsins and calpain) form the heavy chain. D4 contains BK. These domains are common to both HK and LK. D5_H which binds to artificial surfaces and D6 which binds to prekallikrein and factor XI, form the unique light chain of HK. The function of D5_L, the unique light chain of LK, is not known. The activation of the contact system can be better understood on the basis of the multiple interactions between HK and LK with cells, although the *in vivo* activating agent(s) remains unknown. Both HK and LK are known to bind to platelets, neutrophils and endothelial cells through D3, D4 and D5. On the platelet surface HK inhibits the binding of thrombin and its subsequent induction of platelet aggregation by binding to GPIb. On the neutrophil surface HK inhibits adhesion by competing with fibrinogen for binding to CD11b/CD18 (Mac-1). HK binds to several receptors on endothelial cells which mediate BK release and fibrinolysis (See below).

Activation, amplification and relationship of the contact system with other biological systems

Activation of the contact system is believed to be initiated by exposure of factor XII to negatively charged surface or cell membrane where activation of the molecule occurs. A small amount of activated factor XII (factor XIIa) can lead to cleavage and activation of its substrates: PK, factor XI and HK. PK

Table 1 Kallikrein-kinin system

Glycoprotein	Molecular weight (kDa)	Plasma concentration (μM)	Direct binding to cell surfaces
Factor XII	80	0.40	Monocytes
Factor XII _f	28		
Prekallikrein (PK)	88	0.56	
High molecular weight kininogen (HK)	120	0.66	Endothelial cells Platelets Neutrophils
Low molecular weight kininogen (LK)	65	1.30	Endothelial cells Platelets

and factor XI bind in part to cell surfaces through HK. Kallikrein can act on HK to form the active cofactor (HKa) and to release the potent nonpeptide BK. Activated factor XI (factor XIa) will, in turn, trigger the intrinsic pathway of blood coagulation. Activation of the system can be amplified, since kallikrein can activate more factor XII which in turn, will generate more kallikrein (reciprocal activation). In addition, kallikrein will generate a fragment from factor XIIa (factor XII_f) that has the ability to efficiently activate PK that is bound to HK in the fluid phase in the absence of a surface. Activation of the contact system can lead to activation of the fibrinolytic system. Kallikrein, factor XIa, and factor XII_f can generate plasmin from plasminogen *in vitro* but each of these plasminogen activators has less than 1/10 000 the plasminogen activator activity of urokinase. However, there is evidence indicating that kallikrein is an activator of prourokinase and that HKa binds to the urokinase receptor on the surface of endothelial cells, which makes the enzymatic reaction physiologically relevant.

The classical pathway of complement can be activated by the contact system. Factor XII_f can directly activate the C1r and C1s subcomponents of C1. Kallikrein itself has been shown to proteolytically cleave the C1r and C1s subcomponents of complement, leading to their inactivation. Plasminogen, which can be converted to plasmin by kallikrein-activated urokinase, has been shown to be capable of inactivation of the C1s component of C1.

Regulation of the contact system

The major plasma protease inhibitor of activated factor XIIa and factor XII_f is C1 inhibitor (C1-INH) accounting for over 90% of the inhibition of these proteases in plasma. Antithrombin III has some inhibitory activity on factor XIIa. Plasminogen activator 1 (PAI-1) also inhibits factor XIIa. The light chain of plasma kallikrein reacts with protease inhibitors, principally α_2 -macroglobulin (α_2 M) and C1-INH. Although C1-INH and α_2 M account for an equal amount of kallikrein inhibitor in plasma, C1-INH in plasma acts more rapidly than α_2 M. Antithrombin III also inhibits kallikrein but it does so slowly even in the presence of heparin. Protein C inhibitor has also been recognized to be a potent inhibitor of kallikrein. BK is rapidly inactivated by two proteases (kininases). An aminopeptidase cleaves the N terminal lysine from Lys-BK (Kallidin), converting it to BK. Carboxypeptidase N acts on BK to cleave the C-terminal arginine, yielding des Arg⁹-BK. Further degradation occurs through the action of

angiotensin-converting enzyme (a dipeptidyl carboxypeptidase) or by various aminopeptidases.

The kallikrein-kinin system in human disease and experimental models

There is increasing evidence for the role of the kallikrein-kinin system in human disease. The kallikrein-kinin system appears to exert a significant effect in various surface-mediated defense reactions including hereditary angioedema (HAE), systemic inflammatory response syndrome (SIRS), previously known as sepsis, cardiopulmonary bypass, rheumatoid arthritis and inflammatory bowel disease. HAE is a congenital condition associated with a deficiency or defect in C1-INH. Acute attacks of HAE have been well documented to be associated with activation of the kallikrein-kinin system. BK liberation is believed to be a major mediator of the edema seen in this condition. Activation of the kallikrein-kinin system has been postulated to be one of the mediators of SIRS. Historically, studies of patients with SIRS, showed that factor XII, PK and C1-INH were decreased. However, whether activation of the contact system was an early event or a result of complications associated with SIRS was not demonstrated until recently in studies with human normal volunteers given a low dose of *E. coli* endotoxin. Functional prekallikrein levels were significantly lower in the endotoxin group compared with controls as early as 2 h after infusion and remained lower throughout the rest of the experimental protocol (24 h). In an established experimental baboon model of SIRS, irreversible hypotension correlated with activation of the kallikrein-kinin system. Furthermore, treatment with a monoclonal antibody directed against human factor XII which is able *in vitro* to inhibit its autoactivation, prevented the irreversible lethal hypotension induced by *E. coli* in this experimental animal model of SIRS.

Clinical cardiopulmonary bypass (CPB) is performed on over 350 000 Americans each year. During CPB, there is an extensive contact between blood anticoagulated with heparin and the synthetic surface of the extracorporeal circuit. In simulated CPB there is a significant increase in kallikrein-C1-INH complex formation. Further studies have demonstrated that aprotinin, an inhibitor of both plasmin and plasma kallikrein, reduced blood loss after cardiac operations and decreased the elevated postoperative bleeding time. In a simulated extracorporeal bypass model, in which no plasmin is found, aprotinin decreased both kallikrein-C1-INH and C1-C1-INH complexes, resulting in a marked inhibition of the release of neutrophil elastase.

The role of the kallikrein system in inflammatory arthritis has been investigated in a model of acute and chronic relapsing arthritis induced by intraperitoneal injection of proteoglycan-polysaccharide from group A streptococci (PG-APS) into Lewis rats. In this model, PK and HK functional levels were significantly decreased. There was a striking inverse correlation between functional HK concentration and joint enlargement. Further experiments showed in this experimental animal model that rats injected with PG-APS receiving orally a potent plasma kallikrein inhibitor (P8720 or Bz-Pro-Phe-boro-Arg-OH, $K_i = 0.15$ nM) had a significant decrease (61%) in joint swelling with a disappearance of most of the dense neutrophil infiltration and mononuclear cells observed in animals not receiving P8720. The beneficial aspect of P8720 in this experimental animal model could be explained in part by prevention of BK generation from HK by the action of plasma kallikrein.

BK effects its changes in the intramuscular compartment by binding to at least two receptors, the B1 and the B2 receptors. Both of these receptors are G-coupled and this binding of BK stimulates cellular signal transduction. Further studies employing this experimental animal model of acute and chronic relapsing arthritis have shown a beneficial effect when a B2 receptor antagonist was administered to the rats. Interestingly, a deleterious effect was noticed when either therapy with B1 receptor antagonist or a combined B1-B2 receptor antagonist was used. In a similar model of Crohn's disease, P8720 inhibited both acute and chronic gross and histological features including edema, granuloma formation as well as the systemic manifestation of splenomegaly, leukocytosis, anemia and the acute phase reaction.

See also: Asthma; Atopic allergy; Clotting system; Histamine; Prostaglandins.

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KLEBSIELLA, INFECTION AND IMMUNITY

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The most common members of the genus *Klebsiella* are *K. pneumoniae*, *K. planticola*, *K. oxytoca*, *K. terrigena* and *Klebsiella* group 47. Two former species, *K. ozaenae* and *K. rhinoscleromatis* are now considered to be biochemically inactive strains of *K. pneumoniae*. Of these, the most commonly isolated pathogen is *K. pneumoniae*, also known as Friedlander's bacillus. As its name implies, it is usually isolated from cases of pneumonia, but it can infect other parts of the body, such as the urinary tract and wounds. Because of its overwhelming importance, this entry will deal exclusively with *K. pneumoniae*.

Characteristics of the organism and its antigens

K. pneumoniae grows as lactose-fermenting colonies on differential enteric isolation media. It is non-motile, i.e. it does not produce flagellar (H) antigens. Encapsulated *K. pneumoniae* appear quite opaque and mucoid when growing on laboratory agar. These organisms are virulent in humans and animal models. Avirulent *K. pneumoniae* (those which produce no capsules) appear smaller and translucent on similar agar surfaces. *K. pneumoniae* produces both O (somatic) and K (capsular) antigens. The term K antigen comes from the German, *Kapsule*, and was first used to describe the polysaccharide antigens of enteric bacilli. The O antigen is a polymer of repeating oligosaccharide units of several monosaccharides. There are at least eight different O antigen types. The K or capsular antigen of *K. pneumoniae* can be seen by employing the India ink stain, which is comprised primarily of carbon particles. These polysaccharide antigens are used primarily for serotyping. There are at least 77 known antigens. Epidemiologic studies have demonstrated a predominance of certain serotypes in human infections.

Immune responses of the host

In healthy adults, natural host resistance against *K. pneumoniae* is quite high. The progression of this disease and its prognosis are primarily related to age and its predisposing factors. Accordingly, the typical patient is a middle-aged or older man with underlying medical problems, which can include diabetes mellitus, alcoholism or chronic bronchopulmonary disease. Several animal models have been developed,

including rats, mice and monkeys. Classically, the histopathological features of a *K. pneumoniae* lung infection in humans include a massive confluent lobar consolidation consisting primarily of polymorphonuclear leukocytes, a voluminous edema and abscess formation with massive cavitation. Spontaneous recovery is dependent upon the production of type-specific antibodies to the carbohydrate capsule. These antibodies are usually demonstrable in the serum about 1–2 weeks after initiation of the infection. The primary function of these antibodies is opsonization, as *K. pneumoniae* is rapidly killed by phagocytic cells. Type-specific immunity to *K. pneumoniae* infections is long-lasting. Recurrent attacks of *K. pneumoniae* are usually caused by organisms of a different serotype.

The possibility that *K. pneumoniae* is involved in the development of ankylosing spondylitis (AS) is suggested by the demonstration, in AS patients with active disease, of an increased fecal carriage of *K. pneumoniae* as well as the presence of high levels of serum IgA antibodies against *K. pneumoniae*. The etiology of AS apparently involves some genetic predisposition. The disease occurs mostly in males and many AS sufferers possess the HLA-B27 antigen. However, the correlation with known genetic factors is not absolute. Two main theories have been proposed to explain the association of *Klebsiella* with AS. The molecular mimicry hypothesis suggests that HLA-B27 (expressed in approximately 95% of AS patients) cross-reacts with *Klebsiella* and that antibody produced by the patient against these cross-reactive antigen sites will also bind to self epitopes and produce destructive immunity. The second theory states that a *Klebsiella* plasmid encodes the production of a bacterial modifying factor that reacts with HLA-B27, making it susceptible to immunological attacks. Recently, significantly elevated frequencies and titers of antibodies to *K. pneumoniae* capsular types 26, 36 and 50 were detected in sera from AS patients. These results suggest the predominance of *Klebsiella* serotypes 26, 36 and 50 in patients with AS.

Evasive strategies by the organism

The primary virulence factor of *K. pneumoniae* is its carbohydrate capsule (Figure 1). The capsule allows the organism to resist phagocytosis, and hence death

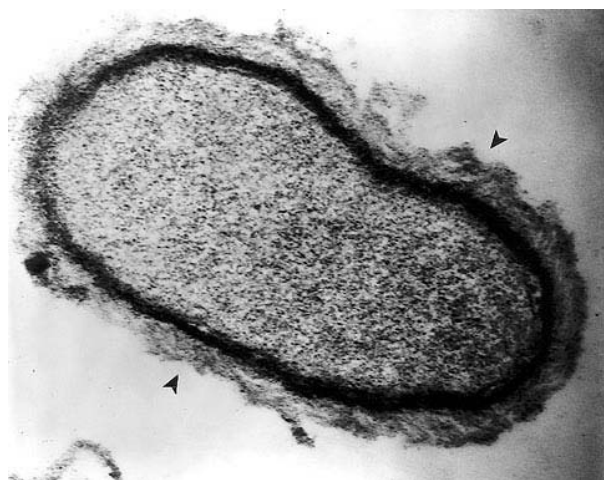


Figure 1 Electron micrograph of *K. pneumoniae* serotype 1. The bacterium is surrounded by the antiphagocytic carbohydrate capsule (arrows). Original magnification $\times 25\,000$.

in professional phagocytes. Encapsulated strains have been shown to be many fold more virulent than nonencapsulated *Klebsiella* in a variety of different animal models. There have been reports of several toxins produced by *K. pneumoniae*, including enterotoxins, by a number of strains isolated from patients with tropical sprue. However, none of these toxins could account for the extensive lung destruction seen in lobar pneumonia produced by *K. pneumoniae*. Probably the best candidate for this devastating pathological effect is the endotoxin produced by this organism. Indeed, a compound referred to as the extracellular toxic complex (ETC) has been described for *K. pneumoniae*. This material is composed of endotoxin, capsule and protein, and the endotoxin portion was shown to contain the toxic moiety. This material was lethal when injected intraperitoneally into mice, and when it was introduced into the lungs of mice, the pathology seen after 24, 48 and 72 hours was similar to the damage caused by an active *K. pneumoniae* lobar pneumonia. All *K. pneumoniae* serotypes examined to date produce the ETC.

Vaccines

Clinical isolates of *K. pneumoniae* express a number of virulence factors, each with the potential to serve as protective antigens in a vaccine. These factors include the capsular polysaccharide (CPS), pili, endotoxin and outer membrane proteins. Of these virulence factors, only the possession of a well-defined capsule seems to be essential for virulence. Therefore, vaccine development for *K. pneumoniae* has emphasized induction of CPS-specific antibody in the host.

It has already been convincingly shown that antibody to CPS is highly effective in preventing experimental *K. pneumoniae* sepsis. The CPS can be obtained from cell-free supernatants by Cetavalon and ethanol precipitation. Trace quantities of endotoxin present in vaccines can be detoxified by 0.1 N NaOH. In clinical trials, vaccinees received 25–50 μg of *K. pneumoniae* CPS. All vaccinees responded with at least a fourfold or greater rise in immunoglobulin G (IgG) and IgM titers. IgG antibody obtained from these individuals was shown to be highly effective in preventing experimental burn wound bacteremia in mice due to the homologous organism. Currently, a polyvalent vaccine containing the CPS from 25 different *K. pneumoniae* serotypes is being tested, with promising results. These findings indicate that a CPS-based vaccine against *K. pneumoniae* is feasible and should be multivalent, eliciting antibodies directed against the 25 serotypes making up 70% of all the bacteremic isolates.

See also: Bacteria, immunity to; Bacterial cell walls; Respiratory and cardiac infections.

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KNOCKOUT, GENETIC

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The term 'knockout' or 'gene disruption' denotes a technology to introduce precise genetic lesions into the genome of the mouse and to produce an animal model with a predefined genetic defect.

Previously, the isolation of mutant animal models has been solely dependent on screening spontaneously occurring mutants or on random mutagenesis using carcinogens. Recent ground-breaking advances in mouse genetics, however, have greatly facilitated this process. Today, it is possible to introduce specific defects into any mouse gene and to study the consequence of the alteration *in vivo*. This has been made possible by technological advances in two areas: 1) the ability to introduce defined changes into the mouse genome using homologous recombination, and 2) the capability to reintroduce genetically altered embryonic stem cells into the germline of the mouse, generating mutant mouse strains. In the following, the current status of the knockout technology and its application for studying the immune system will be discussed.

Gene disruption by homologous recombination

Eukaryotic cells have the intrinsic capability to exchange sequences between two homologous duplex molecules of DNA. This so-called homologous recombination event results in a reciprocal exchange of genetic information so that no nucleotides are lost or added to the participating DNA molecules. Homologous recombination not only takes place between homologous chromosomes but also

between chromosomes and extrachromosomal plasmid molecules that have been introduced artificially into the cells. This mechanism can be exploited to insert plasmid molecules (targeting constructs) into cellular genes in order to alter or destroy them (knockout or gene disruption). Because homologous recombination is a rare event and because most cells will integrate the targeting construct at random into their genome, special selection strategies had to be designed to enrich for homologous recombination. The positive/negative selection scheme employed mostly for gene targeting has been developed by Capecchi and coworkers. It results in inactivation of the target gene by integration of a positive selection marker into the gene coding region (Figure 1).

Initially, a targeting construct is assembled that contains part of the target gene, separated by a positive selection marker. Typically, this marker is the neo gene that confers resistance to the aminoglycoside G418 on cells that have taken up the construct. To enrich for integration of the vector into the target gene via homologous recombination, a negative selection step is employed simultaneously, eliminating cell clones that have undergone random integration of the construct. This is achieved by introducing the thymidine kinase gene of herpes simplex virus (HSV-tk) in the targeting vector. HSV-tk converts inactive nucleoside analogues such as 1-[2-deoxy, 2-fluoro- β -D-arabinofuranosyl]-5 iodouracil (FIAU) into chain terminators that are incorporated into the DNA of the target cell and block DNA synthesis. In the vector described in Figure 1, the HSV-tk gene flanks the homologous gene region and is

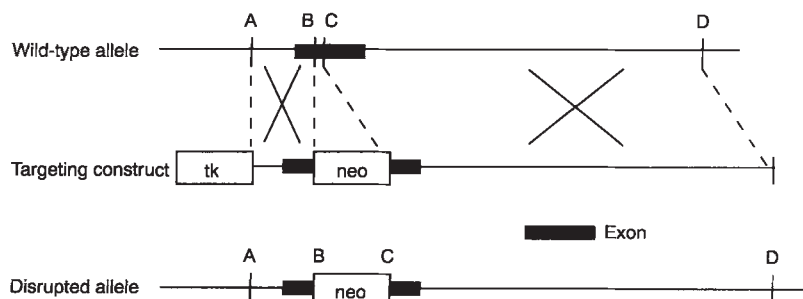


Figure 1 Generation of a gene targeting construct. The gene targeting construct is assembled from DNA segments AB and CD, which are derived from the murine target gene locus (wild-type allele). The neo expression cassette is inserted between fragments AB and CD, disrupting essential exon sequences of the target gene (filled box). The herpes simplex virus thymidine kinase gene (tk) is placed adjacent to the gene homology region. In the event of homologous recombination between targeting construct and wild-type allele, the tk gene will be lost but integration of the neo cassette into the target gene will destroy the gene coding sequences (disrupted allele).

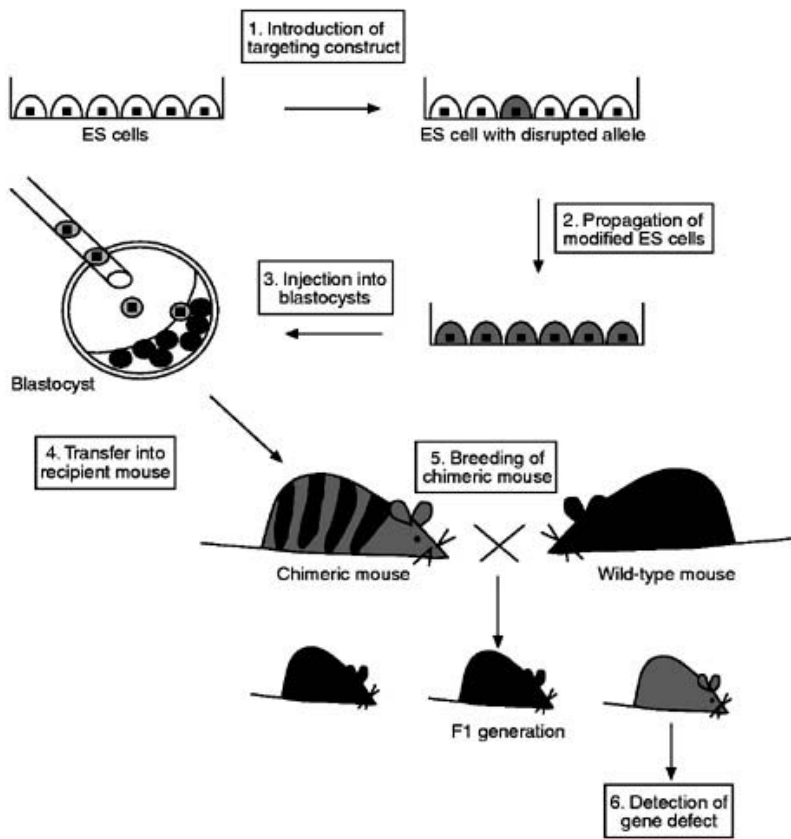


Figure 2 Derivation of mice carrying a gene disruption. The targeting construct is introduced into embryonic stem (ES) cell cultures and individual cells carrying the disrupted target gene are isolated by positive/negative selection using G⁴¹⁸ and FIAU. After propagation, the ES cells are injected into mouse blastocysts, which are transferred into pseudopregnant recipient mice for generation of chimeric offspring. Chimeras are crossed with wild-type mice and animals of the F1 generation are tested for the gene defect, transmitted through the germline of the chimeras.

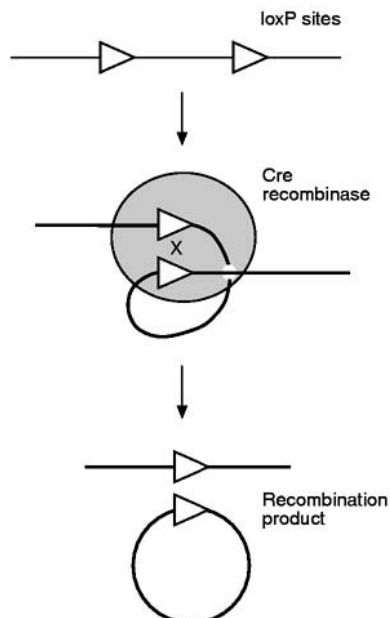


Figure 3 Site-specific recombination by Cre recombinase. Cre recombinase performs site-specific recombination on two loxP sites introduced in tandem into the target DNA. As a result, DNA sequences located between the two loxP sites will be excised from the target DNA and released in the form of a circularized excision product.

lost, when the plasmid is integrated by homologous recombination. In contrast, random integration will frequently result in insertion of the HSV-tk gene into the host cell DNA. These cell clones will be eliminated by selection in FIAU. Using this positive/negative selection strategy, targeting constructs can be introduced into any gene for which homologous DNA sequences are available. Typically, a knockout experiment will generate cells that carry one wild-type and one mutant allele of the target gene.

Generation of mice from genetically modified embryonic stem cells

The capability to introduce genetic modifications such as gene disruptions into mice is based on initial studies by Mintz, extended by Evans and Kaufmann and Martin. Their studies demonstrated that embryonic stem cells (ES cells), derived from the inner cell mass of the early mouse embryos, could be propagated and manipulated in culture without losing their pluripotency. After reintroduction into blastocysts these cells colonized many tissues of the developing embryo including the germline. This offered the

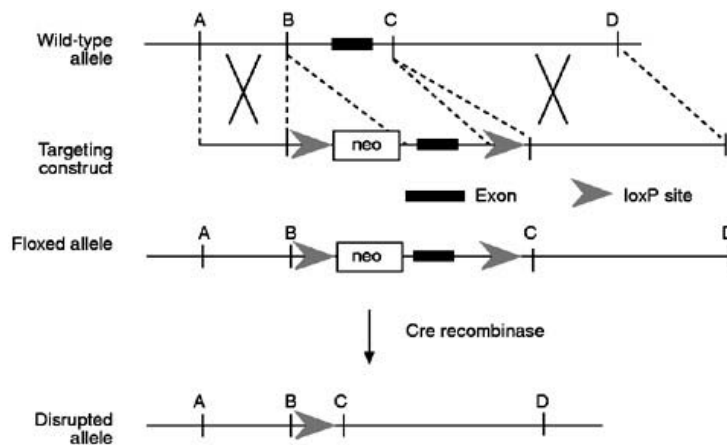


Figure 4 Strategy for Cre recombinase-mediated gene inactivation. A targeting construct is assembled from DNA segments AB, BC and CD of the murine target gene locus (wild-type allele). Essential exon sequences in fragment BC (filled box) are flanked by introns (solid line) containing the neo expression cassette and two loxP sites (arrowheads). Integration of the targeting vector into the wild-type allele does not affect target gene expression because only noncoding intron sequences are modified (floxed allele). However, Cre recombinase activity will cause site-specific excision of exon sequences flanked by the two loxP sites, disrupting the target gene locus (disrupted allele).

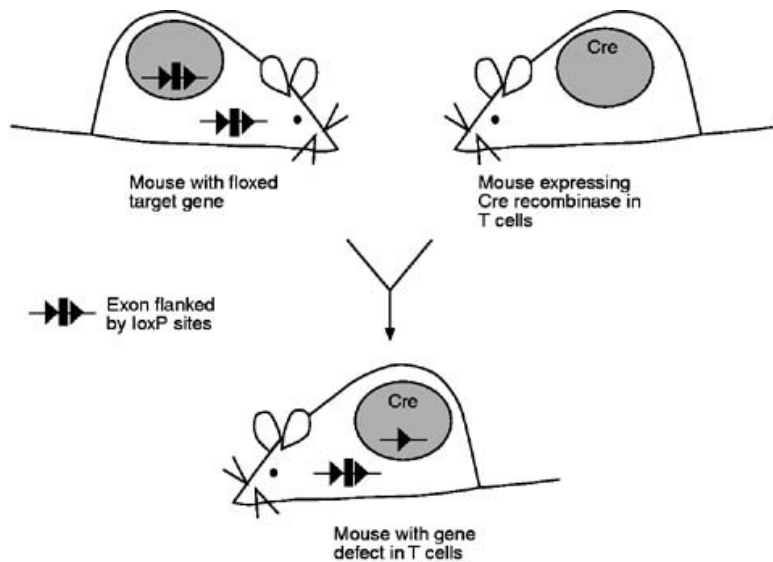


Figure 5 Tissue-specific gene disruption in mice. A mouse carrying the floxed target gene in all tissues is bred with an animal expressing the Cre recombinase gene under tissue-specific promoter elements (e.g. T cells). In the offspring, activity of Cre recombinase will disrupt the target gene in T cells exclusively, leaving all other cell types unaffected.

possibility to perform a gene knockout experiment in ES cell cultures and to introduce the genetic alteration into mice by transferring the genetically modified ES cells into mouse embryos (Figure 2).

In detail, ES cell clones heterozygous for a gene disruption are isolated by positive/negative selection and are injected into the blastocoel cavity of mouse embryos at day 3.5 of gestation using micromanipulation. These blastocysts are subsequently transferred into pseudopregnant foster mothers to develop to term. The chimeric animals obtained are bred to normal mice and their offspring are tested for the presence of the mutant allele that has been transmitted through the germ line. F1 generation animals which are heterozygous for the gene alteration can then be

bred to each other to derive mice homozygous for the mutant allele (Figure 2).

Mouse models for genetic defects of the immune system

The gene knockout technology has been particularly useful to study the function of the immune system. Numerous mouse strains with deficiencies in T cell and B cell function, in cytokines and their receptors or in the development of lymphoid organs have been developed. Analysis of these models not only gave new insights into mechanisms controlling the immune system but also provided valuable animal models of immunologically relevant diseases in

humans such as autoimmunity, immunodeficiency and infections.

Conditional gene disruption, the second generation knockouts

Conventional gene disruption by homologous recombination manifests itself in all cells of an organism and at all stages of ontogenesis. Under certain circumstances however, it might be desirable to induce a gene defect only at distinct stages in development or in a tissue-specific manner. For instance, embryonic lethality caused by disruption of developmentally essential genes precludes the analysis of the gene defects in adult animals. One approach to achieve conditional gene inactivation is based on the activity of the Cre recombinase of the bacteriophage P1, a site-specific recombinase which specifically binds to a 34 bp loxP recognition sequence (Figure 3). If two loxP sites are located in the same orientation on a DNA molecule, Cre recombinase-mediated recombination will result in excision of the intervening DNA region, leaving a single loxP site behind. This system has been adapted to achieve inducible gene disruption in mammalian cells.

One such system is described in Figure 4. A targeting construct is generated that contains a region of the target gene modified by integration of the neo marker and one loxP site in one intron and a second loxP site in an adjacent intron. The modified introns flank an essential exon of the target gene. Using standard protocols, this modification is introduced into the target gene of ES cells via homologous recombination (Floxed allele), and mice carrying two copies of the loxP-modified gene are derived (Figure 2). These animals are phenotypically normal as integration of neo and the loxP sites in the intron sequences most likely does not interfere with the functional expression of the gene. However, induced expression of the Cre recombinase in these mice will result in excision of the exon sequences flanked by the two loxP sites and in disruption of the gene (Figure 4, Disrupted allele).

Controlling the temporal and spatial expression of the Cre recombinase in loxP-modified mice is key to determining the conditions of the gene inactivation. Tissue-specific gene inactivation can be achieved by crossing loxP-modified mice with transgenic mice carrying the Cre recombinase gene under the control of tissue-specific promoter elements. In the double transgenic animals, the target gene is inactivated in cell types expressing Cre recombinase but not in other tissues (Figure 5). This approach has been used by Rajewsky and coworkers to inactivate the gene for DNA polymerase β in T cells exclusively, and to

study the importance of the gene product in the somatic gene rearrangements at the T cell receptor gene locus. Previously, these studies have not been possible because the obligate DNA polymerase β gene knockout resulted in embryonic lethality.

See also: Gene therapy; Herpes simplex virus, infection and immunity; Immunodeficiency, animal models; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; Transgenic animals.

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L

LANGERHANS CELLS

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Langerhans cells are members of the dendritic cell family and are the major antigen-presenting cells of epidermis. Derived from bone marrow, Langerhans cells or their precursors migrate through blood to the suprabasal area of epidermis, where they represent 2–3% of all epidermal cells. Typical markers for these cells include the cell surface expression of CD1a, a major histocompatibility complex (MHC) class I-like molecule, and the pathognomonic Birbeck granule (Figure 1), a tennis racquet-shaped structure observed on electron microscopy. Functionally, Langerhans cells process epicutaneous protein antigens, migrate from epidermis, and present antigenic peptides to T cells in the context of MHC class I and II molecules, either within skin or within the paracortex region of regional lymph nodes. The study of Langerhans cell function during the sensitization and elicitation phases of contact hypersensitivity has proven to be invaluable as a paradigm for the study of immune responses in general, and for the study of cell-mediated delayed-type hypersensitivity reactions in particular. In addition, the biological effects on Langerhans cell function induced by exogenous agents, such as ultraviolet radiation and the human immunodeficiency virus (HIV), have been intensely studied.

History

In 1868, the German medical student Paul Langerhans described cells in human epidermis that stained with gold chloride and exhibited prominent cell surface processes that resembled neuronal dendrites. Because of the morphologic similarities to neurons, Langerhans believed these cells consisted of primitive



Figure 1 Electron micrograph of a human Langerhans cell demonstrating the classic ultrastructural features of this cell. These include an indented nucleus, a relatively electron lucent cytoplasm, and the pathognomonic Birbeck granules (inset), which are shaped like rods or tennis racquets.

nerve tissue. Others proposed the theory that Langerhans cells represented dying melanocytes being sloughed from the skin surface. In the 1960s, the first description of the characteristic ultrastructural

feature of Langerhans cells (i.e. the Birbeck granule), the observation that the tumors of histiocytosis X contained Langerhans cells, and the demonstration of normal Langerhans cells in murine skin experimentally deprived of neural crest components led to the conclusion that these cells were distinct from neurons and melanocytes. Breakthrough observations that Langerhans cells were bone marrow-derived leukocytes with potent antigen-presenting cell properties followed in the 1970s. Table 1 summarizes the historical highlights in Langerhans cell research prior to 1980. Following the correct characterization and classification of Langerhans cells, recent research has focused on the detailed cellular and molecular mechanisms involved in all aspects of Langerhans cell biology.

Cell surface phenotype and cytokine profile

Langerhans cells are the prototypic nonlymphoid members of the dendritic cell family (Table 2). The cells in this family exhibit prominent dendritic morphology, characteristic phenotype (Table 3), and potent antigen-presenting cell capabilities. These features make this family of cells distinct from monocytes/macrophages and other bone marrow-

Table 1 Historical highlights in the study of Langerhans cells prior to 1980

Year	Significant finding
1868	Cells first described by Langerhans and proposed to represent nervous tissue
1948	Popular theory proposed by Masson that Langerhans cells consisted of dying melanocytes
1961	Birbeck and colleagues describe the characteristic ultrastructural feature of Langerhans cells, the Birbeck granule, which distinguishes them from all other cell types
1962	Langerhans cells first stained for ATPase, defining them further as distinct metabolically active epidermal cells
1965	Basset and colleagues note the cells of histiocytosis X tumors exhibit 'virus-like' particles, which are later noted to be Birbeck granules
1968	Breathnach and coworkers demonstrate Langerhans cells, but not melanocytes and neurons, in mouse skin experimentally deprived of its neural crest component.
1977	Three papers published in a single issue of <i>Nature</i> showing that Langerhans cells express cell surface Fc and C3 receptors, as well as MHC class II molecules, firmly establishing Langerhans cells as immunocompetent cells
1979	Bone marrow origin of Langerhans cells clearly established

Table 2 Selected members of the dendritic cell family and their tissue location

Dendritic cell type	Tissue location
Langerhans cells	Epidermis, oral and genital mucosa ^a
Dermal dendritic cells	Dermis
Veiled cells	Afferent lymph
Interdigitating cells	Paracortex region of lymph nodes (T cell-rich areas) ^b
Blood dendritic cells	Blood

^aThis article focuses on epidermal Langerhans cells, as opposed to mucosal Langerhans cells, although these cells share many of the same features.

^bOf note, follicular dendritic cells found in the cortex region of lymph nodes (B cell-rich areas) are not generally considered to belong to the dendritic cell family.

Table 3 Cell surface molecule expression^a by freshly isolated (f) and cultured (c) human^b Langerhans cells (LC) compared to blood dendritic cells (DC)^c and monocytes/macrophages (M/Mø)

Cell surface antigens	fLC	cLC	Blood DC	M/Mø
<i>MHC and MHC-like molecules</i>				
HLA-A, -B, -C	+	++	++	+
CD1a	++	+	-	-
CD1b	-	-	-	-
CD1c	+	+/-	-	-
HLA-DR, -DP, -DQ	+	++	++	+
<i>Costimulatory/adhesion molecules</i>				
B7-1 (CD80)	-	++	++	+/-
B7-2 (CD86)	-	++	++	+
ICAM-1 (CD54)	+	++	++	+
ICAM-2 (CD102)	-	-	-	+
ICAM-3 (CD50)	++	++	++	+
LFA-3 (CD58)	+	++	++	+
CD40	+/-	++	++	+/-
CD11a	-	+/-	++	+
CD11b	-	-	-	+
CD11c	+/-	-	++	+
β ₁ -Integrin (CD29)	-	-	++	+
β ₂ -Integrin (CD18)	+	+	++	+
E-Cadherin	++	+	nd	nd
<i>Fc receptors</i>				
FcγRI (CD64)	-	-	-	-
FcγRII (CD32)	+	-	-	+
FcγRIII (CD16)	-	-	-	-
FcεRI	+	nd	nd	+/-
FcεRII (CD23)	-	+/-	-	+/-
<i>Miscellaneous</i>				
CD83	+/-	+	+	-
CD45	++	++	++	++
CD4	+	+	+/-	+
CD3	-	-	-	-
CD14	-	-	-	++
CD19	-	-	-	-

^aand, not determined; -, no expression; +/-, expression under certain conditions or in a subset of cells; +, low to moderate levels of expression; ++, moderate to high levels of expression.

^bMurine Langerhans cells have a similar antigenic profile.

^cBlood dendritic cells cultured over a 36-hour period as described by Steinman and coworkers.

derived cells. In general, Langerhans cells *in situ* and freshly isolated Langerhans cells represent functionally 'immature' dendritic cells. They express lower levels of cell surface MHC class II molecules and stimulate resting T cells less well in mixed lymphocyte reactions when compared to 'mature' dendritic cells, e.g. peripheral blood dendritic cells. However, upon culturing *in vitro* for 2–3 days, Langerhans cells upregulate MHC class II expression, as well as costimulatory molecules, and become functionally 'mature', i.e. they become potent stimulators in mixed lymphocyte reactions. Table 3 outlines the cell surface antigens expressed by both freshly isolated and cultured Langerhans cells, with a comparison to the antigenic profiles observed on blood dendritic cells and monocytes/macrophages. Of note, cell surface expression of CD1a is a reliable marker for human Langerhans cells (Figure 2); the only other cells that express this MHC class I-like molecule include a subset of cortical thymocytes and a small subset of dermal dendritic cells.

Because of the difficulty in isolating large numbers of relatively pure cells, elucidation of the cytokines produced by Langerhans cells (as well as by other dendritic cells) has been difficult. In particular, the presence of small numbers of contaminating keratinocytes (which produce a wide array of cytokines and growth factors) within selected Langerhans cell populations can confuse this issue. Nevertheless, several investigators have shown that freshly isolated Langerhans cells are capable of producing macrophage inflammatory protein 1 α (MIP-1 α), MIP-2, interleukin 12 (IL-12) and IL-15. In contrast, cultured Langerhans cells produce IL-1 β and IL-6. When carefully controlled for keratinocyte

contamination, it is unclear whether Langerhans cells produce other known cytokines. Interestingly, cytokines produced by surrounding cells (i.e. keratinocytes) can greatly influence Langerhans cell phenotype and function. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes growth of Langerhans cell precursors and potentiates immune function of Langerhans cells, tumor necrosis factor α (TNF α) promotes viability of Langerhans cells in culture and probably the emigration of Langerhans cells from epidermis, and IL-10 can decrease antigen presentation by Langerhans cells.

Antigen-presenting cell function

Tissue location, dendritic morphology, cell surface phenotype, and ability to migrate to T cell-rich areas make Langerhans cells ideally suited as antigen-presenting cells. Figure 3 outlines the generally accepted model for Langerhans cell-mediated T cell activation. Facilitated by the prominent cell surface dendritic processes, Langerhans cells take up protein antigens or protein-hapten complexes within epidermis. They then traverse the epidermal basement membrane and present MHC-restricted peptides to CD4⁺ or CD8⁺ T cells; antigen presentation occurs either within dermis, to memory T cells that have circulated in dermal capillaries, or within the paracortex area of regional lymph nodes, to naive T cells following Langerhans cells emigration through afferent lymphatics. Evidence to support this model has come from both *in vitro* and *in vivo* studies, and in both human and murine systems. Allergic contact dermatitis (e.g. poison ivy dermatitis) is the prototypic clinical disease associated with sensitization to a contact allergen, Langerhans cell antigen presentation and T cell activation as outlined here.

Not surprisingly, freshly isolated Langerhans cells (analogous to Langerhans cells *in situ*), when compared to cultured Langerhans cells (analogous to Langerhans cells that have emigrated to regional lymph node), are more efficient at antigen uptake *in vitro*. Conversely, stimulation of naive T cells, a process that normally occurs within lymph nodes *in vivo*, is best performed by cultured Langerhans cells *in vitro*. The cell surface antigens present on freshly isolated and cultured Langerhans cells, as outlined in Table 3, are consistent with this functional data. For example, the high levels of cell surface MHC and costimulatory molecules present on cultured Langerhans cells are critical for efficient stimulation of naive T cells. Additionally, Birbeck granules, which are structures that may be involved in antigen uptake, are detected only in freshly isolated Langerhans cells.

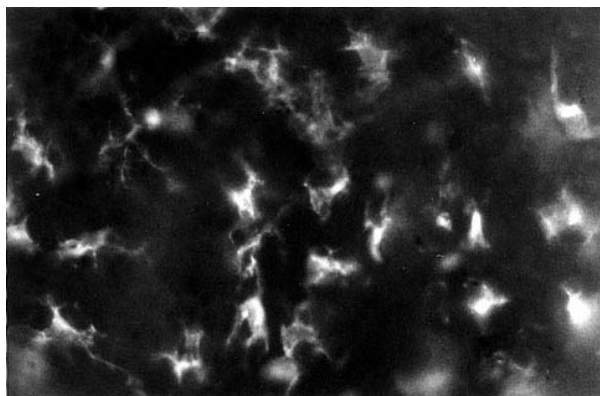


Figure 2 Human Langerhans cells within acetone-fixed epidermis stain with FITC-conjugated monoclonal antibody directed against CD1a. To obtain epidermis, a blister was induced on the skin of the volar forearm using vacuum suction and heat and the blister roof (epidermal sheet) was removed using sterile scissors. Note the characteristic prominent dendritic processes.

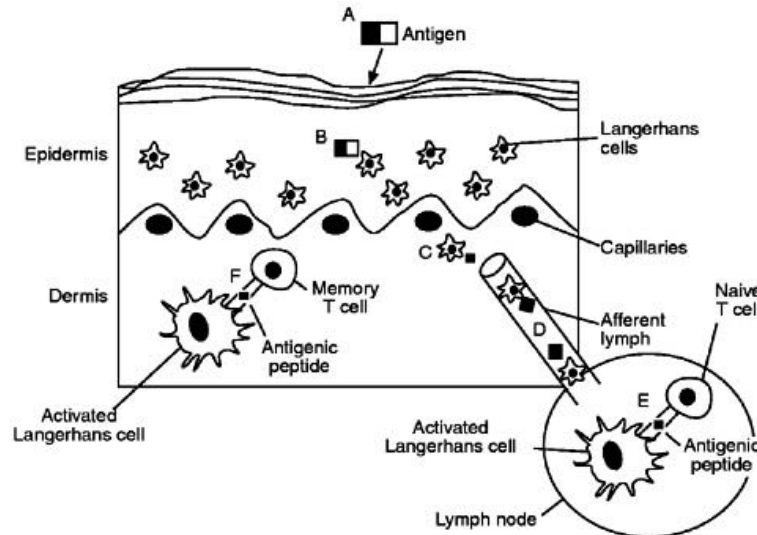


Figure 3 Schematic diagram showing the function of Langerhans cells in mediating antigen-specific T cell activation. Following contact with an antigen or hapten (which becomes antigenic following binding to a protein carrier) on the skin surface (A), epidermal Langerhans cells take up and process antigen within MHC class I or class II compartments (B). Activated Langerhans cells, i.e. Langerhans cells exposed to antigen, migrate from epidermis and display antigenic peptides on their cell surface (C). For primary immune responses, activated Langerhans cells enter afferent lymphatics within skin and travel to regional lymph nodes (D). To facilitate activation of naive T cells, cell surface MHC and costimulatory molecules are upregulated during this migration. Within the paracortex of lymph node, stimulation of naive T cells occurs in an MHC-restricted antigen-specific manner (E). For secondary immune responses, activated Langerhans cells present antigen and stimulate memory T cells, which circulate in capillaries, within the dermis (F).

Current advances

Exposure to ultraviolet radiation can lead to both local and systemic immunologic defects. In particular, Langerhans cell depletion and dysfunction play a central role in ultraviolet light-induced immunosuppression. Ultraviolet light B (wavelength 290–320 nm) can induce Langerhans cell phenotypic changes and death; it also leads to the repopulation of epidermis with CD36⁺ macrophage-like dendritic cells 24–72 h after exposure. Functionally, the lack of normal Langerhans cells and the presence of abnormal Langerhans cells and macrophage-like cells leads to impaired contact sensitivity. Specifically, application of a contact allergen to ultraviolet light B-irradiated skin can induce tolerance for that particular antigen. In addition, it has been proposed that mice and humans susceptible to the development of skin cancer are those susceptible to ultraviolet light B-induced defects in contact sensitization. These studies suggest that short-term immunologic defects induced by ultraviolet light are linked to long-term defects in processing and presentation of tumor antigens. Thus, in addition to foreign antigen recognition, Langerhans cells are probably important in tumor surveillance within skin.

Epidermal Langerhans cells can be targets for HIV

infection both *in vitro* and *in vivo*, however, most studies show that they do not represent a major reservoir for virus. In contrast, Langerhans cells in genital mucosa (where no stratum corneum exists) may be more easily infected by HIV and therefore may be the first cells infected during initial sexual exposure to HIV. Following emigration to regional lymph nodes, HIV-exposed Langerhans cells may then transmit virus to CD4⁺ T cells. In support of this theory, Langerhans cells and other dendritic cells pulsed with HIV *in vitro* transmit high levels of infection to co-cultured T cells. Interestingly, this efficient transmission of virus does not require dendritic cell infection to occur *per se*. That is, these cells can capture HIV within their prominent cell surface dendritic processes in an infection-independent manner; subsequently, captured virus is transmitted to T cells during the process of antigen-specific T cell activation. Additionally, impaired Langerhans cell function has been proposed as an important factor in the pathogenesis of HIV-associated skin diseases, although initial studies to assess the function of Langerhans cells isolated from skin of HIV-infected individuals failed to detect marked defects in antigen presentation.

See also: **Acquired immune deficiency syndrome**

(AIDS); Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; CD1; Contact hypersensitivity; Cytokines; Dendritic cells; Photoimmunology; Skin, contribution to immunity.

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LARGE GRANULAR LYMPHOCYTES

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Studies performed in the 1970s on human peripheral blood mononuclear cells led to the identification of a unique population of non-T, non-B cells bearing receptors for the Fc portion of immunoglobulin G. These cells, originally called third population cells or T₃ cells, are now named large granular lymphocytes (LGLs) based on subsequent studies conducted in several laboratories.

Morphology, phenotypic markers, and lineage(s) of large granular lymphocytes

LGLs are morphologically distinctive in having a low nucleus/cytoplasm ratio, with an indented nucleus

and many azurophilic granules in the cytoplasm. These granules can be easily identified under the light microscope using various cytochemical staining techniques (**Figure 1**) and appear opaque (electron-dense) under the electron microscope. LGLs represent a minor population, ranging from 2 to 15%, of human peripheral blood lymphocytes. Similar cell populations have also been found in the blood and different tissues/organs of mice, rats, and other animal species. In rats, the 'pit cells' (so named because of the presence of numerous cytoplasmic granules resembling fruit pits) identified in the liver sinusoids were also found to be LGLs. Owing to their larger size and lower density, LGLs can be readily separated from small ('resting') lymphocytes by centrifug-

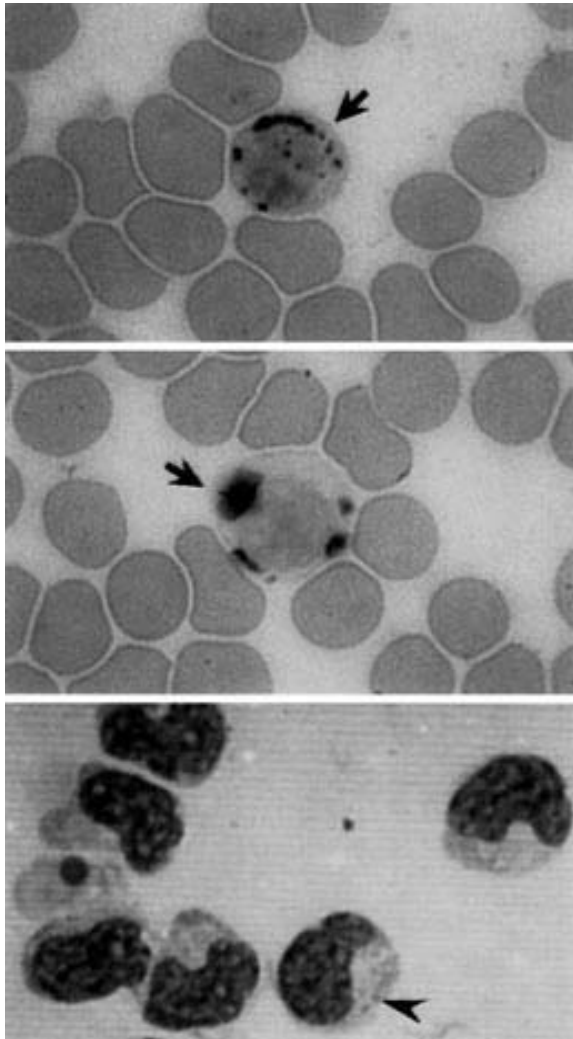


Figure 1 LGLs in human peripheral blood and after isolation. The cytoplasmic granules of peripheral blood LGLs (indicated by arrows; top and middle panels) are visualized by cytochemical staining for α -naphthylacetate esterase activity. Small, discrete granules and occasionally giant granules can be identified. May-Grunwald-Giemsa stain of isolated LGLs (bottom panel) show more clearly their indented nucleus and abundant cytoplasm with azyrophilic granules (indicated by a pointer). Magnification $\times 1000$.

ation on discontinuous Percoll density gradients, allowing for their characterization in detail.

The phenotypic markers expressed by LGLs suggest heterogeneity in this population of cells. Most human LGLs express CD2, CD16 (Fc γ RIII), CD56 (NKH1; Leu-19), but not CD3 and T cell receptor (T cell markers) or surface immunoglobulin and CD19 (B cell markers). Some LGLs, however, do express T cell surface molecules such as CD8. On the other hand, some LGLs have been shown to express a myeloid marker protein recognized by a monoclonal antibody OKM1. In mice, most LGLs are of

the CD3⁺CD4⁺CD8⁺ phenotype but express other surface antigens such as NK-1.1/2.1, LGL-1, and asialoGM₁. Some mouse LGLs also express CD8 (Lyt-2) surface marker. The heterogeneity in the phenotypes of LGLs, while posing an ambiguity of their relationship with other hematopoietic cell lineages, indicates the diversity in their precursor cells (see below). However, recent studies have suggested that LGLs are derived from lymphoid progenitor cells in the bone marrow (Figure 2).

Functions

When the cytolytic activities of different populations of peripheral blood lymphocytes separated on Percoll density gradient were investigated, it became evident that LGLs accounted for virtually all of the so-called 'natural' cytotoxicity. Natural cytotoxicity is thought to be mediated by natural killer (NK) cells which, in contrast to cytotoxic T lymphocytes (CTLs), do not exhibit antigen-specific memory upon immunization. NK cells recognize antigens/target cells in a non-major histocompatibility complex (MHC)-restricted manner. They have also been shown to mediate antibody-dependent cellular cytotoxicity (ADCC). Our understanding of how NK cells recognize/interact with target cells has significantly advanced following the identification of several families of NK cell receptors (e.g. Ly-49 in mice) in the past few years. Remarkable progress has also been made in the elucidation of the cytolytic mechanisms utilized by NK cells and CTLs. Studies have revealed the presence of a number of cytolytic mediators in the cytoplasmic granules of NK cells and CTLs.

Due primarily to the functional correlation outlined above, the terms LGL and NK cell are often considered interchangeable. However, it should be pointed out that not all LGLs are NK cells and not all NK cells exhibit the morphology of LGLs. CD3⁺CD8⁺ CTLs, which are small and have little cytoplasm with none or few granules under resting conditions, can develop LGL morphology upon stimulation by antigens and/or cytokines (e.g. interleukin 2, IL-2). For example, it has been clearly demonstrated that LGLs isolated from mice infected with lymphocytic choriomeningitis virus contain both CD3⁺asialoGM₁⁺ NK cells and CD3⁺asialoGM₁⁺ CTLs. Moreover, small agranular NK cells have been reported in the literature. Therefore, LGL morphology may represent an activation status of lymphocytes rather than a unique feature of NK cells. It is now widely accepted that LGLs are the phenotypic expression of a variety of activated cytolytic cell types, which include NK cells, CTLs,

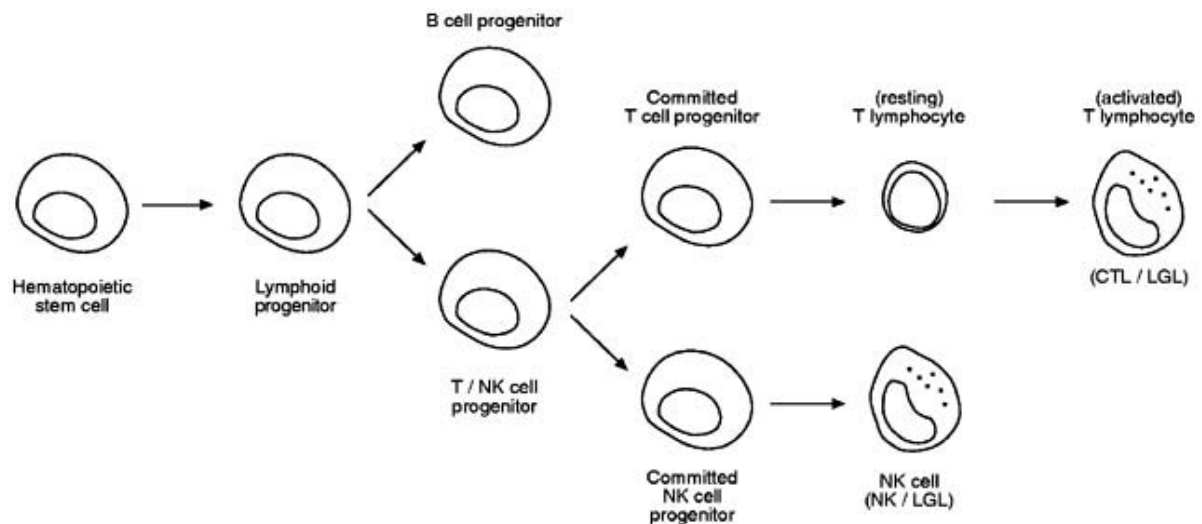


Figure 2 Schematic diagram of the development of LGLs.

and γ/δ T cells. Nevertheless, it should be emphasized that LGLs, regardless of whether they are NK cells, CTLs or γ/δ T cells, are thought to play an important role in immune defense against viral infections or infections with other microbes, as well as in immune surveillance against tumor cells.

In addition to mediating cytolytic activity, LGLs have been shown to secrete a variety of cytokines, such as IL-1, IL-2, interferons, and colony-stimulating factors. These cytokines can stimulate the growth of hematopoietic cells, activate other immune cells, and induce the expression of various genes crucial for immune responses including those encoding MHC. This functional capacity of LGLs indicates their involvement in the regulation of immune responses.

Other laboratory and clinical features

The growth of LGLs appears to be under the regulation of various cytokines, in particular IL-2. Early studies demonstrated that high concentrations of IL-2, in the absence of other stimuli such as antigens or mitogens, can stimulate the proliferation of LGLs but not T lymphocytes. Moreover, LGLs propagated in the presence of IL-2 retain potent cytolytic activity. The capacity of LGLs to respond directly to IL-2 is probably due to the high level of expression of the IL-2 receptor β chain. In addition to IL-2, interferon γ is also capable of stimulating the growth of LGLs. These observations, combined with the fact that LGLs themselves can secrete cytokines, suggest that LGLs are important players in the cytokine network of the immune system.

The remarkable response of LGLs to IL-2 has made it possible to generate lymphokine-activated

killer (LAK) cells *in vitro*. LAK cells, propagated primarily from LGLs/NK cells isolated from peripheral blood, contain more cytoplasmic granules and display a broader range of cytotoxicity against tumor cells than do their precursor LGLs. Similarly, the so-called 'tumor-infiltrating lymphocytes' (TILs), probably of CTL origin and with potent cytolytic activity, can be generated in culture in the presence of IL-2. These mighty killer cells, alone or in conjunction with IL-2, have been used in adoptive immunotherapy of advanced cancer.

Defects in LGL development, growth or functioning may result in clinical disorders. For example, aberrant proliferation of LGLs can lead to LGL leukemia. In addition to the expansion of LGLs, patients with LGL leukemia often develop accompanied abnormalities such as neutropenia (decrease of neutrophils) and rheumatoid arthritis. Hypofunction of LGLs/NK cells has been observed in patients with the Chédiak-Higashi syndrome, a genetic disorder associated with fusion and defective degranulation of lysosomes and cytoplasmic granules in various types of granulated cells. As a consequence of granule dysfunction in hematopoietic cells, these patients exhibit increased susceptibility to infections. In mice, the beige mutation results in a phenotype similar to that of the Chédiak-Higashi syndrome, with anomalies in granule formation in LGLs/NK cells and reduced cytolytic capacity.

See also: Antibody-dependent cellular cytotoxicity; Cytokines; Cytotoxic T lymphocytes; Cytotoxicity, mechanisms of; Effector lymphocytes; Granzymes; Cell-mediated lysis; Immunotherapy of tumors; Innate immunity; Lymphocytes; Lymphokine-acti-

vated killer (LAK) cells; MHC restriction; Natural killer (NK) cells; Perforin.

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LECTINS

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Lectins (*L. legere*, to select or choose) are cell-agglutinating proteins of nonimmune origin that bind mono- and oligosaccharides reversibly and with high specificity. They also precipitate polysaccharides and glycoproteins. The agglutinating and precipitating activities of lectins are very similar to those of antibodies. They can likewise be specifically inhibited by low molecular weight compounds (haptens) which, in the case of lectins, are monosaccharides or oligosaccharides. Indeed, many of the methods of lectin research, as well as the applications of these proteins, are based on immunochemical techniques. Lectins, however, differ from antibodies in several respects. For instance, they are produced not only by animals but by essentially all classes of living organisms and by different types of cell, and are structurally diverse. In their structural diversity, lectins are akin to enzymes, but they are devoid of catalytic activity.

Lectins are of major interest since they act as recognition determinants in many biological processes such as clearance of glycoproteins from the circulatory system, adhesion of infectious agents to host cells, recruitment of leukocytes to inflammatory sites and cell–cell interactions in the immune system. Hundreds of lectins have been purified from plants and other sources, mainly by affinity chromatography on immobilized carbohydrates, and have been characterized. Some serve as invaluable carbohydrate specific tools in biological and medical research, and are used clinically as well.

History

The history of lectins is closely interwoven with that of immunology. Shortly after the first reports appeared on the presence of hemagglutinating proteins in plant extracts – ricin in the castor bean (1888) and abrin in extracts of the jequirity bean (1891) – Ehrlich realized that they would be more useful as model antigens than the bacterial toxins, such as that of diphtheria, which were popular research tools at the time. Although Ehrlich's preparations were very crude, he was able to establish with them some of the fundamental principles of immunology, such as the specificity of the immune response and the transfer of immunity from a mother to her offspring via blood during pregnancy, and via milk after birth. By studying the inhibitory effect of ricin antiserum on the agglutinating activity of ricin, he proved that there was a quantitative relationship between the amount of antiserum and the amount of antigen it neutralized, and thus performed the first quantitative determination of an antibody *in vitro*.

In 1908, Landsteiner observed that the hemagglutinating activities of various seed extracts varied when tested with red blood cells from different animals and concluded that the reactions of plant hemagglutinins 'resemble antibody reactions in all essentials'. The sugar specificity of lectins was discovered in 1936 by Sumner who found that hemagglutination by concanavalin A, the jack bean lectin, was inhibited by cane sugar. With much foresight, Sumner suggested that the agglutination might be the

consequence of a reaction of the lectin with carbohydrates on the erythrocyte surface.

In the 1940s, Boyd and Reguera, and independently Renkonen, discovered that certain seeds contain agglutinins specific for some human blood group antigens. Thus, lima bean lectin was found to be specific for A-type erythrocytes and the lectin from the seeds of *Lotus tetragonolobus* specific for O-type cells. Subsequently, lectins specific for other blood groups were found. Blood type-specific lectins played a crucial role in the demonstration, by Kabat and by Watkins and Morgan, that carbohydrates are the immunodeterminants of the ABO blood group system.

Despite these developments, lectins attracted only limited attention until the early 1960s, when Nowell first reported that the lectin of the red kidney bean (*Phaseolus vulgaris*), known as phytohemagglutinin (PHA), is mitogenic. This discovery had a revolutionary impact on immunology in that it shattered the view, held until then, that small, resting lymphocytes are dead-end cells that could neither divide nor differentiate. It led to increased interest of immunologists in lectins. Thus, interleukin 2 was discovered in 1976, by Gallo, in the growth medium of normal human lymphocytes stimulated by PHA. In the late 1970s, Reisner and Sharon introduced the lectins for lymphocyte fractionation and purging of bone marrow for transplantation across histocompatibility barriers. More recently, lectins have been shown to perform key recognition functions in the immune system.

Molecular properties

Lectins constitute a heterogeneous group of oligomeric proteins that vary widely in their molecular properties (Figure 1). Nonetheless, many of them can be grouped into distinct families that share sequence homologies and similar structural properties, and in some cases also in superfamilies. The largest and most thoroughly studied family is that of legume lectins, which includes concanavalin A, PHA, and the lectins from soybean (SBA) and peanut (PNA). Close to 100 such lectins have been purified, the complete sequences of some 40 of which are known. In addition, the three-dimensional structures of close to ten of these lectins, most in complex with their carbohydrate ligand, have been elucidated.

Typically, legume lectins consist of two or four identical, or almost identical, subunits (or protomers) of 25–30 kDa, each of which contains a single carbohydrate-binding site with the same specificity. A prominent exception is PHA, which occurs as a family of five tetrameric proteins with varying proportions of two classes of subunit, E and L, and different biological properties: E₄ (E-PHA) is a potent hemagglutinin. L₄ (L-PHA) has leukoagglutinating activity and is a potent mitogen; intermediate forms (e.g. E₂L₂ or E₃L₁) possess lower levels of the above activities; E₄ and L₄ also differ in their specificity for oligosaccharides. Other families of plant lectins are those of cereals (e.g. wheat germ agglutinin, WGA) and of solanaceous plants (e.g. potato and tomato lectins). Members of these

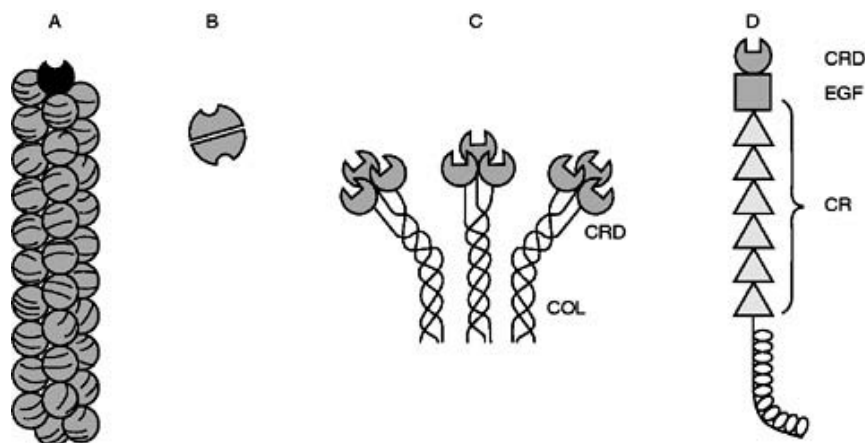


Figure 1 Schematic representation of the overall structure of lectins from different sources. (A) Part of fimbria of *E. coli*. The fimbriae are an assembly of different types of subunit, only one of which (black) contains a carbohydrate-binding site. (B) Dimeric legume lectin (e.g. from peas or from seeds of *Erythrina corallodendron*). (C) Mammalian soluble mannose-binding protein, a collagen lectin. (D) E-Selectin; the coil represents the membrane-spanning and cytoplasmic domains. COL, collagenous region; CR, complement regulatory repeats; EGF, epidermal growth factor-like domain; CRD, carbohydrate recognition domain. The indentations represent binding sites. (Reproduced with permission from Sharon N and Lis H (1995) *Essays in Biochemistry* 30: 59–75.)

families, too, consist mostly of two identical subunits but differ markedly in other properties from those of the legume lectins.

Animal lectins are frequently composite molecules with a wide range of molecular weights, consisting of multiple types of protein modules or domains, only one of which serves as a carbohydrate-recognition domain (CRD) (Figure 1). Three types of CRDs have been discerned: P-type, S-type and C-type, each of them shares a pattern of invariant and highly conserved amino acid residues at a characteristic spacing. The occurrence of such a domain in a protein provides a novel guideline for the identification and classification of new lectins. The P-type CRD has to date been found only in the mannose-6-phosphate receptors. Proteins containing S-type and C-type CRDs are known as galectins (previously S-lectins) and C-type lectins, respectively. The galectins constitute a family of soluble lectins that bind exclusively β -galactosides, such as lactose ($\text{Gal}\beta(1-4)\text{Glc}$) and *N*-acetyllactosamine ($\text{Gal}\beta(1-4)\text{GlcNAc}$). They have a relatively simple structure (molecular weights between 14 and 35 kDa) and occur predominantly in mammals, but have also been described in other vertebrates. Their three-dimensional structure is similar to that of the legume lectins and they may thus be considered as members of the same superfamily. The C-type lectins include endocytic lectins, collectins and selectins. Each of these families shares a common overall architecture, defined by the nature of their domains. In addition to their CRDs, the collectins, for example, contain a collagen-like region, while the selectins – E-selectin (CD62E), P-selectin (CD62P) and L-selectin (CD62L) – contain an epidermal growth factor-like domain and several short repeating units related to complement-binding protein. The collectins are similar in design to complement protein C1q, which also contains a collagen-like domain linked to globular domains involved in the binding of immunoglobulin.

The sialoadhesins are a family of sialic acid-specific cell surface lectins with variable numbers of extracellular immunoglobulin-like domains and are thus members of the immunoglobulin superfamily. They include the sheep erythrocyte receptor of macrophages (referred to simply as sialoadhesin), CD22 found only on B cells and MAG, the myelin-associated glycoprotein.

Bacterial lectins are usually in the form of fimbriae (or pili), hair-like submicroscopic appendages, consisting of an assembly of 5–6 different types of subunit, only one of which has carbohydrate-binding activity, e.g. for mannose (in type 1 fimbriae) or galactose, $\text{Gal}\alpha(1-4)\text{Gal}$ (in P fimbriae).

Carbohydrate specificity

Lectins are classified into a small number of specificity groups (mannose, galactose, *N*-acetylglucosamine, galactose/*N*-acetylgalactosamine, L-fucose and *N*-acetylneuraminic acid (sugars are of the D-configuration unless otherwise stated)) according to the monosaccharide for which they exhibit the highest affinity. The lectins within each group may differ markedly in their affinity for the specific monosaccharide or its various derivatives. Certain lectins bind monosaccharides poorly, but interact strongly with di-, tri- and tetrasaccharides. Lectins that bind oligosaccharides only include the selectins, specific for the sialylated Lewis^x blood group determinant, $\text{NeuAc}\alpha(2-3)\text{Gal}\beta(1-4)[\text{Fuc}\alpha(1-3)]\text{GlcNAc}$ (SiaLe^x) and its positional isomer, sialylated Lewis^a blood group determinant, $\text{NeuAc}\alpha(2-3)\text{Gal}\beta(1-3)[\text{Fuc}\alpha(1-4)]\text{GlcNAc}$ (SiaLe^a), with both L-fucose and sialic acid required for binding; the sialic acid can be replaced by another negatively charged group such as sulfate. The association constants for the binding of monosaccharides and oligosaccharides to lectins are typically in the range 10^3 – $5 \times 10^4 \text{ M}^{-1}$ and 10^5 – 10^7 M^{-1} respectively. These values are of the same order of magnitude as those for the binding of haptens to antibodies and of substrates to enzymes.

Lectin-carbohydrate interactions

Lectins combine with carbohydrates by a network of hydrogen bonds and hydrophobic interactions; coordination with metal ions may also play a role. In general, the combining sites of lectins within a family are structurally similar, but are quite different in different families. For instance, legume lectins, irrespective of their specificity, bind ligands through a constellation of three invariant combining site residues, an aspartic acid, an asparagine and an aromatic amino acid. In the rat mannose-binding protein, a calcium ion serves as the nucleus of the combining site, forming direct bonds with the carbohydrate. Additional bonds are provided by two glutamic acid and two asparagine residues (Figure 2).

Biological activities

Certain lectins are potent mitogens; PHA and concanavalin A, for example, stimulate T cells, while pokeweed mitogen (PWM) stimulates both T and B cells. The mitogenic lectins are polyclonal activators, in that they activate lymphocytes, including memory-type cells, irrespective of their antigenic specificity. Prior to the advent of monoclonal antibodies to cell surface antigens, lectins were the major tool for

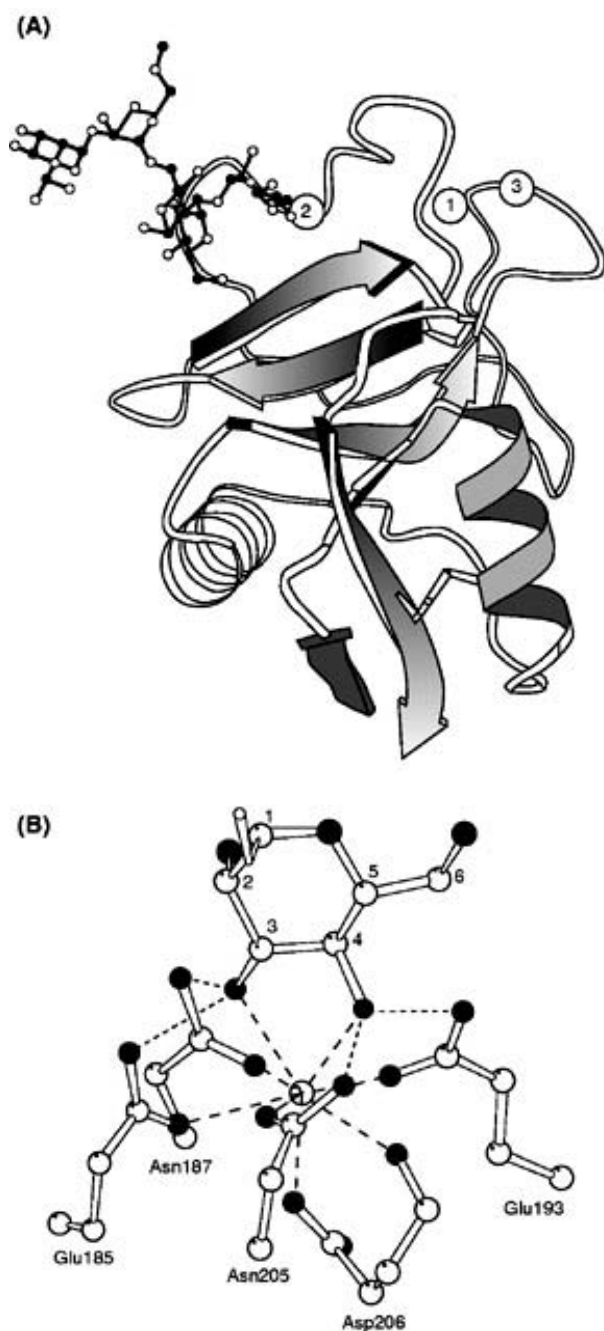


Figure 2 Binding of carbohydrate and Ca^{2+} to the CRD of rat mannose-binding protein. (A) Ribbon representation of the CRD. The β strands are shown as arrows, the α helices as ribbons. (B) Close-up of sugar interacting with CRD. The equatorial 3- and 4-OH groups of the sugar form hydrogen bonds (dotted lines) with two glutamic acid and two asparagine residues, and coordination bonds (dashed lines) with Ca^{2+} . (Reproduced with permission from Weis WI, Drickamer K and Hendrickson WA (1992) *Nature* **360**: 127–134 Copyright 1996 Macmillan Magazines Limited.)

studies of the mechanism of mitogenic stimulation of lymphocytes.

In the presence of a mitogenic lectin, a wide variety of antigenically unrelated target cells are lysed by cytotoxic T lymphocytes, a phenomenon known as lectin-dependent cytotoxicity (in analogy to antigen-dependent cytotoxicity). Another form of lectin-dependent cytotoxicity is the killing of tumor cells by macrophages. This activity resembles that of tumor recognizing antibodies that can induce macrophages to lyse tumors.

Several lectins, e.g. concanavalin A and wheat germ agglutinin, are toxic to mammalian cells in culture and to animals, but their toxicity is at least 1000 times lower than that of ricin and abrin. The cytotoxic properties of such lectins serve for the selection of clones of lectin-resistant cell mutants that are widely employed in studies of the genetics, biosynthesis and function of cell surface carbohydrates.

Functions

The major function of lectins appears to be in cell recognition (Table 1). Considerable evidence for such a role has been obtained for the influenza virus hemagglutinin (a lectin specific for *N*-acetylneuraminic acid) and for bacterial surface lectins (or sugar-specific adhesins) that mediate the binding of the pathogens to host cells, a step essential for the initiation of infection. Inhibitors of bacterial lectins protect animals against experimental infection by the lectin-carrying organisms, providing a basis for the development of antiadhesion therapy of microbial infections. Some bacterial surface lectins may allow the specific binding of the bacteria to human polymorphonuclear cells and human and mouse macrophages in the absence of opsonins, leading to activation of the phagocytes and ingestion and killing of the bacteria; the process has been named lectinophagocytosis, in analogy to opsonophagocytosis. The major receptors for mannose-specific *E. coli* are the leukocyte surface antigens CD11/CD18, which also constitute the receptor CR3 for complement fragment iC3b. The same receptors thus participate in both lectino- and opsonophagocytosis. In invertebrates, it has been postulated that lectins on hemo-cyte membranes are nonself-recognition molecules.

The galectins are believed to function in cell adhesion. These lectins are found both inside the cytoplasm and nucleus of different cells and occasionally also on the cell surface and outside the cell. Their expression is developmentally regulated. The elevated levels of galectins that occur on the surface of metastatic murine and human cancer cells may be responsible for the adhesion of these cells

Table 1 Lectins in cell-cell recognition

Lectin	Role in	Carbohydrate determinant
<i>Microorganisms</i>		
Influenza virus		
Human isolates	Infection	NeuAc α (2-6)Gal
Avian and equine isolates	Infection	NeuAc α (2-3)Gal
Amoeba	Infection	Gal/GalNAc
<i>Bacteria</i>		
<i>E. coli</i> , type 1	Infection	Mannose
<i>E. coli</i> , type P	Infection	Gal α (1-4)Gal
<i>Animals</i>		
Galectins	Cell adhesion (?)	Gal β (1-4)GlcNAc
<i>C-type lectins</i>		
Mannose-binding protein	Host antimicrobial defense	Mannose
L-Selectin	Lymphocyte homing	
E-Selectin	Leukocyte trafficking to sites of inflammation	Sialyl Lewis ^x , Sialyl Lewis ^a
P-Selectin	Leukocyte trafficking to sites of inflammation	Sialyl Lewis ^x , Sialyl Lewis ^a
<i>Sialoadhesins</i>		
Sialoadhesin	Cell adhesion	NeuAc α (2-3)Gal β (1-3/4)GlcNAc; NeuAc α (2-3)Gal β (1-3)GalNAc
CD22		NeuAc α (2-6)Gal β (1-4)GlcNAc
MAG		NeuAc α (2-6)Gal β (1-3)GalNAc

Sialyl Lewis^x, NeuAc α (2-3)Gal β (1-4)[Fuc α (1-3)] GlcNAc; sialyl Lewis^a, NeuAc α (2-3)Gal β (1-3)[Fuc α (1-4)] GlcNAc.

to target organs, a step necessary for the formation of metastases.

The mannose-6-phosphate receptors are responsible for targeting lysosomal enzymes into lysosomes.

The endocytic lectins, a class of C-type lectins, are membrane-bound receptors with different specificities. They appear to be involved in the clearance of glycoproteins, and possibly also of cells (e.g. aged erythrocytes or bacteria), from the circulation. A prominent representative is the mammalian asialoglycoprotein receptor found on hepatocytes, specific for galactose and *N*-acetylgalactosamine. It mediates clearance from the circulation of glycoproteins with complex oligosaccharide units (e.g. ceruloplasmin and α_1 -acid glycoprotein) from which the terminal sialic acid has been removed, exposing galactose residues. Endocytic lectins also play a role in intracellular sorting and trafficking of glycoproteins and in their delivery to the cell surface. The mannose-specific lectin present on the surface of macrophages has been implicated in nonimmune antimicrobial defense. It binds infectious organisms that expose mannose-containing glycans on their surface, leading to their killing by lectinophagocytosis.

A similar function, albeit by a different mechanism, is performed by the soluble mannose-binding protein (MBP) of mammalian serum and liver, a member of the collectin family of C-lectins. The lec-

tin binds to oligomannosides of yeast and fungi, causing activation of complement in an antibody- and Clq-independent manner and subsequent lysis of the pathogens. It also enhances phagocytosis of the invading organisms by acting as an opsonin. Clinical evidence for its importance has come from the identification of an MBP-deficiency syndrome. It results from a mutation of a single amino acid in the collagen-like domain of the lectin and is associated with recurrent, severe bacterial infections in infants.

Adhesive interactions mediated by surface carbohydrates and selectins, another family of C-type lectins, control leukocyte trafficking to sites of inflammation and the migration (homing) of lymphocytes to specific lymphoid organs. The selectins thus provide the best paradigm for the role of sugar-lectin interactions in biological recognition. They mediate the adhesion of circulating leukocytes to endothelial cells of blood vessels, a prerequisite for the exit of the former cells from the circulation and their migration into tissues. L-Selectin, also known as 'homing receptor', is found on all leukocytes and is involved in the recirculation of lymphocytes, directing them specifically to peripheral lymph nodes. The two other selectins (E-selectin and P-selectin) are expressed on endothelial cells when these cells are activated by inflammatory mediators (e.g. interleukin 2 and tumor necrosis factor) released

from tissue cells in response to, for example, wounding, infection or ischemia.

Selectin-mediated adhesion of leukocytes to activated endothelial cells may lead to their harmful accumulation, causing tissue damage, swelling and pain, for instance the inflammation of rheumatoid arthritis or myocardial injury during reperfusion of an ischemic heart. Prevention of adverse inflammatory reactions by inhibition of leukocyte-endothelium interactions, another potential application of antiadhesion therapy, has become a major aim of many pharmacological industries. As shown in animal models, oligosaccharides recognized by the selectins exert protective effects against experimentally induced lung injury. Application of P-selectin- or L-selectin-specific antibodies, or of the selectin ligand sialyl Le^x, attenuates myocardial necrosis after myocardial ischemia and reperfusion. In addition to their involvement in inflammation, selectins may play a role in the spread of cancer cells from the main tumor throughout the body.

The sialoadhesins have been implicated in cell-cell interactions: sialoadhesin and CD22 in those of the immune system and MAG in the process of myelination.

C-type lectins on the surface of natural killer (NK) cells, e.g. Ly49A, appear to act as mediators of carbohydrate-dependent NK-target cell interactions by binding to class I major histocompatibility complex antigens on the surface of the target cells. Such interactions are crucial for delivery of inhibitory signals linked to the prevention of NK cytolytic activity against normal cells.

Applications

Native lectins are used predominantly for applications that are based on precipitation and agglutination reactions or for mitogenic stimulation (Table 2). For numerous purposes, however, lectin derivatives are required. Thus, lectins derivatized with fluorescent dyes, gold particles or enzymes are employed for detection of glycoconjugates in tissue sections, on cells and subcellular organelles, and in investigations of intracellular pathways of protein glycosylation. Lectin binding has been used to demonstrate that membrane receptors for hormones, growth factors, neurotransmitters and toxins are glycoconjugates. Immobilized lectins, such as those that are covalently bound to Sepharose, are indispensable for the purification and isolation by affinity chromatography of glycoproteins, glycopeptides and oligosaccharides. The HLA antigen from normal human cells was first purified to homogeneity with the aid of concanavalin A-Sepharose; immunoglobulin (IgA1) can be easily distinguished from IgA2 by the use of jacalin, the lectin from the jackfruit, *Artocarpus integrifolia*.

Mouse and human cortical (immature) thymocytes differ markedly from the medullary (mature) ones in their surface carbohydrates, as evidenced by the fact that the former are bound and agglutinated by peanut agglutinin (PNA⁺ cells), whereas the latter are not (PNA⁻). Peanut agglutinin therefore provides easy separation of the individual thymocyte subpopulations and makes it possible to examine *in vitro* the developmental and functional relationship between them.

Table 2 Applications of lectins in immunology

Lectin	Source	Monosaccharide specificity	Main application
Concanavalin A	Jackbean (<i>Canavalia ensiformis</i>)	Mannose	T cell mitogen; separation of membrane glycoproteins
DBA	Horse-grass (<i>Dolichos biflorus</i>)	N-Acetylgalactosamine	Blood typing
LTA	Winged pea (<i>Lotus tetragonolobus</i>)	L-Fucose	Blood typing
Peanut agglutinin	Peanut (<i>Arachis hypogaea</i>)	Galactose	Fractionation of human and mouse thymocytes; detection of polyagglutination
Phytohemagglutinin (PHA)	Kidney bean (<i>Phaseolus vulgaris</i>)	^a	T cell mitogen
Pokeweed mitogen	Pokeweed (<i>Phytolacca americana</i>)	^a	B cell mitogen
Ricin	Castor bean (<i>Ricinus communis</i>)	Galactose	In immunotoxins
Soybean agglutinin	Soybean (<i>Glycine max</i>)	N-Acetylgalactosamine	Purging of human bone marrow for transplantation

^a Inhibited only by oligosaccharides.

Selective agglutination by SBA permits separation of mouse B and T splenocytes. However, the main application of this lectin is for purging human bone marrow for transplantation. It is employed routinely for transplantations into children born with severe combined immune deficiency (SCID) with close to 70% success. SBA purging is also used experimentally in bone marrow transplantation of leukemic patients, alongside other accepted techniques for T cell depletion, such as monoclonal antibodies.

Another clinical application of lectins is in blood typing. Thus, the lectins from *Lotus tetragonolobus* and *Ulex europaeus* are employed for identification of blood type O cells, and for the identification of secretors of blood group substances. The lectin from *Dolichos biflorus* is used to distinguish between A₁ and A₂ subgroups and that from *Vicia graminea*, specific for blood type N, to differentiate between M and N cells. In addition PNA, specific for the T antigen, is employed in the differential diagnosis of 'polyagglutination' or ('polyagglutinability'), a condition accompanying certain bacterial and viral infections, in which human erythrocytes become agglutinable by antibodies normally present in the sera of nearly all adults.

Mitogenic stimulation by lectins provides an easy and simple means to assess the immunocompetence of patients suffering from a diversity of diseases (recently, also in cases of the acquired immune deficiency syndrome (AIDS)), and to monitor the effects of various immunosuppressive and immunotherapeutic manipulations. It has been used to examine the effect of stress, both physical and psychological, on the immune system, e.g. sport, weightlessness in space, bereavement. It is also employed for the preparation of chromosome maps for karyotyping, sex determination and detection of chromosome defects, since chromosomes are easily visualized in the stimulated cells.

See also: Adhesion molecules; Agglutination; Bacterial cell walls; Contrasuppression; Hematopoietic stem cell transplantation; Phytohemagglutinin (PHA); Pokeweed mitogen (PWM); Polyclonal activators; Targeting of immunological agents.

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LEGIONELLA, INFECTION AND IMMUNITY

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Legionella pneumophila is a facultative intracellular pathogen that causes Legionnaires' disease in humans. In nature, this organism is commonly present in aquatic habitats, often growing as an intracellular parasite of free-living protozoa. Occasionally, *L. pneumophila* contaminates water distribution systems, resulting in inoculation of humans by inhalation of infectious aerosols or microaspiration of potable water. In susceptible hosts, a fibrinopurulent pneumonia with infiltration of mononuclear and polymorphonuclear phagocytes develops. The infection is progressive and may be fatal, particularly in immunocompromised hosts.

Although *L. pneumophila* is not an obligate intracellular pathogen, progressive disease occurs only when host mononuclear phagocytic cells (MPCs) are permissive to intracellular replication of the organism. In 1980, Horwitz and colleagues demonstrated that *L. pneumophila* replicates in unactivated human MPCs. Subsequent *in vitro* studies demonstrated that *L. pneumophila* also replicates in unactivated MPCs from other animal species, including guinea pigs, rats, nonhuman primates and A/J mice. These species, like humans, develop replicative *L. pneumophila* infections following experimental inoculation. In contrast, other mouse strains, including BDF1, DBA/2, C3H/HeN, C57B1/6, BALB/c and outbred mice, have nonpermissive MPCs; these animals do not support intrapulmonary growth of *L. pneumophila*. Recently, a single autosomal, dominant gene on murine chromosome 13 (designated Lgn-1) was linked to the permissiveness of murine MPCs for *L. pneumophila* replication.

The primacy of intracellular infection in the development of progressive disease has been confirmed by numerous experiments with *L. pneumophila* mutants that are unable to grow in MPCs *in vitro*. Predictably, these mutant bacteria cannot cause progressive infection in animals. The ability of a susceptible host to survive replicative *L. pneumophila* lung infection is dependent on a complex interaction between host MPCs, cytokines and effector cells which facilitate MPC activation and elimination of the pathogen.

Intracellular infection by *L. pneumophila*

L. pneumophila may be ingested by macrophages without opsonization, although they are ingested much more efficiently after exposure to serum and fixation of complement via the alternate pathway. In either case, the bacteria are able to alter the normal phagocytic pathway, avoid intracellular microbiological mechanisms and establish productive intracellular infection.

The uptake of the bacterium into macrophages often involves a novel mechanism of ingestion known as 'coiling phagocytosis', so named because macrophage pseudopods encircle and eventually spiral around the bacterium several times during engulfment (Figure 1, step 2). In these membrane coils, macrophage surface molecules are selectively sorted so that the nascent phagosomal membrane is different from the plasma membrane from which it was derived. Specifically, major histocompatibility complex (MHC) class I and class II molecules and alkaline phosphatase are excluded during uptake, while the CR3 receptor and 5'-nucleotidase are retained in the coiled phagocytic membranes and subsequently eliminated within an hour following bacterial uptake. Ingestion of *L. pneumophila* by amoebae also has novel ultrastructural features. The bacterium is often engaged at the tip of an extended filipod (Figure 1, step 1) and then retracted toward the body of the cell and engulfed. It is not known whether membrane sorting occurs in protozoa; however, Abu Kuwaik and colleagues demonstrated that uptake by the amoeba, *Hartmannella vermiformis*, is a more prolonged process than macrophage uptake, and it requires new protein synthesis. In addition, virulent and avirulent *L. pneumophila* induce distinct sets of amoebal proteins during ingestion.

Once virulent *L. pneumophila* are ingested by either macrophages or protozoa, the phagosomes evade events that normally follow phagocytosis, and the bacteria-containing endosomes become permissive for bacterial replication. In macrophages, normal acidification of the phagosome is attenuated, and the oxidative burst is minimized. During the first 15–60 minutes after uptake, the bacterial phagosome associates with smooth vesicles (Figure 1, step 3). Later, they make intimate contact first with mito-

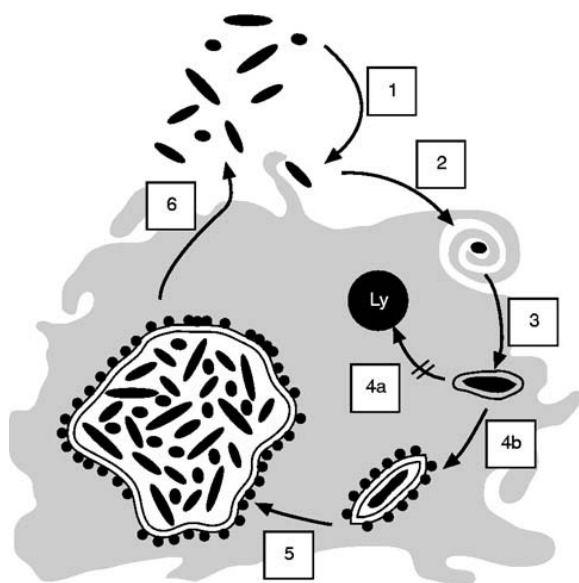


Figure 1 Events associated with intracellular infection by *L. pneumophila*. (1) Bacteria make contact with filipodia (in amoebae) or (2) are taken up by coiling phagocytosis (in macrophages). (3) The bacterium is ingested within a phagosome, which associated transiently with mitochondria and smooth vesicles. Some avirulent mutants are directed to the degradative endocytic compartments (Ly, lysosome) (4a), but most virulent bacteria are encircled by rough endoplasmic reticulum at ~4 hours after uptake (4b). (5) Bacteria begin to divide in this endosome 4–8 hours after uptake. They double in 2 hours, and grow to large numbers within the endosome over the next 24–48 hours. (6) When the internal bacterial load becomes very large, the cell is lysed.

chondria and then with rough endoplasmic reticulum (ER). Viable *L. pneumophila*-containing phagosomes were shown by Horwitz to evade fusion with thorium-labeled lysosomes (Figure 1, step 4b). More recent immunofluorescence microscopy studies confirm that the late endosomal and lysosomal proteins CD63, LAMP-1, LAMP-2 and cathepsin D are frequently associated with *L. pneumophila*-containing phagosomes. The mechanisms by which lysosomal fusion is avoided is not known; however, Berger and Isberg have identified and cloned a bacterial gene (designated *dotA*) that is required for this pathologic process.

By 4 hours after ingestion, phagosomes containing live *L. pneumophila* appear studded with ribosomes as a result of the association with the ER. This pathologic process resembles autophagocytosis, a cellular process that occurs in response to limitation of nutrients. During autophagocytosis, host cells demarcate sections of cytoplasm by encirclement with smooth endoplasmic reticulum. Proteins within the encircled area are degraded and recycled to the viable parts of the cell. The notion that the *L. pneumophila*–ER

association might represent a form of autophagocytosis was proposed in 1995 by Swanson and Isberg. These investigators showed that amino acid restriction of host cells increases the association of ingested *L. pneumophila* with the ER and facilitates intracellular bacterial growth. Since *L. pneumophila* utilize amino acids as their primary carbon source, direct stimulation of host cell autophagy may account for the ER association. Autophagocytic degradation may make simple nutrients available to the growing bacteria, or, alternatively, the enzymes or channels contained in the ER may facilitate bacterial nutrition.

Between 4 and 8 hours after ingestion, *L. pneumophila* begin to multiply. By 24 to 48 hours, endosomes packed with bacteria fill the cell. Cell lysis occurs, and the released bacteria can reinitiate infection in a neighbouring cell (Figure 1, steps 5 and 6).

Iron availability limits the intracellular growth of *L. pneumophila*

Like all pathogenic bacteria, *L. pneumophila* must compete successfully with the host for iron in order to grow. Siderophores and high-affinity iron uptake systems have not been identified in *L. pneumophila*. Ordinarily, MPCs acquire iron by receptor-mediated endocytosis of iron-binding proteins (e.g. transferrin and lactoferrin) and store it in the form of intracellular ferritin. *L. pneumophila* cannot use iron bound to these proteins. Therefore, the growth of intracellular *L. pneumophila* depends on the accessibility of iron from a transient, intracellular pool.

Interferon γ (IFN γ), a cytokine produced by several cell types (e.g. natural killer (NK) cells, CD4⁺ and CD8⁺ T cells, Thy-1⁺ CD4⁺CD8⁺ $\alpha\beta$ T cells and Thy-1⁺ CD4⁺CD8⁺ $\gamma\delta$ T cells) inhibits *L. pneumophila* replication within human MPCs. Byrd and Horwitz discovered that IFN γ inhibits *L. pneumophila* growth by limiting the availability of intracellular iron. This cytokine was shown to downregulate expression of transferrin receptors on the MPC cell surface and to decrease the concentration of intracellular ferritin. Most importantly, the IFN γ inhibition of bacterial growth could be completely reversed by the addition of excess, exogenous transferrin iron, suggesting that iron limitation is the main mechanism by which this cytokine inhibits intracellular *L. pneumophila* infection.

Host defense against primary replicative *L. pneumophila* lung infections

Because IFN γ limits growth of *L. pneumophila* within permissive MPCs, the ability of the

susceptible host to survive a primary replicative *L. pneumophila* lung infection is dependent on the induction of an active cellular immune response. The central role of cellular immunity is consistent with the clinical observation that legionellosis is most severe in immunocompromised patients with cellular immune defects. Similarly, in rodent models of legionellosis, administration of immunosuppressive agents (e.g. cyclosporine, corticosteroids) increase the lethality of *L. pneumophila* infections. Likewise, the enhanced natural susceptibility of guinea pigs to *L. pneumophila* infection may be due to the relative unresponsiveness of the lymphocytes and mononuclear cells of this species to the bacteria.

Humoral immunity plays a relatively minor role in resistance to primary *L. pneumophila* infection. Horwitz and colleagues demonstrated that: 1) *L. pneumophila* are completely resistant to killing by human complement even in the presence of high-titer human or rabbit anti-*L. pneumophila* antiserum; 2) *L. pneumophila* are resistant to killing by human phagocytes in presence of high-titer antibody and complement in spite of markedly enhanced phagocytosis; and 3) antibody- and complement-opsonized *L. pneumophila* multiply in monocytes as rapidly as unopsonized bacteria. In addition, immunocompromised humans are susceptible to recurrent replicative *L. pneumophila* lung infections even when specific serum antibody is present. These studies suggest that, in the absence of an active cellular immune response, antibody to *L. pneumophila* is an ineffective host defense against *L. pneumophila*. Specific antibody may even be detrimental, as it facilitates uptake of the bacteria by permissive MPCs. Furthermore, recent *in vivo* studies in our laboratories, using a murine model of Legionnaires' disease, demonstrate that clearance of the bacteria from the lung during a primary replicative infection temporally precedes the peak production of antibody against *L. pneumophila*.

Since IFN γ exerts a bacteriostatic, rather than bactericidal effect on intracellular *L. pneumophila*, it is assumed that additional effector cells or mechanisms are required to facilitate the elimination of these bacteria from the lung. Binding of *L. pneumophila* to MPCs results in the direct induction of other cytokines, including tumor necrosis factor alpha (TNF α), interleukin 1 β (IL-1 β), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage inflammatory protein 2 (MIP-2). TNF α inhibits intracellular multiplication of *L. pneumophila* in human MPCs *in vitro*, but it is not known whether this cytokine is bactericidal *in vivo*. *In vitro* studies also demonstrate that IFN γ -stimulated NK cells have enhanced capacity to lyse *L. pneumophila*-

infected MPCs. Similarly, cultured human and murine polymorphonuclear leukocytes (PMNs) stimulated with IFN γ and TNF α , also have enhanced bactericidal activity against *L. pneumophila*. Co-stimulation of murine MPCs with IFN γ and TNF α induces nitric oxide synthetase (mac-iNOS) and the production of reactive nitrogen intermediates including nitric oxide (NO). *In vitro* and *in vivo* studies show that NO restricts growth and facilitates the elimination of *L. pneumophila* from rodent alveolar MPCs by: 1) decreasing the concentration of MPC ferritin; 2) amplifying the inhibitory effect of TNF α ; and 3) reducing bacterial viability by a direct toxic effect. In contrast, IFN γ eliminates *L. pneumophila* from cultured A/J mouse peritoneal macrophages by an NO-independent mechanism. It is not known why the effects of NO differ in murine alveolar and peritoneal MPCs. Likewise, the potential role of NO in resistance to human MPCs remains controversial, due mainly to inconsistent demonstration of NO generation by these cells. It is likely, though, that clearance of *L. pneumophila* depends on activation of NK cells, PMNs or macrophages by IFN γ , TNF α , and/or other cytokines.

The cellular sources of IFN γ during primary replicative *L. pneumophila* lung infection have not been identified. *In vivo* studies of BALB/c mice and *in vitro* studies using human peripheral blood mononuclear cells suggest that cells bearing NK markers are induced to make IFN γ in response to virulent *L. pneumophila*. In the A/J mouse model, where IFN γ is required for bacterial clearance, we found that both T and B lymphocytes are recruited into the lung during primary replicative infection. However, mice depleted of both CD4⁺ and CD8⁺ T cells by monoclonal antibody treatment are able to produce IFN γ in the lung and to resolve primary *L. pneumophila* lung infection as efficiently as normal mice. Flow cytometric analysis of lung homogenates from these depleted animals reveal a small but significant population (8%) of Thy-1⁺ CD4⁺ CD8⁺ cells. These cells, which may be CD4⁺CD8⁺ $\alpha\beta$ T cells, or CD4⁺CD8⁺ $\gamma\delta$ T cells, are potential sources of IFN γ .

IL-12, derived from alveolar MPCs and epithelial cells, induces cellular immunity and augments expression of IFN γ from T cells and NK cells. Systemic administration of IL-12 has been demonstrated to increase host resistance to several pathogens, including *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Plasmodium chabaudi* and *Klebsiella pneumoniae*. Our recent studies in A/J mice suggest that IL-12 is rapidly induced in bronchoalveolar lavage fluid following induction of a primary infection and

correlates temporally with induction of IFN γ . The relevance of IL-12 to the control of *L. pneumophila* infection remains to be determined.

Acquired immunity to *L. pneumophila* infection

After resolution of a primary *L. pneumophila* lung infection or vaccination with *L. pneumophila* antigens (e.g. *L. pneumophila* cell membranes or extracellular protease), experimental animals become resistant to rechallenge with the bacteria. Splenocytes derived from guinea pigs or rodents previously infected with *L. pneumophila* undergo blastogenesis following stimulation with *L. pneumophila* antigens, and these animals develop delayed-type, cutaneous hypersensitivity. In human studies, Horwitz and colleagues demonstrated that peripheral mononuclear cells taken from patients convalescing from Legionnaires' disease proliferate in response to *L. pneumophila* and secrete lymphokines which inhibit *L. pneumophila* replication in MPCs derived from a naive host.

The effector cells responsible for resistance to reinfection with *L. pneumophila* have not been definitely identified. We recently demonstrated that resistance to reinfection in the A/J mouse model and showed that it is associated with enhanced recruitment of lymphocytes into the lung. However, depletion of CD4⁺ and/or CD8⁺ T cells in these animals has little effect on this resistance. Therefore, resistance to reinfection appears to depend on effector mechanisms that are largely independent of both CD4⁺ and CD8⁺ T cells.

Studies by Newton and colleagues suggests that T_H1 CD4⁺ T cells may play a key role in the development of acquired immunity in BALB/c mice. In this model, primary lung infection induces resistance to a subsequent lethal bacterial inoculum. Consistent with increased activity of T_H1 CD4⁺ T cells, the acquired resistance is associated with antigen-specific splenocyte proliferation, production of IFN γ , and anti-*L. pneumophila* IgG2a antibodies in the serum. In addition, administration of Δ -9-tetrahydrocannabinol, a compound that disrupts the development of T_H1 responses, renders BALB/c mice susceptible to fatal *L. pneumophila* infections after rechallenge with virulent *L. pneumophila*. Since BALB/c mice are not permissive to the growth of *L. pneumophila*, it is likely that this model reflects protection against the toxic effect of a large bacterial inoculum, rather than growth-limiting immunity. The potential roles of T_H1 versus T_H2 cells in the development of acquired immunity to progressive *L. pneumophila* infection in a permissive host has not been explored.

Similarly, the role of humoral immunity in acquired resistance to *L. pneumophila* in the immunocompetent host is also unclear. Although antibody to *L. pneumophila* plays a minimal role in resistance to primary replicative lung infection, some studies suggest that antibody may assist clearance of the bacteria from the lung during rechallenge. Rolstad and colleagues demonstrated that passive transfer of *L. pneumophila*-specific antibody to naive immunocompetent rats protects against normally lethal inocula of *L. pneumophila*. We demonstrated that *L. pneumophila* opsonized with a monoclonal antibody prior to intratracheal inoculation are cleared more rapidly from the lungs of A/J mice than unopsonized bacteria. Clearance of opsonized *L. pneumophila* is correlated with enhanced phagocytosis by host MPCs. Since IFN γ is rapidly induced in this model, the enhanced clearance may reflect increased phagocytosis by nonpermissive, IFN γ activated MPCs.

Summary

Susceptibility to replicative *L. pneumophila* lung infection is determined by the permissiveness of host MPCs to the intracellular growth of the bacteria. These bacteria grow within macrophages and protozoa by avoiding phagolysosomal fusion and by trafficking to a permissive endosome that associates with the host ER. Intracellular growth is controlled both by genetic factors and by the state of MPC activation. Survival of susceptible hosts after primary replicative *L. pneumophila* infections depends on cellular immune mechanisms, including the release of IFN γ . Acquired immunity to *L. pneumophila* is probably the result of a coordinated response between humoral and cellular immune responses, which facilitates enhanced uptake of the bacteria by activated MPCs.

See also: Bacteria, immunity to; Bacterial cell walls; Phagocytosis; Respiratory and cardiac infections.

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LEISHMANIASIS

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Leishmaniasis is a parasitic human infection of global importance for which no reliable vaccine currently exists. Infection can result in the development of different disease states which constitute a spectrum of pathology from cutaneous lesion to fatal visceral infection. Mouse models of infection, which also display a similar spectrum of disease, have allowed a clear understanding of the mechanisms of susceptibility and resistance to this infection, making experimental leishmaniasis the best model system for the examination of T cell subset polarization and its relationship to pathogenesis.

Characteristics of the organism and its antigens

The intracellular protozoan parasites of the genus *Leishmania* cause a range of clinical infections, termed leishmaniasis. With the exception of Oceania,

leishmaniasis occurs in most parts of the world, with around a third of the world population at risk of infection. An incidence of 400 000 new cases per year has been reported and the worldwide prevalence is believed to be around 12 million cases. The clinical diseases caused by the leishmaniases fall into three distinct categories:

1. *Cutaneous leishmaniasis* (CL) is termed 'Old World' or 'New World' according to geographical boundaries and the parasite species involved, although the diseases are essentially the same. A lesion develops in the skin at the site of parasite inoculation; it can take some time to heal and may also allow secondary infection. These lesions are typically resolved but may leave a disfiguring scar. Old World CL is caused by *L. major* or *L. tropica* and is found in the Middle East, Africa and parts of Asia; it is variously referred to as Oriental sore, Baghdad boil, Delhi

sore and Aleppo button. New World CL is found mainly in Central and South America and is known as Chiclero's ulcer or Bay sore (in Belize, Peru and Venezuela), pian bois or forest yaws (in Brazil, Colombia, French Guyana and Suriname) and uta (in Peru). This disease is caused by the species *L. mexicana*, *L. amazonensis* and *L. panamensis* among others.

There is a more severe form of CL – diffuse cutaneous leishmaniasis (DCL) – which can occur as a result of infection with *L. aethiopica* in the Old World and *L. amazonensis*, *L. pifanoi* or *L. mexicana* in the New World. In this disease, the parasite is not contained at the site of inoculation but spreads to other subcutaneous tissues around the body, where it causes extensive swelling.

2. *Mucocutaneous leishmaniasis* (MCL) or espundia is caused by *L. braziliensis* and is due to a reactivation of disease in mucosal tissues some time after primary infection at another site. Oronasal and pharyngeal mucosa can be destroyed in highly disfiguring leprosy-like lesions.
3. *Visceral leishmaniasis* (VL) is also found in the Old World, caused by *L. donovani* or *L. infantum* infection, and in the New World, caused by *L. chagasi*. The clinical disease is referred to as kala-azar (black disease), dum dum fever or ponos and is a result of parasite spread from the initial skin infection to tissue macrophages in visceral sites, such as the liver, spleen and bone marrow. Typical symptoms include fever, malaise, weight loss, coughing and diarrhea, accompanied by anemia, skin darkening, and hepatosplenomegaly. This disease is usually fatal if untreated.

While the clinical forms of leishmaniasis are distinct, the parasite species causing disease are not as absolute as outlined above. There are incidences of visceral disease caused by normally cutaneous species and vice versa. The clinical disease observed after infection is probably determined by an interplay between parasite factors and host immune responsiveness to the organism.

Diagnosis of active leishmaniasis is primarily based upon the culture of parasites from infected tissue, although some immunoassays suitable for field work are being developed, including those using monoclonal antibodies and enzyme-linked immunosorbent assays. A skin test using a killed whole parasite preparation (leishmanin) is used as a presumptive test but is not fully specific, while other DNA-based

techniques are accurate but not suitable for all field use.

Treatment of leishmaniasis is based upon the use of antimony compounds, notably the pentavalent antimonials, sodium stibogluconate and *N*-methylglucamine antimonate. These drugs require daily intramuscular injection, have severe side-effects, and are not always effective, particularly against cutaneous leishmaniasis. Second-line drugs include the antimicrobials, such as amphotericin B, pentamidine, metronidazole and nifurtimox.

Most forms of leishmaniasis are zoonotic and various animals are reservoir hosts for the parasite (Figure 1); these include wild cats, dogs, jackals, foxes, sloths, hyraxes and rodents, depending on the precise geographical location. The parasite is transmitted to the mammalian host by over 50 species of sandfly of the genera *Phlebotomus* (in the Old World) and *Lutzomyia* (New World). The infective, flagellate form of the parasite (promastigote) is injected by the sandfly as it takes a blood meal from the host, whereupon the promastigote rapidly invades tissue macrophages (Figure 2). Inside the macrophage, the parasite transforms to a nonflagellate form (amastigote) that resists microbicidal damage from the macrophage and divides by binary

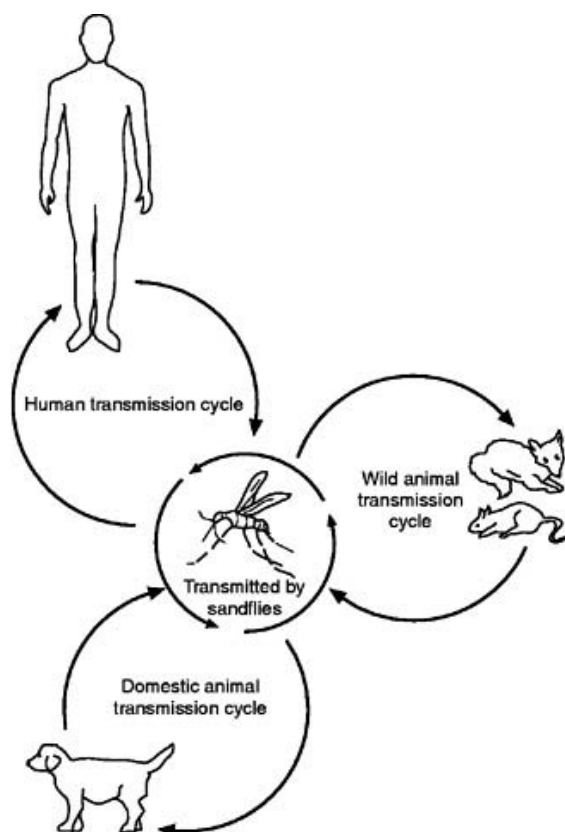


Figure 1 Transmission cycles of *Leishmania*.

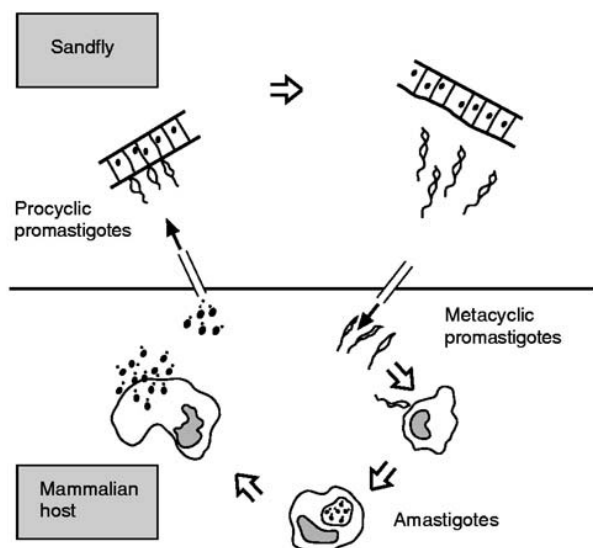


Figure 2 Life cycle of *Leishmania*.

fission before rupturing the cell and invading others. Transmission to another sandfly is achieved with the withdrawal of a blood meal containing free parasites or parasitized macrophages.

In the infected sandfly the amastigote attaches to lectins on epithelial cells of the hindgut and transforms into a noninfectious form of the promastigote, the procyclic promastigote. Here the procyclic parasite matures into the infectious promastigote (metacyclic) by a process, termed metacyclogenesis, that involves alterations to a parasite surface antigen, lipophosphoglycan (LPG), allowing detachment from the gut cells. These parasites are then free to migrate to the sandfly proboscis, ready for transmission to another mammalian host. The life cycle of the parasite within the sandfly can be simulated *in vitro* using cell-free medium, allowing analysis of leishmanial antigens and immune responses after experimental infection.

As mentioned above, alterations to the promastigote surface LPG molecule allow the parasite to complete the life cycle in the sandfly. The LPG molecule has been well characterized. It consists of four defined structural domains: a phosphatidylinositol lipid anchor, a phosphosaccharide core, a number of repeating oligosaccharide units and a short oligosaccharide cap. In addition to its importance in the sandfly, LPG is also likely to be involved in the initial binding and entry of macrophages by the promastigote stage. The major surface glycoprotein on all *leishmania* species is the promastigote surface protease (PSP or gp63). Although the protease activity of gp63 upon a number of molecules, including the T cell surface molecule CD4, has been documented, the natural substrate for this protease is unknown. A

number of other cell surface and cytoplasmic proteins with unknown functions have been described, including the cytoplasmic protein, *Leishmania* homolog of receptor for activated C kinase (LACK), which may form the basis of future vaccination strategies.

Immune response of the host

It is clear from numerous studies that immunity to leishmaniasis, both in human disease and murine experimental infection, is determined by the polarization of CD4⁺ T cell subsets. These divergent T cell responses to infection, clearly seen in different mouse strains, either cure or fail to cure infection with *L. major*. The generation of a T_H2 response to infection in mice, with production of interleukin 4 (IL-4) and IL-10, correlates with susceptibility to disease, while mice which respond with a T_H1 phenotype (interferon γ (IFN γ) and IL-2) resolve lesions and remain refractory to further challenge. The adoptive transfer of T_H1 or T_H2 cell lines or clones has demonstrated the critical importance of these cells with regard to disease outcome.

The susceptible BALB/c mouse strain can be manipulated to cure infection using various immunological interventions, which suggests that overcoming susceptibility may be possible in human infection. Sublethal irradiation, depletion of CD4⁺ cells, neutralizing IL-4 production or injecting mice with the T_H1-stimulating cytokine, IL-12, all confer upon BALB/c mice the ability to control infection. The common factor in these treatments is the downregulation or control of IL-4 production in the susceptible mouse early after infection. IL-4 is necessary at this early stage for the efficient generation of a detrimental T_H2 response to the parasite which will impede the development of a protective T_H1 response via the T_H2 cytokine IL-10 (Figure 3). Indeed, these treatments are ineffective if delayed until later during infection as T_H2 polarization has already occurred by this point. The genetic factors involved in determining early production of IL-4 are unknown and the response to IL-4 early during infection may underlie the nature of susceptibility to disease.

The mechanism by which T_H1 and T_H2 cells exert their protective and nonprotective effects has also been extensively studied. The production of IFN γ is essential for the protective capacity of T_H1 cells, as depletion of this cytokine using monoclonal antibodies exacerbates disease. IFN γ activates macrophages to produce tumor necrosis factor α (TNF α), which synergises with IFN γ to allow killing of resident parasites by the induction of nitric oxide (NO) (Figure 4). The production of NO is catalyzed by the

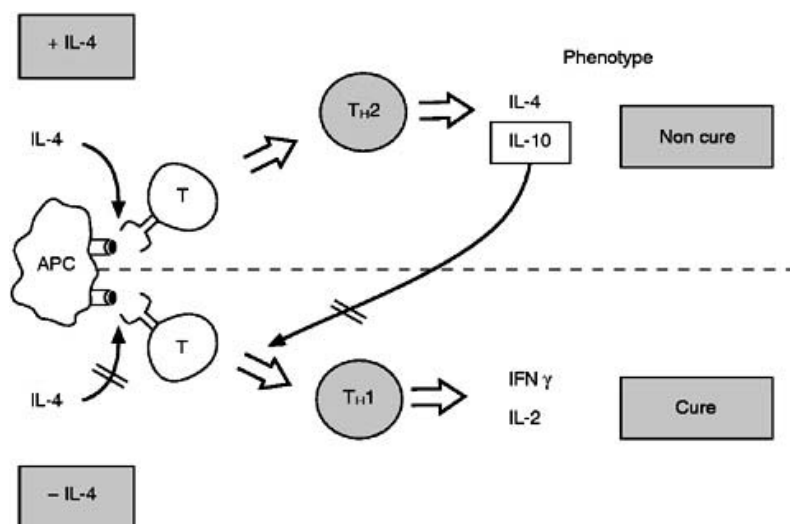


Figure 3 Induction of T_H2 cells in susceptible mice. APC, antigen-presenting cell. Interrupted arrows indicate inhibition.

inducible enzyme nitric oxide synthase (iNOS), whose expression in tissues correlates with disease cure. Blocking NO production *in vivo*, using the inhibitor L- N^G -monomethylarginine (L-NMMA), renders normally resistant mice susceptible to disease, emphasizing the essential nature of this molecule for parasite killing. Resistant mice deficient in iNOS are highly susceptible to *L. major* infection. This was so despite the presence of enhanced T_H1 responses. IL-4 is known to prevent IFN γ -induced NO synthesis by macrophages, thus allowing the survival of the parasite within the host cell.

While the resolution of infection is determined by $CD4^+$ T cell subsets, it is also evident that $CD8^+$ cells, natural killer (NK) cells and $\gamma\delta$ cells can participate in control of infection, although these clearly play a secondary role to $CD4^+$ cells, as adoptive transfer of $CD4^+$ cells from resistant or susceptible mice is sufficient to reproduce the spectrum of disease phenotype outlined above. $CD8^+$ cells are of importance in the resolution of secondary *L. major* infection, as depletion of these cells causes disease exacerbation when cured mice are rechallenged. Early NK cell production of IFN γ has been demonstrated and may be important in the development of T_H1 cells at this critical stage, although the importance of this in

determining disease phenotype is unclear. An increase in $\gamma\delta$ cell numbers has been recorded in both susceptible and resistant mice following infection with *L. major*, which, if depleted using a monoclonal antibody, resulted in the development of larger lesions and increased parasite numbers. It is clear, therefore, that other cellular responses can function in a protective capacity during infection but the ultimate phenotype of disease cure or susceptibility is determined by the $CD4^+$ compartment. In contrast, humoral antibody does not appear to play a significant role in this disease.

Evasive strategies of the organism

The survival of the amastigote stage of the parasite within the environment of the macrophage phagolysosome suggests the presence of mechanisms to resist the normal proteolytic degradation that occurs there. The LPG molecule on the amastigote surface may provide protection against this environment, as LPG deficient strains of *L. major* are killed within 18 hours after internalization. It has also been suggested that the surface protease, gp63, may cleave harmful proteolytic enzymes present in this vacuole, although this has not been clearly demonstrated.

The amastigote stage of the parasite can also interfere with the antigen-presenting function of the infected macrophages, which may impede the detection and activation of infected cells by a protective immune response. This impaired antigen presentation is not due to an inability to process antigen or the lack of major histocompatibility complex (MHC) class II molecules but may reflect an interruption of class II loading with peptide by the presence of amastigotes.

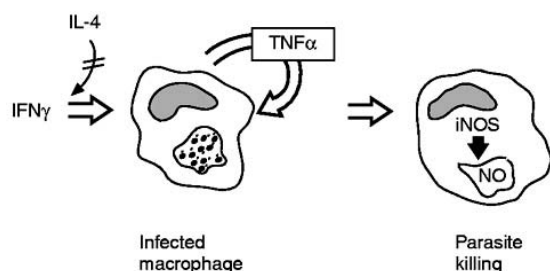


Figure 4 Nitric oxide mediated parasite killing.

While the outcome of leishmaniasis in humans and

mice is balanced between cure of disease or progressive lesion development, depending upon the immune response, it is also clear that the parasite is able to coexist in the mammalian host for some time without producing any observable clinical disease. In resistant mice which cure cutaneous infection, parasites can be cultured 1 year after the lesions resolve. The incidence of leishmaniasis in individuals infected with the human immunodeficiency virus (HIV) also suggests that, years after successful cure of infection, parasites are still present and can reactivate to cause disease in the face of immunosuppression. Therefore, even in the face of a protective immune response and apparent cure of disease, the parasite has the ability to survive and avoid immune-mediated clearance. The mechanism by which this is accomplished is unclear but may involve the parasitization of cells other than macrophages, such as lymph node dendritic cells, in order to avoid macrophage activation.

Vaccination

Human vaccination against leishmaniasis is feasible, as individuals cured of disease remain resistant to reinfection. Indeed, a form of vaccination against leishmaniasis has existed for centuries. Inhabitants of the Middle East would expose the bottoms of their infants to bites from the sandfly vector in order to produce a lesion in a hidden part of the body and thus avoid a potential disfiguring facial scar from later infection. Parasites taken from human lesions, and latterly from *in vitro* cultures, have similarly been administered. There are a number of problems associated with this method when considering large-scale vaccination. Complications accompanying live vaccinations are numerous and serious: around 2–3% of individuals develop large or nonhealing lesions that require treatment. Even in individuals who do not develop these problems the lesions persist for a minimum of 4–5 months. In addition, vaccination of immunosuppressed individuals by this method may have potentially fatal consequences, and vaccination of those with a tendency to develop dermatological complications may be detrimental as there is a clear relationship between live leishmania vaccination and the recrudescence of latent psoriasis or the appearance of new cases. A further complication is the incidence of secondary infection of the lesion, which, in one study, has been documented to occur in 25% of those vaccinated. Finally, the tendency for live parasites to cause immune suppression can lead to a depressed response to other vaccines, such as the triple diphtheria, pertussis and tetanus vaccine, in children for up to 6 months after administration. Because of these extensive complications,

live vaccination is only considered in highly endemic areas and only as a last resort.

Vaccination using a killed preparation of *Leishmania* has been used in Brazil; however, the incidence of disease dropped in this area during the period after immunization, making evaluation of protective immunity difficult. More recent trials with killed parasites coinjected with live bacille Calmette–Guérin (BCG) resulted in a reduction of established cutaneous lesions, indicating that, with an appropriate adjuvant, protective responses could be generated in humans using a killed vaccine.

The route of vaccination is a further complication that has arisen. In the murine model, intravenous vaccination of killed parasites is superior to subcutaneous inoculation. Furthermore, the injection of killed parasites by the subcutaneous route can block the protective effect of intravenous administration. Vaccine delivery systems, such as attenuated *Salmonella*, that deliver antigen directly to the liver and spleen after oral administration may therefore represent an efficient alternative to intravenous delivery, which would be an obstacle to mass vaccination.

While the results of human vaccination studies have not so far resulted in the production of an effective vaccine, the availability of new leishmanial antigens and the development of vaccine delivery systems suited to the induction of T_{H1} responses suggest that a vaccine may be developed in the future. The recent development of DNA vaccination and the cloning of the antigen p24/LACK, which confers complete protection to BALB/c mice when injected with IL-12, suggests that this antigen may be superior to the previous studies using LPG and gp63 and may lead to an effective vaccine strategy for the future.

See also: Cell-mediated immunity; Cytokines; Parasites, immunity to.

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LEPTOSPIRA, INFECTION AND IMMUNITY

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Characteristics of *Leptospira* and its antigens

Leptospirosis is a collective term used to denote all infections of humans and animals by members of the genus *Leptospira*. Leptospire are helical-shaped, thin, flexible, motile spirochetes with semicircular hooked ends (occasionally one or both may be straight). They are 0.1 µm in diameter and 6–20 µm in length and are surrounded by an outer envelope or external sheath which covers the cylindrical body. Two periplasmic flagella are inserted subterminally at opposite ends of the cell, with the free ends overlapping in the center of the cell. The flagella are located between the outer envelope or sheath and the protoplasmic cylinder. Inside the protoplasmic cylinder are the cytoplasmic contents.

Various protective and genus-specific antigens are located in the *Leptospira* outer envelope. Lipopolysaccharide material appears to be an important antigenic component of the outer envelope. Antigenic proteins have been visualized by immunoblotting, although only those proteins whose epitope groups remain recognizable by antibodies are detected. Numerous protein bands are found in the outer envelope or on the surface of intact cells. Major antigenic proteins of 22–26, 31–34, 38, 40–44, 50, 60–64 and 95 kDa are found in the outer envelope. Additionally, antigenic proteins of 18, 24, 35, 42, 66 and 77 kDa vary quantitatively between the serovars studied. *Leptospira interrogans*-specific nonagglutinating, nonopsonic antigen (35 kDa) has also been found in outer envelope preparations, but there is speculation that this may have originated from the inner surface of the outer envelope. A 33 kDa protein found in serovar *grippotyphosa* is presumed to be associated with avirulent cultures, while the 41 and 44 kDa proteins appear to be associated with hamster-virulent isolates. Proteins of molecular

weights 31, 34 and 37 kDa make up the flagella. The heat shock protein (hsp) is 64 kDa and shares common antigenic determinants with a wide variety of bacteria. Seven new proteins were produced by serovar *hardjo* when it was cultivated at 37°C.

Two species of *Leptospira* are recognized on the basis of infectivity of laboratory animals: *Leptospira biflexa*, which comprises the nonpathogenic leptospire commonly found in natural waters, and *Leptospira interrogans*, which includes all the parasitic and pathogenic serovars (serotypes). Typing has traditionally been performed by means of agglutination tests with high-titered antisera. The traditional classification methodologies are being augmented by the use of monoclonal antibodies, and the *interrogans* species is being further subdivided into 'geno-species' by restriction endonuclease analysis and DNA probes. The leptospire genome is approximately 4500 kb, and a circular plasmid of approximately 350 kb has been reported.

Leptospire are aerobic and most are readily cultivated in artificial media containing 10% rabbit serum or 1% bovine serum albumin plus long-chain fatty acids (Tween 80) supplemented with ammonium chloride, ferric sulfate, calcium, magnesium, zinc, thiamine and cyanocobalamin. Optimum cultivation temperature is 30°C at a pH of 7.2–7.5. The optimum incubation time for growth ranges from a few days to 4 or more weeks. Fresh isolates tend to require longer incubation. Generally maximum cell densities are attained in 4–10 days, depending on the inoculum and organism being cultivated. Leptospire are visualized by dark-field or phase-contrast microscopy.

Leptospirosis is a zoonosis of worldwide distribution, affecting a wide variety of domestic and wild mammals. Many serovars occur, predominantly in select animal hosts, but distribution of these serovars

occurs readily. One mammalian species may be the primary reservoir for several different serovars. Parasitic leptospires often reside in the mammalian nephritic tubule, from which they are shed into the urine. The duration and intensity of shedding is often dependent on the host and infecting serovar. Following infection, rodents may shed high numbers of some *Leptospira* serovars for the remainder of their life, while shedding other serovars for a relatively short period of time. Domestic mammals generally shed leptospires for several months, with decreasing intensity up to 6 months.

Infections occur as a result of direct contact with the urine of chronically infected animals or indirectly by contact with the *Leptospira*-contaminated water (streams or ponds) or moist soil. Pathogenic leptospires are able to survive more than 3 months in neutral or slightly alkaline soil or water. Animals and humans acquire leptospires either by direct contact with urine or tissues from an infected animal or indirectly by contact with *Leptospira*-contaminated soil or water. Once the host is exposed, leptospires may enter through mucous membranes of the eye, nose, mouth, genital tract or abraded skin.

Most human cases of leptospirosis occur in young adult men, with a peak incidence of summer and early fall. Indirect contact with an infected animal via contaminated water or soil is a very common cause of human infection other than those individuals whose occupation (veterinarian, abattoir workers, dairy farmers, etc.) places them in direct contact with numerous animals. However, the hazards of swimming in ponds or streams frequented by livestock has been demonstrated repeatedly. In the human, disease transmission generally ends, as human-to-human transmission is very rare, only occurring via transplacental infection of the fetus. Worldwide, rodents are the most common source of human infection. In the USA the most common source of human infection is dogs, livestock, rodents and wild mammals. The most common serovars infecting humans are *canicola*, *pomona*, *ballum*, *icterohaemorrhagiae*, *grippotyphosa*, *australis* and *hebdomidis*.

Domestic animals generally become infected in a manner similar to humans, i.e. via contact with *Leptospira*-contaminated urine, water (used by livestock for drinking), soil, infected tissues or uterine discharge. Leptospires have been demonstrated in semen from cattle and pigs and may be transmitted by natural and artificial insemination. Leptospires have been isolated from cows' milk, however natural transmission via this medium has never been demonstrated.

Pathogenicity

Human leptospirosis is primarily associated with occupational exposure, although the number of cases associated with recreational exposure will probably increase. Once entry is gained, there is immediate spread throughout the body without a significant inflammatory reaction. During this incubation phase, which generally ranges from 10 to 14 days but may be as short as 3 or as long as 30 days or more, leptospires are multiplying in the host. Based on findings in experimental animals, multiplication takes place in the liver and blood. Disease onset is often abrupt. Severe headache and muscle tenderness is common and accompanied by an elevation of 1–3°C in body temperature. During this acute febrile phase, leptospires may be cultured from blood, cerebrospinal fluid and possibly urine. Leptospires can be isolated from the urine starting at 2 weeks following clinical disease. Other signs which may be present include jaundice, hemorrhage, malaise, nausea and vomiting. The extent and severity of lesions depends on the infecting serovar and size of the infecting dose. Following the first week of disease the fever subsides, although intermittent short febrile periods may occur over the next 2 weeks. Coinciding with the termination of the leptospiremic phase is the appearance of specific antibodies. Following the first week of illness, leptospires were found in the urine and are shed intermittently for the following 3 months and possibly longer.

In animals the disease process may take a more acute form or be subtle and chronic. The clinical signs observed in domestic mammals are dependent upon the age and species of the animal and the infecting serovar. Fever does occur in domestic mammals but often goes undetected. Clinical signs may include hemorrhage in mucous membranes and kidney tissue, jaundice, anemia, meningitis, anorexia and increased thirst. Often the first observed sign of leptospirosis in animals is abortion, which usually occurs in the later stages of gestation. However, abortion can and does occur at any time during gestation. When abortion occurs it is several days to weeks following leptospires in the blood. Abortion has been associated with leptospirosis in cattle, swine, sheep and horses. The fetus becomes infected as a result of leptospire migration from the maternal circulation. Other complications occurring as a result of *Leptospira* infection are infertility and neonatal death as a result of congenital infection.

Chronic *Leptospira* infection may result in degenerative changes in the kidneys and/or eyes. The interstitial nephritis which occurs results in substantial kidney damage. Tissue damage does not always

occur during acute leptospirosis in areas where aggregates of leptospires are found. Virulent leptospires appear to attach preferentially to kidney cell cultures *in vitro* although no tissue cell damage was noted after 24 hours of incubation. However, kidneys may exhibit lesions characteristic of hypersensitivity reactions following *Leptospira* infection.

Eye damage, which may occur as a result of leptospirosis, varies from acute to chronic. Total blindness may occur in the chronic form of the disease. The humor of the eye in the affected host may harbor viable leptospires and high levels of specific antibody. Ocular involvement occurs more frequently following multiple exposures to *Leptospira*, probably resulting from cross-reactions between leptospiral antigens and proteins of the cornea and lens tissues, particularly in horses. This could partially explain why the lesions which occur in the eye have characteristics similar to those resulting from hypersensitivity reactions.

Immunity

Freshly isolated leptospires do not readily react with specific antibody. This may help the leptospire evade the host immune system, allowing for multiplication prior to clinical disease. Once entry of the host is accomplished, *Leptospira* seem to further evade the host's immune systems by becoming sequestered in the renal tubules, uterine lumen, eye or brain.

Specific immunoglobulins being to appear 5–14 days after leptospiemia. Immunoglobulin M (IgM) is the predominant immunoglobulin produced initially. This antibody, along with complement and phagocytic cells, initiates the clearing of leptospires from the host. Detectable levels of IgG, which is opsonizing and neutralizing, appear a short time later in the disease and persist for variable periods of time. The effect of cell-mediated immunity during *Leptospira* infection is probably limited, however this needs further evaluation.

Vaccines manufactured for use in animals consist of chemically inactivated whole leptospire cultures. These vaccines contain sufficient quality and quantity of antigenic material to prevent clinical disease, abortion, and to reduce incidence of renal shedding in the immunized host. The immunity induced is serovar specific, and although some serological cross-reactions occur, protection afforded by these antibodies is nonexistent. To circumvent the limitations of immune specificity, specific preparations from several serovars are mixed to make more broadly protective vaccines.

It is important to use locally prevalent serovars whenever possible. In addition, most vaccines should

be prepared from virulent leptospires which make better immunogens than avirulent organisms, as less antigenic mass is required to induce immunity and prevent renal infection and/or death. A critical aspect of any *Leptospira* vaccine preparation is that the antigens located in the outer envelope retain their natural configuration as much as possible because the outer envelope is the primary site of antibody–complement interaction.

Parenteral administration of a vaccine stimulates the production of specific IgM followed by IgG, as monitored by the microscopic microtiter agglutination test. IgM appears to be more efficient at agglutinating and neutralizing leptospires than IgG; however, both are critical to the rapid removal of leptospires from the infected host. Titers may be detectable for 6 months or more, depending on the serovar.

See also: Bacteria, immunity to; Bacterial cell walls; Eye infections; Fever; Renal infections.

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LEUKEMIA

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Virchow applied the term leukemia (from the Greek λευκός white and αἷμα blood) in the mid-nineteenth century to indicate a disease characterized by fever, bleeding, enlargement of the spleen and lymph nodes, and accompanied by an abnormally high number of white cells in the peripheral blood. Leukemias are clonal expansions of lympho-hematopoietic cells. Generally, acute leukemias present with high numbers of immature lympho-hematopoietic cells ('blasts') in blood and/or in the bone marrow. In chronic leukemias, the bulk of the neoplastic population is represented by mature-looking cells. The incidence of acute and chronic myeloid leukemias and of chronic lymphoid leukemias progressively increases with age. Acute lymphoblastic leukemia, however, occurs more frequently in childhood and accounts for a sharp age peak for leukemia at 3–4 years of age.

Understanding of leukemia cell biology has progressed dramatically during the last two decades. Advances in the immunological characterization of normal and leukemic hematopoietic cells, the unravelling of immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements, and the molecular characterization of genetic abnormalities underlying leukemogenesis have increased the accuracy of leukemia classification and provided clues about novel therapeutic strategies.

Acute leukemia

Classification

Morphology and cytochemistry Acute leukemias are classified according to the predominant morphology of the malignant cells. One widely used morphologic classification of acute leukemia was devised in the mid-1970s by a group of French, American and British ('FAB') investigators. There are three main FAB subtypes (L1–L3) of acute lymphoblastic leukemia (ALL) and five (M1–M5) of acute myeloid leukemia (AML); the M1–M3 types reflect discrete stages of myeloid differentiation, while M4 and M5 cases have monocytic components. M6 and M7 denote erythroid and megakaryocytic leukemias, respectively. The precision of leukemia classification can be enhanced by investigating the activity of intracellular enzymes (e.g. myeloperoxidase, esterases and phosphatases) and the distribution of phospholipids and glycogen, by using cytochemical stains. Morphological and cytochemical analyses, however, are not sufficient for an accurate classification of all cases of leukemia. Thus, modern diagnostic procedures usually involve immunophenotypic, karyotypic and molecular studies.

Immunophenotype A few monoclonal antibodies have lineage-restricted reactivity and are, therefore,

Table 1 Immunologic classification of acute lymphoblastic leukemia

Subtype	CD19	CD79	<i>clg</i> μ	<i>slgM</i>	<i>slg</i> κ or <i>slg</i> λ	CD7	CD3 ^a	Frequency (%)
Early pre-B ALL	+	+	–	–	–	–	–	60–65
Pre-B ALL	+	+	+	–	–	–	–	20–25
Transitional pre-B ALL	+	+	+	+	–	–	–	1
B-ALL	+	+	+	+	+	–	–	2
T-ALL	–	–	–	–	–	+	+	15–18

^aCytoplasmic or cell surface.

useful for leukemia classification. These include CD79 for B cells, CD3 for T cells and antimyeloperoxidase for myeloid cells. Diagnostic panels consisting of these and other reagents, such as CD19, CD7, CD13, CD33, antiglycophorin and CD61 unequivocally classify the vast majority of cases of acute leukemia.

Leukemic cells in B-lineage ALL, the most frequent form of ALL, are typically CD19⁺, CD22⁺, CD79⁺ and surface Ig[–] (Table 1). Most cases are CD10⁺ and are termed 'common' ALL, while CD10[–] cases have been called 'null' ALL. 'Common' and 'null' ALL are collectively termed 'early pre-B ALL'. Other molecules often expressed by these cells include CD24, CD34, CD72, HLA-DR and nuclear terminal deoxynucleotidyl transferase (TdT). In 20–30% of B-lineage ALL cases, cells express cytoplasmic mu heavy chains ('pre-B-ALL'; Table 1). The majority of pre-B ALLs are CD10⁺, while about 60% are CD34⁺ and 40% are CD20⁺. Rare cases in which cells express cytoplasmic *and* surface mu heavy chains without kappa or lambda light chains have been called 'transitional pre-B ALL'. In these cases, the surface mu heavy chains are thought to be associated with pseudo light chains. Finally, the blasts in a few (<5%) B-lineage ALL cases express surface IgM and either kappa or lambda light chains ('B-ALL'). Most B-ALLs are the leukemic phase of Burkitt's lymphoma.

In T-ALL, blasts typically express CD3 (in the cytoplasm or on the cell membrane) and CD7 (Table 1). T cell receptor proteins may be found in the cyto-

plasm of T-ALL blasts lacking surface TCR expression. Among other molecules, CD2, CD5, CD6 and nuclear TdT are expressed in >90% of cases while CD1a, CD4, CD8, CD10 and CD21 are expressed less frequently, and HLA-DR is usually negative. Approximately 80% of T-ALL with surface TCR have the $\alpha\beta$ form of the receptor, the remaining cases being TCR- $\gamma\delta$ ⁺.

Malignant cells in AML express myeloperoxidase, CD13 and/or CD33 (Table 2). CD34 expression is more common in cases with less differentiated morphology. HLA-DR antigens are found in most AML cases, but are negative in the majority of cases of promyelocytic leukemia (FAB M3; Table 2). CD117 is also expressed in many AML cases, with the exception of the promyelocytic and the monocytic subtypes. Leukemias whose blasts react with CD14 antibodies usually have monocytic morphology (FAB M4 and M5; Table 2). Monocytic differentiation of AML is also suggested by CD11c, and CD4 expression. These cases are usually CD34[–]. An additional sign of monocytic differentiation is the high membrane expression of Fc receptors. Finally, cases of acute erythroid leukemia can be identified with antibodies to glycophorin A, while cases of acute megakaryocytic leukemia react with CD41b, CD42a and CD61. These subtypes are rare (<1% of acute leukemias).

Cases composed of two separate blast populations expressing either lymphoid or myeloid markers ('mixed leukemias') are extremely rare. The term 'mixed' or 'biphenotypic' leukemia has also been

Table 2 Predominant immunophenotypes according to FAB classification in AML

FAB group	MPO	CD34	HLA-DR	CD33	CD13	CD11b	CD14
M1	+	+	+	+	+	+/-	–
M2	+	+	+	+	+	+/-	–
M3	+	–	–	+	+	–	–
M4	+	–	+	+	+	+	+
M5	+	–	+	+	+/-	+	+

MPO, myeloperoxidase.

applied to more frequent cases in which blasts simultaneously express 'myeloid'- and 'lymphoid'-associated markers. For example, a proportion of ALL cases express CD13, CD33 and/or CD15. In most of these cases, the expression of an array of lymphoid-associated antigens and the rearrangement of Ig or TCR genes strongly indicate a lymphoid origin. In a proportion of AML cases, blasts may express 'lymphoid-associated' markers, such as TdT, CD7, CD19 and/or CD56. The myeloid affiliation of these cases is usually clear and they rarely represent a diagnostic problem.

A distinct group of cases (1–2% of acute leukemias) are undifferentiated morphologically and cytochemically, and show recurrent phenotypic features such as HLA-DR, TdT and CD34 positivity, and lack of myeloperoxidase, CD13, CD33, CD79, CD22, CD19, CD3, glycophorin and CD61. These cases have been named 'acute undifferentiated leukemia'.

Antigen-receptor gene rearrangements and genotypic abnormalities Virtually all cases of B-lineage ALL have IgH gene rearrangements, while approximately half of the cases also show TCR δ and TCR γ gene rearrangements and one-third TCR β gene rearrangements. Virtually all T-ALL cases have TCR β and TCR γ gene rearrangements, and 70% of cases have

TCR δ gene rearrangements, while IgH genes are rearranged in approximately 15% of cases. Rearrangements of the same genes in B- and T-lineage cells are not always identical. For example, TCR δ genes found in B-lineage ALL usually have short junctional regions (i.e. V δ 2–D δ 3 and D δ 2–D δ 3), in contrast to the dominant V δ 1DJ δ 1. rearrangements in T-ALL. IgH gene rearrangements in B-lineage ALL at diagnosis are often biclonal or oligoclonal. Moreover, investigators have found changes in the pattern of rearrangement at presentation and relapse for both IgH and TCR genes, an important potential pitfall when these genes are used to track residual leukemia (see below). Nevertheless, in the majority of cases, at least one major rearranged IgH, TCR γ or TCR δ allele appears to persist from diagnosis to relapse. Oligoclonality and clonal evolution in IgH genes are often due to disruption in the V–N–D portion, while the D–N–J sequence is left unmodified. Rearrangements of the IgH and/or TCR genes can also occur in approximately 10% of AML.

Some of the most common genotypic abnormalities of acute leukemia are summarized in Table 3. In approximately 25–40% of cases of adult ALL and 3–5% of cases of childhood ALL, leukemic cells have a reciprocal translocation between chromosomes 9q34 and 22q11 (Philadelphia (Ph)-chromosome),

Table 3 Some recurrent genotypic abnormalities in acute leukemia

Disease	Chromosomal abnormality	Molecular abnormality	Frequency (%) ^a	
			Adults	Children
Acute lymphoblastic leukemia	t(9;22)(q34;q11)	BCR-ABL	25–40	3–5
B-lineage	t(12;21)(p12–13;q22)	TEL-AML1	<1	25–30
	t(1;19)(q23;p13)	E2A-PBX1	2–3	5–6
	t(4;11)(q21;q23)	MLL-AF4	5	2–4
	t(5;14)(q31;q32)	IL3-IGH	<1	<1
	t(11;19)(q23;p13)	MLL-ENL	<1	<1
	t(9;11)(p21–22;q23)	MLL-AF9	<1	<1
	t(17;19)(q22;p13)	E2A-HLF	<1	<1
	t(8;14)(q24;q32.3)	MYC-IgH	4–5	1–2
T-lineage	–	TAL1 deletion	10–30	20–30
	t(11;14)(p13;q11)	RHOM1-TCR δ	5–10	5–10
	t(1;14)(p34;q11)	TAL1-TCR δ	1–3	1–3
	t(10;14)(q24;q11)	HOX11-TCR δ	1–3	1–3
Acute myeloid leukemia	t(8;21)(q22;q22)	AML1-ETO	5–10	5–10
	t(15;17)(q22;q11–21)	PML-RARA	5–10	5–10
	inv(16)(p13q22)/t(16;16)	CBF β -MYH11	5–10	5–10
	t(9;11)(p21–22;q23)	MLL-AF9	1–5	5–10
	t(9;22)(q34;q11)	BCR-ABL	1–3	<1
	t(6;9)(p23;q34)	DEK-CAN	<1	<1

^aThe frequency within each disease group.

which disrupts the ABL and BCR genes, respectively. Cytogenetically, the Ph-chromosome found in ALL is indistinguishable from that seen in chronic myelogenous leukemia (CML; see below), but they may differ at the molecular level. While the breakpoints on chromosome 22 in CML usually map to the major breakpoint cluster region (M-bcr) of the BCR gene, most Ph-positive ALL patients show a translocation to the minor breakpoint cluster region (m-bcr) of the same gene. About 25% of pre-B ALL have either the balanced form of $t(1;19)(q23;p13)$ or its unbalanced form, $der(19)t(1;19)(q23;p13)$, while most CD10⁺ ALL cases have 11q23 abnormalities and rearrangements of the MLL gene. The TEL and AML1 genes, which are involved in the extremely rare translocation $t(12;21)(p12-13;q22)$, are frequently rearranged in childhood ALL. Cases with TEL-AML1 rearrangements have B-lineage phenotype and account for 25–30% of childhood B-lineage ALL cases. All cases of surface Ig⁺ B-ALL and Burkitt's lymphoma have translocations involving the MYC proto-oncogene on the 8q24 region and the immunoglobulin genes, most commonly the heavy chain gene at 14q32. In T-ALL, nonrandom chromosomal abnormalities include $t(11;14)(p13;q11)$, $t(1;14)(p34;q11)$ and $t(10;14)(q24;q11)$. In addition, 90 kb deletions on chromosome 1p32, affecting the TAL-1 gene, are found in approximately 10–30% of T cell cases.

In AML, the $t(15;17)(q22;q12-21)$ is found in most cases of acute promyelocytic leukemia (FAB M3). The breakpoints of this translocation affect the retinoic acid receptor α gene (RARA) on chromosome 17 and a transcription factor, PML, on chromosome 15. Other molecular abnormalities included the AML1/ETO fusion in the $t(8;21)(q22;q22)$, CBF β -MYH11 in $inv(16)(p13q22)$, DEK-CAN in $t(6;9)(p23;q34)$, and MLL-AF9 in $t(9;11)(p21-22;q23)$.

Biologic indicators of prognosis

Chromosomal abnormalities are strong prognostic indicators in both ALL and AML. In ALL, hyperdiploidy (51–65 chromosomes), detected in approximately a quarter of children with B-lineage ALL but rare in adults, has consistently been associated with favorable outcome in different chemotherapy programs. It appears that hyperdiploid lymphoblasts have a marked propensity to apoptosis, and a higher sensitivity to methotrexate, one of the main components of ALL therapy. Childhood ALL cases with TEL-AML1 rearrangements also have a strikingly good response to chemotherapy. By contrast, $t(9;22)(q34;q11)$ and BCR-ABL rearrangement and 11q23 abnormalities involving the MLL gene are

typically associated with a dismal response to chemotherapy. MLL gene abnormalities are frequent in infant leukemia. AML cases with $t(8;21)(q22;q22)$ often express CD19 and/or CD56 and show a distinctively good response to chemotherapy. Those with $t(15;17)(q22;q11-21)$ abnormality also respond well to therapy, and show a characteristic response to all-*trans*-retinoic acid, which induces the differentiation of the leukemic promyelocytes to more mature forms. By contrast, monosomy 7, 11q23 translocations and $t(6;9)$ are associated with a particularly poor prognosis.

Children with CD10⁺ B-lineage ALL generally fare worse than CD10⁺ cases, an observation that may be explained by the strong association between CD10 negativity and MLL gene abnormalities. By contrast, CD34 expression and lack of CD45 expression have been correlated with favorable treatment outcome in childhood ALL, the latter feature being strongly associated with hyperdiploidy. The prognostic significance of myeloid antigen expression in ALL is controversial. T-ALL is characterized by a high initial leukocyte count at diagnosis, mediastinal masses in one-half of the patients, and a generally poorer prognosis than early pre-B and pre-B types of ALL. However, in multivariate analysis, the effect of T cell phenotype is lost when correction is made for age and leukocyte counts. Subclassifications of T-ALL according to the level of thymic differentiation have generally failed to show relationships with clinical features or treatment outcome. The expression of CD10 in childhood T-ALL has been associated with better clinical outcome, while adults with T-ALL and CD2⁺ blasts appear to have a particularly unfavorable prognosis. In general, immunophenotypic features of AML do not show strong correlations with clinical parameters or treatment outcome. It has, however, been reported that patients whose blasts lack CD15 and/or express CD34 may have a lower complete remission rate. The predictive value of TdT expression is controversial.

Detection of minimal residual disease

Patients with acute leukemia who have achieved clinical remission may still have up to 10^{10} malignant cells (the upper limit of detection by standard morphologic techniques). Postremission therapy is the same for these patients as for those who may have fewer remaining cells, or perhaps no minimal residual disease (MRD). The assumption underlying studies of MRD is that improved assessments of the total body burden of leukemic cells would increase the accuracy of leukemia treatment and advance cure rates.

Sensitive detection of MRD can be approached

with a variety of techniques, including immunophenotyping, and polymerase chain reaction (PCR) amplification of chromosomal breakpoints and antigen-receptor gene rearrangements. MRD detection with immunologic methods exploits the expression of marker combinations that are found on leukemic cells but are not expressed by normal peripheral blood and bone marrow cells. The maximum sensitivity of these methods for detecting MRD is approximately one target cell in 10^4 when flow cytometry is applied. Virtually all cases of T-ALL are amenable to sensitive flow cytometric analysis of MRD, while approximately 40–50% of B-lineage ALL and AML cases express a suitable immunophenotype. In cases of ALL or AML with genetic abnormalities such as those listed in Table 3, PCR can be applied to amplify RNA or DNA sequences unique to the malignant clone. This approach affords the detection of one malignant cell among 10^6 normal cells. PCR amplification of uniquely rearranged Ig and/or TCR gene sequences can also be used for MRD detection. Here, leukemia-specific probes or primers are needed in each case; the sensitivity of this approach ranges from 10^{-3} to 10^{-6} . Prospective studies of MRD with different techniques, currently ongoing, should clarify its clinical significance. In general, the retrospective studies so far reported showed that the absence of detectable leukemic cells does not ensure that a patient has been cured, but their persistence during or off-treatment appears to correlate with occurrence of relapse.

Chronic leukemias

Chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) is a clonal proliferation of hematopoietic precursor cells, with a greatest incidence between 40 and 60 years of age. The t(9;22)(q34;q11), or Philadelphia (Ph)-chromosome, and BCR-ABL rearrangements are characteristic of CML, and occur in all hematopoietic lineages, an indication that the disease originates from a multipotent progenitor cell. Rare cases of CML (<5%) are Ph-negative but in many of these cases (BCR-ABL) rearrangements can be detected with molecular techniques. PCR amplification of BCR-ABL can be used to detect disease recurrence after allogeneic bone marrow transplantation, although positive PCR results have not consistently predicted treatment outcome.

At diagnosis, CML usually features extremely high white blood cell counts (e.g. $>100 \times 10^9/l$); most cells are granulocytes or maturing myeloid forms. This morphologic appearance is typical of the initial phase of the disease ('chronic phase'). However, it is

not stable and invariably transforms into a 'blast crisis', where the bulk of the neoplastic population is represented by immature-looking forms, resembling those seen in acute leukemias. In the majority of patients, these blasts have morphological and phenotypic features identical to those seen in AML, while approximately 30% of cases have lymphoid morphology and B-lineage ALL immunophenotype; T cell blast crises are rare.

Chronic lymphoid leukemias

B-chronic lymphocytic leukemia (B-CLL) is the most common chronic lymphoid leukemia, and accounts for about 30% of all leukemia patients diagnosed in the Western world, but is less frequent in the Orient. B-CLL is a disease of the elderly, unusual under the age of 40. The most consistent chromosomal abnormality is trisomy of the chromosome 12. Patients with a normal karyotype appear to do better than those with chromosomal abnormalities, and multiple chromosomal abnormalities are associated with a more rapid progression of the disease. Leukemic cells in B-CLL and in other B-lineage chronic lymphoid leukemias (i.e. hairy cell leukemia (HCL), and prolymphocytic leukemia (PLL)) typically express CD19, CD79, CD20 and CD37, whereas TdT is absent (Table 4). In B-CLL, the level of Ig expression on the cell membrane is heterogeneous and often weak. Similarly, membrane expression of CD22 may be extremely weak or undetectable. CD23 and CD5 expression are also characteristic of B-CLL while CD25 and CD11c expression together with high-level expression of CD22 are typical of HCL (Table 4). CD5 expression in B-CLL recalls the phenotype of cells found in primary follicles of fetal lymph nodes and spleen, possibly equivalent to murine Ly1⁺ B cells.

Typical cell markers of T cell chronic lymphoid

Table 4 Predominant immunophenotypes of chronic B cell leukemias

Marker	CLL	PLL	HCL
CD19	+	+	+
CD20	+	+	+
CD37	+	+	+
slg	+/–	+	+
CD5	+	–	–
CD11c	–	–	+
CD22	–	+	+ ^a
CD23	+	–	–
CD25	–	–	+
FMC7	–	+	+

^a Typically, high expression.

leukemias are CD2, CD3 and TCR chains. CD3 and TCR molecules often accumulate in the cytoplasm and are weakly expressed on the cell membrane. In addition, natural killer-associated markers such as CD16 and CD57 are usually found in T-CLL. The majority of T-CLL are CD8⁺, CD4⁻ whereas the neoplastic cells in T prolymphocytic leukemia (T-PLL) are commonly CD4⁺, CD8⁻. In T-CLL, CD7 is usually weakly expressed or undetectable and CD25 is absent, while most T-PLL cases are strongly CD7⁺. Adult T cell leukemia/lymphoma (ATLL) is a disease associated with a human retrovirus (HTLV-I) infection and is distributed in geographical clusters in the southern USA, southern Japan and the Caribbean. In ATLL, cells are CD2 and CD3 positive and usually CD4⁺, CD8⁻. Typically, CD25 is strongly expressed.

Advances in therapy

Approximately 70% of children with ALL can be cured with multiagent chemotherapy. Cure rates are much lower (approximately 30%) for adult patients with ALL, and patients with AML of all ages. Chemotherapy of chronic leukemias is generally directed to delay their progression; disease eradication can only be achieved with allogeneic bone marrow transplantation. Current treatment of acute leukemias emphasizes assessment of risk of relapse at the time of diagnosis. Thus, a continuing challenge is how to identify patients who will need more aggressive (and toxic) therapy to prevent relapse. In addition to clinical features, such as patient's age and white blood cell count at diagnosis, several biologic parameters may predict treatment outcome and, therefore, may be useful for risk classification. The use of immunophenotypic and molecular predictors of outcome are promising tools. Moreover, the *in vitro* determination of leukemic cell growth potential, drug sensitivity and expression of molecules associated with drug resistance (e.g. P-glycoprotein, BCL-2) may also prove to be useful. Finally, the ability to detect MRD in patients with acute leukemia would allow more timely, and potentially curative, intervention. Studies of MRD could also be helpful in autologous bone marrow transplantation, where they could provide means of detecting leukemic cells in 'remission' marrows and in peripheral blood autografts.

In parallel to the refinement of conventional therapies, novel antileukemic agents are becoming available. All-*trans*-retinoic acid is widely used in the treatment of promyelocytic leukemia, and interferon α has already produced significant clinical results in CML and HCL. Interleukin 4 has been shown to be

cytotoxic for leukemic lymphoblasts in cases of high-risk ALL and is currently being tested in clinical trials. Toxin-conjugated, human-mouse chimeric and bivalent monoclonal antibodies against various leukemic cell types are being developed and tested in clinical trials for *in vivo* administration.

See also: B lymphocyte differentiation; Bone marrow and hematopoiesis; CD antigens; Chromosome translocations of immune genes; Flow cytometry; Immunoglobulin genes; Lymphoma; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T lymphocyte differentiation.

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LEUKEMIA INHIBITORY FACTOR

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Leukemia inhibitory factor (LIF) is a cytokine with pleiotropic activities. It was originally isolated, purified and cloned on the basis of its differentiation-inducing activity, but was subsequently found to possess a broad spectrum of biological functions (Table 1).

Expression of LIF

The LIF gene is constitutively expressed at low levels in a number of different cell types and the level can be increased by a variety of stimuli, such as lipopolysaccharide (LPS), interleukin 1 α (IL-1 α), transforming growth factor β (TGF β), epidermal growth factor (EGF), tumor necrosis factor α (TNF α), phorbol ester, retinoic acid or infections with cytomegalovirus (CMV).

Structure of LIF

The characterization of the LIF molecule is summarized in Table 2. The structure of murine LIF, as determined by X-ray crystallography, affiliated LIF to the hematopoietic cytokine family. It is composed of an α helix bundle structure, previously observed for several members of this cytokine family. A variant of murine LIF, derived by alternative splicing of signal sequence exons, is targeted to the extracellular matrix.

Table 2 Structural characteristics of LIF

	Mouse	Human
Molecular weight (kDa)	58 000	45 000
Deglycosylated	20 000	20 000
Chromosomal location	11A1–A2	22q12
Genomic structure (kb)	3 exons	3 exons
3' untranslated region	8.7	7.6
mRNA (kb)	3.2	3.2
half-life (min)	4.1	4.1
	30	30

LIF-mediated signal cascades

LIF belongs to the family of IL-6-type cytokines which includes oncostatin M (OSM), ciliary neurotrophic factor (CNTF), interleukin 11 (IL-11), interleukin 6 (IL-6) and cardiotrophin 1 (CT1). These cytokines have diverse and often overlapping effects on a multitude of cell types. The receptors for members of this cytokine family consist of a cytokine-specific ligand-binding chain and the shared gp130 signal transducer chain, which explains the functional redundancy. Downstream of gp130, two signal-transducing pathways have been recognized, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and the Ras/mitogen-activated protein kinase (MAPK) pathway. Both pathways are rapidly activated by LIF in various cell types. In addition, the tyrosine kinases Tec, Fes, Btk

Table 1 Synonyms of LIF

Acronym	Name	Defining action
LIF	Leukemia inhibitory factor	Induction of differentiation of M1 myeloid leukemic cells
D-factor	Differentiation-stimulating factor	
DIF	Differentiation-inducing factor	
DIA	Differentiation inhibitory activity	Inhibition of differentiation of embryonal stem cells
DRF	Differentiation-retarding factor	
ESCGF	Embryonal stem cell growth factor	
	ES cell growth factor	
CNDF	Cholinergic neuronal differentiation factor	Induction of cholinergic neuronal differentiation
HILDA	Human interleukin for DA1-1a cells	Stimulation of myeloid DA1-1 α cell proliferation
MLPLI	Melanoma-derived lipoprotein lipase inhibitor	Inhibition of lipoprotein lipase activity
	Lipoprotein lipase inhibitor	
OAF	Osteoclast-activating factor	Stimulation of bone resorption <i>in vitro</i>
HSF-3	Hepatocyte-stimulating factor 3	Stimulation of acute phase protein synthesis

and Hck were shown to be activated by triggering a signal via gp130. Therefore, in addition to the JAK/STAT and Ras/MAPK signal cascades other pathways are activated, and cross-talk between these pathways has been proposed to exist and to enhance, prolong or specify the cytokine signal.

Functions of LIF

Hematopoietic cells

LIF was identified as a factor capable of inducing the terminal differentiation of the murine myeloid leukemic cell line M1 into macrophages. Activation of the transcription factor STAT3 is essential for LIF-induced differentiation of M1 cells. A number of myeloid leukemic cell lines, which were unresponsive to treatment with LIF, expressed various defects in the JAK/STAT signal cascade, suggesting modulations of this pathway to be a crucial step in leukemogenesis. Although LIF expressed pronounced effects on leukemic cells it had no proliferative activity on normal hematopoietic progenitor cells. Mice with targeted disruption of the LIF receptor ligand-binding chain contained a normal hematologic cell compartment. Targeted disruption of the gp130 signal transducer chain led to hematological disorders during embryogenesis, demonstrating a crucial involvement of gp130-mediated signaling. Attempts to maintain or expand primitive hematopoietic stem cells *in vitro* without concomitant loss of their differentiative and proliferative potential *in vivo* were significantly more successful by culturing the cells in the presence of LIF. The effect of LIF on long-term repopulating stem cells resulted from promoting cytokine expression by bone marrow stroma and may thus be an important tool in blood cell transplantation and transfusion medicine.

Acute phase response

The acute phase response is a systemic reaction against infection or tissue injury, which involves fever, leukocytosis, increased vascular permeability and increased serum levels of acute phase proteins. Acute phase proteins are synthesized in the liver in response to increased levels of certain cytokines including IL-1, TNF α and IL-6. LIF can induce the same set of acute phase proteins as IL-6. This is due to the use of the same signal cascade leading to activation of transcription factors of the STAT family, which then bind to specific control regions of various acute phase genes. In addition, LIF stimulates hepatic triglyceride secretion and mediates changes in lipid metabolism that accompany the acute phase response.

The involvement of LIF in postoperative anorexia and in cancer cachexia is likely to be mediated centrally by decreased food uptake and in addition by its influence on lipid metabolism, both leading to loss of body weight.

LIF is a cytokine released at the site of local injuries, where a recruitment of monocytes and polymorphonuclear cells takes place. Supernatants of monocytes treated with LIF had chemotactic activity for neutrophils and monocytes. LIF acts as an endogenous mediator of cutaneous inflammation and is a mediator of (CMV)-induced inflammatory reactions.

Nervous system

LIF/CNTF has been known to switch the pattern of neurotransmitter expression from the noradrenergic to the cholinergic phenotype. Like CNTF, LIF has profound effects on the survival and maintenance of motor neurons. In addition, LIF may be important during cerebral infection and inflammation. Primary astrocyte cultures express LIF mRNA constitutively, and this expression is regulated by proinflammatory and anti-inflammatory stimuli, suggesting that astrocytes may be an important source of LIF during cerebral inflammation and infection. A rapid upregulation of LIF in the nerve segments, produced by Schwann cells subsequent to nerve injuries, leads to the suggestion that LIF acts as a trauma factor in injured peripheral nerves.

Development

LIF is expressed in a menstrual cycle-dependent manner in human endometrium, with maximal values coinciding with the time of implantation. LIF induces the expression of other cytokines to regulate the temporal and spatial production and activity of proteases and protease inhibitors to create a favorable environment for implantation. By increasing the synthesis of fibronectin LIF acts on human trophoblasts and shifts their differentiation towards the anchoring phenotype.

LIF and cancer

A variety of cancer cells have been shown to respond to LIF by stimulation of proliferation using either a paracrine or an autocrine mechanism. In addition, LIF modulates the capacity of tumor cells to adhere to matrix components, a requisite for a metastatic process, suggesting a potential role in the modulation of tumor progression. The levels of soluble LIF produced by various tumor cell clones correlated with their tumorigenicity.

LIF in transgenic mice

LIF expresses pleotropic functions and thus cannot be categorized according to a single major effect. In addition to the above described functions, LIF is involved in effects, such as the maintenance of totipotent embryonal stem cells in culture, proliferation and differentiation of megakaryocyte progenitor cells, stimulation of new bone formation and calcium release from bones. Targeted disruption of the mouse LIF gene resulted in impaired implantation of blastocysts, but failed to lead to severe defects. This finding can be explained by the functional redundancy of IL-6-type cytokines. Targeted disruption of the specific ligand-binding chain for LIF, which is also used by OSM, CNTF and CT-1, resulted in mice expressing placental, skeletal, neural and metabolic defects and led to perinatal death. Mice without a functional gene for gp130, the signal transducer chain used by all members of the IL-6-type family, died shortly after midgestation. Finally, the lack of the main transcription factor STAT3, used by gp130-triggered signal cascades, resulted in very early embryonic death, demonstrating STAT3 to be a common and crucial endpoint of various receptor-mediated signals.

See also: Acute phase proteins; Hematopoietic stem cell transplantation; Cytokine genes, regulation of; Cytokines; Cytokine receptors; Interleukin 6; Knock-out, genetic.

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LEUKEMIA VIRUSES

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Leukemia is a cancer of blood-forming cells. It can be caused by viruses in humans (adult T cell leukemia) as in animals (mice, cats, cattle, birds, etc.). All known leukemia-inducing viruses belong to the subfamily Oncovirinae of the family Retroviridae. Other retroviruses include the lentiviruses, a subfamily of pathogenic slowviruses (the AIDS viruses, for example) and

the spumaviruses or foamy viruses that cause persistent infections without clinical disease.

The oncoviruses (Gr. *onkos*, mass, bulk) include viruses that infect target cells and lead to their transformation; that is, the virus creates a tumor-producing potential in infected cells. Transformation can occur as an acute phenomenon; it then leads to polyclonal

tumors (like myelomonocytosis induced in the chicken by the avian leukemia virus, MC29). When transformation occurs as a chronic phenomenon, it is a very rare event (1 cell out of 10^{10} – 10^{15} for example). Tumors that develop in the animal are mono- or oligoclonal. They derive from a single (or very few) transformed cell(s).

Acutely transforming retroviruses carry *v-onc* sequences derived from the host proto-oncogenes (*c-onc* sequences).

Conditional and nonconditional mutations affecting oncogenic properties of the virus have been mapped to the *onc* sequences. They code for all or part of the transforming protein; they are not involved in virus replication and are, in most cases, devoid of intron sequences. The latter observation suggests that the recombination event that leads to integration of cellular information occurred as a postsplicing event of the RNA transcript. It could be that recombination took place at the reverse transcription step by a copy choice mechanism. As a consequence, the *onc* information replaces information needed for viral replication and all acutely transforming leukemia viruses known today are replication-defective. They need a nondefective helper for propagation and have been considered as laboratory curiosities rather than widespread pathogens. All however, were isolated from the field.

Nonacutely transforming viruses (inducers of leukemia after a long latency period) carry the information necessary for virus replication. Their oncogenicity is due either to their site of integration in the target cell (avian leukosis virus, ALV; Moloney leukemia virus, MoLV) or to the activity of transactivating proteins that strongly activate virus replication but, at the same time, perturb regulatory circuits that control cell division and/or differentiation (human T lymphotropic virus type I; bovine leukemia virus).

The clinical experience in humans and the experimental data obtained in animals indicate that the development of leukemia is a multistep process. One thus expects that oncogene activation via viral infection is only one step in a cascade. In acute transformation, activation of the whole cascade is a short-duration process. In contrast, infection by nonacutely transforming viruses is supposed to only affect minor determinants of the neoplastic process. A long chronic stimulation is required before overt leukemia appears. Moreover, there are grades in the level of oncogenicity achieved by a given transformed cell. Indeed, DNA transfection experiments suggest that some oncogenes are not able to induce complete neoplastic transformation. However, frank oncogenicity can be achieved if two oncogenes are introduced in the same cell, acting in concert, presumably expressing complementary functions.

Viral carcinogenesis studies have amply demon-

strated that mechanisms leading to cancer include the following five processes:

1. Overexpression of a viral *onc* gene.
2. Capture of a proto-oncogene (*c-onc*) in a retroviral vector and appearance of mutations in the captured gene that make it highly oncogenic (*v-onc*).
3. Combination of two cooperating *v-oncs* in the same provirus.
4. Cooperation between a viral protein and activation of a *c-onc*.
5. Reorganization of the cellular transcriptional processes by a viral transactivation protein.

Overexpression of a viral *onc* gene

In this case, *v-onc* and *c-onc* have identical sequences; *v-onc* is overexpressed due to its viral regulatory environment. Examples include insertion of *myc* in the avian leukemia virus MC29, and in the feline leukemia virus. An analogous state of overexpression can be created if a provirus is inserted upstream of *c-onc* and serves as a strong promoter via its 3' LTR (long terminal repeat). Such a situation is known as 'downstream promotion' of expression. Examples of this situation include a majority of lymphomas induced by Moloney murine leukemia virus and a minority of the chicken lymphomas induced by RAV-1 or ALV.

The Rous-associated virus type 1 (RAV-1), a helper virus that does not carry any oncogene, induces overexpression of *myc* and B cell lymphomas in chickens of the SPAFAS genetic background. The same helper promotes high expression of *erbB* and induces erythroblastic leukemia in chickens of the line 15 I. Obviously host-virus interplay is fundamental in the type of neoplasm induced by the incoming virus. In most cases of downstream promotion, the integrated provirus has lost the 5' part of its genome; transcription starts from the 3' LTR and extends over the neighboring proto-oncogene.

A similar case of overexpression occurs when the enhancing provirus gets inserted downstream of the activated *c-onc*, irrespective of the relative orientations of the provirus and the activated gene. This situation is known as 'regional activation' or 'proviral enhancement'. Regional activation can occur through chromosome breaks, translocations, deletions, insertions, amplifications caused by radiation, chemicals, or just by accident. Many examples of this situation have been described in human leukemia.

Capture of a proto-oncogene (*c-onc*) in a retroviral vector and appearance of mutations in the captured gene that make it highly oncogenic (*v-onc*)

Examples are numerous and include the *onc* genes *v-src* (Rous sarcoma virus), *v-myb* (avian myelo-

blastosis virus) and *v-abl* (Abelson murine leukemia virus). Expression of the encoded protein is a prerequisite to transformation of the target cell and maintenance of the transformed state. Animals that carry tumors caused by such viral agents develop antibodies to the *v-onc* protein (even though it derives from a normal cell constituent playing a role in cell proliferation or cell differentiation).

Combination of two cooperating *v-oncs* in the same provirus

The fact that two active oncogenes may cooperate to confer a growth advantage on the transformed cell is illustrated by the genetic structure of three avian acute leukemia viruses: AEV, with *erbA* (*c-erbA* is the receptor for thyroid hormone) and *erbB* (*c-erbB* is the receptor for EGF, epidermal growth factor); E26, with *myb* and *ets* (the functions of *c-myb* and *c-ets* are not well characterized yet); and MH2, with *myc* and *mil* (*mil* is homologous to the oncogene *raf*, found in the murine sarcoma virus 3611). The *myc*, *myb* and *erbB* genes have been identified separately in other viral isolates as the sole transforming gene. In each case, however, the virus with two oncogenes induces a more pronounced or advanced state of transformation than its counterpart with a single oncogene. In fact, refined experimental systems show that the six *v-oncs* – *myc*, *myb*, *mil*, *ets*, *erbA* and *erbB* – are all separately transforming. Their association in a given virus or their coexpression in a single target cell reinforces the transformed state as appreciated by characteristics such as requirement of specialized culture conditions, colony induction in semisolid medium, requirement for complex liquid medium for proliferation of cells explanted from colonies, and degree of spontaneous differentiation within colonies.

Cooperation between a viral protein and activation of a *c-onc*

The viral protein acts as a mitogen The Friend erythroleukemia is induced in mice by a virus complex containing the defective spleen focus-forming virus (SFFV) and a helper murine leukemia virus. The SFFV envelope gene encodes a membrane glycoprotein, gp55. When expressed at the surface of murine erythroblasts, gp55 is highly mitogenic. It has no effect when it has lost its transmembrane anchor. The first consequence of infection is therefore polyclonal erythroblastosis that leads, however, to cell differentiation and recovery unless the helper is present and contributes to the production of more and more virus particles. Among the numerous integration events, one might concern the Spi-1 site (Spi for SFFV provirus integration site; the Spi-1 product is a transcriptional factor), a site linked with immortalization and tumor

onset. Spi-1 is a presumptive oncogene. Enhanced transcription of Spi-1 follows SFFV integration in the vicinity. The Friend erythroleukemia system combines a mitogenic effect mediated via gp55 at the cell membrane and an insertional activation through enhanced transcription of Spi-1.

Overexpression of a growth factor Another model of cooperation between oncogenes uses *myc*-immortalized monocytes. The cells are superinfected *in vitro* with a CSF-1 (colony stimulating factor)-expressing retrovirus. The result is a strong promotion of tumorigenesis. In this simple model, only two oncogenic activations are required for monocyte/macrophage transformation: immortalization of the cells with *c-myc* and deregulation of the CSF-1 gene. However, not all mechanisms that result in loss of CSF-1 dependence lead to full tumorigenicity, suggesting that, *in vivo*, tumorigenesis may involve multiple secondary events including growth factor independence. Analogous experiments have shown that activation of the macrophage colony-stimulating factor (M-CSF) gene is a secondary event leading to the development of tumors in mononuclear phagocytic cells that have first been immortalized with the *v-fms* oncogene. (*fms* is the *v-onc* found in the McDonough strain of feline sarcoma virus; *c-fms* is the receptor for CSF-1).

Transactivation by a viral gene product

The human T-cell leukemia virus system

The human T-cell leukemia virus type I or HTLV-I is a retrovirus associated with the development of adult T-cell leukemia (ATL). ATL is a malignancy of mature CD4⁺ T-lymphocytes which occurs in 0.1 to 1% of seropositive individuals following prolonged periods of HTLV-I infection. HTLV-I infects CD4⁺ lymphocytes and is also able to transform primary lymphocytes in *in vitro* assays. The peripheral blood from patients with ATL contains transformed lymphocytes which derive from a dominant clone as evidenced by the clonal or oligoclonal integration sites of the HTLV-I provirus.

The mechanism of HTLV-I transformation is different from other retroviruses since HTLV-I provirus is not integrated at specific sites in the genome of the transformed lymphocytes and HTLV-I lacks a typical oncogene. However, HTLV-I codes for a transactivator protein, Tax, which is essential for viral gene expression and is also able to transform cells of both lymphoid and non lymphoid origin. The Tax gene has been implicated in HTLV-I induced leukemogenesis because it transactivates cellular genes involved in the control of T-lymphocyte proliferation, including the genes encoding interleukin 2 (IL-2) and its receptor IL-

2R α , GM-CSF, IL-3, IL-4 and IL-6, as well as the proto-oncogenes *c-sis* and *c-fos*. In addition, Tax immortalizes primary lymphocytes, transforms established rat cell lines and immortalizes primary rat fibroblasts in combination with an activated *ras* oncogene. Furthermore, Tax induces mesenchymal tumors in mice transgenic for the Tax gene and a leukemia of large lymphocytes when the Tax transgene is expressed under the control of a T-cell specific promoter.

Tax activates viral and cellular genes by interacting directly or indirectly with cellular transcription factors, including members of the cyclic AMP response element binding protein (ATF/CREB) and the NF- κ B/cRel families, the two related co-activators CBP and p300 as well as the serum response factor. Activation of HTLV-I gene expression involves direct interaction of Tax with specific members of the ATF/CREB family of transcription factors resulting in enhanced binding of these factors to the regulatory element consisting of three repeats of 21 base pairs in the HTLV-I LTR. This transcriptional regulatory complex subsequently recruits additional factors including the CREB binding protein CBP which bridges upstream transcription factors to the basal transcription machinery.

Tax induction of specific cellular genes including IL-2 and IL-2R involves the stimulation of the DNA binding properties of NF- κ B. NF- κ B is constitutively activated in Tax-expressing cells and in HTLV-I infected cells. This constitutive activation is due to Tax-induced lytic breakdown of I κ B- α which sequesters NF- κ B in the cytoplasm in the absence of specific inducers. Degradation of I κ B- α results in translocation of NF- κ B to the nucleus and subsequent activation of gene expression from specific promoters containing NF- κ B binding sites.

In HTLV-I transformed lymphocytes, Tax exhibits a cytoplasmic diffuse distribution in addition to a nuclear punctate distribution. The discrete nuclear structures containing Tax include components of both basal transcription complexes and splicing complexes, the ATF-01 member of the ATF/CREB family of transcription factors, the two subunits of NF- κ B p50 and RelA as well as both related coactivators CBP and p300. They also include mRNA encoded by a gene specifically activated by Tax suggesting that these nuclear structures are critical for Tax transcriptional and transforming activities.

The Bovine Leukemia Virus system

The Bovine Leukemia Virus (BLV) shares structural homologies with HTLV-I. BLV transforms bovine and ovine B lymphocytes and induces monoclonal leukemia/lymphoma (malignant lymphoma) in 100% of infected sheep with an average latency of 30 months. Only a very limited fraction ($\pm 5\%$) of infected cattle develop tumors, but with a very long latency of about

10 years. Tumor cells always harbor BLV proviral sequences. In sheep tumors, BLV proteins are not expressed, but some degree of expression is found in bovine tumors. It was not established, however, whether expression occurred in transformed cells or in non-tumorous cells, infected by the virus and present in the tumor tissue.

No p53 mutations were found in sheep tumor cells, indicating that this rare event is not required to induce full malignancy of the infected cell in sheep. On the contrary, p53 mutations were uncovered in 50% of BLV-induced bovine tumors. It was also observed that the rate of apoptosis is reduced by 50% in PBMCs of BLV(+) animals as compared to controls. This observation remained valid even in animals carrying a disabled, weakly propagating BLV.

The BLV Tax, R₃ and G₄ viral products play a major role in the transformation process of sheep B cells. Tax phosphorylation occurs at serines 106 and 293 and is required for Tax to be transforming. Mutation of these aa residues into Ala abrogates the transforming potential of Tax but maintains its transactivating capacity for the proviral promoter. BLV viruses deleted for R₃ and G₄ are still infectious but propagate very weakly in sheep. Ten animals infected for more than 5 years did not show any sign of tumor development whilst an average latency of 30 months before onset of tumor was observed in wild type BLV-infected sheep.

Such data assign a role to the regulatory genes tax, R₃ and G₄ in the induction of malignant lymphoma by BLV.

Leukemia viruses are examples of biological plasticity. They have been and still are seeding elements for impressive progress in cancer research. Identification of oncogenes has led to the assumption that onset of a tumor results from alterations of growth induced by lesions in the cell signaling system combined with lesions that perturb the biochemistry controlling the self-renewal and/or the differentiation potential of cells. The time sequence of events seems largely irrelevant, one oncogene can be associated with quite different types of tumors in different tissues. There are many ways that lead to a given end – all paths lead to Rome!

See also: Chromosome translocations of immune genes; Human immunodeficiency viruses; Leukemia; Lymphoma; Retrovirus, infection and immunity.

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LEUKOCYTE ADHESION DEFICIENCY

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Studies of genetic deficiency syndromes have provided important insights into the molecular basis and biology of leukocyte emigration. Both animal and human syndromes are well recognized.

Animal models

A spontaneous mutation in the gene encoding the β_2 -integrin has been described both in dogs and cattle. In the cattle model two point mutations were identified within the gene encoding bovine CD18. These animals suffered from recurrent infections and persistent mature neutrophilia associated with poor growth performance. The carrier frequency of the defective gene among Holstein cattle is approximately 15% in bulls and 6% in cows.

Using methods of homologous recombination in embryonic stem (ES) cells, it is possible to generate strains of mice deficient in specific adhesion molecules. A knockout mouse with CD18 deficiency was found to be viable and fertile, with mild granulocytosis. The mutant mice showed an impaired inflammatory response to chemical peritonitis and delayed rejection of cardiac transplants (Table 1).

In another model, ICAM-1-deficient mice were generated. The animals developed normally and had mild granulocytosis. Deficient mice exhibited abnormalities of inflammatory response especially in impaired neutrophil emigration. In addition, leukocytes from these mice provide negligible stimulation for the mixed lymphocyte reaction. In a recent study using the ICAM-1-deficient mice, mutant mice were resistant to the lethal effects of high doses of endotoxin, which correlated with a significant decrease in neutrophil infiltration in the liver. As the production of various inflammatory cytokines is normal in these mice, the protective effects appear to be related to a

diminution in critical leukocyte–endothelial interactions.

Mice lacking P-selectin develop normally. However, they exhibit striking leukocytosis, diminished rolling of leukocytes in mesenteric vessels and delayed recruitment of neutrophils to the peritoneal cavity after experimentally induced peritonitis. Mice deficient in L-selectin initially have normal levels of leukocyte rolling, but show a significant decline in rolling within the first hour. Furthermore, L-selectin knockout mice have a severe reduction in the number of lymphocytes localized in the peripheral lymph nodes. E-Selectin-deficient mice have a milder defect in neutrophil recruitment, and do not have leukocytosis. Administration of P-selectin-specific antibodies to the E-selectin knockout mice completely blocks neutrophil recruitment, suggesting that in this model some redundancy of function may exist between the two endothelial cell selectins.

Human Leukocyte Adhesion Deficiency (LAD) syndromes

The best way to appreciate the *in vivo* importance of the various adhesion molecules is by examining those rare ‘experiments of nature’ where a specific defect in an adhesion molecule exists. Currently two such syndromes have been described. In the first, LAD I, the integrin family is defective, whilst in the second, LAD II, sialyl Lewis^x, the ligand for selectins, is missing (Figure 1).

LAD I

This syndrome was described more than 10 years ago and is clinically characterized by severe life-threatening infections, chronic neutrophilia and impaired wound healing. Delayed separation of the

Table 1 Adhesion molecule knockout mice

Adhesion molecule	Viability	Leukocytosis	Rolling	Migration	Inflammation
ICAM-1	Good	+++	N	NT	Reduced
β_2 -Integrin (partial)	Good	++	N	Decreased	Reduced
E-Selectin	Good	–	NT	N	Normal
P-Selectin	Good	++	Reduced (early)	N	Reduced (1–4 h)
L-Selectin	Normal	–	Reduced (later)	N	Reduced (6–8 h)
VCAM-1	Nonviable				

N, normal; NT, not tested.

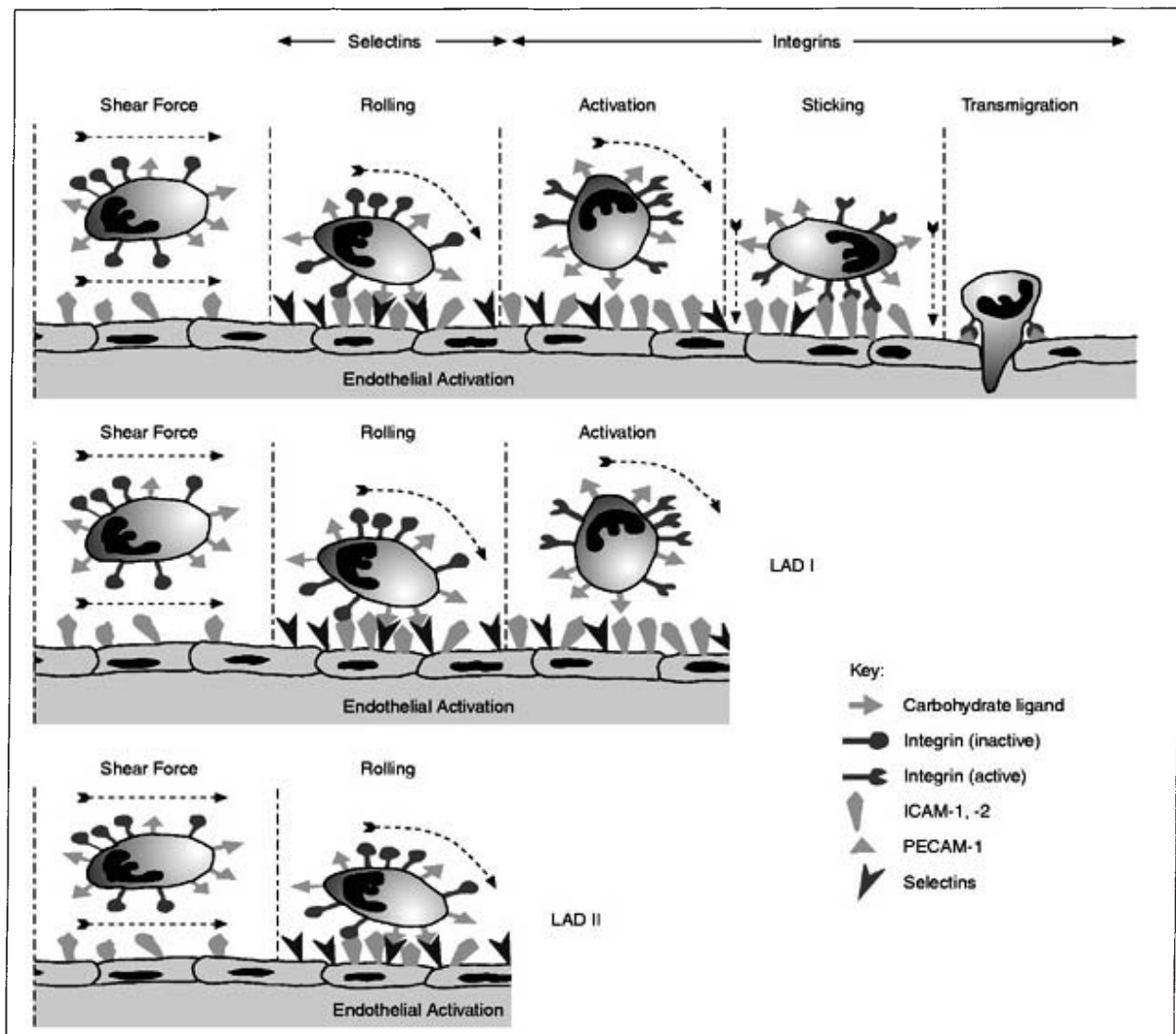


Figure 1 The adhesion cascade: defects in LAD I and II.

umbilical cord, chronic gingivoperiodontitis and lack of pus formation complete the clinical picture. The syndrome is a result of a defect in the expression of the β_2 subunit (CD18) of the integrin molecule. Heterogeneous mutations in the common β_2 chain splicing, frameshift, missense, and initiation codon have been demonstrated to be the molecular basis of LAD I. As a consequence, neutrophils are unable to emigrate from the blood vessels to the tissue since adhesion and transmigration are severely impaired. Biopsies from infected lesions in these patients demonstrated inflammatory infiltrates totally devoid of neutrophils. This histopathologic feature is particularly striking considering that marked peripheral granulocytosis (5- to 20-fold higher than normal) is a constant finding. Although neutrophil motility is the major defect, cellular and humoral immune func-

tion can also be abnormal, but its *in vivo* significance is still unclear.

Clinically, LAD I has been divided into two groups: a severe phenotype and a moderate one. In the severe form no expression of CD18 can be demonstrated and the patients will suffer from life-threatening infections and will die early in life if definite therapy is not instituted. In the moderate form some surface expression (around 5%) exists and these patients have fewer serious infections and survive into adulthood. While antibiotics have some beneficial effect, the definite approach to the treatment of the disease is by bone marrow transplantation which was found to be very successful in LAD I. Since the syndrome is a monogenic disorder involving hematopoietic cells, it is obviously an attractive candidate for curative treatment by gene therapy.

LAD II

This new adhesion molecule deficiency, which was described in 1992, is also characterized by recurrent infections, no pus formation, gingivitis, and pronounced neutrophilia. The severity of the infection resembles the moderate form of LAD I (Table 2). The syndrome is also manifested by mental retardation, growth retardation and exhibits the rare Bombay blood group phenotype. LAD II is due to a congenital defect of endogenous fucose metabolism. This results in an inability to synthesize fucosylated carbohydrate molecules, such as sialyl Lewis^x, the ligand for selectins, on the neutrophils. Neutrophils from LAD II patients express normal levels of β_2 -integrins and L-selectin, but lack sLe^x or other fucose-containing surface antigens. Neutrophils from these patients do not bind to E-selectin expressed on cytokine-activated cultured endothelial cells, or to purified E- or P-selectin, and exhibit a marked decrease in migration to skin chambers or a skin window *in vivo*. Nonetheless, under static conditions the cells adhere normally through integrin-ICAM interactions and can also migrate across endothelial monolayers.

Compelling evidence in support of the current paradigm of leukocyte-endothelial interaction was obtained by using intravital microscopy to study neutrophil behavior in cells from LAD I and LAD II patients. Fluorescein labeled cells were observed during interactions with venules in rabbit peritoneum treated with interleukin 1 to induce E-selectin. Neutrophils from LAD I patients showed normal rolling,

but were unable to stick and emigrate upon chemotactic stimulation. Neutrophils from LAD II patients rolled poorly, and failed to stick and emigrate under the shear forces provided by flow. However, when flow was reduced the cells adhered and emigrated in response to a chemoattractant. This study clearly showed that selectin-carbohydrate ligand interaction initiates low-affinity adhesion, and subsequent activation induces engagement of integrins with endothelial immunoglobulin-like ligands promoting firm adhesion and emigration into the tissue.

See also: Adhesion molecules; Lewis^x/sialyl-Lewis^x (CD15/CD15s); Endothelium; Immunodeficiency, primary; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Knockout, genetic; Neutrophils; Selectins (CD62-E/L/P).

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Table 2 Leukocyte adhesion deficiency syndromes

	LAD I	LAD II
<i>Clinical manifestation</i>		
Recurrent severe infections	+++	+
Neutrophilia		
Basal	+	+++
With infection	+++	+++
Gingivitis	++	++
Skin infection	++	+
Delayed separation of umbilical cord	+++	–
Development abnormalities	–	+++
<i>Laboratory findings</i>		
CD18 expression	!!!	NL
sLe ^x expression	NL	Absent
Neutrophil motility	!!!	!!!
Neutrophil rolling	NL	!!!
Neutrophil adherence	!!!	↓
Opsonophagocytic activity	↓	NL
T + B cell function	↓	NL

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LEUKOCYTE CULTURE

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The last 10–15 years have seen an explosion of new therapeutic approaches, mostly in the oncologic field. The agents developed are blood-derived cells either intrinsically endowed with direct and/or indirect antitumor activity (such as lymphokine-activated killer (LAK) cells, tumor infiltrating lymphocytes (TILs) and dendritic cells (DC)) or developed for hematopoietic cell transplantation purposes (such as CD34⁺ precursors) after high-dose chemo/radiotherapy protocols. These therapies offer promising novel approaches to cancers that have resisted initial treatment protocols as well as treatment opportunities for patients afflicted with the acquired immune deficiency syndrome (AIDS), other immunodeficiencies and genetic disorders. New technologies have therefore needed to be developed for the large-scale expansion of leukocytes to be used as biological therapeutics.

Classically, small- to medium-scale leukocyte cultures (up to 10^9 cells and for research laboratory purposes only) are performed in sterile containers (consisting of polystyrene flasks or Petri dishes) in various commercially available media (the most commonly used for leukocyte cultures being Roswell Park Memorial Institute (RPMI) 1640 and Iscove's modified Dulbecco's medium (IMDM)) supplemented with either heat-inactivated fetal bovine serum (FBS) or human AB serum, antibiotics and, when indicated, specific growth factors (such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), IL-2, etc.). All cell manipulations are performed under laminar flow hoods using sterile supplies and the flasks are kept in humidified 37°C incubators with 5–10% CO₂, depending on the type of leukocyte culture. In such conditions, both cell growth and viability can be checked easily by direct visualization of the culture flask under an inverted microscope.

Using conventional polystyrene flasks for large-scale expansion of blood-derived cells for clinical use (in this case, the cell numbers can rise up to 10^{11} with culture volumes in the range of liters) creates

obvious problems, both at the logistic level (i.e. laboratory space, number of laminar flow hoods, and incubators needed, etc.) and at the practical level (since the handling of these cultures may require a high number of specially trained personnel). Furthermore, because the cultured cells are to be reinjected into humans, important safety issues need to be considered such as insuring that the whole culture process is performed under sterile conditions (also referred to as good manufacturing procedures or GMP), minimizing contamination with microbial and other components (i.e. sera and/or antibiotics) whose presence may induce severe side-effects in humans.

These requirements have led in the last few years to the development of new culture technologies that have moved the traditional culture process from a human-based, rather artisanal level, to a sophisticated industrial level in which the 'human-work' component has been maximally limited. These new culture systems are presently used by various laboratories involved in cellular therapy studies. The aim of this entry is to give to the reader an overview on the latest technologies used for large-scale expansion of leukocytes for clinical use and their major medical applications. However, it is important to keep in mind that this field, boosted by the promising results obtained in different cellular therapy applications, is in continuous and fast development; new products with improved features are regularly appearing on the market with the ultimate goal of allowing the production of the largest possible number of clinical-grade blood-derived cells at the lowest possible price.

Culture media

Historically, mononuclear blood cells have always been cultured *in vitro* in a medium (usually RPMI 1640 or IMDM) containing serum as a source of undefined growth supporter. FBS and pooled human AB serum are routinely used in this system. However, cells intended for clinical use should preferably not be cultured with animal or human sera due to

possible viral transmissions and side-effects, including patients' anaphylactic reactions caused by the presence of xenogeneic/allogeneic proteins remaining on the cell surface. Therefore, when possible, serum-free culture media should be used for cells intended for patient reinfusion. In response to this need, different companies have developed serum-free media (Table 1) specifically designed to support the *in vitro* growth of T cells, such as LAK cells and TILs, or hematopoietic progenitors, such as CD34⁺ cells. In addition, the media used for *in vitro* expansion of blood-derived cells for reinjection into humans must be endotoxin-free (endotoxin units (EU) <0.05 per ml) and, if possible, antibiotic-free. When the addition of antibiotics is required, preference is to be given to antibiotics for which the risk of allergic reactions is low (such as gentamycin sulfate). Consequently, β -lactamic antibiotics (e.g. penicillin) will not be used.

Growth and/or differentiation factors (such as GM-CSF, G-CSF, IL-3, stem cell factor (SCF) for hematopoietic progenitors, IL-2 for LAK cells and TILs, IL-4, GM-CSF and tumor necrosis factor α (TNF α) for dendritic cells) are usually added to the culture media to support the growth and/or potentiate the biological functions of these blood-derived cells. Only commercially available and purified recombinant human growth factors can be used in clinical-grade cultures. Conditioned media containing 'growth factor activities' cannot be used to grow cells for use in humans.

Tissue culture vessels

As described above, traditionally, cultures of blood-derived cells have been performed in polystyrene flasks of different sizes (ranging from 25 to 225 cm²). Although flasks represent an excellent tissue culture vessel for most leukocytes growing either in suspension (such as lymphocytes) or adhering to plastic surfaces (such as macrophages and dendritic cells) and allow monitoring of cell growth by visual inspection under an inverted microscope, they can hold logistic

and practical problems when the number of cells to be cultured becomes very high (>10⁹ cells). These problems include incubator space (Figure 1), time and support personnel required for cell manipulations, and high risk of microbial contaminations. In this latter respect, cell culture bags represent an improvement over flasks by providing a closed system from the time the cells are initially collected from a patient/donor, cultured and then reinfused into the patient. Moreover, the bags are single-use vessels, thus preventing any possibility of patient sample cross-contamination, and can be easily disposed of after use. More importantly, they greatly facilitate the handling of large-scale cultures since a single technician can easily manage up to 50–100 l of tissue culture at a time.

The two companies producing commercial cell

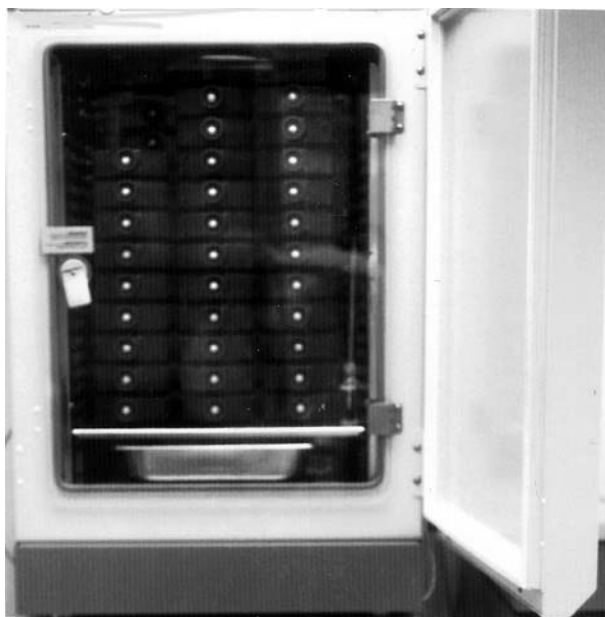


Figure 1 Large-scale expansion of leukocytes in T-175 polystyrene flasks. A standard-size incubator can hold an average of 64 flasks containing a maximum of 150 ml of cell culture each (total capacity per incubator – 9.6 l of cell culture and about 10¹⁰ total cells).

Table 1 Serum-free media for leukocyte growth

Medium	Manufacturer	Applications
AIM-V	Gibco, Grand Island, NY	LAK cells and TILs
X-VIVO-10	BioWhittaker, Walkersville, MD	LAK cells and TILs
HB-LAK	DuPont, Wilmington, DE	LAK cells
HL-1	Ventrex, Portland, ME	LAK cells and TILs
X-VIVO-15	BioWhittaker, Walkersville, MD	Hematopoietic progenitors
Progenitor-34	Life Science Tech., Gaithersburg, MD	CD34 ⁺ progenitors
QBSF-59	Quality Biologicals, Gaithersburg, MD	Hematopoietic progenitors

culture bags are Baxter Healthcare Corp. (Immunotherapy Division, Irvine, CA) and American Fluoroseal Corp. (Silver Spring, MD). Baxter cell culture bags are sold under the trade names of Lifecell and Cryocyte. American Fluoroseal provides the Teflon cell culture bags. They come in different sizes ranging from 50 to 3000 ml maximum bag capacity (with culture volume ranging from 100 to 1500 ml, respectively). Importantly, Lifecell culture bags can be used in conjunction with the Lifecell solution transfer pump (Figure 2), thus allowing a drastic

reduction in the risk of cell contamination, biohazard exposure, feeding time and occupied incubator space. Finally, culture harvesting is also facilitated by direct centrifugation of the cells in the bags using specially designed bag holders that fit into large centrifuge cups, such as the ones used in Sorvall centrifuges.

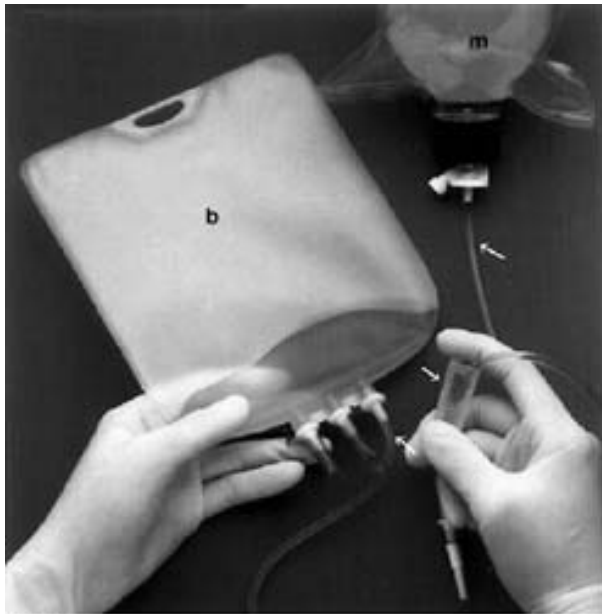
A number of factors need to be considered in the use of plastic cell culture bags:

1. The kinetics of cell growth varies with each cell type, creating specific requirements for culture medium components and/or oxygen and carbon dioxide.
2. Bag surface properties will affect growth. Adherent cells cannot adhere to gas-permeable plastic bags so they cannot be used to grow, for instance, dendritic cells.
3. Gas permeability is a critical factor. Oxygen limitation can occur in culture bags in which the material is not sufficiently gas permeable and the proper feeding schedule is not maintained. Currently, a limited number of bag materials are available that provide the appropriate necessary gas exchange for cell culture. However, depending upon the cell being cultured, an increase in cell proliferation can be achieved by simply altering the incubator gas conditions (10% carbon dioxide and 30% atmospheric oxygen have been shown to be the best gas conditions for LAK cells and TILs grown in Lifecell bags).
4. Appropriate volumes and cell surface ratio should be used. Using the optimal culture medium depth is important for gas diffusion and delivery of oxygen to the cells. In this respect, one should consider that the nominal bag size is usually much greater than the optimal culture volume. Bag cell cultures can be initiated at low volumes and fed during the culture period by simply adding more culture medium and recombinant growth factors to the bags.

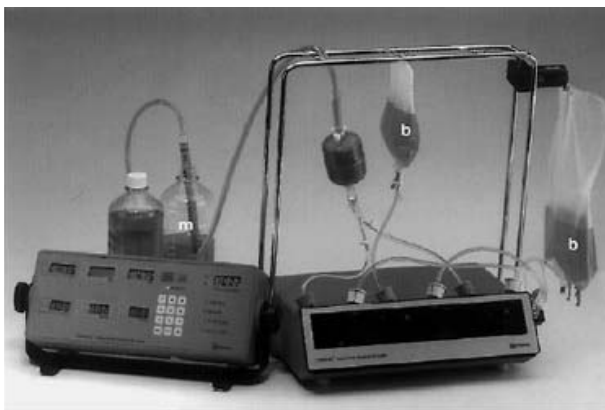
Finally, a disadvantage of cell culture in gas-permeable culture bags is the impossibility of visually checking the growth and viability of the cells while they are inside the bag (Table 2). Periodic sampling of the cultures is therefore needed to monitor their growth.

Tissue culture bioreactors

Currently, two different kinds of bioreactors are available. These bioreactors have been proven by different investigators to grow cells effectively (including blood-derived cells) to clinically useful



(A)



(B)

Figure 2 Large-scale expansion of leukocytes in gas-permeable cell culture bags. Bags can be filled up manually (A) using a gravity transfer set (arrows) or using a solution transfer pump (B). An incubator can hold an average of 30 bags containing a maximum of 1500 ml of cell culture each (total capacity per incubator = 45 l of cell culture and about 2.5×10^{11} cells). b, gas-permeable cell culture bag; m, medium.

Table 2 Comparison of polystyrene flasks versus gas-permeable bags as tissue culture vessels for large-scale leukocyte expansion

	Flask	Bag
Surface area ^a	25–225 cm ²	60–750 cm ²
Culture volume	10–150 ml	20–1500 ml
Cell type	Adherent and suspension	Not indicated for cells that need anchorage
Visual inspection	Possible	Not possible
Risk of contamination	High	Low
Feeding time	Long	Short
Incubator space	Large	Small
Cell harvesting	Laborious/long	Easy/quick

^aSurface area refers only to one side of the vessel.

quantities while significantly reducing cost, time and space requirements. They include the 'hollow fiber cell culture bioreactors' (namely Cellmax 100, Celco Advanced Bioreactors, Kensington, MD) and the 'rotary cell culture system' (Synthecon Inc., Houston, TX). Both systems provide a three-dimensional growth environment that mimics the parental tissue structure and function (Table 3).

Hollow fiber cell culture bioreactors

These bioreactors allow *in vitro* cell growth within artificial capillary beds. As shown in Figure 3, they consist of a standard glass medium bottle serving as reservoir, a stainless steel gear pump, an autoclavable hollow fiber cartridge (in which cells are cultured) connected with two side-port glass bottles (used for cell inoculation and harvesting procedures), medical grade silicon rubber tubing serving as gas exchanger to maintain the appropriate culture medium pH and pO₂, a stainless steel tray of sufficiently small dimensions to enable two such systems to fit within a standard tissue culture incubator (Figure 3), and a pump motor magnetically coupled to the pump that can be lifted from the system when it needs to be removed from the incubators. The pump speed and flow direction are determined by an electronic control unit which sits outside the incubator and is connected to the pump motor via a flat ribbon cable which passes through the gasket of the incubator door.

Cells are inoculated into the extracapillary cartridge space and settled out of the capillary surface. Tissue culture medium, drawn from the reservoir, is pumped through the hollow fiber lumen; the cells are subsequently nourished within the artificial capillary network, in which nutrients and oxygen in the circulating medium readily diffuse through the capillary walls (available in different pore sizes). Metabolic waste products similarly diffuse away from the cells and are diluted in the circulating medium. Because of their high molecular weight, secreted growth factors, which are often cell-specific and feedback-regulated within the sphere of influence of each cell, are preserved within the extracapillary space, thereby maintaining a stable environment similar to the *in vivo* physiological conditions.

In many applications, the combination of physiological concentrations of nutrients, oxygen, growth factors and physical support, and low levels of metabolic waste enable the cells to fill the entire extracapillary space, forming nearly solid cell masses within the network of artificial capillaries. In artificial capillary cell culture systems (as in the bag culture system), the growing cells cannot be visualized within the cartridge capillary bundle. However, useful cellular growth data can be generated by monitoring the depletion of the medium carbon source and the increase in catabolic products (Figure 4). Such measurements are a direct window into the cell

Table 3 Characteristics of different leukocyte culture systems

Culture system	Shear stress	Mass transfer	Dimensionality	Colocation of dissimilar cell types	Cell density (cells ml ⁻¹)
Static (flasks/bags)	None	Adequate 2D	3–4 layers 2D	Limited	0.3–1 × 10 ⁶
Roller bottles	Moderate	Good 2D	3–4 layers 2D	Very limited	1–5 × 10 ⁶
Hollow fiber	None	Good	3D	Limited	10 ⁷ –10 ⁸
NASA bioreactor	Very low	Good	Excellent 3D	Good	10 ⁷ –10 ⁸

2D, two-dimensional; 3D, three-dimensional.

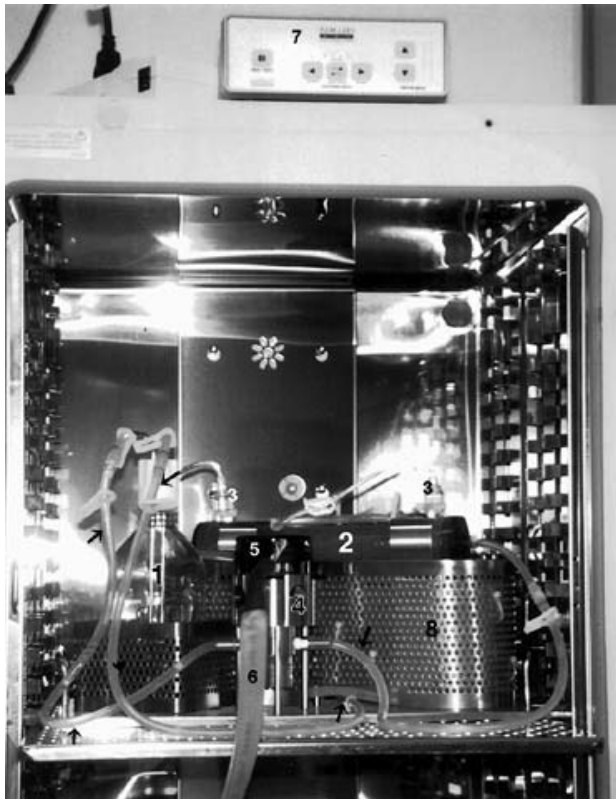


Figure 3 Large-scale expansion of leukocytes in hollow fiber cell culture bioreactors. An incubator can fit two bioreactors. Each bioreactor can maintain up to 10^{11} cells. 1, medium bottle; 2, hollow fiber cartridge; 3, side-port connections to glass bottles for cell inoculation and harvesting; 4, pump; 5, pump motor magnetically coupled to the pump; 6, flat ribbon cable connecting the pump to an electronic control unit (7) which sits outside the incubator and determines pump speed and flow direction; 8, stainless steel tray.

physiology: in fact, glucose consumption and concomitant lactate production are both directly related to cell growth.

Daily maintenance of an extremely high cell number growing in a cartridge is reduced to: 1) daily monitoring of the lactate/glucose concentrations in the reservoir bottle, and 2) cell feeding by simply changing the medium bottle when the medium has become depleted. This refeeding procedure requires approximately 5 min. The extrafiber space is drained periodically to either harvest the culture medium, presumably enriched in nondiffusible products secreted by the cells, or to evaluate the cell number and functional characteristics. This is accomplished in a laminar flow hood by draining the extrafiber fluid by gravity into one of the two side-port bottles. This procedure requires 20–30 min. A single Cellmax 100 cartridge can maintain up to 10^{11} cells, equivalent to 400–1000 T-175 polystyrene flasks. In fact, Cellmax 100 bioreactors have been successfully used by different laboratories for expansion of LAK cells,

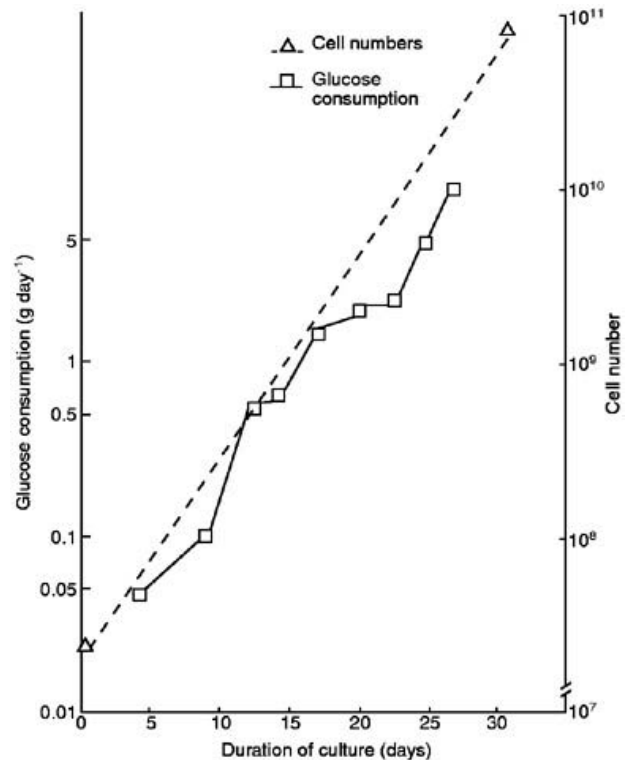
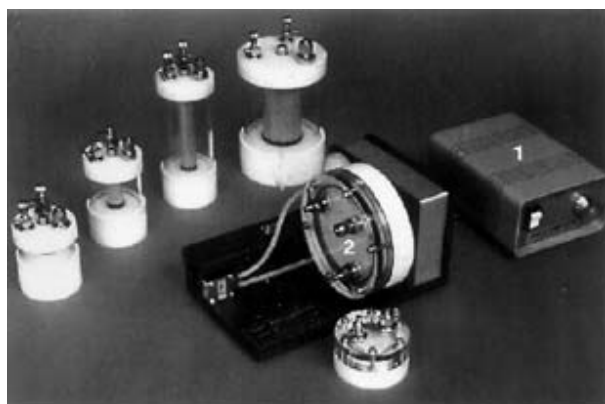


Figure 4 Typical glucose consumption (g day^{-1}) curve of a hypothetical leukocyte culture in hollow fiber cell culture bioreactor showing logarithmic increases over time.

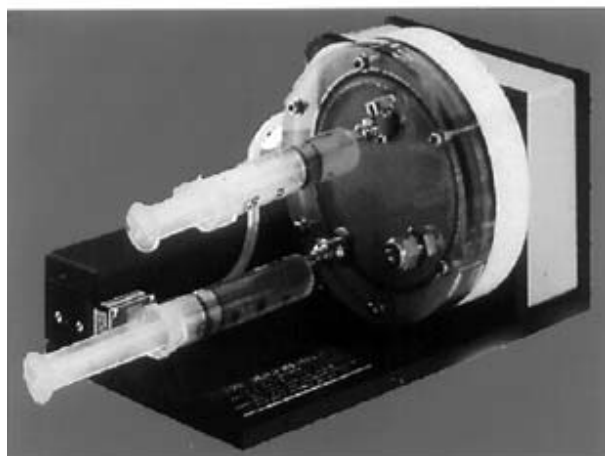
TILs and, more recently, CD34⁺ cells for clinical use (see below).

Rotary cell culture system

These bioreactors are based on principles derived from microgravity research (Figure 5). Most culture systems address one specific parameter, e.g. shear force, at the expense of others, such as mass transfer of nutrients and metabolic wastes, three-dimensionality, and/or cocultivation of dissimilar cell types. The rotary cell culture systems are the first bioreactors designed to simultaneously integrate cocultivation, low shear, high mass transfer, and three-dimensional growth without sacrificing other parameters (Table 3). The microgravity-based bioreactor was developed at the NASA-Johnson Space Center (Houston, TX) and is a zero-head space, aqueous medium-filled bioreactor suspending particles by rotating the vessel wall and integral gas diffusion membrane around the horizontal axis. This rotation can hold particles up to 1 cm diameter in orbital suspension, as the sedimentation forces induced by gravity are balanced by the centrifugal force generated by the vessel rotation. Nonadherent cells may be cultured in suspension while adherent cells are grown on microcarriers. In its simplest form,



(A)



(B)

Figure 5 Rotary cell culture system (NASA bioreactor). (A) Machine disassembled to show the individual components: 1, electronic control unit; 2, rotary system; different size vessel units (white) are also shown in this picture. (B) Machine assembled.

the system has a capacity of 125–500 ml (Figure 5A) and can support the cultivation on microcarriers of masses up to 1 cm diameter and cell concentrations of at least 10^7 cells ml^{-1} . The rotary cell culture system has a wide range of cellular and clinical research applications, including cancer research, *in vitro* toxicology testing and tissue engineering.

The cell types grown in the rotary cell culture system are listed in Table 4. Although lymphocytes to be used in adoptive immunotherapy have been reported by several laboratories to grow successfully in these bioreactors, the major advantage of this cell culture system over the ones described above is the possibility of growing differentiated three-dimensional tissues, thus resembling the parental tissue structure and function more closely than any other *in vitro* culture system currently available.

Table 4 Cell types grown successfully in the rotary cell culture system

<i>Suspension cultures</i>	<i>Adherent cultures</i>
Human lymphocytes	Human keratinocytes
Human hepatocytes	Human breast cancer
Mouse BM stem cells	Human prostate cancer
Human leukemic cells	Human lung cancer
	Human melanoma
	Human skin fibroblasts
	Human melanocytes

Clinical applications

Generation of LAK cells and TILs for use in adoptive immunotherapy

Adoptive cellular immunotherapy offers an alternative therapy for cancer patients who become unresponsive to conventional treatment protocols. The identification and expansion of lymphoid subpopulations able to mediate tumor regression upon adoptive transfer in the tumor-bearing host is an important tumor immunology goal. In the past 10 years, a great deal of attention has been focused on LAK cells generated by expansion of patients' lymphocytes (collected by multiple leukopheresis) in recombinant human (rh) IL-2 for 3–5 days. *In vitro*, these killer cells are capable of lysing a variety of autologous and allogeneic fresh tumor targets in an MHC nonrestricted fashion while sparing cells from normal tissues. Upon adoptive transfer in conjunction with systemically administered rhIL-2, LAK cells were proven effective in clinical trials by reducing metastatic tumor burden in cancer patients. A major problem in these therapies has been the *in vitro* activation of large number of peripheral blood lymphocytes (up to 2×10^{11} cells) obtained from successive leukopheresis. LAK cells for clinical trials have been successfully cultured in polystyrene flasks and roller bottles and, more recently, in large size (up to 3000 ml) gas-permeable culture bags using serum-free media formulations (AIM V and X-VIVO) and high doses (up to 1000 U ml^{-1}) rhIL-2.

TILs are lymphoid cells infiltrating solid tumors, which can be grown *in vitro* with rhIL-2. Theoretically, TILs have been primed against the autologous tumor by prolonged *in vivo* exposure and have a 50–100-fold increased ability to induce tumor regression, as compared to LAK cells. Their lack of efficacy in eliminating the tumor *in situ* is probably due to their insufficient numbers, and/or local tumor-mediated immunosuppression. However, TIL preparation, even using new technologies, is still cumbersome: classically, tumors are enzymatically digested

into single tumor cells and an average of 15% lymphocyte suspensions. The mixture is then cultured in polystyrene flasks with AIM-V or X-VIVO 10 media in the presence of high doses of IL-2 (1000 U ml^{-1}) leading to selective outgrowth of TILs with concomitant tumor cell demise. When the cell cultures reach about 0.5 l, they are usually transferred to culture bags (LifeCell or Teflon) which are incubated in a flat position on perforated shelves without agitation for 3–5 days in 37°C humidified 10% CO_2 /air incubators. Recently, in some laboratories, TIL suspensions harvested from culture bags at a 5×10^8 – 10^9 cell concentration are further expanded to high cell numbers (up to 2×10^{11} cells) required for human clinical injections using hollow fiber bioreactors. The rates of TIL growth in the gas permeable bags currently used and in the Cellmax 100 bioreactors are similar. Likewise, phenotypic and cytotoxic characteristics of TILs obtained from either culture method are not significantly different. However, the use of Cellmax 100 bioreactors is accompanied by an 80% reduction in the technician time and incubator space required, as compared to bag cultures.

Culture of CD34⁺ cells and *in vitro* generation of hematopoietic precursors for bone marrow reconstitution

Bone marrow transplantation (BMT) is increasingly used for the treatment of many malignant and non-malignant disorders. The use of autologous bone marrow cells has the advantage of avoiding the immunological complications of allogeneic BMT, but has the disadvantage of requiring the use of bone marrow, which potentially contains neoplastic cells from patients who have also undergone extensive chemotherapy. These complications may lead, on the one hand, to slow and incomplete marrow recovery and, on the other hand, to higher risk of relapse. Recently, cell-processing systems have been developed for the purification of bone marrow and peripheral blood-derived stem cells used for hematopoietic reconstitution following high-dose chemotherapy treatment protocols. Several clinical studies comparing traditional BMT and CD34⁺ stem cell reconstitution showed for the latter treatment reduced infusional side-effects while maintaining equivalent time for neutrophil engraftment. In this respect, however, following high-dose chemotherapy protocols, a severe neutropenia always develops in spite of reconstitution using autologous stem cell transplantation.

This neutropenia is the leading cause of morbidity and mortality from infections, requiring inpatient supportive care and contributing to the high therapy cost. The use of hematopoietic growth factors has

reduced but not eliminated this period of leukopenia. Studies have demonstrated that the use of peripheral blood stem cells mobilized by chemotherapy and/or growth factors gives rise to faster hematologic recovery than BMT. However, the duration of the severe neutropenia after these transplants is always at least 7 days, irrespective of the method of mobilization or the number of hematopoietic progenitor cells infused. This 7 day interval is now seen as the minimum necessary time for transition from mobilized stem cells to band/mature granulocytes. A solution to further decrease or eliminate this neutropenia is to generate *in vitro* cells capable of resolving the neutropenia.

Several investigators have examined *in vitro* expansion and maturation of hematopoietic precursors in liquid cultures originating from enriched CD34⁺ cell preparations stimulated with IL-3 and/or GM-CSF or G-CSF, or SCF either in gas-permeable plastic bags or in hollow fiber bioreactors. These studies have shown that enriched CD34⁺ precursors can proliferate and differentiate *in vitro*. However, there is not yet certainty that the duration of neutropenia can indeed be shortened by infusion of progenitor cells cultured *in vitro* in any of the listed conditions.

Culture of dendritic cells for use in active immunization

Dendritic cells (DCs) are potent antigen-presenting cells specifically involved in the initiation of antigen-specific immune responses. Because of their potent costimulatory activity, they are well suited to prime naive T cells toward various antigens such as viral peptides or alloantigens. The bone marrow origin of DCs has been shown in transplantation studies and recently described using cord blood and CD34⁺ peripheral blood progenitors *in vitro* in conjunction with a cytokine cocktail (SCF, EPO, IL-1 β , IL-3, IL-6, and GM-CSF) with either TNF α or IL-4. The recent possibility of growing relatively large numbers of these cells in culture (1×10^6 precursor cells can be expanded up to 5×10^7 mature DCs) provides a potent source of antigen-presenting cells from individual patients for vaccination protocols against infectious or tumor-associated antigens.

See also: Hematopoietic stem cell transplantation; Cell separation techniques; Cytotoxic T lymphocytes; Dendritic cells; Immunotherapy of tumors; Lymphokine-activated killer (LAK) cells.

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LEWIS^x/SIALYL-LEWIS^x (CD15/CD15S)

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With the advent of the hybridoma technology, researchers from many areas of biology and medicine embarked on programs of monoclonal antibody production in order to identify functionally important antigens that appear at specific stages of embryonic development, cellular differentiation, and in oncogenesis. It is remarkable that the blood group-related antigen, Lewis^x (Le^x, **Figure 1**), featured in all three of these contexts. Moreover, following these assignments, a fascinating story has unfolded with assignments of important roles to Le^x and related oligosaccharide sequences on glycoproteins and glycolipids as ligands for the selectins. These are the leukocyte-endothelium adhesion molecules which have crucial roles at the initial stages of leukocyte recruitment in inflammation.

Murine stage-specific embryonic antigen 1 (SSEA-1) is Le^x

In the search for embryonic antigens with biological roles, antibodies were raised to the murine teratocarcinoma line, F9, as a representative of relatively undifferentiated embryonic cells. A stage-specific embryonic antigen, SSEA-1, was detected by hybridoma antibody anti-SSEA-1. On the embryo, the antigen appears at the 8-cell stage coincident with the onset of compaction (the tight adhesion of the embryonic cells to one another). In the course of embryonic cell differentiation and development, expression of the antigen diminishes, and in the adult mouse its expression is restricted to limited areas of the nervous, reproductive and genitourinary systems. SSEA-1 was identified as the trisaccharide sequence consisting of fucose joined by α 1–3 linkage to the N-acetylglucosamine of a type 2 blood group backbone (galactose β 1–4 linked to N-acetylglucosamine), and borne on high molecular weight glycoproteins. This

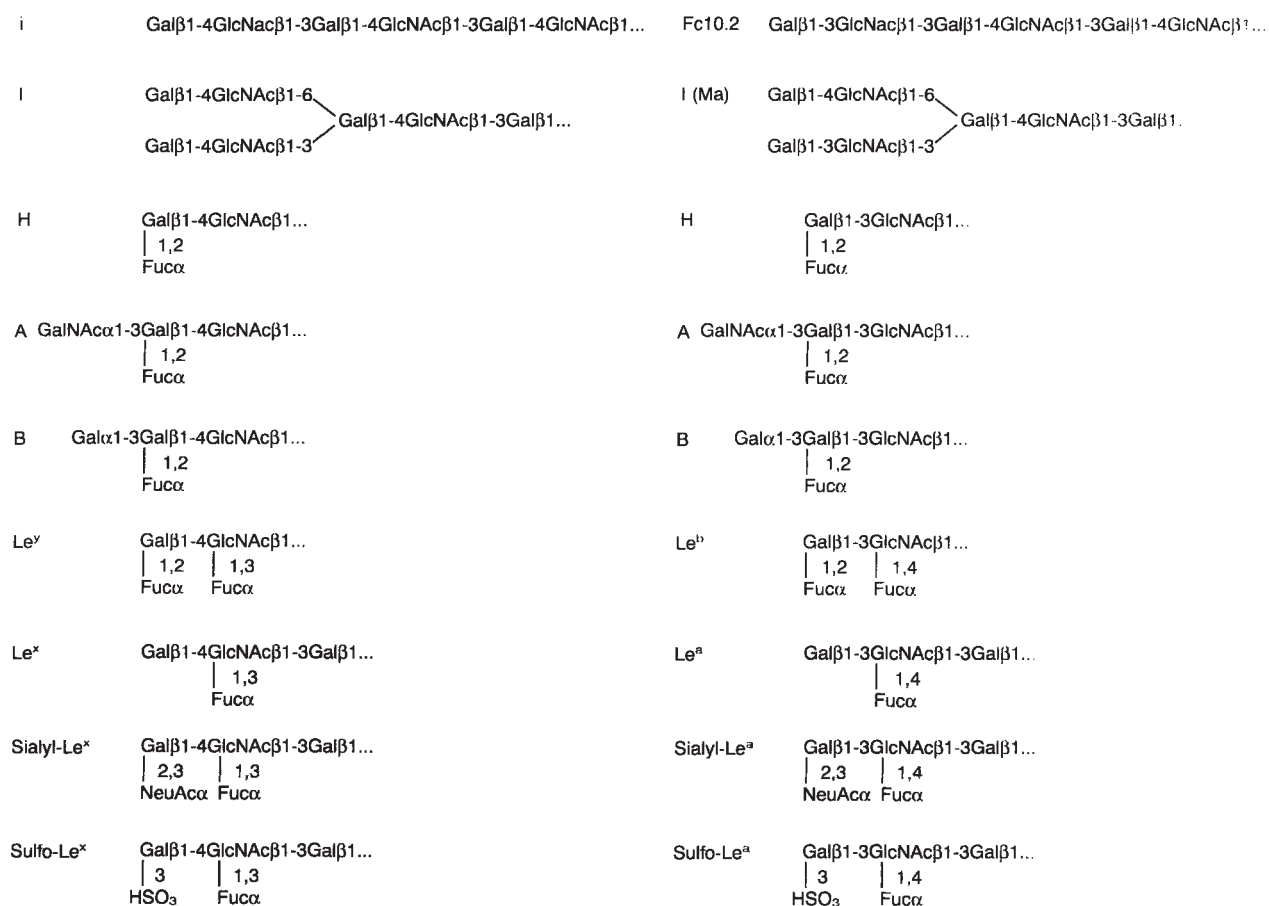


Figure 1 The inter-relationships between the carbohydrate antigens Le^x, Le^y, sialyl- and sulfo-Le^x (based on the type 2 backbone sequence Galβ1–4GlcNAc), their isomers Le^a, Le^b, sialyl- and sulfo-Le^a (based on type 1 backbone Galβ1–3GlcNAc), the i, I and Fc10.2 antigens, and the major blood group antigens H, A and B. The i and I antigenic determinants are expressed on nonsubstituted or accessible, repeating, type 2 backbone sequences, and Fc10.2 on a type 1 disaccharide backbone joined to a type 2 backbone. The 'hybrid' backbone structure with a type 2 and a type 1 branch expresses one of the I antigenic determinants, I(Ma), which consists of the Galβ1–4GlcNAcβ1–6Gal sequence. The major blood group antigens are expressed on either type 1 or type 2 backbones and on additional backbone families (not shown here). Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; HSO₃, sulfate.

structure termed Le^x, is an isomer of blood group Lewis^a (Le^a) which consists of type 1 backbone (galactose β1–3 linked to *N*-acetylglucosamine) which is α1–4 fucosylated at *N*-acetylglucosamine. Further fucosylation of Le^x and Le^a with fucose α1–2 linked to the terminal galactose constitute the Le^y and blood group Le^b antigens, respectively. The inter-relationships of Le^{x/y}, Le^{a/b} with the major blood group antigens H, A and B are shown in Figure 1.

The backbone sequences are antigens in their own right when they are not further glycosylated and are accessible. Thus, the repeated type 2 backbones in linear sequence express the i antigen, and when branched, the large I antigen (Figure 1). Rather ahead of the applications of hybridoma antibodies, the human monoclonal autoantibodies, anti-i and

anti-I, were used as sequence-specific reagents, and some striking changes were noted in the expression of these antigens during early murine embryogenesis, I being the predominant antigen in the early embryo, and i appearing coincident with the first differentiation event in the embryo, i.e. the formation of the primitive endoderm. From the observations with the monoclonal antibodies, together with earlier knowledge that marked changes occur in the expression of the major blood group antigens in human fetal organs at various developmental stages, a picture emerged that some of the changes in antigenicities of glycoproteins and glycolipids of cells during successive developmental stages may be brought about by sequential addition or deletion of monosaccharide residues. This led to the proposal that Le^x, I and i and other oligosaccharides of the blood group family

may have important biological roles as recognition structures. They seemed excellent candidates as 'area codes' which determine cell migration pathways, for example.

A functional antigen in early neural development, L5, is Le^x

L5 antibody, originally raised to glycoproteins of the murine nervous system, was shown to recognize neurons and astrocytes in the cerebellum. Later observations on the chick embryo have suggested that the L5 antigen plays a role during early neural development. The antigen has been detected on multiple brain glycoproteins including Thy-1, and on a chondroitin sulfate proteoglycan. The structure of this antigen has been elucidated recently by generating oligosaccharide probes (neoglycolipids) from N-glycans released from total antigen-positive glycoproteins, and from Thy-1 isolated from the brain. The L5 antigen was shown to consist of Le^x. The various attributes for L5 antigen in the development of the nervous system can therefore be assigned to the Le^x sequence. By other approaches, it has been shown that astrocytes in the developing brain and astrocyte subpopulations in adult cerebellum express the Le^x antigen, and there is increased expression on the cell processes at sites of injury. It has been suggested that Le^x and related sequences may be ligands for carbohydrate-binding proteins that mediate astrocyte–neuron and astrocyte–matrix interactions.

Le^x and sialyl-Le^x are markers of human granulocytes and monocytes, and have been designated CD15 and CD15s, respectively

The production of antibodies to distinguish different human blood cell types and lineages has been a major application of the hybridoma technology. Innumerable antibodies that clearly distinguish granulocytes from other blood cells were among the earliest to emerge. The antigenic determinants of one of these antibodies, VEP8, and the second, VEP9, which, in addition binds monocytes, were shown to be Le^x. These findings highlighted a difference between the human and mouse cellular markers, for, in the adult mouse Le^x is not readily detectable immunochemically on blood cells of any kind. Innumerable antibodies with similar specificities toward human granulocytes have been produced over the years, and are assigned as CD15. Desialylation of human blood cells revealed a strong reactivity of human monocytes with anti-Le^x as well as granulocytes, indicating that monocytes express pre-

dominantly the sialyl-Le^x, and that the sialic acid results in masking of the expression of Le^x antigen. Antibodies that recognize the sialyl form of Le^x (Figure 1) on cells of the myeloid series are now designated CD15s.

Le^x and sialyl-Le^x are tumor-associated antigens

Another intensive area of research that became stimulated by the advent of the hybridoma technology was the search for tumor-associated antigens that might be targets for immunotherapy. Among promising antibodies isolated, by comparing reactivities with tumor cells and their normal counterparts, were those which upon further investigation were found to be directed at carbohydrate sequences of glycoproteins and glycolipids. Disappointingly for those who were hoping to identify targets for a new generation of therapeutic monoclonal antibodies ('magic bullets'), the carbohydrates identified were not unique to the tumors, but were sequences that occur normally on other cells and tissues of the body. Notable examples were antibodies directed to Le^x and sialyl-Le^x. Thus, these oligosaccharides may have been inappropriately expressed on the cancerous cells being investigated, but, as discussed above, they were already established as 'distinctive markers' of myeloid cells among human peripheral blood cells. The conclusions of the present author were that either these findings were a reflection of limitations of the methods of selection of hybridoma antibodies, or they were pointers to roles for the carbohydrate structures not only as area codes but also as ligands for growth and differentiation.

Le^x and Le^a-related sequences as ligands for carbohydrate binding receptors, the selectins

The concept that oligosaccharides can serve as area codes has been corroborated by developments in leukocyte biology and inflammation research, notably from work with the leukocyte–endothelial adhesion molecules, the three selectins. The molecular cloning of these molecules, E- and P- selectins (CD62E, CD62P) of endothelia and L-selectin (CD62L) of leukocytes, and the finding that each contains a lectin-like domain, stimulated intensive research into their oligosaccharide ligands. Knowledge that the E- and P-selectins bind to granulocytes and monocytes focused research on Le^x and sialyl-Le^x as obvious candidate ligands. Indeed, all three selectins can bind to sialyl-Le^x-related sequences when these are displayed in the clustered state on protein or lipid (E-

selectin can also bind the asialo-Le^x sequence, although less avidly). The isomeric sialyl-Le^a-related sequences (found at the surface of epithelial tumors) are rather more strongly bound than the Le^x analogs. In addition, the sulfated Le^a and Le^x sequences (identified on an ovarian cyst adenoma glycoprotein) are also bound avidly. On endothelial venules (sites at which L-selectin-mediated leukocyte adhesion occurs), variously sulfated forms of the Le^x sequence have been identified. Details of the macromolecular carriers of these oligosaccharide ligands are out of the scope of this entry: suffice it to say that oligosaccharides of this family are key players in the selectin recognition systems. Unlike the usual situation of receptor–ligand pairs, however, a picture is emerging of recognition systems that operate as triads: receptors, ligands and carriers. Here the receptors are lectins (the selectins), the ligands are oligosaccharides, but only when they are optimally expressed and presented on carriers (proteins or lipids) are functional counter-receptors formed. Also a possible involvement of these carbohydrate structures in the process of metastases has been suggested, the proposed mechanism being that sialyl-Le^x- or sialyl-Le^a-positive cancer cells hijack the leukocyte adhesion pathway for extravasation.

See also: **Cold agglutinins; Selectins (CD62-E/L/P).**

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LIFESPAN OF IMMUNE CELLS AND MOLECULES

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A complete understanding of the immune system relies not only on knowing the detailed functions and interactions of its cells and molecules, but also on knowledge of the population kinetics of those cells. Our theories of how immune responses are initiated and regulated, and, for instance, how both tolerance and memory are induced and maintained, are based on assumptions about the production of lymphocytes from primary lymphoid organs (bone marrow and thymus) and on the turnover of these lymphocytes in the periphery.

Measurement of lymphocyte lifespan

Most methods of measuring lifespan rely on identifying cells that have gone through cell cycle and, therefore, the usual definition of lifespan is the period elapsing between cell divisions (intermitotic lifespan). This is usually assessed by labeling cells in cycle with identifiable DNA precursors such as tritiated thymidine (³H]TdR) or bromodeoxyuridine (BrdU). Obviously, some cells (e.g. plasma cells) may not divide once derived from a precursor population and leave the system through cell death. However, all cells of the immune system are derived from cycling precursors in primary lymphoid organs, and so the rate of accumulation of labeled cells during continu-

ous infusion of [^3H]TdR can be used as a measure of the 'average lifespan' within a population. This method has its drawbacks: if too much label is used it can have direct toxic effects on lymphocytes, or indirectly cause polyclonal activation due to breakdown of the intestinal wall. If too little label is used, rapid decay of the DNA precursors *in vivo* leads to an underestimate of cycling cells. Other methods are available, such as the selective killing of cycling cells by cytotoxic drugs (e.g. hydroxyurea). Persistence of marked cells after adoptive transfer only gives an estimate of the clonal lifespan, as it does not take into account cell division subsequent to transfer.

Population kinetics of B lymphocytes

Production of B cells in the bone marrow proceeds at an enormous rate. In both mice and rats the bone marrow produces enough B cells to repopulate the peripheral lymphoid organs within 3–5 days (5×10^7 per day in mice, 3×10^8 per day in rats). Most, if not all, of these cells are exported from the marrow into the bloodstream. At face value then, the peripheral B lymphocyte pool (i.e. in spleen, lymph nodes, Peyer's patches etc.) should turn over completely within a few days (Figure 1). However, this assumes that all of the newly produced B lymphocytes have free access into the secondary lymphoid organs. Certainly, there is no barrier for entry into the spleen from the blood supply; however, in lymph nodes, cells must cross high endothelial venules, a process mediated by specific surface molecules that are not

expressed uniformly by all lymphocyte subsets. Several studies have shown that B cells newly emerged from the marrow do not immediately enter the areas in which most recirculating B cells localize, i.e. in lymphoid follicles. Stable incorporation of new B cells into the peripheral pool is estimated at 5–10% of the output of the bone marrow. Therefore, at least 90% of new B cells exit the system very quickly, their lifespan being only 3–4 days. Most of this time is spent in the spleen, probably in the red pulp; entry into the white pulp, and hence to follicles, is restricted.

Encounter with antigen is one of the factors that can mediate entry of new cells into this stable, recirculating pool; following immunization there is a wave of activation and incorporation of newly produced, antigen-specific B cells into the peripheral pool. Interestingly, if antigen is localized only in lymphoid follicles or in lymph nodes, no such wave of incorporation occurs, illustrating the restricted migration path of newly produced B cells. Selection for entry into this pool is not mediated solely by exogenous antigen: comparison of the V gene repertoire of IgM $^+$ IgD $^+$ B cells in the bone marrow (newly produced) and in the spleen (stable, recirculating) reveals a bias in the latter population that suggests a ligand selection process has taken place; however, the biased repertoire of the splenic population is similar in germ-free mice. Competition for niches within the follicular microenvironment seems to work at the level of signaling via immunoglobulin, as self-reactive transgenic (i.e. monoclonal) B cells when

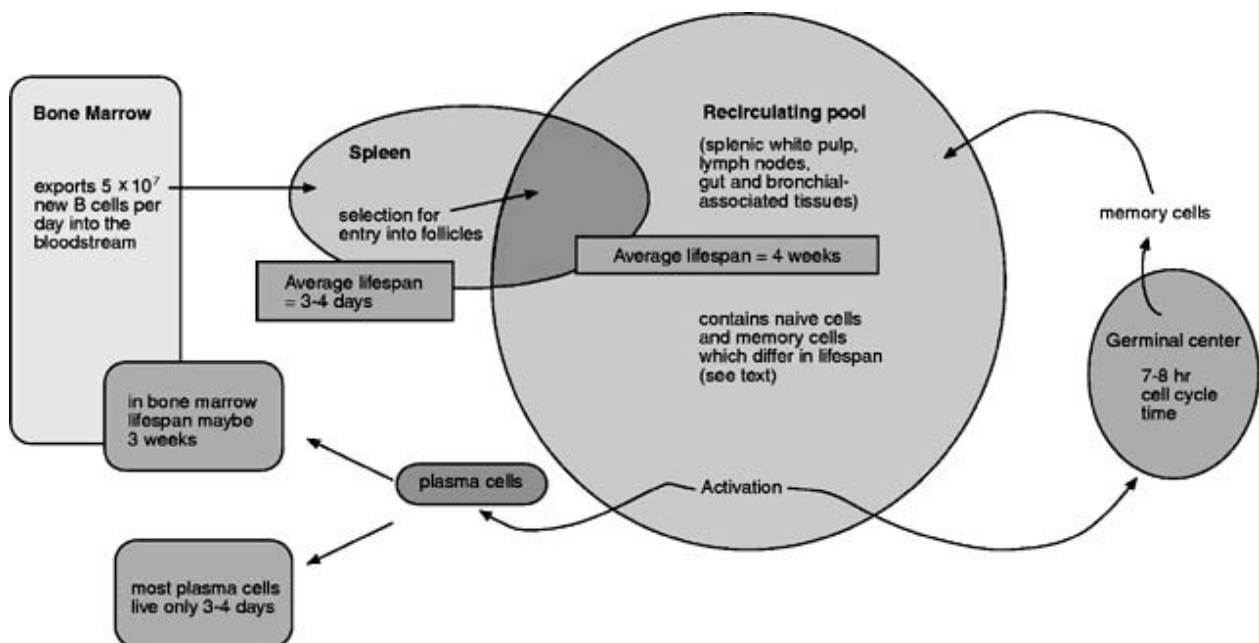


Figure 1 B lymphocyte population dynamics.

exposed to antigen are excluded from follicular entry when in competition with the polyclonal repertoire of normal B cells. Antigen-independent mechanisms of selection may also occur.

The 5–10% of new B cells that become incorporated now begin to recirculate, by which we mean that they move from the spleen into the bloodstream, then cross high endothelial venules to enter lymph nodes or Peyer's patches, migrate to follicles and, after a time, leave via efferent lymphatics, eventually to return to the blood. B Lymphocytes within this recirculating pool have an intermitotic lifespan of several weeks. Determinations of B cell lifespan have varied widely, depending on the method of assessment. In general, the use of cytostatic drugs has led to shorter estimates than those obtained by labeling dividing cells. Controlled use of BrdU in nontoxic doses indicates that the average lifespan of a recirculating B cell is around 4 weeks in mice. These experiments cannot rule out the existence of B cells with much longer lifespans.

Encounter with antigen activates B cells and stimulates cell division. The number of B cells entering this activated pool comprises less than 1% of the peripheral pool per day. Activation of B cells in follicles (by antigen on follicular dendritic cells) leads, in the second week of the immune response, to the formation of germinal centers. B cells proliferate very rapidly within germinal centers with an estimated cell cycle time of only 7–8 h. These structures are important in the clonal expansion of memory cells and generate high-affinity somatic mutants. The strong selection against nonantigen-binding mutants means that only 10% of germinal center B cells ever leave to rejoin the recirculating pool. The rest die by apoptosis and are rapidly engulfed by tingible body macrophages.

The B cells that have undergone antigen-driven proliferation and returned to a resting state are classically thought of as memory cells. While traditionally, memory B cells have been regarded as very long-lived, recent experiments have shown that external factors, such as antigen stimulation, may play a role in their maintenance. The persistence of memory B cells over long periods may not be a reflection of their longevity, but rather the intermittent stimulation they receive from long-term depots of antigen (as immune complexes on follicular dendritic cells). In the absence of antigen, memory B cells seem to have a lifespan that is similar to B cells of the peripheral pool in general; unfortunately there is no consensus on its exact length, estimates varying from 1 to 6 months.

Following antigenic stimulation, a large proportion of B cells differentiate into high-rate anti-

body-producing cells, i.e. plasma cells. The majority of plasma cells survive in this terminal state for only 3–4 days. However, in certain sites, subpopulations of plasma cells can live much longer. In the mouse lamina propria of the gut 50%, and in the bone marrow 90%, of plasma cells secreting immunoglobulin G (IgG) and IgA have lifespans in excess of 3 weeks. All IgM plasma cells are short-lived. The bone marrow is the major source of serum antibody and the production of antibody following immunization can be extremely long (>25 years). This is likely to be due to continual antigenic stimulation and replenishment of the plasma cell pool. However, a very long-lived population of antibody-secreting cells has not been ruled out.

In this section we have considered only the B2-type B cells that make up the vast majority of the recirculating pool. The B1 cells (CD5, Ly1 B cells) may form a separate lineage that predominates in the peritoneum and responds only to T cell-independent antigens. As these cells are not produced in the adult bone marrow beyond 3 weeks of age, they seem to be self-renewing. Their intermitotic lifespan has not been closely studied, but appears to be in the order of a few weeks.

Population kinetics of T lymphocytes

The adult thymus, unlike the bone marrow, exports too few cells to account for the turnover of T cells in the periphery. Between 1 and 2×10^6 T cells leave the thymus each day. By careful use of BrdU the average lifespan of T cells in the peripheral pool is estimated at 4–6 weeks. This means that around 5% of the pool turns over daily; input from the thymus accounts for 0.5–1% (assuming the T cell pool in mice is 2×10^8). Adult thymectomy of mice allows assessment of the turnover of peripheral T cells in the absence of thymic-derived input; when this is done the average lifespan increases to 3–4 months. An average lifespan figure does not take into account the fact that some populations of T cells turn over more rapidly than others. In recent years careful studies have dissected the dynamics of different populations of T cells. Naive T cells (CD45 RA⁺, CD44^{lo}) may have a lifespan of 6 months or more. In contrast, T cells with an activated or memory phenotype (CD45 RA^{lo}/CD45 RO⁺, CD44^{hi}) turn over much more rapidly, with a lifespan of 3–4 weeks (Figure 2). Again these 'memory' T cells are a mixed bag, consisting of bona fide memory cells, as well as recently activated cells and effector cells. At present it is difficult to distinguish these cells. Certainly it is clear that even after long-term infusion of mice with BrdU, 20% or more of memory cells

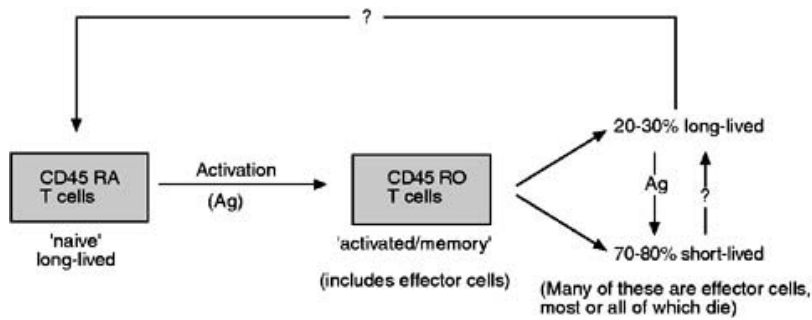


Figure 2 T lymphocyte lifespans.

remain unlabeled, indicating that some of these cells may be long-lived and evidence suggests that some memory T cells revert to a naive phenotype. CD8⁺ T cells appear to have a slightly longer lifespan than CD4⁺ T cells. Based upon the small amount of data available, $\gamma\delta$ T cells differ little in lifespan from $\alpha\beta$ T cells.

Like B cells, the persistence of memory T cells is influenced by continued antigenic stimulation, however, its obligatory role is still controversial. Recent evidence suggests that CD8⁺ T cells may be less antigen-dependent than CD4⁺ T cells. The study of this question and others relating to lymphocyte lifespan may be facilitated by the use of TCR transgenic mice, as it will then be possible to follow the fate of a cohort of antigen-specific cells in the presence or absence of antigen. However, there are pitfalls to avoid with this methodology. Transfer into empty vessels, such as SCID (severe combined immunodeficiency) or RAG1/RAG2-deficient mice may give rise to exaggerated estimates of lifespan as there is no pressure upon the reconstituting cells from repopulating precursors from primary lymphoid organs. Conversely, transfer into intact mice may give very short estimates, although potentially a stable chimerism can be achieved that would allow analysis of the subsequent decay in numbers over time. For reasons not entirely clear, but probably due to encounter with environmental or cross-reactive antigens, T cells expand dramatically after transfer into depleted hosts. For this reason it may be necessary to use TCR transgenic mice that have been backcrossed on to a RAG-deficient background to circumvent antigen non-specific effects on cells with endogenous TCR rearrangements.

Effector T cells are generally thought of as terminally differentiated cells with a short lifespan (3–4 days). However, one theory of memory T cell development proposes that a small proportion of effector cells avoid cell death and become memory cells. It remains to be seen if this is correct or if memory and effector T cells develop along separate pathways of differentiation.

Lifespan of lymphocytes in humans

All of the figures discussed so far in this entry refer to the rodent immune system. There is some evidence that both T and B lymphocytes may live much longer in humans. Studies in patients who have either accidentally, or in the course of treatment, received a heavy radiation dose resulting in characteristic chromosome breakages that preclude mitosis, indicate that lymphocytes can persist for upwards of 10 years without dividing, although the damage to the DNA may itself have caused such longevity. Recently this type of study has been updated with the benefit of markers to identify T cell subsets: The data show that naive T cells (CD45 RA⁺) may have lifespans measured in years but that memory/activated T cells (CD45 RO⁺) turn over within months. As in mice, the reversion of memory cells to a naive phenotype has not been ruled out.

Molecules that regulate lymphocyte lifespan

It is currently thought that lymphocytes, like many other cells in the body, require signals from their environment to prevent the activation of a cell death program (apoptosis). Molecules of the bcl-2 family control the balance between death and survival. bcl-2, bclxL and other members of this family prevent the initiation of the death program that is set in train by molecules such as bax. Most mature, peripheral, recirculating lymphocytes express high levels of bcl-2. Occasionally lymphocytes undergo selection, for instance B cells in germinal centers, and during this process the levels of bcl-2 are dramatically reduced. Rescue from apoptosis is associated with a re-expression of the bcl-2 protein. Evidence from knockout mice suggests that bcl-2 may be less important for the selection that occurs during development of lymphocytes in the bone marrow or thymus, but that it is crucial for their long-term survival in the periphery. In bcl-2 knockout mice the mature, peripheral lymphocyte numbers fall dramatically within a few weeks of birth.

Turnover of accessory cells

The dendritic cells found in T cell areas of secondary lymphoid tissues are termed interdigitating cells. These cells are continually renewed by the influx into lymph nodes of veiled cells via the afferent lymph. [³H]TdR labeling suggests they are replaced from dividing precursors (in the bone marrow) at a rate of about 10% per day. This means that the average lifespan is around 5 days and the whole pool is exchanged within 3 weeks.

Dendritic cells found in B cell follicles are termed follicular dendritic cells (FDC). These cells appear to be extremely long-lived as befits their role in long-term antigen retention. No thymidine uptake has been noted in these cells following long-term infusion. Bone marrow chimeras made between H-2 disparate mice show no replacement of the host FDC population with donor FDC one year after construction, suggesting that FDC are not bone marrow derived and are not of the myeloid lineage (as interdigitating cells are).

The precursors of macrophages, monocytes, are produced in large numbers from the bone marrow. The transit time of these cells in the circulation is between 1 and 2 days, after which time they extravasate. Tissue macrophages do not seem to divide except at sites of inflammation and can persist for long periods (50–70 days). However, there are many different populations of tissue macrophages which may have differing lifespans. For instance, splenic red pulp macrophages turnover completely in bone marrow chimeras within one month, whereas macrophages in the adjacent marginal zone are replaced much more slowly (25% in one month).

Lifespan of immune molecules

Early measurements of the half-life of serum immunoglobulins relied on injecting purified protein labeled with radioactive iodine. This technique produced an estimated half-life of 4 days for 7S molecules. More recently, it has been possible to study half-life without labeling (potentially altering fine structure) by the use of monoclonal antibodies of defined specificity and/or heavy chain allotype. The results of such studies show that in mice IgM has a half-life of 2 days, for IgG3 and IgG1 it is 6–8 days, and for IgG2b it is 4–6 days; IgE has a half-life of 12 h and polymeric IgA 17–22 h. In humans, the use of antibodies in therapy indicates that the half-lives do not differ significantly from these figures.

Other immune molecules have much shorter *in*

vivo half-lives; cytokines decay within minutes, although exhaustive studies on the growing list have not been carried out. Complement components also are not well-studied in this respect but decay with a half-life of several hours, e.g. the half-life of C3 is 50 h.

See also: Apoptosis; B lymphocyte differentiation; Bone marrow and hematopoiesis; Cytokines; Germinal center; High endothelial venules; IgA; IgD; IgE; IgG; IgM; Lymph nodes; Lymphocytes; Memory, immunological; Mononuclear phagocyte system; Proliferation, lymphocyte; Radiation, effects on immune system; Recruitment; Spleen; T lymphocyte clonal expansion; T lymphocyte differentiation; Thymus.

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LIMITING DILUTION ANALYSIS

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Why do we need it?

Progress in understanding how the immune system functions has relied a great deal upon 'readout systems' that require the introduction of antigen at one point in time, and quantitation of 'total response' (e.g. antibody levels) some time later. A large antibody response could have come from a few starting lymphocytes each responsible for high-level production, or it could have come from many lymphocytes producing far less. To fully understand the biology of the cellular events that follow antigen stimulation, one would like to know how many parent (or precursor) lymphocytes in a given starting population were triggered to produce the clones that eventually made the antibody. In other words, one would like to know the frequency of the precursor cells that exist within the starting population of lymphocytes, and the proportion of them that may be used in any response.

There is no simple (e.g. visual) way of being able to identify which of the cells in a large population will go on to produce an active clone. Antigen-specific precursors are rare and morphologically indistinguishable from the silent majority of resting lymphocytes. Limiting dilution analysis (LDA) allows the calculation of precursor frequencies as a retrospective process based on the measurement of the number of active clones generated from a given population of cells. As each active clone is derived from one precursor, then retrospectively one can estimate the frequency of precursor cells that gave rise to those clones.

Estimation of the precursor frequency can indeed be an end in itself, e.g. if one wished to determine the frequency of alloreactive cells that the bone marrow of a potential donor might have toward the graft recipient. Too high a frequency might reasonably predict aggressive graft-versus-host disease and would therefore be unacceptable.

The realization that there was a need for this sort of measurement in immunology, as well as the development of suitable technology to make it possible, led to the first important paper on the subject by Lefkovits in 1972, from which LDA has developed as an extremely powerful tool in immunology.

How is the frequency estimated?

The best way to understand LDA is to consider a specific example (Figure 1). Let us imagine that we have a population of lymphocytes from the blood of a potential bone marrow donor. We want to know the frequency of the precursors of cytotoxic T cells (pCTLs) that may react to the recipient. A simple *in vitro* approach would be to dispense these lymphocytes into many aliquots in microculture trays, each microculture tray getting a different input number. If one adds the best medium and all essential growth factors together with the recipient's irradiated stimulator cells, then any precursor lymphocytes should be activated to grow, and produce clones. Within the clones, the majority of daughter cells eventually differentiate into cytotoxic T cells lethal to the recipient's target cells. A simple chromium release assay can indicate which wells have made cytotoxic T cells and which have not. If the culture conditions have been good enough, then single precursors should have produced sufficient clone progeny to guarantee that all wells containing CTLs would be measurable in the readout.

Limiting dilution analysis relies upon the estimation of negative wells, as clearly these must have had no precursors. A positive well could have had one or more precursors and so is not so useful in the statistical analysis.

The proportion of cultures that fails to respond (i.e. has no pCTL within the lymphocyte aliquot) at any given lymphocyte input can be defined by the expression:

$$F_0 = e^{-u} \quad (1)$$

where F_0 is the number of negative cultures per total number of cultures, e the base of the natural logarithm, and u the average number of precursors (pCTLs) per culture (well). The above expression is the zero term of the Poisson distribution. The full Poisson distribution formula and its derivation is considered in detail in *Limiting Dilution Analysis of the Immune System* by Lefkovits and Waldmann.

If one modifies the above formula, one can derive the following expression:

$$u = -\ln F_0 \quad (2)$$

(this represents the conversion of formula (1) to its logarithmic form). Through this conversion, the for-

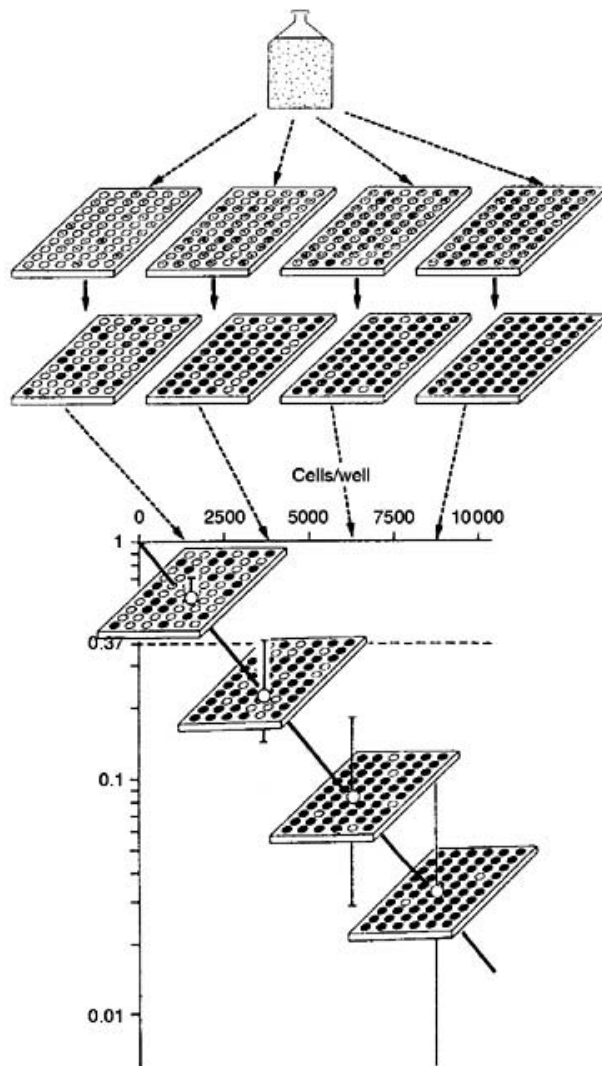


Figure 1 A single-hit curve. Four trays, each of 60 cultures, were inoculated with graded numbers of human lymphocytes (1250, 2750, 6250, 8750 cells per culture). Some of the cells in the inoculum are antigen specific, while the majority are irrelevant third-party cells. The dots in the upper row of trays indicate just the distribution of the antigen-specific cells. Culture conditions were chosen that allowed the cells of interest (dots) to have an optimum chance of proliferating and generating clones of cytotoxic T cells (CTLs). If the original parental T cells are called 'precursor' CTLs or pCTLs, then in the lower row of trays the dots represent the clones arising from the pCTLs. The CTL activity of each well is measured in a chromium release assay on the desired target cells. The cultures which scored positive are shown as 'filled' wells. In the first tray of 60 cultures, 24 responded. Therefore, F_0 is $36/60 = 0.6$. In the next three sets, we see that as cell input per culture well increases, the number and the proportion of negative cultures F_0 decreases. The results are plotted on a semilog plot and they fit a straight line. The vertical bars represent the 95% confidence limits. These represent the limits within which F_0 would be expected to fall 19/20 times. (Reproduced with permission from Lefkovits and Waldmann (1984).)

mula is reorganized around the value that interests us – μ , the average number of precursor cells. In addition, it can be represented graphically, providing a straight line in a semilogarithmic plot. In other words, the negative logarithm of the fraction of nonresponding (negative) cultures is linearly proportional to the mean number of precursors per culture. The resulting straight line has two uses. First, it allows the estimation of frequency by a variety of methods. Second, it acts as a sort of internal control to check that the system is measuring the activity of a single limiting cell type. A straight line means a single-hit curve, which means that only a single cell type is limiting (the pCTL). Any deviations from linearity determined by use of appropriate statistical methods would invalidate that starting assumption, confound the estimate of frequency, and preclude any further dependent analyses.

In the semilog plot in Figure 1, we could apply statistical methods to test the goodness of fit of the data points with the single-hit Poisson model by calculating χ^2 statistics and confidence intervals for the slope. If we assume that the line drawn in the figure is the best-fit line, then we can make a reasonable estimate of frequency from the plot by interpolating at 37% of negative cultures. This is best understood by substituting $\mu = 1$ in the zero term of the Poisson formula (1). We then derive

$$F_0 = e^{-1}$$

$$\text{i.e. } F_0 = 1/e = 0.37 = 37\%$$

There are more sophisticated ways of estimating frequency, and the reader is referred to Taswell. From the example shown here, interpolation at 0.37 reveals a frequency of pCTL of $1/2500$ or 4×10^{-4} .

What may be learned?

The LDA approach is now widely used to estimate precursor frequencies for a wide range of immunological responses (T cell growth, cytokine secretion, helper T cells, pCTLs, B cells at different stages of ontogeny, etc.). It can be manipulated to define the absolute frequency as elicited by the strongest stimuli known, and the operational frequency, i.e. that actually used in the immune response. It can be used to study changes in functional precursors in tolerance (see Desaynard and Waldmann; Good and Nossal) and after immunization.

From the basic frequency data, one can design further, more revealing biological experiments dealing with cell-cell interactions. For example, the first evidence that T cells and B cells have to contact each other to collaborate arose when it was shown that a single T cell (from LDA) could only collaborate with

one of many B cells offered to it. A modification of the procedure termed 'partition analysis' has allowed the investigation of T cells with inhibitory (suppressive) properties that are contained within a population of cells with helper properties.

In vivo, LDA can address a wide range of biological questions of precursor to product relationships. One particularly good example is the estimation of how many T stem cells are required to colonize a thymus lobe.

See also: Cytotoxic T lymphocytes; Cytotoxicity, assays for; Graft-versus-host reaction; Immune response *in vitro*; Statistics, use in immunology.

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LINKAGE DISEQUILIBRIUM

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Linkage disequilibrium is an important concept in genetic studies that aims to identify and/or localize genes related to disease susceptibility. The term linkage disequilibrium is commonly used to indicate that two genes are physically linked, however, the strict definition of the term does not specify close genetic linkage. Linkage disequilibrium is defined as the difference between the observed frequency of a particular combination of alleles at two loci and the frequency expected for random association. This is based on the assumption that, given sufficient evol-

utionary time, the occurrence of random recombination events will result in an equilibrium distribution of alleles at each locus. Thus, the frequency of a particular allele at a given locus will be independent of alleles at other linked loci.

In immunology, the term linkage disequilibrium is often encountered in studies of the HLA complex. The HLA gene complex consists of multiple highly polymorphic loci. Linkage disequilibrium (Δ) is most simply calculated using the equation:

$$\Delta(\%) = (F_{obs} - pq) \times 100$$

where p is the frequency of allele 'a' at locus 1, q is the frequency of allele 'b' at locus 2, and F_{obs} is the observed frequency of alleles 'a' and 'b' occurring together.

Taking a sample population where the gene frequencies of HLA-B8 and HLA-DR3 are 9 and 10% respectively, random recombination of HLA-B8 and HLA-DR3 would result in their presence in the same haplotype at a frequency of 0.9%. However, the observed frequency of HLA-B8/DR3 positive haplotypes is 7%, giving a linkage disequilibrium of 6.1%. Thus HLA-B8 and HLA-DR3 occur together in a haplotype in this population far more frequently than expected. Similar examples of linkage disequilibrium have been observed for alleles of HLA genes in every population studied; the specific alleles involved vary in different populations.

Many diseases have been associated with, or linked to, genes encoded within the HLA gene complex; however, the association between an HLA allele and a given disease is rarely absolute. Therefore, the observed association between a given HLA allele and a particular disease or functional characteristic may reflect some property of a gene closely linked to the tested locus, rather than a property of the locus itself.

Comparison of genetic and physical distances

Genetic distances between loci are determined by the frequency with which recombination events occur between the genes. Genetic distances are expressed in centimorgans (cM) where 1 cM corresponds to a recombination frequency of 1%. It is generally assumed that homologous recombination events can occur at any point in the DNA sequence and therefore genetic distances reflect physical distances. The ability to compare the relationship between genetic and physical distances more directly is now possible because of the technological advances in gene mapping allowing determination of the physical distance between two loci.

In humans, a genetic distance of 1 cM is calculated to be approximately 1200 kb (the average size of the human genome is about 2700 cM and contains approximately 3.3×10^6 kb of DNA). Within the HLA complex, there are regions where genetic and physical distances approximate calculated values and there are other regions where there are marked discrepancies. The physical distance (~1000 kb) between HLA-DR and HLA-B corresponds to the genetic distance (1 cM). By contrast, a recombination frequency of 3% (3 cM) has been observed between the HLA-DB and DQ subregions; these loci are

physically separated by only ~500 kb. Therefore, there is a significantly increased frequency of recombination between HLA-DP and HLA-DQ compared to the physical distance that separates them. These data imply that the properties of any given DNA segment may vary from those of another.

Use of linkage disequilibrium for gene mapping

Two genes may be found in linkage disequilibrium because they are physically close and recombination events do not occur frequently between them, thus linkage disequilibrium analyses have been used in gene mapping studies. Experimental approaches that make use of linkage disequilibrium have been successful in strengthening traditionally performed linkage analyses, particularly those involving genes with few alleles. Certain genes of interest for their role in a particular disease may not be highly polymorphic in a population, therefore the number of informative crosses available for examination may be limited.

In addition to close physical linkage, there are other reasons why two genes may be found in linkage disequilibrium. Two populations with distinct traits may have merged recently. Associations between alleles or traits may be apparent because insufficient time has passed for the genes to segregate randomly. Alternatively, there may be functional interactions between the products of two different loci that depend upon allelic differences. There may be a selective advantage to the co-inheritance of particular alleles of linked loci. The consequence of having a particular allele at one locus may not be observable unless there is a certain allele at another locus because of functional interactions between their products.

Impact of linkage disequilibrium on studies of the HLA complex

Regardless of the basis for linkage disequilibrium, it has an important impact upon analyses of HLA antigens in functional and disease association studies. A historical review of the HLA antigens found to be associated with certain diseases illustrates this point. As new loci within the HLA complex were identified, associations with allelic variants of these new loci in linkage disequilibrium were shown to be stronger than previously observed associations. The number of genes mapping to the HLA complex continues to increase. Due to linkage disequilibrium, functional associations with allelic variants of the newly defined loci may be stronger than those observed with previously characterized loci.

While the HLA gene complex provides the best known examples of linkage disequilibrium, this phenomenon is not limited to HLA. Linkage disequilibrium may also be a consideration when examining other gene complexes encoding immunologically relevant molecules. For example, the immunoglobulin heavy chain and T cell receptor and gene complexes are composed of linked gene segments that show allelic variations. Allotype markers (Gm) for the constant region of immunoglobulin G (IgG) heavy chain are inherited as fixed combinations or haplotypes that have been used as markers for various populations. The stability of Gm haplotypes suggests that linkage disequilibrium may be operative. In contrast, available information on the T cell receptor α and β chain gene complexes suggest that linkage disequilibrium is not a major determinant. Considering the relative physical sizes of T cell receptor α and β gene

complexes, there is extensive heterogeneity observed in the combination of markers present in haplotypes, suggesting that recombination has occurred frequently within the T cell receptor gene complexes.

See also: Allotypes, immunoglobulin; H2 class I; H2 class II; H2 class III; HLA class I; HLA class II; HLA class III region; Immunoglobulin genes; Immunoglobulin structure; MHC disease associations; Relative risk; T cell receptor, $\alpha\beta$.

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LIPOSOMES

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Liposomes are artificially prepared spheres consisting of concentric phospholipid bilayers separated by aqueous compartments. They form when amphipathic phospholipid molecules are dispersed in an aqueous solution. The phospholipids tend to find a conformation in which their hydrophobic fatty acid chains are prevented from direct contact with the water molecules. For that reason, phospholipid bilayers are formed, of which both outer parts are made up of the hydrophilic head groups, whereas the hydrophobic fatty acid chains are located directly opposite to each other and form the inner part of the bilayer (Figure 1).

Part of the aqueous solution, together with hydrophilic molecules dissolved in it will be encapsulated during the formation of the liposomes, whereas lipophilic molecules may be associated with the phospholipid bilayers. Amphipathic molecules, such as the phospholipids themselves, or conjugates made up of a phospholipid molecule and a hydrophilic molecule, attempt to find a conformation with their hydrophobic parts inserted in the bilayers and their hydrophilic parts extended either in the aqueous compartments or on the outer surface of the liposomes.

Because liposomes are biodegradable and may be composed of natural, nontoxic and immunologically inert phospholipid molecules, they have been sug-

gested as promising carriers of drugs. Since liposomes may serve as an immunoadjuvant with respect to encapsulated and surface-associated antigens, they have also been proposed as antigen carriers for application in vaccines.

Different liposomes for different applications

Liposomes can be prepared according to different methods. They may vary in their dimensions, composition (different phospholipids and cholesterol contents), charge (resulting from the charges of the composing phospholipids), and structure (multilamellar liposomes consisting of several concentric bilayers, separated by aqueous compartments or unilamellar liposomes, consisting of only one phospholipid bilayer surrounding one aqueous compartment). For a detailed description of liposome technology and chemistry, the reader is referred to Gregoriadis (1993, see Further reading).

The *in vivo* distribution of liposomes is strongly influenced by their characteristics. Targeting of liposome-encapsulated drugs to particular cells or tissues can be achieved by covalent attachment of monoclonal antibodies, or other appropriate proteins, to the outer surface of the liposomes. One possible approach to the preparation of such *immunolipo-*

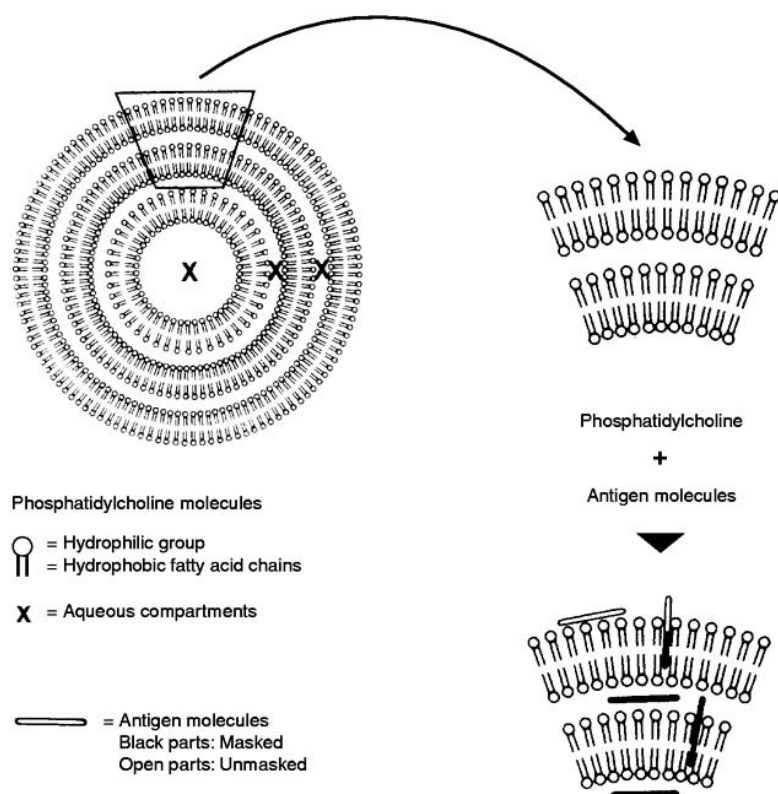


Figure 1 Schematic representation of phosphatidylcholine liposomes and their possible association with antigen molecules.

somes is to incorporate the phospholipid molecule phosphatidylethanolamine in the phospholipid bilayers. This molecule can be covalently coupled to protein molecules by using their amino groups.

Phagocytosis forms the natural fate of the bulk of normal liposomes once they are administered into mammals. As a consequence, liposome-mediated modulation of macrophage function is one of the *in vivo* applications of liposomes. Liposome-encapsulated immunomodulatory molecules such as muramyl dipeptide (MDP) and muramyl tripeptide (MPT) can be used to activate macrophages, which then are able to eliminate metastatic tumor cells (e.g. in the liver) and show an increased capability to eliminate various microorganisms. It is not known whether the activated macrophages directly kill these cells and microorganisms or if their influence is mediated by other cells such as natural killer (NK) cells.

In contrast, some liposome-encapsulated drugs can be used to reduce macrophage activity or even to deplete macrophages completely from tissues or organs. This approach is increasingly chosen for studies aimed at unravelling macrophage functions *in vivo*.

When liposomes are chosen to serve as a depot for drugs (storage and gradual release) or when liposomes are intended for targeting of drugs to non-

phagocytic cells, uptake and clearance by macrophages can be reduced or even avoided by using liposomes with long half-lives in the circulation. Such *long circulating liposomes* can be prepared by coating liposomes with polyethylene glycol polymers or other molecules which inhibit opsonization of the liposomes.

Cationic liposomes can be prepared by the incorporation of cationic phospholipids in the bilayers. This allows a high-yield DNA incorporation and for this reason such liposomes may be more suitable for gene and antisense therapy. *pH-sensitive liposomes*, which destabilize and become fusion-active at mildly acidic pH, may be more suitable for delivery of encapsulated molecules into the cytoplasm of the target cells. Normally, the bulk of liposomes together with their encapsulated material will enter the lysosomal pathway, where it will be exposed to a large panel of lytic enzymes. It is a matter of course that combination of various liposomal features may lead to a product which is even better able to meet the needs of, for example, specific targeting or gene therapy. So, *long circulating immunoliposomes*, which are better able to avoid the cells of the mononuclear phagocyte system, are undoubtedly better in targeting encapsulated molecules to nonphagocytic cells. Also, *pH-sensitive cationic liposomes* may be expected to be better DNA carriers for gene therapy.

The latter liposomes allow a high-yield DNA incorporation due to their positive charge while their pH-dependent behavior promotes their fusion with endosomes, facilitating release of DNA into the cytoplasm.

Liposomes as antigen carriers

Liposomes may act both as carriers for haptens and as an immunoadjuvant for complete antigens. Haptenated liposomes induce a hapten-specific humoral immunoglobulin M (IgM) immune response without the generation of immunological memory. An IgG response is not detected after primary or secondary immunization with haptenated liposomes, and in addition mice depleted of, or deficient in T lymphocytes are able to respond to haptenated liposomes. Thus, haptenated liposomes are T cell-independent (TI) antigens. TI antigens are further divided into two types: TI-1 and TI-2. TI-2 antigens activate B lymphocytes from all the normal mouse strains except those of CBA/N, which carry an X-linked immune defect, and the immature B cells from neonatal mice of all strains. TI-1 antigens, having intrinsic mitogenic properties, stimulate B cells of all mouse strains, including those with the X-linked immune defect. It appears that CBA/N mice respond to haptenated liposomes, and for this reason these antigens are considered TI-1 antigens. However, unlike other known TI-1 antigens, they appear to be nonmitogenic in mice.

In contrast to haptenated liposomes, liposome-associated protein antigens elicit an IgG response in addition to the preceding IgM response, generate immunological memory, and behave as real T cell-dependent antigens. The response is strongly enhanced when the antigen is given in a liposome-associated form compared to free in solution. Antigens can be associated with liposomes either by encapsulation (masked antigenic epitopes) or by linking to their surfaces (exposed antigenic epitopes; see Figure 1). Surface exposition can be achieved by a number of methods for covalent attachment of the antigen to one of the phospholipids used for preparation of the liposomes or by nonspecific adsorption of hydrophobic antigens to the phospholipid bilayers. Antigens can also be entrapped in the liposomes when dissolved in the aqueous solution used for preparation of the liposomes. For most antigens, the latter procedure will result in liposomes with antigen entrapped in their aqueous compartments as well as exposed on their outer surfaces. The ratio between entrapped and exposed antigen may vary to a large extent and will depend on the characteristics of both the antigen, e.g. the presence and distribution

of hydrophobic groups, and the liposomes. It has been demonstrated that encapsulated antigens as well as surface exposed antigens induce both IgM and IgG antibodies. However entrapped antigens tend to stimulate a stronger IgG response, whereas surface exposed antigens tend to stimulate a stronger IgM response against the same antigen.

Liposomes have also been found to be efficacious antigen carriers for the induction of cytotoxic T lymphocytic (CTL) responses. pH-sensitive liposomes are especially able to elicit strong CTL responses. It is postulated that their efficacy in the induction of CTL responses depends on the fact that they are able to deliver the encapsulated antigen into the right intracellular compartment of the antigen-presenting cell (APC).

Dendritic cells, and not macrophages, are generally believed to be the major APC responsible for processing antigens and their presentation to T lymphocytes. This is in line with the observation that an enhanced antibody response is elicited when macrophages are selectively depleted prior to administration of soluble antigens. By competing with dendritic cells for the antigen molecules, macrophages actually reduce the amount of antigen which is available for dendritic cells. However, when large particulate antigens such as red blood cells (RBCs) or liposome-encapsulated antigens are injected, antibody responses are strongly reduced when macrophage depletion precedes antigen administration. Thus, macrophages play a crucial role in the induction of antibody responses against such large particulate antigens. Evidence is also accumulating that macrophages play a role in the induction of CTL responses against liposome-encapsulated antigens. Selective depletion of macrophages by a liposome based 'suicide' approach (see later) blocks the secondary CTL response induced by consecutively administered liposomal antigen. Since it is known that dendritic cell functions are not affected by this method of macrophage depletion, it would appear that macrophages are involved in the induction of CTL responses against liposome-encapsulated antigens.

One of the main advantages of liposomes as an immunoadjuvant is that they are biodegradable. They are ingested by macrophages which consecutively digest the phospholipid bilayers using their lysosomal phospholipases. As mentioned before, it is probable that liposome-mediated targeting of antigens to macrophages forms part of the mechanism of the immunoadjuvant activity of liposomes. Another important advantage of liposomes as an immunoadjuvant is that they may be composed of phospholipids (e.g. phosphatidylcholine) that are nontoxic in the doses used. Liposomes may even diminish tox-

icity of the associated antigens. It has been shown that after attachment of cholera toxin to liposomes containing ganglioside GM1, toxicity of the toxin was eliminated whereas immunogenicity was enhanced. Also the toxicity of lipopolysaccharide (LPS) incorporated with liposomes appeared to be reduced when compared to free LPS. It is not surprising that phospholipids, which form the basic matrix of all cell membranes, are not very immunogenic themselves. Although liposomes may serve as carriers for haptens and as an immunoadjuvant for protein antigens, antibodies against their phospholipid component are not elicited, unless LPS or LPS-derived lipid A is inserted in these bilayers. It has been shown by Alving and coworkers that incorporation of LPS or lipid A greatly enhances the adjuvant activity of liposomes, but, in addition, antibodies to the liposomal phospholipids and even to liposomal cholesterol can also be induced. There is some evidence that these phospholipid-specific antibodies are produced by a distinct lineage of B cells (CD5⁺, B-1 cells), which might also be involved in autoimmune reactions.

Liposome-based immunoassays

Several liposome-based immunoassays have been developed to detect immune-related compounds or analytes of interest. The aqueous compartments of liposomes can be used to carry various reporter molecules and the so-called immunoliposome assays may offer distinct advantages over existing immunoassays depending on the nature of the intended assay. A wide variety of immunoliposome assays have been developed with different approaches and sensitivities. Antibody as well as antigen concentrations can be determined due to the fact that, in the presence of specific antibody, antigen- or hapten-bearing liposomes can fix complement, which in turn may result in the lysis of such liposomes by activated complement and the consecutive release of the encapsulated markers. Based on the finding that antigen-specific lysis can be inhibited by addition of soluble antigen to compete for the binding of antibody to the liposome-exposed antigen, a number of inhibition assays have been developed.

Liposomes for manipulation of macrophage functions

The selective targeting of immunomodulators to appropriate sites of action is an integral and important component of immunotherapy for infectious diseases and cancer. Most attempts to target liposomes to solid tumors have been disappointing

because the phospholipid vesicles are unable to reach organ parenchyma, since they are not able to cross the barriers formed by capillary walls. Also, they are rapidly endocytosed by phagocytic cells. However, taking advantage of this natural fate of liposomes to target immunomodulators to cells of the mononuclear phagocyte system, Fidler and colleagues were able to activate these cells to a tumoricidal or virocidal state. Systemic administration of liposomes containing various immunomodulators has been shown to bring about regression of lymph node, lung and liver metastases, and dramatic prophylaxis of viral infections in rodents.

Macrophages perform a wide variety of functional activities in immune reactions. They have an important role in the clearance of immune complexes and apoptotic T and B lymphocytes and they are involved in processing of particulate antigens. In addition they play a crucial role in the differentiation of several nonphagocytic cells and in the regulation of their functions. These latter roles are dependent upon the production of a number of cytokines and other soluble mediators by the macrophages. Recent studies have shown that, for example, in the liver, macrophages (Kupffer cells) are responsible for the bulk of all lipopolysaccharide-induced interleukin 1 β (IL-1 β), IL-6, IL-10 and IL-12 mRNA expression. Macrophages form a heterogeneous cell population. In the spleen for instance, red pulp macrophages, marginal zone macrophages, marginal metallophilic macrophages, white pulp macrophages and tingible body macrophages show a compartment-specific localization pattern. In addition, they can be distinguished by different surface markers, a different morphology and by differences in their functional specialization.

Selective *in vivo* depletion of macrophages is a widely accepted approach used to investigate whether macrophages are involved in any particular biomedical process. Since the effects of the usual methods for macrophage depletion, such as treatment with silica, carrageenan, antimacrophage antibodies etc. were neither selective nor complete, we developed a new approach, based on the liposome-mediated intraphagocytic delivery and accumulation of the bisphosphonate clodronate. Macrophages ingest the liposome-encapsulated clodronate and then disrupt the phospholipid bilayers using their lysosomal phospholipases (see Figure 2). The encapsulated clodronate is then released within the cell and since clodronate molecules do not easily escape from living cells, the drug is accumulated intracellularly. Finally, the cell death programme is activated, leading to elimination of the cell by apoptosis. Using this so-called liposome-mediated macrophage 'suicide'

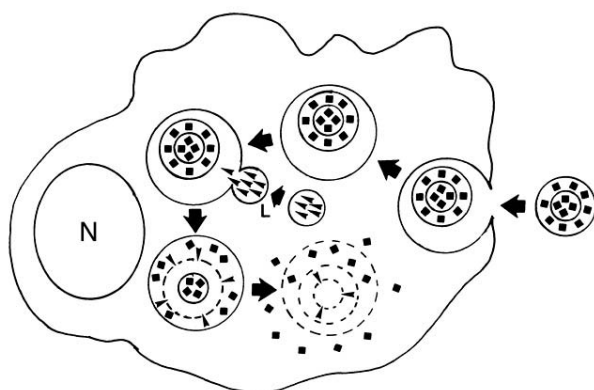


Figure 2 Liposomes, encapsulating clodronate or other water-soluble molecules (squares) in their aqueous compartments, are ingested by macrophages. After fusion of the liposome-containing endosome with lysosomes (L) containing phospholipases (arrowheads), the latter disrupt the phospholipid bilayers of the liposome. The more the concentric bilayers of the liposome are disrupted, the more the encapsulated molecules are released within the cell. N, nucleus.

approach, it has been established for instance, that alveolar macrophages actively suppress the function of dendritic cells in the lung, that liver macrophages play a role in the differentiation of liver-specific NK cells and that testis macrophages play a role in the differentiation of Leydig cells.

It is anticipated that, apart from effects mediated by physical depletion of macrophages from tissues, encapsulated drugs (in sublethal doses for macrophages) may also prove to be useful in the

transient suppression of macrophage activity. This may be particularly relevant to the role of macrophages in the induction of inflammation, since the macrophage-derived cytokines IL-1 and tumor necrosis factor α (TNF α) appear to play a crucial role in the induction of various inflammatory reactions.

See also: Adjuvants; Hapten; Immunoassays; Immunopotential; ISCOM (immuno-stimulating complex); Macrophage activation; Phagocytosis.

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LISTERIA, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

The genus *Listeria* contains seven species, only two of which, *L. monocytogenes* and *L. ivanovii*, are pathogenic. The former is by far the principal pathogen of the groups and will be described in this article. It is widespread in the environment and has been isolated as a commensal and a pathogen from a wide variety of vertebrate species. It causes abortion, meningitis and septicemia in human beings, and encephalitis, abortion and mastitis in ruminants. The usual portal of entry is thought to be the gastrointestinal

tract. This latter point has been amply demonstrated by several large outbreaks, and numerous sporadic cases, of listeriosis in people who ingested *Listeria*-contaminated food products. Listeriosis causes considerable morbidity and a relatively high mortality rate, particularly for the fetus of infected pregnant women and for immunosuppressed adults.

L. monocytogenes is a gram-positive, nonsporulating, β -hemolytic, facultatively anaerobic, catalase-positive rod. It possesses the ability to multiply at 3–45°C and, under some circumstances, to survive short periods at temperatures of 60°C or greater. Organisms grown at room or refrigeration tempera-

ture have multiple flagella and are motile, whereas those grown at 37°C have few flagella and are non-motile. The ability of *L. monocytogenes* to multiply (albeit slowly) at 4°C can be used as an enrichment technique for its isolation from clinical specimens.

Both somatic and flagellar antigens are used to serotype *L. monocytogenes*; nearly all human and animal infections involve serotypes 4b, 1/2b and 1/2a. SDS-PAGE analysis reveals a number of protein bands with T cell-activating and B cell-mitogenic activity. Organisms grown at 20°C produce abundant amounts of a 29 kDa protein that is flagellin. The cell wall contains abundant teichoic acids and lipoteichoic acids. Virulent *L. monocytogenes* produce an extracellular 58 kDa thiol-activated hemolysin that is immunogenic for both T and B cells. Humoral and cellular immune responses against other virulence determinants (internalin, actA, p60) have also been reported.

Immune responses of the host

Because of its relative convenience, reproducibility and safety, murine listeriosis has been widely used as a model for investigating protective cellular immunity to facultative intracellular pathogens. It had been thought that the organism resided principally within macrophages, however more recent data suggest that large numbers of listeriae reside and multiply within hepatocytes in the infected host. Innate resistance to listeriosis in mice is controlled principally by the Lr1 locus on chromosome 2, which is linked with the Hc locus that regulates production of the fifth component of complement. *Listeria*-resistant strains of mice recruit greater numbers of inflammatory phagocytes in response to injection of listeriae and various sterile irritants than do susceptible strains of mice. *Listeria*-immunized mice recruit a greater number of inflammatory phagocytes in response to injected listeriae than do nonimmunized controls. Blood and inflammatory neutrophils and mononuclear phagocytes from several mammalian species kill *L. monocytogenes* *in vitro*, whereas resident macrophages have limited antilisterial activity. Some investigators have reported enhanced antilisterial activity for macrophages isolated from immunized mice, or activated *in vitro* with interferon γ (IFN γ). Other researchers found that macrophages activated *in vivo* or *in vitro* are no better able to kill listeriae *in vitro* than are macrophages elicited by nonspecific inflammatory agents. Both oxygen-dependent and independent bactericidal mechanisms may contribute to killing of listeriae. Some studies demonstrated the importance of reactive nitrogen intermediates for

resistance *in vivo*, or macrophage anti-*Listeria* activity *in vitro*, while others demonstrated no effect.

Host defense against *Listeria* is quite complex, involving a number of cell types and mediators (Figure 1). Depletion of CD4⁺, CD8⁺, TCR $\alpha\beta$ ⁺ or TCR $\gamma\delta$ ⁺ cells results in a more severe infection, but the animals generally recover and eradicate the listeriae from their tissues. In contrast, mice depleted of both TCR $\alpha\beta$ ⁺ and TCR $\gamma\delta$ ⁺ cells demonstrate profoundly reduced resistance, as do mice completely depleted of Thy-1.2⁺ cells. These findings suggest that T cell-mediated immunity is important, but not the sole factor, in resistance to listeriosis. This is underscored by earlier observations that experimentally infected athymic nude mice, or SCID mice, are unable to completely eliminate the *L. monocytogenes* from their tissues. Animals that have cleared a sublethal *L. monocytogenes* infection are resistant to reinfection with 1000-fold greater numbers of *L. monocytogenes* than would prove fatal for naive mice. One can transfer resistance to naive recipient mice with T lymphocytes from the spleen, or other tissues, of immunized mice.

Other cells, particularly neutrophils, make important contributions to anti-*Listeria* resistance. Mice treated with monoclonal antibodies that either prevent extravasation of neutrophils, or deplete them of mature granulocytes, succumb to infection with low numbers of *L. monocytogenes* that are easily eliminated by control mice. Granulocytes interact directly with *Listeria*-infected hepatocytes, and appear to either lyse infected cells or release mediators (e.g. tumor necrosis factor α , TNF α) that make the hepatocytes less permissive for intracellular listerial multiplication. Natural killer (NK) cells have also been implicated in resistance to listeriosis, perhaps via their ability to rapidly release large amounts of IFN γ that can restrict listerial multiplication in hepatocytes and macrophages.

Cytotoxic T cells are activated by *L. monocytogenes* as a result of its intracellular replication cycle, in which it multiplies freely within the cytoplasm of macrophages and other cells. *L. monocytogenes* antigens are thus processed and presented on the cell surface with class I antigens that activate, and serve as targets for, cytotoxic T cells. A series of elegant investigations have delineated the immunodominant portions of *L. monocytogenes* virulence molecules (e.g. hemolysin) and provided a powerful model for studying the specificity and regulation of cytotoxic cell-target cell interactions. The relative importance of cytotoxic cells in resistance to listeriosis is unclear, however, as depletion of CD8⁺ cells reduces, but does not eliminate, resistance to infection.

L. monocytogenes infection results in rapid

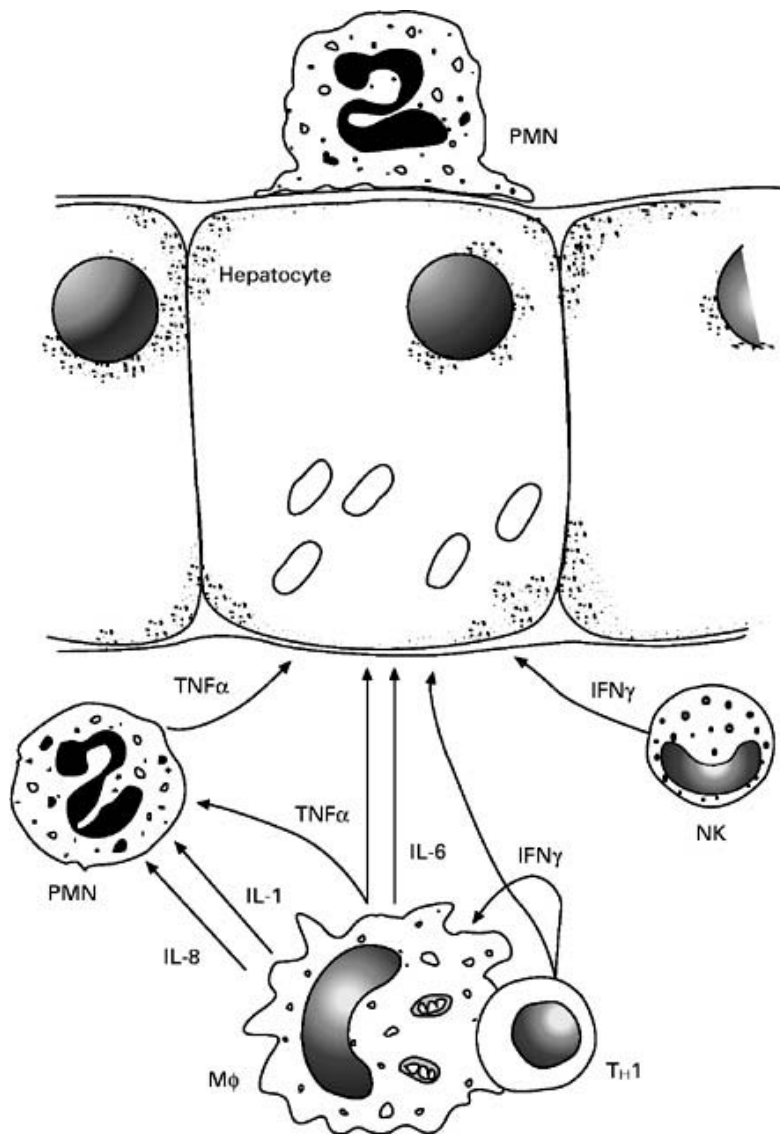


Figure 1 An overview of some of the cells and cytokines likely to be involved in host defense against *L. monocytogenes*. Mφ, macrophage; PMN, polymorphonuclear leukocyte. (Illustration by P.W. Yu.)

expression of IFN γ , IL-1 β , TNF α , granulocyte-macrophage colony-stimulating factor (GM-CSF), and other cytokines. IFN γ and TNF α appear to be perhaps the most important endogenous cytokines for resistance to listeriosis. Resistance to *L. monocytogenes* infection is consistent with the T_H1–T_H2 cytokine paradigm, as IFN γ (T_H1) is required for resistance, and both interleukin 4 (IL-4) and IL-10 (T_H2) impair resistance. The pivotal role of macrophage production of IL-12 in determining whether a T_H1 or T_H2 cytokine response will predominate was first delineated in an *L. monocytogenes* infection model.

Administration of a wide array of recombinant cytokines, particularly those that potentiate the inflammatory response (e.g. IFN γ , TNF α , IL-1 β , IL-12), enhance resistance to *L. monocytogenes* infection.

Evasive strategies of the organism

L. monocytogenes can invade and multiply within mononuclear phagocytes, hepatocytes and epithelial cells (Figure 2). It produces a series of virulence factors that enable it to invade cells, multiply intracellularly, and directly invade adjacent cells. This results in foci of infection that can be readily demonstrated by histopathology. These virulence determinants are positively regulated in a coordinated manner by the product of the *prfA* gene. A surface protein, called internalin, facilitates adherence and invasion of mammalian cells via interaction with E-cadherin (Figure 2, step A). Another *L. monocytogenes* protein (p60), which can be found on the bacterial surface or secreted into the culture medium, also affects interaction with mammalian cells. Once it invades a mammalian cell, *L. monocytogenes* escapes from the

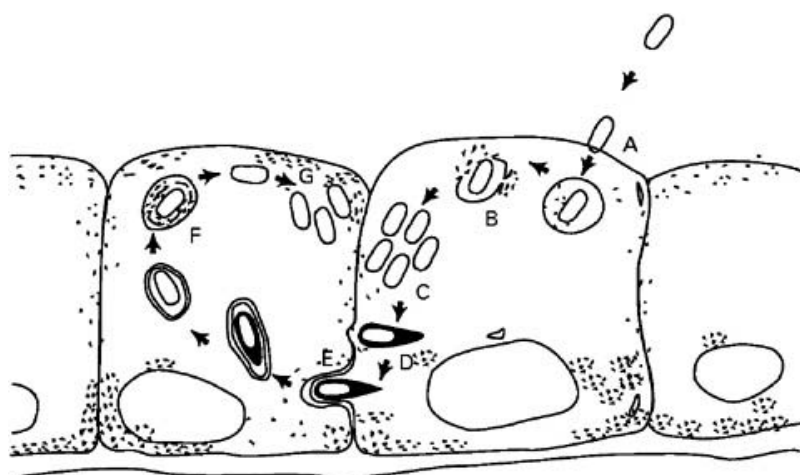


Figure 2 Stages involved in the invasion and multiplication of *L. monocytogenes*. See text for details.

membrane-bound phagosome by the action of its hemolysin and two phospholipases C, one of which has broad activity and the other of which is phosphatidylinositol specific (step B). The listeriae then multiply freely within the cytoplasm (step C) and express a surface protein, called actA, that serves as a nucleation point for the attachment of actin filaments (step D). This occurs in a unidirectional manner such that new actin subunits are continually placed on the surface of the bacterium, resulting in formation of a long (up to 40 μm) actin tail. These actin tails can propel *L. monocytogenes* at speeds of up to 1 $\mu\text{m s}^{-1}$, driving some of them through the cytoplasmic membrane of the original cell and into the cytoplasm of an adjacent cell (step E). The listeriae then escape from the double membrane-bound phagosome in which they find themselves (step F), and repeat the replication cycle (step G). Cellular and humoral immune responses to the hemolysin, internalin, and actA have been demonstrated. Which of these responses is most important for restricting the multiplication of the organism is not clear.

Vaccines

Vaccination of experimental animals is typically achieved by injection of a sublethal dose of viable listeriae. Surviving mice are highly resistant to rechallenge for several months, and then resistance gradually wanes. Infection with other facultative intracellular pathogens may induce nonspecific resistance to listeriosis and vice versa. Injection of killed listeriae generally does not confer substantial immunity, although vaccination with subunits of *L. monocytogenes* in adjuvant has been reported to provide limited protection against experimental challenge. Immunization with large numbers of avirulent *L. monocytogenes*, or with other *Listeria* species, may induce a transient increase in resistance. Use of atten-

uated vaccines in sheep has been reported to lower the incidence of spontaneous listeriosis.

Other strategies have been shown to transiently increase anti-*Listeria* resistance in mice. Various biological response modifiers (e.g. killed *Lactobacillus casei*, trehalose dimycolate, muramyl dipeptide) have been reported to stimulate anti-*Listeria* resistance, perhaps via release of various cytokines. Prophylactic administration of a number of recombinant cytokines (e.g. IFN γ , IFN β , IL-1 α , IL-2, TNF α , GM-CSF and others) afford short-lived enhancement of anti-*Listeria* resistance. The ability of *L. monocytogenes* to multiply freely in the cytoplasm of infected cells has been exploited as a vehicle for eliciting a class I major histocompatibility complex (MHC)-dependent cytotoxic T cell response against other antigens. Recombinant *L. monocytogenes* has been used as a vaccine vector to elicit protective immunity against experimental infection with several viruses, and against tumor cells expressing viral antigens.

See also: Cell-mediated immunity; Helper T lymphocytes; Cytokines; Cytotoxic T lymphocytes; Macrophage activation; MHC restriction; Neutrophils; Vaccines.

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LYMPHATIC SYSTEM

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The lymphatic system is an integral part of the blood vascular system of vertebrates, but in mammals it also plays a crucial part in the transport and distribution of antigens, antibodies and immunologically competent cells. Similarly, it is important in the dissemination of tumor cells and in immune reactions to them.

The word 'lymph' simply means 'a clear fluid' and its existence as an internal body fluid, distinct from blood and other humors, was appreciated by physicians of the early Mediterranean civilizations. A scientific understanding of the composition and formation of lymph only became possible during the last part of the nineteenth century and it was not until the beginning of the twentieth century that Starling gave the subject a sound physiological basis.

In any animal with a substantial blood pressure, fluid and some plasma proteins are forced to leak continually through the walls of the blood capillaries. The primary function of the lymphatic system is to collect this proteinaceous fluid and return it to the blood vascular system, and thus maintain the volume of the circulating blood plasma. The lymphatic system thus transports extravascular macromolecules and it follows that it plays a major part in the transport and distribution of antigenic material. In mammals, the lymphatic vessels that convey the lymph from the peripheral tissues back to the great veins at the root of the neck are interrupted at various points by secondary lymphoid organs, the lymph nodes.

These are richly vascularized structures composed of a meshwork of collagen fibers which are covered with reticular cells. In addition, they are the site of the large-scale extravasation of lymphocytes. The reticular cells enable the node to act as a filter and to arrest much antigenic material that is in the lymph, while the incoming B and T lymphocytes from the blood react to the antigen and transform respectively into antibody-forming cells and activated helper or cytotoxic lymphocytes. Many of these are discharged into the lymph efferent from the nodes and so become distributed systematically.

Methodology

Most of what is known about the function of the lymphatic system has come from experimental work on animals. Although there were some notable experiments in the eighteenth, nineteenth and early twentieth centuries, the procedures were not easy until small, flexible, plastic cannulae became available in the mid-twentieth century. This made it possible to cannulate the major lymph trunks, such as the thoracic duct in rats, guinea pigs, and even mice, and to collect substantial volumes of lymph.

A major problem with this procedure was that it required relatively deep anesthesia as well as the postoperative restraint of the animals in specially designed cages. These factors induce major perturbations in the function of the blood vascular system and the levels of the adrenocortical hormones. Also,

in the ensuing days, the loss to the animals of substantial amounts of fluid, protein, electrolytes and cells rapidly produce a state of metabolic chaos unless elaborate facilities are at hand to make good the deficits at physiological rates. Although such preparations can be a useful source of lymph cells, their value as physiological models is limited.

In the early 1960s Professor Bede Morris of the Australian National University initiated research into the lymphatic physiology of ruminant species, particularly sheep. The latter are docile animals that make no attempt to interfere with cannulae that have been inserted into them; they need no restraint and can be kept in ordinary pens to which they are accustomed. Also, the cannulation of a single peripheral lymph duct causes an insignificant metabolic deficit so that lymph from individual organs can be collected for periods of weeks from unanesthetized animals under physiological conditions. Because of the structure of the ovine placenta, it is even possible to cannulate lymphatic vessels of fetuses *in utero*. These types of preparations have yielded much useful and novel information, and will probably continue to do so. However, the risks of extrapolating data from a single species should not be overlooked.

Some useful data have been obtained from humans but ethical and technical difficulties have prevented systematic studies.

It may be worth noting here that while lymph always contains some lymphocytes, and may occasionally contain all other classes of blood leukocytes as well as some red cells, it never contains platelets. It does, however, contain fibrinogen and will clot unless anticoagulants are added to the containers into which it is collected.

The formation and composition of lymph

Lymph begins as an ultrafiltrate of blood at the arteriolar, high-pressure region of the capillary. Towards the venular end of the capillary, the hydrostatic pressure of the blood is much lower and this, together with the osmotic 'pull' of the blood plasma proteins, allows some of the water and crystalloids of the ultrafiltrate to be reabsorbed (Figure 1). However, the protein component of the ultrafiltrate is not reabsorbed and remains in the tissue spaces until it is carried away in the lymph. By virtue of its own colloid osmotic pressure (sometimes referred to as the 'oncotic' pressure) the protein retains some water and crystalloids with it, and it is this somewhat concentrated ultrafiltrate that constitutes the tissue fluid proper, which is the fluid that enters the peripheral lymphatics.

The composition of the tissue fluid varies some-

what from tissue to tissue, and with the level of activity, and hence blood pressure, of the individual. In general terms, the total protein concentration of the tissue fluid is about 25–30% of that of the blood plasma. It contains the whole range of blood plasma proteins, but their relative concentrations are different from those of the blood. This is because the ultrafiltration process functions as a molecular sieve so that smaller molecules, such as albumin (66 kDa) pass through relatively easily, but immunoglobulin G (IgG) is less well represented (150–170 kDa), and the large IgM molecule (970 kDa) is present at a concentration less than 8% of the blood plasma value.

The physiological basis for ultrafiltration and molecular sieving is unknown. It is explained best by the 'Pappenheimer pore theory', which postulates that the capillary endothelium has the characteristics of a semipermeable membrane provided with 'pores' of graded sizes. The theory envisages that there are more small pores through which albumin can pass than large ones that allow the escape of, for example, IgM. However, the pore theory is really a mathematical abstraction used to explain experimental results and it was not meant to imply that obvious pores exist as observable microanatomical entities. The structural basis of the 'pores' is debatable. Some workers believe that they consist of transient gaps between endothelial cells. Others believe that the real ultrafiltration apparatus resides in the basement membrane which surrounds the outer surface of the capillary endothelium. It has also been proposed that the extravasation of fluid and protein is brought about by the transcytosis of endocytic vesicles that traffic across the cytoplasm of the endothelial cell. So far, there is really no satisfactory detailed explanation of the observation that the ease with which plasma proteins escape from the circulating blood, across the capillary wall and into the tissue fluid, is inversely proportional to their mean diffusion diameters.

Despite these conceptual difficulties, it is relatively easy to measure the permeability characteristics of a given capillary bed. This is done by injecting intravenously a dose of plasma protein, usually albumin, labeled with, for example, a radioisotope. Lymph is then collected from the region under study at appropriate intervals and compared with corresponding samples of venous blood plasma. When the amount of radioactivity per unit weight of protein in the lymph is equal to that in the blood, 'equilibration' is said to have occurred. The elapsed time between the initial injection and the equilibration is known as the 'equilibration time' and gives a relative measure of the permeability of the particular capillary bed.

For most mammalian somatic tissues, the equili-

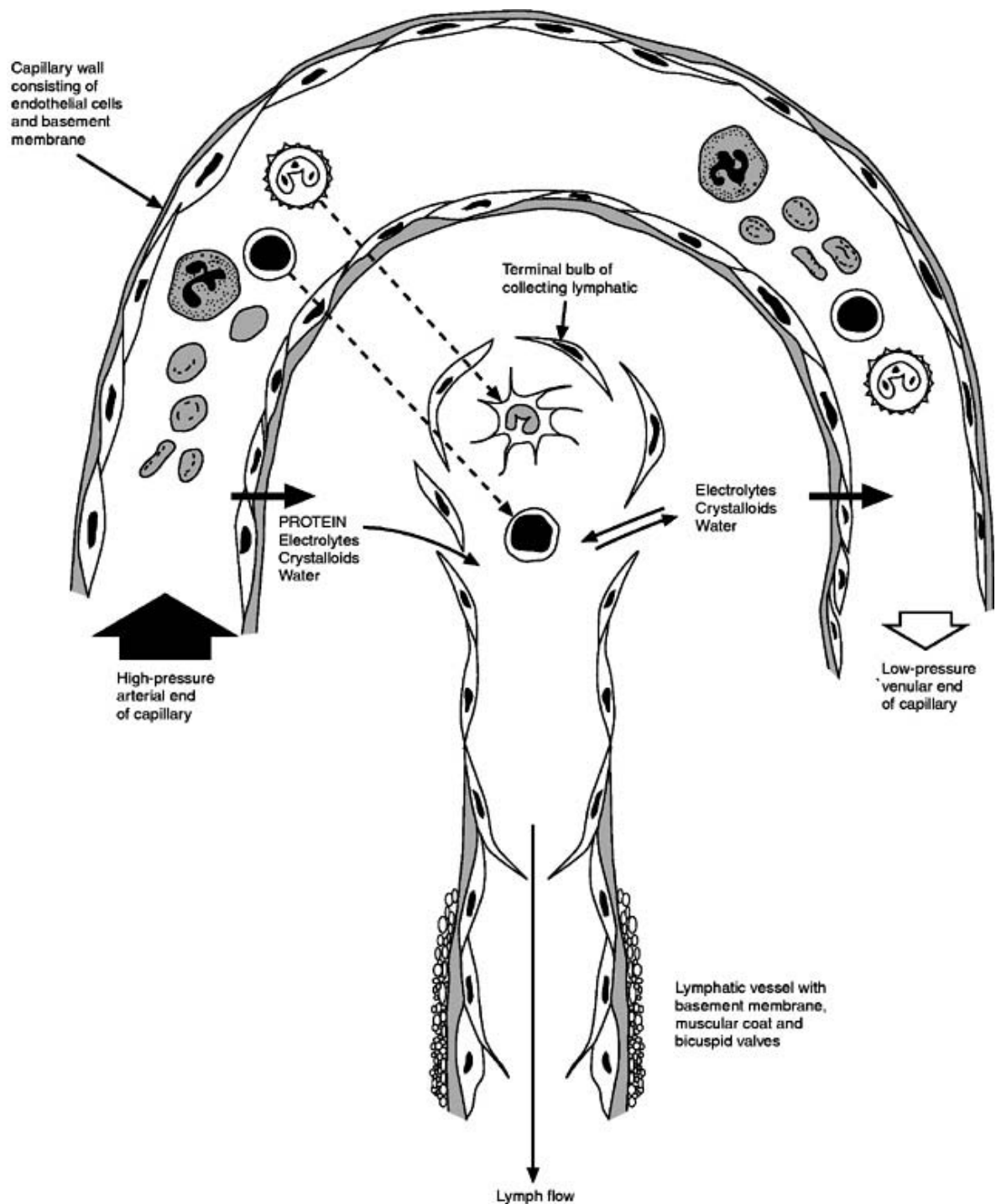


Figure 1 The principles of lymph formation. The high hydrostatic pressure at the arterial end of the capillary forces proteinaceous fluid through the capillary wall. At the venular end the hydrostatic pressure is low, and the colloid osmotic pressure of the plasma proteins causes the reabsorption of water, electrolytes and crystalloids. The protein cannot be reabsorbed and together with some water and solutes, it is drained away by the lymph. Note that the diagram is not to scale and in reality the diameter of the capillary just permits the passage of a red cell, while the larger white cells have to deform to pass through. This imposes a resistance to flow which helps to create the pressure drop between the arterial and venous ends of the capillary. (Reproduced with permission from Hall JG (1988) Lymphatic system in drug targeting. In: Gregoriadis G and Poste G (eds) *Targeting of Drugs, Anatomical and Physiological Considerations*, pp. 15–28. NATO ASI series *Life Sciences*, vol 155. New York: Plenum Press.)

bration time for albumin is between 12 and 24 h, depending on the activity of the individual. There are, however, some major variations. For example, the endothelium of the portal sinusoids in the liver is fenestrated by ultrastructurally observable discontinuities. For this reason, there is no continuous barrier to impede the egress of the plasma proteins; the hepatic lymph plasma has a protein concentration almost as high as that of the blood, and the equilibration time is only 1–2 h.

At the other end of the scale is the central nervous system, which has no conventional lymphatic apparatus at all. The endothelial cells of the intracerebral capillaries are very tightly joined, so virtually no protein escapes. What little protein there is in the cerebrospinal fluid is provided by specialized tufts of vasculature in the choroid plexuses that lie on the floor of the ventricles. Nonetheless, there is a flow of fluid into the confines of the skull and an equivalent amount has to be removed to maintain the status quo.

Fluid is removed in two ways. First, small villous structures from the arachnoid membrane of the brain bulge into the main venous sinuses, and these arachnoid villi can be envisaged as a sort of private lymphatic system for the brain because they transmit excess fluid back into the venous blood. Second, some fluid tracks down the sheaths of the cranial nerves, particularly the olfactory nerves, and so gains entry into the nasal submucosae, where it is absorbed by conventional lymphatic vessels.

Fluid exchange in the lungs presents particular problems. The perfusion pressure is low, the blood pressure in the pulmonary arteries being much lower than in the arteries of the systemic circulation. Accordingly, in spite of the large blood flow, relatively little lymph is formed. Similarly, the low blood pressure makes the lung vasculature vulnerable to embolic phenomena. For example, white cells or tumor cells that are infused intravenously may be arrested temporarily in the lungs because there is not enough pressure to push them through the capillaries at the usual rate.

The movement and propulsion of lymph

Tissue fluid is formed continually, and this causes the tissue tension to rise. As it does so, fluid is forced into the collecting bulbs of the most peripheral lymphatic capillaries. These structures have a rather scanty basement membrane, to which the endothelial cells are loosely attached; these cells act as 'flap valves' which favor the entry, rather than the exit, of fluid. The confluence of lymphatic capillaries forms larger vessels which have a more definite structure. The

basement membrane is continuous and surrounded by coats of smooth muscle, and the vessels are provided with numerous bicuspid valves which prevent retrograde flow (see **Figure 1**). The muscular coats of the lymphatic vessels exhibit intrinsic rhythmic contractions; these generate pulse pressures of several mm Hg, and force the lymph in a central direction. The rate of pulsation of the lymphatic vessels increases in parallel with the pressure inside them, and so a mechanism exists for transporting the lymph at a rate proportional to its rate of formation.

The mechanisms that modulate the lymphatic pulse have yet to be defined accurately; the action of adrenergic nerves, and the local synthesis of prostaglandins and nitric oxide have all been proposed. However the contraction waves themselves seem to be an intrinsic response to the stretching of the muscle coats.

In considering the flow of lymph, it is necessary to distinguish critically between those factors that increase the formation of lymph, and those that actually move it. For example, when a limb is at rest, the blood flow is low and little lymph is formed, there is no fluid to distend the lymphatics and provoke a contraction wave in the larger lymphatic vessels, and so there is little observable lymph flow. If the limb is moved, either actively or passively, some residual tissue fluid is forced into the lymphatics and so promotes active contraction in the lymphatic vessels. Continued passive movements soon exhaust the supply of tissue fluid and lymph flow again becomes sluggish, whereas active movements promote greater blood flow, the formation of more tissue fluid, and thus a continuous flow of lymph.

Anatomical compartments

The lymph of mammals is classified as being peripheral, intermediate or central. Peripheral lymph is lymph which has not yet passed through a lymph node. Intermediate lymph is lymph contained in vessels between lymph nodes. Central lymph is lymph contained in the major lymph trunks like the intestinal, thoracic, right lymph duct or deep cervical duct. Central lymph does not pass through any lymph nodes before it enters the great veins at the root of the neck (**Figure 2**).

Peripheral lymph

True peripheral lymph has a very low content of white cells, usually well under 1000 mm⁻³. Most of these are small lymphocytes of which some 85% are T cells. However, peripheral lymph is distinguished by the presence of 'veiled' dendritic cells, which account for about 10% of the cellular content

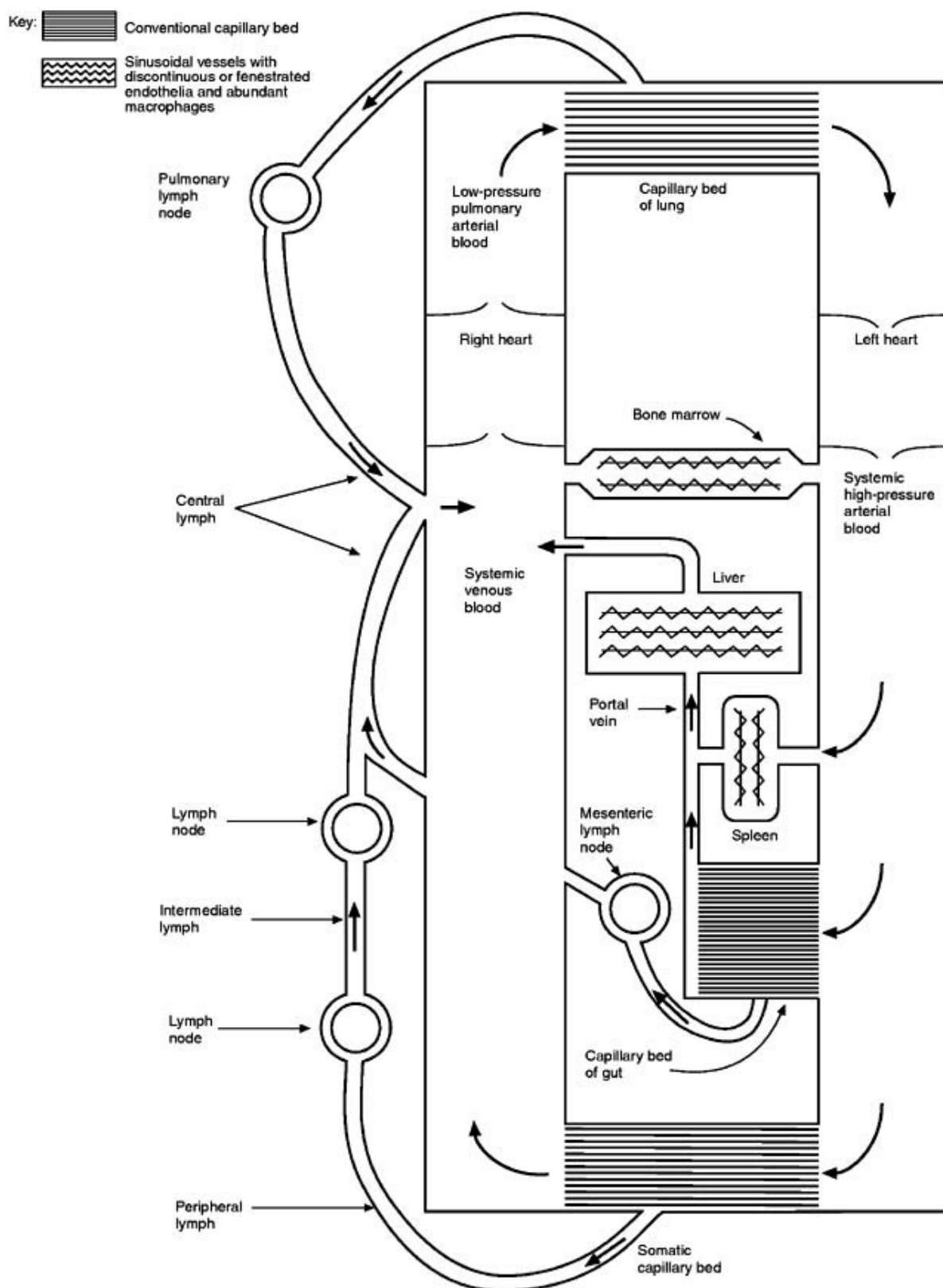


Figure 2 Diagram to show the integration of the blood and lymphatic vascular systems. (Reproduced with permission from Hall JG (1988) Lymphatic system in drug targeting. In: Gregoriadis G and Poste G (eds) *Targeting of Drugs, Anatomical and Physiological Considerations*, pp. 15–28. NATO ASI series *Life Sciences*, vol 155. New York: Plenum Press.)

(Figure 3). Their main function is probably the induction of T cell-mediated immunity.

Intermediate lymph

Intermediate lymph is populated by large numbers (up to $20\,000\text{ mm}^{-3}$) of small lymphocytes, some 75% of which are T cells. Nearly all of these are recirculating lymphocytes that have extravasated from specialized postcapillary venules in the node. In rodents and humans, these specialized venules are recognizable in histological sections because of their cuboidal or 'high' endothelium (high endothelial venules, HEV) between which extravasating lymphocytes may be identified. This high endothelium is not seen in the lymph nodes of ruminants or athymic rats, even though the recirculation of lymphocytes is taking place. Lymphocyte extravasation in sheep proceeds via paracortical venules in lymph nodes. The function of these venules appears to be analogous to HEV in nonruminant species.

Hardly any of the dendritic cells from peripheral lymph appear in intermediate lymph. The majority are retained by the first node to which the lymph stream carries them. In this context, it should be remembered that more deeply seated nodes will receive two sorts of afferent lymph. One will be lymphocyte-rich lymph efferent from more peripheral nodes; the other will be genuine hypocellular, peripheral lymph formed in the adjacent tissues.

The lymphatic system in the gut has a rather special organization. There is an abundant extravasation of lymphocytes in the wall of the gut. This occurs diffusely throughout the length of the intestine, but also in macroscopic aggregates of subepi-

thelial lymphoid follicles that occur at intervals along the antimesenteric aspect of the small gut. These structures, the Peyer's patches, are believed to provide the B and T cells necessary for inducing immune responses which result in the production of IgA, the principal immunoglobulin in the secretion which protects all mucosal surfaces. In ruminants, the ileal Peyer's patch is also the site at which immature B cells undergo proliferation and the genetic rearrangements that endow them with the potential for Ig synthesis. In all mammalian species, these special features endow peripheral intestinal lymph with a content of lymphocytes that is about 50 times greater than that of peripheral lymph from any other organ. The lymph also contains substantial numbers of dendritic cells and, in postnatal animals whose guts have been colonized by bacteria, significant numbers of immunoblasts are also present. The regional nodes of the gut – the mesenteric nodes – are thus perhaps unique in receiving a supply of hypercellular afferent lymph that is already immunologically activated to some degree. It may be, however, that the same applies to those cervical nodes which receive lymph from Waldeyer's ring (i.e. tonsils, adenoids, etc.) but direct evidence is lacking.

There are other noteworthy features of the intestinal lymphatic system. The intestine is the site of the absorption of fatty acids from the diet, and these are processed into minute fat globules – the chylomicra – by the enterocytes which then discharge them into the lymph and give it its characteristic milky appearance.

Central lymph

Central lymph is merely a conglomerate of all the intermediate lymph contained in the various lymphatics that unite to form the major lymph trunks. For the practical purposes of immunology, thoracic duct lymph is the most commonly collected example. It is as well to remember that the thoracic duct is the final common pathway of all lymph generated below the level of the diaphragm, and the majority of this is lymph from the intestine. In sheep, the small lymphocytes from intestinal lymph can be shown to recirculate preferentially through the gut, while those from nonintestinal lymph recirculate mainly through other tissues. At present there is no direct evidence that this distinction applies to humans and rodents. There is less uncertainty about the behavior of the large lymphocytes (immunoblasts) which are present in intestinal, and thus thoracic duct lymph. After being discharged into the blood they extravasate abundantly and rapidly in the lamina propria of the small gut. Many of them can be shown to transform into classical plasma cells which synthesize and



Figure 3 Phase-contrast photomicrograph (approximately $\times 1500$) of small lymphocytes and 'veiled' dendritic cells in peripheral lymph from the leg of a sheep.

secrete antibody. Much of this is of the IgA class; that which is not immediately secreted into the lumen of the gut is swept up into the intestinal lymphatics, conveyed to the blood and transported rapidly into the bile by the active transcytotic vesicles in the hepatocytes.

Transport of antigens

The intravenous injection of a dose of antigen may be experimentally convenient but it is rather unnatural. Apart from some arthropod-borne parasitic and viral diseases, secondary septicemia, etc., the presence in the blood vascular system of significant amounts of microbial antigens is fortunately a rare event. Most naturally occurring infections begin with the penetration into the tissue fluid, via the skin or mucosae, of invading microorganisms. In such cases, the exogenous material is swept up into the lymphatics and conveyed to the regional node. This mechanism is mimicked by injecting antigens either intradermally or subcutaneously.

A foreign soluble protein that finds its way into the tissue fluid will be conveyed almost quantitatively to the regional nodes. Unless pre-existing specific antibodies convert it into immune complexes, or unless it is a lectin or toxin that binds with high affinity to mammalian cells, very little of it will be retained in the node. Most of it will pass through the lymph nodes and enter the central lymph, and thus the blood. For this reason, many ordinary soluble proteins tend to be poorly immunogenic unless they are first aggregated and/or mixed with adjuvant. In the presence of specific antibody, the antigen will accumulate in germinal centers of lymphoid tissues, where it will be presented to B lymphocytes in the form of antigen-antibody complexes on follicular dendritic cells.

Particulate material such as bacteria and viruses tend to be filtered out of the lymph stream more efficiently by the phagocytes of the regional node. This is partly because particles are more susceptible to mechanical filtration, and partly because of the presence of 'natural' antibodies that can react with such materials. Of course, there are exceptions, such as certain pathogenic microorganisms which, almost by definition, are able to elude host defenses unless a state of specific immunity has been induced previously.

Antigens and cell traffic

The arrival at a lymph node, via its afferent lymphatics, of an immunogenic dose of antigen, sets in train an enormously complicated series of cellular

events. In the first 2–3 days after the antigenic stimulus, the output of recirculating lymphocytes in the efferent lymph increases about threefold from a basal level of some $3 \times 10^7 \text{ h}^{-1} \text{ g node weight}^{-1}$. This phase is generally regarded as a phase of 'recruitment' during which those lymphocytes with appropriate immunological specificity are selected from the larger numbers made available by increased blood flow through the node. The lymphocytes thus selected react to the antigens and cytokines present and transform into immunoblasts in the paracortex of the node. Some of these blast cells mature and carry out their various functions within the node itself, but others are discharged into the efferent lymph where, about 4 days after the initial stimulus, they may account for up to 30% of all the cells. These free-floating immunoblasts are able to colonize other lymphoid tissues to which the lymph, and later blood, may carry them. In this way, a state of systemic humoral and cell-mediated immunity may be established even when the antigen itself (which may be potentially pathogenic) remains localized in the node which first received it.

In most mammalian species, the antibodies and cells produced in a lymph node during an immune response are dependent absolutely on the lymphatic system for their initial transport. In pigs, however, the cells trafficking through lymph nodes are able to leave by direct entry into the intranodal blood vasculature. Consequently, there are few lymphocytes in the intermediate and central lymph of pigs. Such exceptions should alert us to the fact that facile generalizations about the behavior of lymphocytes and lymphatics can be misleading given that there are significant differences between mammalian species in the structure and function of the lymphatic apparatus.

See also: Antigen, entry into the body; Lymph nodes; Lymphocyte trafficking; Lymphocytes; Mononuclear phagocyte system; Mucosa-associated lymphoid tissue (MALT); Natural antibodies; Plasma; Porcine immune system; Recruitment.

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LYMPH NODES

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Lymph nodes are encapsulated organs ranging in size (in humans) from a few millimeters to more than a centimeter, and generally ovoid in shape. They are found as groups of nodules along the lymphatic vessels draining superficial areas of the skin or deep viscera. The bases of the limbs, the neck, the retroperitoneal areas in the pelvis and abdomen, and the mediastinum are sites especially rich in lymph nodes (Figure 1).

Lymph nodes are peripheral lymphoid organs colonized by bone marrow-derived B cells and thymus-derived T cells which seed into distinct zones of the lymphoid parenchyma. The organization of the lymph node must favor the interactions between antigens and lymphocytes equipped with specific antigen receptors. For this reason, lymph nodes contain numerous cells capable of processing and presenting antigens (macrophages, dendritic cells, interdigitating cells and veiled cells) and are continuously renewing their cell populations through active lymphocyte and accessory cell trafficking.

Both humoral and cell-mediated immune responses can occur in lymph nodes. Consequently, activated lymphocytes and T or B effector cells are frequently detected. Following an immune response to antigen, memory T or B cells are also generated which, similar to the unstimulated lymphocytes, are located in distinct zones of the lymph node.

Structure of the lymph node

A lymph node consists of capsule, reticulum, lymphoid tissue with lymphocytes, blood and nerve supplies (Figure 2).

The capsule is composed of dense collagenous connective tissue. It has a broad convex surface, and at one point, a deep indentation, the hilus. Afferent lymphatic vessels pass through the convex surface of the capsule and empty into the subcapsular (or marginal) sinus. One efferent lymphatic vessel leaves at the hilus where blood vessels and nerves enter and exit. Connective tissue projections of the capsule into the node are seen as the trabeculae.

The reticulum is a delicate meshwork enclosed by the capsule and trabeculae and consists of reticular cells and fibers. The reticular cells are large, branched fibroblasts which produce the reticular fibers.

Lymphocytes and other free cells lie in this meshwork and form the lymphoid tissue or lymph node parenchyma. Three distinct regions of the lymph node parenchyma extend from the capsule in towards the hilus. These are termed 'cortex', 'paracortex' and 'medulla'.

The cortex is largely occupied by lymphocyte aggregates forming nodules or follicles which are usually spherical or ovoid. Follicles may contain

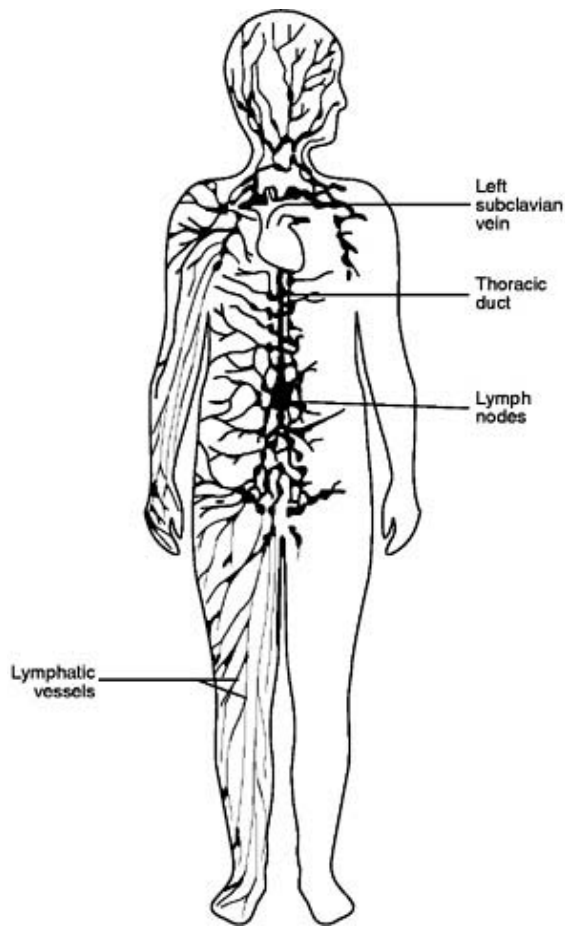


Figure 1 The lymph node network. (Reproduced with permission from Roitt IM, Brostoff J and Male D (eds) (1998) *Immunology*, 5th edn. London: Mosby.)

tightly packed small lymphocytes and are termed 'primary follicles'. Alternatively, they may contain a central area consisting of larger lymphoid cells, macrophages and follicular dendritic cells. This central area is termed a 'germinal center' and the cuff of small lymphocytes that surround it forms the mantle zone. Nodules that develop a germinal center are termed 'secondary follicles'. Fully developed secondary follicles are composed of germinal center lymphocytes, mantle zone lymphocytes and macrophages/antigen-presenting (dendritic) cells. The germinal center is a site of active cell proliferation and cell death. A schematic model of the germinal center is shown in Figure 3. There are three major zones: a dark zone, a basal light zone and an apical light zone, and these are predominantly occupied by centroblasts, centrocytes and secondary blasts respectively. Primary B cell blasts which carry surface immunoglobulin receptors (sIg⁺) enter the follicle and leave as memory B cells or antibody-forming cells (AFCs). Antigen-presenting follicular dendritic

cells are mainly found in the two deeper zones, and cell death by apoptosis occurs mainly in the basal light zone where tingible body macrophages are also located. The lymphoid area between adjacent cortical follicles is called the 'interfollicular cortical tissue'.

The paracortex (or deep cortex) is a lymphoid area which is located immediately underneath the cortex, between the follicles and the medulla. Lymphocytes and lymphoblasts are densely packed in this area which displays a uniform lymphoid structure. Macrophages and antigen-presenting cells are interdispersed amongst the lymphocytes. Antigen-presenting cells are predominantly interdigitating cells similar to those found in the medulla of the thymic lobules. These cells, which belong to the monocyte/macrophage lineage, express membrane class II major histocompatibility complex (MHC) molecules at high density, have poor phagocytic ability and sparse lysosomes, but are very effective in presenting antigens to T cells. Other antigen-presenting cells are found in lymph vessels draining skin areas undergoing inflammation or antigenic stimulation. These are the 'veiled cells'. It appears that these veiled cells are Langerhans cells, which migrate from the epidermis where they are identifiable through the presence of Birbeck granules in their cytoplasm. Veiled cells reach the lymph node and seed into the paracortex where they lose their Birbeck granules and become interdigitating cells.

Another structural feature of the paracortex is the presence of high endothelial venules. These postcapillary venules are the sites where lymphocytes leave the circulation and enter the lymphoid tissue. The wall of these vessels is often infiltrated by small lymphocytes on their way from the blood into the parenchyma of the node. The lymph node medulla consists of cords of cells separated by lymphatic sinuses. Medullary cords are rich in plasma cells, macrophages and small lymphocytes.

Afferent lymphatic vessels enter the lymph node and empty into the large subcapsular sinus which lies directly beneath the capsule. Cortical sinuses run radially as extensions of the subcapsular sinus along the trabeculae, and continue between the medullary cords to converge into the terminal sinus, in the hilus region. One efferent lymphatic channel leaves the node emerging from the terminal sinus. Lymphatic sinuses are lined by endothelial cells and by macrophages termed sinus macrophages which become activated and increase in number during an inflammatory response.

Blood vessels enter the lymph node at the hilus. Arterioles reach the cortex through the trabeculae and break up into a rich capillary plexus. These ves-

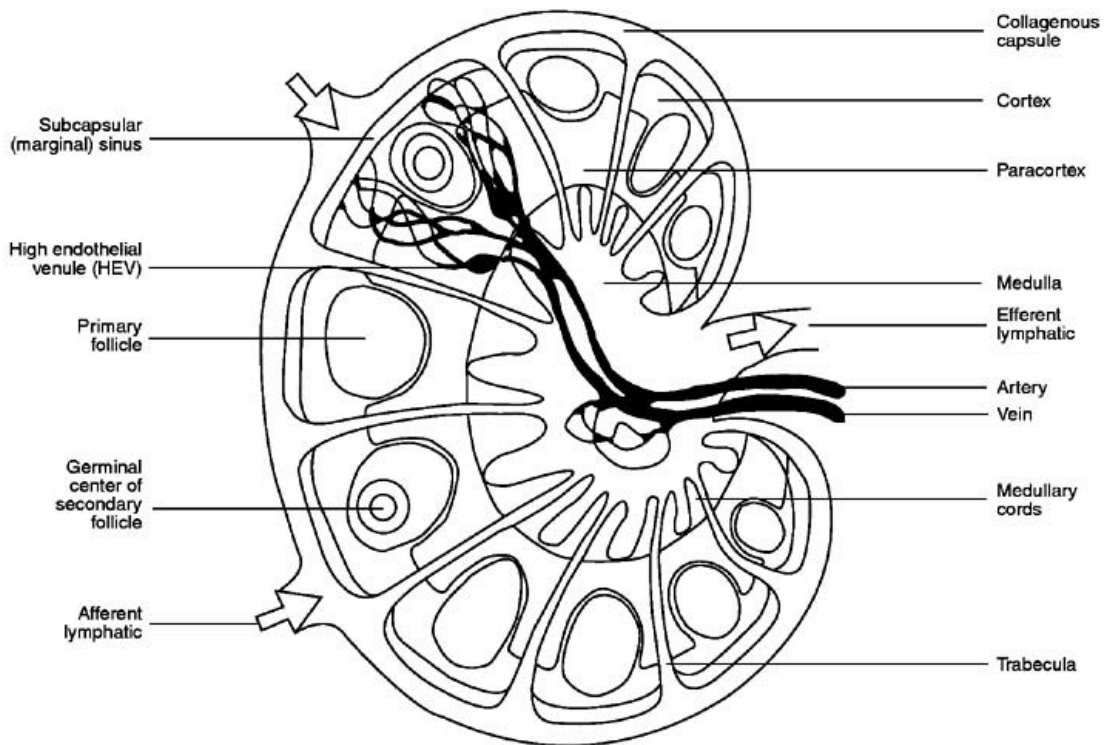


Figure 2 The structure of a lymph node. (Reproduced with permission from Roitt IM, Brostoff J and Male D (eds) (1998) *Immunology*, 5th edn. London: Mosby.)

sels group into venules, tributaries of veins which run from the cortex into the medulla and then leave the node via the hilus.

Functional anatomy of the lymph node

A correlation between structure and function of the lymph node is shown in Figure 4. The lymph node cortex is generally regarded as a B-dependent area. Virgin B cells are found in the primary follicles of the cortex, whereas memory B cells account for the majority of the germinal center cells (centroblasts and centrocytes). Mature B lymphocytes expressing surface immunoglobulin M (IgM) and IgD are predominant in the mantle zone of secondary follicles. B and T cells are found together in the interfollicular cortical tissue and a few T cells with 'helper' phenotype (CD4⁺) are also detected in the germinal centers. Macrophages line the cortical lymphatic sinuses and are interdispersed in the follicular lymphoid tissue. Other macrophages are located within the secondary follicles, particularly between the mantle zone and the germinal center. Follicular dendritic cells are the antigen-presenting cells found in the germinal centers, whereas rare interdigitating cells are present in the interfollicular lymphoid tissue of the cortex.

Resting and activated T cells are the major components of the paracortex, which is therefore referred

to as a T-dependent zone. Antigens are presented to T lymphocytes by interdigitating cells. These are Langerhans cells which have migrated from the epidermis together with other antigen-presenting cells which reach the lymph node via the afferent lymphatic vessels. The paracortex is also an area of active cell traffic due to the presence of numerous high endothelial venules which are the major route for lymphocyte entry from the circulation into the lymph node.

The medulla is mainly a B-dependent zone, although T cells and macrophages are frequently detected in the medullary cords. Medullary B lymphocytes are antibody-producing effector cells, i.e. plasmablasts and mature plasma cells.

Antigens which have penetrated the surface defense barriers usually reach the lymph nodes via the afferent lymphatic vessels (Figure 4). Macrophages lining the lymphatic sinuses may transfer antigens to the T-independent and the T-dependent zones where cells equipped with specific antigen receptors can recognize them. Active recirculation of lymphocytes increases the chance of encounter between antigen and antigen-specific cells.

Stimulation of lymphocytes within the primary follicles by B-dependent antigens results in the development of secondary follicles with formation of germinal centers where antigens are sequestered and

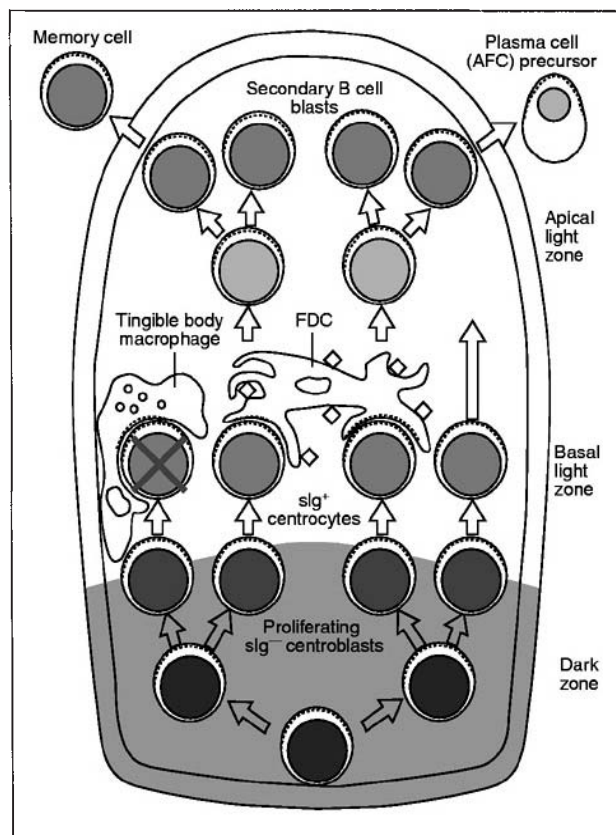


Figure 3 Schematic organization of the germinal center. AFC, antibody-forming cell; FDC, follicular dendritic cell; slg, surface immunoglobulin. (Reproduced with permission from Roitt IM, Brostoff J and Male D (eds) (1998) *Immunology*, 5th edn. London: Mosby.)

memory B cells home. Antigen-activated B cells migrate to the medullary cords where they differentiate into antibody-producing plasma cells. Antibodies produced in the course of a humoral immune response leave the lymph node predominantly via the efferent lymphatic vessel. T-dependent antigens reach the lymph node via the afferent lymph usually carried by antigen-presenting cells (e.g. veiled cells) which home into the paracortical region and stimulate antigen-specific T cells. T cell proliferation and activation results in enlargement of this T-dependent zone. Activated T cells may also leave the lymph node via the efferent lymphatic vessel. Recently, adhesion molecules on both lymphoid cells and

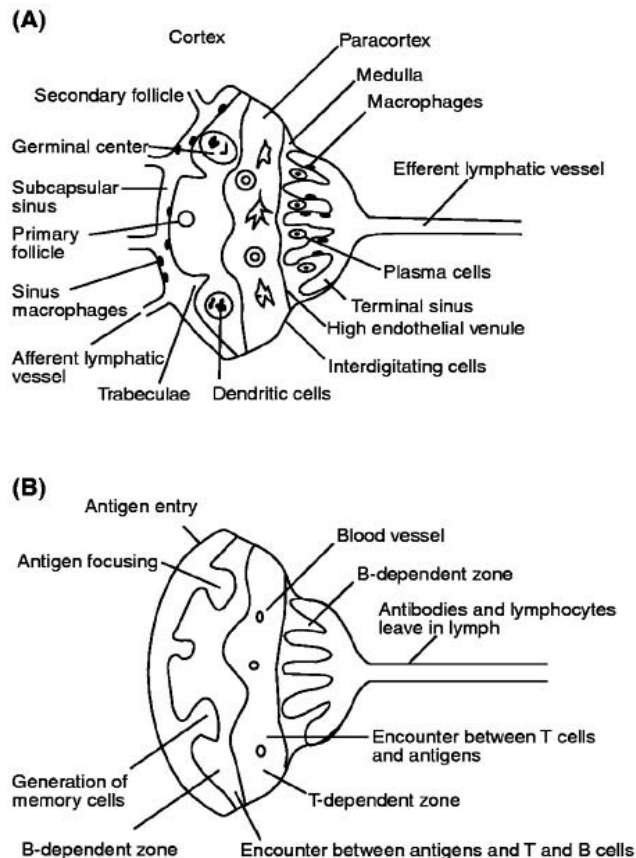


Figure 4 (A) Lymph node structure. (B) Lymph node function.

endothelial cells have been shown to be of major importance in lymphocyte trafficking. These molecules play a role both in the random and nonrandom homing of lymphocytes.

See also: Adhesion molecules; High endothelial venules; Lymphocyte trafficking; Lymphatic system.

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LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN 1 (LFA-1)

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Lymphocyte (leukocyte) function-associated antigen 1 (LFA-1) (CD11a/CD18) is a leukocyte integrin (β_2) adhesion molecule which plays a crucial role in the adhesions of most leukocytes to each other and to other cells, as well as in recognition of some microbes by phagocytes. LFA-1 typically forms adhesions by binding to ICAM-1, a ligand expressed by most tissues, or, on leukocytes or endothelium, also to ICAM-2 or ICAM-3. LFA-1's ability to engage ICAMs can be activated or inactivated within minutes by poorly understood configurational changes in LFA-1 ('inside-out signaling'). Activation is typically under the control of other receptors, for example the T cell antigen receptor in T cells. LFA-1-dependent cell adhesion is temperature, magnesium and cytoskeleton dependent. ICAMs bind to the inserted (I or A) domain of CD11a, where Mg^{2+} plays a critical role bound to a MIDAS motif. LFA-1 itself generates costimulatory signals ('outside-in signaling') that are probably important in leukocyte function. LFA-1 function is essential in most steps in the immune response, both in elicitation and in effector mechanisms, and is a target for pharmacological intervention.

Expression and nomenclature

LFA-1

LFA-1 is expressed on all leukocytes including B and T lymphocytes, monocytes, macrophages, neutrophils, basophils, and eosinophils, but is absent from nonhematopoietic tissues and human platelets. The α chain of LFA-1 is designated CD11a, and the β chain, CD18. However, since historically no other names have been used for LFA-1, both 'LFA-1' and 'CD11a/CD18' are unambiguous.

Leukocyte integrins

The leukocyte integrins are heterodimeric molecules, found on leukocytes, all sharing a common β chain, CD18. In addition to CD11a (LFA-1), they include CD11b (also called complement receptor type 3 or Mac-1, prominent on phagocytes and eosinophils), CD11c and CD11d. The leukocyte integrin α chain genes are linked on human chromosome 16; the gene for CD18 is on human chromosome 21. There are

several other families of integrins, each of which shares a different common β chain. All integrins are adhesion molecules, and all display temperature and divalent cation dependence for function. Although some nonleukocyte integrins require a highly conserved Arg-Gly-Asp (RGD) in their ligands (e.g. fibronectin), none of the ICAMs which bind to LFA-1 contain the RGD sequence.

Structure

CD11a

LFA-1 is an $\alpha_1\beta_1$ noncovalently linked heterodimer, CD11a/CD18 (Figure 1). Mature human CD11a, a glycoprotein of 180 kDa (126 kDa without glycosylation), consists of a 1063 amino acid extracellular domain including 12 N-linked glycosylation sites, a 29 amino acid transmembrane domain, and a 53 amino acid cytoplasmic domain. The extracellular portion contains seven repeats of roughly 50 amino acids each, five of which are in tandem. The extracellular portion also includes a single ~200 amino acid domain similar to domains found in a few nonleukocyte integrins, various receptors found also in invertebrates, some blood proteins, and in collagens and other matrix proteins. This domain is called the A domain because it was the first of five repeated domains, termed simply A through E, identified in von Willebrand factor. In the context of the leukocyte integrins, this same domain is often called the I domain (I for inserted/interactive).

Three of the seven repeated domains include EF-hand-like sequences which are putative divalent cation-binding sites. The I domain includes a different type of divalent cation-binding site called MIDAS (see below). The region containing the I insertion and the repeats has only a single N-linked glycosylation site and a single cysteine, consistent with ligand binding function (see below).

CD18

CD18, the LFA-1 β chain, is a 95 kDa glycoprotein. Mature LFA-1 β consists of a 677 amino acid extracellular domain containing six potential N-glycosylation sites, a 23 amino acid transmembrane domain, and a 46 amino acid cytoplasmic domain. The extra-

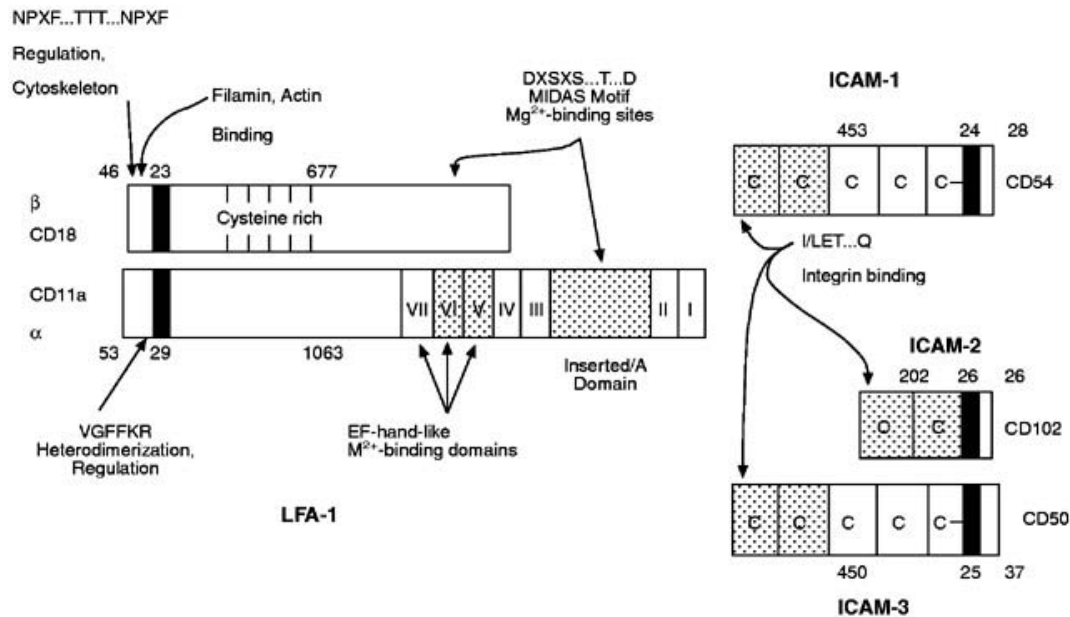


Figure 1 Structure of LFA-1 and its ligands ICAM-1, ICAM-2 and ICAM-3. Stippled regions participate in ligand binding; the MIDAS region of CD18 and other nonstippled regions may also participate. M^{2+} represents Ca^{2+} or Mg^{2+} . Numbers along the chains indicate the number of amino acids in each region. Transmembrane regions are black. C marks domains with similarity to immunoglobulin superfamily constant domains. The β chain of LFA-1 contains four tandem repeats of an eight-cysteine motif. Glycosylation is not shown.

cellular domain contains 56 cysteines, suggesting extensive intrachain disulfide linkage leading to a rigid structure, and may also contain a divalent cation-binding site of the MIDAS type (see below).

Polymorphisms

The mouse and human CD11a polymorphisms which have been noted to date appear to have no functional importance. Some CD18 polymorphisms, however, prevent expression with serious consequences (see LAD-1 below).

Ligands

Three ICAMs

The only ligands identified for LFA-1 (other than microbial ligands, see below) are the intercellular adhesion molecules ICAM-1 (CD54), ICAM-2 (CD102), and ICAM-3 (CD50), all of which bind in a temperature- and magnesium-dependent manner. Antibody blocking experiments are consistent with these being all of LFA-1's ligands. The three ICAMs are each glycosylated integral membrane proteins with a single transmembrane domain, and extracellular domains consisting of tandem head-to-tail immunoglobulin superfamily domains (Figure 1). All have rod-like configurations (ICAM-1 being bent), and in each case, one or two N-terminal domains

(farthest from the membrane) contains the LFA-1-binding site. ICAM-3, at least, can generate cytoplasmic signals, since certain antibodies to ICAM-3 activate LFA-1, VLA-4 and VLA-5.

ICAM-1 and ICAM-3 are heavily glycosylated (90–124 kDa, 55–59 kDa core proteins). ICAM-2 may be as well since it contains six predicted N-linked glycosylation sites. Different cell types glycosylate ICAMs differently. This affects binding of ICAM-1 to CD11b/CD18, but all forms of ICAM-3 appear to bind equally well to CD11a/CD18. CD11b/CD18 binds ICAM-1, but neither CD11b/CD18 nor CD11c/CD18 bind ICAM-3.

ICAM expression

ICAM-1 is expressed in most tissues at low levels, and expression is readily increased by inflammatory cytokines. In contrast, ICAM-2 is expressed primarily on endothelium and leukocytes (except neutrophils), and its expression generally is not responsive to cytokines. ICAM-3 is expressed on leukocytes and Langerhans cells, but not resting nor cytokine-induced endothelium, nor nonhematopoietic tissues. ICAM-3 predominates on thymocytes and resting lymphocytes, which are low in ICAM-1. When neutrophils are activated, they shed the majority of their ICAM-3 into the medium by proteolytic cleavage.

Microbial ligands

CD18 binds to certain bacteria (*Escherichia coli*) and fungi (*Histoplasma capsulatum*) in a temperature- and divalent cation-dependent manner. This is crucial for phagocytosis of the unopsonized forms of these microbes. Rhinovirus binds to ICAM-1 in a divalent cation-independent manner. *Plasmodium falciparum* also binds to ICAM-1, and both microbes depend on this binding for successful infection.

Function

Cell-cell adhesions

Nearly all leukocyte adhesions appear to depend on LFA-1. Some depend almost entirely, and some only slightly. Dependence is evaluated by the ability of LFA-1 α -specific antibody to inhibit the adhesion. In particular, the following adhesions are known to have a major LFA-1 component: T cell–antigen-presenting cell, T cell–B cell (in delivery of help), cytotoxic T lymphocyte (CTL)–target, natural killer cell (NK)–target, and macrophage–tumor cell. Following initial selectin-mediated adhesion and rolling under blood flow shear, neutrophils and other leukocytes employ LFA-1 to strengthen their adhesion to endothelium, and during subsequent transendothelial migration.

LFA-1-dependent adhesions of lymphocytes require magnesium and metabolic energy, and are inhibited by cytochalasins (which induce actin depolymerization). The adhesions are temperature dependent, forming within minutes at 20–37°C, and slowly at 15°C, with no detectable adhesion at 0°C. These characteristics are typical of all integrin-mediated adhesions. In contrast, the component of T lymphocyte adhesion which is mediated by CD2 binding to LFA-3 is independent of temperature and divalent cations.

Immune responses

LFA-1 is crucial to immune responses, consistent with its wide use in critical interactions of immunocytes. A rare congenital deficiency of the LFA-1 β chain, called leukocyte adhesion deficiency type 1 (LAD-1), is characterized, in its severe form, by profound immunodeficiency often fatal during the first 2 years of life. Patients have recurrent infections, granulocytosis, impaired pus formation, and delayed umbilical cord separation. *In vitro*, cells from LAD-1 patients have impaired adhesion of neutrophils and lymphocytes, resulting in deficient neutrophil, NK and CTL function, among others. A defect in the β chain in LAD-1 prevents surface expression of any of the three leukocyte integrins.

Antibodies to CD11a, CD18, or ICAM-1 have shown great promise as immunosuppressive and anti-inflammatory agents in animal models of reperfusion injury after ischemic events, autoimmunity, and transplantation. In contrast, soluble ICAM-1 has been relatively ineffective, perhaps because of low affinity. Although cyclic peptide antagonists of non-leukocyte integrins ('disintegrins') have shown promise in clinical trials, no such peptides are yet identified for CD11a/CD18. Clinical trials with antibodies have not completed phase III (efficacy in placebo-controlled, double-blind studies). Early indications with graft-versus-host (GVH) and cadaveric renal transplants are that antibody to CD11a may be effective as a prophylactic regime, but seems less effective as intervention for acute crises. Anti-ICAM-1 has shown promise in clinical trials with cadaveric renal transplants, liver transplants, and rheumatoid arthritis; trials with stroke and burn are underway. The most dramatic clinical results are for anti-CD11a therapy for bone marrow grafts in immunodeficient children, where the incidence of graft failure was reduced from 90% to 25% (51 cases). Because mouse antibodies, immunogenic in humans, have been used to date, this has prevented assessment of chronic therapeutic regimens; humanized antibodies are under development.

In vitro, LFA-1-specific antibody inhibits mixed leukocyte proliferative responses, T cell proliferation and interleukin 2 (IL-2) production to specific antigen, T cell proliferation to concanavalin A (but not to IL-2, nor B cell proliferation to lipopolysaccharide (LPS)), T cell-dependent antibody responses (but not T cell-independent antibody responses), and CTL and NK-mediated killing.

Molecular basis for function

Effects of antibodies

ICAM-1 and ICAM-3 bind to overlapping but different epitopes on LFA-1. Most antibodies to LFA-1 (either the α or β chains) or ICAM-1 inhibit adhesion function, some distinguishing between different ICAMs. Inhibitory antibodies often bind to CD11a at the I domain or the EF-hand-like repeats. Rare 'functionally neutral' antibodies neither inhibit nor activate adhesion (e.g. TS2/4), and these are thought to bind to nonfunctional epitopes. Some of these bind to the N-terminal region of CD11a (residues 1–57). A few antibodies activate function. Most of these bind to CD18, but rare examples bind to CD11a. Some activating antibodies have been shown to reduce the concentration of magnesium required for half-maximal function.

Divalent cations

LFA-1-dependent adhesion requires magnesium, in contrast to some other integrins and cadherins which are calcium dependent. Physiological $[Mg^{2+}]$, about 3 mM, is sufficient for optimal LFA-1 function with or without calcium. In the absence of magnesium, calcium supports no adhesion, and calcium reduces the efficacy of magnesium. (A synergistic effect of calcium observed under some circumstances could well result from an influx into the cytoplasm, rather than a direct effect on extracellular LFA-1). Manganese supports LFA-1 function with several times the potency of magnesium, and is useful for experimental maneuvers but is not believed to be bound to LFA-1 under normal conditions. The effects of divalent cations on function are complex and probably involve more than one of the five or more putative divalent cation-binding sites in CD11a/CD18.

The I domain and its MIDAS motif

The I domain of CD11a is believed to be the binding site, or a crucial part of the binding site, for ICAMs. A discontinuous magnesium-binding site has been identified in the I domain, termed the MIDAS motif (metal ion-dependent adhesion site). This motif is made up of AspXSerXSer (where X represents any amino acid) plus Thr and Asp in distant parts of the chain, arranged in proximity to the metal ion in the tertiary structure. In CD11a, magnesium is bound to Asp137GlySerMetSer plus Thr206 and Asp239 (Figure 2). CD18 probably also contains a functional MIDAS motif. Mutation of Asp134 or Ser136 in CD18 allowed expression with CD11a or CD11b, but produced loss of ligand binding in each case.

In both CD11a and CD11b, one of the six coordination sites of the magnesium ion is not coordinated by the I domain. This site is likely to bind a negatively charged residue on the ICAM ligand, such as Glu34 in ICAM-1 or Glu37 in ICAM-3, shown by mutagenesis to be critical for binding, and hence probably Glu40 in ICAM-2. It is not clear at present whether this proposed metal-to-ligand bond is essential for ligand binding. Alternatively, the metal may stabilize the necessary binding site, rather than being a critical part of the binding site itself.

Regulation of function

Rapid activation and inactivation

Efficient leukocyte function requires that leukocytes be selective about the cells to which they adhere. Adhesion responses must often be made within seconds to minutes. Early observations showed that mouse CTLs adhere to cognate (specific antigen-

bearing) target cells (forming 'conjugates') but showed little or no adhesion to noncognate targets. The time required for half-maximal conjugate formation was as short as 1 min. LFA-1 plays a crucial role in such conjugate formation, and is under the control of the T cell antigen receptor. More recently, selectins and the VLA-4 integrin have been shown to mediate leukocyte attachment to and rolling on endothelium under blood flow shear stress. LFA-1 does not engage ICAMs under shear stress, but when activated on an already attached, rolling leukocyte, LFA-1 can cause rolling to cease, immobilizing the leukocyte prior to its extravasation by migration through the endothelium (diapedesis).

Human CTL clones (unlike mouse CTLs produced *in vivo*), and B lymphocyte cell lines transformed by Epstein-Barr virus, show promiscuous adhesion which is LFA-1 dependent. This seems likely to result from constitutive LFA-1-activation signals produced by regulatory disturbances during long-term culture or viral gene expression.

The adhesion function of cell surface LFA-1 for ICAM-1 can be changed rapidly, without any change in the number of LFA-1 molecules on the cell surface. This activation or deactivation is normally under the control of other plasma membrane receptors (some of which may be integrins), and may also involve some positive or negative cytoplasmic feedback signals from LFA-1 itself. Antibodies or ligands for dozens of diverse plasma membrane receptors have been shown capable of activating LFA-1. For example, antibody to CD3 activates LFA-1 transiently on T lymphocytes, peaking at about 10 min. In common with many other receptor-controlled cellular events, physiologic activation of LFA-1 involves a complex interaction of tyrosine and serine/threonine kinases and phosphatases, and elevation of cytoplasmic calcium. Cyclic AMP inhibits activation of LFA-1 induced by certain receptors. The cytoplasmic domain of CD18 contains two AsnProX₂Phe sequences which are conserved in six of the eight integrin β chains. Evidence from β_1 and β_3 suggests that these may be important in regulation of function. Membrane-proximal portions of the cytoplasmic domains of both chains, including ValGly-PhePheLysArg in CD11a, appear to be important for heterodimer formation and regulation of function.

Experimental activation

In addition to physiologic activation by receptor cross-talk, LFA1 (and other integrin) function can be activated by several experimental maneuvers. Phorbol esters potently activate LFA-1 function, probably via their well-known activation of C-type protein kinases, since diacylglycerol transiently activates

LFA-1. The LFA-1 cytoplasmic domain serine most heavily phosphorylated in response to phorbol ester, Ser756 in CD18, was mutated without effect on regulation of function. For some cell types, certain kinase inhibitors (e.g. staurosporine) activate LFA-1. Recently it has been reported that the reducing agent dithiothreitol (DTT) induces activation of LFA-1. Ample magnesium (>1 mM) in the absence of calcium (EGTA), or manganese (0.1 mM) in the absence of calcium, activate LFA-1. As mentioned above, certain unusual antibodies to LFA-1 activate it. Some of these methods may activate LFA-1 to a higher quantitative or qualitative level than occurs physiologically.

Mechanism of activation

Activation via receptor ligation or phorbol ester is temperature dependent and requires metabolic

energy. This is called 'inside-out' activation. However, activation by the binding of magnesium or manganese, or by activating LFA-1-specific antibodies, is (by analogy with other integrins) effective on purified integrins, and hence occurs by direct interaction with the extracellular domains. There is evidence consistent with physiologic activation involving an increased affinity for magnesium, hence increasing occupancy of a critical metal-binding site.

The mechanism of activation of LFA-1 is not understood in detail, but must involve conformational changes. Rare antibodies recognize epitopes present only on an activated form of LFA-1, consistent with the expected configurational change. Results suggest that only a minority of the LFA-1 molecules on a cell may be active at one time. Activation of LFA-1 probably involves both a change in intrinsic affinity for ligand, and clustering of LFA-1 molecules on the cell surface, which increases functional affinity through multivalency. Some recent evidence indicates that clustering is calcium dependent, and may be prerequisite but not necessarily concurrent with the final activation event.

The low affinity of soluble ICAM-1 for LFA-1 has hampered studies of intrinsic affinity. One indirect

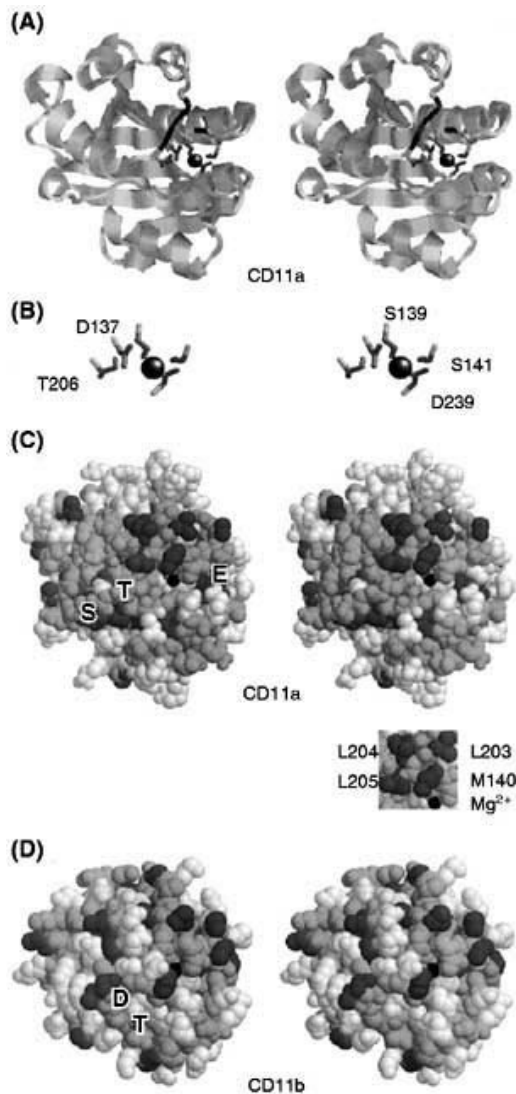


Figure 2 Structures of the ligand- and divalent cation-binding I domains of CD11a. (Stereo pairs for wall-eyed viewing made with the program RasMol.) (A) Backbone cartoon of the I domain of CD11a (atomic coordinate file 1LFA.PDB, from Qu A and Leahy DJ (1995) *Proceedings of the National Academy of Sciences of the USA* **92**: 10277). Mg^{2+} is shown as a black sphere. The five residues of the MIDAS motif which coordinate Mg^{2+} are shown as darkened stick representations. (Some of the coordinations are via water molecules, not shown. Mn^{2+} was the divalent cation in the crystal from which this structure was determined.) The darkened sections of the backbone indicate the positions of the strained hydrophobic ridge residues Met140 and Leu203LeuLeu, which is unique to CD11a and may be important in binding ICAMs. (B) Enlargement of the Mg^{2+} from (A), with the five MIDAS residues identified. (C) Spacefilling representation of the putative ligand-binding face of the I domain of CD11a. Mg^{2+} is black. Backbone and polar side-chains are gray, charged side-chains are white, and hydrophobic side-chains are dark gray. The inset identifies the four dark residues which make up the strained hydrophobic ridge. The letters S, T, E mark Ser245, Thr243 and Glu146, which along with Met140 are the major contributors to species-specific binding of ICAM-1. (From Huang C and Springer TA (1995) *Journal of Biological Chemistry* **270**: 19008–19016.) (D) Spacefilling representation of the putative ligand-binding face of the I domain of CD11b, colored as in (C). Note the absence of the hydrophobic ridge pattern seen in CD11a. Letters D and T mark residues that are crucial for iC3b binding but not for metal binding (Asp248, Thr252). (From McGuire SL and Bajt ML (1995) *Journal of Biological Chemistry* **270**: 25866–25871.) (Atomic coordinate file 1IDO.PDB kindly provided by Amin Arnout. From Lee J-O, Rieu P, Arnaout MA and Liddington R (1995) *Cell* **80**: 631–638.) These and related images may be viewed in color at <http://www.umass.edu/microbio/lfa-1>.

estimate of K_d was approximately 10^{-4} M, and was consistent with 15–30% of the LFA-1 molecules being activated by phorbol ester, achieving a K_d of approximately 10^{-6} M. Integrins which have higher affinities for their ligands than does LFA-1 can be activated in solution (e.g. by manganese or activating antibodies) to bind to ligand columns, suggesting an increase in the intrinsic affinity of a univalent interaction. Recently it has been reported that ICAM-1 is normally dimeric on the cell membrane.

A recent comparison of two crystal forms of the CD11b I domain suggests structures for its active and inactive conformations. In the active conformation, Mg^{2+} is coordinated by uncharged residues (Ser, Thr, water), making it more electrophilic and likely to bind a negatively charged residue in the ligand. In the inactive conformation, Mg^{2+} is coordinated by a charged residue (Asp) making it less likely to bind an acidic group in the ligand. Ca^{2+} , were it to occupy the MIDAS site, is more likely to require coordination by the Asp, hence to stabilize the inactive configuration. The inactive conformation also buries two Phe residues and hides an acidic pocket which could be involved in ligand binding. These configurational changes are similar to those observed during function of G proteins, which are similar in structure to I domains.

It seems certain that the actin filament cytoskeletal system plays a role in LFA-1 function. Cytochalasins (which inhibit actin polymerization) have long been known to be potent antagonists of LFA-1-mediated leukocyte adhesions. Activation of LFA-1 may involve anchoring of LFA-1 to the actin filament system, or actin-dependent clustering of LFA-1 molecules into multivalent plaques. Filamin and α -actinin binding sites have been identified in the cytoplasmic domain of CD18. Evidence from chimeric receptors suggests that the CD18 cytoplasmic

domain sequence Thr758ThrThr, shown to be essential for cell adhesion, is involved subsequent to ligand binding in actin-dependent spreading.

See also: Adhesion molecules; Complement receptors; Cooperation, mechanisms of cellular; Cytotoxic T lymphocytes; High endothelial venules; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Natural killer (NK) cells.

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LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN 3 (LFA-3)

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Lymphocyte (leukocyte) function-associated antigen 3, also termed LFA-3 or CD58, is expressed as either a transmembrane or a lipid-linked cell surface protein. The transmembrane form consists of a 188 amino acid extracellular region, a 23 amino acid transmembrane hydrophobic region, and a 12 amino acid intracellular hydrophilic region ending in the C-

terminus. The lipid-linked form consists of the extracellular portion anchored to the membrane via a phosphatidylinositol membrane lipid. Treatment of cells with a phospholipase specific for phosphatidylinositol will solubilize the phospholipid-linked form of LFA-3. The LFA-3 on erythrocytes is anchored via the phospholipid linkage while other cells, such as

the Epstein-Barr virus transformed B cell line JY, express both transmembrane and lipid-linked LFA-3. The LFA-3 molecule is widely distributed and found on most hematopoietic cells, fibroblasts, epithelial cells, and endothelial cells. The expression of LFA-3 by antigen presenting cells (APCs), including macrophages, dendritic cells and B lymphocytes, implies a role in regulating the immune response.

Interaction with CD2

Initially, LFA-3 was defined by the ability of a monoclonal antibody (mAb) to block human cytolytic T lymphocyte (CTL)-mediated lysis by binding to the target cell surface. However, even before this discovery, a method widely used by immunologists to purify human T cells by forming rosettes with sheep red blood cells utilized the molecular interaction between LFA-3 and its ligand. The CTL-mediated lysis of targets as well as T cell rosetting was also inhibited by mAbs which bound to CD2 on the T lymphocytes. These antibody-blocking studies suggested that CD2 and LFA-3 were both involved with cell-cell adhesion.

Subsequent studies demonstrated that purified LFA-3 incorporated into the lipid bilayer of large lipid vesicles could bind to cells expressing CD2. Protein micelles, another form of multivalent LFA-3 prepared by detergent dialysis of purified phosphoinositol (PI)-linked LFA-3, bound to CD2-expressing cells with a K_d in the range of 0.5 nM. Bivalent LFA-3-Fc, prepared by splicing residues 1-184 of LFA-3 to the hinge and C_{H2} - C_{H3} region of human immunoglobulin G1 (IgG1), would also bind to cells expressing CD2 with a K_d in the range of 150 nM. Soluble monovalent LFA-3 prepared by expression of amino acid residues 1-180 in CHO cells demonstrated weak binding to CD2. In a recent study using surface plasmon resonance technology, the association of soluble LFA-3 to immobilized CD2 was found to occur quickly; however, the dissociation was also very rapid, $k_{off} \geq 4 \text{ s}^{-1}$, resulting in a low affinity, $K_d \approx 15 \text{ } \mu\text{M}$. Since all interactions would not reverse at the same instant, weak interaction between individual LFA-3/CD2 molecules would be stabilized if present as multivalent interactions. Provided the density was sufficient, the fast rate of association would ensure new interactions that would maintain adhesion. The experimental data with bivalent LFA-3, LFA-3 micelles and LFA-3-containing lipid membranes support this idea.

Using fluorescence photobleaching recovery of a planar phospholipid membrane containing purified fluorescently tagged phosphatidylinositol-linked LFA-3, the interaction between CD2 on the T lymphocyte

membrane and LFA-3 in a lipid planer membrane has been quantitated. This study reported the two-dimensional dissociation rate constant to be about 20 LFA-3 molecules μm^{-2} of membrane. (This measurement was based upon the density of LFA-3 in the contact area (bound) and the density outside the contact area (free) to calculate the number of molecules per unit area of membrane to give equilibrium binding.) Most APCs express LFA-3 at levels several fold above this density suggesting that laterally mobile LFA-3 should readily promote adhesive interactions with cells expressing CD2. However, the transmembrane form of LFA-3 has a much slower membrane mobility than PI-linked LFA-3 and would probably require a greater membrane density to achieve the same level of adhesion found for the lipid-linked LFA-3 in model membranes. This and a previous study indicated that the multivalent interaction between LFA-3 and CD2 in different membranes was favorable but dependent upon the membrane density and mobility of the receptor-ligand pair.

Human LFA-3 (CD58) and its receptor, CD2, are both encoded on chromosome 1 and consist structurally of two extracellular immunoglobulin-like domains. There is 21% homology between these two glycoproteins, suggesting that they arose from a single ancestral gene that encoded for a self-binding or homophilic type protein. Interestingly, a homolog of LFA-3 has not been found in mice and rats. However, a protein structurally similar to LFA-3, CD48, which is also encoded on chromosome 1 in humans, appears to be utilized in both rodent species comparably to LFA-3 in humans. In studies using surface plasmon resonance, binding between human soluble CD48 and CD2 was not found; however, binding between rat CD48 and CD2 was demonstrated and determined to have a K_d of about 80 μM . Although somewhat less than the interaction of LFA-3 with CD2, this level of binding in multivalent form should be sufficient to mediate adhesion in the rodent immune system.

The regions of LFA-3 required for binding to CD2 have been mapped by limited mutagenesis of the extracellular domain. Two regions containing charged amino acids in the first Ig-like domain were required. Specifically, amino acids 30, 34 and 84 of LFA-3 were shown to be essential for binding to CD2. Modeling by comparison with the structure of CD2 places these sites in two loop-out regions on one face of the LFA-3 molecule. From the position and complementarity of these charged groups, it appears that the LFA-3-CD2 interaction is mediated in part by interactions between amino acids with opposite charges.

Cell stimulatory signals

Based upon the structure of CD2 and LFA-3, the dimensions of each molecule would position the membrane of the LFA-3-bearing cell about 150 Å from the membrane of the cell expressing CD2. This distance would be optimal to encourage an interaction between the antigen specific receptor (TCR) on T lymphocytes and the peptide antigen presented by major histocompatibility complex (MHC)-encoded proteins on the surface of the APC. Numerous studies have supported this by showing that the interaction of LFA-3 with CD2 enhanced the responsiveness of T lymphocytes to antigen, presented by either class I or class II MHC. Under defined experimental conditions, lipid vesicles containing both LFA-3 and MHC-antigen triggered a several-fold greater response compared to liposomes containing MHC-antigen only. Similar studies indicated that anti-CD3 or lectin was able to trigger a greater T cell response in the presence of cells expressing LFA-3 or LFA-3 micelles. Thus, the LFA-3-CD2 interaction can deliver a costimulatory signal for both naive lymphocytes and previously activated or memory lymphocytes. This costimulation appears to involve a combination of adhesion properties, which promote antigen recognition, as well as delivering a direct costimulatory signal through CD2.

In addition to costimulation in the presence of antigen, it is also possible for CD2 to deliver a stimulatory signal in the absence of antigen. Certain pairs of anti-CD2 mAbs (e.g. T11₂ + T11₃) have been shown to activate T cells in the absence of an apparent antigen signal. It remains unclear whether this is due to an activation site on CD2 whose ligand has not been defined or results from antibody-mediated clustering of cell surface CD2. Recent studies suggest that LFA-3 alone can deliver an activation signal to T lymphocytes and this signal is transmitted through CD2. Proliferation and interferon γ (IFN γ) production by post-activated phytohemagglutinin (PHA)-stimulated T cells in response to interleukin 12 (IL-12) was shown to be dependent on the presence of macrophages expressing high levels of LFA-3. Although LFA-3 was also present on B cells and T cells in the culture, these cells could not support IL-12-driven proliferation and IFN γ production. The apparent reason for this was the (~10 $\times$) higher cell surface expression of LFA-3 on the macrophage surface. CHO cells expressing levels of LFA-3 similar to that on the macrophages would also support the IL-12-mediated responses, suggesting that a high surface density of LFA-3 was all that was required for T cell stimulation under these conditions. This type

of cytokine signaling would drive an IFN γ -dominant response commonly referred to as a T_H1-type immune response.

These data are consistent with a model where cell surface LFA-3 on monocytes or other APCs can mediate adhesion with T cells expressing CD2 and support antigen recognition, perhaps by providing a region for the MHC-peptide accumulation on the APC and TCR accumulation on the T cell. Once an activation signal is delivered by APCs, additional signaling through CD2 will directly drive T cell proliferation or expansion of the responder population. The requirement of a high surface density of LFA-3 on APCs implies that a polar clustering of CD2 is necessary and sufficient to deliver a stimulatory signal to previously activated lymphocytes. Reports indicating that IL-12 production by APCs is mediated by cross-linking of CD40, and the observation that CD40L on T cells was upregulated in response to endothelial cells expressing LFA-3, suggests that additional cellular components may contribute to this activation loop.

The interaction of CD2 with LFA-3 has also been reported to deliver a signal in the other direction, to the cell expressing LFA-3. In this study cross-linking LFA-3 on the monocyte surface induced the production and release of tumor necrosis factor α (TNF α) and IL-1 β . The lack of an extensive cytoplasmic domain on LFA-3 suggests that this signaling must be mediated through interactions with other cellular proteins.

Although the mechanism is far from clear, it has been shown that signals delivered through the TCR and accessory molecules in an imbalanced manner can lead to anergy or even T lymphocyte apoptosis. The human immunodeficiency virus (HIV) envelope protein, gp120, can also mediate anergy and apoptosis, presumably by binding to CD4 and seven-span chemokine receptors. Several studies have implicated the LFA-3-CD2 interaction in the reversal of these forms of anergy and apoptosis. These effects are apparently associated with signaling by CD2. Experiments show that the first domain of LFA-3 (or both extracellular domains) expressed as an Fc fusion protein will inhibit both primary and secondary T cell proliferation to antigen, lectin or anti-CD3 mAb, resulting in an anergic state. It is not clear if this is due to inappropriate signaling through CD2 or to the desensitization and/or blockade of CD2, rendering it unable to function in a normal manner. Consistent with these *in vitro* observations, *in vivo* studies demonstrated an increased survival of allografts in mice treated with anti CD2/anti-CD48, or in nonhuman primates treated with LFA-3-Fc.

Summary

LFA-3 (CD58) is a membrane protein found on nearly all human cells. The interaction between LFA-3 and CD2 is very weak in solution, but when these glycoproteins are present in different membranes, the multivalent action of the receptor/counter-receptor pair leads to effective cell-cell adhesion. In the presence of an antigen signal for T lymphocytes, this binding leads to a costimulatory signal and an enhanced response. Cells with a high density of LFA-3 can support proliferation and cytokine production by preactivated T lymphocytes in the absence of an antigen signal. The ability of LFA-3, through its interaction with CD2, to deliver signals that enhance T cell responses or modulate anergy/apoptosis indicate that regulation of this receptor-ligand pair may have therapeutic potential.

See also: Adhesion molecules; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Apoptosis; CD2; CD59; Cell-mediated immunity; Anergy, T cell; Cooperation, mechanisms of cellular; Cytokines; Cytotoxic T lymphocytes; Human immunodeficiency viruses; Immunoglobulin gene superfamily; Interleukin 12 and its receptor; Rosetting techniques; T lymphocyte activation.

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LYMPHOCYTE TRAFFICKING

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Lymphocyte traffic is essential for the interaction of the full antigen receptor repertoire on the lymphocyte surface with antigens wherever and whenever they enter the body. Lymphocytes migrate continuously from the blood into lymphoid and nonlymphoid organs and back to the blood via lymphatics and venules. On average lymphocytes stay in the blood-stream for only about 30 minutes on each passage. This means that the pool of lymphocytes circulating in the blood pool is exchanged about 48 times per day, and that in humans about 5×10^{11} lymphocytes leave the blood every day (Figure 1). This is in contrast to other leukocytes, for example granulocytes, which circulate for about 20 h in the blood, extravasate only once into the tissues and never return to the circulation again. Therefore, the regulation of the 0.25×10^{11} granulocytes leaving the blood each day into the tissues can only occur at the entry sites – endothelial cells of capillaries and venules. Lymphocyte migration, however, can be regulated during entry, transit and exit. There are indications that the migration patterns of natural killer (NK) cells and monocytes lie between these two extremes.

The detailed migration routes of lymphocytes, their kinetics and the regulation during entry, transit and exit of lymphoid and nonlymphoid organs are all included nowadays in the term lymphocyte trafficking. In the following, some of these aspects will be outlined in more detail and it will be stressed that these features may differ depending on which lymphocyte population is investigated.

Migratory routes of lymphocytes

In 1885 Walter Flemming argued that lymphocytes in lymphatics are not only newly formed cells but also lymphocytes on their way back to the blood. It took about 70 years until Gowans provided experimental evidence for lymphocyte traffic from the blood via lymph nodes into the thoracic duct of rats. Since then many studies have documented that similar mechanisms are at work in lymphocyte traffic in all mammals.

The vast majority of lymphocytes migrate from the blood into organs such as the lung, spleen, liver and bone marrow (Figure 1A). They enter the tissues and return into the blood using normal venules and sinusoids. Entry of lymphocytes into the spleen,

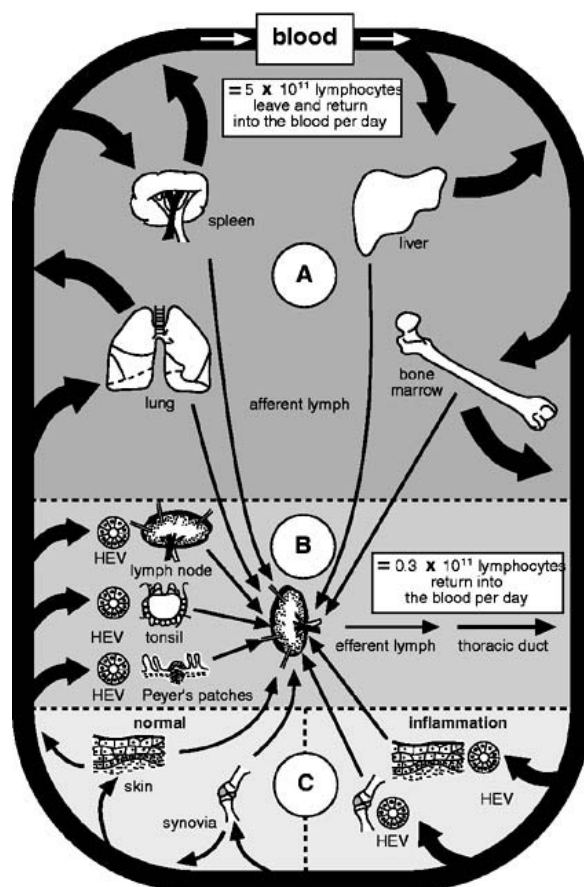


Figure 1 Each day about 5×10^{11} lymphocytes leave the blood to migrate into various organs and return into the blood. (A) Most of the blood lymphocytes enter organs such as the lung, spleen, liver and bone marrow via normal venules and sinusoids. The entry is protease-resistant and the lymphocytes have a short residence time in the organs. The vast majority return into the blood via normal venules. Only a few lymphocytes leave these organs via the afferent lymph. (B) In humans about 0.3×10^{11} lymphocytes migrate daily from the blood into organs such as the lymph nodes, tonsils and Peyer's patches. They use HEVs, the entry is protease-sensitive and the lymphocytes have a long residence time in the tissue. The lymphocytes return into the blood via the efferent lymph. (C) Only very few lymphocytes migrate under normal circumstances into organs such as the skin, synovia, muscle and brain. They enter these tissues via normal venules and their numbers are only related to blood flow. The lymphocytes return into the blood either via normal venules or via afferent and efferent lymph. However, during acute and chronic inflammation HEV-like structures may develop, leading to a large increase in lymphocyte influx, indicated by the thicker arrows.

which is best understood, seems to be via the marginal zone, and subsequently T and B lymphocytes use different routes through the splenic compartments. The majority leave via the splenic vein and not via lymphatics. On a quantitative basis several-fold more lymphocytes recirculate through the spleen and probably each of the other tissues than through all lymph nodes together. It is often overlooked that the bone marrow and the thymic medulla are included in the normal migratory route of lymphocytes. In addition, nonlymphoid organs, such as the lung and liver, contain large pools of temporarily sessile lymphocytes within the vascular bed, the so-called marginal pool (Figure 2). This is not the result of nonspecific trapping but a specific physiological phenomenon, at least for the lung.

Less than 10% of all lymphocytes in the blood migrate into organs such as the lymph nodes, tonsils and Peyer's patches (Figure 1B). They use specialized venules, the so-called high endothelial venules (HEV). The lymphocytes return into the blood via the efferent lymph (classic pathway of lymphocyte recirculation). The transit time of lymphocytes from the blood through lymph nodes into the thoracic duct is longer than that through organs not possessing HEVs.

Only very few lymphocytes migrate under normal circumstances into organs such as the skin, synovia, muscle and brain (Figure 1C). They enter these tissues via normal venules and return into the blood either via normal venules or via afferent and efferent lymph. However, during acute and chronic inflammation HEV-like structures may develop, leading to a large increase in lymphocyte influx. The signals involved in the switch from a normal endothelial cell to a specific exit site for lymphocytes are not known.

There appears to be a further area of complexity. In species like pigs, sheep and calves there are two types of Peyer's patches in the small intestine, which differ in lymphocyte entry from the blood by a factor of up to 10. This heterogeneity has also been found for the different tonsils and lymph nodes and also in the appearance of lymphocyte migration in ontogeny. In addition, it is well-known that within organs, B and T lymphocytes travel differently through the various organ compartments.

The migration described so far is probably indicative of the routes of naive lymphocytes because in most experiments unseparated lymphocyte populations were used which usually contain few activated or memory lymphocytes.

Migratory routes of lymphoblasts

After presentation of the respective antigen and appropriate costimulation, naive lymphocytes become lymphoblasts and start proliferation. The traffic of proliferating large lymphoid cells shows a different pattern. Most typically, after injection immunoglobulin A (IgA) lymphoblasts from thoracic duct, Peyer's patches or mesenteric lymph nodes are preferentially found in the lamina propria of the gut. In addition, injected lymphoblasts from the gut-associated lymphoid tissue, but not from peripheral lymph nodes, are found preferentially in organs with mucous layers, such as the bronchial tract, lacrimal, salivary and mammary glands. It has to be stressed, however, that it is a preferential and not an exclusive location.

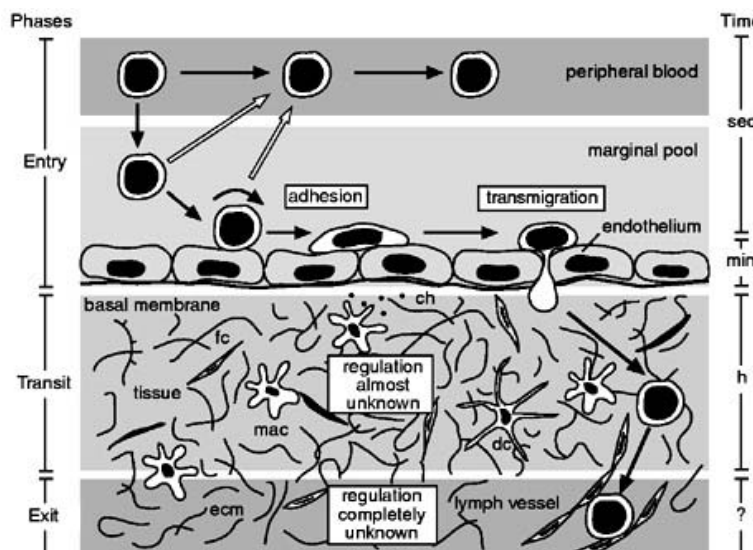


Figure 2 Different steps in lymphocyte migration. The mechanisms involved in lymphocyte adhesion to endothelium are known in considerable detail, although lasting only in the range from seconds to minutes. In contrast, the regulation of lymphocyte migration during transit and exit are not well understood, although these phases last for hours, leaving time for intensive interaction with the extracellular matrix (ecm), fibrocytes (fc), macrophages (mac), dendritic cells (dc) and various chemokines (ch) and cytokines.

Migratory routes of memory lymphocytes

It had been proposed that naive lymphocytes exclusively enter lymph nodes via HEVs (Figure 1B), and memory T lymphocytes enter tissues such as the skin and then finally reach the lymph node via afferent lymphatics (Figure 1C). However, the evidence was indirect. More recent *in vivo* studies using different techniques showed that memory lymphocytes also migrate through HEVs and are found in the efferent lymph. However, it is always problematic to deduce functions from phenotype analysis (as it is necessary to identify naive and memory lymphocytes in migration studies), and therefore the terms naive and memory have to be used with caution. Thus, only when the traffic of marked lymphocyte subsets is followed *in vivo* is it possible to define the traffic routes used by various lymphocyte subsets.

Techniques to study lymphocyte migration

As in other processes *in vivo*, effective physiological study of the routes followed by migrating lymphocytes calls for minimal and innocuous intervention. First, use of lymphocytes from the blood or lymphatics, typically the thoracic duct, has the advantage that those lymphocytes are already in transit, in contrast to those extracted from lymphoid organs, which are a mixture of sessile and mobile lymphocytes. The cells must be labeled to be traced and this can be done by using isotopes, such as ^{51}Cr , [^3H]-cytidine or [^3H]-lysine to label all lymphocytes, or [^3H]-thymidine and [^{125}I]-uridine to label mainly lymphoblasts since these isotopes are only incorporated in proliferating lymphocytes. Fluorescent dyes (e.g. fluorescein isothiocyanate, FITC) have proven valuable labels for tracing lymphocytes *in vivo* and *in vitro*. Used in combination with identification of surface markers, resting and activated lymphocyte subsets can be identified among migrating cells, both in suspension and also in tissue sections. In species in which congenic strains exist (e.g. mouse, rat, pig), lymphocytes can be transferred without any *in vitro* labeling procedure and the route of injected cells through lymphoid organs followed by identifying the cells with monoclonal antibodies. Thus, using adequate techniques the detailed routes of migration of lymphocyte subsets through lymphoid and non-lymphoid organs and their subcompartments are being identified.

Alternatively, *in vitro* techniques have proved very useful for the molecular study of lymphocyte adhesion to blood vessel endothelia. For example,

lymphocyte binding to HEVs of lymphoid tissues can be analyzed on cryostat sections. HEV cells from lymph nodes in rodents can also be cultured *in vitro*, and the interaction between lymphocytes and these endothelial cells can be studied in more detail. Cultured human umbilical venous endothelial cells (HUVEC) are often used to study the mechanisms involved in lymphocyte binding. However, it has to be considered that this endothelium might differ in major aspects from the endothelia in lymphoid organs. A major advantage was the establishment of *in vivo* videomicroscopy, allowing the analysis of migrating lymphocytes under physiological shear forces.

In all the different techniques used to study lymphocyte traffic, the extent to which the methodology may have altered the physiological processes has to be considered and compared critically.

Regulation of lymphocyte migration

Migration can be regulated during entry into, transit through and exit from lymphoid and non-lymphoid organs (see Figure 2). The vast majority of studies were and still are concerned with regulation of lymphocyte traffic during entry.

Entry

When lymphocytes enter a lymph node, Peyer's patches or tonsils from the blood, only certain specific sites in the vascular bed are used for migration into the parenchyma. These preferential sites for binding and emigration of lymphocytes are the post-capillary venules in the paracortex, which in most species have a high endothelium. Initially it was assumed that migration into peripheral lymph nodes is mediated by a single molecule on the lymphocytes, called a 'homing receptor', and the corresponding molecule on the HEV, the 'addressin'. Another pair of molecules was thought to be responsible for migration into Peyer's patches. However, it is now clear that a steadily increasing number of receptor pairs exists for the different lymphoid tissues. There is a partly overlapping sequence of interaction of adhesion molecules on lymphocytes and endothelial cells. Some adhesion molecules are expressed constitutively and others are inducible by chemokines and cytokines. Lymphocytes are found in the peripheral blood (circulating pool), and also near to or in intermittent contact with the vessel wall (marginal pool; Figure 2, Table 1). The emigration through the vessel wall can be divided into several steps: 1) Lymphocytes make contact with the endothelium and, under appropriate conditions, start to roll on the endothelium, also called tethering. 2) By factors coming

Table 1 Factors influencing lymphocyte entry

Steps during entry	Properties	Implications
Marginal pool (reversible)	Cellular composition	Determines cell type available for immigration
Adhesion to endothelium (reversible)	Shear force	Physical properties important for the availability of adhesion molecules
	Cell charge	
	Length of adhesion molecules in relation to other surface molecules e.g. CD45: 300 nm; L-selectin: 30 nm	
	Position of adhesion molecules	Mainly involved in the early phase of adhesion
	at the tip of microvilli e.g. L-selectin and $\alpha_4\beta_7$ -integrin	Mainly involved in the late phase of adhesion
	at the cell base e.g. LFA-1 and CD44	
	Change of adhesion molecule conformation e.g. LFA-1 within seconds	Facilitates or inhibits adhesion molecule function
	Shedding of adhesion molecules e.g. L-selectin within seconds	Facilitates or inhibits adhesion molecule function
	Release of chemokines	Alters functional state of adhesion molecules
	Rolling on already adherent neutrophils, lymphocytes, platelets	Makes adhesion molecules available which are normally not present on the endothelium
Transmigration (irreversible)	Type of endothelium, e.g. flat or high	Selective entry of leukocyte subsets, e.g. normally only lymphocytes but no granulocytes migrate through high endothelium
	Site of transmigration, e.g. inter- or intracellular	
	Induction of degrading enzymes, e.g. gelatinase for basal lamina	

from the endothelial cells or from beneath the basal membrane, lymphocytes become activated, also called triggering, resulting in strong adhesion to the endothelial cell. 3) Finally the lymphocytes start the transmigration, also termed diapedesis. The transmigration is associated with a dramatic change in the morphology of the lymphocytes, e.g. they lose the microvilli on their surface and the cytoplasm forms a uropod. These phases are regulated by different pairs of adhesion molecules, and factors possibly influencing this process are listed in Table 1. A further interesting aspect is the time schedule: lymphocyte activation takes place in a few seconds, strong adhesion in a few minutes but the transmigration lasts several minutes (Figure 2).

The role of adhesion molecules has been elucidated by studies *in vitro* and *in vivo* using inhibitory antibodies against adhesion molecules on both lymphocytes and endothelial cells. More recently, experiments in mice deficient in certain adhesion molecules have extended our knowledge about the role of individual molecules in different lymphoid organs. L-Selectin knockout mice, for example, not only show blocking of lymphocyte entry into peripheral lymph nodes (as expected) but also a severe reduction in entry into Peyer's patches. Thus, it has become obvious that one should not speculate on a functional role of an adhesion molecule only based on its pres-

ence on a cell type. As an example, it has been documented that the adhesion molecule MAdCAM-1, initially thought to be specific for migration into Peyer's patches, is also expressed on cells of the marginal zone of the spleen, but blocking this molecule had no effect on lymphocyte migration into the spleen. So far, no specific molecules have been identified regulating lymphocyte traffic to organs without HEV, such as the spleen, bone marrow, lung and liver.

Recent data showed that some adhesion molecules are concentrated at the tip of the microvilli, whereas others are located at the cell base, indicating different roles in the interaction with the endothelium (Table 1). In addition, adhesion molecules are shed from the cell surface and found as soluble molecules in the serum. The regulatory and potential therapeutic role of these soluble molecules has to be studied in more detail.

Transit and exit

After a lymphocyte has left the blood and migrated via an intercellular route between endothelial cells, the transit through the lymphoid organ starts (Figure 2). Lymphocytes will interact with the extracellular matrix, e.g. with fibronectin, laminin, collagens, and also interstitial cells such as fibrocytes, macrophages and dendritic cells. This transit lasts for hours and enables intercellular contacts and effects of cytokines

and chemokines in a paracrine fashion. Very little is known about regulation of the transit through the organs. Finally, most lymphocytes will leave the lymphoid organ again and exit into venules or lymphatics. No regulatory factors have been characterized for this step so far. However, there are indications that this step is carefully regulated. For example, sheep lymphocytes efficiently used the HEVs within pig lymph nodes to exit into the blood, a route normally not taken by sheep lymphocytes. In addition, pig lymphocytes migrated from lymph nodes of fetal lambs into lymphatics, a route not taken by pig lymphocytes but normal for sheep lymphocytes. This suggests that the mechanisms regulating lymphocyte migration are well conserved, at least between these two species which diverged from a common ancestor about 50 million years ago.

Conclusions

The continuous traffic of lymphocytes through lymphoid organs is an essential component of the immune system: there are only few immunocompetent lymphocytes for a specific antigen and this traffic vastly increases their probability of meeting the antigen in the appropriate microenvironment around accessory cells and other lymphocyte subsets which are important for the initiation of effective immune responses. Lymphocyte traffic in general is not dependent on antigens. Sheep fetuses have normal lymphocyte recirculation through lymphoid tissues and into the thoracic duct, although they are secluded from external antigens by their particularly impervious placenta.

The entry into lymphoid organs through HEVs is regulated by a series of sequentially involved receptor–ligand interactions by several adhesion molecules. Certain models, e.g. the preferential route of ‘memory’ and ‘naive’ lymphocytes, have had to be revised recently. Much less is known about factors that may regulate transit within the lymphoid organs and the exit from the organ. Cytokines may play important roles in all these steps of transit through an organ. Little is known about the regulation of lymphocyte traffic to lymphoid organs without HEVs and to nonlymphoid organs such as the lung and liver.

See also: Adhesion molecules; Chemotaxis of lymphocytes; Endothelium; Fluorochrome labeling; High endothelial venules; Lifespan of immune cells and

molecules; Lymphatic system; Proliferation, lymphocyte.

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LYMPHOCYTE TRANSFORMATION TEST

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The lymphocyte transformation test is based on the observations that, in response to activation with mitogens, antigens, superantigens and antibodies to components of cell membranes, lymphocytes synthesize proteins such as immunoglobulins, cytokines and growth factors, enter the cell cycle, synthesize DNA and replicate, and undergo metabolic and morphologic changes. T lymphocytes are activated by mitogens such as phytohemagglutinin and concanavalin A, superantigens, anti-CD3 and antigens that are presented by antigen-presenting cells. B Lymphocytes are activated by anti-immunoglobulins, bacterial lipopolysaccharides and staphylococcal protein A.

The lymphocyte transformation test has become one of the most widely used *in vitro* tests for evaluation of lymphocyte functions in clinical immunology. The general procedures used in this test are also used to study the biochemical events that accompany lymphocyte activation, effector functions of activated lymphocytes and to produce clones of specific antigen-responsive lymphocytes.

Historical background

Perhaps the first use of mitogens in lymphocyte biology was described by Peter Nowell in 1960 when he reported that phytohemagglutinin, a protein from the red kidney bean, *Phaseolus vulgaris*, would stimulate DNA synthesis and cell proliferation in cultures of blood lymphocytes. The first applications to immunologic studies were described by Bach and Hirschhorn and associates in the early 1960s, and soon the test was applied to studies of lymphocytes from patients with immunodeficiency syndromes, malignancies and other disorders of the immune system. It is now one of the most widely used tests for *in vitro* studies of lymphocyte functions in clinical immunology. The clinical studies have shown that there is usually a close concordance between antigen-induced lymphocyte proliferation *in vitro* and expression of delayed-type hypersensitivity to the same antigen.

Procedures

Lymphocyte transformation test using human peripheral blood lymphocytes

1. Venous blood is aseptically collected into heparinized (20 units ml⁻¹) syringes or tubes.
2. The heparinized blood is diluted with an equal volume of Hank's balanced salt solution (HBSS) or phosphate-buffered saline (pH 7.4) (PBS) and then layered over a mixture of Ficoll and Hypaque that has a specific gravity of 1.077. Sterile preparations of Ficoll-Hypaque are commercially available. The tubes are centrifuged at 500 g at room temperature for 30 minutes. The mononuclear cells are concentrated between the uppermost plasma layer and the middle layer of Hypaque-Ficoll. They are recovered by aspiration with a sterile pipet. Erythrocytes and granulocytes sediment to the bottom of the tube.
3. The mononuclear cells must be washed at least twice with tissue culture media or HBSS to remove the Hypaque-Ficoll. At this point an aliquot of the cell suspension is removed for a cell count and differential. Normal subjects usually yield about 1 million lymphocytes per milliliter of whole blood and the final product contains 75–85% lymphocytes and 10–20% monocytes. The distribution of T lymphocytes, T lymphocyte subsets and B lymphocytes in the final product is proportional to the ratios of these cells in the original specimen.
4. The cells are suspended in tissue culture medium (RPMI 1640 is commonly used) that has been supplemented with fresh glutamine (200 mM), penicillin (100 units ml⁻¹), streptomycin (100 µg ml⁻¹) and heat-inactivated serum (5%). Depending on the goal of the experiment, the serum may come from a fetal bovine calf, the original blood donor or a pool of group AB human sera. A cell density of 10⁶ cells ml⁻¹ is common. There is a good deal of individuality in the fine points of human lymphocyte transformation tests, with some workers preferring HEPES-buffered media and gentamicin or amphotericin B as additional antibiotics.
5. The cells are distributed into culture trays or culture tubes in concentrations of 200 000 cells per well in round-bottom 96-well trays or 10⁶ cells

ml⁻¹ in culture tubes. The stimulating agent is added in a volume of 10–50 µl and the culture vessels are incubated at 37°C in an atmosphere of 5% CO₂ and 95% air. It is best to do dose-response studies in which several doses of the stimulating agent are used. For example, phytohemagglutinin (PHA-M) can be used in concentrations of 0.1, 1.0 and 10 µg ml⁻¹. With lymphocytes from normal donors, half-maximal responses are obtained with doses of 0.3–0.5 µg ml⁻¹ of PHA-M. Concanavalin A is used in doses of 0.5, 5.0 and 10 µg ml⁻¹ and pokeweed mitogen is used in doses of 1.0, 10 and 30 µg ml⁻¹.

6. Most clinical studies are intended to examine the responses of a patient's lymphocytes to antigens from infectious agents such as herpes simplex, mycobacteria or *Candida albicans*, or toxins such as tetanus toxoid or staphylococcal enterotoxins. Many of these preparations are not standardized for potency and dose-response experiments are necessary. For example, our laboratory uses ultraviolet-irradiated herpes simplex virus in doses of 10⁶, 10⁷ and 10⁸ plaque-forming units (PFU) ml⁻¹; tuberculin PPD in concentrations of 1.0, 10 and 30 µg ml⁻¹; *Candida albicans* in doses of 1, 10 and 100 µg ml⁻¹; and tetanus toxoid in doses of 0.01, 0.1 and 1.0 limit flocculation units (LFU) ml⁻¹. Each dose is studied in triplicate and a set of unstimulated cells serves as the control for spontaneous activity.
7. The duration of culture varies with the stimulant used. For mitogens, peak responses with human cells occur on day 3; for antigens peak responses occur on day 5 or day 6; and when allogeneic cells are used as the stimulant in mixed leukocyte reactions, peak responses occur on day 6 or day 7. In these last experiments the stimulating cells are 'blocked' by irradiation or incubation with mitomycin C (50 µg ml⁻¹ for 20–30 minutes at 37°C), after which the mitomycin is removed by washing.

Lymphocyte transformation test using murine lymphocytes

In many studies it is desirable to study responses by a subpopulation of cells from a lymphoid organ, and it is necessary to selectively remove the cells of interest from complex mixtures. It is also desirable to remove erythrocytes from suspensions of spleen cells.

1. Lymphoid tissues from donor mice are aseptically collected and placed in cold tissue culture medium. The organs are minced with sterile scissors,

and single cell suspensions of lymphoid cells are obtained by gently pressing the tissue through 60 mesh stainless steel screens or 200 µm mesh nylon screens with the plunger from a 3 ml hypodermic syringe. The cells are suspended in a test tube in tissue culture medium and clumps of 'debris' are allowed to settle by standing the tube at an angle of 60° in an ice bath. After 10–15 minutes the cells are collected by decantation.

2. For spleen cell suspensions it is desirable to remove erythrocytes. The cells are suspended in 2–3 ml of fetal calf serum and 5 volumes of EDTA-ammonium chloride solution is added. After incubation on ice for 20 minutes the lymphoid cells are collected by centrifugation at 700 g for 10 minutes and washed twice with tissue culture medium.
3. At this stage the cell mixtures contain T lymphocytes, B lymphocytes and accessory cells (and other cells if thymus was used). They may be cultured at this stage or processed further to collect specific cells of interest. This can be done by 'positive selection', in which cells are preferentially removed from the mixture, or 'negative selection', in which the undesired cells are removed, leaving the cells of interest (Table 1). In general, positive selection is more specific, while negative selection serves to enrich the fraction of cells of a particular subtype.

Table 1 Selection of specific cell populations

Positive Selection

1. Panning by incubation of cells on plastic surfaces that are coated with monoclonal antibodies against immunoglobulin for B lymphocytes, anti-Thy-1 for T lymphocytes, or anti-CD4 or anti-CD8 for T lymphocyte subsets
2. Collection of selected cell populations on antibody-coated magnetic beads
3. Collection of antibody-coated cells by fluorescence-activated cell sorting (FACS)
4. Collection of dendritic cells and macrophages on plastic surfaces

Negative Selection

1. Depletion of adherent cells. Cell mixtures are incubated with nylon wool or Sephadex G-10 in small columns. B Lymphocytes and accessory cells adhere to the columns but T lymphocytes do not. They are collected by elution
2. Depletion by cytotoxicity. Cell suspensions are incubated with monoclonal antibodies against the undesired cell population. The sensitized cells are lysed by addition of complement. Anti-Thy-1 antisera remove T lymphocytes and anti-immunoglobulin or anti-MHC class II antisera remove B cells and accessory cells

Culturing murine lymphocytes

In general, murine lymphoid cells require enriched media with larger concentrations of glucose (Iscove's media or Dulbecco's MEM with high glucose), 5–10% fetal bovine serum, L-glutamine and 2-mercaptoethanol (5×10^{-5} M). Accessory cells such as irradiated or mitomycin-treated syngeneic spleen cells provide nutrients for optimal cell proliferation and are required as antigen-presenting cells in studies of antigen-induced proliferation. A mouse spleen yields $1\text{--}2 \times 10^8$ cells with approximately 33% of these cells being T lymphocytes.

The cells are suspended in the complete medium at a concentration of 10^6 cells ml^{-1} and distributed into wells of tissue culture trays (flat bottom) or culture tubes. The wells contain 200 000 cells and tubes contain $1\text{--}2 \times 10^6$ cells. If the cells have been separated from accessory cells it is necessary to add 'feeder cells' in the form of irradiated or mitomycin-treated syngeneic spleen cells with approximately 10^6 feeder cells per well or 5×10^6 cells per tube.

At this stage the stimulants may be added to the cultures, and the cells are ready to be incubated at 37°C in CO_2 -air.

Mixed leukocyte reactions

Responses to alloantigens are measured in cultures containing responder cells and allogeneic stimulator cells that have been treated with irradiation or mitomycin C. The treated cells cannot contribute to the proliferation or transformation response, but serve as sources of alloantigens. B cells, dendritic cells and macrophages serve this function.

Proliferation responses are usually maximal at day 6 or 7. This test is widely used for pairing donors with recipients for bone marrow transplantation.

Lymphocyte transformation tests with whole blood

With infants and small children it is often difficult to remove enough blood for detailed studies of lymphocyte responses to antigens and mitogens; however, an assay using whole blood can be used for screening such subjects. First, one should do a leukocyte count and differential to determine the number of lymphocytes. Then a sample of blood from a heel stick or a small volume of heparinized venous blood is diluted with 9 volumes of tissue culture media, as described above, and the cell suspension is distributed into culture trays or tubes. Often, there are so few cells that only a single dose of each antigen or mitogen can be evaluated; the dose that produces optimal responses in normal subjects should be used.

The culture conditions and harvesting are the same as described above, except that just prior to har-

vesting the cultures, the erythrocytes must be lysed with distilled water, EDTA-ammonium chloride solution, or a commercial lysing solution such as Zap-Isoton® (Coulter). If this is not done, the color from the hemoglobin will quench the counts per minute (c.p.m.) in the liquid scintillation spectrometer.

Quantitation of lymphocyte transformation responses

Since the introduction of the lymphocyte transformation test, the most widely used readout has been measurement of DNA synthesis with radioactive tracers such as ^3H - or ^{14}C -thymidine. The isotope is usually added during the final 4–16 hours of culture. The cells are then collected on filter paper disks, washed to remove unincorporated radioactivity and the incorporated radioactivity is measured with a liquid scintillation spectrometer.

Data from such experiments may be presented in a number of ways including net c.p.m. in which mean c.p.m. from unstimulated cultures are subtracted from mean c.p.m. of stimulated cultures, or as stimulation ratios (stimulation index) in which the mean c.p.m. in stimulated cultures is divided by the mean c.p.m. in unstimulated cultures. It has been argued that the mean c.p.m. should be logarithmically transformed to consider the exponential nature of cell division.

It is recommended that publications present the raw data to readers.

Nonisotopic assays for cell proliferation

The problem of disposing of radioactive waste has led to development of nonradioactive assays for DNA synthesis in lymphocyte cultures. 5-Bromo-2'-deoxyuridine (BrdU) is incorporated into the newly synthesized DNA and incorporation of BrdU is enhanced by addition of the thymidylate kinase inhibitor, 5-fluor-2'-deoxyuridine. The incorporated BrdU is detected with an antibody to BrdU and a peroxidase-conjugated second antibody in a standard ELISA assay. The reagents for this assay are available as commercial kits.

Alternative assays for transformation responses

An obvious shortcoming in the thymidine incorporation assay is that it only measures events during the later hours of the experiment. With the growing interest in the biology of lymphocyte growth and activation, new assays have been developed that measure events that occur soon after exposure of the cells to ionophores, mitogens and antigens. Other assays quantitate functional responses by activated cells such as secretion of immunoglobulins by B lymphocytes and cytokines by T lymphocytes.

- Changes in cellular ionized calcium (Ca^{2+}). Cells are loaded with a fluorescent dye such as Indo-1-AM and the cells are studied in a flow cytometer. Following addition of the stimulus the change in fluorescence profiles are monitored. Changes in calcium concentration in as few as 1% of the cells can be detected and may occur within 1–2 minutes after addition of the stimulus.
- Cell cycle analysis. Activated cells enter the cell cycle and synthesize DNA, which can be monitored by treating the cells with propidium iodide, an agent that reacts with double-stranded DNA. Changes in DNA synthesis can be detected in less than 24 hours if adequate numbers of cells are responding. The sensitivity of this assay greatly exceeds the traditional thymidine incorporation assay.
- Expression of cell membranes and receptors. Shortly after activation, B lymphocytes, macrophages and T lymphocytes express greater numbers of major histocompatibility complex (MHC) molecules and T lymphocytes express interleukin 2 (IL-2) receptors. These changes can be demonstrated within 24 hours after activation by staining cells with the appropriate monoclonal antibodies and performing FACS analysis.
- Cytokine production. Activated T lymphocytes secrete factors such as interferon γ and interleukins (IL-2, IL-4). Significant concentrations of cytokines can be detected by ELISA as early as 12 hours after activation.
- Immunoglobulin (Ig) synthesis and secretion. B lymphocytes secrete IgM, IgA, IgG and IgE in response to activation with anti-immunoglobulin, lipopolysaccharide, pokeweed mitogen or other activators. It usually requires 4–5 days to produce amounts of immunoglobulin that can be detected by radioimmunoassay or ELISA.

Clinical applications

The test is most commonly used to investigate suspected immunodeficiency in patients with unusual susceptibility to certain infectious diseases. Patients with severe immune deficiencies such as severe combined immunodeficiency (SCID) have little or no lymphocyte transformation responses to mitogens or antigens, even when they have normal numbers of lymphocytes in their blood. In other disorders, such as chronic mucocutaneous candidiasis, responses to mitogens may be normal, but the patients' lymphocytes do not respond to antigens, especially antigens from *Candida albicans*.

The test is also useful for identifying serum factors that inhibit normal lymphocyte transformation

responses. In these cases (such as patients with recurrent vaginal candidiasis, chronic mycobacterial infections or Hodgkin's disease) the proliferation responses are normal in normal serum but subnormal in autologous serum.

Recently, superantigens such as bacterial enterotoxins have been implicated in certain autoimmune diseases and toxic shock syndrome. These agents activate large numbers of T lymphocytes to proliferate and secrete cytokines.

The lymphocyte transformation test has also been useful for studying mechanisms of lymphocyte activation and in identifying critical cell interactions (e.g. CD40–CD40 ligand) and defects in signal transduction.

See also: Alloantigens; Antigens; Antilymphocyte serum; B lymphocyte activation; Cell separation techniques; Cytokines; Delayed-type hypersensitivity; Fetal calf serum; Lectins; Leukocyte culture; Mixed lymphocyte reaction (MLR); Phorbol esters; Phytohemagglutinin (PHA); Pokeweed mitogen (PWM); Polyclonal activators; Proliferation, lymphocyte; Statistics, use in immunology; Tissue typing; T lymphocyte activation; T lymphocyte clonal expansion; Viability, methods of assessing leukocyte.

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LYMPHOCYTES

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The small lymphocyte has a nucleus of mainly condensed chromatin and a scant cytoplasm that contains relatively few organelles. While we now know that this unremarkable morphology is associated with a wide phenotypic and functional heterogeneity, in the 1950s there were very diverse views about these cells, being regarded by some as multipotential stem cells and by others as long-lived cells that were terminally differentiated. In less than 50 years this situation has radically changed: not only is the central role of the lymphocyte in immunity established, but our understanding of these cells continues to grow at a truly impressive rate.

By the mid-1960s studies by Medawar and by Gowans had established that graft rejection, graft-versus-host reactions and antibody formation were all mediated by the progeny of small lymphocytes that underwent blast transformation when stimulated by antigen. Further, the demonstration by Gowans that lymphocytes recirculate from blood to lymph by passage through specialized endothelial cells in lymph nodes resolved the paradox that large numbers of these cells enter the lymph each day but are not a short-lived population.

While these studies established the role of the lymphocyte in the adaptive immune response they did not explain how a single cell type could have such a diverse range of functions. However, by 1967 the work by Miller on the effects on the immune system of neonatal thymectomy and of Mitchell and Miller on the role of thymus-derived lymphocytes in the generation of antibody responses demonstrated that small lymphocytes, despite their uniform morphology, consisted of subsets of cells with different functional attributes and different tissues of origin. This functional specialization provided an explanation of the experiments by Mitchison on the hapten-carrier effect in which he showed that the induction of hapten-specific antibody synthesis by lymphocytes of bone marrow origin (B cells) required the 'helper' activity of thymus-derived lymphocytes (T cells). These experiments, at least in retrospect, suggested what is now an established fact, specifically that B cells and T cells require different types of antigenic stimulation in order to respond.

The applications of classical serology and the study of B cells in experimental systems soon made two significant contributions to the study of lympho-

cyte heterogeneity: B cells were shown to express surface immunoglobulin which further experiments showed had the same antigen specificity as that secreted by the plasma cell progeny of these B cells. In contrast, T lymphocytes did not express surface immunoglobulin but a lineage-specific marker was discovered in the mouse whose thymocytes and peripheral T cells were found to express the Thy 1 antigen. The clonal nature of B cell surface immunoglobulin provided an explanation of the demonstration by Nossal (1960) that no plasma cell was found to produce antibody of more than one specificity and confirmed Burnet's clonal selection theory of antigen recognition.

The next major development came from studies of cytotoxic T lymphocytes which help to eliminate viruses by killing virus-infected cells. In 1974, Zinkernagel and Doherty found that these cytotoxic T cells killed their targets only if they shared the same haplotype of the major histocompatibility complex (MHC). This phenomenon of MHC restriction led to a series of experiments to determine whether T cells expressed two receptors, one for self-MHC and the other for antigen or whether T cells recognized 'altered self'. The altered self hypothesis was supported by the finding, in 1986, by Guillet and coworkers, that short peptides bound to MHC molecules were recognized by antigen-specific T cells. However a full explanation of MHC restriction had to await the X-ray crystallographic studies by Bjorkman and her colleagues which showed in 1987 that antigen-specific receptors on a T cell recognize a complex determinant made up of an antigenic peptide locked into the antigen-binding site of an MHC molecule. Those T cells that recognize MHC class I-peptide complexes express the CD8 cell surface antigen and their target cells present peptides derived from cytoplasmic proteins. In contrast, T lymphocytes that recognize peptide in association with class II express CD4 and in this case the antigenic peptides originate from extracellular proteins that are taken up by the class II positive antigen-presenting cells (APCs). The MHC restriction exhibited by cytotoxic lymphocytes with respect to MHC class I antigens is now known to be also a characteristic of CD4⁺ T cells interacting with peptides bound to MHC class II. Thus T cells that provide B cell help become activated by MHC class II-peptide complexes on APCs

and subsequently the activated T cells help when they recognize the same complexes on B cells.

In both APCs and B cells antigen is processed internally into peptides which are loaded into MHC class II molecules and transported to the cell surface. However, whereas the uptake of antigen by APCs is nonspecific, the uptake by B cells is antigen-specific and is mediated by the immunoglobulin receptors on the B cell surface. This dual recognition of antigen, conformational determinants of proteins by B cells and peptide determinants by T cells, has probably evolved to ensure an extra degree of specificity control in the antibody response. This view is supported by the observation that self-tolerance at the T cell level is more extensive than it is for B cells.

The further study of the immune system was immeasurably facilitated by two technological advances. In the 1950s immunologists had been quick to make use of the fact that the ability of the immune system to generate antigen-specific antibodies provided them with a valuable way of studying the distribution of antigens within tissues and the source of immunoglobulin-producing cells. However the full exploitation of antibodies as immunological tools became possible only when, in 1975, Köhler and Milstein developed monoclonal antibodies (mAbs). The great value of these reagents was not only that they were antigen-specific but also, as Williams showed in 1977, they could be raised against unknown antigens that might constitute only a minor part of a complex mixture of immunogens. With mAbs it was soon shown that small lymphocytes were phenotypically much more diverse than the applications of classical serology had suggested and the CD4/CD8 dichotomy, to which reference has already been made, is but one example of this. The identification by mAbs of different subsets of lymphocytes made possible detailed functional studies of the roles of these different cell types and helped to establish lineage relationships between them. Further, they could be used to specifically interfere with intermolecular interactions and thus give indications of the functions of these molecules. Antibodies identified by such studies have become potential therapeutic agents.

The second major advance was the development of gene sequencing by Sanger in 1975 and of recombinant DNA technology by Sambrook and many others. An early consequence of the application of this methodology was the characterization of antibody genes by Tonegawa and others, and later of the T cell receptor genes by Davis, Mak and others. The nature of the diversity among B cell receptors at the protein level had been largely established by classical amino acid sequencing by Milstein and others but

a detailed understanding of the genetic basis of this diversity awaited the ability to sequence the genes themselves. The results of DNA sequencing showed that both B cells and T cells generate their repertoires of receptors by random rearrangements of DNA segments obtained from a large pool of genetic elements. (The process can be considered loosely akin to the generation of a dictionary of words by the use of only 26 letters.) Further diversity is generated during the process of gene rearrangement, by introducing or deleting a few base pairs at the junctions between the genetically encoded elements. Finally, among B cells, but not T cells, the receptor genes may undergo somatic mutation during an immune response.

The applications of gene sequencing and recombinant DNA technology, which enables genes to be expressed at the protein level and mutants and chimeric molecules to be generated at will, has had an impact on cellular as well as molecular immunology. It has proved possible to develop transgenic mice that express an experimentally introduced gene and to produce mutant mice in which a selected gene has been eliminated by homologous recombination. By the application of these new techniques at least a partial understanding in molecular terms has been obtained of lymphocyte homing, of lymphocyte effector functions and of self/nonself discrimination. It is known that lymphocytes interact with each other and with other cell types, such as antigen-presenting cells, endothelial cells and virus-infected target cells both through cognate cell/cell interactions and via the synthesis and binding to surface receptors of soluble mediators termed cytokines. Many of the molecules involved have been cloned at the DNA level and expressed as recombinant proteins. These reagents provide material both for basic biochemical studies and for the derivation of specific mAbs that block their effector functions. The *in vivo* and *in vitro* use of these reagents, commonly cytokines and antibodies to them, has already enabled immunologists to selectively promote cell-mediated or humoral responses to antigens and have provided insights into the way that the immune response is physiologically regulated. A major advance has been the recognition that different subsets of CD4⁺ T cells exist, which produce distinct combinations of cytokines that mediate either humoral or cell-mediated immunological functions. Curiously it has recently been found that the experimental use of immunological mediators has been anticipated by pathogenic viruses which have captured genes from their mammalian hosts and use the products of these genes to their own advantage. It is highly probable that some lymphocyte genes will be first discovered in viruses.

In the review of lymphocytes, in the first edition of this encyclopedia, Gowans pointed out that our understanding of the function of these cells has developed only over about the last 40 years. There has been a most impressive expansion in knowledge in that interval but despite this progress there is some way to go before we can give a comprehensive explanation, in molecular and genetic terms of the beneficial effects of vaccination, a prophylactic procedure that was introduced by Jenner 200 years ago.

Acknowledgements

The text of this article is, in part, adapted from the entry 'Lymphocytes' by JL Gowans, which appeared in the first edition of the *Encyclopedia of Immunology*.

See also: Bursa of Fabricius; Lymphatic system; Lymph nodes; Lymphocyte trafficking; Mucosa-associated lymphoid tissue (MALT); Spleen; Thymus.

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LYMPHOKINE-ACTIVATED KILLER (LAK) CELLS

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In response to exogenous activation stimuli, e.g. interleukin 2 (IL-2), some T lymphocytes as well as natural killer (NK) cells increase in size, become more granular and acquire the ability to bind to and kill a wide spectrum of tumor cells, virally infected cells and even some normal tissue cells. Such IL-2-activated effector cells have been referred to operationally as lymphokine-activated killer (LAK) cells. In studies using blood from normal human donors, the phenomena of NK and LAK activities were shown to be closely related; however, in some cancer patients or for cells from other organs, LAK cells may consist of both NK and T cells at various stages of activation and in various proportions.

Operationally, LAK cells are defined as lymphocytes that mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity against a broad array of targets following *in vivo* or *in vitro*

activation with IL-2 alone or in combination with other cytokines. The LAK phenomenon was originally described by Grimm and colleagues in 1982, and since then it has become clear that LAK cells do not represent a novel lymphocyte type, as initially suggested. More recent studies have demonstrated that LAK cells are derived from either NK or T lymphocytes, although in the blood of normal human donors, NK cells are the predominant precursor cells. B cells or monocytes do not give rise to LAK cells. Recently, several other surface markers with different molecular weights and functional properties, such as DM1, VGO1 and LAK1, have been described to identify human LAK cell precursors. Among T cells, a small subset of CD3⁺ CD56⁺ lymphocytes (generally less than 1% of total T cells), which mediate non-MHC-restricted cytotoxicity, seems to be the primary source of LAK cell precursor

sors. On long-term culture with IL-2 or in the presence of CD3-specific antibodies, the contribution of this T lymphocyte subset to LAK activity appears to be substantial. In LAK cells the large granular lymphocyte (LGL) morphology (Figure 1) is characterized by a reniform nucleus and abundant cytoplasm rich in azurophilic granules, which contain cytolytic and some enzymes possibly involved in the cytolytic process mediated by LAK cells. A lysosomotropic amine, 1-leucine-methylester, is effective in inhibiting generation of LAK cells by removing granular lymphocyte precursors. Like NK cells, LAK cells are a source of multiple cytokines and release appreciable amounts of some cytokines, such as tumor necrosis factor α (TNF α) and interferon γ (IFN γ). On the basis of phenotype, origin and functional characteristics, NK and LAK cells represent cytotoxic effectors at different stages of activation: the former in the poised state, ready to accept an activation signal when it is delivered, the latter in the highly active 'killer' state after delivery and processing. When human peripheral blood mononuclear cells (PBMNCs) are incubated *in vitro* with IL-2, a proportion of these cells acquire a property of adherence to glass or plastic. These plastic-adherent cells have a phenotype of NK cells, i.e. are CD3⁺ CD56⁺ CD16⁺ LGLs, and they have been named A-NK cells, for activated, adherent-NK cells. This property of adherence to plastic has been used to isolate and culture highly enriched A-NK cell preparations from human PBMNCs. Vujanovic and colleagues have shown that A-NK cells are more cytotoxic against solid tumor cell masses than nonadherent LAK cells, have a greater ability to migrate into tumor spher-

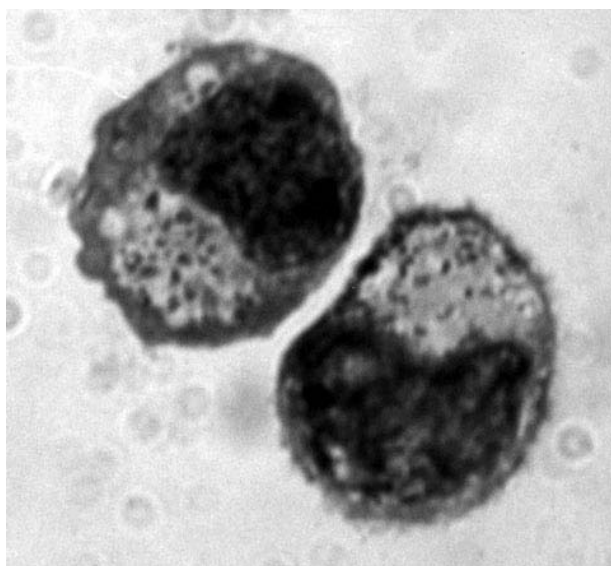


Figure 1 Photomicrograph of natural killer (NK) cells, with morphology of large granular lymphocytes.

oids, and their precursors in blood can be isolated with a monoclonal antibody to an epitope on N-CAM.

To measure LAK activity, ^{51}Cr -labeled NK-resistant tumor cells, e.g. the human lymphoblastoid cell line Daudi, are used as targets. Effector cells may be fresh PBMNCs prepared by Ficoll-Hypaque centrifugation of human blood, body fluids, bone marrow or disaggregated lymphoid tissues. *In vitro*, IL-2-activated effectors may be generated (usually after culture for 3–4 days) and then assayed for NK or LAK activity. The cytotoxicity assays must be performed at multiple (at least four) effector (E) to target (T) ratios, e.g. E:T from 50:1 to 5:1, in order to analyze the dose-response relationship between the percentage of specific lysis and the number of effector cells present. The assay is technically simple and reliable when performed in a clinical laboratory setting.

A 4 hour ^{51}Cr -release assay involves incubation of a constant number of labeled target cells with graded numbers of effector cells in triplicate wells of a microtiter plate. Killing is quantitated by measuring the amount of ^{51}Cr released into the supernatant. The results of the assay can be expressed as a percentage of specific lysis or, more conveniently, as lytic units (LUs), defined as the number of effector cells required to kill a predetermined number of target cells. The number of effector cells in an LU depends on their potency or lytic ability, with smaller numbers of cells indicating more potent effectors. LUs are calculated on the basis of lytic curves, established by plotting the percentage of specific lysis for each E:T ratio and then transforming the resulting curve into LUs. Various approaches have been developed for transformation of cytotoxicity data into LUs. These systems are available as computer programs.

On a routine basis in a clinical laboratory, LAK cell assays can be performed reliably but only if stringent quality control measures are established to assure reproducibility and minimize daily variability of these assays. We have recently described a program for controlling the performance of the NK assay in a clinical laboratory, and have implemented similar requirements for the LAK assay. Measurements of LAK cells are strongly influenced by the quality of cells used in the assay. Therefore, storage of the effector cells, their cryopreservation, the presence of human serum and even the temperature of cell storage may influence to a considerable degree the cytolytic activity of effector cells. For these reasons, we prefer to perform LAK cell generation on fresh rather than cryopreserved cell suspensions, in fetal calf serum rather than human serum, and on cells collected within 24 hours of the performance of

the assay. The use of cryopreserved cells for LAK cell generation is not recommended because of variable and often considerable losses in the ability to respond to activators *in vitro*, which are observed with cells of many individuals.

Human NK cells can be induced to adhere to the plastic surface of a culture flask after 24 hours of incubation of peripheral blood lymphocytes (PBLs) with recombinant IL-2 (rIL-2). In response to exogenous IL-2, some NK cells in PBLs, presumably those that express the p75 β chain of the IL-2 receptor, adhere to the plastic before T lymphocytes do. In this way, activated NK cells can be captured on plastic and separated from T lymphocytes, whose activation and expression of surface adherence molecules require a longer period of time. Studies by Melder and coworkers have shown that cellular adhesion molecules are involved in the attachment of NK cells to plastic because this adherence can be reduced markedly by monoclonal antibodies to the CD11 and CD18 adhesion molecules. Incubation of A-NK cells in the presence of IL-2 and conditioned medium results in their proliferation, which may continue for 2–3 weeks in culture, yielding large numbers of highly purified NK cells (generally 80–99% purity) expressing high levels of antitumor cytotoxicity. Although nylon wool-passed PBLs are generally used for A-NK cell generation, human splenocytes and tonsils as well as liver-infiltrating lymphocytes have also been used to generate A-NK cells with comparable levels of purity and antitumor cytotoxicity as those of A-NK cells obtained from PBLs. Overall, the generation of A-NK cells from NK cells in response to exogenous IL-2 has several characteristics: induction of adherence to plastic; increased antitumor cytotoxicity against NK-resistant target cells; and proliferation in the continued presence of IL-2. Because human A-NK cells can be generated in substantial numbers, e.g. 2×10^9 A-NK cells from leukapheresed normal donors, they represent an excellent source of enriched NK cells for study of their characteristics. These cells have recently been used to investigate the process of activation of NK cells, the expression of the CD18/CD11 family of adherence molecules on their surfaces, the role of Fc γ RIII in cytotoxicity mediated by A-NK cells, *in vitro* production of cytokines by these cells, and their movement on plastic as well as *in vivo* in the microvasculature of tumors and normal tissues.

Human A-NK cells have been generated from the blood of patients with various malignancies, including ovarian, hepatocellular, colon, and renal cell carcinomas, metastatic melanoma, and glioblastoma, as well as in patients with acute lymphocytic or myelo-

genous leukemia. In many cancer patients, proliferative and cytotoxic properties of A-NK cells are equivalent to those seen with A-NK cells generated from normal human PBLs. However, in patients with bulky disease or those previously treated with chemotherapy or radiotherapy, the ability to generate A-NK cells appears to be impaired. Also in certain types of cancers, the phenotype of expanding adherent-LAK cells may be different from that of A-NK cells obtained from normal individuals. For example, in glioblastoma, particularly in patients maintained on relatively high doses of steroids, we found that adherent-LAK cells had a phenotype associated with non-MHC-restricted T cells, i.e. CD3⁺CD56⁺. Nevertheless, these glioblastoma patients' adherent-LAK cell cultures had levels of antitumor cytotoxicity that were markedly higher than those obtained with unseparated PBLs cultured in the presence of IL-2. In most patients with cancer, however, and in contrast to IL-2 cultured PBLs (which are mixtures of IL-2 activated NK cells and T cells), A-NK cells represent homogeneous populations of activated NK cells.

Approaches to immunotherapy with LAK or A-NK cells

Adoptive transfers of LAK cells and IL-2 to tumor-bearing animals were effective in inducing the regression of established lung, liver and subcutaneous metastases of immunogenic and nonimmunogenic sarcomas or adenocarcinomas. To achieve therapeutic effects with LAK cells plus IL-2, it was necessary to administer sufficient numbers of LAK cells and high enough concentrations of IL-2 and to reduce tumor burden by previous surgery or by including chemotherapy. Therapy with LAK cells of IL-2 administered as single agents had little or no antimetastatic effects in animals bearing non-immunogenic tumors. On the other hand, high doses of IL-2 alone could induce appreciable tumor reduction in animals bearing immunogenic tumors. Basically, the experience with LAK cells plus IL-2 in animal models indicated that this form of therapy could be effective against a metastatic tumor if the tumor burden were first reduced by surgery or chemotherapy. Complete elimination of existing micrometastases and long-term survival could only be achieved in animals receiving all three treatment modalities – surgery, chemotherapy and immunotherapy.

On the basis of results obtained in animal models treated with LAK cells plus IL-2 and of well-documented *in vitro* induction of LAK activity in PBLs from patients with cancer, phase I clinical trials were

performed to determine the toxicity and antitumor efficacy of this form of immunotherapy in patients with advanced malignancies. The results from these early trials, some performed with IL-2 alone and others with a combination of LAK cells and IL-2, have not been as encouraging as originally expected. More than 70% of patients with advanced malignancies treated with immunotherapy did not respond, and some patients demonstrated severe toxicities that were subsequently linked to the high doses of IL-2 administered. On the other hand, 25–30% of patients with metastatic melanoma and renal cell carcinoma unresponsive to conventional therapy responded to immunotherapy with LAK cells plus IL-2. As more experience with adoptive immunotherapy was acquired, it became clear that improvements in this form of therapy are necessary and may indeed result in better overall response rates. The advantages and disadvantages of continuous versus bolus administration of IL-2 have been debated. Lowering the concentration of IL-2 administered to patients with cancer in order to eliminate the vascular leak syndrome has been considered. Attempts were made to prolong the half-life of IL-2 *in vivo* by using IL-2 complexed with polyethylene glycol (PEG-IL-2) or by manipulating infusion schedules. Rosenberg and colleagues recently reported the results of a randomized comparison of treatment with high dose IL-2 alone versus LAK cells plus IL-2, and found that administration of LAK cells plus IL-2 has additional clinical therapeutic efficacy above that achieved with IL-2 alone. LAK cells may not represent an optimal population of cells for therapy. The LAK cells are heterogeneous populations of IL-2-activated autologous mononuclear cells, and their *in vivo* distribution and therapeutic efficacy can be variable, depending on their cellular composition and *in vitro* activation achieved.

Once methods for generating large numbers of A-NK cells were developed, preclinical studies were performed to assess the antimetastatic activity of these cells. In several different syngeneic tumor models, the number of lung and liver metastases was found to be substantially reduced after treatment with moderate doses of A-NK cells plus IL-2. *In vivo* support of the A-NK cells with PEG-IL-2 appeared to provide particularly beneficial results. In a human metastatic tumor model in nude mice, purified human A-NK cells plus IL-2 were shown to have very potent and rapid antimetastatic therapeutic effects. Based on such promising preclinical therapeutic results, clinical trials with autologous A-NK cells and moderately high doses of IL-2 have been initiated. These treatments have been well tolerated and some therapeutic efficacy has been seen.

To understand the basis for *in vivo* antitumor effects and to increase therapeutic efficacy, detailed studies have been performed to evaluate the *in vivo* distribution and function of adoptively transferred A-NK cells. By use of fluorescent-labeled A-NK cells, it has been possible to demonstrate the selective accumulation of the transferred effector cells at sites of tumor metastases (Figure 2). Efforts are now being directed towards maintaining or enhancing the antitumor functions of such tumor-infiltrating A-NK cells in order to maximize their therapeutic efficacy.

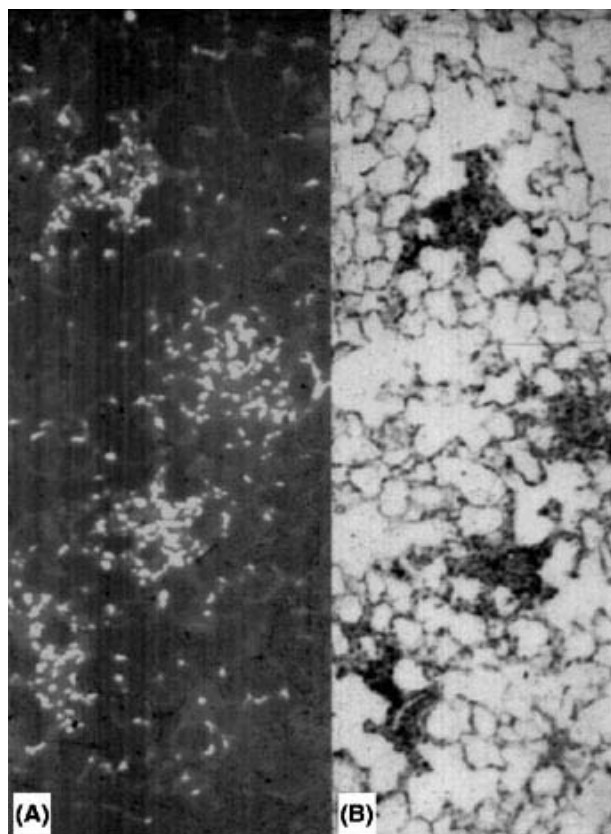


Figure 2 Localization of fluorescent-labeled murine A-NK cells, 18 hours after adoptive transfer intravenously to a mouse bearing metastases of B16 melanoma. (A) Distribution of fluorescent A-NK cells in lung; (B) stained adjacent section of lung, showing several micrometastases.

See also: Cytokines; Cytotoxicity, mechanisms of; Effector lymphocytes; Immunopotential; Immunotherapy of tumors; Interleukin 2; Natural killer (NK) cells; T lymphocytes; Tumors, immune response to.

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LYMPHOMA

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Lymphomas are neoplasms (tumors) arising from lymphoid tissue. They display the basic characteristics of neoplastic growth (purposeless, progressive, parasitic), and are distinguished further by being commonly multicentric, due to early spread, even in their most benign forms. Spreading reflects the normal migratory behavior of the cells of origin; in con-

trast, the spread (metastasis) of an epithelial cancer is a criterion of malignancy.

The vast majority of human lymphomas of recognized lineage arise from T or B lymphoid cells, and are usually confined to a restricted range of maturation. Only an occasional tumor is seen to arise from natural killer (NK) cells, mononuclear phago-

cytes (monocytes and macrophages), and the various specialized antigen-presenting cells such as dendritic cells. Lymphoma presents usually in a major lymphoid organ – bone marrow, thymus, lymph node, spleen or mucosal tissue – but as normal lymphoid cells are virtually ubiquitous throughout the body it is not surprising that their tumors sometimes appear first in other organs such as brain.

The diversity of lymphomas, coupled with arcane nomenclatures, is on first acquaintance quite discouraging. However, they have an academic importance far greater than their incidence (about 5% of human cancer) might warrant. The ease with which their constituent cells can be isolated and studied in the test tube, coupled with our current understanding of the markers and functions of normal lymphoid cells, means that we have a greater knowledge of these tumors than of any other. Some, such as Burkitt's lymphoma, exhibit well-characterized karyotypic abnormalities with associated oncogene activation, and so acquired a major role in unravelling the molecular basis of malignancy. A T cell leukemia/lymphoma was the first – and remains the only – human tumor to be associated with an RNA retrovirus. The immunoglobulin (Ig) products of secreting B lymphomas, known collectively as myeloma proteins, were of great importance in the unravelling of Ig structure and genetics. Now the rearrangements of genes for Ig and the T cell antigen receptor (TCR), with the resulting idiotypic epitopes in their protein products, provide excellent criteria for clonality and for divergence of the original neoplastic clone. It is also of some interest that an animal lymphoma, Marek's disease of fowl, was the first tumor for which an effective vaccine (directed against the oncogenic chicken herpes virus) was developed.

Nomenclature and classifications

Modern use of immunologic markers often detects neoplastic lymphoid cells in the blood of patients presenting with a mass of solid lymphoma. If such cells are present in numbers sufficient to give an abnormally high lymphocyte count, the case can be regarded as one of leukemia. Conversely, a patient presenting with a frankly leukemic picture might be troubled later in the disease by solid accumulations of neoplastic cells. Clearly there is no sharp dividing line between solid lymphomas and lymphoid leukemias, as has been recognized in the recent Revised European American Lymphoma (REAL) classification. However, in most current texts, and in this entry, the two forms are treated separately in deference to current clinical practice.

Another problem of nomenclature arises with neoplasms of Ig-secreting B lymphoid cells, of which the most important representatives in humans are multiple myeloma and macroglobulinemia. These have some features sufficiently distinctive to lead to their being considered apart from lymphomas in most texts. In this entry the term lymphoma will be used to embrace all neoplasms of lymphoid cells, including the leukemias and Ig-secreting tumors.

To classifying lymphomas there is no end. The varying roles of viruses, the often multiple genomic lesions, and the interactions with normal tissues combine to present tumors of bewildering variety, with apparently unique features associated with each patient. Nevertheless those common features which can be discerned have led to insights into pathogenesis, and often reflect significant differences in prognosis and responses to therapy, so that the efforts of lymphoma taxonomists have been well justified. Each person approaching the subject must decide upon the depth of classification which their interests dictate. The basic classification of the commoner human lymphomas set out in Table 1 provides the major headings in this entry. Much further along the spectrum of complexity are the 50 or so types and subtypes of lymphoma listed in the well-researched and eminently expandable REAL classification. Unfortunately a vast amount of clinical data are locked into the Working Formulation, a classification of non-Hodgkin lymphomas (NHL) in widespread use since 1982 which omits references to cell lineages and has proved poorly adaptable to recent advances.

Lymphoid leukemias

Acute lymphoblastic leukemia (ALL)

This group represents the common leukemias of childhood. The commonest variant is pre-B ALL. Here the cells lack surface B markers, but rearranged Ig genes, sometimes accompanied by cytoplasmic μ chains, identify their B lineage. Other variants include B, T and null cell ALL, the last having no known lineage-defining features. All varieties exhibit a cellular morphology and markers typical of immature lymphoid cells. A well-known marker of imma-

Table 1 A basic classification of human lymphoma

<i>Leukemia</i>	<i>Solid lymphoma</i>
Acute lymphoblastic	Hodgkin's disease
Chronic lymphocytic	Non-Hodgkin lymphoma
	Myeloma and macroglobulinemia

turity is the nuclear enzyme terminal deoxynucleotidyl transferase (TdT), whose normal role is to help diversify the antigen-receptor site in both T and B cells. ALL of childhood is one of the more successful arenas for modern chemotherapy: 5-year survivals are now at 60–70%, but achieved at the cost of considerable toxicity.

Chronic lymphocytic leukemia (CLL)

CLL in its common B cell form is the commonest of all human leukemias, with an incidence rising steeply with advancing age. The course of the disease is usually – although by no means always – fairly indolent, and it is probably true to say that B cell CLL found incidentally on medical examination after the age of 70 is of little actuarial consequence. The neoplastic cells, or the vast majority of them, resemble mature small lymphocytes with scanty cytoplasm. Their surfaces display B cell markers, including sparse surface Ig of uniform idiotype. They also often display CD5, which among normal cells is present on T cells and a small subset of B cells. Whether this means that the leukemia arises from the small B cell subset, or simply reflects circumstances which might induce any B cell to display the marker, is not apparent. The cell numbers in the blood can raise the lymphocyte count up to 200-fold. Cellular infiltrates enlarge and architecturally distort lymph nodes and spleen, and eventually pack out the marrow. Commonly death comes from marrow failure, from infection facilitated by immune deficiency, or from some unrelated age-associated problem.

Several uncommon variants of B cell CLL are recognized. The prolymphocytic variant displays larger cells with prominent nucleoli and abundant surface Ig. There is a tendency to infiltrate the spleen rather than lymph nodes, and a relatively malignant course. The cells of hairy cell leukemia display B cell markers, but have prominent surface villi and a capacity for phagocytosis – features which have not yet been identified among any normal B cell populations. This rare leukemia is also of interest because it will melt away (but not disappear entirely) in the face of interferon therapy.

In what may be called lymphoma spillovers, one sometimes encounters CLL-like blood pictures developing in the course of neoplasms which exist predominantly as solid lymphomas.

In the Western world T cell CLL usually presents with cells of 'large granular' morphology – medium lymphocytes with cytoplasmic azurophil granules. The appearance of such cells in the blood is often given the alternative designation T lymphocytosis, a term deliberately noncommittal as to whether the condition is neoplastic. There is, in fact, considerable

confusion at present regarding the significance of a blood picture of large granular lymphocytosis, which possibly can reflect at least three different conditions: CD8 cell CLL, NK cell CLL, and lymphocytic hyperplasia in response to chronic infection or other antigenic stimulus. Thus sometimes the lymphocytic cells display the surface molecule CD8 and a clonal rearrangement of the T cell receptor (TCR) genes, indicative of T cell neoplasm. Sometimes surface CD16 and other NK markers are present. Sometimes the picture is rendered more difficult by the presence of cells of mixed phenotype.

In southern Japan, the Caribbean basin and sub-Saharan Africa there occurs a relatively malignant adult T cell leukemia/lymphoma associated with the retrovirus HTLV-I (human T cell leukemia virus type I). In some areas this might represent the commonest form of leukemia. The viral infection is endemic in the tumor areas. The virus can spread from mother to child (mainly via milk), by blood transfusion, and probably at a low rate by sexual activity. Infection may be present for more than 30 years before the tumor appears, and the lifetime risk for carriers of the virus is probably not more than 1%. The cells are larger than in common CLL, are usually of CD4 phenotype, and display a relatively high density of receptors for the cytokine interleukin 2 (IL-2). Often there are skin infiltrations, lytic bone lesions and a marked hypercalcemia.

The Sézary leukemia is a well-known, although rare, neoplasm of CD4 lymphocytes which diffusely infiltrate the skin. The cells have characteristic deeply grooved 'cerebriform' nuclei. The infiltrated skin becomes red and can exfoliate. (Mycosis fungoides is a related lymphoma of CD4 cells, with skin infiltration which initially is patchy and indolent; the leukemic component is absent.) T cell prolymphocytic leukemia presents a clinical picture resembling that of the B cell type, but is rarer. The cells are usually of CD4 phenotype.

Hodgkin's disease

This tumor or group of tumors accounts for about a third of solid lymphomas in Western societies. It is an enigma in that although more successfully treated than other lymphomas (by chemotherapy and precisely controlled radiotherapy) its nature, i.e. the lineage of the neoplastic cell, remains a mystery. This cell (the Hodgkin cell, called a Reed–Sternberg cell when in bi- or multinucleate form) accounts in the vast majority of instances for only a tiny proportion (<3%) of the cells making up the tumor masses. It appears to be lymphoid in nature and frequently displays activation markers. However there has been no

consistent occurrence of T or B markers, nor of rearrangements of genes for Ig and the T cell receptor. Generally the predominant histological feature is a complex and variable inflammatory infiltrate comprising lymphocytes, macrophages, eosinophils, neutrophils and fibrous tissue. Such an appearance suggests a high local release of cytokines.

An interesting clinical feature is that the tumor is often seen to spread from a single original site. In this regard it differs from the non-Hodgkin lymphomas which are usually multifocal at the time of diagnosis. This difference means that the management of Hodgkin's disease depends more heavily upon an initial clinical 'staging', i.e. assessment of spread. The histological picture in Hodgkin's disease is also an important predictor of the clinical course. The commonly used Rye classification recognizes four variants called, in order of worsening prognosis, lymphocyte-predominant, nodular sclerosing (the most common type), mixed cellularity, and lymphocyte-depleted. Some evidence has recently been presented that the relatively uncommon lymphocyte-predominant type may in reality be a B cell lymphoma in most instances, and would be better considered apart from Hodgkin's disease.

Some epidemiological and genomic data suggest that the Epstein-Barr virus (EBV) has a contributory etiological role in many cases of Hodgkin's disease, particularly the mixed cellularity variety. In the AIDS-associated form of the disease the presence of EBV in the neoplastic cells is evident in virtually all cases.

Non-Hodgkin lymphoma (NHL)

This complex group of tumors have been seen to arise from all the known lymphoid lineages (B, T, NK, mononuclear phagocytic, dendritic). The lineage of origin in a given case can usually be identified without difficulty by a modern laboratory. However such information is rarely prominent in the clinical notes, first because it is not particularly discriminatory – more than 80% of NHL in Western societies arises from the B lineage – and secondly because clinicians, encouraged by classifications such as the Working Formulation, have long correlated prognosis and management with the 'grade' of tumor (low, intermediate or high) suggested by conventional histology. However closer studies of tumor behavior, and the advent of immunotherapy, are encouraging greater interest in tumor lineage. Table 2 sets out a lineage-based subdivision of NHL, simplified from the REAL classification, which will guide our discussion here. Distinctions between the various categories depend upon histology, and upon cell

Table 2 Subdivisions of non-Hodgkin lymphoma

B Cell NHL
B Lymphoblastic
B Lymphocytic
Mantle-cell
Follicle-center
Diffuse large B cell
Burkitt's
Mucosa-associated (B cell MALT lymphoma)
T Cell NHL
T Lymphoblastic
Enteropathy-associated (T cell MALT lymphoma)
Other peripheral T cell lymphomas

markers seen either in tissue sections or dispersed cells. Some of the rarer tumors have been omitted, as have the Ig-exporting B cell lymphomas which are discussed in the next section.

B cell NHL

B lymphoblastic lymphoma is seen histologically to be composed of diffusely distributed immature cells similar to those of pre-B ALL, and the two tumors can probably be regarded as belonging to a single disease spectrum.

B lymphocytic lymphoma can similarly be regarded as a tissue equivalent of CLL, with scattered tumor masses in which infiltrates of small lymphomas efface normal lymphoid architecture. Although the leukemic component is by definition inconspicuous initially, there is a tendency to progress to a final mixed leukemia/lymphoma with widespread tumor throughout nodes, spleen and marrow. Mantle cell lymphoma is a similar tumor which usually behaves more aggressively. It is composed of small to medium lymphocytes which, like those of CLL and B lymphocytic lymphoma, display the surface molecule CD5. The cells have a mainly diffuse distribution, but sometimes congregate around normal follicular centers to form huge mantles. This striking arrangement is apparently imposed by the normal cells upon the neoplastic ones. In this tumor a characteristic translocation between chromosomes 11 and 14 is thought to activate the oncogene *bcl-1*.

Follicle-center lymphomas (follicular lymphomas) are the largest group of NHL occurring in Western societies. Morphologically the cells resemble a segment of that range of lymphocytes which occur normally in the germinal centers of secondary lymphoid follicles. They show a variable tendency to organize themselves into follicles. In general, among the spectrum of follicle-center lymphomas, the larger the cells

and the more diffuse their organization the more aggressive the tumor. In some of the tumors there is a heavy admixture of normal T cells, whose role is obscure.

Follicle-center lymphomas are almost invariably multicentric at presentation. The clinical course varies markedly with the nature of the tumor, but most cases are fairly indolent and 5-year survivals may be expected in more than 50%. Some patients may be left untreated for a number of years, a course chosen by some physicians because, although responses to chemotherapy can be expected, extensive and prolonged remissions are unusual. Waxing and waning of the tumor masses may occur. Eventual progression of the disease is sometimes accompanied by conversion to a more malignant-looking histology, and it is likely under these circumstances that an emerging subclone has suffered additional genomic damage.

The characteristic karyotypic abnormality of follicle-center lymphomas, seen in up to 85% of cases in some series, is a reciprocal translocation between chromosomes 14 and 18. This moves the *bcl-2* oncogene from its normal position on chromosome 18 into the Ig heavy-chain locus on chromosome 14. There follows an overproduction of the *bcl-2* protein, which acts as a suppressor of apoptosis. The cell thereby resists the apoptotic fate of most normal follicle-center cells, a property which must contribute to growth of the tumor. Superimposed genomic damage such as mutation of the p53 suppressor gene has been associated with more aggressive behavior.

Diffuse large B cell lymphomas represent a heterogeneous group of aggressive tumors to which a variety of other names (including centroblastic and immunoblastic) have been given. The cells are larger than those of B lymphoblastic lymphoma and have a high proliferative rate. In some cases a translocation, of variable type, has been reported to activate the oncogene *bcl-6*. The patients often present with a single mass, and can respond well to (and even be cured by) chemotherapy.

Burkitt's lymphoma is a highly malignant extranodal tumor occurring mostly in children. It is composed of B lymphocytes of medium size which resemble follicle-center blasts, but are rarely seen to form follicles. The lymphoma is endemic among children in the tropical uplands of Africa and New Guinea. In these areas the tumor cells regularly contain the EBV genome. In Western societies the tumor occurs only sporadically and is associated with EBV in only a minority of cases. In both situations one sees a characteristic disturbance of the genome in which the *myc* oncogene (or at least its two coding exons) is translocated from chromosome 8 to one of the Ig chromosomes (14, or less commonly 2 or 22).

The high tropical incidence of the tumor probably reflects something like the following chain of events.

1. Early infection with malaria and other parasites leads to a hyperplastic lymphoid system.
2. Infection with EBV also occurs very early in life, possibly acquired via the mother's milk or saliva.
3. The virus promotes mitosis in infected B lymphocytes, but is unable by itself to promote that relentless, environmentally independent growth which constitutes neoplastic autonomy.
4. The parasite-preoccupied lymphoid system cannot generate a sufficiency of the cytotoxic T cells which normally rid the body of EBV-infected B cells.
5. The exuberant B cell mitosis greatly enhances the chance of the chromosomal translocation with *myc* activation which is probably the major determinant of the neoplastic transformation.

Again a superimposed mutation in the p53 suppressor gene may contribute to oncogenesis in some cases, and other genetic lesions have occasionally been described.

Mucosa-associated B cell lymphomas (B cell MALT lymphomas) have been described in gut, salivary glands, thyroid and ocular adnexa. The histology tends to recapitulate that of the normal mucosa-associated lymphoid tissue (MALT), and the neoplastic cells tend to home to mucosal sites. Paradoxically most of these tumors arise in the stomach, which normally contains no organized lymphoid tissue. However such tissue can be acquired as a result of chronic infection with *Helicobacter pylori*, which has been found in association with more than 90% of the lymphomas. Low-grade tumors have been seen to regress after eradication of the bacterium. The association suggests that other causes of chronic mucosal inflammation can also predispose to MALT lymphoma.

Immunodeficiency-associated lymphomas

B cell NHL, in a variety of guises, is the most important tumor to emerge at increased incidence in states of immunodeficiency. Such states are now seen most often in association with AIDS or immunosuppression for organ grafting. The frequency with which NHL emerges appears to reflect the degree of immunosuppression, so that it is commoner following cardiac than renal grafts.

In most cases of immunodeficiency-associated lymphoma there is genomic and antigenic evidence of EBV in the tumor cells. In some cases a polyclonal lymphoproliferation is seen initially, from which a

clearly monoclonal tumor may later emerge. The AIDS-associated lymphomas often exhibit also the *myc*-activating translocations and histological appearances typical of Burkitt's lymphoma.

Despite the EBV association the post-transplant tumors are rarely of Burkitt type. More commonly the histology is of diffuse large B cell lymphoma, often with plasmacytoid cells present. A variety of genomic lesions have been described. Immuno-deficient states in general can also present pleomorphic lymphomas difficult to classify.

T cell NHL

T lymphoblastic lymphoma is a childhood tumor, the tissue-equivalent of T cell ALL with the cells indistinguishable. It tends to involve the thymus, to progress rapidly, and to terminate in leukemia. For reasons entirely unknown it afflicts predominantly boys.

Enteropathy-associated T cell lymphomas are generally seen as jejunal tumors associated with celiac disease. The latter is a malabsorptive state of puzzling pathogenesis, with features of both hypersensitivity (resolution upon elimination of gluten proteins from the diet) and autoimmunity (a strong association with certain major histocompatibility complex (MHC) class II alleles, the presence of reticulinspecific antibodies, and heavy lymphocytic infiltration of the jejunal epithelium). It seems likely that the tumor is preceded by the celiac disease and arises from the infiltrating lymphocytes. The tumor cells are of various sizes, sometimes pleomorphic. Jejunal ulceration and perforation often supervene.

Other T cell lymphomas are often lumped together as peripheral T cell lymphomas. They are a heterogeneous and confusing group, from which a few striking tumors stand out. Angioimmunoblastic lymphoma displays amid the neoplastic cells an expansion of high endothelial venules, the specialized vessels of T cell areas in lymphoid tissue through which lymphocytic migration from the blood occurs. In angiocentric lymphoma the neoplastic cells invade and occlude blood vessels, leading to ischemic necrosis. Anaplastic large-cell lymphoma is marked by a consistent and striking display of CD30, an activation antigen first observed in Hodgkin's disease. The cells are unusually large, have a variable display of T cell antigens, and often involve nodal sinuses and extranodal sites.

Ig-secreting tumors

Logically these tumors could be included among the non-Hodgkin's lymphomas, but clinical features related to their site of occurrence (myeloma) or

consequences of their protein secretion (macroglobulinemia) usually lead to their being considered apart. A secreted monoclonal Ig accumulating in plasma is called a 'paraprotein'. A secreted molar surplus of its light chain, escaping through the glomerular filter to appear in urine, is called 'Bence Jones protein'.

Macroglobulinemia (of Waldenström) is a lymphoma defined by the accumulation of an IgM paraprotein in large amounts in plasma. Pleomorphic small lymphocytes, some plasmacytoid, widely infiltrate lymphoid tissue so as to enlarge the spleen and lymph nodes and displace normal marrow. They are readily detectable in the blood by virtue of their monoclonal surface IgM. The osteolytic lesions seen in myeloma are rare. Typically the secreted monoclonal IgM is present in plasma at $>10 \text{ mg ml}^{-1}$, and can reach much higher levels. This can cause a striking rise in plasma viscosity, with the possible consequences including retinal hemorrhages and cerebral catastrophes. Such risks sometimes merit an emergency plasmapheresis to lower the paraprotein concentration. A bleeding tendency is common and might reflect a variety of paraprotein activities – raised viscosity, binding to clotting proteins, and interference with platelet functions. Other problems, for example cold hemagglutination and polyneuropathy, can be due to an autoantibody activity of the IgM.

Multiple myeloma is a tumor of plasma cells, sited in the marrow. Usually it is highly malignant. The cells spread diffusely throughout the marrow, and also form discrete nodules which erode the surrounding bone. The characteristic, sharply demarcated bone defects are due to osteoclast activation, possibly by cytokines (IL-1 β and tumor necrosis factor β (TNF β)) released by the tumor cells. In about 75% of cases there is an obvious plasma paraprotein (in order of frequency IgG, IgA, IgD, IgE), in about 50% a Bence Jones protein excretion exceeding 500 mg day^{-1} , and in perhaps 10% a clinically significant light-chain amyloidosis (associated with tissue deposition of the tumor light chains or fragments thereof). The plasma protein may be associated with troubles similar to those described for macroglobulinemia, but these are uncommon. More often the problems of this distressing disease relate to bone destruction, renal failure and immunodeficiency. The disease commonly presents with bone pain or pathological fractures. The kidney can be afflicted by hypercalcemia associated with bone destruction, by extensive protein deposition in tubules associated with Bence Jones proteinuria, by infection, and by amyloidosis.

Solitary myeloma, and myeloma at an extra-

osseous site (plasmacytoma) are occasionally seen. The latter form can readily be induced in the peritoneal cavity in BALB/c mice, a study which has made major contributions to Ig genetics and monoclonal antibody technology.

See also: Bence Jones proteins; Epstein-Barr virus, infection and immunity; Leukemia; T lymphocyte differentiation; B lymphocyte differentiation; Leukemia viruses.

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LYMPHOTOXIN

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Lymphotoxin (LT, also known as TNF β) was originally described as a cytotoxic activity present in culture supernatants of activated lymphocytes. Ruddle and Waksman termed the material 'cytotoxic factor'; Granger and colleagues called it 'lymphotoxin'. In addition to its cytotoxic activity, LT appears to play a role in several biological processes. *In vitro*, LT induces gene expression of immune molecules such as major histocompatibility complex (MHC) and adhesion proteins, stimulates B cell growth, acts as an osteoclast-activating factor, and stimulates the replication of human immunodeficiency virus (HIV). *In vivo*, LT plays a critical role in lymphoid organ development, and has also been implicated in the pathogenesis of the autoimmune diseases multiple sclerosis and insulin-dependent diabetes mellitus. LT may also play a role in protection from infection.

It has recently been determined that LT can be produced as either a soluble protein or surface-associated protein. The soluble form of lymphotoxin is composed of three LT α monomers. It is produced after antigen activation of cytotoxic CD8 T cells and the T_H1 subset of CD4 cells. It is also produced constitutively at a high level by several human T lymphotropic virus type I (HTLV-I)-infected cell lines, by

several murine malignant transformed cell lines that represent early stages of B cell development, by human myeloma cells, and by human Epstein-Barr virus-transformed B cell lines. There have also been reports of its production by cells of the central nervous system, including astrocytes. LT α is not generally produced by macrophages. Analysis of the LT α genes and their proteins indicates strong conservation between several animal species with a homology of approximately 70% at the amino acid level of a secreted mature glycosylated protein of approximately 169 amino acids in length. The molecular weight of the LT α monomer is about 25 kDa, while the secreted trimeric complex is approximately 60–70 kDa in size.

In 1991, Browning and Ware and their colleagues determined that LT was expressed on the surface of T cells. Biochemical characterization of this surface form of LT indicated that LT α subunits associated with a 33 kDa type II transmembrane protein whose gene was subsequently cloned and called LT β . The predominant form of the surface LT on T cells is a heterotrimer of one LT α subunit with two LT β molecules (LT $\alpha_1\beta_2$). LT β mRNA is constitutively expressed by lymphocytes with the spleen and the

thymus expressing the highest level of LT β *in vivo*. It appears that LT α expression is required for appropriate assembly and transport of LT β to the cell surface. The LT $\alpha_1\beta_2$ trimer possesses some cytotoxic activity although much less than LT α . It is not known whether an LT β_3 molecule exists and whether it possesses biologic activity.

LT α and LT β are also related to tumor necrosis factor α (TNF α), sharing sequence and structural characteristics as well as a tight genetic linkage. TNF α , LT α and LT $\alpha_1\beta_2$ all bind to members of the TNF receptor family. These receptors are type I transmembrane proteins with cysteine-rich repeats in the extracellular domain. LT α and TNF α both bind to the type I (TNFRI) and type II (TNFRII) TNF receptors that are also referred to as p55 and p75 respectively. As noted above, because of its relatedness to TNF α and its common receptor usage with TNFRI and TNFRII, LT α is also termed TNF β . TNF α , after release by TNF α convertase (TACE), and LT α trimers bind to and cause clustering of the TNFRI and TNFRII receptors, and this clustering is important for transmission of a signal to the target cell. The crystal structure of the LT α trimer with TNFRI indicates that three receptors are bound by each trimer. LT $\alpha_1\beta_2$ binds to the LT β receptor – originally called TNF receptor-related protein. A schematic representation of the TNF/LT ligand receptor system is shown in Figure 1.

Biologic roles

The role of LT α in lymphoid organ development was established through the analysis of mice made deficient in that protein through homologous recombination. LT α -deficient mice are completely devoid

of peripheral and mesenteric lymph nodes (Figure 2) as well as Peyer's patches. Though the thymus appears normal, there is marked splenic disorganization with a loss of T and B cell areas and germinal centers. It has not yet been established whether LT exerts its effects on lymphoid organ development through the secreted LT α trimer or through the surface-associated LT α/β complex. It has been assumed by some authors that LT exerts its effects in lymphoid development as an LT α/β complex because both TNFRI- and TNFRII-deficient mice have normal lymph nodes. This invokes a role for another receptor, which could be LT β R. An alternative explanation is that there is another LT-specific receptor which has not yet been identified. The role of LT β in lymphoid development will be revealed with the analysis of LT β -deficient mice. However, the organization of lymphoid tissue is probably quite complex, involving several TNF family members and their receptors. This is evidenced by the TNFRI-deficient mice who, although they have normal lymph nodes, lack Peyer's patches and germinal centers.

LT also plays a role in the initiation and elaboration of inflammation. LT α mRNA has been detected during several inflammatory processes in humans and mice. Its proinflammatory properties are most clearly demonstrated in a transgenic mouse model in which LT α is expressed in the pancreas under the control of the rat insulin promoter (RIP). These mice contain leukocytic infiltrates around the pancreatic islets – the site of transgene expression (Figure 3). The mechanism of the LT-induced mononuclear accumulation includes induction of adhesion proteins on the surface of endothelial cells that facilitate the egress of leukocytes to the site of transgene expression and may also involve the induction of

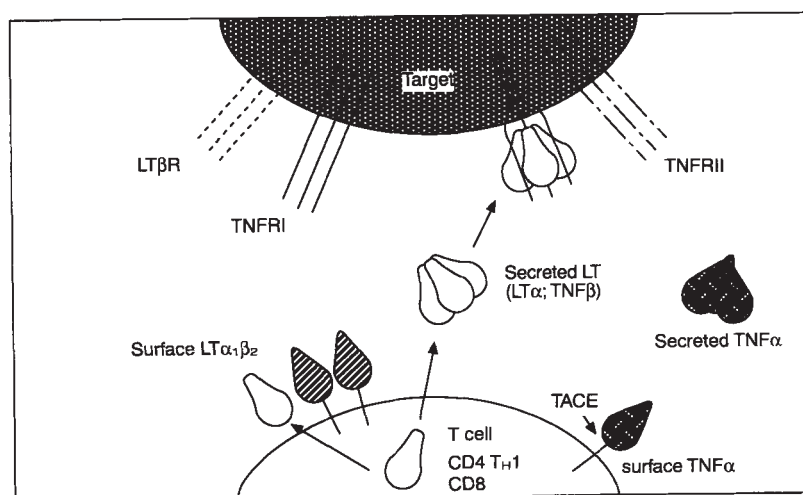


Figure 1 Schematic of TNF/LT ligand receptor system.

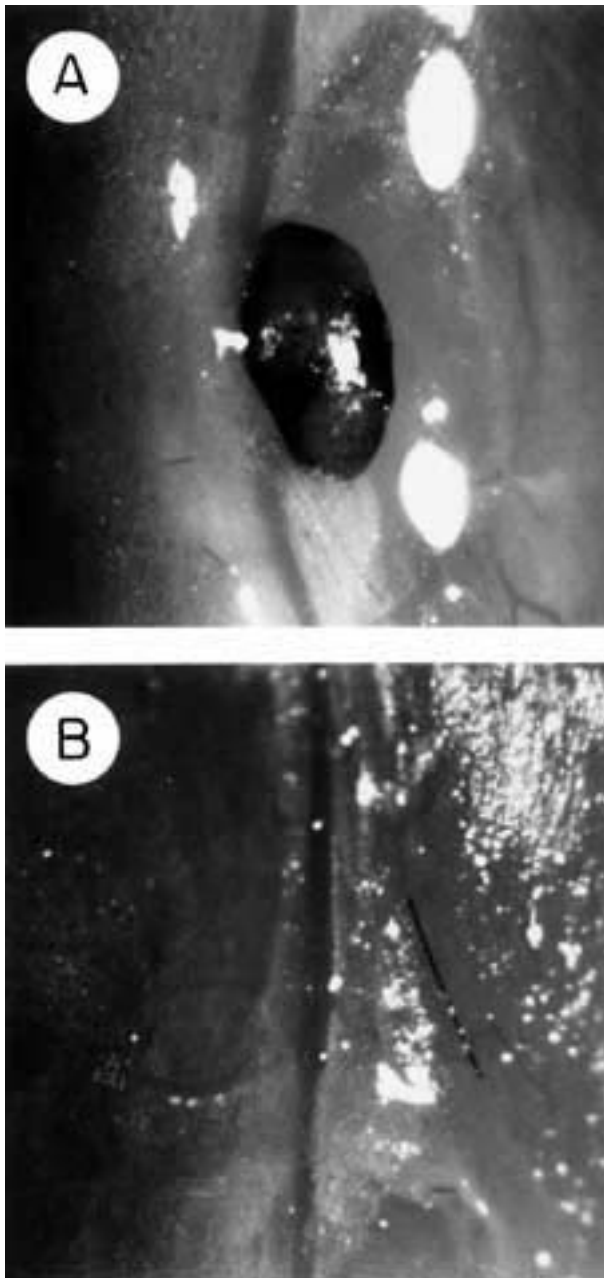


Figure 2 $LT\alpha$ -deficient mice lack lymph nodes. (A) Lymph node of a normal mouse. (B) The same region of an $LT\alpha$ -deficient mouse that lacks lymph nodes. (Reproduced with permission from De Togni *et al* (1994) *Science* 264: 703–707. © 1994 American Association for the Advancement of Science.)

chemokines that chemotactically recruit lymphocytes.

$LT\alpha$ is a mediator of killing by cytolytic T cells, 'helper-killer' T cells, natural killer cells, and lymphokine-activated killer cells. It is produced by such cells after stimulation by antigen presented by MHC, their natural targets, or high levels of interleukin 2. Other factors made by such cells that also contribute to killing and may synergize with LT to accelerate

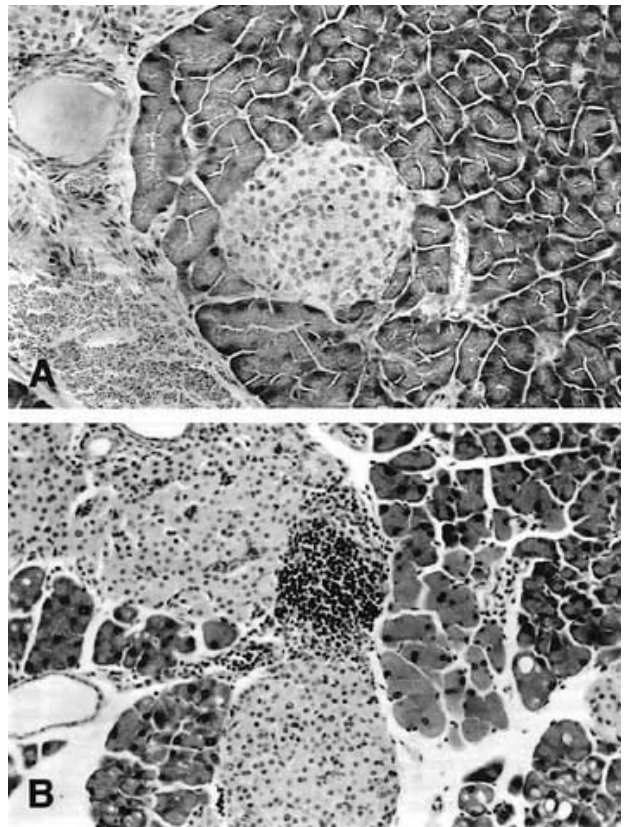


Figure 3 Expression of $LT\alpha$ from the rat insulin promoter (RIP) induces a mononuclear infiltration around the islets of Langerhans in the pancreas. (A) Normal islet of nontransgenic mouse. (B) Inflamed islet of RIP- $LT\alpha$ transgenic mouse.

the process include interferon γ (ITNF δ), TNF α , perforin and serine proteases. The ability of LT to induce DNA fragmentation by apoptosis, a characteristic of cytolytic but not antibody plus complement-mediated killing, further implicates it in cell-mediated killing. Through its cytotoxic activity, LT probably contributes to graft rejection and to manifestations of graft-versus-host reactions. LT kills virus-infected target cells more effectively than non-infected cells, in part because of its synergism with IFN γ . This property suggests that a crucial biologic role for LT may include defense against viruses, particularly through eliminating virus-infected cells.

$LT\alpha$ may also contribute towards the pathogenesis of autoimmune diseases. The ability of $LT\alpha$ to synergize with IFN γ and interleukin 1 in destroying β cells of the islets of Langerhans suggests a role in insulin-dependent diabetes mellitus (IDDM). Observations of induction of myelin swelling by LT, its cytotoxicity against oligodendrocytes and its production by cells of the central nervous system *in vitro* and in multiple sclerosis plaques, implicate it in demyelinating diseases. Its role as an inflammatory

mediator also suggests its involvement in such diseases with their prominent mononuclear infiltrates. LT α production is associated with the ability of T cells to transfer experimental allergic encephalomyelitis, a murine model of an autoimmune demyelinating disease. Suggestive evidence for the contribution of LT to autoimmune disease susceptibility in humans comes from genetic linkage studies that demonstrate an association between a polymorphic allele of LT α with increased risk of diabetes.

Gene structure and regulation

The lymphotoxin genes map within the MHC in humans on chromosome 6 and in the mouse on chromosome 17. They map between the complement (class III) genes and class I MHC genes in close proximity to the TNF α locus (Figure 4). The protein products of these LT/TNF genes share 50% identity at the amino acid level and have common structural motifs. These observations suggest that LT α , LT β and TNF α have arisen through a process of gene duplication.

Expression of LT is regulated at several levels. The promoter and 5' negative and positive regulatory elements of the LT α gene have been identified. Positive transcriptional elements include an NF κ B element. LT α expression is also regulated at the post-transcriptional level. A sequence located in the 3'

untranslated sequences of the gene, TTATTAT, has been implicated in mRNA destabilization. LT α mRNA accumulates to high levels in cells in the presence of cycloheximide, suggesting the existence of a labile repressor of mRNA, perhaps a ribonuclease. LT α has a conventional signal peptide. In contrast to TNF α , which can be displayed as a membrane-bound molecule and then released, LT α is transported through the cell and secreted or stored in granules in cytolytic T cells, or forms the LT α / β complex as noted above. The promoter region of LT β shares several features in common with the LT α promoter, including the presence of a NF κ B-binding site, indicating that LT α and LT β expression may be induced by common signals. However, the LT β promoter also contains transcription factor-binding sites that are not contained in LT α , implying that the two genes can also be regulated independently. The lack of lymphoid organs in the LT α -deficient mice indicates that this gene is expressed during development.

Signaling mechanism

At least one of the two molecular classes of TNF receptors of 55 and 75 kDa have been found on most cell types, with the exception of erythrocytes and resting T cells. They bind to LT α with high affinity. Dissociation constants (K_d) ranging between 10^{-9} and 10^{-11} M have been reported. The number of

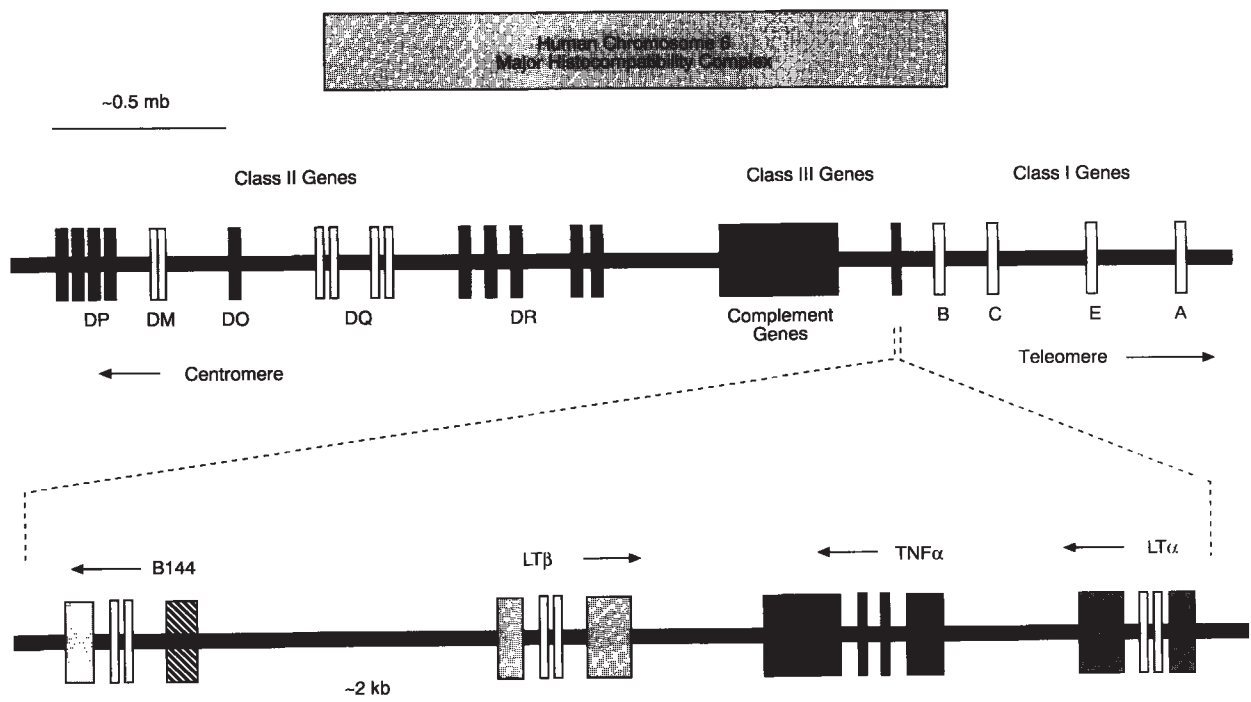


Figure 4 Genetic map of the tumor necrosis factor/lymphotoxin (TNF/LT) locus in the human MHC. (Reproduced with permission from Ware *et al* (1995) In: Griffiths and Tschopp (eds) *Pathways for Cytolysis*, pp 175–218. Basel: Springer-Verlag.)

receptors found on individual cells range from 2000 to 50 000. Though the presence of a receptor is required for a response to LT α , the number of molecules expressed by a cell does not necessarily reflect its sensitivity to LT α . Nevertheless, susceptibility to killing by LT α can be enhanced by treatment of targets with IFN γ and this does result in increased binding of LT α , presumably due to increased numbers of receptors.

Less is known about the properties of the LT β R. LT β R mRNA has been detected during embryogenesis as well as in the lung, kidney, liver, spleen and lymph node of the adult mouse, and in several tumor cell lines. The expression of LT β R during embryogenesis suggests it may mediate a signal for lymphoid organ development through binding to the LT $\alpha_1\beta_2$ complex. It is also possible that an as yet unidentified receptor for LT α also plays a role in lymphoid organ development.

Several intracellular signaling events have been implicated in the TNF/LT pathway, including protein kinases, ceramide, proteases, lipases and induction of gene transcription. However, since the TNF receptors contain no intrinsic enzymatic activity, it has been difficult to determine the intracellular events that occur following ligand binding. Recently, regions of the TNFR1, TNFR2 and the LT β receptor have been identified that are necessary for the transmission of signal to the target cell. An 80 amino acid region in TNFR1 is necessary for the cytotoxic activity of TNF. This region has been termed the 'death domain' and is also found in other members of the TNF receptor family that mediate apoptosis. An intracellular protein, TRADD, has also been identified that binds to the TNFR1 through the death domain and initiates the cytotoxic activity. TNFR2 and LT β R similarly possess regions in their cytoplasmic tails that bind intracellular proteins involved in the signal cascade. Both of these receptors bind to proteins called TRAFs. The TRAF (TNFR-associated factor) proteins associate with the receptor and form an intracellular complex that activates NF κ B and subsequent gene expression.

In conclusion, the role of LT in biological processes is quite complex and not fully understood. Its expression is required for the development of lymphoid organs and contributes towards the elaboration of inflammatory processes that are essential for clearance of infectious agents. However, LT expression can also be harmful, contributing towards the pathogenesis of autoimmune diseases. It thus appears that the effects of LT *in vivo* may depend upon appropriate expression levels at the correct time in development or during immune responses. The generation of mice deficient in LT α has allowed for the characterization of the role of LT in immune function as well as its contribution towards autoimmunity. Further data with LT β -deficient mice will provide information regarding the individual roles of secreted LT α and the membrane-associated LT α/β complex.

See also: Cytokine assays; Cytokine genes, regulation of; Cytokines; Cytokine receptors; Cytotoxic T lymphocytes; Cytotoxicity, mechanisms of; Effector lymphocytes; Experimental autoimmune encephalomyelitis (EAE); Graft rejection; Graft-versus-host reaction; Lymphokine-activated killer (LAK) cells; Tumor necrosis factor α .

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M

MACROPHAGE ACTIVATION

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The modern concept of the 'angry macrophage (Mø)', the immunologically activated cell generated during infection of the host by intracellular pathogens such as bacille Calmette–Guérin (BCG) and *Listeria*, dated from the work of Mackaness and his colleagues at the Trudeau Institute in the 1960s. Whilst T lymphocytes become activated by antigen-specific pathways, their production of Mø-activating lymphokines, later shown by various workers to be mainly interferon γ (IFN γ), results in enhanced antimicrobial activity directed nonspecifically against a range of microorganisms. This interaction between T lymphocytes and Mø lies at the heart of cellular immunity and delayed-type hypersensitivity. Subsequent studies have shown that a specialized antigen presenting cell related to Mø (the dendritic cell), plays a key role in the activation of T lymphocytes by foreign antigens and that T helper lymphocyte subset 1 (T_H1) responses are mainly responsible for Mø activation, whereas T_H2 responses modulate T_H1 and Mø activation. The major antimicrobial products of the activated Mø include reactive oxygen and nitrogen metabolites, and the range of targets can be extended to viruses including human immunodeficiency virus (HIV), and possibly tumor cells. The importance of Mø activation in host defence and pathogenesis of tissue injury has been underlined by experimental 'knock out' of key genes in the above pathway, and in genetic and acquired diseases in humans.

The Mø also plays a central role in innate immunity, in the absence of T cell activation, and interacts with specific antibody to enhance humoral defense mechanisms. The immunobiology of Mø can be compared with that of neutrophils and other myeloid cells, as well as natural killer (NK) cells and cytotoxic T lymphocytes. Some of the differences and similarities of Mø responses compared with those of other cells will be brought out below.

The definition of Mø activation given above is a relatively strict one and there are circumstances where it may break down. For example, microbial particulates and products, especially lipopolysaccha-

rides (LPS), have complex direct and indirect effects on Mø defense functions. Whilst well able to interact with and amplify IFN γ -induced responses of Mø, these agents also 'activate' a range of antimicrobial systems in the absence of IFN γ . Phagocytic stimulation via plasma membrane receptors involving specific antibody (FcR), other opsonins (e.g. complement) and opsonin-independent recognition of particulates profoundly alter the pattern of Mø gene expression, with overlap and differences from the IFN γ -induced modulation associated with immune, T cell-dependent activation. There is marked heterogeneity of the Mø phenotype within the host reflecting intrinsic changes in their differentiation and extrinsic modulation by cytokines and other factors in their microenvironment. The wide repertoire of plasma membrane receptors and biosynthetic responses, combined with extensive endocytic activity and vacuolar specialization, involve a complex set of Mø-specific and nonspecific gene products and signaling pathways which are still far from unraveled. Some simplifying concepts are now, however, well established and will be summarized here.

Principles of Mø activation

Cell differentiation and recruitment result in heterogeneity of Mø function *in vivo* – resident, recruited (elicited or primed) and reactivated Mø

Monocytes enter the circulation after production from hematopoietic stem cells and more committed precursors in bone marrow, a process that is controlled by various growth and differentiation factors, e.g. colony-stimulating factor 1 (CSF-1), granulocyte-macrophage CSF (GM-CSF) and interleukin 3 (IL-3), as well as inhibitors such as macrophage inflammatory protein 1 α (MIP1 α). The monocytes enter many tissues constitutively in the absence of inflammation, and differentiate into mature, nonproliferating, so-called 'resident' Mø populations. These Mø display a nonactivated phenotype; they express various receptors which allow them to interact with

adjacent cellular and matrix ligands at specific locations (**Figure 1**). Their main functions include trophic interactions with other developing hematopoietic cells and the ability to react to local injury and infection by phagocytosis, production of chemokines and vasoactive molecules, and activation of plasma proteinase cascades. These responses initiate inflammation and result in the recruitment of additional leukocytes, including monocytes themselves. Monocytes can be recruited to local sites of inflammation in large numbers, responding to a range of cytokines and other mediators that influence their adhesion to endothelium, diapedesis and subsequent migration. This process involves adhesion molecules (L- and E-selectins, β_2 -integrins and CD31) and chemokines that act on monocytes alone, or also on neutrophils or T lymphocytes. Recently recruited monocytes retain surface and other properties, e.g. type 3 complement receptor (CR3) and major histocompatibility complex (MHC) class II antigens and can be induced to produce a range of secretory products including tumor necrosis factor α (TNF α) and proteinases such as plasminogen activator (PA), that promote inflammation and repair. Once cells have been recruited in this manner, it is convenient to distinguish two types of inflammatory tissue M ϕ depending on their further exposure to IFN γ : those that become 'immunologically primed' or not ('elicited cells'). The immunologically primed M ϕ are able to respond to local surface triggers, e.g. phagocytosis of microorganisms or interaction with LPS, display an enhanced cytotoxic potential and are

thus fully activated for host defense; elicited M ϕ share many properties with activated M ϕ because of their recent recruitment, but do not express high levels of MHC class II antigens or produce the full range of cytotoxic mediators. Other distinguishing features include differential downregulation by IFN γ of mannose receptors, lectins that recognize a range of microbial ligands. Both elicited and activated M ϕ can aggregate and interact with persistent particulates (foreign bodies, microorganisms, respectively) and other host cells to form granulomata. Recruited M ϕ in diffuse or focal infiltrates thus vary in their phenotype, longevity and turnover; it is likely that Fas/Fas ligand, as well as TNF α /TNF α receptors (TNFR I,II), regulate apoptosis and resolution of M ϕ -rich lesions. Resident M ϕ are longer-lived cells, but even terminally differentiated M ϕ such as microglia in the brain, can be induced to express markers of activation by appropriate stimuli. Locally reactivated M ϕ are soon augmented by the influx of larger numbers of freshly recruited monocytes which become difficult to distinguish from pre-existing M ϕ as they all differentiate in a similar microenvironment. Finally, activation is a reversible process, and cells may undergo deactivation.

Two-stage induction of M ϕ activation – a spectrum of priming

As noted, IFN γ -primed monocytes or M ϕ depend on a further local trigger to initiate full expression of cytotoxicity, e.g. a respiratory burst and production of highly reactive oxygen species (**Figure 2**). The

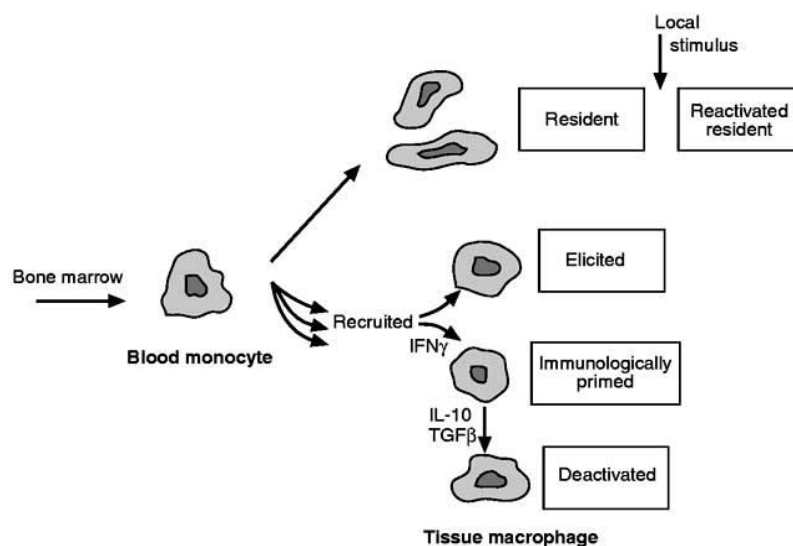


Figure 1 Heterogeneity of macrophages with regard to activation. Resident M ϕ can be stromal, adhering to tissue ligands and interacting trophically with many cell types in their microenvironment, or less adherent, e.g. in serosal cavities. Heterogeneity results from cellular differentiation and extrinsic modulation by cytokines and other molecules induced by interactions of host cells, often macrophages themselves, with foreign antigens, injurious stimuli and pathogens.

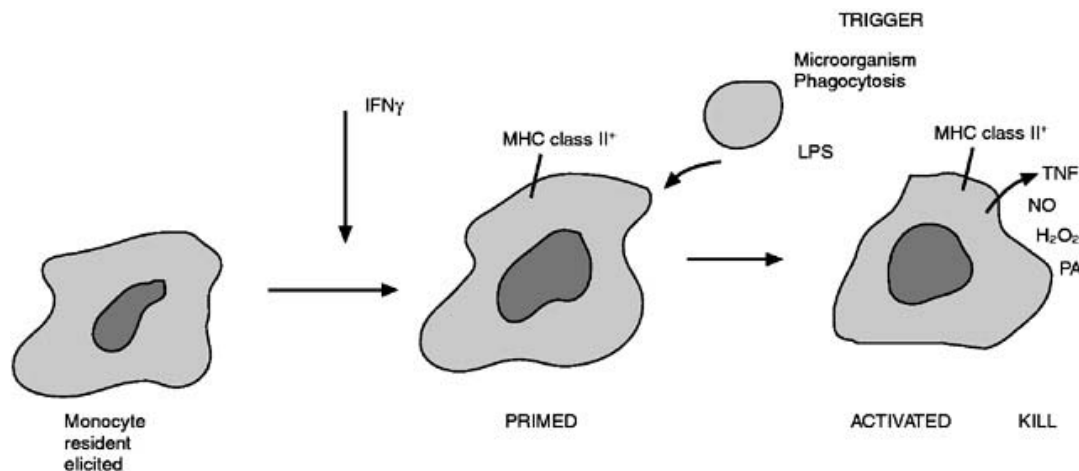


Figure 2 Two-stage activation of macrophages. Different stages of resident and recruited Mø require action of IFN γ to become primed. Local stimuli, often microbial products, trigger surface changes and production of cytokines such as TNF α and reactive oxygen metabolites. Neutral proteinases such as PA can be induced by IFN γ and their release triggered by phagocytosis, but are also induced by nonactivating cytokines, for example CSF-1.

requirement for priming by specific cytokines allows subtle control of appropriate gene expression in the Mø, which is biosynthetically more adaptable than neutrophils, for example, which store preformed products or can activate microbicidal pathways directly. T_H1 and T_H2 lymphocytes, NK, dendritic and other local cells, as well as Mø themselves, play a major role in regulation of Mø priming via cytokines (IL-12, IL-18, IFN γ , IL-4/13, IL-10, IL-6, transforming growth factor β (TGF β), IFN α/β) and other mediators (glucocorticoids, prostaglandins); cell surface molecules (e.g. MHC class II/CD4, adhesion molecules such as integrins and ICAMS, and immune accessory molecules such as B-1/B-2) also control hematopoietic and other cellular interactions (Figure 3).

Recent studies have shown that Mø priming and activation cover a phenotypic spectrum of cytokine actions on Mø (Figure 3 and Table 1) in which it is possible to distinguish not only the polar opposites of activation (IFN γ) and deactivation (IL-10), but also an intermediate phenotype, termed alternate activation (IL-4, IL-13). This spectrum of Mø properties is summarized in Table 1 and indicates considerable diversification of function, depending on their roles in cellular versus humoral immunity, inflammation and repair. It is likely that these described phenotypes, which are mainly based on *in vitro* studies, with isolated cells and individual cytokines, coexist *in vivo*, and that the net outcome of Mø activities within tissues will depend on their reciprocal interactions.

Plasma membrane triggers vary widely in their effects

Mø express a wide range of surface receptors for opsonins and direct microbial recognition as well as for migration, adhesion, cytokine responses and cellular interactions (Table 2). These vary considerably in their ability to trigger Mø biosynthetic and secretory responses; e.g. receptor for Fc fragment of immunoglobulin G (FcR) induces a respiratory burst and arachidonate release, whereas CR3 does not, in various experimental models. Other receptors, such as scavenger receptor type A (SR-A), may downregulate Mø responses to LPS, either by competition among different receptors for LPS (SR-A versus CD14, the receptor for LPS-binding protein), or by postmembrane interactions that are not yet clear. Another question that is not fully resolved is to what extent surface ligation and clustering of receptors is sufficient to trigger Mø secretion, compared with ingestion of phagocytic targets. Differential expression by activated Mø of plasma membrane receptor levels, e.g. reduced mannose receptor, may also not correlate with their ability to mediate secretory responses. Finally, there is still little understanding of receptor turnover and changes in intracellular compartments (endosomal, lysosomal) that accompany immune activation. Receptor-mediated endocytosis is strikingly influenced by different cytokines, and receptor molecules can be induced to undergo proteolytic cleavage and shedding as well as degradation by triggering agents.

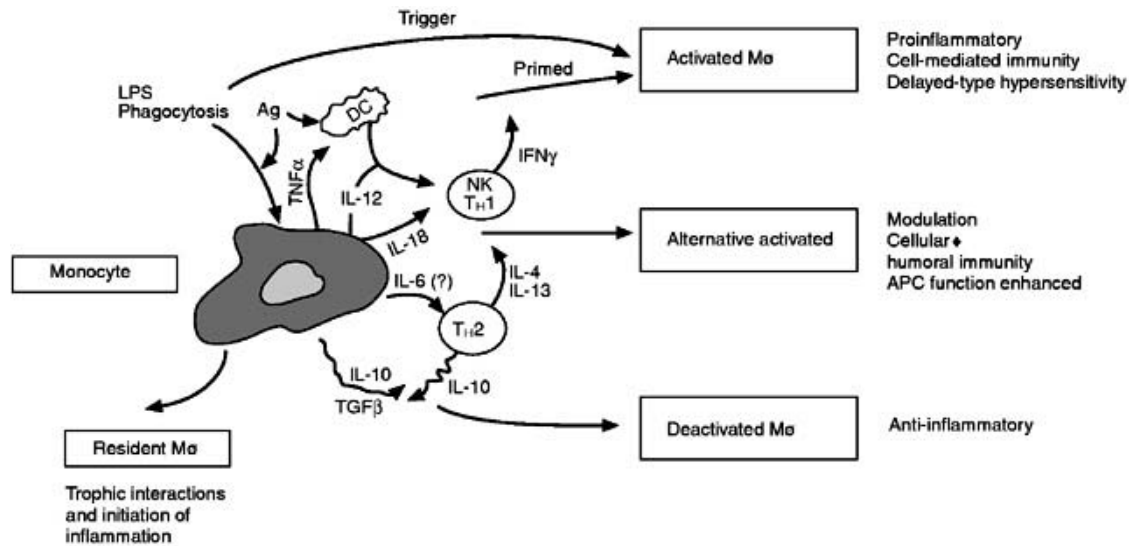


Figure 3 A somewhat speculative synthesis of interactions among macrophages, microbial agents and other immune cells, resulting in distinctive Mφ activation phenotypes. Initial contact with foreign antigens and organisms may involve Mφ as well as dendritic cells (DCs), which are specialized to present processed antigens to naive T cells. Mφ and DCs both produce IL-12; IL-18; an IL-12-like IFN γ inducing factor, has less defined sources and target cells. The role of different antigen-presenting cells (APCs) in regulating T_H2 versus T_H1 responses is still unclear. IL-6 has recently been put forward as a candidate. Note that Mφ can produce both pro- and anti-activation signals; TNF α influences DC migration and maturation, as well as performing other effector functions. IFN γ and IL-4 have additional cross-inhibitory and self-amplificatory activities.

Table 1 Spectrum of Mφ activation: phenotypic effects of T_H1 and T_H2-type cytokines on phenotype

	T _H 1-type	T _H 2-types		Significance
	IFN γ	IL-4/13	IL-10	
MHC class II	++	+	–	Immune cell interactions
Respiratory burst	++	(–)	–	Cell-mediated immunity
NO	++	(–)	–	Tissue injury
TNF α	++	(–)	–	Proinflammatory
IL-1				
IL-6				
Mannose receptor	–	++	0(+)	Phagocytosis/endocytosis e.g. antigens
Growth	–	+	0	Local growth of Mφ in immune lesions
Fusion	0	++	0	Giant cell formation – granulomata
Growth factor secretion	–	++	+	Healing of lesions

Diverse biosynthetic and secretory responses are protective, as well as injurious, and are tightly regulated

As a result of differentiation, recruitment, cytokine actions and plasma membrane stimuli, there are complex changes in Mφ gene expression and secretory activities (Table 3). Intracellular signaling pathways in Mφ are still poorly understood, as are the roles of myeloid-specific and other transcription factors that regulate gene expression by Mφ in different functional states. Nuclear factor kappa B (NFκB) and related molecules have received much attention,

mostly in simple model systems or cell lines rather than in primary Mφ *in situ*. IFN γ , by itself, induces a range of changes in expression of Mφ-specific and other genes, at the transcriptional level; inducible products such as TNF α , IL-1 β and IL-6 are controlled transcriptionally and translationally, as well as at post-translational stages, e.g. by processing at the cell surface mediated by specific metalloproteinases. Characteristic 3' AU-rich sequences contribute to instability of mRNA of several such inducible cytokines. Products such as lysozyme are more uniformly induced on activated Mφ populations by

Table 2 Selected plasma membrane receptors of activated macrophages

Receptor	Ligand(s)	Functions	Comments
FcR (several)	IgG, IgE	Opsonic phagocytosis, ADCC, release of inflammatory mediators	Ig superfamily FcR can downregulate hypersensitivity
CR3	iC3b, ICAM-1 promiscuous	Opsonic phagocytosis, non-opsonic phagocytosis, migration (?), adhesion, Mø activation	β_2 -integrin. May not signal secretion from Mø, promotes neutrophil apoptosis Essential for granuloma formation
Scavenger receptor (SR-A)	Selected polyanions, LPS, lipoteichoic acid	Endocytosis, phagocytosis, adhesion	Protects host against LPS
CD44		Adhesion, migration	
Sialoadhesin	Lectin: NeuAc α 2,3Gal β 1,3GalNAc on sialoproteins and gangliosides	Interactions with hematopoietic cells including T lymphocytes	IgSF Expressed by stromal and granuloma Mø Downregulated by IFN γ , IL-4, IL-13
Mannose R	Lectin: mannosyl glycoconjugates	Endocytosis, phagocytosis, secretion	Multiple C-type carbohydrate recognition domains Downregulated by IFN γ Upregulated by IL-4, IL-13
Cytokine receptors	IFN γ	Mø priming, activation, induction MHC class II, TNF α , iNOS, respiratory burst	Essential for granuloma formation, resistance to mycobacteria, other pathogens and opportunistic microorganisms
	IL-4	Alternate activation, stimulate MR endocytosis and APC functions	Similar to IL-13
	TNF α	Proinflammatory, cytotoxicity, apoptosis, granuloma formation, antimicrobial activity, DC migration and maturation	Distinct type I (p55) and II (p75) receptors Septic shock and tissue injury
	IL-10	Deactivation, anti-inflammatory, promotes humoral immunity	Autoregulation by Mø and T _H 2 cells. Can induce Mø genes, as well as inhibit
CD14	LPS-binding protein	Transduces LPS signaling activation, TNF α release	GPI-linked Septic shock soluble form

In addition there are receptors for other cytokines, chemokines and adhesion (CD31, selectins, other integrins), proteases, protease inhibitors, peptides and drugs found on monocytes and/or Mø and not well characterized in activated Mø. (See Gordon and Hughes (1997) for further details.)

poorly understood mechanisms, and serve as excellent markers of Mø activation *in situ*, compared with more limited expression of TNF α . Neutral proteinase activities can be enhanced by downregulation of inhibitors, as well as by induction of enzyme gene expression; their function close to the cell surface is promoted by plasma membrane binding sites, and regulated by endocytic uptake, binding to extracellular ligands such as connective tissue proteins, or inhibitors such as α_2 -macroglobulin which limit their activity to pericellular sites.

TNF α is a central cytokine responsible for many of the local and systemic actions of activated Mø. It is essential for granuloma formation and host

defense, but when produced in excessive amounts, e.g. by primed Mø in response to acute LPS challenge, it contributes to septic shock, vascular damage and death. In chronic infections, it may contribute to host wasting and other manifestations of increased catabolism.

Reactive oxygen and nitrogen metabolites play a major role in the ability of activated Mø to restrict a range of intracellular and other pathogens, as shown by studies in mice and humans, and contribute to local tissue damage. Expression of the various components of the NADPH oxidase and I-NOS is independently regulated, although considerable overlap in responses to cytokines, including IFN γ and

Table 3 Selected secretory products of activated macrophages

<i>Product</i>	<i>Function</i>	<i>Comments</i>
Enzymes		
Lysozyme	Antimicrobial	Constitutive <i>in vitro</i> , inducible <i>in vivo</i> ; myelomonocytic and Paneth cells
Urokinase	Plasminogen activation and fibrinolysis, neutral proteinase activation	Inducible by macrophage activation, phagocytosis and cytokines
Collagenase } Elastase }	Connective tissue catabolism	Inducible
Angiotensin-converting enzyme	Pressor	Steroid inducible
Acid hydrolases (many)	Lysosomal digestion	Mainly intracellular
Cytokines		
IL-1 β } TNF α }	Multiple local and systemic host defense functions, e.g. endothelium, leukocytes, connective tissue	Endogenous pyrogens, tissue injury, weight loss. Superinducible
IFN α/β	Antiviral, immune modulation	
IL-6	Acute phase response	
IL-10	Inhibitor proinflammatory cytokines and APC functions	Also product T _H 2 cells
IL-12	Stimulates IFN γ production by NK and T _H cells	
FGF	Fibroblast growth	
TGF β	Inhibitor activation of macrophages and other targets	
GM-CSF	Granulocyte, macrophage and dendritic cell growth and differentiation	
IL-8	Granulocyte chemoattractant	Chemokine family
MIP1 α/β	Chemoattractant, hematopoietic regulator	Chemokine family
MCP1	Monocyte recruitment	Chemokine family
Rantes	Recruitment of monocytes and T _H cells	
Complement proteins		
Most components classic and alternative pathway	Local opsonization and complement activation	Other sources, e.g. liver
Coagulation factors		
Several, including tissue factor	Local initiation and regulation of clotting	Other major sources
Adhesion, matrix molecules		
Fibronectin, thrombospondin, proteoglycan	Localization, migration, modulation of cellular interactions and phagocytosis	Other major sources
Transport proteins		
Transferrin, B ₁₂ -binding protein, apolipoprotein E.	Transport Fe, vitamin, lipid	Other sources
α_2 -Macroglobulin	Proteinase inhibitor	
Bioactive lipids		
Cyclooxygenase and lipoxygenase products of arachidonate	Mediators of inflammation, e.g. effects on leukocytes, small vessels	Macrophage has high-level arachidonate in membranes therefore potent source
PAF	Platelet activation	
Reactive oxygen intermediates		
Superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl radicals	Killing and stasis of microorganisms and cells by activated macrophages	Tissue injury, modulated by free radical scavengers
Reactive nitrogen intermediates		
Nitric oxide, nitrites, nitrates	Killing, microbial, parasitic, and cellular targets by IFN γ -activated macrophages	iNOS inducible, compared with low levels of NOS constitutive in other tissues.
		Note: NO not readily detectable in human monocyte/macrophage culture systems
Defensins		
Polypeptides	Antibacterial	Produced only by some macrophages as well as PMNs and Paneth cells

Some of these have not been demonstrated as products of immunologically activated versus other simulated M ϕ .
Adapted from Gordon and Hughes (1997).

IL-10, is present. Secretory activities of activated Mø are tightly regulated by multiple external and intrinsic factors. Products of activated Mø such as IL-10 and TGF β , as well as IFN α/β , prostaglandins and antiproteases, all serve to limit injurious Mø activities.

Intracellular pathogens utilize different strategies to subvert Mø activation

Microorganisms, including bacteria, viruses and parasites, utilize different uptake pathways and adopt a range of survival strategies to evade killing by activated Mø (Figure 4). The failure to trigger a respiratory burst may be connected to their survival; once within the Mø, an organism may interfere with maturation of phagosomes, inhibit acidification and fusion with lysosomes, or lyse the vacuolar membranes. IFN γ can reverse some of these processes, even when added after uptake of an organism. The vital role of IFN γ and its receptor in resistance to various infections has been confirmed in mice and humans, including mycobacterial infection of subjects and experimental animals genetically lacking these molecules. It should be stressed that cytokines such as IL-10 that deactivate Mø and thus enhance susceptibility to infections and inflammatory injury, as in inflammatory bowel disease, are also able to inhibit entry or growth of other pathogens, such as HIV-1. It is therefore necessary to define the role of

individual cytokines in Mø 'activation' in relation to challenge by particular organisms.

Activation of Mø can mediate immunosuppression

If Mø are persistently activated by repeated antigenic stimulation, or fail to eradicate an intracellular pathogen, the cells themselves can become refractory to subsequent stimulation. One consequence may be a failure to produce a respiratory burst in response to a further surface stimulus. The mechanisms are poorly understood and include receptor downregulation as well as inhibition of intracellular signal transduction pathways. An *in vitro* example of failure to reactivate Mø to a repeated stimulus is seen after pretreatment with phorbol ester (PMA), which activates protein kinase C irreversibly. Activated Mø through cell-cell and secretory interactions with T lymphocytes and dendritic cells can also mediate immunosuppression. Examples include inhibition by alveolar Mø, via nitric oxide of antigen-induced proliferation of T lymphocytes. Other Mø products (reactive oxygen metabolites, TNF α , prostaglandins) have also been implicated in antigen nonspecific immunosuppression.

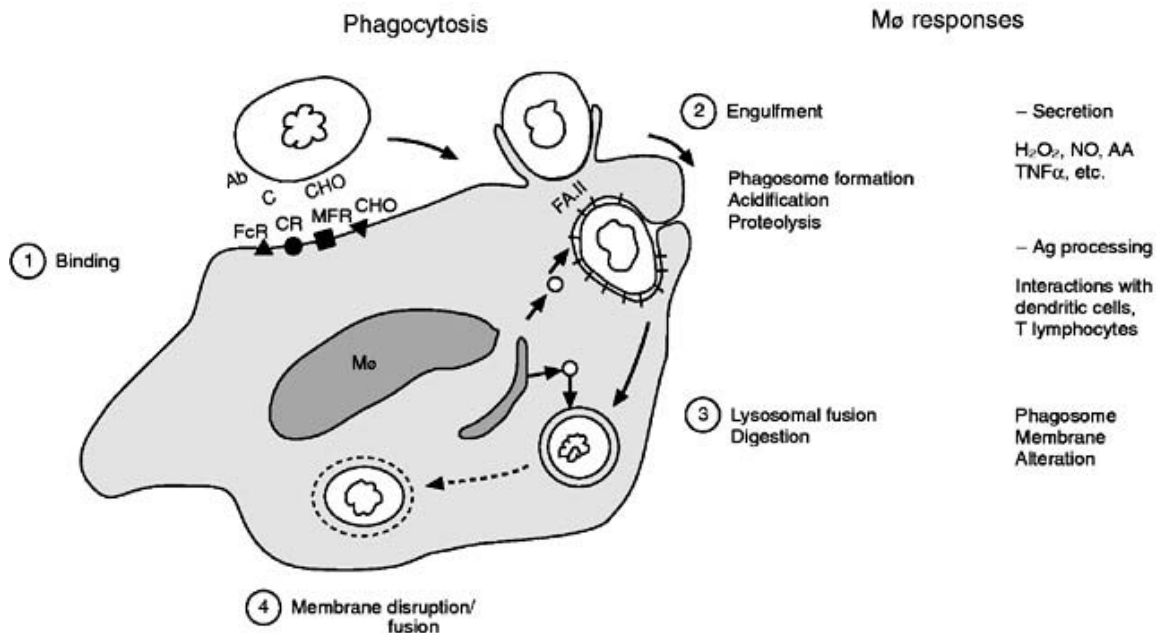


Figure 4 Stages of uptake of a hypothetical pathogen by macrophages. Receptors involved in phagocytic recognition are shown, as are potential steps of evasion of host killing mechanisms. Some of the Mø responses are listed. FA-11 (macrosialin, the murine homolog of CD68) is a pan-Mø glycoprotein abundantly expressed in phagosomes. Selected phagocytic stimuli alter the glycoforms of this membrane molecule profoundly, possibly contributing to phagosomal membrane resistance to intravacuolar enzymes and acidification.

Conclusions

Defective or exaggerated activation of Mø contributes to pathogenesis of a wide range of immunological and other diseases. Key molecules involved in this process provide suitable targets for therapy. Many questions regarding the selective expression of Mø genes and their regulation by extrinsic and intrinsic cellular processes remain for further study. The concepts outlined above should serve as a guide for further dissection of this complex process, and its relation to other aspects of Mø function.

See also: Adhesion molecules; Adjuvants; Antigen, entry into the body; Antigen-presenting cells; Antigens, cell surface; BCG; Biozzi mice; Complement, alternative pathway; Cell-mediated immunity; Chemokines; Cytokine assays; Cytokine genes, regulation of; Cytokine receptors, soluble; Cytokines; Cytokine receptors; Cytotoxicity, mechanisms of; Dendritic cells; Endotoxin (lipopolysaccharide (LPS)); Fas (CD95) and fas ligand; Fc receptors; Glucocorticoids; Granulocyte-macrophage colony stimulating factor (GM-CSF); Granuloma; Immunopathology; Inflammatory bowel disease; Interferon α ; Interferon β ; Interferon γ ; Interleukin 1 and its receptors; Interleukin 4; Interleukin 10 and its receptor; Lifespan of immune cells and molecules; *Listeria*, infection and immunity; Macrophage colony stimulating factor (CSF-1); Microbicidal mechanisms, oxygen-dependent; Microbicidal mechanisms, oxygen-independent; Microenvironment; Monocyte chemotactic protein 1 (MCP-1); Monocytes; Mononuclear phagocyte system; *Mycobacteria*, infection and immunity; Myeloid antigens; Neutrophils; Nitric oxide; Phagocytosis; Sarcoidosis; Septic shock; Spleen; Stromal cells; Targeting of immunological agents; Tolerance, peripheral; Tumor necrosis factor α ; TNF receptors; Viruses, immunity to; Chemotaxis of macrophages and monocytes.

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MACROPHAGE COLONY-STIMULATING FACTOR (CSF-1)

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CSF-1/macrophage colony-stimulating factor (M-CSF) stimulates the survival, proliferation and differentiation of mononuclear phagocytic cells, from the determined but undifferentiated monoblast through to the mature macrophage. It is the primary regulator of the production of these cells, including osteoclasts and also regulates cells of the female reproductive tract. CSF-1 is synthesized by a variety of different cell types, including fibroblasts, endothelial cells, bone marrow stromal cells, osteoblasts, keratinocytes, astrocytes, myoblasts and, under the control of the female steroid hormones during pregnancy, by uterine epithelial cells. It is homodimeric and secreted as an 80–100 kDa glycoprotein or a 130–160 kDa chondroitin sulfate proteoglycan or

is expressed on the cell surface as a biologically active, membrane-spanning glycoprotein of 68–86 kDa.

Both the secreted forms are found in the circulation. The cell surface form is involved in local regulation and the proteoglycan form may be sequestered to particular sites. The effects of CSF-1 are mediated by the CSF-1 receptor (CSF-1R) that is expressed on osteoclasts and tissue macrophages and their progenitors, as well as embryonic cells, decidual cells and trophoblast. Local and humoral regulation by CSF-1 is summarized in **Figure 1**. Autocrine or paracrine regulation by CSF-1 may be involved in the development or progression of myeloproliferative diseases and neoplasms of the ovary, endometrium

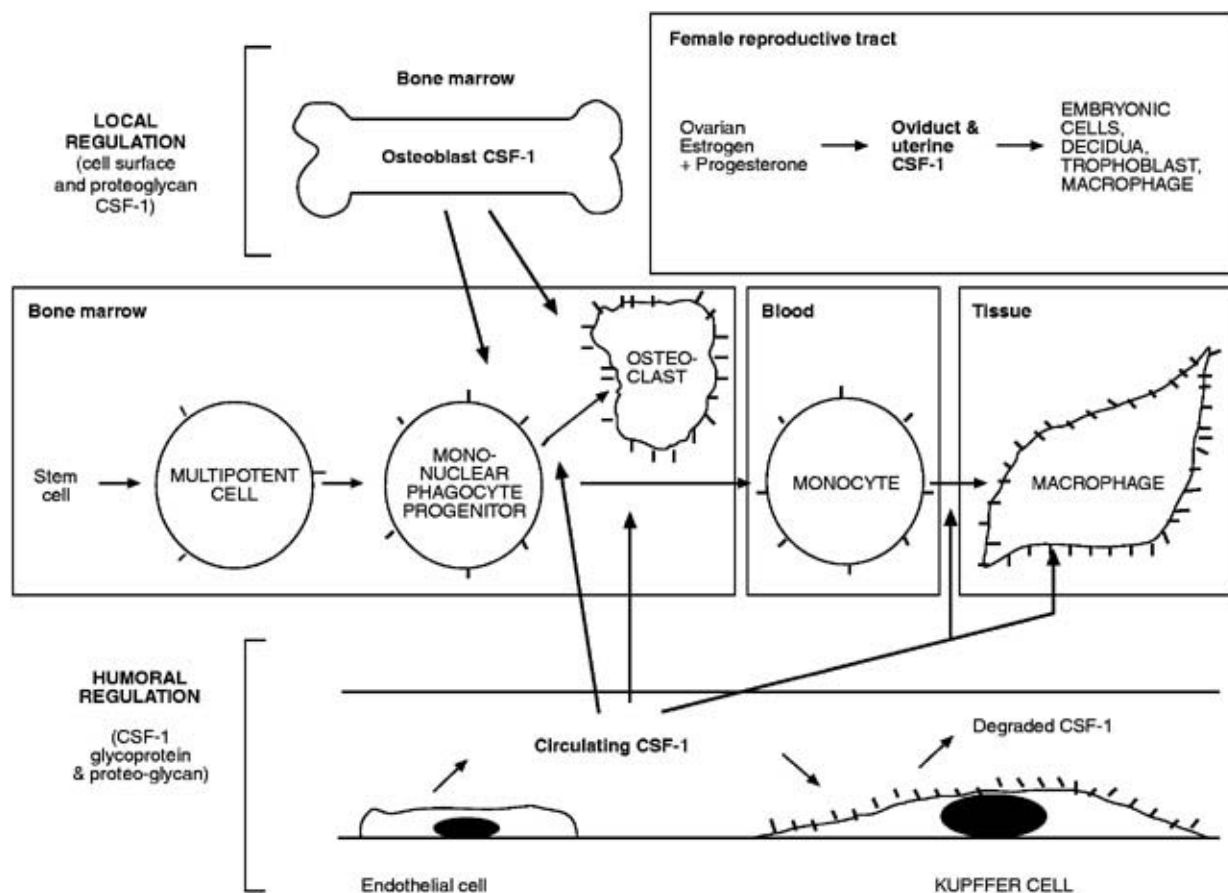


Figure 1 Humoral and local regulation by CSF-1. Target cells are labeled in upper case. Spikes on cells reflect relative CSF-1 receptor density.

and breast for which elevated levels of CSF-1 are a tumor marker.

CSF-1 genomic organization, biosynthesis and structure

The genomic organization and biosynthesis of the various forms of CSF-1 are summarized in Figure 2. Only the N-terminal 150 amino acids of the full-length 522 (human) or 520 (mouse) amino acid CSF-1 precursor are required for *in vitro* biological activity. The crystal structure of this region reveals that the CSF-1 monomer has an antiparallel four α -helical bundle/antiparallel β ribbon structure, similar to the structure of other cytokines, e.g. GM-CSF. The CSF-1 dimer is formed by disulfide bonding two monomers end-to-end, yielding a very flat, elongated structure.

The CSF-1 receptor and CSF-1 signal transduction

The CSF-1R is encoded by the *c-fms* proto-oncogene product. The CSF-1R gene is localized to human chromosome 5q33.3 and to mouse chromosome 18. The human CSF-1R gene is 58 kb in length and comprises 21 introns and 22 exons. Exon 1 encodes non-translated sequences and is 26 kb upstream of the signal peptide encoding exon 2. Gene transcription occurs in a tissue-specific fashion from two distinct promoters. Transcription is initiated upstream of exon 1 in placental trophoblast. However, in macrophages it is initiated immediately upstream of exon 2. The receptor possesses a highly glycosylated extracellular ligand binding domain (493 amino acids), a hydrophobic transmembrane domain (25 amino acids) and an intracellular tyrosine kinase domain

(436 amino acids). The extracellular domain of the CSF-1R is characteristic of members of the immunoglobulin gene superfamily and the tyrosine kinase domain is interrupted by an interkinase domain of 73 amino acids.

Signaling by the CSF-1R is initiated by binding of CSF-1 ($K_d = 0.1$ nM), leading to the stabilization of a dimeric receptor–ligand complex, transphosphorylation of the CSF-1R on multiple tyrosine residues, association of several proteins with the receptor and the tyrosine phosphorylation of cytosolic and membrane-associated proteins. Extracellular domain disulfide bonding, further tyrosine phosphorylation and multiubiquitination of the receptor precede internalization and intralysosomal destruction of the receptor–ligand complex. Proteins that associate with the activated receptor include Shc, Grb2, Cbl and the phosphatidylinositol phosphatase, SHIP. The juxta-membrane region of the CSF-1R has been implicated in internalization. The C-terminal tail of the CSF-1R has been shown to negatively regulate signal transduction and its truncation, in conjunction with point mutations in the extracellular domain in the *v-fms* product, leads to ligand-independent signaling and transforming activity.

CSF-1 biology

Circulating CSF-1 is believed to be derived from endothelial cells lining the small blood vessels. Ninety-four per cent of circulating CSF-1 (half-life 10 min) is cleared by CSF-1R-mediated endocytosis and intracellular degradation by Kupffer cells of the liver and splenic macrophages, only 5% being filtered through the kidney. This clearance mechanism represents a coarse negative feedback control on macrophage production, whereby the number of sinusoidally located end cells determines the steady-state concentration of circulating growth factor. In addition, CSF-1 is synthesized locally by osteoblasts and epithelial cells of the female reproductive tract. By virtue of local synthesis of the long-lived cell surface CSF-1 and of localized sequestration of the secreted proteoglycan CSF-1, both these forms may participate in short-range regulation of target cells (Figure 1).

The mouse mutation, osteopetrotic (op), is an inactivating mutation in the CSF-1 gene. While many of the overt phenotypes of the op/op mouse (Table 1) are significantly corrected by restoration of circulating CSF-1, failure to qualitatively rescue several phenotypes by daily injection with human recombinant CSF-1 (e.g. Table 2), indicates that CSF-1 also acts locally. Studies in op/op mice have established the central role of CSF-1 in the regulation of

Table 1 Overt phenotypes of the CSF-1 null op/op mouse

Osteopetrosis due to reduced numbers of osteoclasts
Reduced bone marrow cellularity
Reduced dermal thickness
Female reproductive defects:
Extended estrus cycle
Reduced pregnancy rate
Reduced postimplantation survival
Failure of lactating mammary gland development
Male reproductive defects:
Reduced testosterone production
Reduced mating capability
Reduced numbers of viable sperm
Reduced numbers of tissue macrophages
Reduced auditory and visual processing
Toothlessness
Reduced weight

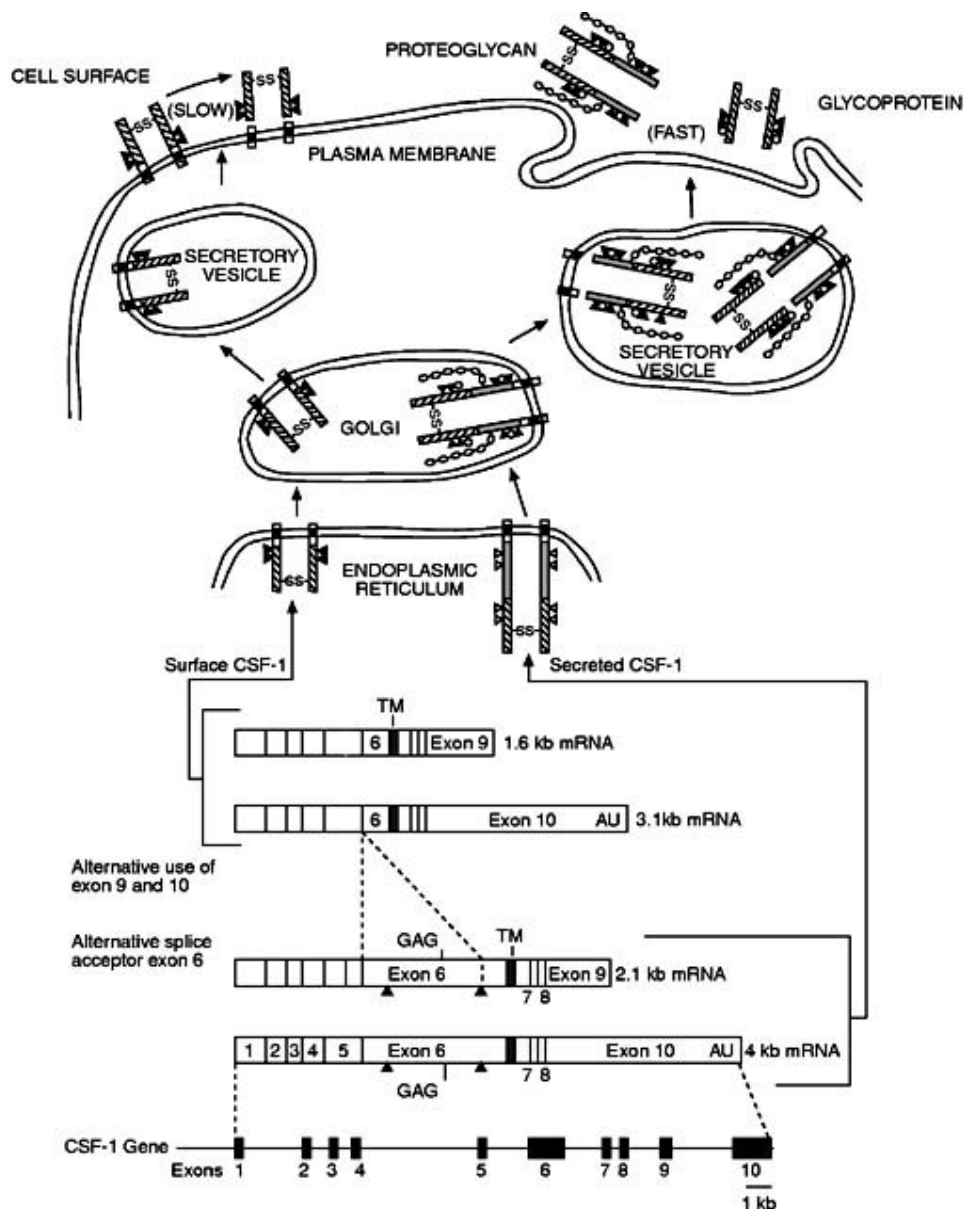


Figure 2 CSF-1 genomic organization, expression and structure. The CSF-1 gene is localized to human chromosome 1p13–p21 and mouse chromosome 3. The bottom half of the diagram shows the intron–exon structure and four representative human cDNA clones that have been sequenced. Similar mouse clones have been isolated. Exons (1–10) and the transmembrane domain (TM, cross-hatched), are indicated. The 4 kb and 2.1 kb mRNAs arise from alternative usage of untranslated regions encoded by exons 9 and 10, the latter exon encoding putative mRNA instability sequences (AU). The 1.6 kb and 3.1 kb mRNAs are products of a splicing reaction that results in the use of a short form of exon 6. The approximate intracellular proteolytic cleavage sites (arrowheads) and the chondroitin sulfate glycosaminoglycan addition site (GAG) are also shown. The top half of the diagram shows the processing of CSF-1 homodimers encoded by both the short and long coding regions. Striped regions represent those present in the mature secreted or released glycoprotein forms while both striped and filled regions are present in the major secreted proteoglycan form. N-linked (filled arrowheads) and O-linked (open circles) glycosylation sites, the chondroitin sulfate chain (linked, open hexagons) and the transmembrane domain (filled region) are shown.

osteoclast progenitor cell differentiation and tissue macrophage production (Table 2).

Local CSF-1 expression in the mouse female reproductive tract coincides with CSF-1R expression. CSF-1 regulates the estrus cycle, pregnancy rate, postimplantation embryonic viability and branching

morphogenesis of the lactating mammary gland. While CSF-1 is not absolutely required at any stage during pregnancy, it affects many stages and, if assessed by their ability to produce viable offspring beyond weaning, op/op females are almost completely infertile. CSF-1 regulates the development of

Table 2 Requirement of CSF-1 for tissue macrophage development and effect of restoration of circulating CSF-1 on macrophage development in op/op mice

Tissue	Criterion for detection	CSF-1 requirement	Effect of postnatal circulating CSF-1
Muscle	F4/80	Complete	None
Tendon	F4/80	Complete	None
Dermis	F4/80	Complete	Partial/none ^a
Periosteum	F4/80	Complete	None
Synovium	F4/80	Complete	None
Kidney	F4/80	Complete	Complete
Retina	F4/80	Complete	Complete
Peritoneal cavity	NSE ^b	Complete	None ^c
Pleural cavity	NSE	Complete	None ^c
Adrenals	F4/80	Partial	None
Bladder	F4/80	Partial	Partial/none ^a
Salivary gland	F4/80	Partial	Partial
Bone marrow macrophage	F4/80	Partial	Partial
Liver	F4/80	Partial	Complete
Stomach	F4/80	Partial ^d	Partial/none ^a
Gut	F4/80	Partial ^d	Partial/none ^a
Spleen	F4/80	Partial	Complete
Langerhans	F4/80	Independent	None
Thymus	F4/80	Independent	None
Lymph node	F4/80	Independent	None
Bone marrow monocyte	F4/80	Independent	None
Spleen	MOMA-1	Complete	Complete

^a Localized subpopulations exhibited different responses to restoration of circulating CSF-1.

^b Nonspecific esterase.

^c Local administration of CSF-1 restores subpopulation.

^d Localized subpopulations within these tissues are completely dependent.

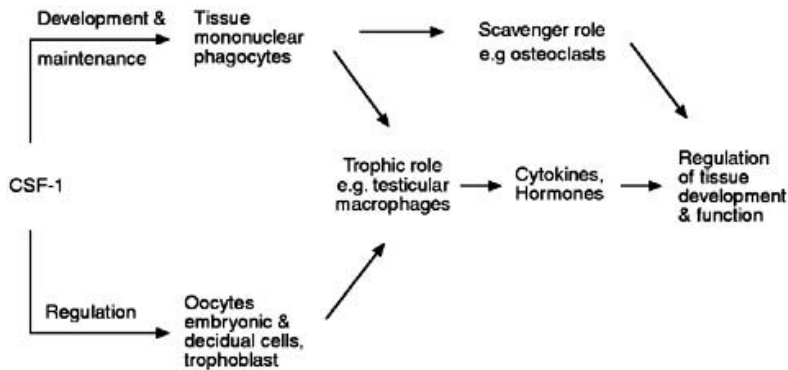


Figure 3 Regulation by CSF-1 of target cells that have trophic and/or scavenger roles in tissues.

testicular macrophages that normally contact the testosterone-producing Leydig cells. Associated with an absence of these testicular macrophages, male op/op mice have a low libido, abnormal Leydig cell morphology and reduced testosterone levels.

The expression of the CSF-1R appears to be restricted to mononuclear phagocytes, their progenitors and cells of the female reproductive tract. Thus the pleiotropic nature of the op/op phenotype suggests that one group of target cells, macrophages,

have important tissue regulatory roles. Tissue macrophages that require CSF-1 for their development are found primarily in tissues undergoing rapid morphogenesis or tissue remodeling (Table 2). A current hypothesis to explain the broad biological effects of CSF-1 is that CSF-1 regulates the development of cells that have important trophic and/or scavenger roles for the development and/or function of the tissue in which they reside (Figure 3).

CSF-1 and the CSF-1R in neoplasia

Viral oncogenic forms of *fms* cause feline fibrosarcomas. The 5' end of the CSF-1R is used approximately 20% of the time as an integration site for Friend murine leukemia virus-induced mouse myeloid leukemias. Autocrine regulation by CSF-1 is a characteristic of all irradiation-induced leukemias in SJL/J mice and of some *myc* retrovirus-induced mouse tumors. Circulating levels of CSF-1 are elevated in patients with myeloid and lymphoid malignancies and carcinomas of the ovary, endometrium and breast. In ovarian cancer, elevated levels of CSF-1 in the circulation or ascitic fluid predict a poor prognosis. At least in some cases, the malignant cells themselves coexpress CSF-1 and the CSF-1R, raising the possibility of autocrine/paracrine growth control by CSF-1 in selected human neoplasias.

See also: Bone marrow and hematopoiesis; Colony stimulating factors; Cytokine genes, regulation of; Cytokines; Granulocyte-macrophage colony stimulating factor (GM-CSF); Macrophage activation; Monocytes; Mononuclear phagocyte system.

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MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF)

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Macrophage migration inhibitory factor (MIF) has an important place in the history of molecular immunology. It has the distinction of being the first lymphokine activity described and as such was the forerunner of the whole new field of molecular immunology. In spite of this, MIF itself has been surprisingly elusive. The name MIF is applied to (at least) two major entities, both of which have some difficulties in definition. MIF was first used to describe a soluble lymphoid cell activity associated with delayed-type hypersensitivity (DTH). However the name has more recently been applied to a specific protein and most current references in the literature are to this protein. To what extent these two definitions overlap is not presently clear.

MIF as a lymphokine activity

The earliest definition of MIF was as a DTH-related activity. Using an *in vitro* assay for inhibition of migration of normal macrophages from a capillary tube, a soluble fraction produced by antigen-stimulated sensitized guinea pig lymphoid cells was defined as MIF independently by Bloom and Bennett, and by David in 1966. However molecular definition of MIF presented difficulties. A key problem, which became apparent as the field matured, is that a wide variety of factors including several cytokines, mitogens and toxins can inhibit macrophage migration. Furthermore, different cell types exhibit random migration which may be inhibited by different factors. Indeed, early attempts to characterize MIF led to the identification of multiple size and charge species. Specific cytokines, in particular, interferon γ (IFN γ) and interleukin 4 (IL-4) have been identified with migration inhibition activity.

One approach to defining MIF was to raise antisera against MIF activity fractions produced by human, mouse and guinea pig lymphoid cells. Although different antisera could block the elicitation of a delayed-type skin reaction, or could detect the expression of immunoreactive material in capillary endothelium a few minutes after stimulation by allergen or toxin, it is not clear to what they were actually reacting. When the human 'MIF' monoclonal antibody 1C5 was used by the Sorg laboratory to purify proteins from con A-stimulated human

peripheral blood monocytes, two proteins of 8 and 14 kDa were identified. These were cloned and were named MRP8 and MRP14, for MIF-related protein. MRP8 and MRP14 are calcium-binding proteins with interesting properties, but it is evident that they do not have MIF activity and they are now referred to as myeloid-related proteins.

MIF as a 12.5 kDa protein

The second, modern phase of MIF studies began with an elegant strategy to identify a protein with MIF activity. This employed expression cloning from a human T cell hybridoma. Pools of expression cDNA clones were assayed for MIF activity, eventually leading to purification of a single clone encoding a 115 amino acid, 12.5 kDa protein by Weiser and others. This protein was designated MIF, but its discovery was followed by some confusion in the field. Although several papers describing the activity of recombinant MIF from this source were published, some had to be withdrawn when preparations were found to be contaminated with phytohemagglutinin which is a potent inhibitor of macrophage migration. Adding to the confusion, what later turned out to be the same gene product was identified by Mikayama and others as glycosylation-inhibiting factor (GIF), a factor involved in regulation of the immunoglobulin E (IgE) antibody response.

In spite of this, the 12.5 kDa protein has retained the name MIF and has become the focus of a rapidly growing number of studies from several different laboratories. The protein does seem to have MIF activity, although this may be sensitive to sources or methods of purification or synthesis. For example, MIF lymphokine activity was detected in native rat liver MIF and recombinant mouse MIF, while both native bovine MIF from brain and recombinant human GIF/MIF apparently lacked detectable MIF activity.

So far, there is no evidence for more than one expressed MIF sequence in any species. For example, mouse GIF is identical to the MIF cloned from mouse eye lens and from BALB/c 3T3 cells, while human GIF is identical to human MIF from eye lens and both differ by only one nucleotide in cDNA sequence and one amino acid in protein sequence from the

original human MIF cloned from T cells, which appears to contain a sequence error or cloning artifact.

A pituitary hormone

In an extensive series of papers from Bucala and coworkers, important new immune system activities have been ascribed to the MIF protein. This group first reported the synthesis of MIF in the anterior pituitary gland and its release during endotoxemia and went on to suggest that MIF is a pituitary stress hormone regulating systemic inflammatory responses. They then expanded on the role of MIF by identifying it as a 'counter-regulatory hormone' for the effects of glucocorticoids in the immune system. Glucocorticoids are a natural part of the response to infection or tissue invasion, acting to modulate inflammatory and immune responses. *In vitro*, MIF can oppose the inhibitory effects which these steroids have on tumor necrosis factor α (TNF α), IL-1 β , IL-6 and IL-8 production in stimulated monocytes, while *in vivo* it was shown that MIF is able to suppress the protective effects of steroids in lethal endotoxemia. In terms of the mechanism of the MIF effect, these authors show that when MIF is used to counteract glucocorticoid inhibition of T cell proliferation, IL-2 and IFN γ synthesis is promoted.

Bucala and coworkers have also identified a regulatory role for MIF in the acute respiratory distress syndrome (ARDS). They find that MIF augments the secretion of proinflammatory TNF α and IL-8 in alveolar cells in ARDS patients, an effect which is blocked by anti-MIF antibodies. Interestingly, another recent report from the same group, shows that MIF colocalizes with insulin in secretory granules in the β cells of the pancreas. Neutralization of MIF with antibody reduced insulin secretion while recombinant MIF potentiated insulin release. Antisense MIF RNA expression was reported to inhibit MIF protein synthesis and to decrease glucose-induced insulin release in the INS cell line. A common thread of both these studies is a role for MIF in promoting secretion of other hormones and cytokines.

A mediator of immune privilege

The anterior chamber of the eye is a classic site of immunological privilege. The tissues of the anterior chamber, such as the corneal endothelium and the lens, lack major histocompatibility complex (MHC) class I antigens. This places them at risk of cell-mediated lysis by infiltrating natural killer (NK) cells.

The anterior chamber is filled with a circulating fluid, the aqueous humor, which promotes rapid suppression of NK cell-mediated cytotoxicity of corneal endothelium. This activity resides in a 12.5 kDa protein which was identified as MIF. The NK inhibitory activity of aqueous humor was abolished by MIF-specific antibody while recombinant mouse MIF inhibited NK cell-mediated lysis. These results suggest another novel function for MIF in regulating NK cell-mediated lysis and sustaining immune privilege (Apree *et al*, submitted).

Roles outside the immune system

Aside from its role as a secreted factor in the immune system, there is evidence for an intracellular mode of action for MIF in many cell types. MIF is widely expressed in many tissues regardless of immune status, including immunologically privileged sites such as the lens and brain. MIF was identified as a highly abundant protein associated with cell differentiation in the acapsular, immune-privileged eye lens in the embryonic chick. It was also cloned from human and mouse lens, confirming its identity with MIF from other sources. Northern blots for human and mouse showed that MIF mRNA is highly abundant in many tissues, including lens and brain and MIF protein was also isolated from bovine brain cytosol.

The spatial and temporal pattern of expression of MIF in lens cells as well as in various other organs suggests that MIF may have a general role in cell growth and differentiation. This idea is supported by the discovery by Lanahan and others that MIF is a delayed early response gene. When growth factors activate cell surface receptors, the signal is transduced to the nucleus where immediate early genes, such as *c-myc*, *c-fos* and *c-jun* are rapidly activated. These factors lead to the induction of the second wave, the delayed early genes. MIF is induced in mid-G₁ phase of the cell cycle in mouse fibroblast cells stimulated by serum or by purified growth factors including platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). MIF induction thus precedes downstream events such as DNA synthesis and cell division. Interestingly, lens cell proliferation and differentiation is thought to be controlled by a gradient of FGF. These and other results suggest that MIF may be part of the growth factor response in several cell types.

The MIF gene

At one time it was proposed that there is a multigene family for MIF-like proteins, which might help to

explain the diversity of properties described. However, it is now clear that the human genome contains only a single gene for MIF, which has been cloned, sequenced and mapped to chromosome 22q11.2. The human gene for MIF is remarkably small for an intron-containing gene; three exons and two introns occupy only 800 bp. In mouse, similarly, there is a single, functional gene on chromosome 10. However, in contrast to human, the mouse genome also contains multiple processed pseudogenes.

MIF as an enzyme

Another contributor to the identity crisis surrounding MIF was a report that MIF is functionally and evolutionarily related to the detoxification enzyme glutathione *S*-transferase (GST). However, it was soon demonstrated that the sequence similarity proposed was not significant. Furthermore, native MIF from bovine brain and recombinant mouse MIF were shown to lack GST activity. From these results and from recent X-ray structural studies, it now seems a relationship with GST can be dismissed.

Nevertheless, a different enzyme relationship for MIF has unexpectedly emerged. This came about with the identification of a rat protein capable of catalyzing an unusual pathway of melanin synthesis, *D*-dopachrome tautomerase (DDT). DDT and MIF are clearly related, albeit distantly. Remarkably, even though MIF shares less than 30% amino acid identity with DDT, it was found that MIF too can catalyze an isomerization of *D*-dopachrome. This is hardly likely to be the physiologically relevant activity of either protein but it does suggest that MIF may exert its effects through an enzymatic mechanism. Interestingly, DDT has precisely the same gene structure as MIF and is closely linked to MIF in both human and mouse genomes. This is certainly consistent with a history of gene duplication and may also indicate some coordination in expression.

The idea that members of the MIF/DDT family are enzymes found additional support from X-ray crystallographic analyses of MIF. This revealed striking structural similarity to two small bacterial isomerases. These bacterial proteins have very little sequence similarity with MIF and DDT and their relationship would not have been predicted from sequence alone. Even so, all four proteins conserve an N-terminal proline residue which has been implicated in catalytic activity.

In the crystal structure, MIF is a trimer, something which was not expected from previous biochemical and biophysical studies. The core structure of each MIF subunit consists of two α helices with an overlying four stranded β sheet. The subunit contains an

approximate two fold symmetry axis, suggesting that MIF arose by duplication of an ancestral unit. Two extensions from the core structure (the N-terminal peptide and a surface loop) contact other subunits in the trimer, forming additional β sheet interactions. Conceivably, flexibility in these loops might allow different multimerization states in solution and perhaps different binding activities.

What is MIF

The original definition of MIF as an activity may not be as useful as it once was. Many factors are now recognized which can influence random migration in different cell types. The focus of most attention has now shifted to a protein which carries on the name MIF, even though this may not turn out to be completely appropriate. MIF does seem to be able to inhibit macrophage migration, but it also has several other, perhaps more important, roles in immunology and in other areas of biology. Does the apparent pleiotropy of MIF reflect distinct separate roles played by the same small protein or are these all aspects of a single, underlying function? Conceivably MIF has a single, central function, perhaps as an isomerase with a key role in cellular differentiation. Indeed, parallels with cyclophilin are suggested. Whether autocrine, purely intracellular or paracrine, MIF may be part of a mechanism to initiate differentiated state responses in many cells. Depending on the cell type, different responses are elicited. In this way, one activity for MIF could be associated with a wide variety of phenotypes.

See also: Cell-mediated immunity; Cytokines; Delayed-type hypersensitivity; Eye, autoimmune disease; Glucocorticoids; Interferon γ ; Interleukin 4; Recruitment; Chemotaxis of macrophages and monocytes

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MALARIA

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Malaria is a devastating disease caused by protozoan parasites (*Plasmodium* spp.) that have a complex life cycle in a mosquito and a vertebrate host. Human malaria is caused by four species. Each stage of the life cycle is morphologically distinct and many immunogenic proteins are synthesized at each stage. The disease is caused by the asexual stage in the bloodstream. The parasite has a number of ways of evading a protective immune response, but immunity to the parasite and the disease it causes does develop after repeated exposure. Several strategies for vaccine design and development are being pursued.

Characteristics of the organism and its antigens

The four *Plasmodium* species that infect humans are *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Of these, *P. falciparum* is largely responsible for acute and often fatal malaria, but there is significant morbidity associated with each malaria infection and a

large proportion of the world's population is at risk from the disease. It has been estimated that malaria is a public health problem in areas where 40% of the world's population live and the disease has severe social and economic consequences for these communities. There has been a recent resurgence of the disease due to the abandonment or breakdown of control measures and to an increasing resistance of the vector to insecticides and falciparum malaria to chemotherapy. There are few new drugs for chemotherapy likely to be available in the immediate future. An alternative approach to control the disease, by vaccination, would be an attractive alternative if effective. The potential of vaccines has stimulated considerable research into the immunology of the disease, and has driven attempts to identify antigens that will induce protective immune responses. In addition to studies in humans, several models using nonhuman malaria parasites or human malaria parasites in unnatural hosts have been exploited experimentally; for example, rodent malaria parasites in

laboratory animals have been used to dissect the immune response to the parasite.

When an infected female *Anopheles* mosquito takes a blood meal sporozoites are transferred from the salivary glands and are carried in the bloodstream of the host to the liver where they invade hepatocytes to begin the exo-erythrocytic (EE) stage of development. Each parasite develops through a trophozoite form and then undergoes nuclear division (exo-erythrocytic schizogony) leading to the production of several thousand merozoites which are released and invade red blood cells. The parasite enters by invagination of the erythrocyte plasma membrane and develops within a parasitophorous vacuole. Asexual multiplication occurs in the infected blood cell, each parasite developing through a trophozoite form into a multinucleated schizont. Further merozoites differentiate in the blood stage schizont and are released by rupture of the infected cell, allowing them to attach to and invade further red cells. Red blood cells infected with mature *P. falciparum* parasites are sequestered from the peripheral circulation by cytoadherence to endothelial cells. Some parasites within red cells differentiate into the sexual forms, gametocytes, which are ingested by the mosquito during a blood meal. Gametogenesis occurs within the mosquito gut to yield macro- and microgametes and after zygote formation the resultant diploid parasite develops into an ookinete which penetrates the mosquito gut wall and forms an oocyst. Within the oocyst further sporozoites develop and on release these migrate to the salivary glands of the mosquito, permitting further infection of the mammalian host.

Each stage of the parasite is morphologically distinct, and although many antigens may be shared between stages others are expressed in a stage-specific manner (Table 1). Since protective immunity

appears to be stage specific, the important immunogens are probably the stage-specific proteins. Proteins on the surface of the parasite, in the vacuole that surrounds the intracellular parasite, and in the apical organelles (rhoptries, micronemes and dense granules) that are involved in cell invasion and colonization, as well as those on the surface of the infected erythrocyte and mediating cytoadherence, are of particular importance.

The circumsporozoite protein (CSP) expressed on the surface, and thrombospondin-related anonymous protein (TRAP) expressed in the micronemes of sporozoites appear to mediate homing to the liver. In the liver stage are found antigens unique to this stage, as well as antigens expressed by the sporozoite and asexual blood stage. The mature EE stage merozoites resemble blood stage merozoites both morphologically and antigenically.

Many proteins synthesized by the asexual blood stage have been identified as antigens using antibodies produced during natural infection. Synthesis of many of the proteins is stage specific, for example many merozoite antigens are synthesized during schizogony (the last 18 h of the 48 h intracellular cycle of *P. falciparum*). There are at least eight proteins on the merozoite surface and several of these are derived from a high molecular mass precursor, merozoite surface protein 1 (MSP-1). The apical secretory complex of merozoites, consisting of paired rhoptry organelles and associated micronemes, is involved in red cell invasion and contains a variety of proteins, including the erythrocyte-binding proteins (EBPs) that are involved in cell recognition and attachment. The parasite extensively modifies the host erythrocyte during intracellular development and several proteins appear to function at the infected red cell membrane. Erythrocyte membrane protein 1 (EMP-1) mediates cytoadherence and is expressed on the

Table 1 Examples of protein antigens, their location and stage specificity

Stage	Location	Protein
Sporozoite	Surface Micronemes	Circumsporozoite protein (CSP) Thrombospondin-related anonymous protein (TRAP)
Infected liver cell	Vacuole	Liver-stage antigen 1 (LSA-1) Sporozoite and liver-stage antigen (SALSA)
Merozoite	Surface Micronemes Rhoptries	Merozoite surface proteins (MSPs) Erythrocyte-binding proteins (EBPs) Apical merozoite antigen 1 (AMA-1) Rhoptry-associated proteins (RAPs)
	Dense granules	Ring-infected erythrocyte surface antigen (RESA)
Infected red blood cell	Surface Parasitophorous vacuole RBC cytoplasm	Erythrocyte membrane protein 1 (EMP-1) Serine-rich protein (SERP) Histidine-rich proteins (HRPs)
Gamete/ookinete	Surface	Pfs 45/48; Pfs 230; Pfs 25

outside of the infected cell membrane, while other proteins appear to interact with the cytoskeleton. Several soluble proteins accumulate in the parasitophorous vacuole and on schizont rupture are released into the plasma. Several of these proteins contain extensive stretches of highly repetitive amino acid sequence, and some of these repeat structures are related to each other, so that extensive antibody cross-reactivity is observed. Both EBP and EMP-1 contain host receptor-binding domains, so called Duffy-binding ligand (DBL) domains. EMP-1 is coded by the var gene family and undergoes antigenic variation.

On the surface of gametes and ookinetes several proteins have been identified. Neither of these stages is present in the blood, but the gametes appear to share these antigens with gametocytes. Other antigens are also shared with the asexual blood stages.

Immune responses of the host

As stated above, the malaria parasite is present in the vertebrate host as two distinct extracellular forms (sporozoites and merozoites) and as intracellular stages within hepatocytes and erythrocytes. Several immune mechanisms could therefore control the different parasite stages and it is probable that no single effector mechanism is responsible for protective immunity in natural infection.

The highest numbers of parasites and the most severe consequences of infection are found in young children in areas of hyperendemic transmission of *Plasmodium falciparum*. Older children often tolerate higher parasitemia without overt clinical disease and with increasing age the incidence of both disease and parasitemia decreases. However it is unlikely that even immune adults eliminate all parasites. The reasons for the slow acquisition of clinical immunity are not yet clear but may be related to extensive antigenic variation of parasite molecules on the surface of infected erythrocytes (see below).

Various experimental models have been developed to examine the interactions between the malaria parasite and the host. None is ideal since in many cases the parasite is not a natural pathogen of the particular host. However these models do highlight the principal means by which a vertebrate host can recognize and eliminate a complex protozoan parasite. Not surprisingly, malaria infections stimulate all components of the immune response in humans and experimental animals: CD4⁺ and CD8⁺ αβ T cell receptor (TCR) T cells, γδ TCR⁺ T cells, macrophages, natural killer (NK) cells, B cells and antibody production are all rapidly induced.

The pre-erythrocytic stages of the parasite include

the extracellular sporozoite and the intracellular forms in hepatocytes. Both stages stimulate the immune system. In humans and in models, CD4⁺ and CD8⁺ T cells and antibodies specific for surface antigens of the sporozoite and some liver-stage antigens can be found after infection. The effectiveness of antibodies to surface antigens of the sporozoite such as CSP in preventing invasion of hepatocytes *in vitro* has been shown with *P. falciparum*, and *in vivo* in rodent and simian model infections. However, only a large amount of specific polyclonal or monoclonal antibody is effective in clearing sporozoites. It is therefore likely that antibodies need to be present prior to infection and in high concentrations in order to control the parasite at this stage. In endemic areas antibodies to CSP develop slowly and high titers of antibody are found only in a few individuals despite the development of clinical immunity. This suggests that antibody to CSP may not be a major protective mechanism in naturally acquired immunity. Humans, monkeys and rodents vaccinated with irradiated sporozoites can be protected against sporozoite challenge. This vaccination procedure induces both antibodies and specific T cell responses, and early studies suggested that protective immunity is due to mechanisms other than ant sporozoite antibodies since there was no correlation between the presence of anti-CSP antibodies and immunity to challenge infection. In addition, immunity in vaccinated rodents could be abrogated by the removal of CD8⁺ T cells. The recent availability of gene-targeted mice with a range of defined immunodeficiencies has confirmed that immunity induced by irradiated sporozoites can be mediated directly by CD8⁺ T cells, CD4⁺ T cells and in some cases by γδ T cells. The effector pathway appears to be via interferon γ (IFNγ) and nitric oxide (NO) in most cases, and not perforins, granzymes or Fas/Fas ligand. The specificity of the protective CD8⁺ T cell response in natural infection is uncertain. CD8⁺ T cells recognizing several different sporozoite and liver-stage antigens have been found in peripheral blood of immunized humans and in the lymphoid organs of rodents.

The next stage of the malaria life cycle takes place within erythrocytes (see Figure 1). Merozoites are released from infected hepatocytes and exist for a short period of time as extracellular forms in the blood. The intraerythrocytic forms – asexual replicating parasites and developing gametocytes – are probably subject to the same immune effector mechanisms. However, since distinct proteins may be expressed in the asexual and sexual forms, the specificity of the response may differ. It is unlikely that infected erythrocytes are recognized directly by T cells as the MHC molecules necessary for recognition

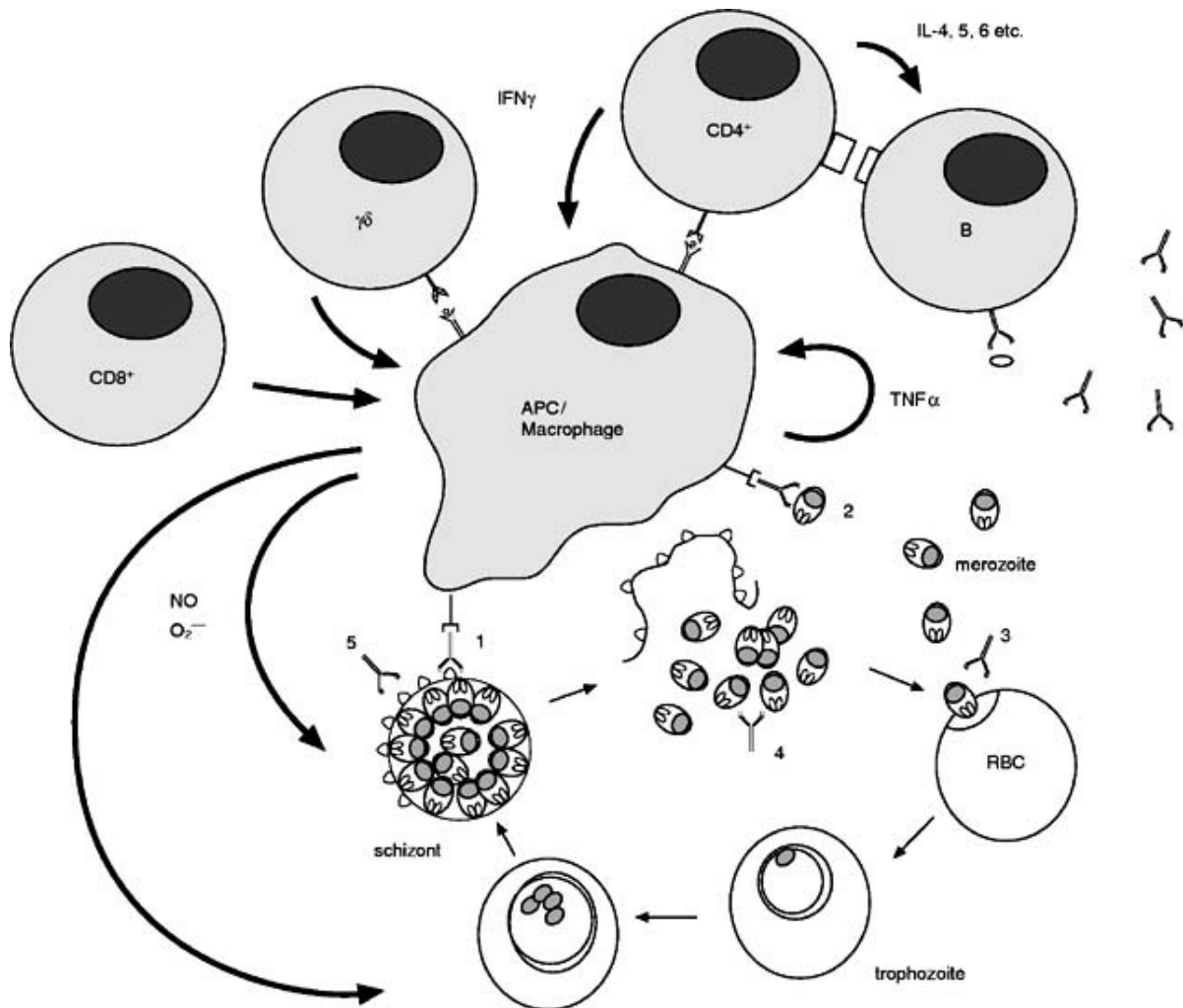


Figure 1 The asexual blood stage of the malaria parasite and possible mechanisms of immunity. Merozoites attach to and invade red blood cells (RBC), and develop through the trophozoite form into the multinucleate schizont. The schizont binds to host cells through proteins inserted into the RBC membrane (cytoadherence). At the end of schizogony merozoites are released and invade new erythrocytes. Antibody may bind to the infected erythrocyte (1) or the merozoite (2), leading to phagocytosis or antibody-dependent cell inhibition; block merozoite invasion of RBC (3); agglutinate free merozoites (4); or interfere with cytoadherence (5). It is possible that the intracellular parasite can be killed by nitric oxide or reactive oxygen radicals released by macrophages.

are not present on mammalian erythrocytes. Thus, as far as the immune system is concerned, the entire erythrocytic phase of the parasite cycle is essentially extracellular. On this basis it would be predicted that antibodies and therefore $CD4^+$ T cells are important in protective immunity. In rodent models where components of the immune system have been inactivated by specific gene targeting, it has been shown that $CD4^+$ T cells are crucial for the development and maintenance of a protective immune response. $CD8^+$ T cells and $\gamma\delta$ T cells appear to play only a minor role in controlling acute blood stage parasites. Detailed analysis of the $CD4^+$ T cell response in rodent erythrocytic infections has shown that both malaria-specific T_H1 and T_H2 cells are activated, each with distinct kinetics. T_H1 cells are predominant

during early acute infection and T_H2 cells are the major responding population later in chronic infection. This led to the hypothesis that parasites could be eliminated by antibody-independent as well as antibody-dependent means. Indeed, it has been shown that macrophages activated by $IFN\gamma$, a key cytokine produced by T_H1 cells, and tumor necrosis factor α ($TNF\alpha$), are able to kill intraerythrocytic parasites. Killing is thought to be brought about by toxic mediators such as nitric oxide and reactive oxygen radicals. Although induction of a large inflammatory response *in vivo* can result in control, and in some cases elimination, of blood stage parasites, mice defective in $IFN\gamma$, $TNF\alpha$ and inducible nitric oxide synthase are able to control and eliminate malaria parasites. By contrast, blood-stage infections

in B cell-deficient mice clearly show that B cells are essential for controlling a primary infection and for resistance to challenge infections. Evidence for the role of CD4⁺ cells and B cells/antibody in human infections is sparse. Specific CD4⁺ of both T_H1 and T_H2 type can be measured in immune individuals, but there are no data yet on the kinetics of these different responses, and their critical role can only be inferred from the rodent models. $\gamma\delta$ T cells and, to a lesser extent, CD8⁺ T cells are activated in human infection but there is no evidence for their role. The species- and strain-specificity of protective immunity against blood-stage parasites implies that specific antibody rather than nonspecific cellular effector mechanisms are important. Antibodies specific for a wide range of parasite proteins are detectable in plasma, some with high titer, during the acquisition of immunity. However, to date, there is no single T cell or antibody response that correlates with protective immunity.

The mechanisms by which antibody can effect elimination of blood-stage parasites are several (Figure 1). Antibodies to surface or rhoptry proteins of merozoites or surface molecules of infected erythrocytes will bring about agglutination or lysis (with complement) and may block invasion of erythrocytes or destroy the infected cell. Successful passive immunization of humans using immune IgG works best with cytophilic antibodies suggesting that antibody-dependent phagocytosis or a type of antibody-dependent cellular cytotoxicity or inhibition may be important. Direct evidence for this role in naturally acquired immunity in humans or animal models is still lacking.

There can be pathological consequences of an immune response to the malaria parasite. Tropical splenomegaly is associated with elevated IgM levels and uptake of immune complexes by phagocytic cells in the liver and spleen. It is most commonly found in regions of high malaria transmission. Nephrotic syndrome associated with *P. malariae* infections in children is related to immune complex deposition in the kidney glomeruli. Many of the features of severe malaria such as hypoglycemia, severe anemia and cerebral malaria could be the consequence of a dysregulated cytokine response. Production of cytokines such as TNF α can be induced in macrophages by the action of parasite components released on schizont rupture (malaria toxins). High levels of plasma TNF α in humans correlate with a poor prognosis in cerebral malaria, and neutralization or elimination of TNF α in a rodent model of cerebral malaria abrogates disease. The very pronounced IFN γ response from CD4⁺ T cells and $\gamma\delta$ T cells observed in acute and convalescent human and rodent infections could

augment the inflammatory responses of macrophages and thus contribute to severe disease.

Evasive strategies by the organism

In order to survive in the host, the malaria parasite needs a number of strategies to avoid the immune response. Shedding of the surface coat of sporozoites when complexed with antibodies may be one avoidance mechanism. Selection of peptide sequences on key molecules that lack the correct motifs for major MHC alleles would be a very effective way of ensuring that the molecule is poorly immunogenic. In this regard, it has been shown that human CSP has long regions of repeated amino acids that bind only to a very restricted number of H-2 alleles. Some molecules expressed on the surface of the parasite, on the parasite-infected erythrocyte, or released as soluble proteins, are polymorphic and coded for by a series of alleles containing repetitive structure (e.g. MSP-2). On top of this some proteins (e.g. AMA-1) may be antigenically very diverse due to numerous single amino acid substitutions. MSP-1 is the target of antibodies inhibiting the proteolytic processing of this molecule that is essential for erythrocyte invasion, but other antibodies binding to MSP-1 block the binding of these host-protective antibodies. One of the most important mechanisms of immune evasion may be antigenic variation of EMP-1, mediated by differential expression of the var gene family.

Vaccines

Studies in a variety of experimental systems have demonstrated that it is possible to protect against challenge infection by vaccination. The complexity of the life cycle means that there are several stages that can be targeted by vaccination strategies (Table 2), although a multivalent, multistage vaccine may be necessary. Vaccines directed solely at the sporozoite stage would need to be capable of inactivating all incoming sporozoites if subsequent liver- and blood-stage infection were to be prevented. Similarly, anti-liver-stage immunity would need to be essentially complete to prevent the clinical disease. Vaccination with sexual-stage antigens, either of the gametes or ookinete (which is not normally exposed to the host immune system) is designed to prevent transmission to the mosquito and, therefore, to protect the population rather than the immunized individual. The ideal requirements for all vaccines is to provide sterile immunity but vaccines that are partially effective may be acceptable, for example one that reduces the blood-stage parasitemia below a level at which it induces pathological and clinical effects. Work has

Table 2 Vaccines against different targets will have different outcomes

Target	Effectiveness	Infection	Parasitemia	Disease	Transmission
Pre-erythrocytic stages	Full	+	+	+	+
	Partial	—	—	reduced (?)	—
Asexual blood stages	Full	—	+	+	+
	Partial	—	reduced	reduced	—
Sexual stages	Full	—	—	—	+
	Partial	—	—	—	reduced
Antidisease	Full	—	—	+	—
	Partial	—	—	reduced	—

A fully effective vaccine against pre-erythrocytic stages would prevent infection (+) and all subsequent stages; an asexual blood-stage vaccine would prevent blood-stage parasitemia (+) and abolish disease pathology (+); a sexual-stage vaccine would only affect transmission (—); and a fully effective 'antidisease' vaccine would prevent disease (+), but have no effect on infection, blood-stage parasitemia or transmission. A partially effective pre-erythrocytic-stage vaccine may reduce disease in semi-immune individuals.

focused on defining protein antigens that may form the basis of a synthetic or recombinant subunit vaccine, and on delivery systems such as effective adjuvants or DNA-based strategies. For an effective vaccine it may be necessary to combine different proteins including those from different stages into hybrid molecules or mixtures (with the additional manufacturing and testing constraints that this imposes). The full extent of malaria antigen diversity and the nature of mechanisms for generating it are not defined yet. It will be important to establish whether the slow acquisition of natural immunity to malaria is due to the need for exposure to many antigenic types within the population. In this context the extent of cross-protection against different strains needs to be elucidated for any vaccine product.

There are no vaccines currently available for large-scale use against malaria. Recent studies in human volunteers with a recombinant hepatitis B surface antigen particle containing part of the CSP, together with a strong adjuvant, have shown that high antibody levels and protection against sporozoite challenge can be achieved.

Extensive field trials of SPf66, a polymerized synthetic peptide containing sequences from a number of *P. falciparum* antigens, have shown that vaccination with this product produces no consistent effect on the occurrence of clinical disease and no effect on parasite prevalence or density. Promising results with recombinant proteins based on MSP-1 have been obtained in experimental models. Thus, although some encouraging results have been obtained, much more fundamental research on the basis of immunity to malaria, the developments of assays that correlate with *in vivo* protection, and extensive studies in humans, are needed before it is clear that an effective vaccine is a realistic goal.

See also: Parasites, immunity to.

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MARSUPIAL IMMUNE SYSTEM

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The Marsupialia are a widely divergent mammalian order which are phylogenetically distinct from Eutheria. It is an order in which the female has two uteri and two lateral vaginae. The young are born at a very early stage of development and continue their growth while permanently attached to a teat, which is usually enclosed within a pouch or marsupium.

Many groups or families of the order have been recognized in both Australia and the Americas. Because the marsupials are born at a very early stage of growth, development in the pouch is akin to fetal *in utero* growth. Therefore, access to the pouch young has allowed study of many aspects of development which is not readily available in the Eutheria.

Studies of the immune system, both anatomically and physiologically, have been limited to only a few of the more than 100 known species. Much of the work has been on four species: the Virginian opossum (*Didelphis virginiana*), the South American short-tailed opossum (*Monodelphis domestica*) and two Australian species, the brush-tail possum (*Trichosurus vulpecula*) and the quokka (*Setonix brachyurus*). These are considered typical of marsupials; it is assumed, as in placental mammals, that the structure and function of the immune system are similar regardless of the species. What differences are present probably reflect different dietary habits and different evolutionary pressures within their respective environments.

The pouch young

Maternal antibody

The very early developmental stage of the newborn pouch young means that they have only basic but essential physiological processes. Most eutherian newborn are immunologically competent at birth but they are antigenically inexperienced. In contrast, marsupials are not only antigenically inexperienced but also immunologically incompetent. Furthermore, they have to survive in a pouch environment which contains potential pathogens. The development of immune competence and the passive protection of maternal antibody are therefore important requirements. The various time stages relevant to immune development are shown in Table 1.

There is no passage of immunoglobulins during fetal life. This is perhaps surprising in view of their immune incompetence at birth. In *Setonix*, as in most of the Marsupialia, the placenta has a yolk sac (choriovitelline)-endometrial attachment; the allantois does not form part of the attachment. The yolk sac-uterine attachment in *Setonix* appears to form a complete barrier to immunoglobulins, even though they have been detected in uterine fluid.

The main exception to this type of attachment appears to be in the bandicoot (*Perameles* and *Isodon* spp.) where there is a true allanto-chorionic placenta in addition to the yolk sac placenta. Neonatal development is comparatively well advanced at birth in the bandicoot as compared to other marsupials. How this relates to immunoglobulin transfer is not known, but studies in placental mammals have shown that where immunoglobulin transfer between mother and fetus prior to birth does occur, there is an allanto-chorionic placenta, such as in primates, or an inverted yolk sac placentation, such as occurs in rodents. In domestic mammals with epithelio-chorionic attachment (horse, cow, pig, sheep), the

Table 1 Developmental stages in the growth and immune responsiveness of the quokka (*Setonix brachyurus*) and the Virginian opossum (*Didelphis virginiana*)

	<i>Setonix</i>	<i>Didelphis</i>
Time in pouch	180	60–65
Time of initial Ig in pouch young	1–2	1–2
Period of maternal antibody transfer	Pouch term (approx.)	
Time of initial humoral response	10	8
Time of initial cellular response	20	12
Cervical thymus		
First presence of lymphocytes	3	NA
First presence of Hassall's corpuscles	10	NA
Thoracic thymus		
First presence of lymphocytes	3–5	2–4
First presence of Hassall's corpuscles	60	–
Time to reach full maturity	–	32
Lymph nodes		
First presence of lymphocytes	5	6–7
Definitive germinal centers	80–100	65
Bone marrow		
First presence of lymphocytes	–	10–12
Spleen		
First presence of lymphocytes	–	17–22

All times expressed in days.
NA, not applicable.

placenta is impermeable to antibodies and where the attachment is endothelio-chorionic (cat, dog), there is about 5–10% transfer of the required immunoglobulin.

Once neonates reach the pouch, attach to the teat, and begin to suckle, immunoglobulins are faintly detected in their serum within 24–48 h (Table 1). As in placental mammals, the transfer in the milk is confined principally to the immunoglobulin G (IgG) class, although IgA and IgE classes are probably also transferred. Some variation in the level of maternal antibody being absorbed has been noted, and with *Didelphis* it has been suggested that the passive transfer does not make an important contribution to the immunological status of the pouch young. More observations are needed to confirm this, but it would be surprising if *Didelphis* were markedly different from other species, where the level of maternal transfer is an important contributor to pouch young immunity. Antigenic stimulation of specific antibody production in the mother is followed by a long delay before transfer of the specific antibody to the young. This may reflect the initial IgM response which does not cross the maternal milk. Absorption of antibody from maternal milk is believed to continue until the young leave the pouch (Table 1). This is in contrast to placental mammals, where, for example, maternal

antibody is absorbed by the foal, calf, lamb, and piglet for about 24 h, the dog for 10–12 days, the rat for 21 days and the hedgehog for 41 days.

Pouch environment and development of immunity

The pouch normally contains a wide range of microorganisms which might be expected to be a problem for an antigenically inexperienced and immune-incompetent neonate. However, it has been shown that there is a reduction in the pouch flora of pregnant *Setonix*. It is not clear whether the antibacterial properties of pouch secretions are due to immunoglobulins or other substances. This 'cleansing' process allows time for the adaptation of the pouch young to its environment and the opportunity to become immune competent. In *Setonix*, the gut of the pouch young is first colonized at about 10 days, which is the time at which some immunocompetence is first apparent (Table 1).

The ability to develop and maintain an immune response is dependent on thymic maturation. The Marsupialia include species with thymic tissue in both intrathoracic and superficial ventral cervical locations. The cervical thymus has its origin in the cervical sinus, whereas the thoracic thymus originates in the third and fourth pharyngeal pouches. There are three distinct groupings based on thymic location, namely thoracic only, both thoracic and cervical, and cervical only. The reason for this variation is not clear. Those with two distinct glands are herbivorous, have a small number (1–4) of pouch young and a large deep pouch. These include many Australian species including *Trichosurus* and *Setonix*. Most families that have carnivorous, insectivorous or omnivorous diets have large litters, a shallow or no pouch, a short pouch term and a thoracic thymus only. All the American marsupials, including *Didelphis* and *Monodelphis*, are in this latter group, which also includes *Antechinus*, *Isodon* and *Perameles* spp. from Australia. The only family grouping with cervical thymus glands only are the wombats, *Vombatus* and *Lasiorninus* spp.

The deep-pouched species have a rich and varied bacterial profile, whereas species with only a thoracic thymus have much less diversity in the bacterial flora in the pouch. However, it has been shown that in *Setonix*, the cervical gland plays a definite role in the development of immune responses. Removal of the gland does not cause a compensatory increase in the thoracic gland but removal of the cervical thymus before day 10 and total thymectomy before day 20 depresses the blood lymphocyte count and delays the onset of immune competence. Total neonatal thymectomy does not stop the subsequent development of immune competence in the adult, even though the

development of other lymphoid tissues is delayed following the thymectomy. Functionally, the role of the cervical gland is probably, at least in part, similar to that of the thoracic gland.

Thymic development in the pouch young is very rapid and appears to correspond to the development of immune competence, which occurs very early after entering the pouch. Immune competence of *Setonix* appears to increase in proportion to the development of the cervical thymus, which becomes functional about 30 days before the thoracic gland and reaches full size at about the time the young leave the pouch. However in the American marsupials the thoracic gland reaches maturity at an early age (Table 1). Whether this also applies to the bandicoots (where immunoglobulin transfer across the allantoic-chorionic placenta may occur) or whether development of their thoracic gland is delayed is not known. The cervical gland can develop to a large size and it has been suggested that the size of the cervical thymus would be difficult to accommodate in an intra-thoracic location.

In most instances, detectable antibody is present within 7 days of challenge. Challenge prior to 8–10 days of age is unlikely to elicit a response. The degree or level of antibody response appears to be dependent on the nature of the antigen. Some antigens appear to provoke an adult-like response early in life, whereas others tend to show an increasing magnitude of response in proportion to age; reaching adult levels at the time of leaving the pouch. This would suggest that there is an inverse relationship between the degree of immune response and the presence of maternally derived antibody. Similar findings to this have also been noted in various species of kangaroos (*Macropus* spp.).

The development of cellular immunity appears to parallel the development of humoral immunity. The results of lymphocytic stimulation tests, for example, support the conclusion that both humoral and cellular immunity develop throughout the pouch term and reach full maturity at the end of pouch life.

The adult immune response

Lymphoid organs

Involution of the thymus is similar in its progression to that of eutherian species. The structure and cellular morphology of the spleen, lymph nodes and bone marrow are also similar to those of the placental mammals. A remarkable happening regarding involution of lymphoid organs occurs in the marsupial mice (*Antechinus swainsonii* and *A. stuartii*). Studies have shown that the thymus undergoes involution before the onset of puberty. In the males, the stress

response to breeding leads to involution of the spleen and lymph nodes; the gut being unaffected. At the end of the breeding season, there is an abrupt and absolute mortality of the males in which it is apparent that the involution of organs associated with immunity plays a role. This 'die back' is unique to the species. Demonstration of cytomegalovirus and adenovirus particles in the tissues of many marsupial species in association with stressful situations suggests that there is a fine balance between health and compromised immunity.

Humoral response

There is evidence to support retardation in the initial phases of the humoral response. This is based on observations where the response to an inoculated bacteriophage by a rabbit produced more antibody, more rapidly than *Didelphis*. Similar observations have been made in koalas (*Phascolarctos* spp.), where it is thought to be due to a delay in B cell activation because B cell numbers are present in similar proportion to those in placental mammals. In *Monodelphis* it has been shown that the primary immune response consists almost equally of IgM and IgG. However, in general, the humoral response in marsupials has the primary features that typify an immune response in placental mammals, including the ability to mount antibody-based or immediate hypersensitivity responses.

Cellular immunity

As in eutherian species, the initiation of the ability to mount a cellular response is dependent on the thymus, although there is evidence to support the possibility of differences in the effector mechanisms of marsupials. This has been reported in *Didelphis* and *Setonix*. In the latter, a study has been made of the delayed hypersensitivity response to 2,4-dinitrofluorobenzene from both topical application and intradermal injection. It showed that a more prolonged regime was required to achieve a eutherian-like response following topical application and a longer latent period was observed with the intradermal administration. This reduced delayed hypersensitivity expression, particularly in contact hypersensitivity, has also been shown to occur in *Trichosurus*, where the use of oxazolone failed to induce a contact hypersensitivity. It has been found that the skin of *Trichosurus* has low numbers of Langerhans cells as compared to the guinea pig – the latter had 4–5 times the density. A lack of, or a reduced response to, lymphokines has also been suggested.

The lymphokine responsiveness is of interest because of the many reports of mycobacterial infections in various marsupial species. While most of

these infections have resulted in typical granulomatous reactions characteristic of tubercle bacilli, there have been exceptions. The most important is the infection of *Trichosurus* with *Mycobacteria bovis*. This usually results in a disseminated systemic infection in which granuloma formation, a normal characteristic of the disease, is an infrequent finding. A similar finding is seen in *Didelphis* with *Mycobacteria intracellulare* infection. It has been shown that *Trichosurus* has a cell-based response to mycobacterial antigens, but a study with a specific cytokine, macrophage chemotactic factor (MCF), showed that the *Trichosurus* macrophages did not respond to MCF. This was despite the fact that an MCF was produced by *Trichosurus* macrophages that was effective against macrophages of the guinea pig. This unusual finding requires further investigation, but it may explain why tuberculosis in the brush-tail possum is a disseminated disease.

Conclusions

At birth, the pouch young of the Marsupialia are antigenically inexperienced and immune incompetent. There is, by necessity, a rapid development of immune competence. In many species this is mediated by a rapidly developing thymus in a cervical location. Some species may have maternal transference via the placenta but mostly, maternal antibody transference commences at the time of initial suckling and continues until the young leave the pouch, by which time immune competence is at the adult stage of development.

Adult marsupials have, with some variation, lymphoid organs and responses, both humoral and cellular, comparable to those of eutherian species. Observations have suggested that there may be initial retardation in the humoral response and that aspects of the cellular response may be refractory to some cytokines.

See also: Cell-mediated immunity; Humoral immunity; Maternal antibodies; Maturation of immune responses; *Mycobacteria*, infection and immunity; Thymus.

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MAST CELLS

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Mast cells and basophils were first described, by Paul Ehrlich more than 100 years ago, as granular cells that stain metachromatically with basic dyes. Mast cells are found in connective tissues, whereas basophils are present in the blood. Both cells have many similarities, although they are distinct cell types. Both cells synthesize and store histamine, proteoglycans and proteases within their granules and have surface receptors (FcεRI) that bind immunoglobulin E (IgE) with high affinity. The binding of multivalent antigen to this cell-bound IgE results in degranulation and the release of mediators, producing an immediate hypersensitivity reaction. Basophils and mast cells are sparsely distributed in the blood or tissues.

Basophils and mast cells have surface receptors, such as integrins, that mediate binding to other cells and to the extracellular matrix. There are changes in adherence of these cells after stimulation that may not only control their migration and localization at sites of inflammation but also regulate their function.

Characteristics and heterogeneity of mast cells

Mast cells are derived from multipotential stem cells that are in the bone marrow. The precursors enter the circulation and appear as ungranulated lymphoid cells that are CD34⁺, c-kit⁺, Ly-1⁺, CD14[−] and CD17[−]. These precursors undergo differentiation after entering a particular tissue and their phenotypic characteristics depend on the local microenvironment. In mice, microenvironmental factors critical for mast cell development include stem cell factor (SCF, also called c-kit ligand). C-kit is a receptor tyrosine kinase present on mast cells that binds, and

is activated by, stem cell factor which is produced by fibroblasts, both as a cell surface protein and as a soluble secreted product. Mast cells are distributed in connective tissues, often adjacent to blood vessels and beneath epithelial surfaces (e.g. the gastrointestinal and respiratory tracts, and the skin). The mature mast cells do not circulate.

Ultrastructurally, mast cells have many long thin projections which appear as villi by scanning electron microscopy. The nucleus is round; there are some mitochondria but the endoplasmic reticulum and the Golgi apparatus are not prominent (Figure 1). Mast

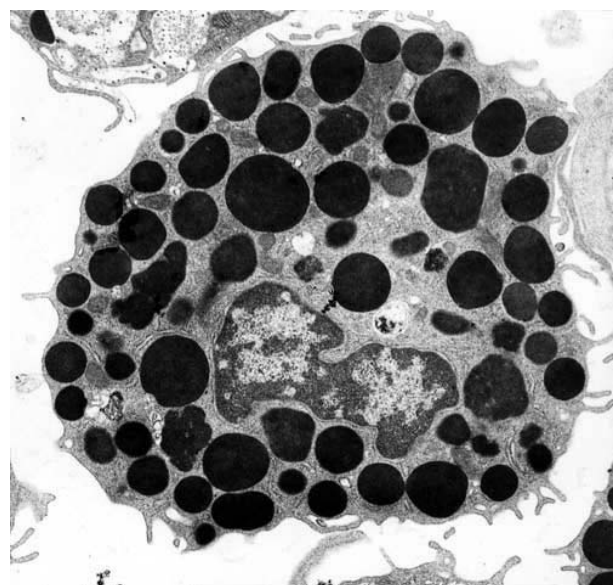


Figure 1 A rat mast cell. Note the prominent granules. (Electron micrograph kindly provided by Drs Constance Oliver and Celia Jamur.)

cells also contain intermediate filaments and lipid bodies. The granules are heterogeneous in substructural pattern; some have a scroll-like appearance, while others have an amorphous electron-dense matrix.

In rodents there are at least two populations of mast cells: the 'mucosal type' (MMCs), which is considered immature, and the 'connective tissue type' (CTMCs), which is more mature. The CTMCs, as the name implies, are distributed in connective tissues, e.g. peritoneum, lung, skin. These cells stain red with the combined alcian blue/safranin O stain owing to their content of heparin. Their proliferation and differentiation is probably influenced by the fibroblasts in connective tissues (i.e. by c-kit ligand). They have a long lifespan (probably several years) and have prominent granules that contain a large amount of histamine. The MMCs, which have also been called atypical mast cells, are prominent in the gastrointestinal mucosa. They differ from CTMCs in that they stain blue with the alcian blue/safranin O dyes because their granules contain mainly chondroitin sulfate proteoglycan. They are smaller than the CTMCs, contain fewer granules with less histamine, and have a short lifespan (a few weeks). CTMCs and MMCs have a common precursor that matures into the different types, depending on the microenvironment. Even after morphological differentiation, mast cells can transdifferentiate between the two different phenotypes. Interleukin 3 (IL-3) stimulates the proliferation and differentiation of hematopoietic progenitor cells to generate neutrophils, macrophages, megakaryocytes and mast cells. The culture of mouse bone marrow or spleen cells with this factor generates MMCs; the addition of fibroblasts and therefore stem cell factor causes a phenotypic change to the connective tissue type of mast cell. The two types of mast cell express different proteases.

The two types of mast cell respond differently to secretagogues and pharmacological inhibitors of release. Both types respond to IgE receptor-mediated activation. However, the mucosal cells respond poorly to several secretagogues including basic compounds. The two types of mast cell release different arachidonic acid metabolites; the CTMC releases predominantly prostaglandin D₂ (PGD₂), whereas the MMCs release more leukotriene C₄ (LTC₄) and other products of the lipoxygenase pathway.

Two types of mast cells have been identified in human tissues. The majority of the mast cells on mucosal surfaces, such as the intestinal mucosa and lung alveolar wall, contain tryptase (MC_T). In contrast, the majority of mast cells in the connective tissue and in the dermis contain tryptase, chymase and carboxypeptidase (MC_{TC}).

Mediators from mast cells and basophils

The secretory granules of these cells contain preformed mediators that are released when the cells are stimulated. The *biogenic amines*, such as histamine and serotonin, are major components of the secretory granules. Human mast cells contain histamine but not serotonin; however, serotonin is also present in the mast cells of other species, e.g. rodents. Histamine is produced from the amino acid L-histidine by the cytoplasmic enzyme histidine decarboxylase and then stored in granules. Human mast cells contain 2–3 pg of histamine per cell. After histamine is released from basophils or mast cells it binds and activates specific receptors on other cells, such as bronchial and gastrointestinal smooth muscle. This results in contraction of these smooth muscles and in enhanced vascular permeability. Serotonin, or 5-hydroxytryptamine, is formed by decarboxylation of the hydroxylated tryptophan molecule; in rodents it causes edema and plays an important role in anaphylactic reactions. *Neutral proteases* are also a major component of the secretory granules of mast cells. Several different proteases have been found in mast cells, including tryptase, chymase and carboxypeptidase. Tryptase is in mast cells but not basophils. The characteristic metachromatic staining of mast cells is due to the presence of *proteoglycans* in their granules, i.e. heparin or chondroitin sulfate-type that differ in their carbohydrate linkage. Human mast cells contain heparin and chondroitin sulfate E, whereas basophils have chondroitin sulfate A. Proteoglycans are involved in packaging of preformed mediators in granules. Heparin is an anticoagulant and has several other biological effects. A number of other enzymes including β -hexosaminidase, β -glucuronidase, β -D-galactosidase, arylsulfatase, superoxide dismutase and peroxidase are present and released from the secretory granules. Chemotactic factors for eosinophils, neutrophils and monocytes are also released from mast cells or basophils. These include histamine, which is chemotactic for eosinophils, and small and large peptides.

A number of *lipid mediators* are generated after these cells are stimulated. During secretion arachidonic acid is released from phospholipids as a result of the activation of phospholipase enzymes. The arachidonic acid is then metabolized, either along the cyclooxygenase pathway with the formation of the prostaglandins (predominantly PGD₂ but also thromboxanes), or by the lipoxygenase pathway with the formation of the leukotrienes (predominantly LTC₄). Platelet-activating factor is a low molecular weight phospholipid generated from alkyl phospholipids in the cell. These lipid mediators have

a number of potent biological activities. Mast cell lines and basophils synthesize and release *cytokines* when stimulated, including IL-3, IL-4, IL-5, IL-6, IL-8, tumor necrosis factor α (TNF α), interferon γ (IFN γ), several chemokines and granulocyte-macrophage colony-stimulating factor (GM-CSF).

The high-affinity receptor for IgE

There are two types of receptors for IgE on cells: mast cells and basophils have a high-affinity receptor for monomeric IgE (Fc ϵ RI), while other cells (e.g. lymphocytes, eosinophils, macrophages) have receptors that bind IgE with much lower affinity (Fc ϵ RII, CD23). Structurally, the two receptors are different.

The Fc ϵ RI consists of four polypeptide chains; an IgE-binding α chain of 45–60 kDa, a 33 kDa β subunit and a homodimer formed of disulfide linked γ chains. The different receptor subunits are not covalently linked but have some covalently linked lipids that may be important in their association in the membrane. The binding of IgE to Fc ϵ RI is a simple reversible bimolecular reaction with a high affinity and is not inhibited by immunoglobulins of other classes. Generally, IgE from one species binds only to the Fc ϵ RI of the same or closely related species. However, an exception is the binding of mouse IgE to the human Fc ϵ RI. The Fc ϵ RI are distributed diffusely over the surface of the cell and monomeric IgE–receptor complexes are freely mobile in the membrane. There are large numbers of receptors on basophils and mast cells (best estimates are over 10^5 per cell).

Stimuli for secretion from mast cells or basophils

The physiologically important stimulation of basophils and mast cells is through the high-affinity IgE receptor; the IgE binds through the Fc portion of the molecule while the two Fabs are available for interacting with antigen. Activation of the cell is due to bridging of the IgE molecules by antigen. Receptor-bound IgE can also be cross-linked with anti-IgE antibodies or mitogens. Besides Fc ϵ RI, mast cells have Fc γ RII and Fc γ RIII that bind IgG with much lower affinity than the binding of IgE to the Fc ϵ RI. These receptors for IgG may activate or regulate mast cell secretion. Mast cells/basophils can also be activated to release mediators by other secretagogues, some of which act by binding to surface receptors. These secretagogues include C5a anaphylatoxin, formylmethionine-containing small peptides that are related to bacterial chemotactic factors,

members of the IL-8 family of chemokines, the eosinophilic granule major basic protein, the ionophores, phorbol myristate acetate, polycationic compounds (e.g. polymyxin B or compound 48/80) and neuropeptides (e.g. substance P). Some cytokines induce histamine release by binding to IgE on the cell surface. Several lymphokines modulate IgE-mediated histamine release at low concentrations and induce degranulation at high concentrations.

Biochemical mechanisms of mediator release

Mast cells are stimulated to secrete mediators by antigen reacting with IgE molecules bound to receptors on the cell surface. The release is a rapid secretory (necytotoxic) process. Optimal conditions for the reaction depend on the concentration of IgE on the basophil/mast cell surface, the concentration of the antigen and the affinity of the IgE for the antigen. The bridging of two IgE molecules on the cell surface initiates the cell-triggering signal. The cross-linking of a very small proportion of the total surface IgE is sufficient for cell activation (probably bridging less than 100 IgE molecules, representing 1% of the total receptors on the cell surface).

A number of morphological changes occur during mast cell secretion. The bridging of receptors transforms the microvillous surface of basophils to a plicated appearance; the cells spread and become more adherent. The degranulation of human mast cells results in swelling of the individual granules, a change in the electron-dense granular contents and the formation of interconnected granules with the granule closest to the cell surface fusing with the plasma membrane and thus open to the extracellular medium. There are prominent cytoplasmic filaments close to the granules.

Various biochemical events have been recognized during basophil/mast cell activation, either by direct measurements or by the observation of the effect of inhibitors on the release process. The measurement of a biochemical change is the most unequivocal result.

The Fc ϵ RI subunits lack any known enzymatic activity and thus this receptor depends on associated molecules for transducing intracellular signals. The extracellular domain of the α chain of Fc ϵ RI binds IgE, whereas its relatively short cytoplasmic domain probably does not play a role in cell signaling. In contrast, the COOH-terminal cytoplasmic domains of both the β and the γ subunits are important in Fc ϵ RI-mediated signal transduction. The cytoplasmic domains of the β and the γ subunits of Fc ϵ RI contain the ITAM motif (immunoreceptor tyrosine-based

activation motif) that is critical for signal transduction. This motif is also present in subunits of the T cell and B cell antigen-receptor complexes. The γ component of Fc ϵ RI appears to be the essential receptor subunit for signaling cells for secretion.

Many intracellular processes are regulated by the phosphorylation-dephosphorylation of proteins. Protein tyrosine phosphorylation is an early and critical signal for Fc ϵ RI-induced degranulation. Fc ϵ RI associates and activates several nonreceptor protein tyrosine kinases. Lyn, a member of the *src* family of kinases, is associated with the inner surface of the plasma membrane and with the β subunit of Fc ϵ RI in nonstimulated cells. Aggregation of the receptor results in activation of Lyn. This activation may be due to the dephosphorylation of Lyn at its regulatory site by a protein tyrosine phosphatase that may be CD45. The activation of Lyn results in autophosphorylation of Lyn and in the phosphorylation of the tyrosine residues in the ITAMs of both the β and γ subunits of the Fc ϵ RI. Phosphorylation of the two tyrosines in the ITAM causes more Lyn binding to Fc ϵ RI and also renders both β and γ accessible for interacting with SH₂-containing molecules. Tyrosine phosphorylation of the γ subunit appears to be essential for propagating the signal for degranulation. The phosphorylated ITAMs recruit the cytoplasmic tyrosine kinase Syk that binds to mostly the γ subunit of the receptor by its two SH₂ domains. This results in the activation of Syk, its autophosphorylation and in propagation of downstream signaling events.

The activated Syk tyrosine kinase phosphorylates phospholipase C- γ 1 and phospholipase C- γ 2, which results in increased catalytic activity. These phospholipases catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in the generation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. These second messengers release Ca²⁺ from internal stores and activate protein kinase C respectively. Both events are essential for Fc ϵ RI-mediated secretion.

Following Fc ϵ RI aggregation there is an increase in the cytoplasmic calcium concentration due to the mobilization of Ca²⁺ as a result of the binding of inositol 1,4,5-trisphosphate to specific intracellular receptors. This initial rise in [Ca²⁺]_i is followed by the sustained influx of Ca²⁺ from the extracellular medium. The increase in [Ca²⁺]_i regulates Ca²⁺/calmodulin-dependent events. One of these downstream enzymes is calcineurin, a calcium/calmodulin-dependent serine phosphatase that is sensitive to the immunosuppressive drug cyclosporine.

Aggregation of Fc ϵ RI also activates other cytoplasmic protein tyrosine kinases. One of these is Btk (Bruton's tyrosine kinase) which associates with and

is phosphorylated by protein kinase C. Another protein tyrosine kinase is FAK (focal adhesion kinase, pp125^{FAK}), a kinase that is present in many cells and is activated by adhesion. FAK is tyrosine phosphorylated after cell stimulation and may play a role in the late steps of degranulation. The aggregation of Fc ϵ RI also induces the tyrosine phosphorylation of many other proteins.

The early tyrosine phosphorylation events activate the Ras protein kinase cascade. In this pathway the adaptor protein Shc binds to the tyrosine phosphorylated Fc ϵ RI components, recruits Grb2 which in turn recruits Sos (the mammalian homolog of the *Drosophila* son of sevenless). Sos is constitutively associated with Grb2 in a complex that is cytosolic but gets membrane associated after cell activation. Sos is a guanine nucleotide exchange factor that activates Ras to the GTP form by the promotion of guanine nucleotide exchange. Vav also may have similar guanine nucleotide exchange factor activity. Activation of Ras by the dissociation of GDP and the binding of GTP results in stimulation of downstream kinases. Ras is deactivated by GTPase-activating proteins (GAP) which results in the hydrolysis of GTP. In mast cells Ras activates the Raf pathway. Activated Ras binds Raf, a cytosolic serine/threonine kinase, and recruits it to the plasma membrane and results in its activation. Raf then activates Mitogen Activated Protein (MAP)-kinase kinases by phosphorylating it on Ser residues. The MAP-kinase kinases then activate MAP kinase by phosphorylating them on both Tyr and Thr residues. The substrates of the MAP-kinases include transcription factors such as *c-Jun*, *c-Fos* and cytoplasmic phospholipase A₂. Phosphorylation of the transcription factors of the *c-Jun* and *c-Fos* families allows their nuclear migration and the induction of new genes. The Ras pathway thus leads to both the release of arachidonic acid and nuclear events such as the induction of cytokine genes.

The protein kinase C (PKC) family of molecules phosphorylate proteins on serine or threonine residues and are essential transducers of signals for secretion. The RBL-2H3 mast cells contain the α , β , δ , ϵ and ζ isoforms of PKC. After Fc ϵ RI aggregation PKC translocates to the membrane fractions. The β or δ isozymes of PKC isozymes are required for optimal Fc ϵ RI-mediated secretion, whereas the α and ϵ isozymes inhibit the receptor induced hydrolysis of inositol phospholipids. Activated PKC phosphorylates several proteins, including myosin light chain and the γ subunit of Fc ϵ RI.

Cytoskeletal reorganization plays a role in the movement of granules and the degranulation process. These changes are secondary to the rise in

[Ca²⁺]_i, phosphorylation of cellular proteins and/or activation of GTP-binding proteins. Two *ras*-related small GTPases, *rho* and *rac*, appear to play a role in secretion. The cytoskeleton is also involved in the large-scale clustering and capping of aggregated FcεRI.

The activation of phospholipase C and phospholipase A₂ in stimulated cells results in the release of arachidonic acid from cellular phospholipids. The activity of cytosolic phospholipase A₂ is regulated by phosphorylation by MAP kinase and PKC. The arachidonic acid released is metabolized by either the cyclo-oxygenase or the lipoxygenase pathway. The products of the lipoxygenase pathway are the leukotrienes (LTC₄, LTD₄, LTE₄, LTB₄), whereas the cyclo-oxygenase pathway results in the formation of prostaglandin D₂. There is also the formation of some thromboxane A₂ and prostacyclin.

See also: **Allergens; Anaphylatoxins; Arachidonic**

acid and the leukotrienes; Basophils; Exocytosis; Fc receptors; Histamine; Hypersensitivity reactions; IgE; Interleukin 3; Platelet-activating factor (PAF); Serotonin.

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MATERNAL ANTIBODIES

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The fundamental rule of the immune system is to protect against the numerous potential pathogens which are present in the environment. The memory of earlier invasions of pathogens allows the immune system to mount an early and vigorous response when invaded for the second time. Newborn and young are particularly susceptible to infection as the development of the immune system is incomplete and little is committed to immunological memory. To offset this vulnerability the mammalian young benefits from the mother's experience and the newborn is protected from certain diseases by maternal antibodies that are transferred *in utero* and via the breast milk.

The transferred maternal antibodies can also lead to overt disease in the fetus and newborn child. Examples of this are isoimmunizations and neonatal disease caused by transfer of autoantibodies from mothers having an antibody-mediated autoimmune disease.

There is, however, not only the passive transfer of immunity and the memory of the individual, there is also the memory of the tribe. Early signals reaching the developing immune system strongly influences the functional status of the adult immune network. Such signals are maternal antibodies, self antigens

and external antigens that are present during a critical period. A strong analogy is found in the development of the nervous system. The initial system is genetically programmed and the connectance greater than in the corresponding adult system. The final structure and function depends on the selective enhancement and stabilization of some pathways caused by external stimuli. An imprint of the environment is thus integrated into the initial system. Thus, in individuals with the same basic repertoire, the presence of different self antigens, different maternal immunoglobulins and external antigens will drive the immune system into different functional states. The most important functional effect of this prenatal and neonatal priming is the transfer to the child of maternal immunoglobulins representing the mother's immunological memory, thus making the child's immune system ready to react early and vigorously when meeting a pathogen for the first time.

Evidence for the effect of maternal immunity on the immunological competence of the offspring comes from experiments involving foreign as well as self antigens and antibodies. In the case of foreign antigens both enhanced antibody responses and specific hyporesponsiveness have been reported in the

progeny of mothers making an immune response during pregnancy. The same fundamentally different effects can be caused by maternal antibodies. In contrast, only relative protection against autoimmunity rather than susceptibility to disease has been described in the very few studies made of the offspring of mothers with autoimmune diseases.

Effect of maternal immunity on the child

Transfer of antibodies

In most mammals, the offspring receives maternal immunoglobulins both *in utero* and in the neonatal period via the mother's milk. In humans, transfer of immunoglobulin G (IgG) via the placenta has been detected as early as 38 days gestation. This transfer remains stable until week 17 and then progressively increases towards term. Only IgG antibodies pass the placenta. The transport process involves binding of an intact Fc portion to specific placental membrane receptors, typically of the Fc γ type III variety (CD16) that contain binding sites for the Fc region of IgG as well as antigenic determinants that can be recognized by the antigen-binding site of IgG. These receptors have low affinity for aggregated or complexed IgG. Their affinity is strongest for IgG1 and IgG3, less for IgG4, least for IgG2 and none for IgM and for IgA. The receptors are especially concentrated on the area of the syncytiotrophoblast that makes direct contact with the maternal circulation. The selectivity of the transport process also relates to the target epitopes (e.g. neutralizing antibodies to measles reach a higher relative concentration in the fetus than mumps antibodies), to the antibody avidity and to its functions (especially opsonic activity). There is a close positive correlation between the concentrations of IgG in the child and that found in the mother, the IgG concentration in the newborn child usually being 10–15% higher. An exception is children born to mothers with very high IgG levels in whom the transport mechanism of the placenta limits the transfer and the child will be born with relatively less IgG than the mother. This limited placental transfer may interfere with transfer of tetanus-specific antibodies from vaccinated mothers to the child. It has also been implicated as a cause of neonatal susceptibility to infection in children in the Third World.

The newborn is usually resistant to certain infections if the mother has had the disease. Examples are viral infections such as rubeola, rubella and measles. The time of the greatest susceptibility to such infections occurs when the passively transferred antibodies have decreased and before the child's own immunoglobulin production is sufficient, in humans at 3–6 months of age. Induction of antibodies in

pregnant women by vaccination can also prevent infections such as diphtheria, pertussis and tetanus in the newborn.

In rodents, the breast milk is the quantitatively most important source of antibodies. The pups have special receptors for IgG in the intestine that take up IgG from the milk to the fetal circulation.

In humans, the colostrum and fresh breast milk contains an abundance of host-resistance factors, both specific and nonspecific. The most abundant immunoglobulin in these fluids is secretory IgA. The concentration of IgA is highest in colostrum and peaks during the first 3–4 days postpartum. A fully breastfed child receives about 0.5 g of secretory IgA per day. This secretory IgA protects the infant from a variety of enteric and respiratory pathogenic microorganisms. Antibodies to bacteria, viruses, parasites and fungi have been identified. Most of the antibodies present in breast milk are directed against organisms that colonize, or are pathogenic to, the gastrointestinal and respiratory tracts. The antigenic exposure resulting in milk IgA antibodies seems thus to occur primarily in the gut and in the lungs. From these locations lymphocytes which switch to become committed to IgA production home to the sites of secretory IgA synthesis, such as the intestinal mucosa and the salivary and the mammary glands. By this elegant mechanism, the enteromammary link, the baby is provided with mother's milk which contains secretory IgA antibodies against all the microbes in the mother's milieu which the infant is also likely to meet.

Human milk also contains IgG and IgM as well as secretory IgA but in lower concentrations. The IgG antibodies in milk, especially the IgG3 and IgG4 antibodies, may be directed against a number of *E. coli* antigens. The distribution of IgG subclasses in colostrum and milk differs from that in serum, and milk contains more IgG1 and less IgG2. This increase of the milk IgG1 may provide an additional protection against viral infections.

Protection in the breastfed infant against *Vibrio cholerae*, *Shigella* and *Campylobacter* relates to the content in the mother's milk of secretory IgA antibodies against these pathogens. Protection against severe rotavirus diarrhea is also suggested as is protection against *Helicobacter pylori* infections. Studies have also shown that breastfeeding can prevent otitis media and this protection may relate to the fact that the milk secretory IgA may decrease nasopharyngeal colonization with *Haemophilus influenzae*.

Also factors other than immunoglobulins may protect against infections. Lactoferrin, an iron-binding protein, that functions in a bacteriostatic manner by depriving microorganisms of iron, is abundant in

breast milk and organisms with high iron requirements such as coliforms and yeast are inhibited by this substance. Lysosyme, an enzyme that is synthesized by macrophages in the milk, can damage the cell wall of certain bacteria and also has anti-inflammatory properties.

In the presence of human milk, certain bacteria cannot adhere to retropharyngeal cells as they otherwise do. This effect is due to the presence in human milk of receptor analogs. These structures are identical to cell surface components which various microorganisms use for attachment to epithelial cells as an initial step in the establishment of an infection.

In rats the IgG antibodies against food antigens in the milk inhibit an IgE response in the offspring against the same antigens after weaning. There are some data also in humans that high levels of antibodies to cow's milk in the mother's milk, are associated with decreased risk to develop cow's milk allergy.

Breast milk contains macrophages, T and B lymphocytes, neutrophils and epithelial cells. Lymphocytes in the breast milk produce IgA and various cytokines such as interferons that may provide protection against viral infections. The response of milk T cells to some antigens is different from that of peripheral blood T cells. Milk T cells are usually highly reactive to organisms invading the gastrointestinal tract. Interestingly, the milk T cells seem to have the phenotypic and functional characteristics of memory T cells and contain twice as many T cells bearing $\gamma\delta$ receptors as peripheral blood.

There is also evidence that human milk contains agents that may work as immunomodulators. Retrospective epidemiologic studies suggested that breastfed infants are at less risk of developing type I diabetes mellitus, lymphoma and Crohn's disease later in childhood. In the case of diabetes there is evidence suggesting that antibodies to a 17 amino acid peptide from bovine albumin cross-react with a protein (p69) found on the surface of certain pancreatic islets. It is also of interest that maternal renal allografts have a better survival in individuals who were breastfed rather than those that were not breastfed.

The human fetus is capable of synthesis of antibodies of all isotypes starting at the 15–20th week of gestation. The fetus produces small amounts of IgG antibodies but very little IgM and IgA antibodies, unless challenged with an antigen, i.e. an intrauterine infection. In such cases, especially the IgM level can reach that of adults. Production of antibodies in the fetus *in utero* occurs also in response to maternal immunization. Immunization of pregnant women with tetanus toxoid has been shown to induce an active antibody response in the

fetus, probably at least partly due to transplacental passage of the antigen. Maternal anti-idiotypic antibodies against antimicrobial antibodies may induce synthesis of antibodies against the same microbes in the fetus. This may be the explanation for the presence of antibodies against antigens not available to the fetus, such as antibodies against poliovirus in children to immunized mothers.

Priming of the immune system by maternal antibodies

Maternal antibodies have dual effects. They supply both passive protection and they prime the immune system of the child for subsequent exposure to antigen. In mice and rats, neonatal priming with idiotype and anti-idiotypic antibody protects from subsequent infection with the original microbial antigen. This has been demonstrated for *E. coli* K13, *Trypanosoma rhodesiense*, rabies virus, hepatitis B virus, *Mycobacterium leprae*, influenzavirus and *Plasmodium falciparum* malaria.

Little is known about the immune priming by maternal antibodies in humans. Antibody responses to vaccination with pertussis-diphtheria-tetanus vaccine are greater in babies who had passively transferred antibodies from the mother at birth. There is also evidence for immune priming in babies to mothers with *Plasmodium falciparum* malaria. Circumstantial evidence, such as the devastating effect of introduction of new microbes in populations previously spared, is likely to reflect the importance of prenatal immune priming.

The acquisition of self tolerance occurs during ontogeny of the immune system and in experimental animals the effect of antibodies during the fetal or neonatal period is usually a long-lasting suppression. Fetal or neonatal injection of antiallotype or anti-idiotypic antibodies commonly leads to the disappearance of the target allotypes and idiotypes. Neonatal BALB/c mice given small amounts of anti-idiotypic antibody are chronically unresponsive to immunization with the original antigen. Similarly, antiallotype-induced allotype suppression in rabbits and chickens and anti-isotype isotype suppression in mice have been demonstrated.

In analogy to the priming for infections described above, also enhancing effects by administration of antibodies can be demonstrated. The net effect depends on the amount of the antibody injected or transferred and the stage of development of the fetus.

Early observations of maternally induced tolerance in human disease showed that rhesus-negative women were less likely to produce anti-D antibodies when pregnant with a Rhesus-positive fetus if their mother was rhesus-negative. The long-term survival

of maternal renal grafts was also better than for paternal grafts. In a study of hyperimmunized patients with broadly reactive leukocyte antibodies waiting for a renal transplant, it was shown that 50% of the patients had not formed antibodies against the noninherited maternal class I antigens, whereas antibodies against noninherited paternal class I antigens almost always were present.

Very little information is available concerning the effect of maternal active autoimmune disease. The offspring of mice with autoimmune hemolytic anemia induced by immunization with rat red blood cells were unresponsive to subsequent immunization with the cross-reactive self antigen on mouse red blood cells but not to foreign antigens on rat red blood cells. Paternal disease did not affect the progeny. In another study on rats, the progeny of mothers with experimental autoimmune encephalomyelitis during lactation were resistant to subsequent induction of the disease.

In humans, the relative resistance to insulin-dependent diabetes in children to diabetic mothers as compared to children with a diabetic father is likely to represent the same maternally induced tolerance induction to autoantigens during ontogeny.

Diseases caused by maternal antibodies

Isoantibodies

Antibodies against blood group antigens can cause hemolytic disease of the newborn. The mild hemolytic disease caused by ABO incompatibility occurs almost exclusively in children of mothers belonging to group O. It is the most common antibody-mediated perinatal disease with an incidence varying from 1 to 5% in different ethnic populations. Though laboratory findings of this disease are common, only a minority of the children require treatment.

Before the discovery that hemolytic disease due to rhesus-incompatibility and anti-D antibodies could be prevented by postnatal treatment of mothers with anti-D antiserum, the incidence was between 0.6 and 0.7%, with 60% of the children requiring therapy and a fetal mortality of around 12%.

Thrombocytopenia and neutropenia in the newborn child can also occur due to maternal isoantibodies, but these disorders are much rarer than hemolytic disease.

Autoimmune diseases

Transfer of maternal IgG antibodies across the placenta may give rise to autoimmune diseases also in the fetus and newborn child. Such disease is most

often transient, disappearing as the maternal antibodies are catabolized. These neonatal effects have been observed in a range of autoimmune diseases and the materno-fetal model has been crucial in confirming an autoimmune basis of many such disorders. The materno-fetal model also provides a unique, naturally occurring, experimental system which should be valuable in gaining an understanding not only of the pathogenic process in autoimmune diseases but also of the priming influence of the maternal immunity on the developing immune system. The incidence of fetal or neonatal disease in children to mothers with antibody-mediated autoimmune diseases is not 100% but ranges from 5 to 60% for the most common disorders, indicating that factors other than the maternal antibodies are of importance. Recent data in human autoimmune disease suggest that the fetal contribution to expression of neonatal disease may be a very important factor.

Autoimmune hemolytic anemia

In this disease, autoantibodies and complement are bound to erythrocytes, causing lysis or adherence to macrophages followed by phagocytosis. The autoantibodies are either of IgG type and commonly directed against rhesus antigens, or cold agglutinins, mostly IgM, directed against the I or i blood group antigen. The risks to the fetus are hypoxia due to maternal anemia and hemolysis induced by maternal IgG antibodies. Incidence of severe neonatal hemolytic anemia has been estimated at 35–40% in the offspring of affected mothers.

Autoimmune thrombocytopenic purpura

The disease is relatively common in women of child-bearing age and characterized by thrombocytopenia caused by autoantibodies, usually of the IgG isotype. The antibodies bind to circulating thrombocytes that are either lysed or are destroyed by phagocytosis. The level of platelet-bound IgG correlates well with severity of the disease. The condition was early recognized as a cause of neonatal thrombocytopenia. The maternal levels of thrombocytes and platelet-bound antibodies do not correlate well with the fetal platelet count which makes blood sampling from the fetus necessary to predict the outcome. The incidence of the neonatal disease is 60%, with a mortality of about 15%, principally due to intracranial hemorrhage. Neonatal thrombocytopenia also occurs in mothers cured by splenectomy, since the relevant antibodies are still present in the mother.

Thyroid diseases

Autoimmune thyroid disorders are common in

women of childbearing age. There is a whole spectrum of autoantibodies that affect thyroid function, ranging from antibodies that stimulate the TSH receptor to those blocking TSH binding to the receptor. In Graves' disease the net result of the antibody action is usually a stimulation of thyroid hormone production and hence hyperthyroidism. Graves' ophthalmopathy, which sometimes occurs in the absence of thyroid disease, may be due to autoantibodies reactive with orbital antigens.

Stimulating and blocking antibodies may interact with the fetal thyroid and depending on their affinity and relative titers, lead to varying alterations in thyroid function. Fetal and neonatal hyperthyroidism occur in 5% of women with Graves' disease. It is commonly present before birth and then manifested as fetal tachycardia. In rare cases, autoantibodies blocking the binding of TSH can cause transient hypothyroidism. The duration of untreated neonatal thyroid diseases is generally less than 3 months – the survival time of maternal immunoglobulins. The neonatal disease occurs also in children of treated, euthyroid women.

Hashimoto's thyroiditis is an inflammatory disease characterized by antibodies against thyroglobulin and thyroid peroxidase. Transferred maternal antibodies can cause transient impairment of thyroid function, and even permanent residual damage to the child thyroid gland has been described.

Diabetes mellitus

Diabetes mellitus type I is caused by an autoimmune attack on the pancreatic β cells. The disease-specific antibodies against insulin and against islet cells usually precede overt diabetes by years, by which time islet cell antibody has generally disappeared. Although the islet cell antibodies react with the cell surface to inhibit insulin secretion, β cell destruction is probably mediated mostly by T cells. This is supported by the lack of transplacental antibody effects in maternal diabetes. Transient neonatal diabetes with a pattern entirely in accordance with a causative transplacental factor, has, however, been described in babies of mothers who have no history of diabetes. Since the autoantibodies in diabetes usually disappear with the start of overt disease, it is conceivable that only women in a prediabetic stage can give birth to affected babies.

In diabetes, it has for long been known that the relative risk of developing diabetes is about three times higher for the child of a diabetic father than for the offspring of a diabetic mother. This difference is not caused by a selective loss of diabetes-susceptible fetus or by perinatal death. Thus, exposure *in utero* to an affected mother can protect a fetus

from developing diabetes later in life. Induction of immunologic tolerance to the autoantigens of the β cells is a plausible mechanism for this protective effect.

Myasthenia gravis

Myasthenia gravis is caused by antibodies against the acetylcholine receptor of the skeletal muscle endplate. These antibodies disturb neuromuscular function, leading to increased muscular fatiguability. In experimental myasthenia gravis in rabbits, the autoantibodies cause degenerative changes and immaturity of the receptors in the offspring. Children of myasthenic mothers are in rare cases born with contractures of the joints due to immaturity of the muscles caused by underdevelopment of the neuromuscular junction.

About 12% of children of myasthenic mothers develop neonatal myasthenia with neuromuscular symptoms noted usually on the first day of life. The duration of clinical disease is usually 1–6 weeks. The transmission defect as determined by electrophysiological examination may persist for several months, indicating damage to the acetylcholine receptors.

In most infants with neonatal myasthenia gravis the half-life of the acetylcholine receptor antibodies is longer than the expected 20 days. In contrast, healthy children have a much shorter half-life than expected for the maternally transferred autoantibody. A correlation between the absence of anti-idiotypic antibodies against the receptor antibodies and neonatal disease has also been described. The short half-life in healthy children is conceivably due to an anti-idiotypic response in the child, leading to faster elimination of receptor antibodies and/or suppression of antibody-producing cell clones.

In myasthenia, the fetal contribution to the disease may be important. Analysis of the idiotypic specificity of the receptor antibodies has shown fetal production of antibodies bearing idiotopes not found in the mother. This triggering of autoantibody production in the child may explain the persistence of receptor antibodies and the electrophysiological signs of disturbed neuromuscular function in children with neonatal disease.

There is also evidence for a long-term effect of the maternal antibodies on the antibody repertoire of the child. Low concentrations of receptor antibodies are commonly found in healthy children of myasthenic mothers with an incidence of around 40%. In such children studied at 2–2.5 years of age, the majority, 70%, have an idio type repertoire not found in the mother. Thus, in this case, exposure to idiotopes on maternal immunoglobulins leads to tolerance

induction and suppression of the corresponding clones in the developing child.

Nonorgan-specific autoimmune diseases

Within the group of nonorgan-specific autoimmune diseases there are several multisystem diseases with overlapping features. Such diseases are systemic lupus erythematosus, progressive systemic sclerosis, mixed connective tissue disease, rheumatoid arthritis, Sjögren's syndrome and dermatomyositis. Generally, these diseases are characterized by involvement of multiple organs and a wide range of immunological abnormalities. Production of autoantibodies against a variety of nonorgan-specific antigens is the rule. These autoantigens include single- and double-stranded DNA, histones and non-histone nuclear proteins and RNA-protein complexes such as Sm, U1-RNP, Ro(SS-A) and La(SS-B). Antibodies against cell surface structures and certain phospholipids are also common. Tissue damage is in most cases related to immune complex deposition and only a few of the autoantibodies have been proven to be of pathogenic importance.

A complex of neonatal manifestations may appear in children to mothers with certain of these antibodies. The neonatal lupus syndrome includes transient lupus dermatitis, hepatosplenomegaly and hematologic abnormalities such as thrombocytopenia and hemolytic anemia. The most serious and only permanent manifestation of this syndrome is closely correlated to maternal Ro and La antibodies. These antibodies are directed against soluble tissue ribonucleoprotein antigens. The presence of especially Ro antibodies is associated with congenital complete heart block due to diffuse endomyocardial fibrosis and fibrosis and calcification of the cardiac conduction system, especially in the region of the atrioventricular node. Antibodies reactive with fetal cardiac tissue are present in a significantly higher proportion in mothers of babies with congenital complete block than in controls. Anti-La antibodies especially have been shown to cross-react significantly with laminin and to bind to the surface of fetal myocytes but not to normal adult hearts. This suggests that the cross-reaction between laminin and La but not Ro may be important and that laminin may act as a target for specific maternal autoantibodies and contribute to the pathogenesis of congenital heart block at a critical stage during fetal cardiac development. Mothers of affected children always have Ro or La antibodies, but the reverse is not true, suggesting that other factors are necessary to induce the condition. Also in neonatal lupus syndrome there may be a fetal contribution to the pathogenesis. Idiotypes of DNA antibodies different from that of

the mother have been found in newborns. The hearts of affected children show deposition of immunoglobulins of isotypes not transferred from the mother, suggesting that autoantibody synthesis is also occurring in the child.

Some manifestation of the neonatal lupus syndrome is seen in the offspring of 7–38% of mothers with Ro/La antibodies. Complete congenital heart block has a lower incidence in these children, probably around 5%. Many mothers delivering infants with the neonatal lupus syndrome are asymptomatic but more than half will eventually display autoimmune disease.

The presence of antibodies against certain phospholipids is common in patients with nonorgan-specific autoimmune diseases. These antibodies are associated with thrombosis, thrombocytopenia and recurrent fetal loss. Two clinically important antibodies are recognized – the lupus anticoagulant and the cardiolipin antibody. Both these antibodies belong to the broadly reactive family of phospholipid antibodies and bind to phosphodiester bonds in negatively charged phospholipids. They sometimes have identical binding sites but can also show fine differences in the antigen specificity.

The lupus anticoagulant inhibits phospholipid-dependent coagulation tests. Such antibodies are found in about 25% of patients with systemic lupus erythematosus and to varying extents in other autoimmune disorders. There is a prominent clinical association with thrombosis and recurrent fetal loss due to extensive infarction and necrosis of the placenta. A study has shown that more than 90% of untreated pregnancies in women with lupus anticoagulant are lost.

Antibodies against cardiolipin are present in about 50% of patients with systemic lupus erythematosus. The association between recurrent fetal death and cardiolipin antibodies is clear, but not as strong as for lupus anticoagulant.

Intervention by maternal antibodies

The maternal immune status deeply influences the immune capacity of the newborn, both by passive transfer of immunity and by priming of the developing immune system. The use of vaccination of pregnant women to protect the child is already much used and will be developed to encompass more pathogens in the future.

A more specific approach to detection of disease and to specific therapy in antibody-mediated diseases of the newborn will follow the unraveling of the mysteries of self tolerance. Recognition of precipitating factors and manipulation of the immune system by

modulation of specific antibody responses and induction of tolerance may in the future lead to specific immunological therapy of both the mother and the child.

See also: **Anemia, autoimmune hemolytic in human; Idiopathic thrombocytopenic purpura; Idiotype network; IgG; Insulin-dependent diabetes mellitus, human; Neuromuscular junction autoimmunity; Neonatal immune response; Ontogeny of the immune response; Rh antigens; Rheumatoid arthritis, human; Rheumatological disorders; Sjögrens syndrome; Systemic lupus erythematosus (SLE), human; Thyroid autoimmunity, human.**

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MATURATION OF IMMUNE RESPONSES

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Maturation of adaptive immune responses involves a comprehensive system development of multiple components at both the protein and cellular levels in response to the introduction of foreign antigen (immunogen) into an immunocompetent host. Maturation of an immune response commences with recognition of a pathogen or foreign material, either particulate or soluble, and subsequently proceeds through a progressive manifestation of both specific humoral and cellular immune responses mounted to eliminate the material. Stimulation of humoral immune responses leads to production, secretion and circulation of soluble antibodies which react specifically with the antigen. Induction of cell-mediated immunity involves various immunological reactions mediated by stimulated T lymphocytes and can include direct interaction of the T cells with infected or transformed cells. Maturation events associated with cellular immune responses are in general less well defined than those related to humoral responses.

Specific antibody induction is analogous to the basic stimulus–response phenomenon: antigen acts as a stimulus to which cells constituting or mediating the immune system respond in various ways. Physiologically, the ultimate goal of the immune response is antigen elimination from the host. This is achieved efficiently through various maturation processes which are perceived as occurring in two stages. During the first stage, exogenous antigens (primarily proteins) are encountered by phagocytic cells and

internalized within intracellular endocytic vacuoles via various pathways. Antigen uptake, processing and presentation is usually a function performed by various cells including macrophages, dendritic cells and B cells. Antigen is subsequently processed prior to extracellular presentation to the appropriate T lymphocytes in a specific substructural form (peptide) consistent with a stimulus effect. In the humoral response the second phase is evidenced by production and secretion of soluble antibody molecules. Specific interactions of antibody protein products with antigen may promote a second burst of phagocytic activity, digestion, and accelerated antigen clearance because immune complexes function efficiently as reinitiators of the immune response.

Antigenic stimulation produces cellular mitogenic and differentiation effects. Antigenic stimuli are classified as either T cell-dependent or T cell-independent and the immune maturation process differs with the two types of stimuli. T cell-dependent antigenic stimulation usually involves foreign proteins which initiate a complex pathway involving T and B lymphocytes. The cellular pathway involves significant amplification because each activated cell can subsequently stimulate multiple cells at each step in the pathway. Amplification is also due to the fact that antigen-stimulated T and B lymphocytes display an accelerated rate of cell division concomitant with a progressive lymphocyte-to-plasma cell differentiation process. When antigenic stimuli are mediated

by antigens involving T helper cells, the resultant B cell immune response is characterized by immunoglobulin M (IgM) to IgG isotype switching and affinity maturation (which reflects somatic mutation events). In contrast, T cell-independent stimulation results in an immune response deficient in both class switching and affinity maturation. T Cell-dependent responses result in enhanced hyperimmune responses upon restimulation while T cell-independent responses conform to undulatory patterns of similar magnitude upon re-exposure to the antigen.

Processed and presented antigen can also be recognized by the T cell receptor-CD8 complex of cytotoxic T cells. Responding cytotoxic T cells are stimulated to divide and differentiate as certain genes are sequentially activated. The dividing cytotoxic T cells eventually differentiate into separate memory and effector T cell populations. The effector T cells destroy target cells such as malignant tumor cells.

Immune competent hosts are capable of producing different antibodies to millions of foreign antigens and a diverse array of antibodies even to the same antigen. Antigenic stimulation usually involves activation of several different lymphocytes (both B and T) which recognize each epitope but may vary in terms of fine specificity and affinity. After antigenic stimulation, blast cells appear in lymphoid organs. These immature plasma cells or triggered B lymphocytes divide rapidly and the progeny eventually become morphologically and functionally specialized. The phenomenon of multiple lymphocytic stimulations results in a polyclonal immune response to the antigen. Polyclonal activation is not always accomplished instantaneously and can occur via sequential events indicative of maturation processes.

Immunoglobulin class switch characteristic of the maturation process is transacted at the gene level. Antigen-stimulated B lymphocytes produce IgM molecules for a relatively short period of time followed by IgG molecules. Since this occurs within the same cell, and is a function of immunoglobulin gene arrangement, the process is referred to as class switching and is a critical parameter in the maturation of a T cell-dependent antibody response. IgG molecules are the principal component of the memory response, although some IgM is synthesized during secondary immune responses. IgG antibodies are of significantly higher affinity than IgM molecules, which represents another characteristic of the mature immune response.

The temporal-dependent affinity increase in the antigen-specific antibody population is a random somatic hypermutation process which occurs during rapid cell division within the variable domains of antibody molecules. The process results in an

increase in the average affinity of the heterogeneous antigen-specific antibody population. Humoral immune responses which exhibit affinity maturation characteristically differ depending on the biochemical nature of a T cell-dependent foreign antigen.

The extent of affinity maturation also depends on the individual host- or species-related immune response genetic repertoire. Proliferation of B cells secreting high-affinity antibodies is regarded as a selective cellular competition for limited amounts of antigen. Thus, clonal expansion of antibody-secreting lymphocytes results from selection pressures exerted by decreasing antigen concentration, with preferential proliferation of specific B lymphocytes possessing relatively high-affinity Ig receptors, particularly in the germinal centers of secondary follicles. Low-affinity clones are probably eliminated by cellular apoptosis.

It is evident that the phenomenon of affinity maturation is a dynamic process at the antibody level, but the extent to which the average affinity increases depends on the biochemical nature of the antigenic determinant. The biological ramifications of affinity maturation are numerous. High-affinity antibodies are most protective in various diseases caused by pathogenic microorganisms. High-affinity antibodies also provide long-term immunity, since low concentrations of high-affinity antibodies remain effective as protective antibodies. In immune responses characterized by significant affinity maturation and achievement of highly avid antibodies, the corresponding antibody titer determined in various assays can be attributed more to intrinsic affinity than to antibody concentration. The latter is consistent with the *in vivo* protective effects of high-affinity antibodies.

Maturation results in immunological memory which is a unique feature of the adaptive immune response. Immunological memory represents a mechanism whereby the immune system responds faster and more efficiently to subsequent exposures to an antigen relative to the first exposure. Immunological memory postulates that a large number of primed cells with preferential selection for high affinity are stored due to the rapid cell division which occurred as a result of the initial antigenic stimulus. The larger number of cells represent a reserve that the immune system relies upon to provide long-term protective immunity. Sustenance of immunological memory represents successful completion of the various factors involved in maturation of the immune response.

In recent years, a new facet involved in immune response maturation has been identified based conceptually on the immune system acting in the context of control mechanisms as a regulatory network of

interacting antibodies and lymphocytes. In general terms, the immune response to an antigen can be regulated via intricate and complex networks. These Ig networks are based on responses to antibody-variable domains in both the nonliganded (idiotype) and liganded (metatype) states. In addition, an auto-immune response against circulating immune complexes (presumably of high affinity) exists which assists in the clearance of immune complexes. This response, termed the antimetatype response, is specific for the unique conformation of the variable domains of antibodies in the liganded state. Sequential events that occur after the initial antigen stimulation and lead to a regulatory dynamic equilibrium (at the protein and cellular levels) are considered part of the immune response maturation processes.

See also: **Affinity; Affinity maturation; Antibodies, synthesis; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; B lymphocyte activation; Humoral immunity; Idiotype network; Memory, immunological.**

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MEMBRANE-ASSOCIATED CYTOSKELETON: ROLE IN REGULATING IMMUNE CELL FUNCTION

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The term 'membrane skeleton' refers to the array of cytoskeletal elements which underlie and interact with the plasma membrane and/or internal membrane vesicles of cells. All eukaryotic cells, including lymphocytes, contain such a membrane skeleton system. Based on a variety of cytochemical, biochemical and molecular biological evidence, it has been established that transmembrane linkages occur between intracellular actin/myosin filaments and surface

receptors. It is believed that the formation of a direct connection between the membrane skeleton and transmembrane proteins may be one of the earliest events to occur following ligand–receptor interaction. This entry focuses on the molecular interactions between certain lymphocyte membrane proteins and two membrane-associated cytoskeletal proteins, fodrin (a spectrin-like molecule) and ankyrin, during lymphocyte signal transduction and cell adhesion.

Interaction between CD45 and the cytoskeleton in lymphocyte activation

The leukocyte common antigen CD45 is a transmembrane glycoprotein tyrosine phosphatase that is extensively expressed on all nucleated hematopoietic cells. The extracellular region of CD45 shows remarkable polymorphism among different leukocyte lineages. The CD45 protein contains an extracellular domain for the ligand binding, a single transmembrane domain and a large cytoplasmic domain that displays an intrinsic protein tyrosine phosphatase (PTPase) activity.

Certain cytoskeletal proteins (e.g. fodrin and spectrin) have been found to be closely associated with CD45 as a complex. Recently, the fodrin-binding domain of CD45 has been mapped by deleting various portions of the cytoplasmic region, followed by the expression of these truncated cDNAs using an *in vitro* transcription/translation system. The results of these experiments indicate that the CD45's fodrin-binding domain resides between amino acids 825 and 939. Construction of a fusion protein encoding the region between amino acids 825 and 939 shows that this particular sequence itself is sufficient for fodrin binding. Further analyses indicate that the amino acid sequence ($^{930}\text{EENKKKNRN}^{939}\text{S}$) in CD45 has good sequence homology with the spectrin-binding domain found in the MSP1 glycoprotein of the malarial parasite. Biochemical studies, using binding competition assays and a synthetic peptide containing the sequence ($^{930}\text{EENKKKNRN}^{939}\text{S}$), support the conclusion that the sequence between amino acids 930 and 939 is a critical part of CD45's fodrin-binding domain. Further studies indicate that this sequence is also involved in the fodrin-induced upregulation of CD45 PTPase activity. Therefore, it is suggested that fodrin binding to this domain is required for the onset of CD45-mediated signal transduction and leukocyte activation.

Interaction between CD44 and the cytoskeleton

CD44 isoforms belong to a family of glycoproteins known to comprise a variable extracellular domain, a single spanning 23 amino acid transmembrane domain and a 70 amino acid cytoplasmic domain. These molecules are expressed in a wide variety of tissues and cells. In humans CD44 is encoded by a single gene on chromosome 11p which contains 19 exons. Most often, alternative splicing occurs between exons 5 and 15, leading to an insertion in tandem of one or more variant exons (v1–v10, or exons 6 through exons 14 in human cells) within the

membrane proximal region of the extracellular domain. The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N- and O-glycosylations and glycoaminoglycan (GAG) additions. CD44 isoform expression has been shown to be closely correlated with malignancy of lymphoid cells.

The most common hematopoietic form, CD44s (CD44 standard form), contains exons 1–4 (N-terminal 150 amino acids – extracellular domain), exons 5, 15 and 16 (membrane proximal 85 amino acids), exon 17 (transmembrane domain), and a portion of exons 17 and 19 (C-terminal 70 amino acids – cytoplasmic domain). The extracellular domain of CD44s binds specifically to certain extracellular matrix (ECM) molecules such as hyaluronic acid (HA) – the major extracellular glycosaminoglycan found in mammalian ECM. CD44s–HA interaction has been shown to induce intracellular signal transduction events, cell aggregation, cell proliferation, cell migration and cell adhesion. The cytoplasmic domain of CD44s (approximately 70 amino acids long) is conserved in most of the CD44 isoforms and is clearly involved in the binding of cytoskeletal proteins such as ankyrin. The ankyrin binding site(s) is expressed at a very early stage in the biosynthesis of CD44. Furthermore, the binding interaction between CD44 and ankyrin is highly specific and regulated by several factors including protein kinase C-mediated phosphorylation, palmitoylation and GTP binding. The fact that ankyrin preferentially accumulates underneath lymphocyte CD44 capped structures suggests that the formation of an ankyrin–CD44 complex is related to ligand-induced lymphocyte activation.

Furthermore, the ankyrin-binding domain of murine and human CD44 has been mapped using deletion mutation analyses followed by expressing in the eukaryotic cell systems. The results of these experiments indicate that the ankyrin-binding domain resides in the cytoplasmic domain of CD44 (i.e. amino acids 306–320 in mouse CD44, and amino acids 304–318 in human). Most importantly, CD44's ankyrin-binding domain is required for HA-mediated binding, cell adhesion and signal transduction.

Interaction between Ca^{2+} channels and the cytoskeleton

Cytoskeletal proteins are known to be important participants in multiple functions involving cell regulation. Because the cytoskeletal network (e.g. microfilaments, microtubules and intermediate filaments) can potentially form a physical link

between the plasma membrane, organelle membranes and nuclear membrane, it has been suggested that they are involved in transmitting important regulatory signals during cell activation by agonists. Recently, it has been found that inositol 1,4,5-trisphosphate (IP₃) receptor (a Ca²⁺ channel required for internal Ca²⁺ release), and the cytoskeletal proteins such as ankyrin, are preferentially accumulated within ligand-induced lymphocyte capped structures. The binding between ankyrin and the IP₃ receptor appears to be very specific. Further analyses indicate that the amino acid sequence, 'GGVGDVLRKPS', in the IP₃ receptor shares a great deal of structural homology with the ankyrin-binding domain located in certain well-characterized ankyrin-binding proteins such as the cell adhesion molecule, CD44. Biochemical studies, using competition binding assays and a synthetic peptide identical to 'GGVGDVLRKPS' (a sequence detected in rat brain IP₃ receptor (amino acids 2548–2558) and mouse brain IP₃ receptor (amino acids 2546–2556)), indicate that this 11 amino acid peptide binds specifically to ankyrin (but not fodrin or spectrin). Furthermore, this peptide competes effectively for ankyrin binding to IP₃ receptor-containing vesicles and/or purified IP₃ receptor; and it blocks ankyrin-induced inhibitory effects on IP₃-binding and IP₃-mediated internal Ca²⁺ release in mouse T lymphoma cells. These findings suggest that this amino acid sequence, 'GGVGDVLRKPS', which is located close to the C-terminus of the IP₃ receptor, resides on the cytoplasmic side (not the luminal side) of IP₃ receptor-containing vesicles. This unique region appears to be an important part of the IP₃ receptor ankyrin-binding domain and may play an important role in the regulation of IP₃ receptor-mediated internal Ca²⁺ release during lymphocyte activation.

In addition, the ryanodine receptor (another type of Ca²⁺ channel required for internal Ca²⁺ release) is found to be closely associated with ankyrin. Biochemical analysis indicates that specific, high-affinity binding ($K_d = 0.075$ nM) occurs between the ryanodine receptor and ankyrin in lymphoid cells. Most importantly, the binding of ankyrin to the Ca²⁺ release vesicles significantly blocks ryanodine binding and ryanodine-mediated inhibition of internal Ca²⁺ release. These results suggest that the cytoskeleton plays a pivotal role in the regulation of Ca²⁺ channel (e.g. IP₃ receptor and ryanodine receptor)-mediated internal Ca²⁺ release during lymphocyte activation.

Taken together, these findings strongly suggest that the membrane-associated cytoskeletal proteins, such as fodrin and ankyrin, play a pivotal role in regulating early signal transduction (including Ca²⁺ mobilization and tyrosine phosphatase activity) and cell adhesion required for lymphocyte function.

See also: CD44; CD45 (the leukocyte common antigen); Motility of immune cells.

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MEMORY, IMMUNOLOGICAL

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Memory is a critical property of the immune system which has evolved to provide efficient defense against pathogens. For centuries it has been known that a state of resistance was frequently induced during an illness so that it was unusual for an individual to become symptomatic a second time. This was particularly evident for diseases like smallpox with its characteristic skin rash. This state of resistance

became known as a state of 'immunity' (*l. immunitas*, 'to be exempt from duty to the state'), and was subsequently widely exploited in smallpox infection using a procedure known as 'variolation'. An individual at risk was inoculated with material from smallpox pus or scabs to deliberately induce a mild infection which then conferred immunity against the normally severe natural infection.

Since then the conceptual understanding of immunization has progressed and vaccination has had a major impact on public health. The aim of vaccination is generally to induce immunological memory which is as robust and sustained as that induced by natural infection. The longevity of immunological memory is illustrated by the measles epidemic which affected the entire population of the Faroe islands in 1846 except for a few elderly people who had been previously infected 65 years before. This natural experiment also effectively demonstrated that re-exposure to the measles virus was not necessary to maintain protective immunity for many years.

It is clear that the basis for immunity is the capacity of the immune system to retain a memory of the original infective agent so that on re-encountering it, the immune response is enhanced, usually preventing overt infection and averting a fatal outcome. However, the consequences of immune memory are not invariably beneficial. For example a second infection with dengue virus is usually more severe than the first. A noninfectious example of the detrimental effects of immunity is sensitization to an allergen.

Immunological memory, like neurological memory, is multifactorial and the precise molecular and cellular mechanisms are only partially understood. Both major compartments of lymphocytes, the B cells and T cells, contribute to immunological memory. Ultimately, immunological memory exploits efficient interactions of memory T cells with memory B cells.

T cell memory

Consider, for example, a simplified scenario of the development of contact hypersensitivity to an antigen such as poison ivy. At the first exposure to the antigen, the immunologic response is usually modest and may be barely discernible. Nevertheless, at the second or subsequent exposure, severe inflammation may result from a similar amount of antigen. The cell type which mediates this kind of delayed type hypersensitivity (DTH) is a CD4⁺ T cell which recognizes the foreign antigen introduced to the skin and produces inflammatory cytokines. At the first exposure, the individual has only naive T cells. Naive T cells probably are not sensitized in the skin at the site of antigen entry, but rather in the regional lymph nodes. Such nodes are ideal sites for the initial stimulation for several reasons:

1. There are specialized antigen-presenting cells (APC) in the skin (Langerhans cells) which take up antigen and transport it to regional lymph nodes. In the lymph node antigen is 'presented' very efficiently by these specialized APCs (either

by transfer from the Langerhans cells or further differentiation of that cell).

2. Naive T cells are not present in skin (and most other tissues) in significant numbers, but circulate profusely through regional lymph nodes where the microanatomy facilitates encounter of T cells with APCs. The large number of naive T cells migrating through the lymph node and the high frequency of T cell encounters with APCs assures effective stimulation of the rare naive T cell specific for the particular antigen such as poison ivy.
3. The lymph node provides a specialized micro-environment which facilitates the stimulation of naive cells. *In vitro* studies suggest that naive T cells need special conditions for stimulation and the milieu within the lymph node is rich in the appropriate adjunctive signals derived from cytokines, specialized extracellular matrix or cell surface ligands.

During a primary response, the T cell stimulation may not be evident because it is occurring in the regional lymph nodes rather than the site of antigen exposure: the skin. The specific naive T cells, which are small, are undergoing activation, clonal expansion and differentiation into primary effector cells. Therefore, what had been rare cells become more abundant, by at least several orders of magnitude. The activated effector cells enter the circulation and migrate to the site of original antigen exposure where several mechanisms are employed to deal with the 'infectious agent'. These include lysis of infected cells, local release of cytokines and production of T cell-dependent antibodies. Within a few days the amount of antigen has diminished significantly and the immune response becomes quiescent. The effector T cells are now redundant and most disappear as a result of cell death by means of apoptosis. Importantly, some effector cells survive and become memory cells. A fundamental question is why most effector cells die via apoptosis. Their elimination is probably triggered by the acquisition of sensitivity to ligation of apoptosis-inducing molecules such as CD95 (Fas) and loss of contact with critical cytokines required for growth.

Because of the huge diversity of T cell receptors, and the complexity of typical antigens, each antigen can typically be recognized by multiple rare T cells. Those which expand most efficiently will become the predominant memory cells. The naive T cells with the highest antigenic affinity will preferentially expand. This, together with the increased expression of adhesion molecules, is probably the main reason

that memory T cells have higher affinity for antigen than naive T cells.

T cells differentiate from a surface phenotype characteristic of naive cells, to a surface phenotype characteristic of memory/effector cells. These surface changes alter the way the T cell interacts with its environment. Of particular importance are changes in levels of expression of adhesion molecules. Expression of multiple adhesion molecules is increased as the naive cell becomes a memory/effector cell. Such changes profoundly change the rules of cell-cell interaction which influence migration as well as stimulation. Also, intracellular regulatory pathways are also altered as a T cell becomes a memory/effector cell.

During a subsequent rechallenge with antigen, overt skin inflammation occurs. This results from an infiltration of various inflammatory cells into the area which is initiated by specific T cell recognition. Memory T cells migrate through skin in modest numbers. Recognition of antigen on an APC in skin by a single memory T cell may be sufficient to initiate the cascade of events which constitute the inflammatory response. The memory T cell is easy to trigger via its antigen-specific receptor. Consequently, the nomadic memory T cell moving through skin is poised to respond efficiently to antigen. The ability of memory T cells (much more than naive T cells) to migrate into normal tissue reflects their increased expression of adhesion molecules which enables them to bind endothelium. This memory cell influx is amplified during the inflammatory response when cytokines produced by T cells (e.g. interferon γ (IFN γ) and tumor necrosis factor α (TNF α)) induce expression of adhesion ligands on endothelial cells (such as VCAM-1) to which memory cells bind preferentially. Thus, the dramatic difference between the primary response and the rechallenge in this clinical situation is largely determined by differences between naive and memory T cells. In the immune individual there is a new population of memory T cells for the specific antigen, which occur at higher frequency, can migrate into tissue and can be triggered more readily.

How does the individual maintain memory over decades? Two distinct mechanisms are likely to contribute. First, individual memory T cells may have an extremely long lifespan. Data from humans with severe radiation exposure suggest that individual T cells can persist without dividing for decades. Second, there are specialized mechanisms for retaining antigen in lymphoid organs for extended periods of time. Such prolonged presence of antigen fosters the preferential expansion of high-affinity T cell

clones and contributes to the long-term presence of sizable frequencies of memory cells.

Much of the recent progress in understanding naive and memory T cells has been made possible by the identification of cell surface markers which reliably discriminate between them. Although a variety of molecules are differentially expressed by these subsets, the most useful and widely used are different forms of the CD45 molecule which differ due to differential RNA splicing. Immature or naive T cells may be identified by their expression of the high molecular weight isoform of the CD45 molecule known as CD45RA (or CD45R in older nomenclature). Mature or memory T cells express the low molecular weight isoform of CD45 known as CD45R0. Although CD45 isoforms are, in effect, the benchmark, other molecules have also been informative markers. In the mouse the cell surface glycoprotein Pgp-1 is differentially expressed; naive cells are Pgp-1 dull and memory cells are Pgp-1 bright. Murine studies have also employed the differential expression of CD45RB as a naive/memory discriminator. In human studies memory cells were noted to have enhanced expression of CD29 (the β chain of the VLA family of integrin molecules), CD58 (LFA-3) and CD26 (Tp103) which identify distinct but functionally related subsets. All of these molecules have been used as alternative markers of memory T cells by different research groups.

The conclusion that the T cell subsets can be distinguished by markers such as the CD45 isoforms is based on the following lines of investigation.

1. T cells from newborns are almost entirely of naive phenotype, and the percentage of T cells of memory phenotype increases progressively with aging. This fits the concept that the newborn has had minimal antigenic exposure, and that memory cells accumulate over time as a consequence of antigen exposure.
2. The term 'recall' response refers to immunologic responses of individuals who have been previously exposed to the specific antigen. For example, strong T cell proliferation to antigens such as tetanus toxoid or *Candida* are observed *in vitro* only with cells from individuals previously exposed to these antigens. Any putative marker of naive versus memory T cells should allow separation of T cells into one subset which mounts a strong recall response and another which fails to. The markers discussed in the preceding paragraph generally behave in this predicted manner.
3. Memory cells are believed to differentiate from naive cells *in vivo*. This expectation fits with the

in vitro findings that following activation naive cells generally acquire the memory phenotype characterized by the foregoing markers. This conversion has also been noted for single cells with clonal analysis.

The phenotypic definition of naive and memory T cell subsets with CD45 isoforms as described above is likely to be an oversimplification that will need to undergo further refinement as new data emerge.

Differential expression of molecules that define naive and memory T cells has not been useful in differentiating between memory and effector cells. An effector CD4⁺ T cell actively secretes cytokines in contrast to a memory CD4⁺ T cell which has to be stimulated with antigen to elicit cytokine production. The relationship between memory and effector T cells is not well-defined at present.

Concepts of differential migration are an important part of the foregoing description of naive and memory T cells. Some of these concepts derive from immunohistologic studies of the cells present in murine and human tissue. It is apparent that memory cells are the predominant ones present in most tissues including skin, intestinal mucosa and on the surfaces of lung mucosa. Further, kinetic studies of migration into sites of dermal inflammatory responses demonstrate selective migration of memory cells. These studies are complemented by studies in sheep which provide a particularly useful experimental model because of their well-developed lymphatic system. Sheep studies elegantly document transit of memory cells through tissue into afferent lymphatics and of naive cells through lymph nodes into efferent lymphatics. Presumably this pattern of recirculation optimizes encounters of the appropriate T cell with antigen.

There is experimental evidence to suggest that memory T cells are easier to activate than naive cells. This has been best illustrated by several groups using the proliferative response of human T cells to antibodies against the T cell receptor (TCR) complex. No biochemical mechanism to explain this has been identified and there are comparable amounts of TCR molecules on both subsets. It is plausible that the acquisition of memory by a T cell may share common biochemical features with memory acquisition in other cell systems. For example, in neural models there is evidence that the properties of protein kinase C, a key enzyme in cellular activation, are altered with memory acquisition. There is some evidence to suggest that the CD45 molecule itself has an important role in regulation of T cell activation as a consequence of its cytoplasmic portion which is a tyrosine phosphatase. One important explanation

for the enhanced triggering of memory T cells is their increased expression of adhesion molecules, as mentioned above, most of which are capable of also providing activation signals.

In the DTH scenario outlined above, CD4⁺ cells were the focus. It is apparent, however, that there is similar diversity among memory (as well as naive) CD8⁺ cells. There are memory cytotoxic T cells which, like the DTH cells, undergo clonal expansion and conversion of surface phenotype. Effector CD8⁺ cytotoxic T lymphocytes (CTLs) have spontaneous cytotoxic activity, whereas memory CTLs need restimulation with specific antigen to become active.

B cell memory

Antibody responses, like DTH responses, differ dramatically between naive individuals and individuals who have been primed by exposure to antigen. Induction of a primary antibody response requires a significant dose of antigen and gives rise largely to low-affinity antibody of the immunoglobulin M (IgM) isotype which appears typically at day 5. In contrast, the secondary response that results from re-exposure to that antigen appears sooner and is marked by a higher titer of mainly IgG antibody with higher affinity for antigen. The secondary response can be evoked with a lower antigen dose than that required for priming.

The differences between primary and secondary antibody responses reflect contributions both of helper T cells and of B cells. B cells, like T cells, can be classified into naive (or virgin) and memory (or primed) subsets based on whether they have undergone stimulation by antigen. For the sake of simplicity in this discussion, we start by assuming the simple model that when a naive B cell is stimulated by antigen, some of its progeny differentiate into plasma cells (which actively produce antibody) and the remainder of its progeny differentiate into memory cells. Many of the principles of B cell memory are similar to those outlined above with regard to T cells: clonal expansion, clonal selection, differentiation of surface phenotype and change in responsiveness to stimulatory signals.

Clonal expansion of B cells is conceptually similar to that with T cells. The frequency of B cells specific for any particular antigen is low and the proliferation which results from antigen stimulation amplifies the number of cells reactive with a given antigen. This increase in frequency of antigen-specific cells partially explains the faster kinetics and enhanced magnitude of a secondary antibody responses. Clonal selection also operates, and is probably even

more important for B cells than for T cells for two reasons:

1. The mechanism of clonal selection is an extremely efficient one. The surface immunoglobulin (sIg) on B cells competes for soluble antigen; the B cells whose sIg has the highest affinity will capture the available antigen and be triggered by it (together with other signals).
2. Unlike T cells, B cells undergo dramatic diversification of their receptor (Ig) by a process of random somatic mutation as they undergo clonal expansion. Therefore, as the response develops, clonal selection is essential for choosing for further expansion those B cells with the most avid Ig.

Memory B cells express an altered cell surface phenotype. The most characteristic feature is change in immunoglobulin isotype. Naive B cells express surface IgD, as well as IgM. After stimulation they lose their IgD; they also undergo isotype switching from IgM to IgG (and for some eventually IgE or IgA) when provided with appropriate help by T cells. Although many other changes in B cell phenotype have been noted, it has not been straightforward to distinguish those which accompany acute activation from those which consistently identify memory cells. Memory B cells also have a reduced number of Ig receptors which increases the stringency of the clonal selection by making it more likely that antigen in limiting amounts will only trigger cells with high-affinity receptors.

The site of memory B cell production appears to be the germinal center of the lymph follicle. Resting naive B cells are thought to be unable to enter lymph follicles but following initial activation their migration properties are altered so that late in the primary response they have that capacity. There, the B cell encounters antigen that is complexed to both antibody and complement on the surface of specialized APCs known as follicular dendritic cells. These cells, which have long complex cytoplasmic processes, are capable of interaction with several different lymphoid cells and antigen may be detected unmodified on their surface some 12 months after primary immunization. It seems that these cells can form a 'network' through which there is a continuous recirculation of migrating B cells. This arrangement allows for the continued encounter and stimulation of specific memory B cells with retained antigen over long periods which may be critical in maintaining immunity.

The foregoing discussion assumes that a single lineage of B cells differentiates into both plasma cells and memory cells; this bifurcation could be dictated

by differences in the lymphokine and cellular milieu that individual progeny encounter. An alternate possibility is favored by recent data. It proposes that there are two distinct lineages stimulated in the primary response: one lineage produces plasma cells in the primary response but not memory cells; the other generates memory cells which differentiate into plasma cells only during subsequent responses.

Synergism due to cooperation of different kinds of memory cells

The foregoing sections discuss memory T cells and memory B cells in isolation. In reality, immunologic responses in a primed individual involve cooperation of a variety of different kinds of memory cells. For example, memory helper T cells produce interleukin 2 (IL-2) which facilitates the response of memory cytotoxic T cells. Similarly, memory (primed) T cells provide help to memory B cells. Such cooperativity results in marked increases in efficiency of responses.

A special feature of T/B cooperativity relates to the capacity of B cells to act as APCs. This is of special significance when only small amounts of antigen are present; B cells are unique among APCs since their surface Ig enables them to concentrate antigen more than 1000 times as efficiently as is possible by simple pinocytosis. So one can envisage in a secondary immune response a memory B cell which expresses high-affinity antibody concentrating minimal amounts of antigen. A cognate interaction of high efficiency may then ensue with a memory T cell enhanced by its adhesiveness. This may then result in the prompt production of effector T cells and high-affinity antibody; production of the latter is augmented by the helper function of the memory T cell, notably the production of cytokines such as IL-4 that are potent in influencing B cell function.

Perspective and implications

A more profound understanding of the mechanisms of immune memory will provide the basis for more rational and effective strategies to manipulate the immune system to produce longstanding immunity against threatening pathogens. This is relevant not only to organisms to which there is no effective vaccine at present (notably malaria parasites and human immunodeficiency virus (HIV)) but also many organisms to which there are existing vaccines that warrant improvement in terms of efficacy and/or ease of administration. Derangements of the cellular and molecular processes that are the foundation of immune memory probably contribute to the pathogenesis of many disease states. This is clearly true for

allergic conditions and indeed may be valid for many other diseases which are presently categorized as being autoimmune in nature. Again, understanding of the mechanism of immune memory may lead to novel therapies for both prophylaxis and therapy of these and other related diseases.

See also: Affinity maturation; B lymphocytes; CD45 (the leukocyte common antigen); Helper T lymphocytes; Clonal selection; Delayed-type hypersensitivity; Germinal center; Lifespan of immune cells and

molecules; Lymphocyte trafficking; T lymphocytes; Vaccines.

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MERCURY AND THE IMMUNE SYSTEM

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Mercury is a compound without any known physiological action in the human organism. It is instead a member of a group of highly toxic metals and has effects on, for example, enzyme function and calcium ion channels at concentrations only slightly above those normally found in healthy subjects. Exposure to mercury occurs not only through environmental pollution but also deliberately, for example in various therapeutic preparations. Mercury is known to be particularly toxic for the kidneys and central nervous system and effects on the reproductive system have also been suggested. A carcinogenic effect may exist, judging from kidney tumour formation in methyl mercury-exposed male ICR mice and mercuric chloride-exposed male F344/N rats. Interesting effects of this metal on the immune system have been demonstrated. Mercury is allergenic, affects production of immunoglobulin and induces multispecific antibodies and autoimmune reactions. Mercuric chloride is a polyclonal activator of both B and T lymphocytes. A clear genetic influence on the immunological effects of mercury exists. This is of particular interest considering the suspected great individual differences in sensitivity to mercury in humans. That the polyclonal activation of T cells is genetically controlled is quite unique.

Exposure to mercury

To most people exposure to mercury occurs mainly through the consumption of fish from polluted lakes and mercury release from amalgam fillings. The

relative contributions of these mercury sources were evaluated to be in the same order of magnitude but depend, of course, on food preferences, living region and dental status. Certain categories are more heavily exposed to mercury, such as dental healthcare personnel and some industrial workers. High mercury concentrations were, for example, found in pituitary glands from dental staff. A correlation has been shown to exist between number of amalgam fillings and the plasma level of mercury. Amalgam fillings release mercury as vapor, which is then inhaled and absorbed, oxidized (ionized) and bound to cellular proteins. In fish, mercury is in the form of methyl mercury, formed by methylation by bacteria in polluted lakes. Methyl mercury can be biotransformed to inorganic mercury, and it was shown in Wistar rats that the spleen was of major importance for this action, presumably involving macrophages. People are also exposed to mercury through its use as a bactericidal agent and in dermatological and ophthalmic practice (antiseptic solutions, cosmetics, eye drops), nasal drops and thermometers. Mercury compounds are furthermore used as preservatives in preparations for parenteral use and have been used as antisyphilitic agents, diuretic agents, as purgatives and in antitussive therapy. Metallic mercury was used to treat some forms of ileus in the eighteenth century and was suspected to be still in use in folk medicine in some cultures even in modern times. It has also been used in pressure therapy of limbs in various conditions. During a

part of the twentieth century organic mercury compounds were used for treatment of seeds.

Mercury levels in humans

It is of interest to relate the concentrations of mercury occurring in humans to those affecting the immune system in experimental animal studies. Plasma levels of mercury detected in healthy humans is in the order of $1\text{--}10\text{ ng ml}^{-1}$. In contrast, whole blood mercury levels of persons living in a polluted region were reported to exceed $12\text{ }\mu\text{g ml}^{-1}$. Symptoms of mercury intoxication were already seen at a blood level of 20 ng ml^{-1} . Mercury intoxication occurring in patients after use of mercury-containing ointment was associated with plasma levels of $1\text{ }\mu\text{g ml}^{-1}$. Concentrations of less than $10\text{ }\mu\text{g ml}^{-1}$ were reported to be cytotoxic for human lymphocytes, methylmercuric chloride being considerably more toxic than mercuric chloride. In the acute phase after removal of amalgam fillings, plasma levels may increase substantially above normal, and later fall to lower than initially. It has been calculated that individuals with many amalgam restorations absorb as much as $10\text{ }\mu\text{g}$ mercury a day. *In vitro*, optimal mitogenic concentrations were reported to be $10\text{ }\mu\text{M}$, corresponding to $3\text{ }\mu\text{g ml}^{-1}$. For comparison, splenomegaly and increased serum immunoglobulin with antinuclear antibody formation was induced in SJL mice after treatment with repeated injections of 1.6 mg mercuric chloride. Thymic involution was induced in Wistar rats with injections of 1 mg mercuric chloride per kilogram body weight. In guinea pigs, local injection of $10\text{ }\mu\text{g}$ mercuric chloride caused reduced viability and inhibited proliferation of lymphocytes in the thymus. Less than 1 ng ml^{-1} methyl mercury causes calcium-mediated hyperpolarization of mouse (ddY strain) thymocytes.

Effects of mercury on the immune system

Little is known about the principal mechanism by which mercury affects the immune system. An effect on the selection mechanisms in the thymus has been suggested, allowing potentially autoreactive cells to escape into the periphery. A direct mitogenic action on lymphocytes seems to be well established and could result from the chemical effects of this heavy metal on some cell membrane components, such as calcium channels, or direct action on nuclear proteins. Other possible mechanisms would be increased expression of major histocompatibility complex (MHC) class II molecules on cell surfaces, with consequences for antigen presentation, or alterations

of surface antigens into immunogenic forms. A well-documented toxic action might cause release of cellular proteins inducing autoimmune reactions.

Mercury has an affinity for SH-groups and may interact with enzymes and other cellular proteins. In several species (such as humans, guinea pigs, rats, mice and rabbits) *in vitro* culture of lymphocytes (from blood, thymus, lymph nodes or spleen) in the presence of mercuric chloride results in blast transformation and increased mitotic activity. At least in human and murine lymphocytes increased DNA synthesis and mitotic activity is found after treatment with concentrations of mercury compounds close to toxic levels. In BALB/c mice the mitogenic effect seemed to be macrophage dependent. In humans, the responsiveness was reported to increase with age. In guinea pigs, thymocytes from newborn animals were the most easily stimulated by mercury, whereas in older animals spleen cells responded better. In BN strain rats, mercury induces proliferation of both T helper and B lymphocytes in peripheral lymphoid organs, and over-production of different immunoglobulin (Ig) isotypes, preferentially IgE. An early thymic atrophy and increased lymph node weight are also seen.

As shown in BN rat spleen cells, the effects of mercuric chloride are associated with increased production of interleukin 2 (IL-2). In accordance with *in vitro* results, individuals occupationally exposed to mercury have an increased absolute number of T lymphocytes which correlated with the time of exposure. Of particular interest are the different effects of mercury on T helper cells, T suppressor cells and B cells, obviously of importance in understanding the imbalance of the immune system after such treatment. In BN rats, autoreactive T helper cells were initially induced by mercury, and later the appearance of T suppressor cells was demonstrated. These latter cells may be responsible for the self-limiting nature of this autoimmune response. Mercuric chloride is responsible for inhibition of suppressor T cells in PVG/c rats, which develop immune complex glomerulonephritis and antinuclear antibodies. In contrast, in LEW (Lewis) rats, MRC OX-8⁺ cells (suppressor cells) are increased and associated with immunosuppression. These suppressor cells are possibly responsible for mercury-mediated protection against experimental allergic encephalomyelitis in these animals. It seems that in nonresponding strains there is no proliferative response among T helper cells.

In some studies, very low levels of methylmercuric chloride or mercuric chloride inhibit RNA and DNA synthesis, mitogen responses and immunoglobulin production. They furthermore cause downregulation

of some receptors, reduced viability and signs of apoptosis. It has been reported that different immunoglobulin isotypes are differentially affected, IgG1 being the most easily inhibited. Methylmercuric chloride and, to a lesser extent, mercuric chloride also may inhibit activation of mouse (BALB/c and C57BL) lymphocytes, both by mitogen and by MLC stimulation. In guinea pigs, *in vivo* experiments with mercuric chloride resulted in inhibition of thymocyte proliferation (as in Wistar rats) but at the same time decreased negative selection, possibly resulting in formation of an increased fraction of potentially autoimmune cells.

The mechanism by which mercury causes these effects is not known. According to studies with mouse (strain ddY) lymphocytes, methyl mercury increases intracellular Ca^{2+} . It was also shown to be incorporated into lymphocyte nuclei and to increase phosphorylation of nuclear proteins. Some evidence indicates that interaction with immune function is due to inactivation of glutathione. Antigen-specific sensitization to mercury can occur in both humans and experimental animals. BALB/c mice are readily sensitized to mercury by epicutaneous exposure. Certain inbred guinea pig strains can also be sensitized to mercury.

Autoimmune renal disease

One of the more interesting effects of mercury is the induction of autoimmune renal disease. In rabbits, and certain susceptible rat (BN, Wistar, MAXX, PVG and other) and mouse (A.SW, SJL, BALB/c and C3H.NB) strains, mercury may induce an autoimmune glomerulonephritis. In humans, the glomerular dysfunction caused by mercury has also been suggested to have an autoimmune component. In susceptible laboratory strains the disease is associated with T lymphocyte-dependent polyclonal B cell activation, resulting in the formation of antibodies against a broad range of antigens which can include glomerular basement membrane (GBM), immunoglobulins, DNA, myeloperoxidase, sheep erythrocytes, TNP, laminin, type IV collagen, entactin and nucleolar components. Both granular and linear IgG deposits along the glomerular basement membrane and detachment of endothelial cells are seen, as well as proteinuria. In the susceptible rat strain BN it is a self-limiting autoimmune response, and the animals are rendered resistant to rechallenge with mercuric chloride. CD8^+ T cells were of importance in maintaining resistance to mercuric chloride. Immunoregulatory T lymphocytes bearing the marker RT6 have been suggested to be of significance in the recovery process, as their number in peripheral lymphoid

organs inversely correlates with the autoimmune response. They were not affected in a similar way in the nonresponding LEW rats. All manifestations of mercuric chloride-induced nephritis in BN rats could be prevented by treatment with cyclosporine. Mercury also induces the formation of immune complexes which cause a mesangial immune complex glomerulonephritis and immune complex deposition in the vessel walls of the spleen and liver. In H2^s strains of mice (such as A.SW and SJL) mercury causes formation of autoantibodies against the highly conserved nucleolar protein fibrillarin. Interestingly, autoantibodies against this antigen are found in patients with scleroderma.

Genetic influence

An interesting aspect of mercury is the clear strain-dependence of its effect on the immune system. For instance, increased immunoglobulin levels are found upon mercury administration to A.SW mice, to some extent in C57BL/6 mice, but not at all in DBA/2 mice, and this difference between strains is correlated to their susceptibility to autoimmunity after exposure to mercury. Another mouse strain susceptible to induction of autoimmunity by mercury is SJL mice, whereas strain B6 is resistant. Proliferative enlargement of draining lymph nodes due to mercuric chloride was seen in most mouse strains, however DBA/2 and C3H nu/nu mice were totally unresponsive. Stimulation of DNA synthesis occurs in spleen cells of both A.SW and BALB/c mice, but not in DBA/2 mice. Antinucleolar antibodies can be induced by mercuric chloride in SJL, B10.S and Swiss ICR mice but not in A/J male mice. The genetic control of mercury responsiveness is thought to be multifactorial, involving both MHC and non-MHC genes. Formation of antinuclear and antinucleolar autoantibodies in mice is linked to H2. A.SW, SJL and B10.S (H2^s) strains are responders. Expression of H2 I-E may have a suppressive effect. The susceptibility in SJL mice to form antinucleolar antibodies resided in the H2 I-A locus and was codominantly inherited in a cross with resistant mice carrying the H2^b and H2^d haplotypes. The development of autoantibodies to chromatin and histones could not be mapped to the H2 complex. Differences between strains of mice in the response to mercury may also depend on in which form mercury is administered. For instance, strain differences in the popliteal lymph node response to mercuric chloride were not paralleled by similar differences in response to methylmercuric chloride. According to the isotype pattern, the immunoglobulin response of BALB/c, but not SJL, mice is mainly T cell dependent. A low response in

C57BL/6J mice could be increased by using Freund's complete adjuvant.

In rats, the BN and MAXX strains are susceptible to induction of autoantibodies to GBM and autoimmune renal disease by mercury, whereas the LEW and Marshall 520 (M520) rats are relatively resistant. This difference is paralleled by a difference in serum IgE response, as it is in mice, and a difference in lymph node response, in that lymphadenopathy is seen only in BN rats. Thus, whereas mercury causes autoimmunity of BN rats, it instead causes immunosuppression in LEW rats, with increased numbers of suppressor cells and inhibition of local graft-versus-host reactions. Furthermore, mercuric chloride decreases the appearance of splenic interferon γ (IFN γ)-producing cells in BN rats, but not in LEW rats. Different strains of rats also respond differently to mercury as regards phagocytosis or release of hydrogen peroxide from granulocytes and macrophages. In humans, an unusual nephritis associated with GBM autoantibodies exists and is HLA-DR associated. As in mercury-treated BN rats, the production of autoantibodies is spontaneously self-limiting. This condition is therefore an interesting parallel to the mercury-induced renal disease in other species.

As for guinea pigs, contact hypersensitivity to mercury can be induced in strain XIII of guinea pigs but not in strain II.

Mercury-induced, immune system-related disease in humans

Allergic contact dermatitis is a well-established complication of mercury exposure. It may result from handling of metallic mercury and/or topical administration of mercury-containing agents. Allergic contact dermatitis has also been described in persons exposed to mercury-containing antiseptic solutions, cosmetics or eye drops, or following inhalation of mercury vapour. Allergic contact dermatitis to mercury appears to be one of several causes of the so-called baboon syndrome. Low amounts of mercuric chloride were shown to induce certain morphological changes in skin, such as glycogen deposition, appearance of lysosome-like bodies and electron-dense material in cells of the epidermis. In humans, mercuric chloride is a strong sensitizer but also a skin irritant, reducing its use in dermatological testing procedures. Oral lichen planus has been suggested to mirror existence of mercury-specific lymphocytes, as

a high proliferative reactivity in lymphocytes to mercury was noted in such patients, and as removal of amalgam was followed by disappearance of mucosal lesions. A reduced ability of granulocytes to kill ingested microorganisms was noted in workers of a mercury industry. Interference with myeloperoxidase activity and formation of mercury-NADPH complexes were suggested. In addition, the chemotactic migration of these cells was reduced.

Although a toxic influence of mercury on the human kidney is well known, the evidence for a possible immunological damage to kidney function remains weaker. An interesting observation in mice is upregulation of Ly-6 (the murine equivalent of human CD59), a possible target molecule in allogeneic responses, by mercuric chloride. Furthermore, there is extensive cross-reaction between BN rat and human GBM antigens. It has been suggested, therefore, that mercury may also induce autoimmune glomerulonephritis in humans. Both subnormal levels and increased levels of immunoglobulins and some other serum proteins have been reported in workers exposed to mercury. There have also been reports of immediate-type hypersensitivity reactions (urticaria, anaphylaxis, asthma) linked to mercury.

See also: Antinuclear antibodies; Autoantigens; Autoimmune disease, induced animal models; Autoimmune diseases; Autoimmunity; Contact hypersensitivity; H2 class II; HLA class II; Immune complexes; Immunotoxicology; MHC disease associations.

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MHC DISEASE ASSOCIATIONS

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The association of a disease with a genetic marker means that the frequency of a given allele at a specific locus is either increased (positive association) or decreased (negative association) among patients relative to controls. In case-control studies, a positive association indicates that the gene in question confers an increased susceptibility to the disease or is in linkage disequilibrium with a nearby allele that does. Similarly, a negatively associated gene is thought to confer protection or to be in linkage disequilibrium with another gene that is causally involved in the disease process. The relative strength of observed disease associations can be estimated by a variety of statistical measures (see below). In humans, a large number of different diseases are associated with specific alleles of loci in the HLA region (Table 1).

The human major histocompatibility complex (MHC), known as the HLA region, is located on the short arm of chromosome 6 (Figure 1) and encodes two distinct classes of highly polymorphic cell surface molecules (termed class I and class II antigens) that present peptide antigens to T lymphocytes, initiating both cellular and humoral immune responses. Over the past 30 years, a variety of diseases have been associated with alleles at the HLA loci, defined initially by serologic typing as HLA antigens. The

frequency of a specific HLA antigen (e.g. HLA-B27) can be greatly increased among patients with a specific disease (e.g. ankylosing spondylitis), an observation with important implications for differential diagnosis, identification of disease susceptibility and prognosis. The observed HLA disease associations can reflect, in principle, either some aspect of the HLA class I or class II molecules' function in the immune system or simply that the HLA loci are closely linked to and in linkage disequilibrium with other disease-conferring loci. Different HLA-associated diseases can be explained by either one or the other of these basic models (see below).

Most of the diseases that show significant HLA associations are autoimmune or inflammatory disorders; in some cases, the observed HLA association was critical in suggesting the autoimmune nature of the disease, as in the distinction between type I diabetes (insulin-dependent diabetes mellitus or IDDM), a disease involving the autoimmune attack of the pancreatic islet β cells, and type II diabetes (non-insulin-dependent diabetes mellitus or NIDDM), a metabolic disease. Some autoimmune diseases, such as Reiter's syndrome are elicited in individuals with a predisposing HLA type as a consequence of bacterial infection. Several cancers and several infectious

Table 1 A selected list of HLA class II-associated diseases

	HLA antigen	Allele or haplotype	Putative autoantigen
<i>Autoimmune</i>			
IDDM	DR3	DRB1*0301-DQB1*0201	Insulin
(Type I diabetes)	DR4	DRB1*04-DQB1*0302 (The highest risk DR4 alleles are DRB1*0405, *0402, and *0401)	Glutamic acid decarboxylase-65 IA-2
	DR2 (protective)	DRB1*1501-DQB1*0602	
Multiple sclerosis	DR2	DRB1*1501-DQB1*0602	Unknown
Pemphigus vulgaris	DR4	DRB1*0402-DQB1*0302	Pe V antigen complex
	DR6	DRB1*1401-DQB1*0503	
Celiac disease	DR3	DRB1*0301-DQB1*0201	α -Gliadin
	DR7	DRB1*0701-DQB1*0201	
Rheumatoid arthritis (RA)	DR4	DRB1*0401, DRB1*0404, DRB1*0405	Unknown
Pauciarticular juvenile RA	DR8	DRB1*0801	Unknown
	DPw2	DRB1*0201	
Myasthenia gravis	DR3	DRB1*0301-DQB1*0201	Acetylcholine receptor
Stiffman's syndrome	DR3	DRB1*0301-DQB1*0201	Glutamic acid decarboxylase
<i>Unknown etiology</i>			
Narcolepsy	DR2	DRB1*1501-DQB1*0602	—

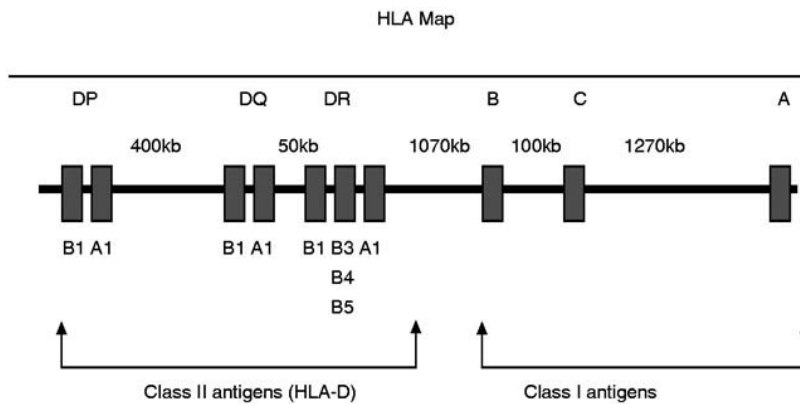


Figure 1 Map of the expressed genes of the HLA class I and class II regions.

diseases have also been associated with specific HLA types, perhaps reflecting the role of the immune system in responding to the tumor or to the pathogen. In addition, some diseases like narcolepsy, a sleep disorder of unknown etiology and with no obvious connection to the immune system, show a strong HLA association.

The initial reports of HLA disease associations all relied on the serologic definition of class I and class II antigens, and to a lesser extent, the cellular definition of HLA class II antigens by the mixed lymphocyte reaction was also used. More recently, polymorphism at the HLA loci has been analyzed at the nucleotide sequence level, revealing a wealth of allelic diversity, and simple and rapid polymerase chain reaction (PCR)-based typing methods have been applied to studies of HLA disease associations. The capacity of PCR-based typing to distinguish alleles relative to serology has resulted in a much more precise understanding of disease associations; there are about 15 serological DR specificities but >150 DRB1 DNA sequence-defined alleles. Because DNA typing can reveal where and how alleles differ, the application of these methods has provided insight into the contribution of individual polymorphic amino acid residues to disease susceptibility. For example, the DR4 specificity can be encoded by any of 22 DRB1 alleles; some DR4-associated diseases (e.g. IDDM, rheumatoid arthritis (RA)) are positively associated with specific DR4 alleles, and negatively associated with others. The negatively and positively associated alleles can differ by as little as a single codon.

The HLA loci

The HLA loci on the short arm of human chromosome 6 (Figure 1) encode two distinct classes of highly polymorphic cell surface molecules that bind and present processed antigens in the form of peptides to T lymphocytes; recognition by the T cell of

the HLA-peptide complex, along with a costimulatory signal results in T cell activation. The class I molecules, HLA-A, -B and -C, are found on most nucleated cells. They are cell surface glycoproteins that bind and present peptides derived primarily from endogenously synthesized proteins (e.g. viral and tumor peptides) to CD8⁺ T cells. These heterodimers consist of an HLA-encoded α chain associated with the non-MHC-encoded monomorphic polypeptide, β_2 -microglobulin. These loci are highly polymorphic; the numbers of alleles at the HLA-A, -B and -C loci are shown in Table 2.

The class II molecules are encoded in the HLA-D region (Figure 1). These cell surface glycoproteins consist of HLA-encoded α and β chains associated as heterodimers on the cell surface of antigen-presenting cells such as B cells and macrophages. Class II molecules serve as receptors for processed peptides; however, these peptides are derived predominantly from membrane and extracellular proteins (e.g. bacterial peptides) and are presented to CD4⁺T cells. The HLA-D region contains several class II genes and has three subregions: HLA-DR, -DQ and -DP. Both the HLA-DQ and -DP regions

Table 2 Allelic diversity at the HLA class I and class II loci

Locus	No. of alleles
HLA-A	83
HLA-B	185
HLA-C	42
DRA1	2
DRB1	184
DRB3	11
DRB4	8
DRB5	12
DQA1	18
DQB1	31
DPA1	10
DPB1	77

The number of alleles listed here includes silent variants and is based on the 1996 WHO Nomenclature Report.

contain one functional gene for each of their α and β chains. The HLA-DR subregion contains one functional gene for the α chain; the number of functional genes for the β chain varies from one to two. All individuals express a DRB1-encoded polymorphic polypeptide that is found on the cell surface in association with the DRA-encoded polypeptide. The other functional class II DRB genes, DRB3, DRB4 and DRB5 encode polypeptides which are found on the cell surface in association with a DRA-encoded polypeptide but at a lower level and only in certain class II haplotypes. In general, the DRB3 locus is found on haplotypes where DRB1 is *03, *11, *12, *13 or *14; the DRB4 locus is found on haplotypes where DRB1 is *04, *07, *09 and the DRB5 locus is found on haplotypes where DRB1 is *02 (either *15 or *16).

With the exception of the DRA and the DPA1 loci, the genes encoding the functional class II molecules are highly polymorphic (see Table 2). Analysis of the HLA class II structure has shown that these polymorphic amino acid residues line the peptide-binding cleft and interact directly with the peptide and/or the T receptor. The extensive polymorphism at both the class I and II loci and its localization to the peptide-binding groove have led to the notion that HLA polymorphism is maintained in the population because different allelic products have the capacity to bind different peptides; consequently, the more alleles an individual or a population has, the better it is able to cope with a variety of infectious pathogens.

Allelic sequence diversity

The allelic sequence diversity of the HLA class I and class II loci is the highest among mammalian coding sequence polymorphisms, with the number of alleles at some loci approaching 200 as of 1996 (see Table 2). The functional significance of this extensive polymorphism as well as the genetic mechanisms and the evolutionary forces that have generated and maintained this sequence diversity are the subject of many immunological and genetic investigations. For the HLA class II genes, the loci encoding the α and β chains of the DR, the DQ and the DP antigens, virtually all of the polymorphism is localized to the second exon. This exon encodes the α -helical 'walls' and the β -pleated sheet 'floor' of the peptide-binding groove formed by the $\alpha\beta$ heterodimer. Among the α chain loci (e.g. DRA), only the DQA1 locus, with over 15 alleles, shows extensive polymorphism. The β chain loci, however, are highly polymorphic. Population surveys in a variety of human populations have identified over 77 alleles at the DPB1 locus and over 184 at the DRB1 locus. A small number of these alleles are identical in amino acid sequence and differ

only at the nucleotide level through silent substitutions. The other DRB loci (i.e. DRB3) show a relatively modest number of alleles (Table 2) although some of the alleles at DRB3 differ at many different sites.

In contrast, the HLA class I HLA-A, -B and -C antigens contain polymorphic $\alpha 1$ and $\alpha 2$ protein domains that comprise the peptide-binding groove encoded within a single heavy chain. The $\alpha 1$ and $\alpha 2$ domains are encoded in the second and third exons respectively. Like the class II loci, the class I loci are highly polymorphic: population studies have revealed 80 alleles at the HLA-A locus, 175 alleles at the HLA-B locus, and 38 alleles at the HLA-C locus. In general, a given population contains only a subset of the class I or class II alleles that have been identified worldwide and different populations have different allele frequency distributions.

Nomenclature

In the WHO nomenclature for HLA alleles, the locus (e.g. DRB1) is followed by an asterisk (*) and by two digits that identify the allele group (e.g. DRB1*04 which, in this case, corresponds to the serologic DR4 type) and two digits that identify the allele or DR4 subtype (e.g. DRB1*0401, DRB1*0402, etc.). An additional digit is used to designate silent variants, that is, different nucleotide sequences that encode the same amino acid sequence (e.g. DRB1*08041 and *08042). For some loci, all the alleles within a single allele group designation (e.g. HLA-B*15) do not necessarily encode the same serologic type.

HLA typing

Serologic and cellular methods

Historically, HLA typing has been performed by using a combination of the serologic microcytotoxicity and, in special cases, mixed lymphocyte reaction (MLR) assays. In the microcytotoxicity assay, which can be used to type both class I and II antigens, an antiserum (or monoclonal antibody) is mixed with live lymphocytes and allowed to bind to the cell surface molecules. Specific binding is then detected with the addition of complement which lyses the cells and allows the uptake of a dye. The microcytotoxicity assay requires viable cells and uses antisera obtained primarily from individuals who have been sensitized to HLA differences such as multiparous women (women who have had multiple pregnancies) or individuals who have received multiple transfusions. Consequently, these reagents are limiting in quantity and difficult to standardize. Although, as noted above, >150 DRB1 alleles (different amino acid

sequences) have been identified, serologic reagents can distinguish only 15 different groups of DR molecules encoded by these alleles.

DNA-based techniques

Over the last decade, molecular genetic techniques have been used to isolate the genes encoding the HLA class I and class II molecules and to characterize their genomic organization. The initial approach to HLA typing at the DNA level involved the restriction fragment length polymorphism (RFLP) method; provided that several restriction enzymes were used, this technique could often subdivide HLA class II serotypes. However, RFLP typing failed to distinguish much of the HLA class II sequence polymorphism.

The development of the PCR in the mid-1980s greatly facilitated the analysis of sequence polymorphism at both the class I and II loci. By generating billions of copies of the target sequence, this technique enabled the use of simple, nonradioactive methods to analyze sequence information. Based on the available database of class II allelic sequence diversity, a variety of relatively simple and rapid PCR-based methods has been developed to carry out HLA class II typing at the DNA level. The first approaches utilized labeled sequence-specific oligonucleotide (SSO) probes to hybridize to PCR products amplified from the sample and immobilized on a nylon or nitrocellulose filter, the 'dot blot' method. Under appropriate hybridization and wash conditions, these SSO probes would bind only to the complementary sequence in the amplified DNA and were able to distinguish single nucleotide differences. Given enough primers and probes, the SSO method is, in principle, capable of distinguishing all of the alleles at a given HLA locus. It has now been applied to typing all of the class II loci and, more recently, to the class I loci.

Another PCR-based approach, based on the specificity of primer extension rather than probe hybridization, has also been applied to HLA typing. This method is known variously as allele-specific amplification (ASA), sequence-specific priming (SSP), and the amplification refractory mutation system (ARMS). Here, a specific primer pair is designed for each polymorphic sequence motif or pair of motifs and the presence of the targeted polymorphic sequence in a sample is detected as a positive PCR, typically identified as a band on a gel. If the PCR is negative, the sample is assumed to lack one or both of the specific motifs. Allele-specific amplification can be used in conjunction with SSO probe typing for high-resolution typing. Other PCR-based methods such as PCR-RFLP, PCR-SSCP (single-

strand conformation polymorphism), and PCR-DHA (directed heteroduplex analysis), have also been developed but are not widely used.

Recently, a new approach to SSO probe analysis of HLA polymorphism has been developed that facilitates DNA-based HLA typing. The conventional dot blot involved an immobilized PCR product that is hybridized to each of many labeled SSO probes. The 'reverse blot' (or immobilized probe) method is based on the hybridization of PCR product, labeled with biotinylated primers during the amplification, to an array of immobilized probes on a membrane. This procedure requires only a single PCR and a single hybridization reaction to obtain information from the entire SSO probe panel; all of the probe reactivity information is contained on a single membrane, making it amenable to automated data interpretation.

Possible explanations of MHC disease association

Although most diseases associated with HLA are autoimmune or inflammatory in nature, HLA antigens may also influence the progress of disease caused by certain infectious organisms. In addition to autoimmune disorders, such as IDDM and multiple sclerosis (MS), diseases shown to have an HLA association include several cancers such as cervical carcinoma (CC), Hodgkin's disease (HD), and nasopharyngeal carcinoma (NPC). These cancers all have a viral association; CC and the precancerous cervical lesions are caused by human papillomavirus (HPV) while HD and NPC are both associated with Epstein-Barr virus (EBV). Relatively few studies of infectious diseases have revealed HLA associations, although specific HLA-B and HLA-DRB1 alleles have been associated with resistance to severe malaria in African populations, and other HLA alleles have been associated with leprosy, tuberculosis and hepatitis. In general, the fundamental mechanisms underlying the HLA disease associations are not fully understood.

Some diseases are associated with specific HLA alleles because these alleles are in linkage disequilibrium with disease-causing mutations or polymorphic variants at a nearby locus. For some autoimmune diseases, immune-related MHC genes other than the HLA class I or class II loci appear to be involved. For systemic lupus erythematosus (SLE), complement deficiencies appear to play a role in pathogenesis; the C4A null allele in linkage disequilibrium with DR3 may account for the observed DR3 association with SLE in Caucasians. Several HLA-associated diseases that are not autoimmune disorders, such as congeni-

tal adrenal hyperplasia (CAH) or hereditary hemochromatosis (HH) are now known to be caused by mutations in linked non-HLA loci. CAH is the result of mutations in the gene for 21-hydroxylase (CYP21B) between HLA-B and DRB1, while HH is due to a mutation in a newly discovered locus (HFE) of unknown function telomeric of HLA-A that is apparently related to the HLA class I family. The common HH mutation (Cys282Tyr) is in strong linkage disequilibrium with HLA-A3.

For most of the HLA-associated diseases, however, the observed association is likely to be attributed to variation in the amino acid sequence of the HLA molecules themselves. The model that an associated HLA allele is directly involved in disease pathogenesis rather than serving as a linked marker is supported by the example of celiac disease, an inflammatory disorder, resulting in malabsorption in the small intestinal mucosa, precipitated by the ingestion of gluten. The DRB1*0301-DQA1*0501-DQB1*0201 haplotype is associated with celiac disease in all populations studied. In some populations, heterozygotes for the haplotypes DRB1*0701-DQA1*0201-DQB1*0201 and DRB1*1101-DQA1*0501-DQB1*0301 are also associated with celiac disease. The observed disease association with DR7/11 heterozygotes suggests a model in which *trans*-complementation of the α chain encoded by DQA1*0501 and the β chain encoded by DQB1*0201 form the same DQ heterodimer as that encoded *in cis* on the DR3 haplotype. This model of *trans*-complementation is consistent with the DQ molecule itself being responsible for the pattern of HLA association with celiac disease rather than the contribution of a linked non-HLA gene.

Peptide binding

The chief function of the HLA class I and class II molecules is to bind antigenic peptides, and present them to CD8⁺ and CD4⁺ T cells, respectively. The susceptibility to some autoimmune diseases conferred by certain HLA alleles could simply reflect the ability of a particular HLA molecule to bind and present a specific peptide derived from an autoantigen. Supporting evidence for this model is found in many human autoimmune diseases, as well as the corresponding animal models.

Multiple sclerosis (MS) is an inflammatory demyelinating autoimmune disease of the central nervous system in which myelin proteins have been implicated as autoantigens recognized by pathogenic autoreactive T cells. In certain strains of mice, immunization with myelin basic protein (MBP), induces experimental allergic encephalomyelitis (EAE), which serves as a model for the T cell autoimmunity

seen in MS. T cells recognize only a limited number of possible epitopes on an antigen, which are termed immunodominant. Studies of MBP T cell epitopes in EAE have demonstrated that susceptible mouse strains recognize an immunodominant epitope; in the EAE-susceptible I-A^u mouse strain (B10.PL), the N-terminal MBP Ac1-11 is immunodominant, whereas in the EAE-susceptible I-A^s mouse strain (SJL), MBP 81-100 is immunodominant. The I-A molecules, the homolog of the human HLA-DQ, of these two mice strains differ in the amino acid sequences that comprise the peptide-binding groove. It has been demonstrated that MBP Ac1-11 binds to I-A^u molecules, but not to I-A^s molecules, and that immunization of I-A^u mice with MBP Ac1-11 can induce EAE. In humans, the HLA region has been implicated as a susceptibility locus for MS, and MS patients have an increased frequency of the DRB1*1501-DQB1*0602 haplotype relative to controls. Studies of the role of human MBP (hMBP) as an autoantigen in MS, and of the epitope specificity of hMBP-reactive T cells have demonstrated that some hMBP peptides bind with highest affinity to molecules encoded by those HLA class II alleles found to be increased in MS patients (DRB1*1501). These studies have implicated hMBP 84-103 as a possible immunodominant determinant.

In rheumatoid arthritis (RA), up to 80% of patients express DR4. The DR4 serologic specificity is present on 22 different DRB1 molecules; however almost all DR4-RA patients possess either the DRB1*0401 (Dw4), DRB1*0404 (Dw14), or DRB1*0405 (Dw15) alleles of the DR4 serogroup. All of these alleles differ from each other in amino acid residues that lie within the peptide-binding groove. Recent evidence suggests that molecules encoded by the genetically susceptible DR4 alleles bind different peptides in their peptide-binding grooves than those encoded by nonsusceptible alleles (such as DRB1*0402), although the nature of the autoantigen in RA is still unknown. Similarly, in IDDM, peptide elution studies have also added indirect support that HLA-DQ and HLA-DR polymorphisms (and I-A in the NOD mouse model of IDDM) influence susceptibility to IDDM by affecting the peptides presented to T cells by these class II molecules.

Another HLA-associated disease consistent with a peptide-binding model is the autoimmune dermatologic disease pemphigus vulgaris (PV). PV is associated with the DR4 subtype, DRB1*0402-DQB1*0302 as well as with the DRB1*1401-DQB1*0503 haplotype. Peptides derived from the putative autoantigen, Pe V antigen complex, have been shown to

bind to the DRB1*0402-encoded DR molecule but not to other DR4 molecules.

T cell subsets (T_H1/T_H2)

Although selective autoantigen-peptide binding and presentation can readily explain many HLA-disease associations for susceptible alleles in autoimmunity, protective HLA allele associations in autoimmunity require different models. In studies of IDDM among Caucasians, DRB1*1501-DQB1*0602 appears to be strongly protective; studies of IDDM in other ethnic groups have demonstrated additional protective alleles or haplotypes – such as DRB1*1402-DQB1*0301 in Mexican-Americans. Siblings of diabetics carrying a protective DRB1*1501-DQB1*0602 haplotype can be shown to produce high titers of autoantibodies to islet cell antigens such as glutamic acid decarboxylase (GAD-65), but do not develop diabetes, indicating that disease protection does not necessarily mean lack of an immune response to autoantigens.

Class II-restricted CD4⁺ T helper cells differentiate into two distinct populations following antigenic stimulation: T_H1 and T_H2 cells. These distinct subpopulations each produce its own set of cytokines and mediate separate effector functions. T_H1 cells produce interleukin 2 (IL-2), IL-12, tumor necrosis factor β (TNF β) and interferon γ (IFN γ), which are important in cell-mediated immunity; T_H2 cells produce IL-4, IL-5 and IL-10, stimulating antibody formation and possibly suppressing cell-mediated immunity. Preferential stimulation of one T cell subset over the other appears to play a role in autoimmunity, although the mechanisms that determine whether an immune response will be predominantly T_H1 or T_H2 are not yet understood. In the mouse EAE model, studies have shown that EAE is induced by T_H1 cells, and that T_H2 cells can prevent induction of disease, as well as abrogate established disease. Cytokines mediating inflammatory responses have been demonstrated in RA and the islet cell damage in IDDM appears to be mediated by T_H1 cells.

Therefore, while susceptible and resistant HLA alleles may encode molecules that present peptides from the same autoantigen, they most likely bind different subsets of peptides and stimulate different subsets of T cells. It has been demonstrated that altered peptide ligands can control CD4⁺ T cell differentiation. Peptide/class II complexes that interact strongly with a T cell receptor favor the generation of T_H1 cells, whereas complexes that bind weakly favor the priming of T_H2 cells. An additional model for a class II protective role has been proposed, suggesting that susceptible and protective class II alleles compete for peptides during antigen processing of

autoantigens, resulting in reduced levels of susceptible class II/autoantigen complexes for T cell activation.

T cell repertoire

In addition to their role in antigen presentation, class I and class II molecules also function to shape the developing T cell repertoire in the neonate. Although developing thymocytes in the fetal thymus are positively selected to react with peptides in the context of self-MHC, thymocytes that react strongly with self-MHC are clonally eliminated. In the mouse, entire subsets of T cells expressing certain T cell receptor (TCR) V_β genes (such as $V_\beta14$) are clonally eliminated in mice expressing an endogenous mouse mammary tumor virus and an I-E molecule, the homolog of the human HLA-DR. In this manner, the development of autoimmune diseases could be negatively or positively influenced through HLA allelic polymorphism by eliminating certain V_β expressing T cells from the periphery (termed a 'hole' in the T cell repertoire), as well as by selecting the repertoire of T cells available for antigen reactivity.

The removal from the periphery of entire subsets of V_β -expressing T cells has not been demonstrated in humans as it has been in mice. However, both mice and man have polymorphic T cell receptor V genes, with some haplotypes carrying insertion/deletion polymorphisms among the T cell V_β genes. While studies have demonstrated that these polymorphisms affect the resulting peripheral T cell repertoire, no study has shown convincing evidence that either peripheral T cell V_β gene use, or germline V_β polymorphism play a major role in human autoimmune disease.

Molecular mimicry

The sharing of antigenic epitopes at either the B cell or T cell level between a host and a pathogen has been termed 'molecular mimicry'. Originally, this was limited to discussions of a region of amino acids encoded within a pathogen with some degree of homology to either a class I or class II structural polymorphism, but now includes any similarity between pathogens and self antigens. In this model, the sharing of identical amino acid sequences present in a host class I or class II molecules or other self antigens with, for example, a viral pathogen could result in a lack of response or 'tolerance' to these viral epitopes, leading to an altered immune response to the virus. Alternatively, the initiation of an immune response to this virus might lead to response to the autoantigenic target within the host, leading to an attack of those tissues expressing the self antigen and the HLA product.

Some examples of molecular mimicry have been described. Molecular mimicry between HLA-B27 and two molecules in *Klebsiella* sp. – nitrogenase and pullulanase D – has been reported, and elevated immune responses to *Klebsiella* sp. have been demonstrated in patients with ankylosing spondylitis. Similarly, the haemolysis molecule encoded in *Proteus* sp. has some homology to DR1 and DR4 molecules, and an elevated response to *Proteus* sp. has been described in RA patients. Whether these autoimmune diseases are actually caused by these immune responses remains to be determined.

Myelin basic protein (MBP) shares amino acid sequence motifs with a number of pathogens, including measles, hepatitis B, influenza virus, and adenovirus. A number of these pathogens have been shown to stimulate MBP-reactive T cell clones from MS patients. These studies have indicated that sequence similarity may be necessary only at a few critical amino acids comprising a T cell epitope. In animal models, conservation of only four of 11 amino acid residues was sufficient to induce EAE following immunization. These experiments demonstrate that tolerance to a self antigen such as myelin could be broken by an immune response to a microbe.

Regulation

Another proposed mechanism for HLA disease associations suggests that it is the genetic variation in regulatory regions affecting gene expression, such as promoter sequences, rather than coding sequence polymorphism that is responsible for the observed pattern of associations. This model postulates that differences in the level of cell surface expression of HLA molecules may have a major effect on their ability to present specific peptide antigens; these differences could, in principle, be manifested at the level of constitutive expression, expression in specific tissues, such as the thymus, or induced expression in response to signals such as interferon γ . In order to account for the observed disease associations, this hypothesis requires linkage disequilibrium between the coding sequence variation identified by serological typing or DNA-based typing and regulatory sequence polymorphism. At present, no compelling evidence for the involvement of regulatory mechanisms in HLA disease associations has been reported.

In general, the potential mechanisms postulated to underly HLA disease associations that are discussed above need not be mutually exclusive.

Population genetics, linkage disequilibrium and linkage

For some diseases, a given HLA allele may be strongly associated with disease in some populations but not in others. This apparent discrepancy may reflect the different distribution of alleles in different populations. For example, DR3 (DRB1*0301-DQB1*0201) is associated with IDDM in most populations but not in Japan, where DR3 is extremely rare. Another possible explanation for discrepancies in the observed disease associations in different populations are differences in the patterns of linkage disequilibrium (see below). For example, DPB1*0401 is increased among IDDM patients in China but not in other populations. This association can be attributed to strong linkage disequilibrium with DR3 in Chinese populations; these alleles at DRB1 and DPB1 are not in linkage disequilibrium in other populations. For diseases involving an infectious pathogen, differences in HLA associations in different populations could, in principle, be attributed to genetic differences in the pathogen. In the Gambia, DRB1*1302 appears to confer resistance to severe malaria whereas in Kenya, DRB1*0101 but not DRB1*1302 appears to be protective. The most common strain of *Plasmodium falciparum* responsible for malaria in these two African countries is different; this difference may account for the difference in the observed HLA associations.

In some cases, the differing incidences of a given disease in different populations or countries can be attributed, at least in part, to the differences in the frequency of the relevant HLA alleles. Pemphigus vulgaris, for example, is frequent among Ashkenazi Jewish populations; one explanation may be that the disease-associated DR4 allele, DRB1*0402, is the most common DR4 allele in this population but is an infrequent DR4 subtype in other populations. For some HLA-associated diseases, several different alleles may confer susceptibility to varying extents; thus, if a high susceptibility allele found in a variety of populations is rare or is absent in a given population, the most strongly associated allele in this population may be one that appears to be neutral or weakly predisposing in the other populations.

As noted above, the phenomenon of linkage disequilibrium (LD), the nonrandom association of particular alleles at linked loci, can complicate the interpretation of disease association studies. Some DRB1-DQA1-DQB1 haplotypes (specific combinations of linked alleles) are very much more frequent than any other combination of alleles. In any given population, the number of observed haplotypes is typically a very small proportion of all possible

haplotypic combinations. Within a given population, the association of particular DRB1, DQA1 and DQB1 alleles can be almost absolute. For example, DRB1*0301 (found in Africans, Caucasians and, more rarely, in Asians) is coupled, almost invariably to DQA1*0501 and DQB1*0201 whereas DRB1*0302 (found only in Africans) is coupled to DQA1*0401 and DQB1*0402. The set of DRB1*0405-containing haplotypes illustrate the population-related patterns of linkage disequilibrium. In Caucasians, this allele is coupled to DQA1*0301 and DQB1*0302. Among Africans, DRB1*0405 can be coupled to DQA1*0301 and DQB1*0201 and in the Japanese, it is coupled to DQA1*0301 and DQB1*0401. In the Philippines, DRB1*0405 is coupled to DQA1*0101 and DQB1*0503.

Strong linkage disequilibrium between closely linked loci such as DRB1, DQA1 and DQB1 (DQA1 and DQB1 are only 12 kb apart) may reflect a lack of crossing over between the loci or, more likely, selection for particular combinations of alleles. In principle, population admixture may also create linkage disequilibrium patterns but this is unlikely to account for the extensive disequilibrium found in virtually all human populations. The presence of linkage disequilibrium in human populations can, in any given population, make it difficult to identify which gene on a disease-associated haplotype is responsible for the observed association. Thus, a disease could show a strong association with a given DRB1 allele (e.g. DRB1*0301), but alleles at nearby loci (e.g. DQA1, DQB1, HLA-B or other non-HLA loci) that are in linkage disequilibrium with DRB1*0301 could, in principle, be responsible for the observed disease association. Some HLA haplotypes reveal patterns of linkage disequilibrium that extend toward the centromere to DPB1 and toward the telomere to HLA-B and HLA-A. These so-called 'extended haplotypes' (e.g. A1, B8, DRB1*301, DQB1*0201, DPB1*0101) have been taken as evidence for strong selection for particular haplotypic combinations. Because the patterns of linkage disequilibrium for alleles at the HLA loci can vary in different populations, studies of the same disease in different populations can be very instructive in helping to identify which gene or combination of genes on a given haplotype may be responsible for the observed disease association.

In addition to association studies, the role of genes in the HLA region in the pathogenesis of certain diseases has been demonstrated by linkage analysis. Linkage analysis follows the cosegregation of alleles with disease in large multiplex pedigrees. Many disease genes have 'incomplete penetrance' and, similarly, for alleles conferring disease predisposition,

many unaffected individuals in a pedigree may carry the susceptibility alleles. Given the confounding effect of incomplete penetrance for conventional cosegregation studies, a highly informative approach has been to focus on the analysis of the genetic markers only among affected sib pairs. The null hypothesis of no linkage would predict a random distribution among affected sib pairs for chromosomes or haplotypes detected with a given genetic marker (i.e. an HLA locus or a variable number tandem repeat locus). The expected random distribution for affected sib pairs is 25% with two identical-by-descent (IBD) haplotypes, 50% for those containing one IBD haplotype and 25% for those containing zero IBD haplotypes. Excess haplotype sharing among affected sib pairs relative to the distribution predicted by the null hypothesis is evidence of linkage. This approach (termed nonparametric) has the virtue of not requiring assumptions about parameters such as genetic dominance or recessivity, or estimates of penetrance, as do conventional (parametric) linkage analyses.

In general, linkage studies have properties distinct from association studies. In case-control studies, a positive association for a given marker and a specific disease will be obtained only if the marker allele is in linkage disequilibrium with the true disease gene, that is only if the marker locus is physically close to the disease locus, usually significantly less than 1 cM (1% recombination). In linkage studies, however, the marker need not be in linkage disequilibrium with the disease locus, only close enough so that recombination is uncommon. Linkage methods are expected to have reduced power when the disease allele is frequent in the general population. In a genome-wide scan for disease genes, when a genetic region has been identified by linkage methods, association (or linkage disequilibrium) methods are useful for homing in on a much smaller region. In terms of sample acquisition, a large number of family samples, however, is usually harder to obtain than samples for case-control studies.

Studies of multiplex families or of affected sib pairs also allow one to estimate the proportion of the total genetic risk attributable to a given genetic region defined by a specific marker; this approach involves calculating a value (λ_s) from the ratio of expected to observed affected sib pairs that share zero haplotypes. For IDDM, for example, the observed proportion of affected sib pairs sharing zero HLA haplotypes is about 0.06; the expected proportion is 0.25, yielding a λ_s for HLA of about 4.1. A proportion of about 50% for the total genetic risk for IDDM attributable to the HLA region can be calculated, making certain assumptions, from these values.

Risk estimates

The strength of disease associations can be assessed by a variety of risk estimates. The most commonly used risk estimate for HLA disease associations is the odds ratio or relative risk, the disease risk of an individual with a particular genetic marker relative to someone without the marker. This risk estimate, calculated as an odds ratio (see **Table 3**) from the frequency of a given HLA antigen, allele or genotype among patients and controls, is sensitive to the population frequency of the genetic marker. An epidemiologic risk estimate for prospective studies defines relative risk slightly differently (see **Table 3**). Retrospective HLA case-control studies almost invariably use the term 'relative risk' to denote the odds ratio. The statistical significance of the relative risk estimate (odds ratio) is often expressed as the 95% confidence interval around the calculated estimate. This risk estimate is independent of the disease incidence and provides no information on the likelihood of disease, given the marker. The risk of someone with the marker actually getting the disease is termed the absolute risk and is the product of the ratio of the marker frequency in patients and controls and the disease incidence (**Table 3**). Because the two risk estimates are defined differently, it can be instructive to consider both in evaluating disease association data. For example, in the case illustrated in **Table 4**, the frequency of one allele (Marker 1) is significantly increased among patients relative to controls and the frequency of another (Marker 2) allele is only very slightly increased. If one were to consider both alleles together as a marker (Marker 3) because, for instance, both alleles share a particular amino acid residue, the relative risk for the combined allele marker will go up but the absolute risk will go down

Table 3 Definition of risk estimates

Marker	
+ -	
Disease	+ a b
	- c d
a = number of patients with marker	
b = number of patients without marker	
c = number of controls with marker	
d = number of controls without marker	
Odds ratio (relative risk ^a)	$= \frac{ad}{cb}$
Absolute risk	$= \frac{a/(a+b)}{c/(c+d)} \times \text{disease incidence}$
Etiologic Fraction (population attributable risk)	$= \frac{(a/(a+b) - c/(c+d))}{1 - (c/(c+d))}$

^aThe relative risk used by epidemiologists for prospective studies differs slightly from the odds ratio used in case-control studies and is defined as $a/(a+c)/b/(b+d)$. For rare diseases where a and b are small relative to c and d , this expression approximates the odds ratio.

as a consequence of mixing a 'high risk' and a 'neutral' allele. Thus, it is inappropriate to argue that, because the relative risk is greater for the amino acid residue as a marker than for the high-risk allele that the susceptibility conferred by the high-risk allele is due to this shared amino acid residue.

A third measure of association, the 'etiologic fraction' or the 'population attributable risk', estimates the proportion of the disease incidence attributable to the marker (**Table 3**). This statistic, which is related to the normalized disequilibrium, D' , is unaffected by the frequency of alleles in the general population. In studies involving the genomic mapping of disease genes, this statistic is sometimes referred to as ' p excess' and estimates the association between a marker locus and nearby disease alleles.

The statistical measures to assess the significance of an observed disease association typically utilize the 2×2 table comparing the marker frequency in patients and controls and the chi-square test (Fisher's exact). When many comparisons are carried out, as is usually the case for the multiple alleles at an HLA locus, the likelihood of a chance association for one of the alleles must be considered. One approach to minimize the probability of a false positive association (type I error) due to multiple comparisons is to multiply the P value obtained for the associated marker by the number of comparisons made (Bonferroni's correction) yielding a P_c (corrected P value). This correction is conservative and, while minimizing type I error, may lead, for studies with moderate sample size but many alleles, to type II error, the failure to detect a true association. Another approach is to compare the allele frequency distributions of patients and controls rather than the frequencies of each individual allele. This is known as a 'heterogeneity' test (e.g. the G-test) and yields a P value for the probability that the two distributions are not different (homogeneous) and indicates which individual alleles have contributed to the heterogeneity.

Examples and issues in HLA disease associations

Virtually all HLA disease associations share certain general properties.

Predisposition to disease

The associated genes are 'normal' allelic variants rather than disease-causing mutations; they confer increased or decreased susceptibility to disease rather than the disease itself. Most individuals carrying the associated genes will not get the disease, indicating that additional environmental or genetic factors or

Table 4 Distribution of susceptibility alleles in patients and controls and risk estimates

	Patients	Controls	RR	AR	EF
<i>Frequency of marker</i>					
Marker 1	0.40	0.10	6.0	4.0 × disease incidence	0.33
Marker 2	0.50	0.40	1.5	1.25 × disease incidence	0.16
Marker 3	0.90	0.50	9.0	1.8 × disease incidence	0.80
<i>For ankylosing spondylitis</i>					
HLA-B27	0.94	0.09	158.4	10.4 × disease incidence	0.93

possibly stochastic events are required to elicit or trigger disease pathogenesis in predisposed individuals. The role of environmental 'triggers', such as bacterial or viral infections (or stochastic events), is evident from the observed concordance rates among monozygotic (MZ) twins. For IDDM, for example, the concordance rates for MZ twins are about 40–70%, depending on the HLA genotype.

Multiple genes

For most HLA-associated diseases, multiple genes, in addition to those in the HLA region, are involved in disease susceptibility. The polygenic nature of these disease associations can be inferred from comparing the concordance rates of HLA-identical siblings to those of MZ twins. For IDDM, for example, the concordance rates for HLA-identical sibs are 10–20% while those for MZ twins are 40–70%, implying that genes outside the HLA region are also involved. For IDDM, it has been estimated that about 45–50% of the total genetic risk can be attributed to the HLA region, based on the analysis of affected sib pairs. In addition, genome-wide scans in multiplex families have identified non-HLA genetic regions that may confer susceptibility to IDDM.

Combinations of MHC genes

In many cases, multiple genes within the HLA region appear to be involved in disease pathogenesis. For the DR4-associated susceptibility to IDDM, specific combinations of alleles at DRB1, DQA1 and DQB1 are required to confer high risk. Moreover, for IDDM, there is a *hierarchy* of risk ranging from highly susceptible to neutral to protective for different alleles, haplotypes and genotypes. In Caucasians, the DRB1*1501-DQB1*0602 haplotype is strongly protective for IDDM. Among susceptible genotypes, the heterozygote DR3/DR4, DQB1*0302 has the greatest risk for IDDM, significantly greater than the DR3/3 and DR4/4 homozygotes. One possible explanation for the dramatic DR3/4 effect on IDDM risk is the formation of *trans*-complementing DQ molecules encoded by the DQA1 locus of the DR3 haplotype (*0501) and the DQB1 locus of the DR4

haplotype (*0302) and those encoded by the DQA1 locus of the DR4 haplotype (*0301) and the DQB1 locus of the DR3 haplotype (*0201). For some diseases, alleles at the DPB1 locus, as well as those in the DR-DQ region appear to be involved in susceptibility. For pauciarticular juvenile rheumatoid arthritis, DPB1*0201 as well as DR8 (DRB1*0801) are strongly associated with disease and may be independent risk factors.

Interaction of host HLA genotype with pathogen genotype

For some HLA-associated diseases that are caused by an infectious pathogen, the HLA association may depend on the genotype of the pathogen. Invasive cervical carcinoma and severe cervical dysplasia result from infection with high-risk human papillomavirus (HPV) types such as HPV-16 or HPV-18. Only a small proportion of infected individuals, however, progress to cervical disease. Certain HLA types (HLA-B*07, DRB1*1501-DQB1*0602, DPB1*0401) are associated with cervical dysplasia and invasive cancer, but only with HPV-16-mediated disease.

The role of individual amino acid residues

The ability to identify specific alleles associated with a given disease has allowed the comparisons of associated sequences with other closely related alleles and made possible the identification of specific polymorphic amino acid residues that may be involved with the disease pathogenesis. Disease-associated alleles often differ from nonassociated alleles at single amino acid residues or at a single polymorphic motif, implicating these positions as critical in some aspect of immune function, such as peptide binding and presentation. For example, the PV-associated DQB1*0503 allele differs from *0501 and *0502 only at position 57; DQB1*0503 encodes Asp whereas *0501 encodes Val and *0502 encodes Ser at this position. The substitution of a negatively charged residue for a neutral residue at position 57 is thought to affect the formation of a salt bridge between the α and β chain. In IDDM, there is a

general correlation of DQB1 alleles with Asp at position 576 and low risk for IDDM; there are, however, several exceptions to this pattern. In general, the risk associated with an allele cannot be predicted by a single amino acid position but only by *combinations* of motifs assembled as alleles, haplotypes, and genotypes.

Summary

A wide variety of human diseases have been associated with specific alleles at the HLA class I and class II loci. In a small subset of these diseases, the association can be attributed to linkage disequilibrium with a linked non-HLA locus. For the majority of these diseases, however, it appears that specific HLA molecules confer increased susceptibility or protection from disease. In many cases, it is specific combinations of alleles at HLA loci that appear to determine disease risk and for some diseases, there is a hierarchy of risk depending on the HLA genotype. The immunological mechanisms underlying the disease associations are still not fully understood but are likely to involve the manner in which peptides are bound and presented to the T cell by the HLA molecules encoded by the disease-associated alleles. DNA-based typing methods, capable of distinguishing the many allelic variants within a serologic HLA type, have been highly informative in identifying specific alleles, haplotypes and genotypes and in estimating the disease risk for the associated markers. These studies promise to have significant

clinical value in differential diagnosis, prognosis and in the identification of predisposed individuals; the identification of at-risk individuals offers the hope of a better understanding of pathogenesis and, ultimately, of disease prevention.

See also: Autoimmune diseases; Autoimmunity; *bm* Mutants; H2 class I; H2 class II; H2 class III; Immune response (Ir) genes; Linkage disequilibrium; Molecular mimicry; Relative risk; Tissue typing.

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MHC, EVOLUTION OF

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The presence of major histocompatibility complex (MHC) class I and class II genes has been demonstrated in all classes of jawed vertebrates (gnathostomes); no trace of these genes has thus far been found in jawless vertebrates (agnathans, represented by hagfish and lamprey) or in nonvertebrate chordates (cephalochordates, urochordates). Whether agnathans truly lack MHC genes or possess genes that are very different from those of the gnathostomes remains an unanswered question. From the rate of nonsynonymous substitutions it can be calculated that the class I and class II genes diverged more than 500 million years ago and hence before vertebrate radiation, but this estimate has a large range of error.

In all jawed vertebrates that have been studied, the organization of the MHC genes (and, by implication,

of the MHC molecules) is the same: 1) the genes fall into two classes (I and II); 2) in each class there are multiple gene families; 3) in each family there are two types of genes, A and B, encoding the α and β polypeptide chains of the MHC heterodimers, respectively; 4) the exon-intron organization is largely conserved in the various genes of the same class; 5) the polymorphism, when present, is concentrated in the same exons (in class I genes in exons 2 and 3, in class II genes in exon 2); and 6) within the variable exons, the polymorphism is concentrated into 3–4 short segments. These observations, particularly the last two, imply that the function of the MHC molecules has been conserved for at least 400 million years, since the emergence of the jawed vertebrates. The focusing of the polymorphism onto the

putative peptide-binding regions (PBRs) in all gnathostome classes suggests that the MHC function has been, from the beginning, to bind peptides, presumably for presentation to T cell receptors (TCRs), and thus to initiate the anticipatory (adaptive) immune response. It can therefore be expected that MHC and TCR (and by extension also immunoglobulins, Ig) emerged simultaneously, and that their emergence marked the appearance of the anticipatory response. By this reasoning, the anticipatory immune system is an innovation restricted to vertebrates or perhaps to chordates, as experimental observations indeed indicate.

The primordial MHC gene seems to have been assembled from two or three other genes, each of which originally had a different function. One of the contributing genes came from the immunoglobulin superfamily, another from a family of genes encoding structures with an α helix rising from a β -stranded platform (= MHC fold), and the third from a gene encoding membrane-anchoring and possibly signal-transmission structures. The identity of the gene family that donated the peptide-binding domains characterized by the MHC fold is not known. The HSP70 family has been proposed as a possible candidate, but newer data on the tertiary structure of HSP70 proteins do not support this suggestion. Another possible candidate is the proteasomal gene family, but here, too, no significant structural similarity to the peptide-binding domains of the MHC molecules has been found. In the tertiary structure, the MHC peptide-binding domains superficially resemble proteins of the interleukin 8 (IL-8) family, and in the primary structure there is a weak sequence similarity between MHC proteins and the endothelial cell protein receptor (EPCR). The significance of these similarities is obscure.

It is generally assumed that the primordial class I and class II genes arose from a common ancestor by gene duplication. Whether this ancestor had the structure of a class I or class II gene is controversial. The most parsimonious hypothesis seems to be that the ancestor resembled a class II gene and that the first MHC molecules were homodimers in which each protein chain contributed one half of the peptide-binding module. A duplication of this gene and subsequent divergence of the two copies produced the primordial class II A and B genes encoding, respectively, the α and β chains of the class II heterodimers. A second round of duplication followed by a deletion–fusion process then created a primordial class I gene with two exons specifying the two halves of the peptide-binding module. The product of this gene came to associate noncovalently with a single domain encoded in another member of the immunoglobulin superfamily, the β_2 -microglobulin gene.

The process of duplication (expansion) and deletion (contraction) of the class I and class II genes has continued throughout subsequent MHC evolution and has led to the emergence of new MHC gene families and the demise of old ones. In bony fish, for instance, every major order possesses different MHC class I and class II gene families, each derived from a separate ancestor. The families persist for a variable length of time. In eutherian mammals, for example, the class II DR, DQ, DP, DN/DO, and DM families are shared by the different orders, whereas the primate class I A, B, C family is restricted to this order and possibly only to one suborder. Within each MHC class I or class II family, different genes are functional for a variable length of time. Thus, for example, while orthologous DQB and DPB genes have apparently remained functional for the entire period of primate evolution, functional DRB genes have been replaced at least three times by their paralogs: in prosimians, New World monkeys, and Old World monkeys (together with apes). The non-functional copies, can, however, persist as pseudogenes for a long time. The primate DRB6 gene, for example, may have been nonfunctional for more than 30 million years.

The average substitution rates at the synonymous and nonsynonymous PBR sites of the MHC genes are 1.2×10^{-9} and 5.9×10^{-9} per site per year, respectively. The synonymous rate is lower and the nonsynonymous rate higher than that of most non-MHC genes. The MHC genes therefore contain a region (PBR) that evolves faster than most other genes, while the rest of the MHC gene evolves at a comparable or a lower rate. In contrast to other genes, however, the MHC genes do not contain regions highly conserved over long evolutionary periods. Although some amino acid residues at certain positions are invariant or nearly so in the encoded proteins, they are scattered, and sharing of long stretches between distantly related molecules is rare. Cloning of MHC genes from unrelated taxa is therefore always a challenge not obviated by the steady accumulation of sequences from a variety of vertebrates.

MHC genes are subjected to both positive (balancing) and negative (purifying) selection. The positive selection is restricted to the nonsynonymous PBR sites, whereas the negative selection affects various nonsynonymous sites scattered over the rest of the coding sequence. The intensity of the positive selection has been estimated to be less than 0.02. (For comparison, the selection intensity exerted by the causative agent of malaria on the hemoglobin S allele is 0.3.) A selection coefficient this low will therefore be difficult to measure experimentally in

any but the most prolific vertebrate species. The selection is presumably effected by parasites or would-be parasites via the interaction of parasite-derived peptides with the PBR of functional MHC molecules. The mechanism of selection is believed to be heterozygous advantage (an MHC heterozygote being protected from more parasites than an MHC homozygote), although a case for frequency-dependent selection (protection effected for as long as an allele is rare) has also been made. The intensity of the negative selection can be expressed in terms of a functional constraint on the non-PBR site. On a scale on which 0 indicates a complete conservation (no amino acid replacements are allowed) and 1 a complete absence of conservation (any substitution is allowed as in a nonfunctional gene), the MHC genes score a functional constraint value of 0.33, the average for non-MHC genes being 0.189. Hence, functional MHC genes are less constrained in terms of purifying selection than most non-MHC genes.

Balancing selection is responsible for the maintenance of MHC alleles in the population, without fixation (attainment of frequency of 100%), for periods in excess of those expected for neutral alleles. This long allele persistence time has two major consequences. First, during their tenure in the population the polymorphic alleles are in a position to diversify by additional mutations. A polymorphic allele incorporates an additional nonsynonymous mutation at a PBR site, or a synonymous mutation on average every 1–4 and 3–5 million years, respectively. Alleles differing in up to 10% of their coding sequence are a common appearance at functional MHC loci. Second, the allelic lineage persistence time can be longer than the lifespan of a species and the MHC polymorphism is therefore transmitted from ancestral to descendant species in a trans-specific manner. Some MHC allelic lineages have been shown to be more than 30 million years old; these must have 'survived' several rounds of speciation.

Old allelic lineages are often characterized by the presence of diagnostic motifs – short, largely invariant stretches of sequences idiomatic to a particular group of alleles. Sequence motifs, which are usually part of the PBR, are normally indicative of shared ancestry, particularly if a given group of alleles has more than one motif in common. Evidence is accumulating, however, that identical or very similar motifs can also arise independently in different genes, presumably by selection. Some motifs, particularly those shared by the MHC genes of very distant taxa, therefore represent instances of convergent evolution, possibly driven by the need to respond to similar parasite-derived peptides.

Although the class I and class II loci occur in clus-

ters, they are, in all vertebrates thus far studied, intermingled with other loci. The latter are often referred to as class III loci although they are occupied by a heterogeneous group of mostly unrelated genes. In bony fish (represented by the zebrafish), on the other hand, the class II loci are dispersed to two different chromosomes, the class I loci are on yet another chromosome, and each of the clusters is associated with class III loci different from those in mammals. The LMP2 and LMP7 loci, which in mammals are part of the class II cluster, are in the zebrafish in the class I cluster. Since the LMP loci are functionally tied to the class I loci, the zebrafish arrangement might be ancestral to that found in mammals. The zebrafish MHC organization rules out the suggestion that class I and class II must be linked to coordinate their expression. Which of the two types of MHC organizations (the human or the zebrafish type) is more ancient remains an unresolved question.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; MHC peptide-binding specificity; β_2 -microglobulin; H2 class I; H2 class II; H2 class III; HLA class I; HLA class II; HLA class III region; Immunoglobulin gene superfamily; MHC disease associations; MHC, functions of; MHC restriction; Mixed lymphocyte reaction (MLR); Phylogeny of the immune response; T cell receptor, evolution of.

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MHC, FUNCTIONS OF

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Genes mapping to the major histocompatibility complex (MHC) encode the cell surface glycoproteins (class I and class II) recognized by self-monitoring and alloreactive T lymphocytes, the LMP2 and LMP7 components of the proteasome complex, two chains that comprise the transporter associated with antigen-processing (TAP) complex, the α and β chains of the H2M (mouse) or HLA-DM (human) molecule, the C4 component of the complement cascade, and the α and β forms of tumor necrosis factor (TNF). The MHC class I molecules were initially identified as the strong transplantation antigens, while the class II glycoproteins were first described as the immune response (Ir) gene products. In addition to the class I genes H2-K, H2-D and H2-L (mice) and HLA-A, -B, and -C (humans), there are multiple loci for class Ib genes, H2-Q, T, and M (mouse) and HLA-E, F, G, H, I and J (human). DNA sequence analysis has also demonstrated the existence of many more genes that have not been associated with any product. Whether there is an underlying biological reason why this set of genes should be inherited as a complex has not been clarified.

T cell targeting and effector function

The class I and class II MHC glycoproteins perform a central immunological function in focusing CD8⁺ and CD4⁺ T lymphocytes, respectively, to the surface of appropriate cells. The underlying principle is the same in both cases. The α/β T cell receptor (TCR) heterodimer recognizes a peptide bound into an active site located in the most external domains of the MHC glycoprotein. In the adult, the 10^7 or more possible TCR specificities are capable of monitoring self MHC molecules that are associated with a great variety of nonself peptides derived, for instance, from invading viruses or bacteria. The CD8 and CD4 molecules are considered to play an accessory role in this cell-interaction process by acting as coreceptors for the TCR and by further stabilizing TCR/MHC interactions by binding to conserved regions on the MHC class I or class II proteins.

The anatomical distribution patterns of the MHC molecules determine the spectrum of T cell targeting. For example, MHC class I glycoproteins are expressed on nearly all nucleated cell types throughout the body. This means that virus-infected cells in the various organ sites can signal evidence of abnormal

cell events, such as viral infection, to recirculating CD8⁺ virus-immune T cells. These lymphocytes can potentially kill virus-infected target cells and thus terminate the progression of the infectious process. An exception to this rule is found in the brain where some cells, especially sensory neurons, remain MHC class I negative. This may be the reason that herpesviruses can persist, for instance, in the trigeminal ganglia.

Under normal circumstances, the class II molecules are distributed mostly on B lymphocytes, dendritic cells and macrophages, all of which are found in lymphoid tissue. This serves to focus the CD4⁺ T cells to the lymph nodes and spleen. A primary function of the CD4⁺ T cell subset is to secrete cytokines, such as interleukin 2 (IL-2) and IL-4, which promote the growth and differentiation of other lymphocytes, such as B cells. Further, the responses of the CD4⁺ lymphocytes are necessary for T-dependent antibody production. As such cytokines function essentially at short range, it is essential that the CD4⁺ helper T cells and B cells make direct contact through TCR/MHC interactions.

The part played by the MHC in normal immunity can be illustrated by giving a somewhat speculative account of what happens during the primary response to a poxvirus. The virus invades through the skin and is immediately transported, either as free infectious particles or in the cytoplasm of 'veiled cells' (a type of dendritic cell), via the lymphatics to the regional lymph node. Some cells, including macrophages, are infected. Virus uncoating follows, the replication process is initiated, and new virus proteins are made. A proportion of the protein is degraded to peptide fragments, perhaps by the proteolytic proteasome complex. Some of these peptides are transported into the endoplasmic reticulum (ER), via the TAP complex, where binding to intracellular class I protein occurs if the peptides have sufficient affinity for the class I molecule. The class I-peptide complex is then transported to the cell surface, where it can be recognized by CD8⁺ T cells expressing the appropriate α/β TCR. Such lymphocytes are present in unstimulated lymphoid tissue at a frequency of $<1:600\,000$ and increase to as much as 1:2000 within 6 days after primary exposure to the virus. The CD8⁺ cytotoxic effectors are first detected on day 5, when they exit the lymph node and localize

to other tissues where there are infected cells expressing the same class I–peptide complex.

Some of the virus that enters the lymph node will be bound by B cells expressing surface immunoglobulin (Ig) specific for determinants on the viral coat glycoproteins. This Ig–virion complex is internalized and degraded in lysosomes. Peptides derived from proteins that are both internal and external to the virus particle can then potentially associate with MHC class II molecules and be expressed on the cell surface where there is a concomitant increase in the total level of class II glycoprotein expression. As a consequence, helper CD4⁺ T cells specific for any viral peptide bound to self class II proteins are focused on to the B lymphocytes that can potentially secrete neutralizing antibody specific for the determinant recognized initially on the invading pathogen. The interleukins produced by these helper T cells promote the growth and differentiation of the responding B lymphocytes.

The class I and class II molecules thus act as ‘signposts’ to direct the CD8⁺ and CD4⁺ lymphocytes to appropriate locations, first in lymphoid tissue and later in organ sites of virus growth. Patterns of cytokine secretion are central to these processes. The level of cell surface MHC class I expression is enhanced by interferon- α /interferon- β (IFN α / β) which is produced by many virus-infected cells. An even more potent stimulus for both class I and class II expression is IFN γ , which is secreted by activated CD8⁺ and some CD4⁺ T cells. In the brain, for instance, astrocytes can be induced to become MHC class I and class II positive following exposure to IFN γ , while sensory neurons remain negative. Such astrocytes can function as potent stimulators of CD4⁺ T cells.

Although much less is understood regarding the role of the class Ib proteins in protecting individuals from infectious pathogens, some recent evidence has revealed that these molecules may play an important part in generating immune responses to certain classes of bacteria. When identifying the nature of the maternally transmitted minor histocompatibility antigen, Mta, murine H2–M3 complexes were shown to bind a mitochondrially derived peptide that contained an N-formyl methionine at the N-terminus. This modification of the peptide is unique in that it is produced only in eubacteria, mitochondria and chloroplasts. It was later shown that, during infection with the gram-positive bacteria *Listeria monocytogenes*, CD8⁺ T cells were generated that recognize M3 molecules associated with an N-formylated peptide derived from a bacterial protein. As the MHC class Ib molecules are far less polymorphic than the conventional class I proteins, understanding

the CD8⁺ T cell responses to these antigens provides insight to design vaccine strategies for populations where the classical MHC molecules are genetically diverse.

Polymorphism, duplication, repertoire and tolerance

Obviously, this ‘self-surveillance’ system only operates if ‘nonself’ peptide(s) can associate with MHC molecules to form an immunogenic complex, i.e. one for which there is a complementary TCR. This is not always the case. Evidence of a complete absence of response can be found for particular MHC molecules, even with large viruses such as vaccinia. Evolution has compensated for this possibility in several ways. The first is that both the H2 and HLA systems are extremely polymorphic. The possibility of utilizing more than 100 allelic forms of the MHC proteins means that there is little likelihood of a total failure of T cell response to a particular pathogen throughout the species. Also, there are new alleles that arise within populations to add even more diversity. For example, studies of small native populations in North and South America have revealed that the HLA-B locus has the potential to evolve rapidly in limited populations. In this instance, new loci were generated by intralocus recombination. Whether this reflects a change in response to a pathogen that was introduced to that particular population is uncertain. The second mechanism is the duplication of genetic loci that seem to be functionally identical. A heterozygous individual expresses as many as six class I glycoproteins (H2-K, H2-D and H2-L in the mouse and HLA-A, -B and -C in humans). MHC class II complexes comprise two transmembrane glycoproteins, α - and β -chains. In the mouse there are two isotypes of class II molecules, I-A and I-E, while humans express three types of class II molecules, HLA-DP, -DQ and -DR.

This duplication of class I and II MHC loci is not devoid of potential negative consequences for immune responsiveness. The problem is that the immune system must learn to be tolerant of self peptides presented in the context of self MHC molecules. The larger the number of self MHC glycoproteins, the greater is the deletion of potential TCR specificities with high affinity for self. This process of negative selection of the TCR repertoire occurs, at least initially, in the thymus. There is good evidence that the presence of a particular MHC complex during thymocyte ontogeny results in the deletion of T cells which express a TCR that has too high an affinity for the MHC molecule. The consequence may, for instance, be the effective deletion of

all T cells expressing a particular TCR V_β gene. This can obviously act to decrease the pool of potential T cell responder specificities. However, it is also likely that a major role of the thymus is to select positively for thymocytes expressing TCR with a low or intermediate affinity for self class I or class II MHC glycoproteins, as precursors for mature T cells that can potentially recognize the same MHC molecule modified by a foreign peptide. Whether the apparent convergence in the existence of the class I MHC loci in mice (H2-K, H2-D and H2-L) and humans (HLA-A, -B and -C) as a trinity is indicative of an optimum evolutionary balance between the need to maintain self-tolerance and the capacity for immune responsiveness, or is simply a reflection of the nature of an ancestral gene pool, is not clear.

Immunological surveillance and graft rejection

Holes in the TCR repertoire resulting from the need to maintain self-tolerance may not normally constitute a problem for the specific host response to viruses or bacteria, as any pathogen is likely to incorporate a great many nonself peptides that can potentially associate with one of the self MHC glycoproteins. This thinking can legitimately be extended to the B cell lymphoma induced by Epstein-Barr virus which, at least for Caucasians, may normally be held in check by T cells. However, the effectiveness of such surveillance for tumors that are not induced by viruses is likely to be very much constrained, especially as there is no obvious reason why a particular oncogenic change should be characterized by the expression of novel peptides that bind to MHC molecules, unless the level of some poorly expressed gene is upregulated to a point where sufficient peptides from that protein are generated and available to bind MHC molecules. Also, the selection pressure exerted by immune T cells on tumors that are immunogenic can lead to the emergence of variants that no longer express the target MHC class I molecule. Further, to initiate an immune response the responding T cell must receive not only a signal from a cognate TCR/MHC interaction, but also costimulatory signals from accessory molecules expressed on the tumor cell. Gene therapy studies have suggested that strategies to express MHC and/or costimulatory molecules on tumor cells may help to initiate a tumor-specific immune response *in vivo*.

The account so far has concentrated on the part played by the MHC in the monitoring of self by T lymphocytes, the phenomenon known as MHC restriction. However, the MHC is named for, and

was historically defined by, the phenomenon of allo-reactivity that is the central feature of graft rejection. Current thinking is that the high frequency of T cells ($>1:1000$ spleen cells in the mouse) specific for any foreign MHC glycoprotein reflects that a TCR which recognizes a particular self-derived MHC-peptide complex will also bind to one or other nonself MHC molecules (alloantigens). However, this does not necessarily mean that alloreactivity is simply an epiphenomenon of the self-surveillance mechanism. It is possible that the fine tuning of host response represented by the thymic T cell-MHC interactions has derived in evolutionary time from a much more primitive mechanism analogous to that operating, for instance, to prevent one colony of corals from parasitizing another. The precursors of the TCR genes are likely to have been a considerably less diverse set of genes encoding cell interaction molecules.

Possible physiological functions

The MHC may also have additional functions which are not directly related to the specific immune response. There is support from both rat and mouse experimental systems for mating preference between MHC-different individuals. Perhaps particular MHC-peptide complexes have pheromone-like properties when, for instance, they are excreted in urine. If this is so, it would provide an obvious mechanism for promoting heterozygosity and thus polymorphism. One problem with this concept is that wild mice maintain in discrete populations, or demes, and tend not to mate with mice from a different deme that is in the immediate vicinity. Further functions for which some evidence has been presented in the past include an influence on patterns of lymphocyte recirculation and the capacity to influence the association of somatic cells other than T lymphocytes. However, there are now a number of other cell surface molecules that may be better candidates for such roles.

Cellular and humoral immunity

The primary role of the MHC in immunity is to alert T lymphocytes to changes in the surface integrity of other cells, so that they can be dealt with in a way that is appropriate for maintaining the integrity of the milieu interieur. The MHC class I and class II molecules define the nature of self to the immune system. If discovered today, the MHC might be more appropriately named the 'self-surveillance complex'. Unlike the Ig molecules, which can be produced in

large numbers by a single plasma cell, the T cells themselves are the effectors of cell-mediated immunity. Antibodies bind well to foreign molecules (e.g. viral coat proteins), and do not normally interact with the MHC glycoprotein-peptide complex which targets recirculating T lymphocytes. The MHC is thus the key operator in a 'law of conservation of T cell function'. The comparatively rare helper and effector T cells are focused on to the cell surface, do not recognize free virus particles, and are not blocked by the much more prolific Ig molecules.

See also: **Alloantigens; Antigen-presenting cells; CD4; CD8; Cell-mediated immunity; Graft rejection; H2 class I; H2 class II; HLA class I; HLA class II; Immune response (I_r) genes; Immune surveillance; Interferon α ; Interferon β ; T cell receptor, $\alpha\beta$; Thymus; Tolerance, central; Tolerance, models; Tolerance, peripheral.**

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MHC PEPTIDE-BINDING SPECIFICITY

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Major histocompatibility complex (MHC) molecules are highly polymorphic membrane glycoproteins that bind peptide fragments of proteins and display them for recognition by T cells. Their function is to present self antigens in the thymus for tolerance induction and foreign antigens in the periphery for immune response. MHC peptide recognition by T cells is thus the means by which the organism discriminates between self and nonself.

One of the hallmarks of class II MHC molecules is their extraordinary allelic polymorphism. Each allelic form of MHC molecules binds a large array of different peptides to ensure T cell-mediated immunity against the spectrum of pathogens, and yet T cells must be tolerant to a sufficient number of self proteins to avoid the possibility of potentially harmful immune responses against self antigens. Recent advances have increased our understanding of the molecular events in peptide interaction with MHC molecules and allowed for the systematic

identification of antigenic sites in natural self and foreign proteins.

The MHC peptide-binding groove

The molecules encoded in the MHC complex form a family of highly polymorphic glycoproteins. Of these MHC proteins, the best characterized are the class I (HLA-A, -B, -C in humans and H-2K, -D, -L in mice) and class II (HLA-DR, -DQ, -DP in humans and I-A, I-E in the mice) molecules which present peptides to CD8⁺ and CD4⁺ lymphocytes, respectively. Class I molecules are dimers of a membrane-inserted heavy chain and a soluble light chain, the β_2 -microglobulin. Class II molecules are $\alpha\beta$ dimers of similar size, both of which are transmembrane proteins. The three-dimensional structure of several class I and class II molecules has been determined by X-ray crystallography. The class I peptide-binding site is formed by the two membrane-distal domains of the heavy

chain and is composed of a floor of eight β strands flanked by two α helical walls. The organization of the class II peptide binding site is similar to the class I binding site, even though it is formed by the membrane-distal domains of both class II chains, to which each contribute one α helix and four β strands. The β sheet floors and helical walls define a groove of suitable dimension for occupancy by 8–10 peptide residues in both MHC classes.

Despite an overall similarity of the class I and class II groove, detailed differences with major functional consequences have been identified. The class I cleft is blocked completely at both ends by bulky amino acid side-chains, while the class II cleft is open due to a replacement and/or a repositioning of these blocking side-chains. Consequently, the majority of class I binding sequences are short peptides in the range of 8–10 residues. In contrast, the open class II cleft allows peptides to extend out, enabling as a result, the binding of a broader range of peptide lengths, typically 12–24 residues.

Sequence-independent MHC peptide binding

MHC molecules must be able to bind a large array of different peptides to ensure T cell-mediated immunity to the antigenic universe. This broad binding capacity arises from the interaction between conserved MHC residues and the peptide main chain, thereby providing a sequence-independent affinity for peptide ligands. For class I, the binding of the peptide main chain involves topographically clustered MHC residues at both ends of the binding groove. These residues interact with the free N- and C-terminal groups of the peptide. The critical contributions of both termini to the affinity of peptide ligands, and the fact that both ends of the groove are closed (see above), explain the strong bias for the stable binding of short peptides. For class II, the location and the identity of the conserved residues interacting with the peptide main-chain are very different. Instead of being clustered at the ends of the cleft, they are distributed along the class II groove. Consequently, the peptide main chain is kept in close contact with the MHC cleft, resulting in a relatively flat conformation of class II-bound peptides. In contrast, prominent kinks are observed in class I-bound peptides, and even a limited amount of bulging is accommodated while the N- and C-terminal binding is maintained.

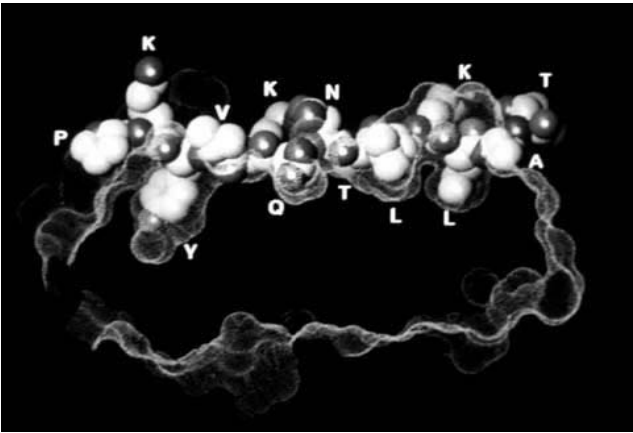


Figure 1 Pockets in the HLA-DR1 peptide binding site. Side view of the molecular surface of the HLA-DR1 peptide-binding site with the influenza hemagglutinin peptide 306–318. Peptide side-chains are labelled in one letter code. (Reproduced, by copyright permission, from *Nature* 1994; **368**: 220.) (See also color **Plate 5**.)

Sequence-dependent MHC peptide binding

The sequence-independent network of hydrogen bonds between conserved MHC residues and the peptide main-chain gives rise to a broad, but not unlimited, peptide-binding capacity. Indeed, most natural peptide sequences lack the characteristics necessary to bind to MHC molecules, because peptide main-chain interactions are not the only mode of MHC binding. Some of the peptide side-chains contact residues within the MHC cleft and increase the overall binding affinity and specificity of the associated peptides (anchor residues); others interfere with residues of the MHC cleft and reduce binding (inhibitory residues). These sequence-dependent interactions are due to the irregular surface of the MHC cleft. MHC side-chains protrude into the cleft and form pockets or ridges, resulting in strong preferences for interaction with particular amino acid side-chains (**Figure 1**).

Notably, most pockets in the MHC groove are

Table 1 MHC motifs

MHC molecules	Peptide positions								
	1	2	3	4	5	6	7	8	9
HLA-A*0201	–	L	–	–	–	V	–	–	V
		M							L
HLA-B*2705	–	R	–	–	–	–	–	–	L
									F
HLA-DRB1*0101	Y	–	–	M	–	A	–	–	L
	F			L		G			M
HLA-DRB1*1101	W	–	–	M	–	R	–	–	–
				L		K			

shaped by clusters of polymorphic MHC residues and are thus of distinct chemical and size characteristics in different MHC alleles. For example, a negatively charged side-chain in one MHC molecule may preferentially interact with positively charged peptide residues, while a positively charged side-chain in another MHC molecule may only bind to negatively charged peptide residues; similarly, a small side-chain in one MHC molecule may greatly extend a pocket and allow for interaction with many different amino acid residues, while a large amino acid side-chain in another MHC molecule may completely block access to this pocket. MHC alleles can therefore be characterized by differences in the precise nature and position of polymorphic pockets, thereby playing a major role in allele-specific peptide binding.

MHC peptide-binding motifs

The interaction of peptide side-chains with pockets of the MHC cleft imposes sequence requirements on the binding peptides. These requirements are summarized in peptide-binding motifs. The characterization of large, MHC-selected peptide pools was a breakthrough for the analysis of MHC binding motifs, for it allowed the generalization of rules for peptide binding to MHC molecules. Class I motifs were analyzed by the pooled peptide sequencing technique; that is, the elution of endogenous class I peptide pools and their subsequent analysis by Edman sequencing. The length restriction of class I-bound peptides leads to a natural alignment of class I-bound peptides, thus allowing the identification of position-dependent preferences for particular amino acid side-chains (class I anchors) by sequencing total mixtures of the eluted peptides. Class II motifs were identified by the analysis of large peptide pools selected from M13 bacteriophage peptide display libraries comprising several million random nonamer peptides. Sequence analysis of the DNA encoding the displayed peptides led to the identification of class II anchors. Both pool sequencing and screening of phage libraries have been used for the identification of several allele-specific class I- and class II-binding motifs, respectively. These motifs generally consist of 2–4 anchor positions that are at fixed distances from one another (Table 1).

Prediction of MHC-binding peptides

The discovery of motifs through pool sequencing and phage library screening would imply simple rules for peptide–MHC interaction. Much evidence has indicated, however, that these motifs were only the tip of the iceberg, and that the rules for MHC peptide

binding are much more complex because: 1) inhibitory residues are as important for MHC peptide binding as anchor residues and binding studies have revealed both position- and allele-specific properties of such inhibitory residues; 2) apart from the main anchor positions identified by pool sequencing and phage library screening, additional ‘secondary’ anchors were found to be important for MHC peptide binding, thus rendering difficult the prediction of MHC binding sequences by using ‘simple’ MHC motifs. Consequently, new quantitative approaches have been developed that allow for a more complete characterization of MHC peptide-binding specificity: the effects of all natural amino acids at each peptide position are quantitated by using multiple-peptide synthesis technology, together with new high-flux *in vitro* binding assays. The resulting quantitative motifs are thus like ‘fingerprints’ of the MHC clefts, as they reflect, as the sum of all peptide side-chain values, the architecture of the polymorphic groove. Effective predictive algorithms for identifying antigenic determinants in proteins or producing custom peptides with defined binding properties have been developed. These methods involve systematically scoring the positive and negative effects of a full set of amino acid substitutions on binding to class II molecules at each position of a peptide, and representing the results for any specific sequence as the sum of the values for each position. These new methods represent a powerful new approach to picking out regions of large proteins representing dominant determinants for use in peptide-based vaccines.

MHC antagonists

The identification of rules for peptide binding to MHC class II molecules could also have interesting applications in the treatment of autoimmune disease.

Autoimmune diseases result from the activation of self-reactive T cells by autoantigens. A striking characteristic of autoimmune diseases is the increased frequency of certain HLA class II alleles in affected individuals. Moreover, as demonstrated in rheumatoid arthritis and insulin-dependent diabetes mellitus, for example, alleles that positively associate with autoimmune diseases share unique amino acid residues in the hypervariable regions involved in peptide binding. Although the pathogenesis of autoimmune diseases is not known, one possibility is that disease-associated HLA class II molecules are able to bind and to present autoantigenic peptides to T cells. These peptides activate autoreactive CD4⁺ T cells, which in turn induce a cascade of chronic inflammation.

Blocking the antigen-presenting activity of disease-associated HLA class II molecules could represent an important way to prevent and possibly treat autoimmune diseases.

The knowledge of the anchor position, as well as the other general rules for peptide binding to MHC molecules, should prove very useful for the design of MHC-specific antagonists. This strategy ought to be directed towards the development of a low molecular weight antagonist, possibly nonpeptic, that blocks the peptide-binding site of disease-linked class II MHC molecules. Such an antagonist is expected to interfere with the presentation of antigenic peptide ligands, including those involved in the activation of autoimmune T cells.

See also: Antigen-presenting cells; MHC disease associations; MHC, functions of; T cell receptor, recognition by; Vaccines.

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MHC RESTRICTION

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Although the concept that multiple cell types must interact to generate an immune response was firmly established in the 1960s, the possible role of cell surface antigens such as the major histocompatibility complex (MHC) in restricting intercellular interactions was not considered until a series of experiments was performed in the 1970s, which established that virtually all intercellular communication in the immune response, including the regulation of antigen-specific T lymphocyte proliferation, antibody production, delayed hypersensitivity, and the generation of cytotoxic T lymphocytes (CTLs) required that the interacting cell types share specific regions of the MHC. Subsequent studies have established that this requirement for MHC compatibility between interacting cells is intimately related to the ontogenetic development of T lymphocytes in the thymus.

Macrophage/T lymphocyte interaction

Primed T lymphocytes and T cell lines/clones can be stimulated to proliferate *in vitro* by antigen only if a specialized antigen-presenting cell (APC) is also present in the culture. Cells that perform APC function include dendritic cells, macrophages and activated B lymphocytes. In 1973, Rosenthal and Shevach took antigen-primed CD4⁺ T lymphocytes from one strain of inbred guinea pig and attempted to stimulate them to proliferate with antigen on the surface of macrophages from another strain. Antigen-induced T cell proliferation could only be observed when the macrophages and T lymphocytes were derived from the same strain. One possible explanation for the failure of histoincompatible combinations of macrophages and T cells to interact was that the mixed leukocyte reaction which was produced when allogeneic combinations of macrophages and

T cells were cocultured led to inhibition of cellular interaction. However, macrophages from F1 animals were fully capable of presenting antigen to T lymphocytes from either of the parental strains even though a low-level mixed leukocyte reaction was observed in such cultures. The conclusion drawn from these studies was that MHC identity was required for effective macrophage/T lymphocyte interaction. These studies in the guinea pig model were confirmed and extended with congenic and recombinant mouse strains to establish firmly that the products of the I-region of the MHC (class II antigens) were responsible for mediating this interaction. In addition, studies on the role of the macrophage in the induction of the T helper cell *in vitro* demonstrated similar genetic restrictions involving I-region products. The transfer of delayed hypersensitivity *in vivo* also required that the donor and recipient be MHC compatible. In this situation, it is necessary for the resident APC population in the recipient skin, the Langerhans cells, to share MHC antigens with the T lymphocytes of the donor.

One of the most instructive experiments performed during the course of studies investigating MHC restriction in macrophage/T lymphocyte interaction was derived from the analysis of the response of T cells to antigens, the response to which is controlled by specific immune response (Ir) genes. MHC-linked Ir genes had been previously identified that controlled the capacity of certain strains of a species to recognize simple antigens. Although macrophages from either parental strain were capable of presenting complex antigens such as ovalbumin to F1 T cells, primed T cells from F1 animals made by mating responder and nonresponder parents failed to recognize simple antigens, the responses to which are controlled by specific Ir genes associated with macrophages from the nonresponder parent. Thus, if strain A was a responder to a given antigen, while strain B was a nonresponder, (A × B)F1 T cells which are responders could only be activated by antigen in association with strain A, but not strain B macrophages. The unresponsiveness of the F1 animal to antigen associated with APC of the nonresponder parent can now be explained by the failure of antigenic peptides from simple antigens to bind to the MHC class II molecule of the nonresponder parent so as to form an effective complex that can be recognized by the T cell receptor. In contrast, complex antigens can be broken down to multiple peptides and some of these are likely to bind to the polymorphic MHC class II molecules of all members of a species, resulting in complexes which can be recognized by the TCR from any strain.

T lymphocyte/B lymphocyte interaction

The first experiments to demonstrate directly the failure of cooperation of histoincompatible T and B lymphocytes to generate an antibody response were performed by Katz and coworkers. In these studies, F1 recipients were injected with carrier primed T cells from one of the parental strains, irradiated 24 h later, and then injected with B lymphocytes from the other parent primed to a hapten on a heterologous carrier. The reconstituted animals were then immunized with the appropriate hapten-carrier conjugate and the antihapten response was measured 7 days later. If the carrier-primed T lymphocytes and hapten-primed B lymphocytes were derived from the same parental strain, an antihapten antibody could be measured. The failure of histoincompatible helper T lymphocytes to collaborate with hapten-specific B lymphocytes was not secondary to an 'allogeneic effect' because B lymphocytes derived from F1 animals could cooperate with T lymphocytes derived from either parental strain; presumably, under these latter conditions, the interaction of parental strain T lymphocytes with F1 B lymphocytes would have been accompanied by as great an 'allogeneic effect' as that seen when T and B lymphocytes from MHC incompatible strains were used. Detailed genetic mapping studies demonstrated that the genes which controlled T lymphocyte/B lymphocyte interaction also mapped to the I-region of the MHC.

One of the major problems in the analysis of MHC restriction on T/B interaction resulted from the demonstration that macrophage/T lymphocyte interaction is restricted. It is therefore possible that the observed restriction on T/B interactions was secondary to a restriction on macrophage/T lymphocyte interaction which occurred early in the priming of the helper T lymphocyte. In order to resolve the issue of whether macrophage/T cell interaction controls the subsequent ability of primed T helper cells to interact with B cells, F1 T cells were primed *in vitro* with parental strain macrophages and then tested for their ability to collaborate with B cells syngeneic to the macrophage donor or with B cells derived from the other parental strain. Unfortunately, this experimental protocol resulted in conflicting results when performed in multiple independent laboratories. In some cases, restriction could only be observed at the level of macrophage/T cell interaction, with T/B interaction completely unrestricted, while in other studies clear-cut restrictions in both interactions were documented. The solution of this dilemma required the use of T cell clones and an in-depth analysis of the heterogeneity of the responding B cell population. Thus, there appear to be two distinct B

cell populations which are capable of interacting with primed T helper cells. One population can interact with histoincompatible helper T cells, requires only T cell-derived growth factors, high concentration of antigen, produces mainly IgM antibody, and does not require that the hapten and carrier be linked. In contrast, a second population of B cells requires genetically restricted T cell help, needs only low concentrations of antigen to differentiate into a plasma cell, produces mainly IgG antibody, and requires that the hapten and carrier molecules be linked.

CTL target cells

Following the demonstration of the requirements for MHC compatibility for macrophage/T lymphocyte interaction and for T lymphocyte/B lymphocyte interaction, it was shown by Zinkernagel and Doherty for virus-infected cells, by Shearer for targets modified with the TNP (trinitrophenyl) hapten, and by Bevan for targets bearing minor histocompatibility antigens, that the killing of antigen-bearing target cells by CD8⁺ T cells was also restricted by MHC antigens. In the virus studies, mice were infected with live virus and 7 days later their spleen cells were tested for their ability to kill virus-infected macrophages of the same or an allogeneic strain. T Cells derived from a virus-primed mouse would only kill target cells from the same strain and not allogeneic strains. In contrast to the results of studies with CD4⁺ helper or proliferating T cell populations, the restriction on the interaction of CD8⁺ CTLs with antigen-bearing target cells was mediated by the products of the K and D regions of the H2 complex (MHC class I antigens).

One of the original interpretations for the MHC restriction of macrophage/T lymphocyte and T/B lymphocyte interaction was that the interacting cells had to share a particular gene product (cell interaction model). This model was based on the idea that the interactions of immunocompetent cells involved like-like interactions and that the recognition of the specific antigen by the T cell receptor was independent of the molecules mediating cell-cell interaction. The results of their studies on the role of the MHC in restricting the interaction of CTLs with virus-infected targets led Zinkernagel and Doherty to propose an alternative model which stated that the antigen-specific T cell receptor was not specific for antigen, but recognized either modifications in the MHC class I antigens induced by viral proteins during the process of virus infection (altered self model) or that the nominal antigen formed a complex with the MHC antigens that was recognized as a unique

antigen by the T cell receptor (complex antigenic determinant model).

Studies with T cells from F1 animals formed the basis of support for these models which required the associative recognition of the nominal antigen and the MHC antigen. Thus, if F1 animals were immunized with a virus, the resultant virus-specific population of CTLs would lyse targets from either of the parental strains. However, if F1 T cells from a virally infected animal were transferred to one of the parental strains which was then infected with the virus, the resultant restimulated CTL population would only lyse target cells derived from the parental strain in which the T cells were boosted.

This experimental result is very difficult to reconcile with the cell interaction model, which states that a single F1 T cell would bear cell interaction molecules of both parental types and should, following boosting in one or the other parental strain environments, be capable of recognizing virus-infected targets derived from either parental strain. A much more likely explanation is that the F1 T cell population is composed of two distinct subpopulations, one of which is specific for viral determinants in association with MHC antigens of one parent, while the second is specific for viral determinants in association with MHC antigens from the other parent. Later studies demonstrated that CD4⁺ proliferating and helper T cells from F1 animals could also be divided into two distinct populations which were restricted in their interactions with macrophages or with B lymphocytes from one of the other parental strains, respectively.

The 1996 Nobel Prize in Physiology or Medicine was awarded to Peter Doherty and Rolf Zinkernagel 'for their discoveries concerning the specificity of the cell mediated immune defence'. These cellular studies formed the basis for the biochemical and molecular dissection of the polymorphism of MHC antigens which directly demonstrated that peptides derived from antigens are bound by the polymorphic regions of the MHC antigens and then displayed to the T cell receptor as a complex in the context of exposed sites on the α helices of the MHC molecules.

MHC restriction is learned in the thymus

It is now clear that the interaction of CD4⁺ T lymphocytes with either macrophages or B lymphocytes and the interaction of CD8⁺ CTLs with target cells involves co-recognition by a single T cell receptor of a complex of antigen and MHC class II molecules in the former case and a complex of antigen and MHC class I molecules in the latter case. The issue that must now be considered is whether this restriction is

created during the process of T cell differentiation in the thymus or whether the restriction is the result of priming to antigen in association with self MHC molecules in the periphery. This question has been resolved by the creation of chimeric animals in which F1 bone marrow cells are allowed to differentiate in lethally irradiated parental hosts. The results of these studies have demonstrated that CD8⁺ F1 T cells which develop in one parental environment are restricted to the recognition of antigen in association with MHC class I antigens of that parental strain. Similar studies with CD4⁺ T helper cells demonstrated that T cells from F1 to parent chimeras only possess the ability to cooperate with B cells and macrophages sharing MHC class II antigens with the MHC antigens of the parent used to construct the chimera. Thus, a contraction of the F1 T cell repertoire occurs during differentiation in the parental environment, as F1 bone marrow which differentiates under normal conditions in the F1 environment is capable of giving rise to T cells which can recognize antigen in association with MHC antigens expressed by either parental strain.

The critical host tissue that was responsible for inducing this restriction was shown to be the thymus and thymic epithelial cells were the critical cell type which determined MHC restriction. Additional support for this 'thymic selection' model for the generation of MHC restriction has been obtained from studies of mice bearing a transgenic T cell receptor. Mice bearing a T cell receptor from a CTL clone originally derived from a strain of mice of MHC type 'A' were crossed with different mouse strains either bearing MHC 'A' or not. Only those offspring bearing MHC 'A' had a large number of peripheral T cells expressing the transgenic T cell receptor, strongly suggesting that the recognition of MHC 'A' in the thymus during T cell differentiation was required for the expression of the transgenic TCR. The process of 'positive selection' involves the delivery of survival and differentiation signals to CD4⁺CD8⁺ thymocytes and is associated with commitments to the CD4⁺CD8⁻ and CD4⁻CD8⁺ lineages.

Collectively, these studies demonstrate that MHC restriction is an acquired or learned characteristic which occurs during T cell differentiation in the thymus in the absence of a foreign antigen. Following immunization of an animal, only those T cell clones specific for the foreign antigen and the learned MHC antigen are expanded. However, a number of studies have also demonstrated that a normal animal does contain T cells which recognize foreign antigens in association with MHC antigens not present in the

thymus during T cell differentiation. The thymic selection model can also accommodate this data if one integrates our understanding of the generation of MHC restriction with our concepts of why normal animals have high frequencies of T cells capable of recognizing alloantigens. All T cells are capable of recognizing foreign antigens in association with self MHC molecules and a certain fraction of these self-restricted T cell receptors (those responsible for alloreactivity) are also capable of recognizing foreign MHC antigens with their associated self peptides. It is therefore not surprising that the normal T cell repertoire can also contain some receptors capable of recognizing foreign antigenic peptides in association with foreign MHC antigens.

See also: Antigen-presenting cells; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; B lymphocyte, antigen processing and presentation; Cooperation, mechanisms of cellular; Cytotoxic T lymphocytes; Thymic epithelium: potential role in regulatory T cell tolerance; Immune response (I_r) genes; MHC, functions of; T cell receptor, αβ; T cell receptor, γδ; T cell receptor, recognition by.

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MICROBICIDAL MECHANISMS, OXYGEN-DEPENDENT

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Phagocytes respond to stimulation with a burst of oxygen consumption, and much, if not all, of this extra oxygen consumed is converted to highly reactive oxidants which are toxic to ingested organisms and to certain extracellular targets. The stimulation may be physical, as when phagocytes adhere to a surface, or chemical, with a large variety of soluble or particulate agents being capable of initiating a respiratory burst. The particulate stimuli, as, for example, a microorganism, are generally coated with molecules such as products of the activation of complement (C3b or C3bi) and/or specific antibody which recognize receptors on the surface of the phagocyte. The soluble stimuli include phorbol esters, which are particularly potent, chemotactic factors such as the microbial product formyl-methionyl-leucyl-phenylalanine (fMLP), the complement activation product C5a, leukotriene LTB₄, certain lectins such as concanavalin A, lipopolysaccharide (LPS, endotoxin), certain cytokines as, for example, tumor necrosis factor α (TNF α , cachectin) and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as a number of other agents.

Physical and chemical stimuli, or two chemical stimuli, may combine to produce a stimulation which is greater than the additive effect of each alone. For example, many chemical stimuli increase the adherence of phagocytes through the increased surface expression of one or more members of a family of adherence glycoproteins (integrins) which consist of a variable α chain (CD11a, b or c) and a common β chain (CD18), thus amplifying the stimulation. TNF α , normally a weak stimulus, produces an amplified and prolonged stimulation of the respiratory burst when neutrophils are adherent to endothelial cells or a surface coated with serum or certain connective tissue proteins. Some chemical stimuli, for example LPS or interferon γ (IFN γ), while weak stimuli by themselves, can greatly increase the response of phagocytes to a number of second chemical stimuli. This process is termed priming.

The respiratory burst results from the assembly in the plasma membrane of a complex electron transport system which transfers electrons from NADPH on the cytoplasmic side of the membrane to oxygen in the extracellular fluid (or, when the plasma membrane is invaginated to form the phagosome, in the

intraphagosomal space) (Figure 1). This enzyme system has been called the phagocyte oxidase (phox) or the NADPH oxidase of phagocytes. Assembly occurs in part by incorporation of components present in cytoplasmic granules by a degranulation process, and in part by the translocation of cytosolic components.

Activation of the oxidase by intact cells is complex and incompletely understood; requirements differ to some degree with the nature of the stimulus. Stimulation is generally but not always initiated by the binding of the stimulant to specific high-affinity receptors on the surface of the cell. Examples include receptors for the Fc region of immunoglobulins and for complement products (C3b, C3bi) which recognize ligands on opsonized particles, the formyl peptide chemotactic receptor, etc. The mechanism of signal transduction following receptor-ligand interaction involves, for some stimuli, GTP-binding protein-induced activation of phospholipase C which hydrolyzes (poly)phosphatidylinositol releasing diacylglycerol and inositol 1,4,5-triphosphate (IP₃). Diacylglycerol activates cytosolic protein kinase C with translocation to the membrane and IP₃ can mediate Ca²⁺ mobilization from endoplasmic reticular stores. Phorbol esters, e.g. phorbol myristate acetate (PMA) or certain synthetic diacylglycerols, e.g. 1-oleoyl-2-acetyl-glycerol (OAG) bypass the early steps of signal transduction to activate protein kinase C directly. Activation of cyclic AMP-dependent protein kinase (protein kinase A) or tyrosine kinase also may occur. As a result of protein kinase activation, a component (or components) of the oxidase is phosphorylated.

As a consequence of stimulation, at least three cytosolic components of the oxidase, p47^{phox}, p67^{phox}, and p21^{rac} are translocated to the plasma membrane where they combine with a unique membrane-associated cytochrome *b* to form the active NADPH oxidase. An intracellular pool of the cytochrome *b* is present in the membranes of the specific (secondary) granules of neutrophils and is incorporated into the oxidase complex when the membrane of the granule fuses with the plasma membrane following stimulation. The cytochrome *b* is a heterodimer composed of a small 22 kDa α subunit (p22^{phox}) tightly linked noncovalently to a heavily glycosylated large 91 kDa β subunit (gp91^{phox}).

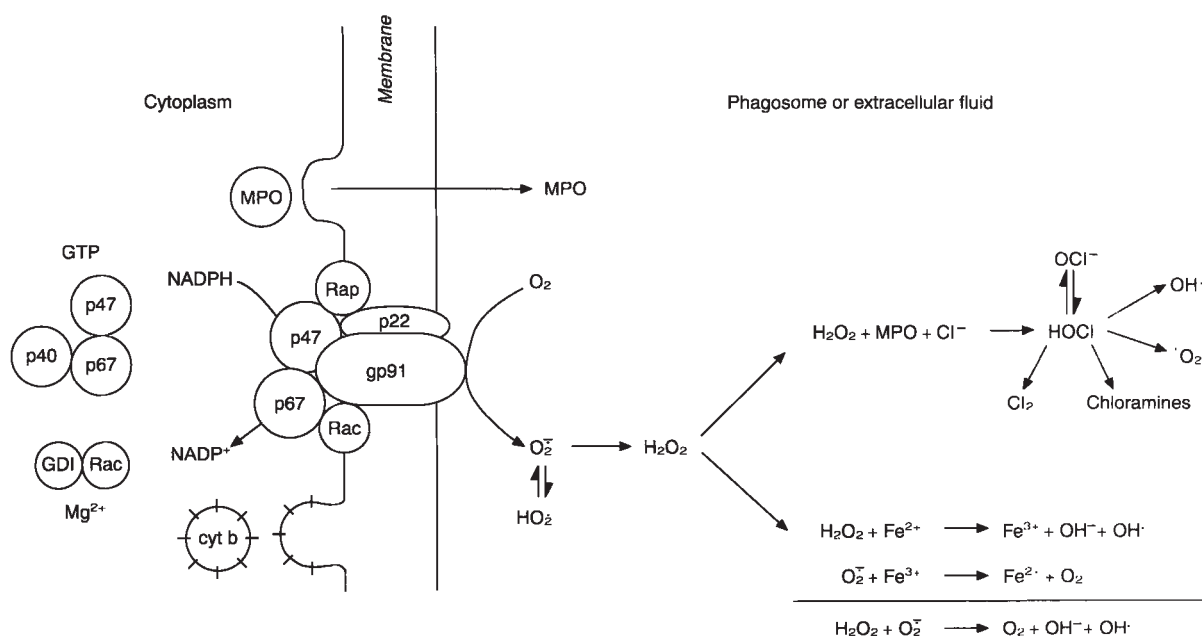


Figure 1 Oxygen-dependent antimicrobial systems of phagocytes.

gp91^{phox} has a transmembrane configuration with the C-terminus extending into the cytoplasm and the glycosylated portion extending into the extracellular (or intraphagosomal) space. The cytochrome contains two heme groups and flavin adenine dinucleotide (FAD) and has a characteristic absorption peak at 558 nm (and thus the name flavocytochrome *b*₅₅₈). Flavocytochrome *b*₅₅₈ contains both NADPH- and FAD-binding sites in the C-terminal third of the gp91^{phox} subunit which extends into the cytoplasm and has an unusually low midpoint potential (-245 mV) which allows it to directly reduce oxygen, suggesting that flavocytochrome *b*₅₅₈ contains all of the elements necessary for the transfer of electrons from NADPH through FAD to oxygen. The gene for the β subunit is located at position Xp91.1 on the short arm of the X chromosome and is expressed only in myeloid cells. It encodes a 65 kDa polypeptide which contains a number of N-linked glycosylation sites. The gene for the α subunit is autosomal in location and is expressed in a wide variety of cells. Flavocytochrome *b*₅₅₈ is associated in the membrane with a low molecular weight GTP-binding protein Rap1A with unknown function.

The cytoplasmic components of the oxidase appear to be regulatory molecules acting on flavocytochrome *b*₅₅₈ to convert it from a dormant to an enzymatically active NADPH oxidase. p47^{phox} and p67^{phox} exist in the cytoplasm as a complex in association with a 40 kDa protein, p40^{phox}, of unknown function. p47^{phox} is a 47 kDa cationic protein with several serine phosphorylation sites. Its

phosphorylation at multiple sites is required for translocation to the membrane. Binding of the 67 kDa cytosolic component (p67^{phox}) is dependent on prior translocation of p47^{phox}. p21^{rac} is a member of the *rho* class of *ras*-related small GTP-binding proteins which is present in the cytoplasm as a complex with an inhibitory molecule, GDP dissociation inhibitor or *rho* GDI which maintains *rac* in an inactive GDP-bound state. Activation of the oxidase is associated with the dissociation of the *rac*-*rho* GDI complex with the release of an active GTP-bound *rac* which migrates to the membrane. There are two highly homologous forms of *rac* (*rac1* and *rac2*), with *rac1* reported to be the regulatory component of the guinea pig macrophage NADPH oxidase and *rac2* serving the same function in the human neutrophil oxidase. p47^{phox}, p67^{phox} and p40^{phox} contain domains homologous to a noncatalytic region of *src* tyrosine kinase (*src* homology 3 or SH3 domain) which binds to proline-rich sequences mediating the protein-protein binding required for the association of p47^{phox} and p67^{phox} with each other, with p40^{phox} and with flavocytochrome *b*₅₅₈. The activated oxidase is associated with submembranous cytoskeletal components.

In cell-free preparations of phagocytes, activation of the NADPH oxidase can be achieved by the addition of an anionic detergent such as arachidonic acid, sodium dodecyl sulfate or other *cis* unsaturated fatty acids. Under these conditions, NADPH oxidase activity requires NADPH, oxygen, a cell membrane fraction, a cytosolic fraction, Mg²⁺, GTP, and under

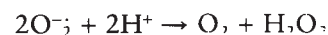
some conditions, ATP. Reconstitution of the phagocyte NADPH oxidase has been achieved with recombinant flavocytochrome b_{558} , p47^{phox}, p67^{phox} and p21^{rac}.

Chronic granulomatous disease (CGD) is an inherited disease characterized by repeated and severe infections with pyogenic organisms. It is associated with a phagocytic cell microbicidal defect in which some microorganisms are ingested normally but are not killed, due to a defect in the respiratory burst oxidase. CGD can result from different genetic lesions affecting one or another of the components of the oxidase. The majority of patients with CGD (56%) have X-linked inheritance and almost all of these patients lack spectroscopic evidence of flavocytochrome b_{558} . Although the primary lesion is in the gene encoding for the β chain, both the α and β chains of cytochrome b_{558} are absent, indicating that the β chain is required for the stable expression of the α chain. In rare cases of X-linked CGD, cytochrome b_{558} is present in normal amounts, but has a single Pro-His substitution at residue 415 of the 91 kDa subunit, which renders the oxidase non-functional. The remaining CGD patients (44%) exhibit autosomal recessive inheritance. The phagocytes of most of these patients contain normal levels of cytochrome b_{558} but lack either p47^{phox} or, less frequently, p67^{phox}. Rare autosomal recessive CGD patients lack cytochrome b_{558} , probably due to a defect in the 22 kDa cytochrome b_{558} subunit. The addition of IFN γ to neutrophils or monocytes from selected CGD patients *in vitro* or the subcutaneous injection of IFN γ into some CGD patients results in a partial correction of the metabolic and microbicidal defect. Gene therapy, in which the appropriate normal gene is introduced into defective cells, is under study.

The initial product of the respiratory burst is the superoxide anion (O_2^-), which exists in equilibrium with the perhydroxyl radical (HO_2), with a pK_a of 4.8. O_2^- is predominantly a reductant, but also can act as an oxidant. Since many biologically important compounds react rather sluggishly with O_2^- , it has been proposed that O_2^- does not have the necessary reactivity to be directly toxic to cells. However, the chemical reactivity of O_2^- is increased considerably in a nonpolar environment, as in the hydrophobic region of membranes where the reactions of O_2^- are not in competition with the proton requiring dismutation reaction. Further, HO_2 is a considerably stronger oxidant than is O_2^- , raising the possibility that a localized fall in pH as might occur in a phagosome or at a membrane surface would cause a shift in the $O_2^- \rightleftharpoons HO_2$ equilibrium toward the more potent protonated form. Finally, the relatively low reactivity

of O_2^- may allow it to diffuse through biologic fluids and the anion channels of certain membranes to be toxic at a distance through the formation of more reactive oxidants.

When two molecules of O_2^- interact, one is oxidized and the other reduced in a dismutation reaction with the formation of H_2O_2 .



This reaction occurs either spontaneously or catalyzed by superoxide dismutase (SOD). Spontaneous dismutation is optimal at pH 4.8 and falls as the pH increases where catalysis by SOD becomes more significant. Although an early rise in intraphagosomal pH has been reported, most studies have indicated that the pH within the phagosome falls to levels where spontaneous dismutation is rapid, suggesting that catalysis by SOD may be unnecessary for H_2O_2 formation. There is no evidence that leukocytic SOD is released into the phagosome; SOD, however, can be introduced there as a component of the ingested organism. Although H_2O_2 formed by phagocytes has toxic properties, its reactivity is relatively low when compared to toxic products formed from it, such as hypohalous acids and hydroxyl radicals ($OH^\cdot$).

The toxicity of H_2O_2 is greatly increased by reaction with peroxidase and a halide. There are a number of distinct peroxidases in mammalian tissues which differ in their primary structure, heme prosthetic group, and in the halide requirement for microbicidal activity. The peroxidase of neutrophils and monocytes (myeloperoxidase, MPO) is present in a subpopulation of cytoplasmic granules (azurophil or primary granules) and is discharged into the phagosome by the degranulation process which follows the phagocytosis of microorganisms or other particles. Although the MPO-mediated antimicrobial system is effective with chloride, bromide, iodide and the pseudohalide thiocyanate, the physiologic halide is chloride. MPO reacts with H_2O_2 generated either by the respiratory burst or certain ingested microorganisms, to form a complex (compound I) which oxidizes chloride to hypochlorous acid ($HOCl$) (Figure 2). Compound II is an inactive form of peroxidase formed by an excess of H_2O_2 . O_2^- can reduce compound II back to the active peroxidase with restoration of activity and may function in this way in the phagosome. O_2^- also reacts with MPO to form compound III (oxypoxidase) which can decay to MPO, be converted to compound II by H_2O_2 or be catalytically active. $HOCl$ is in equilibrium with its ionized form hypochlorite (OCl^-) with a pK_a of 7.3 and, at acid pH, $HOCl$ can react with excess chloride to form

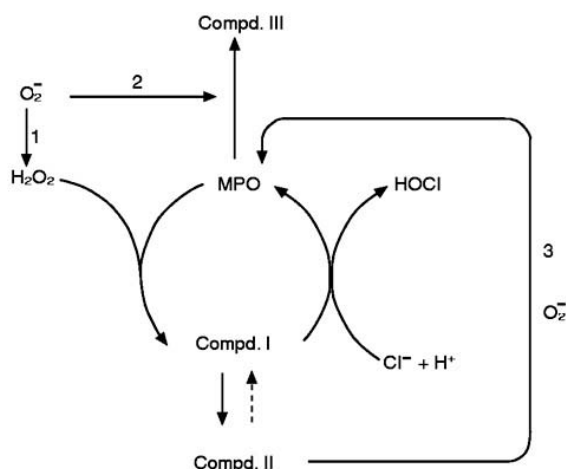


Figure 2 MPO complex formation. MPO forms three complexes: compounds I, II and III. O_2^- can influence the reactivity of MPO by: 1) forming H_2O_2 by dismutation which reacts with MPO to form compound I, 2) reacting with MPO to form compound III, and 3) converting the inactive compound II to native MPO. (Reprinted with permission from Klebanoff SJ (1992) *Oxygen metabolites from phagocytes*. In Gallin JI, Goldstein IM and Snyderman R (eds) *Inflammation: Basic Principles and Clinical Correlates*, 2nd edn. New York: Raven Press.

chlorine. These are powerful oxidants which are highly toxic to microorganisms. Hypochlorous acid can react with certain nitrogen-containing compounds to form monochloramines and dichloramines with retention of some oxidizing activity and toxicity.

In addition to its toxicity to a variety of intraphagosomal targets (e.g. bacteria, fungi, protozoa), the MPO system can be released to the outside of the cell, to be toxic to adjacent microorganisms (bacteria, fungi, viruses, protozoa, helminths), and normal and malignant mammalian cells, and to inactivate certain extracellular substances, e.g. $\alpha 1$ -proteinase inhibitor, leukotrienes and chemotactic factors. Although the peroxidase system is generally inhibitory in nature, it can stimulate certain cells to exhibit their secretory functions (platelets, mast cells) and can activate certain enzymes (procollagenase, progelatinase) through its oxidative action.

When monocytes mature into macrophages, either *in vitro* or *in vivo*, they lose their granule peroxidase. The respiratory burst also is markedly depressed and, as a result, a number of ingested organisms can survive and replicate, producing disease. Activation of the macrophage by $IFN\gamma$ or certain other cytokines partially restores the respiratory burst and cidal activity.

Eosinophil peroxidase (EPO), which is present in the matrix of the cytoplasmic granules of eosinophils, can utilize chloride, but less efficiently than MPO. Bromide appears to be the physiologic halide

for the peroxidase- H_2O_2 -halide system in this cell type and the product of its oxidation, hypobromous acid, like HOCl, has broad reactivity. Thiocyanate also has been reported to function with EPO. MPO and particularly EPO are strongly basic proteins which can bind to the target by ionic bonds and in this way be carried into phagocytes which lack a granule peroxidase (e.g. macrophages).

Lactoperoxidase (LPO) cannot oxidize chloride and appears to utilize thiocyanate in an antimicrobial system in milk and saliva. The source of H_2O_2 may be microbial metabolism, adjacent phagocytes or soluble enzyme systems such as xanthine oxidase in milk. Hypothiocyanous acid is the toxic species formed.

The oxidants formed by the peroxidase system can oxidize the target cell or molecule at a variety of chemical sites (sulfhydryl groups, iron-sulfur centers, sulfur-ether groups, unsaturated fatty acids, terminal amino groups, peptide bonds, etc.) and the binding of the halide in covalent linkage to the target (halogenation) also occurs. The nature of the lesion which causes cell death is not known; perhaps no single lethal lesion will be found.

A number of lines of evidence suggest an important role for peroxidase-derived oxidants in the toxic activity of phagocytes.

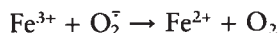
1. MPO, H_2O_2 and a halide form a highly toxic system.
2. MPO is released and H_2O_2 is formed by stimulated neutrophils, and chloride is present in adequate amounts. MPO and H_2O_2 and the products of halide oxidation can be detected in the phagosome.
3. A high proportion of the oxygen consumed by stimulated neutrophils can be accounted for by the formation of HOCl.
4. A role for H_2O_2 in the toxic activity of neutrophils is suggested by the protective effect of catalase and by the partial reversal of the microbicidal defect in CGD neutrophils by H_2O_2 .
5. The requirement for MPO for optimum microbicidal activity is suggested: a) by the decrease in the microbicidal activity of normal neutrophils by certain peroxidase inhibitors; b) by the defective microbicidal activity of neutrophil cytoplasts (which lack nuclei and are greatly depleted of granules and thus MPO, but have an intact respiratory burst), unless MPO is added; and c) by the microbicidal defect in neutrophils from patients with hereditary MPO deficiency, which lack MPO. The microbicidal defect is not as severe as in CGD, and most (but not all)

patients are clinically well. In contrast to CGD, the respiratory burst in MPO deficiency is greater than normal. Thus, the increased accumulation of nonperoxidase-derived oxidants may compensate in part for the loss of MPO in microbicidal activity.

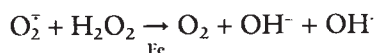
A second mechanism by which the toxicity of H_2O_2 is increased is by its reduction to hydroxyl radicals ($\text{OH}^\cdot$). H_2O_2 reacts with Fe^{2+} (Fenton's reagent), or another trace metal, to form $\text{OH}^\cdot$ as follows:



When the iron concentration is limiting, the reduction of the Fe^{3+} formed is required for the continued formation of $\text{OH}^\cdot$. This can be accomplished by O_2^- (as well as certain other reducing agents) as follows:



with the overall reaction being:



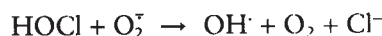
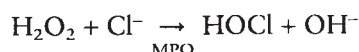
which has been called the iron-catalyzed Haber-Weiss reaction, or the superoxide-driven Fenton reaction.

The Haber-Weiss reaction is limited by the very low solubility of Fe^{3+} due to the formation of polynuclear hydrated iron complexes. Certain iron chelators can maintain Fe^{3+} in solution, and when there is the retention of an iron aquo-coordination site which can be oxidized and reduced, the complexed iron remains catalytically active in the Haber-Weiss reaction. The chelator, for example, ethylenediamine tetraacetic acid (EDTA), is thus stimulatory. Other chelators, e.g. deferoxamine, bind to all six coordination sites of iron, displacing water. These complexes are catalytically inactive and the chelator thus inhibits the Haber-Weiss reaction. A biologically relevant chelator which facilitates the iron-catalyzed Haber-Weiss reaction has been sought. Lactoferrin or transferrin are stimulatory when fully saturated with iron, but are inhibitory when unsaturated or partially saturated (their natural state). Other chelators reported in some studies to facilitate iron-dependent $\text{OH}^\cdot$ formation include ferritin, phosphate, phosphonucleotides, DNA, and α -picolinic acid. When the pH falls, the solubility of Fe^{3+} increases, and under these conditions (which may exist in the phagosome), the formation of $\text{OH}^\cdot$ may not require a chelator.

O_2^- and H_2O_2 are formed in large amounts by

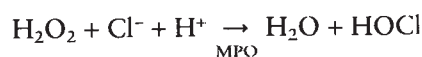
stimulated phagocytes, and it might be anticipated therefore that $\text{OH}^\cdot$ generation by the Haber-Weiss reaction would occur. However, the formation of $\text{OH}^\cdot$ by phagocytes remains controversial. The presence of free iron appears to be the limiting factor in phagocytes, since only a small amount of $\text{OH}^\cdot$ was detected by electron paramagnetic resonance (EPR) spectroscopy unless iron was added. Both the chelating effect of released lactoferrin, and the scavenging of H_2O_2 by myeloperoxidase decrease the formation of $\text{OH}^\cdot$ by neutrophils. Potential sources of iron include iron released from the ingested organism or from iron transport or storage proteins. As a result of its high reactivity, $\text{OH}^\cdot$ is readily scavenged and thus must be formed in close proximity to its target site. H_2O_2 released by phagocytes can, however, move to distant sites where reaction with local iron may produce damage.

Recently it has been proposed that $\text{OH}^\cdot$ is formed in small amounts by stimulated neutrophils by an MPO-dependent mechanism as follows:

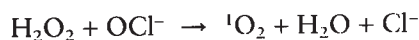
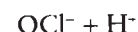


MPO catalyzes the H_2O_2 -dependent formation of HOCl which reacts with O_2^- to form $\text{OH}^\cdot$.

Singlet oxygen ($^1\text{O}_2$) is formed when an unpaired valence electron of oxygen is raised to an orbital of higher energy with an inversion of spin. There are two forms of $^1\text{O}_2$, delta and sigma. Delta $^1\text{O}_2$, while having a lower energy above ground state than sigma $^1\text{O}_2$, has a considerably longer lifespan (approximately 2 μs) and is the reactive form of $^1\text{O}_2$ in solution. It is a strong electrophile which reacts with compounds in areas of high electron density to form oxygenated products. A well-established mechanism for the formation of $^1\text{O}_2$ is by reaction of H_2O_2 with OCl^- . Since both reactants are formed by neutrophils their interaction to form $^1\text{O}_2$ has been proposed as follows:



↓



The formation of $^1\text{O}_2$ by the spontaneous dismutation of O_2^- and by the Haber-Weiss reaction also

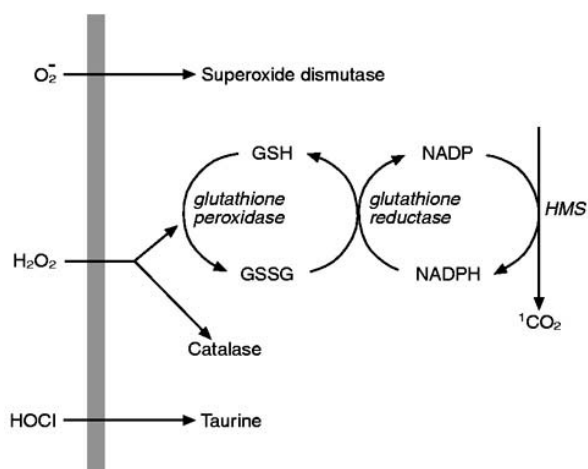


Figure 3 Scavengers of reactive oxygen species in phagocytes. GSH, reduced glutathione; GSSG, oxidized glutathione; HMS, hexose monophosphate shunt. (Reprinted with permission from Klebanoff SJ (1992) Oxygen metabolites from phagocytes. In: Gallin JI, Goldstein IM and Snyderman R (eds) *Inflammation: Basic Principles and Clinical Correlates*, 2nd edn. New York: Raven Press.

has been proposed. However, attempts to demonstrate 1O_2 formation by intact neutrophils have been controversial. It is unclear whether 1O_2 is formed in amounts sufficient to contribute significantly to the antimicrobial effect.

Murine macrophages can be induced by a number of cytokines to form nitric oxide ($NO^\cdot$). A cytosolic nitric oxide synthase is formed in these cells which, in the presence of NADPH, tetrahydrobiopterin and oxygen, oxidizes the guanidino nitrogen of L-arginine to form L-citrulline and $NO^\cdot$. $NO^\cdot$ can react with O_2^- to form the peroxynitrite ion ($ONOO^-$) which, when protonated, decomposes to form a strong oxidant with the properties of $OH^\cdot$. Ultimately, nitrite and nitrate are formed. $NO^\cdot$ can oxidize iron-sulfur centers of proteins with resultant iron loss and the peroxynitrite ion is a potent oxidant of sulfhydryl groups. Although the formation of $NO^\cdot$ and its involvement in the microbicidal and cytotoxic activity of murine phagocytes has been widely reported, its formation and role in human phagocytes has been controversial.

Microorganisms and mammalian cells, including phagocytes, can be protected from the oxidants formed by stimulated phagocytes by a number of mechanisms (Figure 3). Sensitivity to H_2O_2 correlates with the level of the H_2O_2 -scavenging enzymes catalase and glutathione peroxidase. Glutathione oxidized by glutathione peroxidase is reduced by glutathione

reductase and NADPH generated by the hexose monophosphate shunt which serves to dissipate excess H_2O_2 . Sensitivity to O_2^- and to oxidants dependent on O_2^- for their formation is affected by SOD levels and taurine or similar scavengers may neutralize the toxicity of $HOCl$ or other halide oxidation products formed by the peroxidase system.

In summary, phagocytes produce a number of reactive oxygen species on stimulation. Some, e.g. $HOCl$ or $OH^\cdot$ are labile oxidants which are highly reactive but short-lived and function under conditions in which diffusion distances are short, and protein and other scavengers are excluded, such as in the phagosome, or when the phagocyte is adherent to an antibody and/or complement-coated surface. Others, e.g. H_2O_2 or chloramines, are less reactive but more stable, and can penetrate into biological fluids rich in proteins and other scavengers, or across membranes, to be toxic at a distance under conditions in which the labile oxidants are readily scavenged. These agents, acting alone or synergistically with proteinases or granule basic proteins, contribute, along with oxygen-independent mechanisms, to the toxic effects of phagocytes.

See also: Chronic granulomatous disease; Macrophage activation; Microbicidal mechanisms, oxygen-independent.

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MICROBICIDAL MECHANISMS, OXYGEN-INDEPENDENT

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Historical background

The ability of certain leukocytes to ingest and kill microorganisms was established over a century ago by Eli Metchnikoff, a comparative zoologist who coined the terms 'phagocytosis' and 'phagocyte'. Metchnikoff had recognized the central importance of phagocytosis in host defense and vigorously proselytized the community of fin-de-siècle protoimmunologists on its behalf. In 1891, Hankin reported 'it is possible to obtain from cells that are or can become phagocytes a substance having bacteria-killing powers'. Many investigators attempted to define this substance in the ensuing years. Although the rudimentary technology available for protein purification generally restricted the success of early efforts, it did not preclude the creation of memorable names, such as 'leukins' and 'phagocytin', to describe these uncharacterized molecules. With the exception of the noteworthy discovery of lysozyme by Sir Alexander Fleming in 1922, the modern delineation of oxygen-independent microbicidal mechanisms began in the 1960s when Zeya and Spitznagel reported that rabbit and guinea pig neutrophils contained a group of relatively small 'lysosomal cationic proteins' that exhibited a broad spectrum of antimicrobial activity *in vitro*. Although the significance of this discovery was overshadowed by the concomitant delineation of oxidative microbicidal mechanisms in phagocytes and by the delay in finding the human counterparts of these peptides ('defensins'), interest in the endogenous antimicrobial molecules of phagocytes has increased sharply during the past few years. Because much of the available information about nonoxidative ('oxygen-independent') mechanisms was derived from work on human neutrophils, these cells will be a principal focus of this section. More recently, antimicrobial peptides and proteins have been demonstrated in epithelial cells and secretory glands of the genitourinary, respiratory and digestive tracts, suggesting that such molecules play important roles in defending moist, mucosal surfaces throughout the body.

Neutrophils

Neutrophils (PMNs) are short-lived, highly mobile phagocytes that can ingest bacteria or fungi and kill

them by a variety of oxidative and nonoxidative mechanisms. The oxidative mechanisms, which are described in a separate entry, are mediated by molecules whose formation requires superoxide or hydrogen peroxide. In human neutrophils, the antimicrobial oxidants are believed to include hydroxyl radicals, hypochlorous acid and chloramines. In some species, PMNs and macrophages can also form reactive oxides of nitrogen that possess antimicrobial properties. The nonoxidative mechanisms of neutrophils are mediated, in large part, by potent antimicrobial polypeptides, most of which reside within the cell's numerous cytoplasmic granules. Acting conjointly with the neutrophil's oxidants, these endogenous antibiotics may be delivered directly to microbes sequestered within phagocytic vacuoles or they may be secreted outside the neutrophil to inactivate extracellular microbes. Microorganisms isolated within phagocytic vacuoles can also be deprived of nutrients, subjected to harsh and unfavorable ionic conditions and low pH, and exposed to potentially noxious surfactants, such as lysolipids. Because the principal peptide antibiotics in human azurophil granules typically exist in concentrations of 1–5 μg per 10^6 PMNs, fusion of only 1% of these granules to a phagosome 1 μm in diameter may result in intraphagosomal concentrations as high as 20–100 mg ml^{-1} for *each* antimicrobial polypeptide. Under such conditions, interactions and reactions may occur that are not readily predictable from information derived from test-tube experiments.

Approximately a third of the 4500 granules in the cytoplasm of a typical mature human PMN contain myeloperoxidase, and are referred to as 'primary' or 'azurophil' granules. These granules contain most of the PMN's nonoxidative antimicrobial effectors, including defensins, cathepsin G, azurocidin and bactericidal/permeability increasing factor (BPI). The neutrophil's peroxidase-negative granule populations contain the majority of its lysozyme (muramidase), as well as other antimicrobial molecules, such as the α -helical peptide known either as 'FALL-39' or 'hCAP-18' and a 14 kDa secretory phospholipase A_2 .

Because azurophil granules are lysosome-like, their internal contents probably remain inert as long as the surrounding granule membrane remains intact. Degranulation, the fusion of granules with the

peripheral or perivacuolar plasma membrane, can insert granule membrane contents into the cell's or phagosome's membrane and delivers the granule's antimicrobial contents into the extracellular or phagosomal compartments. In contrast to macrophages, the human neutrophil's phagocytic vacuoles are not as strongly acidic. Studies with fluorescent pH probes indicate that newly formed neutrophil phagosomes are neutral to mildly alkaline for 15–45 minutes, with subsequent acidification to approximately pH 6.0. Initial vacuolar alkalization and the respiratory burst are linked temporally and perhaps mechanistically.

Calprotectin

The cytoplasm of human neutrophils contains remarkably high concentrations of 'calprotectin', a member of the widespread S-100 protein family. Human neutrophil calprotectin is a heteropolymer, composed of light (MRP8) and heavy (MRP14) chains. It constitutes over half of the total cytosolic protein present in human neutrophils. Although calprotectin is not secreted from intact neutrophils, its release from dead and dying neutrophils creates high concentrations of the protein in pus or abscess fluids, which also contain large amounts of neutrophil-derived antimicrobial peptides and proteins as well as microbicidal nucleohistones and histone fragments.

Calprotectin has also been called 'L1 protein', 'calgranulins A and B' or 'MRP8/MRP14'. In addition to neutrophils, it is also found in reactive tissue macrophages, in nonkeratinizing squamous epithelia and in reactive epidermal cells. The C-terminal portion of calprotectin's heavy chain, MRP14, contains a sequence identical to a peptide originally described as a neutrophil immobilizing factor, 'NIF'. Calprotectin concentrations of 50–250 $\mu\text{g ml}^{-1}$ inhibit growth by *Staphylococcus aureus*, *Staph. epidermidis* and *Escherichia coli*, and lower concentrations (4–32 $\mu\text{g ml}^{-1}$) inhibit growth by *Candida albicans*, possibly by depriving the *Candida* cells of zinc. Calprotectin purified from rat inflammatory peritoneal cells was markedly cytotoxic for mitogen-stimulated lymphocytes and various tumor cell lines, in which it induced apoptosis. It is likely that calprotectin protects neutrophils, and the other cells that express it, against microbes that invade the host cell's cytoplasm directly or by lysing phagosomal membranes.

Defensins

Classical alpha (α)-defensins are small, cysteine- and arginine-rich antimicrobial peptides with a mass of

3.5–4.0 kDa. They contain 29–34 amino acid residues, including six invariant cysteines that form three intramolecular disulfide bonds. Slightly larger antimicrobial peptides, now called beta (β)-defensins, were discovered somewhat later in the epithelial cells that line the bovine trachea and tongue and in avian and bovine neutrophils. In humans, a β -defensin (hBD-1) is produced by cells in the kidney, male and female genitourinary tracts, trachea and small airways, placenta, pancreas and salivary glands. hBD-1 is not produced by neutrophils or other cells derived from the bone marrow. Individual defensin molecules have a triple-stranded β -sheet structure and form dimers with six-stranded β sheets. Assembly of defensins into multimeric structures may be necessary for them to exert their antimicrobial and membrane-perturbing effects.

Defensin genes show extraordinary multiplicity and provide compelling evidence of the rapid evolution of the sequences that encode the mature defensin peptide. The genes for human α - and β -defensin lie within 150 kb of each other on the short arm of chromosome 8. This observation, and other recent studies suggest that α - and β -defensins arose, via multiple gene reduplications and mutations, from a common gene existent in a progenitor common to birds and mammals. Myeloid α -defensin genes contain three exons, whereas enteric α -defensin genes contain only two. The primary structures of several α - and β -defensins are shown in Figure 1.

Defensins are synthesized as 93–100 amino acid long, nonglycosylated prepropeptides. The amino acids destined to form the mature defensin peptides constitute the C-terminal portion of their respective precursors. After cotranslational removal of a highly conserved 19 residue signal sequence, the resulting prodefensin undergoes sequential proteolytic cleavages that yield the mature defensin molecules that are stored within the neutrophil's azurophil granules. The defensin propeptide is polyanionic and may protect defensin-producing cells from autocytotoxicity. It also plays a key role in the intracellular trafficking that targets defensins to the azurophil granule compartment of the neutrophil.

Since the first description of 'lysosomal cationic proteins' in rabbit and guinea pig neutrophils, more than 50 different α -defensins and 20 different β -defensins obtained from avian and mammalian sources have been identified and sequenced. Although α -defensins are exceptionally abundant constituents of human, rabbit, guinea pig and rat neutrophils, they are not found in those of mice, horses, pigs or cattle. Human neutrophils contain four defensins, called HNP-1, -2, -3 and -4 (Figure 1). α -Defensins constitute 5–7% of the total cellular

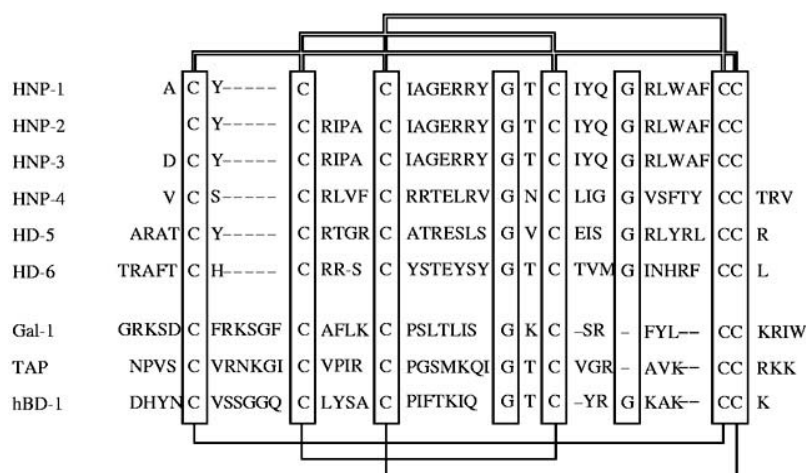


Figure 1 Sequences of selected defensins. α -Defensins HNP-1, -2, -3, and -4 (human neutrophils) and HD-5 and -6 (human small intestinal Paneth cells) are α -defensins. TAP (tracheal antimicrobial peptide) and Gal-1 (gallinacin-1) are present in bovine tracheal epithelial cells and chicken leukocytes, respectively. hBD-1, a human β -defensin, is found in many human epithelial cells and secretory tissues. Amino acids are shown in standard single letter code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Conserved cysteine and glycine residues are boxed, and dashes (gaps) have been introduced in the actual sequences to maximize the alignment between α - and β -defensins. The lines above the sequences show the slightly different cysteine disulfide pairing patterns found in α - and β -defensins.

protein of human neutrophils and 30–50% of their azurophil granule protein, but are absent from human monocytes, lymphocytes, lung macrophages and eosinophils.

Two additional α -defensins, HD-5 and HD-6 (Figure 1), are produced by the small intestinal Paneth cells of humans. These secretory and phagocytic cells line the base of the small intestine's crypts, just below the mitotic zones that are responsible for replenishing effete intestinal epithelial cells. Paneth cells also contain lysozyme and type 2 phospholipase A₂. The mixture of intestinal defensins, lysozyme and phospholipase A₂ that Paneth cells secrete undoubtedly constitutes a formidable barrier to microbes attempting to invade intestinal crypts.

Both α - and β -defensins kill many gram-positive bacteria, gram-negative bacteria and fungi *in vitro*. Several α -defensins can also inactivate herpes simplex and other enveloped viruses. The microbicidal activity of defensins towards many fungi and gram-negative bacteria is inhibited by divalent cations and by the NaCl concentrations found in extracellular fluids. Their activity against gram-positive bacteria is less affected by their ionic environment. Human neutrophil defensins act most efficiently against metabolically active microbial targets, whereas the more cationic defensins of rabbit or rat neutrophils can also kill quiescent, stationary-phase microorganisms. Consequently, human defensins manifest optimal bactericidal activity when the targets are provided with nutrients, such as glucose for *Staph.*

aureus or more complete media for most *E. coli* strains.

Human defensins form voltage-regulated ion channels in artificial 'black-lipid' membrane bilayers and they sequentially permeabilize the outer and inner membranes of metabolically active *E. coli*. Their requirement for active microbial metabolism implicates the participation of the target cell's transmembrane electromotive force in defensin-mediated antimicrobial activity. The bacterial gene (*phoP*), essential for mouse virulence by *Salmonella typhimurium*, also influences the organism's susceptibility to defensins.

Defensins can be cytotoxic to mammalian cells in culture, and this property may be involved in inflammatory processes or antineoplastic defenses. The cytotoxic potential of defensins is mitigated by the presence of serum, which contains several proteins that bind defensins, including α_2 -macroglobulin, α_1 -antitrypsin, α_1 -antichymotrypsin, antithrombin 3 and antiplasmin. Some defensins cause the degranulation of mast cells *in vitro* and many can inhibit protein kinase C and other kinases. Human defensins HNP-1 and HNP-2 are potent and selective stimuli for chemotaxis by mononuclear cells (T lymphocytes and monocytes), hence their extracellular release may promote the recruitment of these cells into inflamed or infected lesions *in vivo*. Low concentrations of defensins were reported to enhance fibroblast and epithelial cell growth *in vitro*, suggesting that defensins might promote tissue repair and

regeneration *in vivo*. Certain α -defensins, most notably rabbit defensin NP-3a (also called 'corticostatin'), bind reversibly to the adrenocorticotropin hormone (ACTH) receptor of adrenal cortical cells and competitively inhibit ACTH binding.

Bactericidal/permeability increasing proteins (BPI)

BPI is a 55–60 kDa, lysine-rich azurophil granule protein that accounts for less than 1% of total human PMN protein. BPI binds to bacterial lipopolysaccharide (LPS) with high affinity, and can mitigate several potentially toxic effects of LPS *in vitro*. Although BPI is bactericidal for rough gram-negative bacteria, including *E. coli* and *S. typhimurium*, it lacks activity against gram-positive bacteria and fungi. BPI exerts maximal bactericidal activity at neutral pH, is relatively insensitive to ionic strength, but is inhibited by millimolar concentrations of calcium or magnesium ions.

The molecular organization of BPI includes an antibacterial N-terminal domain that is both highly cationic and amphiphilic in nature, and a slightly acidic C-terminal domain that contains three hydrophobic regions which might anchor BPI to the azurophil granule membrane and prevent its external secretion. These domains are separated by a hydrophilic and proline-rich region which may serve as a hinge. BPI was reported to bind rapidly to the outer membrane of susceptible gram-negative bacteria and to cause increased outer membrane permeability to hydrophobic molecules and selective activation of enzymes that degrade envelope phospholipids and peptidoglycans. More recently, BPI was also shown to affect the integrity of the inner bacterial membrane.

BPI shows substantial homology to LBP, a lipopolysaccharide-binding protein that is synthesized by hepatocytes and circulates as an acute phase reactant in the plasma of humans and animals. BPI and LBP share the ability to bind the lipid A region of LPS and are immunologically cross-reactive; however, LBP lacks bactericidal activity. The genes for BPI and LBP are located in the same region of the long arm of chromosome 20.

Autoantibodies to BPI are commonly present in the serum of patients with inflammatory bowel disease or primary sclerosing cholangitis, a disorder of small bile ducts. What role, if any, these antibodies play in the pathogenesis of these disorders is unknown.

Type II phospholipase A₂

By definition, phospholipase A₂ (PLA₂) is an enzyme that can remove fatty acids from the middle (*sn*-2) carbon atom of phosphoglycerides. Although several different phospholipase A₂ enzymes occur in mammalian cells, only one of these – a 14 kDa type 2 PLA₂ that is also called secretory PLA₂ (sPLA₂) – has potent microbicidal properties. sPLA₂ differs substantially from the type 1 PLA₂ found in pancreatic secretions, both in its preference for phospholipid substrates such as phosphatidylethanolamine, which are prominent in bacterial membranes, over phosphatidylcholine, and in significant aspects of its primary structure.

sPLA₂ is present in secretory (nonazurophil) granules of human neutrophils and in the secretory granules of small intestinal Paneth cells. The entry of sPLA₂ into the neutrophil's phagocytic vacuoles has been demonstrated by immunoelectron microscopy. sPLA₂ is secreted extracellularly from neutrophils exposed to secretagogues, such as phorbol esters or calcium ionophore, and by Paneth cells exposed to bethanecol (a parasympathomimetic agonist), bacteria or LPS. Serum sPLA₂ is greatly elevated in patients with bacterial infections or sepsis, and rises sharply within 3 hours of the experimental administration of LPS to uninfected patients. High concentrations of sPLA₂ are also present in human tears.

Serine proteases and their congeners

Human PMN granules contain several neutral proteases, and congeners that manifest microbicidal activity *in vitro*. The most prominent of these are cathepsin G (a chymotrypsin-like enzyme), elastase and an enzymatically-inactive elastase congener, 'azurocidin'. It has been suggested that translocation of serine proteases to the external surface of activated neutrophils, where they are unaffected by protease inhibitors, may facilitate chemotaxis and their ability to exit the vascular compartment and enter sites of inflammation. Neutrophils of patients with Chédiak-Higashi syndrome contain greatly reduced amounts of both cathepsin G and elastase, deficiencies that may contribute to their impaired bactericidal performance *in vitro* and to the frequent infections sustained by these patients *in vivo*.

Cathepsin G is a 27 kDa, variably glycosylated cationic protein that is found in the azurophil granules of human PMNs. It also occurs, but in much smaller amounts, in human blood monocytes. The five exon cathepsin G gene is located on chromosome 14, adjacent to the genes for lymphocyte granzymes B and H and mast cell chymase 1. Cathepsin G kills

gram-positive and gram-negative bacteria as well as certain fungi, and is especially effective against *Neisseria gonorrhoeae*. The bactericidal activity of purified cathepsin G is inhibited by increasing ionic strength or serum concentration. Enzymatically-inactive cathepsin G retains bactericidal and fungicidal activity.

The human neutrophil elastase gene also has five exons, but is located on the short arm of chromosome 19, extremely close to genes for azurocidin, proteinase 3 and granzymes A, B, H and M. Human neutrophil elastase exists as a mixture of four isoenzymes that differ only in their carbohydrate content. Elastase can degrade bacterial cell wall protein, potentiate the bacteriolytic activity of lysozyme, and potentiate the microbicidal activity of cathepsin G *in vitro* by a nonenzymatic mechanism. Theoretically, it might also cleave the antimicrobial 25 kDa N-terminal portion of BPI from the intact holoprotein molecule and participate in trimming the cathelin domain from hCAP-18/FALL-39 to generate the antimicrobial peptide it carries. Weak intrinsic antibacterial activity of elastase has been demonstrated towards the gram-negative bacterium *Acinetobacter* 199 A, but detailed studies with other bacteria have not been reported.

Azurocidin (also called CAP-37) is a 29 kDa protein that shares considerable N-terminal sequence homology with the human neutrophil's elastase and cathepsin G. Because it contains glycine instead of serine at the expected catalytic site, azurocidin lacks protease and peptidase activity and does not bind [³H]di-isopropyl fluorophosphate. Purified azurocidin kills *E. coli*, *Streptococcus faecalis* and *C. albicans in vitro*, showing optimal activity under acidic conditions (pH 5.5) in low ionic strength media. It displays considerable activity against deep rough mutants of *S. typhimurium* and, at 5 µg ml⁻¹, is also active against many other gram-negative bacteria, although *Proteus mirabilis*, *Proteus vulgaris* and *Serratia marcescens* are relatively resistant. Like defensins, azurocidin is a chemoattractant for T lymphocytes and monocytes. Autoantibodies to azurocidin (and other neutrophil granule proteins) are found in some patients with vasculitis.

Lysozyme

Lysozyme was described by Fleming, who noted both its wide distribution and its remarkable ability to lyse certain gram-positive bacteria, such as *Micrococcus lysodeikticus*. Lysozyme is a very cationic protein of approximately 14.4 kDa. Its enzymatic activity is directed against the β1-4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid resi-

dues, which stabilizes many bacterial cell walls. Not only is lysozyme a component of PMN, monocyte and macrophage granules, it is also found in blood plasma, tears, saliva and airway secretions.

Unlike the other major granule proteins of neutrophils, lysozyme is proportionally distributed between both azurophil and specific granules. The PMNs of certain mammalian species are relatively lysozyme deficient and leukocyte lysozyme deficiency occurs sporadically in rabbits and routinely in cattle and rhesus monkey without any apparent adverse consequences. The human lysozyme gene has been localized to chromosome 12, and its organization with four exons and three introns is homologous to the lysozyme gene in chickens as well as to the human α-lactoglobulin gene.

Other gram-positive bacteria susceptible to the lytic action of lysozyme include *Bacillus subtilis* and *Bacillus megaterium*. Most gram-negative bacteria are resistant, unless also exposed to the synergistic bactericidal activity of antibody and complement or hydrogen peroxide-ascorbic acid. This resistance has been attributed to modifications of the peptidoglycan of resistant gram-positive species and to the protective effect of the outer membrane's LPS layer in gram-negative bacteria. *C. albicans* is also killed by lysozyme, especially when incubations are conducted in solutions of very low ionic strength. There is considerable evidence that lysozyme, like cathepsin G, can exert bactericidal activity by nonenzymatic mechanisms. For example, the oral bacterium *Str. sanguis* is killed by lysozyme without lysis, and inactivation of muramidase activity by sulfhydryl reduction has no effect on its bactericidal potency. The ability of lysozyme (and lactoferrin) to bind proteins modified by advanced-glycation-end(AGE)-products may contribute to the impaired resistance of diabetics to certain infections. AGE-product binding was attributed to a single AGE-binding domain in lysozyme (and two such domains in lactoferrin), each domain containing a 17-18 amino acid, cysteine-bounded, hydrophobic loop motif. Possibly such domains exist in lysozyme and other antimicrobial molecules to enhance their ability to bind to the glycosylated surfaces of host cells or microorganisms.

Lactoferrin

Lactoferrin is a 78 kDa, slightly basic, single-chain glycoprotein that can bind one or two molecules of ferric iron. It is a principal component of the neutrophil's specific granules, but is absent from azurophil granules. Free lactoferrin is found in human serum, tears, semen, milk and other secretions. The

lactoferrin gene has been localized to chromosome 3q1–23, nearby to genes that encode transferrin, a homologous protein that is the principal iron-binding glycoprotein of serum, and transferrin receptors. Three forms of lactoferrin have been described (α , β and γ), two of which also possess RNAase activity. Intestinal cells and mononuclear phagocytes have surface receptors for lactoferrin but do not synthesize it. Certain bacteria (e.g. *N. gonorrhoeae*, *N. meningitidis* and *Moraxella catarrhalis*) also possess lactoferrin receptors, and can use these to obtain iron.

Since bacteria require iron in order to synthesize essential components such as cytochromes and iron-sulfur proteins, the tight binding of Fe^{3+} by lactoferrin and its high local concentrations may leave insufficient free Fe^{3+} to support bacterial population growth. Although the bacteriostatic effects of lactoferrin can be abolished by saturating the medium with iron, bacteria subjected to iron-free apolactoferrin are often not rescued by subsequent exposure to iron-containing growth media. In addition to microbiostatic effects mediated via iron depletion, lactoferrin can also exert bactericidal effects against many microbes. Lactoferrin or lactoferricin-treated gram-negative bacteria were noted to release LPS, develop electron-dense 'membrane blisters' and become sensitized to lysozyme – all of which suggest the causation of outer membrane damage. Lactoferrin was shown to bind with high affinity to bacterial LPS, and also binds to bacterial porins and glycosylated proteins. These properties were found to reside in the N-terminal portion of the lactoferrin molecule. Exposure of lactoferrin to pepsin (its normal fate when ingested in milk) releases an antimicrobial polypeptide, 'lactoferricin', that is also derived from the N-terminal lactoferrin residues. This peptide was reported to kill many bacteria and *C. albicans*, but was ineffective against *Proteus* and *Serratia* species and *Pseudomonas cepacia*. Lactoferrin had also been noted to inhibit the adherence to and invasion of epithelial cells by certain microorganisms, and may also contribute to host defense in this manner.

Cathelin-associated peptides

The neutrophils of pigs, cattle and sheep contain an array of structurally-diverse antimicrobial peptides, all of which are synthesized at the C-terminal portion of precursors that contain a highly conserved 'cathelin' domain, approximately 100 amino acids long. Some of these cathelin-associated peptides were predicted to have amphipathic α -helical structures, while others formed compact, defensin-like β -sheet structures that were stabilized by intramolecular cystine disulfide bonds. Other cathelin-associated pep-

tides are incredibly rich in proline or tryptophan residues, and some are cyclic. In pigs, the genes for cathelin-associated antimicrobial peptides contain four exons, the first three of which encode almost the complete cathelin domain, while exon IV specifies the last few cathelin residues (including its conserved valine cleavage site), followed by codons for the mature peptide. The highly conserved 5' flanking sequences of this gene family contained NF κ B, IL-6, GM-CSF and NF-1 binding motifs, suggesting that peptide synthesis may respond to mediators generated early during infection.

A cathelin-associated peptide called either FALL-39 or hCAP-18 has also been detected in humans. It is synthesized at the carboxy-terminal portion of a 170 amino acid, cathelin-containing precursor and it is stored in the specific granules of mature human neutrophils as a 19 kDa propeptide. Substantial amounts of mRNA for this peptide were also detected in human testis. A 39 amino acid residue peptide containing the inferred mature peptide has been synthesized, and was found to exhibit antimicrobial activity and to form a perfect amphipathic α -helix throughout much of its length.

Eosinophils

Although eosinophils are usually considered as major contributors to host defenses against helminths, they resemble neutrophils in possessing chemotactic, phagocytic and microbicidal activity. Eosinophils kill bacteria and fungi by oxidative and nonoxidative mechanisms, although the composition of their molecular armamentarium differs from that of neutrophils. For example, instead of myeloperoxidase, which catalyzes the generation of hypochlorous acid and chloramines in neutrophils, eosinophils contain a structurally distinct peroxidase that produces hypobromous acid and bromamines. The principal nonoxidative microbicidal polypeptides of eosinophils include major basic protein (MBP) and eosinophil cationic protein (ECP), both of which also kill helminths and protozoa. MBP is a small, basic and arginine-rich peptide (pI 10.9, M_r 13.8 kDa) that comprises up to 50% of the total protein in guinea pig eosinophil granules. ECP is a larger molecule (M_r 18–21 kDa) that contains a high content of arginine, proline and cystine (pI > 11). Unlike MBP, which occupies the crystalloid core of the eosinophil's distinctive cytoplasmic granule, ECP is located in the granule's matrix. ECP shows sequence homology to ribonucleases and demonstrates ribonuclease activity. ECP and MBP killed *E. coli* by similar but nonidentical mechanisms, each marked by a sequential attack on the organism's outer and inner membranes. ECP

also forms relatively nonselective pores in artificial model membranes.

Mononuclear phagocytes

Although monocytes and macrophages play crucial roles in host defense against facultative and obligate intracellular parasites, their nonoxidative mechanisms for killing intracellular organisms or inhibiting their replication are not well understood. This may be due to the inherent complexity of the mononuclear phagocyte system, interspecies differences and the multiple effects of activation, differentiation and immunoregulatory cytokines on macrophage composition and function. It may also be that macrophages follow 'just-in-time' manufacturing principles for producing antimicrobial peptides and proteins, rather than stockpiling them in cytoplasmic granules in the manner of neutrophils and eosinophils. Strong evidence supporting the existence of nonoxidative antimicrobial mechanisms has been adduced from studies with diverse organisms, including *Toxoplasma gondii*, *Trypanosoma cruzi*, *Chlamydia psittaci*, *Candida albicans*, *Aspergillus fumigatus*, *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Most macrophages contain lysozyme and type 2 phospholipase A₂, and some, such as rabbit alveolar macrophages, contain defensins. There is evidence for histone-like antimicrobial proteins in various macrophages, although their significance for antimicrobial function remains to be established. Under some circumstances, macrophage enzymes such as arginase and indoleamine 2,3-dioxygenase

may exert antimicrobial effects by depleting the environment of arginine and tryptophan, respectively. The possibility that macrophages recycle and use antimicrobial molecules initially synthesized by other cells, such as neutrophils, warrants consideration and is supported by some experimental evidence and the presence of receptors for neutrophil-derived molecules, such as lactoferrin and BPI, and the ability of macrophages to acquire neutrophil-derived molecules by pinocytosis.

See also: Degranulation; Eosinophils; Microbicidal mechanisms, oxygen-dependent; Monocytes; Mononuclear phagocyte system; Neutrophils; Phagocytosis.

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MICROENVIRONMENT

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We define 'microenvironment' as an organized, local association of cells providing an interactive, dynamic structural or functional compartmentalization. Microenvironments may promote or regulate interactions among cells and/or molecules through biologically active molecules. The influence of microenvironments may be expressed at the organ, tissue, cellular or molecular levels.

Microenvironments within organisms evolved to subserve function. The relationship between microenvironment and the functional elements are complementary. In order to understand mechanisms operative in cellular functions, it is important to be

aware of the characteristics of the environment which may influence function.

The complex cellular and molecular interactions involved in the regulation of immune responses have generally been studied *in vitro*. Frequently, it is an implicit assumption that cells perform the same roles *in vivo* as they do *in vitro*. It should be appreciated that lymphoid organs and tissues are highly organized and that this functional architecture is destroyed in the process of cell isolation. To quote Fossum and Rolstad: 'The components must ultimately be explored in their natural context... as the whole being more than the sum of

its parts', and the whole alone represents the '*in vivo* veritas'.

Examples of microenvironments in the immune system

The thymus, a primary lymphoid organ, has two main microanatomical divisions: the cortex and the medulla. Each represents a distinct microenvironment. Maturing thymocytes acquire immunological competence as they migrate from the cortex to the medulla. The thymic cortex, where thymocytes (T cells) begin their functional differentiation and proliferation, occurs in a specially protected microenvironment. Cells in the cortex are protected from foreign, antigenic influence. This microenvironmental protection is provided by the 'blood-thymus barrier'.

Secondary lymphoid tissues and organs demonstrate increasing levels of complexity in organization. This results in increased compartmentalization of cells, each compartment typically containing cells of different types and assortment, and forming a distinct microenvironment. With the exception of diffuse lymphoid tissue composed of a random distribution of lymphoid cells in loose connective tissues, a common feature of the organization of secondary lymphoid tissues is the presence of lymphoid follicles (also called nodules). These structures represent the functional building blocks of the immune system responsible for the development and maintenance of humoral immunity. Lymphoid follicles are found as solitary nodules or as aggregates (e.g. Peyer's patches) associated primarily with the mucosa of respiratory and digestive tracts. In the lymph node, lymphoid follicles are located in the cortex and, in the spleen, in the splenic white pulp.

The microenvironment surrounding lymphoid nodules may vary according to their location. For example, in Peyer's patches, one of the mucosal-associated lymphoid tissues (MALT), lymphoid follicles are intimately associated with the mucosal epithelium of the ileum. In lymph nodes, a close association exists between the lymphoid follicle and the subcapsular (or marginal) sinus (a lymphatic channel) of the lymph node. In the spleen, lymphoid follicles are surrounded by the marginal sinuses (blood sinuses). In all cases these microenvironmental associations control the access of antigens to the B cells of lymphoid follicles, but in each case by somewhat different cellular mechanisms.

In the epithelium overlying Peyer's patch follicles, specialized M cells transport antigen through an intracellular route from the intestinal lumen to B lymphocytes of the follicles for sensitization. Other

epithelial cells exert influence at the molecular level on the immunoglobulin A (IgA) produced by sensitized B cells of the MALT. For example, during the intracellular transport of the IgA antibody to mucosal surfaces by epithelial cells, an IgA-associated glycoprotein molecule termed 'secretory component' is produced by proteolytic cleavage of the epithelial cell poly-Ig receptor. Secretory component protects the IgA from break down by the lysosomal system of the epithelial cell during intracellular transport and also from the enzymes found on mucosal surfaces. The evolution of this remarkable cooperation of IgA-producing cells with epithelial cells is a good example of microenvironmental influence at the molecular level.

A lymphoid follicle is a compartment of primarily B cells which represents a unique microenvironment. Upon antigenic stimulation, lymphoid follicles develop germinal centers, which constitute the primary sites of B memory cell production. This response to antigenic stimulation is referred to as the 'germinal center reaction' and is dependent on the transport of antigen to the lymphoid follicle and on the interaction between T and B cells, which in turn depends on the unique intrinsic organization of the lymphoid follicle microenvironment. This is a dynamic environment and its organization is intimately related to one of its cellular components, the follicular dendritic cell (FDC). FDCs are nonphagocytic cells capable of trapping and retaining antigen on the surface of their dendrites in the form of immune complexes which function in the maintenance phase of humoral immunity. The functional role of the lymphoid follicle microenvironment in the development of the germinal center reaction is best illustrated by the successive cellular events leading to the production of B memory cells. As an example, we will focus on the cellular events as they occur in the lymph node (Figure 1).

The lymph node lymphoid follicles are closely associated with the subcapsular sinus (Figure 1). At its deep or paracortical poles, lymphoid follicles are in the proximity of high endothelial (postcapillary) venules draining blood from the net of capillaries surrounding each follicle. In immune animals, antigen arriving at the lymph node via afferent lymphatics enters the subcapsular sinus in the form of immune complexes. From there, antigen is transported by a cell-mediated process to the lymphoid follicle (see Figure 1). Recirculating antigen-specific clones, on arriving at the high endothelial venules in the blood, recognize this specialized endothelium and migrate through the wall of the venule to the lymphoid follicle. These recirculating lymphocytes normally leave the follicle via the lymph node medullary

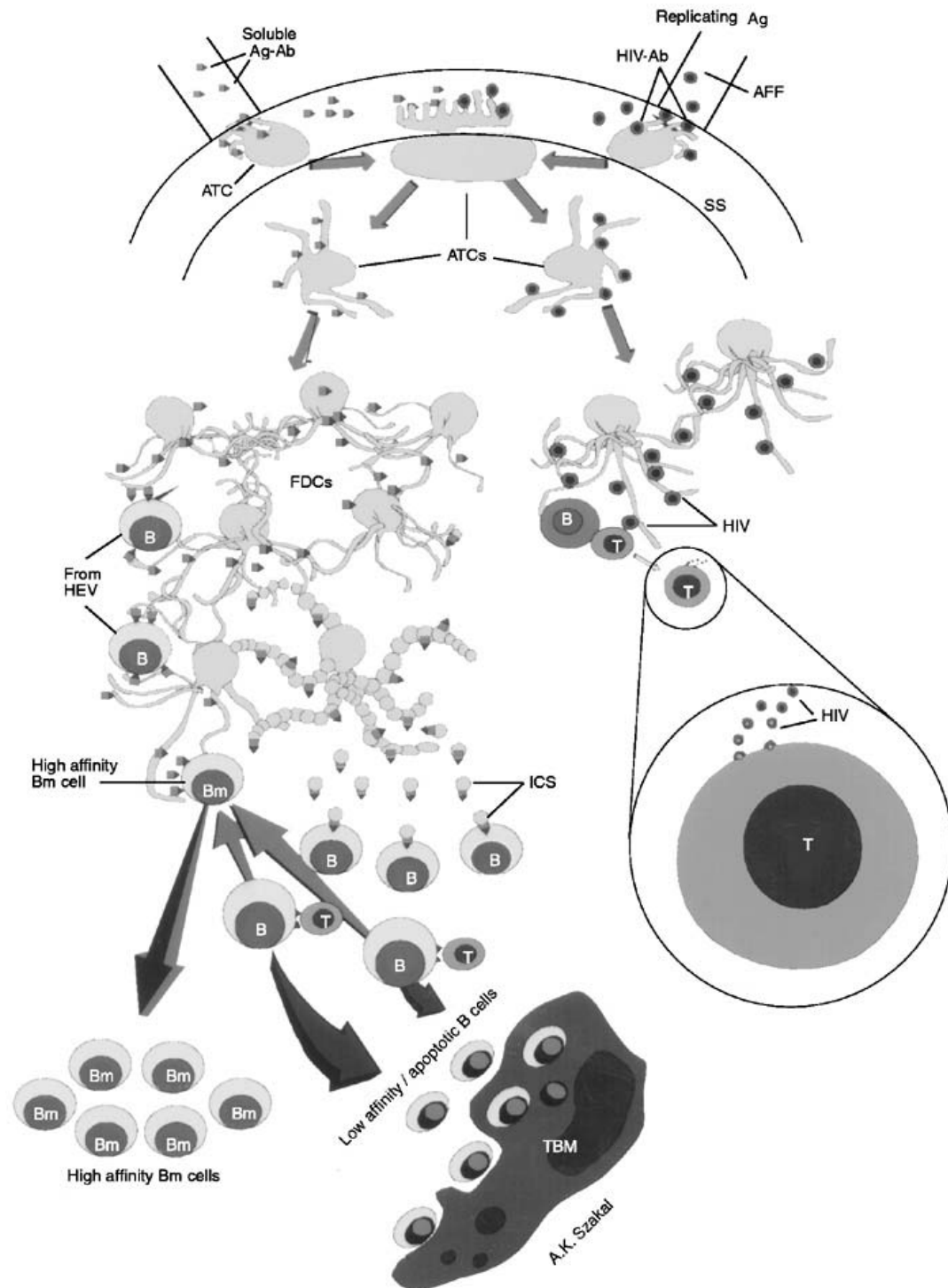


Figure 1 Development of microenvironment during the germinal center reaction. Based on current concepts, the left side illustrates the trapping of soluble immune complexes by FDCs, and the cellular events leading to the production of B memory cells in the germinal center. The right side illustrates the trapping of a replicating antigen such as the retrovirus, HIV, and the role FDCs play in the infection of CD4⁺ T cells. For interpretation of events, see text. SS, subcapsular sinus; Ag-Ab, antigen-antibody complexes; HIV-Ab, HIV-antibody complexes; ATCs, antigen-transporting cells; FDCs, follicular dendritic cells; B, B cell clone (antigen-specific B cells); HEV, high endothelial (postcapillary) venule; ICS, immune complex-coated bodies released by FDCs; T, T cell (CD4⁺); High-affinity Bm cell, high-affinity mutation progeny of B cell; Low affinity/apoptotic B cells, dying (low-affinity mutation) progeny of B cells; TBM, tingible body macrophage phagocytosing apoptotic B cells; Bm, B memory cells of germinal centers; AFF, afferent lymphatics.

cords and sinuses to return to the blood. However, the antigen-specific lymphocytes are retained in the follicle by a special cellular 'spongework', formed by FDCs. This spongework develops as a sequel to the transport of antigen from the subcapsular sinus (see Figure 1). The antigen is transported on the surface of nonphagocytic antigen-transporting cells of varying dendritic morphology. These cells are thought to be precursors of FDCs. On arrival at the lymphoid follicle, the spongework, a three-dimensional network or reticulum, is established by the FDCs intertwining their dendritic processes (see Figure 1).

This spongework is generally referred to as the 'antigen-retaining reticulum' since its component FDCs are capable of retaining antigen on their surfaces in an intact form for months to years. It is located in the region of the lymphoid nodule closest to the subcapsular sinus. The spaces within the spongework are filled mostly by B lymphocytes from the recirculating pool. Some T helper cells are also found here. According to consensus, antigen-specific B cells are removed from the recirculating pool of lymphocytes by binding to the antigen on the surface of FDCs. Thus, this spongework of FDCs forms a specialized microenvironment that brings together antigen, specific B cells, and T helper cells and provides an optimal framework for their interaction.

The antigen-retaining reticulum is a dynamic structure. It develops rapidly during the first 3 days after the introduction of antigen into the region of the animal drained by the lymph node. Electron microscope studies show that during this time the initially smooth, elongated filament-like dendritic processes of these 'octopus-like' FDCs mature to become 'beaded'. These beads or bodies, through an interaction with other FDCs, concentrate immune complexes on their surfaces to become immune complex-coated bodies or iccosomes, which are then released and dispersed in the microenvironment (see Figure 1). This process is accompanied by edema, resulting in an increase in the intercellular spaces, apparently to facilitate iccosome dispersion and cell traffic. The free iccosomes, shown to be many times more immunogenic than free immune complexes, are endocytosed by the recruited B cells. The endocytosed antigen is fragmented in lysosome-like vesicles of the B cells. These antigen fragments then become associated with the Golgi apparatus which is known to be functional in antigen re-expression on the cell surface. Functional studies show that these B cells can present the antigen efficiently to T helper cells (see Figure 1), which are also present in the microenvironment of the antigen-retaining reticulum. This process lasts for about a week. As a sequel to antigen presentation, T cell lymphokines

(interleukins) active within the microenvironment induce proliferation and maturation of B cells. This represents the initiation of germinal center development, leading to B cell memory.

Germinal center development is represented by the proliferative response of specific B cells in the lymphoid follicle which increases through day 10 after antigen exposure and then gradually declines. This proliferative expansion of B cells establishes another compartment and microenvironment. The germinal center developed is situated toward the medullary pole of the lymphoid nodule beneath the antigen-retaining reticulum of FDCs. Soon after the initiation of germinal center development, macrophages typically found in germinal centers migrate into this region. These cells are termed tingible body macrophages (TBM) on account of the stainable (tingible) remnants of phagocytosed germinal center B cells (see Figure 1). Thus, the microenvironment of germinal centers is represented by the proliferating (IgD⁺, IgM⁺) B cells and TBMs. The significance of TBMs in germinal centers was recently related to affinity maturation of B cells. The germinal center microenvironment has the features required for affinity maturation. These include: 1) availability of antigen on nearby FDCs for selection of high affinity B cells; 2) rapid proliferation of B cells allowing for expression of V gene mutations; 3) elimination of B cells in large numbers by TBMs (i.e. B cells not rescued by binding of antigen due to lack of specificity or mutation to decreased affinity for the antigen) (see Figure 1). Thus, affinity maturation could be explained in terms of the germinal center microenvironment.

We have stated that microenvironments evolved to subserve function. This function ultimately may be the elimination of specific microorganisms. Some microbes, however, have evolved through the age-old association with the defensive mechanisms of a microenvironment to subvert its protective features to their own ends. This appears to be the case with retroviruses. The trapping of retroviruses by FDCs, as antigens, was first demonstrated by Szakal and Hanna in 1967. A follow-up study, using Rauscher leukemia virus, showed that B cells were at risk of infection when coming in contact with the FDC-retained virus. That this follicular microenvironment plays a very important role in the acquired immune deficiency syndrome (AIDS) pathogenesis induced by the retrovirus, human immunodeficiency virus (HIV), was only recently realized.

Shortly after primary infection, HIV viremia occurs and the immune system responds with virus-specific cells and antibodies. Virus is complexed with antibody and trapped on the extracellular surfaces

of FDCs in much the same manner as conventional antigens (see right side of **Figure 1**). Throughout most of the disease process, HIV remains on the FDC reticulum. However, eventually the FDCs are destroyed, the lymphoid tissues involute and systemic HIV replication and marked immunosuppression ensue. In 1993, Fauci and colleagues and Haase and coworkers showed that throughout the clinically latent phase of the disease (typically lasting several years), active HIV infection is ongoing in, and virtually confined to, lymphoid follicles. It was postulated that virus on FDC might infect CD4-bearing T cells migrating into germinal centers to participate in T cell-dependent humoral responses. However, it remained to be shown that FDC-trapped HIV was infectious, since it was complexed with antibodies, including some with neutralizing activity. Recently, our group showed that HIV immune complexes on FDC are highly infectious. Even more remarkably, when HIV is complexed with very high levels of neutralizing antibodies, infection persists when FDCs are present. In contrast, the same concentrations of neutralizing antibody and virus do not cause infection in the absence of FDCs, thus supporting the importance of the follicular microenvironment. Other features of this specialized microenvironment that may also contribute to HIV pathogenesis might include:

1. the high degree of cellular activation and potent costimulatory signals present there;
2. the ability of FDCs to retain antigens and viruses for long periods in an unprocessed or 'active' form; and
3. protection from antiretroviral agents which are designed to attack replicating intracellular virus but not the extracellular (nonreplicating) FDC-associated HIV. A better appreciation of specialized microenvironments may help us in future years to devise improved intervention strategies for retrovirus-induced disease.

Microenvironments are ubiquitous in the body. They are not limited to the above examples selected to illustrate the importance of the concept of microenvironment under normal physiological and in pathological conditions. Some microenvironments are well-characterized, others are yet to be defined. The knowledge of the function of any system is inseparably bound to its microenvironment.

See also: Affinity maturation; Antigen, entry into the body; B lymphocyte, antigen processing and presentation; Bone marrow and hematopoiesis; Clonal selection; Cooperation, mechanisms of cellular; Cytokines; Germinal center; High endothelial venules; Immune complexes; Lymph nodes; Mucosa-associated lymphoid tissue (MALT); Recruitment; Spleen; Thymus.

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MINOR TRANSPLANTATION (HISTOCOMPATIBILITY) ANTIGENS

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Snell discovered the existence of minor transplantation antigens at the same time that he discovered H2, the major histocompatibility complex (MHC) of the mouse. This discovery came from experiments using the classic genetic approach of crossing,

backcrossing and intercrossing two genetically dissimilar inbred mouse strains and examining the ability of tissues from the progeny of these crosses to stimulate a graft rejection response in one of the parental strains. Another, complementary, test was

to examine the ability of the progeny to reject a tumor of parental strain origin. The results of these tests indicated the pattern of inheritance and co-dominant expression of transplantation antigens from one strain when placed by breeding on the background of the other strain.

Snell found that the strongest (hence major) transplantation antigen segregated in a Mendelian fashion as a single locus, which he named H2 because it was numerically the second one he described from these experiments. His collaborative work with Gorer established that the antibodies Gorer had made by immunization with tissues between different mouse strains were directed against Snell's H2 antigens.

However, the genetic experiments which led to the discovery and subsequent characterization of the major transplantation (or histocompatibility, as termed by Snell) antigens, H2 in mouse, also showed that there were a large number of other transplantation (histocompatibility, H) antigens independently segregating in the backcross mice. These were given numbers (H1, H3, etc.) in the order in which they were discovered. Snell and Bailey isolated each by continued backcrossing, to generate the minor H congenic strains, each of which consisted of the selected trait, the minor H antigen (as measured by grafting or tumor rejection) of one, donor strain, expressed on the genetic background of the other, recipient parental strain. A congenic strain is therefore identical with the recipient parental strain except for the chromosomal segment incorporating the donor gene whose product has been selected for.

In reality, that chromosomal segment is of variable length and contains other 'passenger' genes. Where these are polymorphic between donor and recipient parental strains, their presence allowed the chromosomal mapping of a number of minor transplantation antigen genes. It is these and other similarly derived minor H congenic strains which have established the *in vivo* definition of minor H (transplantation) antigens, and there are now several dozen of them.

The strength of each antigen can be measured by the length of time it takes to reject skin grafts exchanged between the congenic and the parental strains. The alleles of some (e.g. H1, H3, H4, H7 and H25) are quite strong and give rejection times of between 2 and 4 weeks (in comparison with H2 mismatched grafts, where the rejection time is 1–2 weeks). Generally, there appear to be a limited number of alleles of each minor H antigen and this also distinguishes them from the highly polymorphic H2 antigens. The alleles of a particular minor H antigen in different inbred strains can be determined by grafting *in vivo* in genetic complementation experi-

ments or *in vitro* using minor H-specific, MHC-restricted cytotoxic T cells (see below).

MHC-restricted responses to minor H antigens *in vivo* and *in vitro*

In the mid-1970s, several months after MHC-restricted responses to viruses by cytotoxic T cells were first reported by Zinkernagel and Doherty, two laboratories reported similar MHC-restricted cytotoxic T cell responses specific for minor H antigens. One was that of Bevan, who in secondary mixed lymphocyte culture (MLC), raised MHC-restricted responses to the multiple H antigens by which two MHC-matched inbred strains differed. The other was that of Simpson, who likewise found cytotoxic T cell responses in secondary MLC to the male-specific transplantation antigen, H-Y, to be MHC-restricted.

Subsequently, MHC-restricted minor H-specific cytotoxic T cell responses have been obtained by immunizations between minor H congenic and their recipient parental strains. Alleles of loci stimulating rapid *in vivo* responses generally give good cytotoxic T cell responses following secondary MLC *in vitro* but the strongest *in vitro* responses are obtained from MHC-matched strains differing at multiple minor H loci, presumably due to the activation of a large number of different clones of different specificities. Both Tomonari and Roopenian have isolated T cell clones specific for various minor H antigens present on the immunizing cell from murine secondary MLC and these include not only class I-restricted CD8 but also class II-restricted CD4 clones.

The work of Matzinger has shown that *in vivo* responses to minor H antigens are also MHC-restricted. There is good evidence that both *in vivo* and *in vitro* it is necessary to have concomitant activation of both CD8⁺ and CD4⁺ T cell subpopulations in order to generate either a graft rejection or a cytotoxic T cell response against minor H antigens.

As a corollary, at least two epitopes, restricted respectively by MHC class I and II molecules, and probably encoded by different genes, are necessary to stimulate *in vivo* graft rejection. This has been demonstrated in mice by genetic and molecular genetic approaches.

In humans, the genetic analysis of T cell responses to minor H antigens is much more difficult, because here we are dealing with an outbred population in which the MHC restriction elements (HLA encoded) necessary for the recognition of the minor H antigen are independently segregating, and it is likely that individuals will be heterozygous for alleles of transplantation antigens. Human families are also small and one cannot do the genetic experiments possible

in mice. However, it is quite clear from clinical interventions involving exchange of organs or tissues between MHC-matched but genetically non-identical humans that graft rejection responses include components directed against transplantation antigens encoded outside the MHC. Thus, it is still necessary to use immunosuppression for kidney grafts from HLA-matched donors and for bone marrow transplants from HLA-matched siblings which can give rise to intractable graft-versus-host disease directed against non-MHC transplantation antigens. It is from such patients that Goulmy has isolated *in vitro* HLA-restricted T cell clones with specificities that look remarkably similar to those of the minor H antigens in mouse. One such specificity is the male-specific minor H antigen, H-Y, others are five specificities named HA-1 to HA-5 whose segregation in families and in unrelated individuals expressing the appropriate HLA restriction molecules has been studied.

Molecular identification of minor H antigens

Minor H antigens in mice and humans appear to be recognized *in vivo* and *in vitro* by T cells but not antibodies. This has made it difficult to explore their molecular nature. However, over the past 6 years, initially from the work of Rammensee and colleagues, it became clear that the various epitopes of minor H antigens were peptides derived from endogenous proteins, loaded during the biosynthesis of MHC molecules into their peptide-binding grooves and then expressed at the cell surface. It is thus polymorphic differences of the proteins from which the MHC-binding peptides are derived which are recognized by T cells, in much the same way as they recognize peptides derived from viral proteins in a virus-infected cell. More recently, certain minor H peptides and their encoding genes have been identified by the use of two complementary approaches:

1. the elution of peptides from MHC molecules and
2. expression cloning using T cell clones to detect the products of transfected genes.

The peptide elution approach identified the first human minor H component, HA2, and one of the H-Y peptide epitopes in humans, whilst the expression cloning approach has identified several different H-Y peptide epitopes in mice as well as an increasing number of tumor-specific transplantation antigens in humans and mice, from the pioneering work of Boon's laboratory.

Selection of the T cell repertoire: involvement of minor H antigens

The generation of the T cell repertoire during differentiation in the thymus involves selection. T Cells which express rearranged T cell receptor (TCR) genes encoding high-affinity self-MHC plus peptide-reactive receptor molecules are removed by apoptosis (negative selection). Those which express TCR with a lower affinity for self-MHC molecules are positively selected and exported to the periphery. There they can become activated and clonally expanded by encounter with antigen-presenting cells having novel peptides, for example derived from viruses or bacteria, bound by their MHC molecules, if the novel peptide-MHC complex creates a higher affinity interaction with the TCR. Those TCR expressed in the thymus that have too low an affinity for self-MHC molecules are not selected and die 'from neglect'. It is therefore clear that MHC-binding self-peptides expressed in the thymus can play an important role in shaping the T cell repertoire and can thereby contribute to the capacity to mount effective immune responses to pathogens and certain tumors, and to the propensity to mount autoimmune responses.

Aspects of this are well-illustrated by the first T cell receptor transgenic mouse described, with a virtually monoclonal T cell population expressing a receptor specific for an H-Y epitope presented by H2D^b molecules. Female transgenic mice expressing H2D^b molecules show characteristic thymic development with generation of single positive CD8⁺ T cells from their double positive, CD4⁺8⁺ precursors and export to the periphery of CD8⁺ cells, whereas in females of H2 haplotypes lacking H2D^b, positive selection is faulty, and few thymocytes progress past the immature double positive, CD4⁺8⁺ stage. Male transgenic mice expressing the H2D^b molecule show dramatic negative selection, with apoptosis removing most of the CD4⁺8⁺ immature thymocytes, and reducing the thymus to less than 10% of the size and cellularity of that in females.

An example of non-MHC genes which may encode polymorphic proteins affecting the propensity to develop autoimmunity has been found from studies of the NOD (non-obese diabetic) strain of mice. A number of chromosomal localizations of genes predisposing to susceptibility or resistance to diabetes have been identified from the extensive backcross studies of Todd and Wicker. It is possible that some of these may act by affecting T cell repertoire selection, for example by negative selection, similar to that caused by the expression of a cross-reactive self-antigen of BALB/c (H2^d) origin causing clonal deletion and thus nonresponsiveness to the

copolymer GT, to which DBA/2 (also H2^d) are responders, but BALB/c and the F1 hybrid are not.

Pathological and physiological roles of minor H antigens

Minor transplantation antigens were discovered experimentally by skin and tumor grafting and are manifest clinically as impediments to the exchange of therapeutic grafts of MHC-matched tissues or organs between genetically dissimilar individuals. Graft-versus-host disease following bone marrow transplantation can cause severe pathology, and host-versus-graft can lead to the rejection of donor organs of tissues. It is possible that in the future the identification of immunodominant minor transplantation antigens may lead to better avoidance or treatment of these consequences of transplantation. A physiological role of minor transplantation antigens, i.e. self-peptides bound by self-MHC molecules, may be reflected by T cell repertoire selection. This however may be merely a consequence of evolutionary selection for variants of many genes involved in diverse functions, but whose protein products are sampled as MHC-binding peptides. The T cell immune system is highly evolved to detect these, as they do not differ qualitatively from peptides of viral or bacterial origin.

See also: Congenic mice; H-Y, the male-specific transplantation antigen; Inbred strains; Superantigens; Tolerance, peripheral; Transplantation.

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MIXED LYMPHOCYTE REACTION

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Historical background

Until the early 1960s small lymphocytes were regarded as short-lived resting cells of obscure function. In 1962 Gowans and his colleagues observed that purified small lymphocytes transferred from thoracic duct lymph of rats were able to transform into blast cells when injected into irradiated mice. This experiment provided the first unequivocal evidence that small lymphocytes were capable of being triggered to enter cell cycle. Two years later, independent studies by Bain and colleagues and by Bach and Hirschorn demonstrated that a mixture of blood leukocytes taken from two unrelated donors generated large numbers of blast lymphoid cells after several days in tissue culture. Bain and colleagues made the critical discovery that blast transformation failed to occur between cells from monozygotic twins. Shortly afterwards other workers demonstrated that the mixed leukocyte reaction (MLR) was demonstrable in a number of different species and that the strongest reactions occurred with disparity at the major histocompatibility complex (MHC), i.e. the HLA complex in man and the H-2 complex in mice. With the discovery that small lymphocytes were divisible into thymus-derived (T) and bone marrow-derived (B) cells, the MLR was shown to reflect the proliferative response of T cells, with few or no B cells involved as responding cells.

Technical features

When untreated lymphoid cells from two different individuals are cultured together, the resulting MLR is bidirectional. Thus, with a mixture of homozygous MHC-incompatible *a* and *b* lymphoid cells, the response involves *a* T cells reacting to *b* alloantigens plus *b* T cells responding to *a* antigens. In accordance with the classic laws of transplantation immunity, only *a* T cells proliferate when homozygous *a* lymphoid cells are cultured with heterozygous (*a* × *b*)F₁ cells. Bidirectional responses occur with a mixture of (*a* × *b*)F₁ and (*a* × *c*)F₁ cells.

To ensure that the MLR is unidirectional, one of the two populations of lymphoid cells is generally treated with mitomycin C (an inhibitor of DNA synthesis) or subjected to irradiation. Such treatment of the stimulator population destroys or inactivates T cells in this population but does not interfere with

the immunogenicity of the treated cells. As discussed below, the stimulatory function of lymphoid cells is restricted to specialized antigen-presenting cells (APCs). These cells are highly radioresistant and do not divide in culture.

As with any assay system conducted *in vitro*, the magnitude of the MLR depends critically upon the quality of the tissue culture conditions used. To obtain high responses it is essential to use fully viable cells and a tissue culture medium containing all essential nutrients; addition of serum (1–10%) and 2-mercaptoethanol is usually found to be critical. As responder cells, any population containing a high proportion of T cells can be used. Buffy coat white blood cells are employed as responders (and stimulators) for MLR in humans. For rodents, lymph node (LN) cells give near-optimal data, although most investigators tend to use spleen cells. MLRs with spleen cells, although adequate, are lower than with LN cells (because of the lower content of T cells in spleen) and are often associated with high background responses, i.e. responses with syngeneic stimulators. The best results are obtained with purified T cells as responder cells.

Typical MLRs are usually conducted in 96-well plates in 200 µl volumes, using 5×10^4 to 2×10^5 responder cells and 5×10^5 allogeneic versus syngeneic stimulator cells. Proliferative responses are quantitated by adding [³H]thymidine (³HTdR) (1 µCi per well) to the cultures after 1–6 days and harvesting the cultured cells 6–24 hours later to measure ³HTdR incorporation. With low doses of responder cells, e.g. 5×10^4 , responses generally reach peak levels on day 6 or later. With higher doses of responders, peak responses occur earlier.

For rodents, the magnitude of the MLR depends critically upon the health of the animal colony. Rodents with chronic viral infections generally give rather low MLR, probably because of toxic cytokines or other mediators released by infected macrophages. In this situation, addition of the prostaglandin inhibitor, indomethacin, to the culture often improves the results.

Target antigens

Primary MLRs are directed almost exclusively to gene products of the MHC. The MHC encodes two broad classes of cell surface molecules, termed class

I and class II. Class I molecules are expressed on virtually all cell types. Class II molecules, by contrast, show a much more limited distribution with high expression restricted to B cells, macrophages and dendritic cells; in humans, class II molecules are expressed on T cells, but only after activation. Both classes of MHC molecules show extensive polymorphism. According to the literature, class II alloantigens provide a much stronger stimulus for primary MLRs than class I alloantigens. As discussed later, however, it has recently become apparent that class I alloantigens can elicit strong MLRs under certain conditions.

The mouse provides the one exception to the rule that MLRs are directed almost exclusively to MHC alloantigens. In this species, very powerful MLRs can be stimulated by Mls determinants. These antigens display limited polymorphism and represent endogenous superantigens encoded by mouse mammary tumor virus (mtv) genes. Only a small proportion of mtv antigens elicit strong primary MLRs; these particular mtv antigens are termed Mls antigens. Since mtv antigens bind to T cell receptor V_{β} epitopes, MLR to mtv antigens are V_{β} specific. It should be noted that H-2 and Mls molecules are unrelated and are encoded on different chromosomes. To date, Mls molecules have not been described in other species.

For MHC alloantigens, there is increasing evidence that MLRs are not directed to MHC epitopes *per se* but to a broad array of self peptides bound to MHC molecules. Most of these peptides are presumably derived through breakdown of various intracellular proteins involved in cell metabolism.

Precursor frequency of participating T cells

The precursor frequency of unprimed T cells responding to typical non-MHC antigens, e.g. viral or foreign protein antigens, is extremely low (≤ 1 in 10^5). Responses to MHC alloantigens, by contrast, involve a very high proportion of T cells. Working with rat lymphocytes in the late 1960s, Wilson, Blyth and Nowell estimated that 2–3% of unprimed T cells responded to stimulator cells from an MHC-different rat strain. Subsequent studies of others with mice gave similar data.

In view of the extensive polymorphism of MHC molecules, it is probable that the vast majority of T cells have the potential to display alloreactivity for at least one of the many different MHC alloantigens expressed in the species as a whole. In support of this idea, many T cell clones with defined self MHC-restricted specificity (specificity for antigen com-

plexed to self MHC molecules) have been shown to display dual reactivity for particular MHC alloantigens. Since T cells recognize conventional (non-MHC) antigens and MHC alloantigens via the same antigen-specific $\alpha\beta$ T cell receptor (TCR), these two classes of antigens are presumed to express cross-reactive epitopes. Why T cells have such a high precursor frequency for MHC alloantigens, however, is still something of a mystery.

Ontogeny

As with responses to other antigens, the response of T cells to MHC alloantigens is limited to mature lymphocytes. Most thymocytes are immature, and MLRs with unseparated thymocytes are 20–50-fold lower than with extrathymic T cells. The limited response observed with thymocytes is restricted to the minor (5–10%) subset of mature thymocytes. Mature T cells are not evident in the thymus until relatively late in development (just before birth in mice) and early thymocytes are incapable of giving an MLR. Some immature thymocytes do have specificity for MHC alloantigens (and self antigens) but these cells are highly susceptible to tolerance induction when exposed to these antigens. As first shown for skin graft rejection by Medawar and colleagues, exposure of early T cells to MHC alloantigens results in a state of specific immunological tolerance: T cells specific for the MHC alloantigens are deleted in the thymus, with the result that the T cells exiting from the thymus are unable to produce an MLR to these antigens. A similar mechanism causes T cells to show unresponsiveness to self MHC molecules.

Response of $CD4^+$ versus $CD8^+$ cells

The expression of $CD4^+$ and $CD8^+$ cell surface 'accessory' molecules divides mature T cells into two functionally distinct subsets. Whereas most thymocytes express both CD4 and CD8 molecules, the expression of these molecules on mature extrathymic T cells is mutually exclusive. CD4 and CD8 molecules augment T cell signaling by focusing src family kinases such as $p56^{lck}$ in the vicinity of the TCR. In addition, CD4 and CD8 molecules bind to nonpolymorphic sites on MHC molecules. Such binding is believed to strengthen the interaction of the TCR with the polymorphic (antigenic) sites on MHC molecules. The binding specificity of CD4 and CD8 molecules is MHC class specific. Thus CD4 molecules bind selectively to class II molecules, whereas CD8 molecules bind to class I molecules. As discussed below, the MHC class specificity of CD4 and CD8 molecules strongly influences the allospecificity of T cells bearing these molecules.

When monoclonal antibodies (mAbs) specific for CD4 versus CD8 molecules became available about 15 years ago, it became possible to prepare highly purified populations of CD4⁺ and CD8⁺ cells. Testing the MHC class specificity of these T cell subsets is most easily addressed by using stimulator cells expressing only one class of alloantigen, e.g. class II alloantigens but not class I antigens. The mouse is ideal for such studies as a wide variety of H-2 recombinant and H-2 mutant strains are available. For example, to examine responses directed selectively to H-2 class II alloantigens one can employ the combination of C57BL/6 (B6) (H-2^b) and bm12; these two strains are identical except for three amino acid differences in the I-A^b (class II) molecule. Similarly, the combination of B6 and the class I (K^b) mutant bm1 can be used to study responses directed selectively to class I alloantigens. When B6 T cells are separated into subsets and tested for MLR against bm12 versus bm1 stimulator cells (Figure 1), B6 CD4⁺ cells give high MLR to class II-different bm12 stimulators but almost no response to class I-different bm1 stimulators. Conversely, B6 CD8⁺ cells respond very strongly to bm1 stimulators but give negligible responses to bm12. These data indicate that the alloreactivity of CD4⁺ and CD8⁺ cells shows stringent MHC class specificity, with CD4⁺

cells controlling anti-class II responses and CD8⁺ cells accounting for anti-class I responses.

The evidence that purified CD8⁺ cells can produce a primary MLR deserves comment as the literature is entrenched with the dogma that the response of CD8⁺ cells depends critically upon the presence of cytokines derived from CD4⁺ cells. This view is clearly untenable in the B6 anti-bm1 response because the MLR of B6 CD8⁺ cells responding to bm1 stimulators is not affected by adding CD4-specific mAb to the cultures or by depleting the stimulators of T cells (a possible source of cytokines). It should be stressed, however, that the capacity of murine CD8⁺ cells to produce primary MLRs in the absence of added cytokines shows considerable strain variation. Thus, whereas some strains (e.g. B6) produce very high responses, other strains (e.g. DBA/2) give substantially lower responses. Similar findings apply with rats. The reason for this strain variation is unclear. In humans, MLR by purified CD8⁺ cells tend to be quite low unless purified dendritic cells are used as APCs. It is worth noting that MLRs by CD8⁺ cells characteristically show abbreviated kinetics and can be missed unless early time points are examined. Responses generally reach a peak on day 3–4 of culture and fall to background levels by day 6. The abrupt decline in the MLR by CD8⁺ cells may reflect the responder cells rapidly differentiating into cytotoxic cells which then destroy the APC, thereby removing the stimulus for the MLR. Alternatively, the decline in the MLR may be a manifestation of rapid consumption of interleukin 2 (IL-2) by the responder cells.

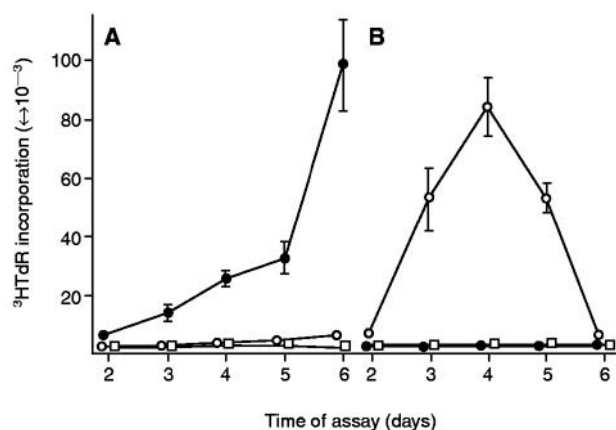


Figure 1 Time response of primary MLR by small doses (1×10^5) of B6 CD4⁺ cells (A) or B6 CD8⁺ cells (B) responding to T cell-depleted (Thy-1⁻) irradiated (1500 rad) spleen stimulators (5×10^5) from class I-different (B6 × bm1)F₁ (○), class II-different (B6 × bm12)F₁ (●) or syngeneic B6 (□) mice. T cell subsets were prepared by a combination of MaB plus complement treatment followed by positive panning at 4°C; under the conditions used the T cell subsets do not pick up antibody during the panning procedure, i.e. CD8⁺ cells are not coated with anti-CD8 mAb. The data show the mean responses of triplicate cultures; ³HdR (1 mCi ml⁻¹) was added 18 hours before harvest. It is evident that B6 CD8⁺ cells respond only to bm1 stimulators whereas CD4⁺ cells respond only to bm12 stimulators; note that the MLR by CD8⁺ cells peaks early and falls to background levels by day 6. (Data from Sprent and Schaefer (1989).)

The syngeneic (autologous) MLR

By definition, MLRs to MHC alloantigens are calculated with respect to the background response, i.e. the proliferative response to autologous or syngeneic stimulators. With unseparated T cells as responders, the proliferative response to self stimulators is usually quite low, although responses can reach appreciable levels late in culture. With purified T cell subsets as responders, the syngeneic (auto) MLR is far higher with CD4⁺ cells than CD8⁺ cells. Indeed, in the later stages of the response, the auto-MLR by CD4⁺ cells can approach the allo-MLR. The critical question of why CD4⁺ cells give a high auto-MLR in culture has yet to be resolved. In particular, it is still uncertain whether the auto-MLR reflects incomplete induction of self tolerance or simply a tissue culture artifact (e.g. a response to antigens or mitogens in the serum supplement).

Nature of APCs for MLRs

For sensitized T cells responding to MHC alloantigens, a number of different cell types such as macrophages (Mφs), dendritic cells (DCs) and B cells express APC function. Which of these cell types have APC function for unprimed T cells is controversial. One school of thought holds that unprimed T cells can be stimulated by only one type of cell, namely DCs. Others argue that, although DCs do express extremely potent APC function for unprimed T cells, significant APC function can also be provided by Mφs and B cells. For CD8⁺ cells, some workers (this writer) maintain that many different cell types, including various class II-negative cells, can express potent APC function for MHC alloantigens. Others refute this view and attest that only DCs can stimulate CD8⁺ cells.

The resolution to the issue of which particular cell types express APC function for unprimed T cells is likely to hinge on defining the precise signals required for T cell activation. In this respect, it is now well established that primary T proliferative responses require costimulatory signals delivered through T cell contact with molecules such as B7 (B7-1, B7-2) and ICAM-1 on APCs; these molecules are expressed at a particularly high level on DCs. Costimulation boosts signaling via the TCR and is crucial for the production of growth factors such as IL-2. Although costimulation is probably important for all primary responses, recent work suggests that the requirement for costimulation is less stringent for high-affinity T cells than for low-affinity cells. This could explain the different findings on the role of DCs in MLRs. Thus, with their high expression of costimulatory molecules, DCs could be crucial for stimulating T cells with low-to-average affinity for

antigen, whereas other cell types could be sufficient for stimulating high-affinity T cells.

Biological significance

The MLR is merely one of many techniques that can be used to quantitate the response of T cells to alloantigens. The limitation of the MLR is that it measures only one step of T cell induction, i.e. proliferation. To study the *differentiation* of T cells responding to alloantigens one has to use other techniques, e.g. the generation of cytotoxic lymphocytes, lymphokine production, etc. Nevertheless, the MLR is a relatively easy and inexpensive assay and this technique has produced a great deal of invaluable basic information on various aspects of the immune response, including the nature of the antigens recognized, the specificity of CD4⁺ and CD8⁺ cells and the features of APCs. The usefulness of the MLR is likely to continue for many years.

See also: H2 class I; H2 class II; Immune response *in vitro*; MHC, functions of; MHC restriction; Superantigens; T lymphocytes.

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MOLECULAR MIMICRY

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The term 'molecular mimicry' was introduced in 1964, by Damian, to describe antigen sharing between parasite and host, as a way for the parasite to avoid destruction by the immune processes of the host. In the 1980s and 1990s, the term has acquired a more specific significance, in that it refers to antigens shared between an immunocompetent host and an external agent, and this leads in some cases to

pathological consequences whereby antibodies produced against the external agent will bind to the self antigens of the host and cause inflammatory damage. Rheumatic fever, ankylosing spondylitis and rheumatoid arthritis are three diseases that could be considered to provide good examples of this type of molecular mimicry.

Rheumatic fever

It has been known since the 1920s that there is a genetic element in the incidence of rheumatic fever, in that the disease is more prevalent in individuals having blood groups A and B than in those with blood group O, and Glynn has pointed out that this observation has been made in many different countries.

Rheumatic fever is a disease where the etiological mechanism has been worked out. *Streptococcus pyogenes* microorganisms infect the upper respiratory tract and, 2–3 weeks later, a generalized serum sickness-like disease develops, with fever, arthritis, sometimes chorea and carditis. Kaplan and Meyeserian demonstrated in 1962 that the damage is caused by crossreactive antibodies, which attack the cardiac valves, because self antigens in cardiac tissues resemble the antigens found in *S. pyogenes*. So here is an example of an autoimmune disease that has been triggered by an infection, because there is molecular mimicry between the human host and the bacterial infectious agent.

All blood groups have a common precursor, the H gene, which gives rise to the H substance. Individuals with blood group O express in their red cell membrane the H substance, which has a terminal carbohydrate residue (L)-fucose. Watkins, in 1966, showed that individuals with blood group A have another gene, whose gene product is a glycosyl-transferase, which adds N-acetyl-(D)-galactosamine to the preterminal galactose of the H substance. Thus individuals with blood group A have two terminal residues, N-acetyl-(D)-galactosamine and (L)-fucose. Blood group B individuals have a gene which adds (D)-galactose to the preterminal galactose of H substance, giving rise to two terminal residues, (D)-galactose and (L)-fucose. In examining the Haworth formulae of these sugars, the substitutions at every carbon atom are different between (L)-fucose and N-acetyl-(D)-galactosamine, except for the connecting first carbon atom. Heymann and colleagues showed in 1964 that the terminal sugar residue in streptococci is N-acetyl-(D)-glucosamine, which is attached to the backbone of a rhamnose polymer. The only difference between N-acetyl-(D)-glucosamine and N-acetyl-(D)-galactosamine is the hydroxyl group, which is in a different orientation on the C-4 atom of galactose. Thus, other things being equal, blood group A resembles the streptococcal antigen more than blood group O. Similarly with blood group B, where (D)-galactose is structurally closer to N-acetyl-(D)-glucosamine of *S. pyogenes* than (L)-fucose of blood group O. Therefore any antibodies produced in blood group A individuals, following infection

with *S. pyogenes*, will bind to tissue antigens containing blood group A substance and cause reactive inflammation.

Goldstein and coworkers showed in 1969 that cardiac tissue contains molecular groups similar to blood group A substance. Thus antistreptococcal antibodies should bind to such crossreacting antigens, explaining the higher frequency of rheumatic fever in individuals possessing such blood groups. It would appear that molecular mimicry provides an explanation for the higher frequency of rheumatic fever in individuals with blood groups A and B compared with individuals with blood group O. Zabris-kie and colleagues have extended this concept and showed that the chorea of rheumatic fever can also be explained by a molecular mimicry model between streptococcal antigens and basal ganglia.

Ankylosing spondylitis

Ankylosing spondylitis (AS) is a chronic inflammatory arthritis, characterized by exacerbations and remissions, affecting primarily the axial skeleton and the sacroiliac joints. In 1973 it was demonstrated by two independent groups that the class I major histocompatibility complex (MHC) molecule HLA-B27 was present in over 90% of patients with AS, while its frequency in the general Caucasian population was of the order of 8%. Clearly the HLA-B27 molecule was somehow involved in the development of the disease, but there are many healthy individuals who possess HLA-B27 but do not develop the disease.

Studies using identical twins showed that when one of the twins had AS, the chance or concordance rate of the other twin developing AS was well below 50%. A low concordance rate in identical twin studies clearly suggests the interaction of an environmental factor acting in conjunction with a genetic factor to produce the disease.

Any theory proposed as a solution to the causation of AS must also explain why individuals possessing particular antigens, such as HLA-B27, have a greater chance of developing the disease. Currently two main theories have been proposed to explain the association of HLA-B27 with AS. The two theories are the receptor theory and the molecular mimicry theory.

The receptor theory states that the HLA-B27 molecule acts as a receptor cavity for some, as yet undefined, external polypeptide, which then somehow increases the susceptibility to develop the disease. No such polypeptide has yet been described. Until such a polypeptide is identified, the validity of the receptor theory will remain untested. The molecular mimicry theory is so far the only theory that has provided a

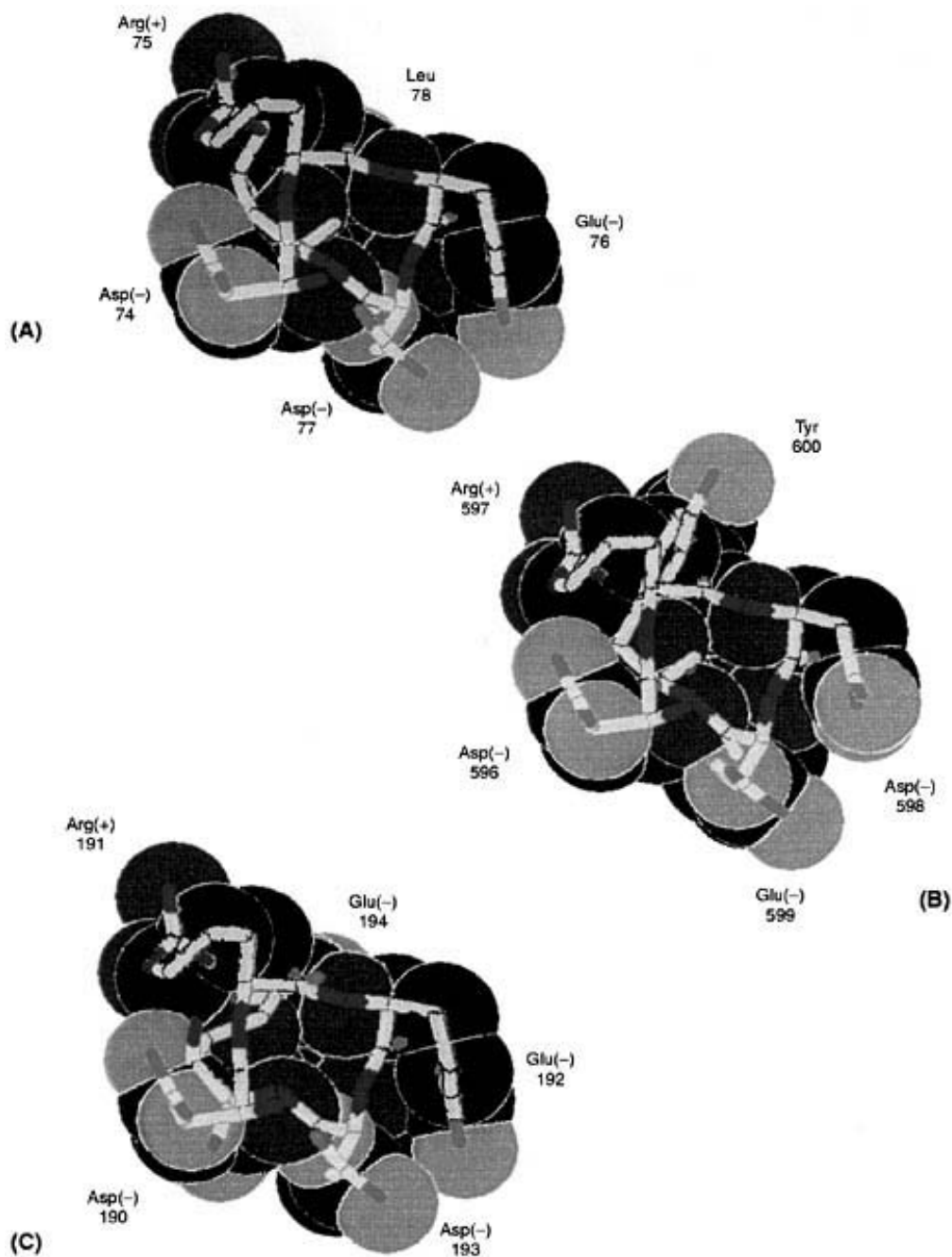


Figure 1 Comparison of space-filling models. (A) HLA-B27.5 (B*2705) (DREDL) predicted from known crystallographic structure; (B) *Klebsiella pneumoniae* pulD secretion protein (DRDEY); (C) *Klebsiella pneumoniae* nitrogenase enzyme (DREDE). (Reproduced with permission from Dr M Fielder, PhD thesis (1995). Molecular modeling by Dr C Etteläe.)

specific etiological agent which may be involved in the causation of AS.

Molecular mimicry and AS

The molecular mimicry theory states that the HLA antigen has a sequence that resembles biochemically and immunologically a similar sequence, located in

some environmental agent, such as a bacterium or a virus. The molecular mimicry model was first proposed by Ebringer, in 1976, at the HLA and Disease Congress, which was held in Paris. Sera obtained from rabbits immunized with HLA-B27-positive lymphocytes reacted with a number of gram-negative bacteria, such as *Klebsiella*, *Enterobacter*, *Salmon-*

ella and *Yersinia*. A search for these microbes in fecal samples obtained from AS patients during active phases of the disease revealed that only *Klebsiella* could be isolated from such patients.

In 1980, Avakian and coworkers reported that allogeneic, anti-HLA-B27 tissue typing sera showed increased binding for *Klebsiella* antigens using three different techniques: radiobinding assay, radioimmunoassay and hemagglutination. Van Bohemen later showed that an anti-HLA-B27 monoclonal antibody (M1), produced by Grumet, reacted with *Klebsiella*, *Shigella* and *Yersinia* strains. Kono and coworkers described an anti-*Yersinia* monoclonal antibody which reacted with 12 out of 12 B27-positive lymphoblastoid cell lines, but with only 4 out of 31 B27-negative ones. However, three out of the four reactive B27-negative cell lines carried HLA-B7, an antigen that crossreacts with HLA-B27. They then went on to show that a second anti-HLA-B27 monoclonal antibody (M2), again produced by Grumet, bound specifically to a 70 kDa component of *Klebsiella*, while no such reactivity was demonstrated by five other monoclonal antibodies. In 1987, Schwimmbeck, Yu and Oldstone identified a hexamer sequence, Gln-Thr-Asp-Arg-Glu-Arg (QTDRED), which was present in amino acid residues spanning positions 188–193 of the nitrogenase enzyme found in *Klebsiella pneumoniae* and the same sequence also occurs on the outer lip of the $\alpha 1$ domain of the main Caucasian HLA-B27 molecule. Furthermore antibodies to this shared sequence were found in the sera of AS patients.

Fielder and coworkers, in 1995, identified a second molecular complex in *Klebsiella* microorganisms which showed crossreactivity with self tissues. Molecular mimicry was shown to be present between two sequences of *Klebsiella pneumoniae*: pulD secretion protein (DRDE) with HLA-B27 and pulA (pullulanase) enzyme (Gly-X-Pro) with types I, III and IV collagen respectively (Figure 1). Furthermore, antibodies to both pullulanase and types I and IV collagen were observed in AS but not rheumatoid arthritis patients when compared with controls. It is relevant to note that type I collagen is present predominantly in tendons and bone. Antibodies cross-reacting between pulA and type I collagen could explain the deposition of fibrous tissue in the axial skeleton, thereby accounting for the main pathological feature of this disease, namely its spinal manifestations. Type IV collagen has been reported to be present in the basement membrane of uveal tissues but it remains to be determined whether crossreactivity with *Klebsiella* pulA is involved in the uveitis found in AS patients.

The molecular mimicry between HLA-B27 and

Klebsiella bacteria isolated from AS patients has been defined down to the level of similarity of four amino acids, found in both the suspect bacteria involving two separate molecules and the genetically susceptible population. Thus the term 'molecular mimicry' could not be said to be inappropriate when studying crossreactivity between bacterial antigens and HLA molecules. Since both the bacteria and antibodies to the bacteria have been demonstrated in AS patients, maybe molecular mimicry could provide a pathogenetic model for the causation of the disease.

Molecular mimicry and pathology of AS

The identification of a shared tetramer sequence between HLA-B27 and *Klebsiella* has confirmed the molecular mimicry model.

If there is crossreactivity between HLA antigens and bacteria, then infection by such microorganisms will lead to the production of antibodies, which will have both antimicrobial as well as anti-self or autoimmune activity. Only a small proportion of the subset of the antimicrobial antibodies will also have anti-self or autoimmune activity. Those bacterial antigens carrying the shared sequences will be immunogenic, especially around the edges of the shared sequence, because it is at these sites that the immune system will not recognize that it is dealing with a self antigen.

If, for example, the bacterial shared sequence is spanning residues 188–193 of the nitrogenase molecule, then the edge antigenic pentamer 187–188–189–190–191 will not be recognized as a self antigen because of the haptenic effect of the nonself amino acid at position 187, and yet the antibody produced may well bind to HLA-B27.

When present in sufficient quantities, such antibodies might activate the complement cascade and cause cytotoxic death with consequent stimulation of the inflammatory cascades, which will eventually result in localized tissue damage. There is thus no breakdown of tolerance and the evocation of bacterial antibodies having also anti-self or autoimmune activity is part of the normal immune response when encountering partially crossreacting antigens.

A classical example of this process, where one haptenic amino acid can cause autoimmune disease, is shown by autoimmune hemolytic anemia, which occurs in hypertensive patients treated with α -methyldopa. Yet the process is not self-sustaining, because once the drug is withdrawn the autoimmune hemolytic anemia subsides. A similar mechanism may be considered to occur in AS and the pathogenetic process can be illustrated as follows:

1. *Infection* occurs in the ileum and ascending

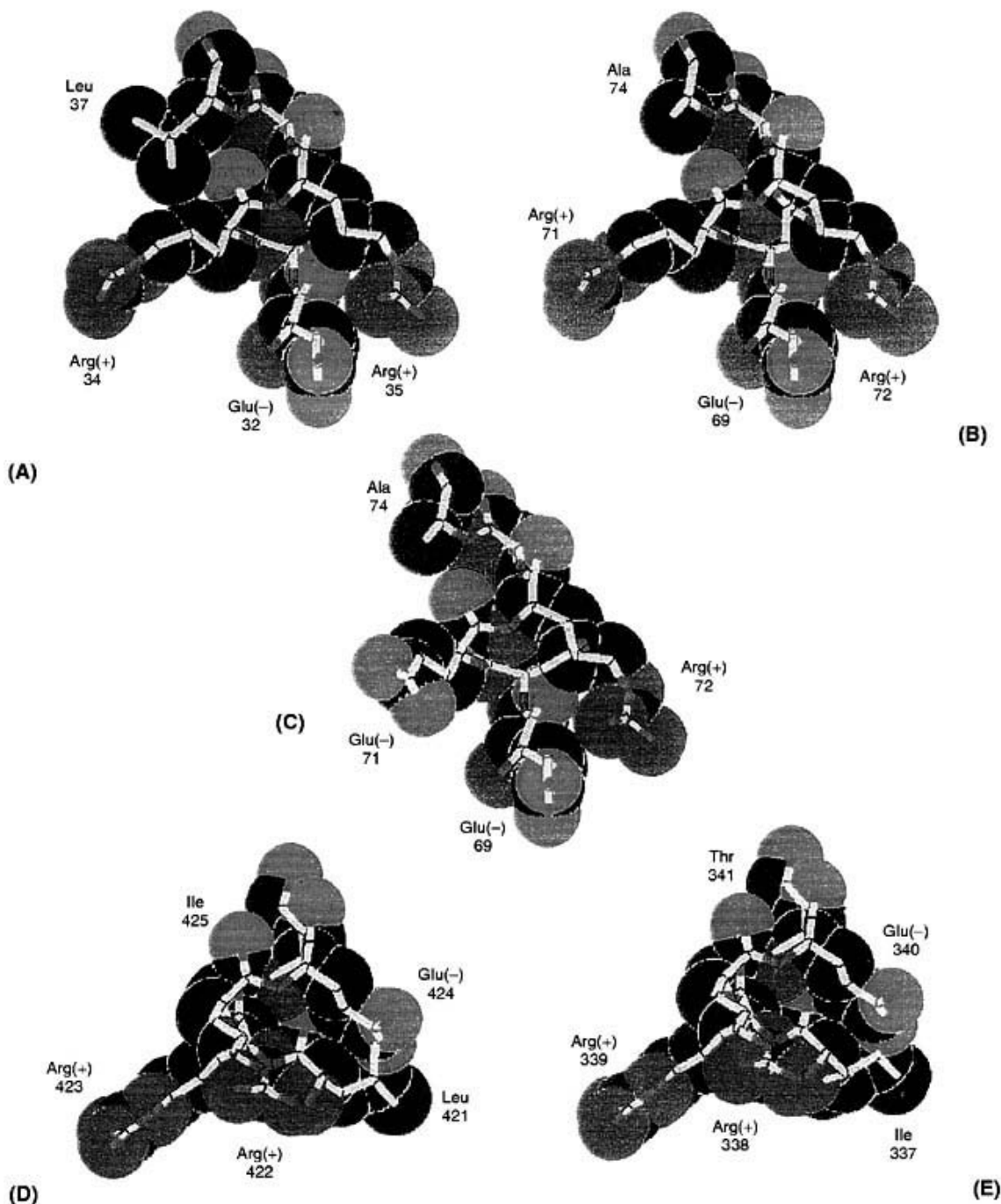


Figure 2 Comparison of space-filling models. (A) ESRRAL sequence of *Proteus mirabilis* hemolysin predicted from known crystallographic structure; (B) EQRRAA sequence within DRB1*0101 (HLA-DR1), predicted from known crystallographic structure; (C) EDERAA sequence of DRB1*0402 (HLA-DR4/Dw10); (D) predicted LRREI sequence of $\alpha 2(XI)$ collagen; (E) predicted IRRET sequence of *Proteus mirabilis* urease. (Reproduced with permission from Wilson *et al* (1995). Molecular modelling by Dr C Ettelaie.)

colon, where the bacteria replicate; their quantity will depend on the dietary intake of the patient. Elegant ileocolonoscopy studies by Mielants have shown that over 50% of active AS patients have histological evidence of gut inflammation. This infection is at a different site

from the eventual location of the arthritic lesions in AS.

2. *Antibody* against *Klebsiella* is produced in the lymph nodes draining the lower ileum and colon; the nodes are located in the mesentery which is attached to the anterior aspect of the lumbar

spine and sacroiliac joints. Elevated levels of antibodies to *Klebsiella* and to no other bacteria were first reported by Trull and coworkers and subsequently confirmed by other groups. Thus a high concentration of anti-*Klebsiella* antibodies is being produced by lymph nodes that are anatomically or geographically located near the main pathological sites in AS, namely the lumbar spine and sacroiliac joints. A subset of these antibodies will bind to cells carrying HLA-B27.

3. *Inflammation* will be triggered by those anti-*Klebsiella* antibodies binding to HLA-B27 molecules and, because of complement activation, cell death and tissue damage will occur. The end-result of inflammation is fibrosis and this is the main pathological lesion of AS.
4. *Chronic disease* will supervene because repeated cycles of infection will produce recurrent waves of anti-*Klebsiella* antibodies, which will cause tissue damage. Eventually this will manifest itself as a generalized fibrosis around the lumbar spine and we call this disease 'ankylosing spondylitis'.

Is there any evidence which suggests that the pathological processes in AS occur through such a molecular mimicry mechanism? In 1989, Husby and coworkers showed that rat antisera raised against *Klebsiella* nitrogenase enzyme antigens containing the shared QTDRED hexamer reacted with 13 out of 14 synovial biopsies obtained from HLA-B27-positive AS patients but with none out of seven biopsies obtained from HLA-B27-negative individuals suffering from either rheumatoid arthritis or osteoarthritis.

The following year, Baines and coworkers showed that rabbit anti-*Klebsiella* sera bound to a greater extent to lymphocytes obtained from 30 B27-positive AS patients and 21 B27-positive healthy controls compared with the binding by lymphocytes from 37 B27-negative healthy controls. Finally, Ewing and coworkers demonstrated, using synthetic peptides, that higher antibody activity was found around the edges of the shared hexamer sequence.

Since anti-*Klebsiella* antibodies are found in AS patients and since xenogeneic anti-*Klebsiella* antibodies bind preferentially to HLA-B27-positive cells, it is not too speculative to suggest that in the presence of high antibody titers the characteristic lesions of AS may arise. One obvious way of testing this theory is to eliminate the antibacterial antibodies and determine whether further pathological damage can be stopped.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis affecting predominantly the hands and feet but it can involve almost any joint. The mean age of onset is later than in AS, usually in the forties, but it can occur at any age. It is three times as common in women as men and any model proposed to explain this disease must account for this characteristic feature.

RA occurs predominantly in individuals carrying HLA-DR1, some subtypes of HLA-DR4 (DW4, DW14 and DW15) and HLA-DR6 (DW16), but not in individuals carrying some other HLA-DR4 subtypes (DW10 and DW13). An examination of these susceptible HLA sequences demonstrated that the common denominator was the sequence QRRAA, present on the DR β 1 chain, encoding positions 70–74. The sequence closely resembles that found in HLA-DR4(DW4) individuals where there is a conservative substitution at position 71 from arginine to lysine.

In 1992, Ebringer and coworkers showed that there was molecular mimicry between the RA 'susceptibility sequence' EQRRAA and the sequence ESRRAL found in *Proteus* hemolysin. Furthermore, elevated titers of specific antibodies to *Proteus mirabilis* in RA patients have been demonstrated by several groups. Wilson and coworkers went on to show that two separate sequences of *Proteus mirabilis* showed molecular mimicry with human self antigens: ESRRAL of *Proteus* hemolysin with EQRRAA of the HLA-DR1/DR4 susceptibility sequence and IRRET of *Proteus* urease (positions 337–341) with LRREI of α 2 chain of type XI collagen (positions 421–425) (Figure 2). Type XI collagen is a component of hyaline cartilage frequently found in small joints and since erosions are a common characteristic of this disease, it could explain the anatomical locations of these lesions. Furthermore, specific antibodies were found, in RA patients, against the ESRRAL sequence of the susceptibility sequence DR1/DR4 but not against the EDERAA sequence found in HLA-DR4(DW10), a subtype not associated with this disease. Finally, RA patients had elevated titers of specific antibodies to purified *Proteus* hemolysin and urease when compared with AS patients and healthy controls. In a coded study involving 89 subjects, Wilson and coworkers found that *Proteus* could be isolated more frequently from the urine of RA patients attending an outpatient clinic compared with controls. A correlation was found between the anti-*Proteus* antibody titer and the number of colony-forming units grown from the urine specimens.

By 1997, specific elevations of anti-*Proteus* antibodies had been reported from ten different countries throughout the world and the perception was emerging that this microbe is a strong candidate for the etiological agent of this severe arthritic condition.

Conclusion

Molecular mimicry has been defined down to the level of shared carbohydrate and amino acid residues in three important diseases: rheumatic fever, ankylosing spondylitis and rheumatoid arthritis. It is possible that a similar mechanism may operate in other human and animal diseases.

See also: ABO blood group system; Cardiac disease, autoimmune; *Klebsiella*, infection and immunity; MHC disease associations; *Proteus*, infection and immunity; Rheumatoid arthritis, human; Rheumatological disorders; *Streptococcus*, infection and immunity.

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MONOCLONAL ANTIBODIES (mAbs)

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Polyclonal antiserum raised against any individual molecular antigen consists of an assortment of antibodies of a variety of classes binding to different epitopes on the antigen with a diverse range of affinities, the proportion varying from animal to animal and within the one animal from bleed to bleed. In monoclonal antibody (mAb) production, the cell producing a single one of these antibodies is selected and propagated in large amounts. This gives a standard reagent

available indefinitely in large quantities. The cell line which makes such an antibody is called a B cell hybridoma. The relevant techniques are conceptually simple and involve the fusion of a B lymphocyte secreting antibody of the desired specificity with a myeloma (plasmacytoma) line which has the capacity for indefinite growth and division. The resultant hybrid then carries the antibody-secreting capacity of one parent and the immortality of the other.

The first production of monoclonal antibodies was reported by Kohler and Milstein in 1975 and led to the award of the 1984 Nobel Prize to these two scientists together with Neils Jerne.

Methods of making monoclonal antibodies

General

While the generation of a mAb is a fairly simple process, the generation of a useful one is often more complex. The fusion process itself takes only a few minutes and involves the incubation of the two types of cell together with a fusogen which is generally polyethylene glycol (PEG) (Figure 1). Hybrids which secrete antibodies of the desired specificity are detected by screening assays that are as close as possible to the final anticipated application of the antibody, although if this is to be a complex application such as *in vivo* use fully relevant screening is not possible.

Cells to be fused

The main requirements for the generation of a good monoclonal antibody are 1) a source of large numbers of suitably activated B lymphocytes from a hyperimmune subject and 2) a fusion partner from (ideally) the same species which is immortal and which has plasmacytoma/myeloma characteristics, i.e. a well-developed endoplasmic reticulum and the capacity to make large amounts of the required antibody after fusion, but which lacks the capacity to make antibody coded by its own genes. This last point is important since otherwise the fused cells make not only antibody from both parents, but also hybrid antibodies involving the combination of the different chains of the two and the capacity of the fused cells to make the required monoclonal in large amounts is corrupted.

The fusion partner must also be made sensitive to a drug such as aminopterin or azaserine so that those cells which do not acquire the drug resistance genes by fusion can be eliminated; this is a comparatively simple process.

In rodent systems the above conditions are fairly readily achieved. The spleen from a hyperimmune animal is probably the best source of B lymphocytes and nonsecreting, drug-sensitive myeloma lines of high fusion efficiency from both the mouse and rat are readily available. In the BALB/c mouse, the most commonly used cell lines are the P3-X63-Ag8 6.5.3 and Sp2/0 cell lines and in the rat the Lou Y3 Ag 1.2.3 line is the most successful (although this retains the capacity to make a kappa light chain). Lympho-

cytes from the Armenian or Syrian hamster can also be fused with mouse myeloma lines to yield stable hybrids.

A small number of groups have been working on generating monoclonal antibodies from bovine, ovine or rabbit sources in more recent years but the rodent systems remain dominant.

Selection and cloning

After fusion, the cells are grown in selective medium. The usual medium for rodent fusions is hypoxanthine, aminopterin and thymidine (HAT). Only cells which have the enzyme hypoxanthine phosphoribosyl transferase (HPRT) and thymidine kinase (TK) can grow in this medium, and the myeloma cells, which have been selected to lack one of these two enzyme activities, therefore die if they have not acquired them by fusion. Spleen cells generally only survive for 10–14 days in culture and thus only the hybrid fused cells survive.

Small clones of hybridomas emerge from the debris of the dead spleen and myeloma cells 5–20 days after the fusion and are assayed to detect those which make antibody of the required specificity. These are then cloned on feeder cells at a density of one hybridoma cell per well in 96-well tissue culture plates so that the final antibody is indeed monoclonal and that the cell secreting it is stable. The antibody-producing cells can then be grown in bulk in the ascitic fluid of rodents or in large culture vessels depending on the quality and quantity required. Antibody purification from the other components of the culture medium is accomplished by standard biochemical or immunochemical purification systems.

Methods for generating human monoclonal antibodies

Human monoclonal antibodies are required for most therapeutic uses as rodent ones are immunogenic and thus ultimately ineffective in humans. They have, however, proved difficult to generate, mainly because it is usually not possible for ethical reasons to hyperimmunize human subjects and frequently the only available source of B lymphocytes is peripheral blood in which the state of activation of the cells is less suitable for fusion.

There are three main ways in which this problem may be overcome (Figure 2):

- (a) A conventional mouse hybridoma is made from an ordinary hyperimmunized BALB/c mouse, and the antibody-coding genes are then manipulated so that the constant regions are of human rather than murine origin. A further sophistication of this technique is to also 'humanize' the

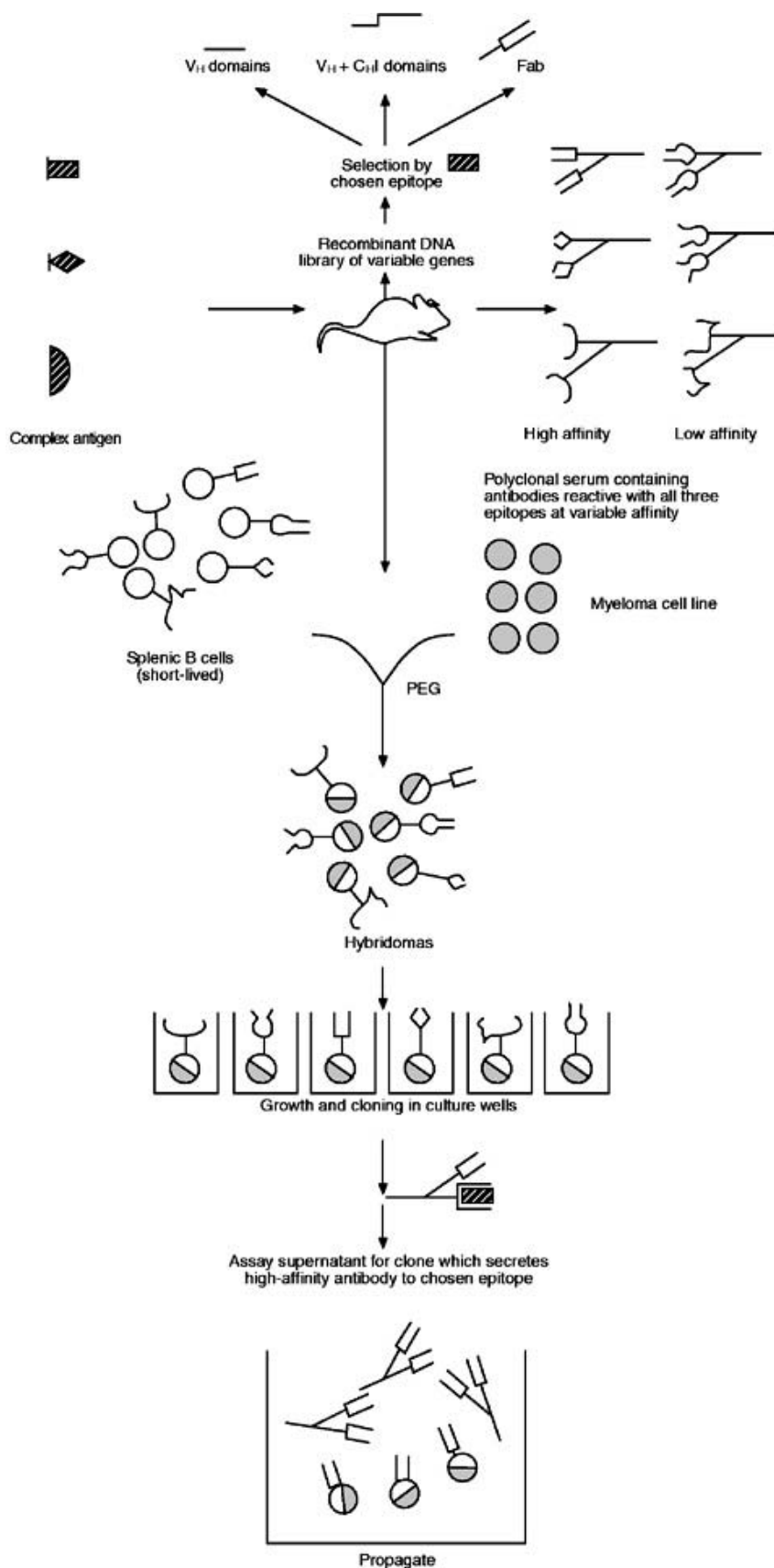


Figure 1 Methods of producing a monoclonal antibody.

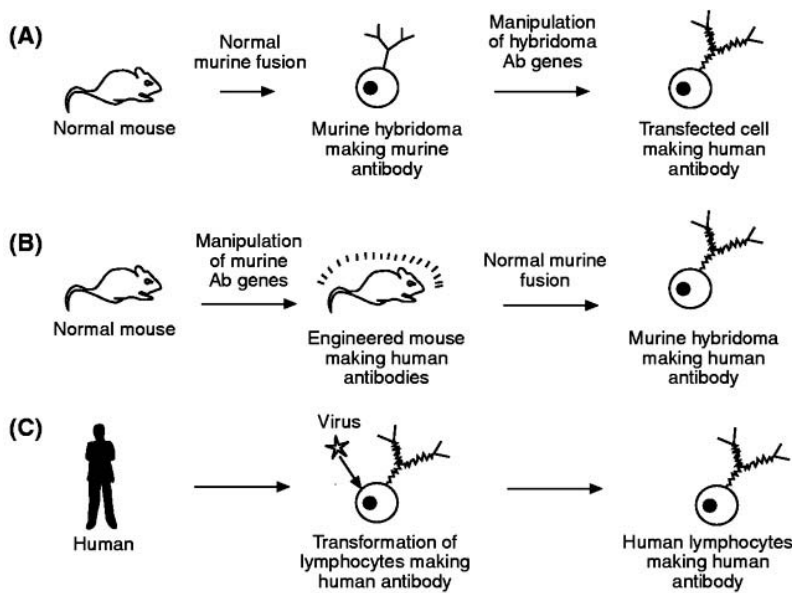


Figure 2 Strategies for generating a human monoclonal antibody. (A) From a conventional mouse hybridoma by manipulation of its constant region genes. (B) From a transgenic mouse carrying human immunoglobulin genes. (C) Transformation by Epstein-Barr virus. Note that in (A) and (B) the response reflects that of the mouse to a human antigen, whereas in (C) the human response to self-antigens (e.g. rhesus, autoantigens) is immortalized.

framework regions of the mouse antibody leaving only the CDRs (complementarity determining regions) of murine origin. Such antibodies elicit little or no immune response in humans.

- (b) A genetically engineered mouse is hyperimmunized. Originally, the SCID (severe combined immunodeficiency) mouse was used as it could be, to some extent, populated with an artificial human immune system. More recently, two groups have produced mice with parts of the murine heavy and light chain immunoglobulin loci replaced by the human loci. Such mice, when hyperimmunized, produce murine B lymphocytes making human antibodies and these can then be used in a normal mouse fusion yielding a murine hybridoma making human antibodies.
- (c) Use of Epstein-Barr virus (EBV) to immortalize human B lymphocytes which carry its CD21 receptor. This is the only method which gives hybridomas making a genuine human response to human antigens, as strategies (1) and (2) immortalize the murine response to the antigen. Clearly the mouse immune system may see many foreign determinants on a human cellular antigen and these may mask its response to the single antigen that the human immune system sees as foreign. The most obvious example is the rhesus blood group antigens which are masked in the mouse by its response to the human ABO system but which are strong antigens in humans. The EBV method can also be used to interrogate the antibody profile and V gene usage in autoimmune conditions by immortalizing the B lymphocytes present at the site of the

lesion. EBV transformants grow well but tend to cease antibody secretion unless this is stabilized. They are therefore frequently 'backfused' after initial cloning to a more stably secreting myeloma line, frequently of mouse origin. The resultant antibody remains human.

Alternative recombinant methods

Newer recombinant methods involve the creation of gene expression libraries from (usually) immunized mice or humans. These may contain the variable regions, or variable plus partial constant region of the heavy or light immunoglobulin chains, and Fab libraries from a combination of the two. A very large number of clones can be screened by inexpensive filter binding methods. In addition, the antibodies can be expressed on the surface of filamentous DNA bacteriophage and selected by affinity chromatography on antigen.

Properties of monoclonal antibodies

Affinity and specificity

Among the most important properties of monoclonal antibodies are their defined nature and their availability over indefinite periods of time. Monoclonal antibodies can also be reagents of exquisite specificity with the ability to identify small amounts of antigen in complex mixtures, giving very low background staining in diagnostic systems. However, they cannot necessarily be assumed to have such properties and it is wise to obtain as much information as possible about the systems in which any mAb has been characterized before using it. Many

commercially available mAbs are poorly characterized and appear to have been bought in by some firms more to swell their catalog repertoire than to offer a useful reagent. In general, a good mAb should have a high intrinsic affinity for the chosen antigen. This means that mAbs of the immunoglobulin M (IgM) class, which have high avidity but often low intrinsic affinity, should be avoided in most cases, the exceptions possibly being in ABO blood grouping where agglutinating IgM mAbs tend to be employed and are well-characterized for use in the specific diagnostic systems employed.

Potential cross-reactions

Monoclonal antibodies can cross-react in two different ways. The first is the expected one where two dissimilar antigens have elements of structural identity. For example, a mAb to lactate dehydrogenase will obviously cross-react with isocitrate dehydrogenase if its epitope is at the common nicotinamide adenine dinucleotide (NAD)-binding site but will not do so if its epitope is at the lactate-binding site. This type of cross-reaction can be investigated at an early stage in screening and the desired antibody can be selected. The second type of cross-reaction is more difficult to detect at early stages of mAb production. This is where a mAb can bind to two or more totally dissimilar antigens, probably using different amino acids in the CDRs, although for steric reasons, it usually cannot bind both at the same time. Antibodies which do this tend to be of the IgM class with low intrinsic affinity for either antigen and this is why such antibodies should be avoided where possible. The phenomenon is particularly evident with polyvalent antigens such as single-stranded DNA and intracellular structural proteins and also where antigen has been plated at high density on an ELISA plate, thus driving a low-affinity interaction forward to give a positive result. Clearly, since these cross-reactions occur with molecules dissimilar to the selecting antigen they are difficult to identify on screening but it is important to try to do so. For example if a mAb selected to bind to a tumor cell for *in vivo* use has even a very low additional capacity to bind to, say, collagen or some other abundant body protein, it is likely to be diverted in its clinical application. While there is a view that such polyspecific antibodies are only secreted by certain types of B lymphocyte expressing the CD5 marker, polyspecificity is a potential feature of any antibody with low intrinsic affinity for the selecting antigen.

It is also possible for monoclonal antibodies to be overspecific in, for example, diagnosis of microbial antigens where a single mAb may only detect a parti-

cular serotype. For this reason, selected mAbs are often pooled to make a comprehensive diagnostic kit.

Diagnostic applications of monoclonal antibodies

In immunological research

Monoclonal antibodies have had wide application in both basic and applied immunological research. For the former, the reader should refer to the cluster of differentiation (CD) system, which is entirely defined by mAbs and now encompasses more than 200 cell surface markers, to appreciate the immense value of mAbs in identifying phenotype, developmental stage and activation status of cells of the immune system. In combination with flow cytometric analysis (FACS) these CD-specific mAbs can be used to detect the appearance or absence of cell populations during antigenic stimulus or infection, for example in monitoring the decline of CD4⁺ cells during the progression of AIDS (acquired immune deficiency syndrome) infection, or the increases in cell surface density of lymphokine receptors during lymphocyte activation.

In general

Monoclonal antibodies are now extensively used in diagnosis in many fields and the estimated annual world market is in excess of US\$3 billion. In most cases, the double sandwich mAb technique where a mAb directed to one part of the antigen under test is used to 'capture' it and a mAb to a different epitope, linked to a detecting system is used to provide the signal has overtaken radioimmunoassay. Diagnostic kits utilizing mAbs for the detection of human chorionic gonadotropin (hCG) in pregnancy or luteinizing hormone (LH) during ovulation are generally available and other home diagnostic kits for bacterial infections including streptococcal infections of the throat and venereal disease have been developed, although these are not on general sale in most countries.

Most mAb-based kits utilize a color reaction as the final detection system but these can readily be adapted by luminescent or bioelectronic mechanisms to biosensors which can beep, flash or trigger mechanisms which generate further intervention without direct human involvement in the relevant decisions. Such systems may prove of value in continuous monitoring of environmental hazards or in process control during, for example, large-scale production of antibiotics.

In the standard laboratory environment, mAbs are now routinely used to determine ABO blood group

antigens and blood and tissue levels of protein and polypeptide hormones. The diagnostic market has, however, been modified over the last decade by the development of routine polymerase chain reaction (PCR) facilities in most laboratories. PCR is very much more sensitive than immunological methods for techniques such as HLA typing and pathogen diagnosis.

Therapeutic applications of monoclonal antibodies

General

Monoclonal antibodies have not yet realized the optimistic expectations of the last two decades in terms of therapy. Scientific understanding of most human diseases has also developed significantly during this period, as has conventional pharmacology, so that monoclonal antibody therapy no longer appears to be appropriate for many of the major diseases in comparison to smaller, cheaper, drug molecules which can more readily penetrate tissues and intracellular compartments.

Transplant rejection/autoimmune disease

The first monoclonal antibody to be licensed by the Food and Drug Administration (FDA) for therapeutic use in humans was OKT3, a mouse antibody which targets all T cells through the cell surface CD3 complex. It is used in acute allograft rejection only where conventional immunosuppressive drugs such as cyclosporine and FK506 have failed, and its cost is very much greater than either of these drugs. Rejection of this foreign mouse antibody is rare since it functions to immunosuppress the patient.

In the case of autoimmune disease which is generally not life threatening, such drastic measures are usually inappropriate and much interest has centered on the removal of specific compartments of the immune system to downregulate the autoimmune response without destroying that patient's ability to fight routine infections. Monoclonal antibodies to CD2, CD5, CD7 and CDw52 have all been put through preliminary clinical trials, as have antibodies to various cytokines and their receptors, but no clear candidate therapy has emerged and for any that may do so, the cost per patient will be a relevant issue.

Bacterial infections

Extracellular bacteria and bacterial endotoxins are the natural target for antibodies and consequently mAbs should have an ideal therapeutic application in such cases. However, low-cost antibiotics are available for the treatment of the great majority of

bacterial disorders so that mAbs cannot compete. Such drug resistance as occurs tends to be in the poorer communities where mAb therapy is not within accessible cost range.

The lipopolysaccharide (LPS) endotoxins, however, provide a potential target since these are the causative agents of gram-negative sepsis in burns and postoperative patients in developed nations and the condition is often fatal before the host antibody immune response has developed. Gram-negative sepsis is caused by a wide range of bacteria, all of which have the lipid A component in common in their LPS endotoxins. Two antibodies, one human IgM and the other murine IgG, both claimed to be specific to Lipid A, and thus all gram-negative endotoxins, were developed for this purpose by two different companies in the late 1980s. Lipids are poor antigens and it remains possible that such antibodies had multiple specificities to the highly antigenic carbohydrate moiety of the LPS. Clinical trials gave ambivalent results, with neither antibody being commercially successful to date. The best reagent would almost certainly be a human IgG antibody to lipid A which could readily be produced by modern technology.

Tumor therapy

In the early days of mAb development, it was anticipated that the potentially high specificity of mAbs would mean that they could be employed not only to identify small metastatic populations of tumor cells but also to kill them, either alone or in conjunction with a cytopathic agent, while leaving normal cells unharmed. This hope has largely not yet been realized and indeed many of the claims of tumor-associated epitopes defined by mAbs in the early 1980s remain unsubstantiated. There are several reasons for this lack of success. For example, the major tumors of humans are those of the epithelial cells of the colon, breast, lung and prostate, and it is now clear that many of the oncogenes activated within such cells code largely for intracellular proteins which are inaccessible to antibody therapy. This view is strengthened by the fact that such common tumors show no increase in frequency in immunosuppressed individuals. Chemotherapeutic strategies have, meanwhile, advanced considerably and offer more chance of success at lower cost in many of these tumors.

In the case of tumors of the immune system, the situation is slightly different. Tumor-specific idiotypic markers are identifiable in B cell lymphomas and the CD antigen surface characteristics of any group of tumor cells may also be used as a mAb target. Uniquely, in the hematopoietic and immune system, it is possible to consider the use of

mAbs temporarily to eliminate all populations other than stem cells with consequent eradication of the bulk of the tumor cells and their clonogenic precursors carrying the activated oncogene. Therapeutic strategies using human–rodent chimeric mAbs have been directed towards this goal for some years now with limited success, perhaps because the originating cell is closer to the hematopoietic stem cell than the one which bears the targeted antigen. Further immunological research may uncover more appropriate targets.

It is possible that, as in many of the diagnostic systems where mAbs are now pooled to form a highly defined polyclonal reagent, or where multi-drug therapy is common in many cancers, antibody-based tumor diagnosis and therapy may also require a pool of antibodies to different epitopes for optimal effect.

Other applications

These tend to encompass improvements in technology achieved by transfer of experimental systems from polyclonal to monoclonal application and include, for example, catalytic mAbs which perform

reactions for which there is no natural enzyme system, albeit at low efficiency as yet, and the use of mAbs in antigen purification.

See also: Affinity; Antibodies, specificity; Antiserum; B lymphocytes; Catalytic antibodies; CD antigens; Enzyme-linked immunosorbent assay (ELISA); Epstein-Barr virus, infection and immunity; Hybridomas, B cell; Immunoassays; Leukocyte culture; Lymphoma; Polymerase chain reaction (PCR) amplification; Tumor antigens.

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MONOCYTE CHEMOATTRACTANT PROTEIN 1 (MCP-1)

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Monocyte chemoattractant protein 1 (MCP-1) is encoded by human chromosome 17q11.2–q21.1 and is also known as MCAF (monocyte chemotactic and activating factor). MCP-1 belongs to the chemokine β family as it has a C–C (cysteine–cysteine) amino acid sequence, as opposed to the C–X–C structure of the chemokine α family to which interleukin-8 (IL-8) belongs. The most prominent features of MCP-1 are its chemotactic effect towards monocytes *in vivo* and *in vitro* and its activating effect on monocytes.

MCP-1 shows 21% amino acid sequence homology to IL-8. There is about 55% homology between the human MCP-1 and the murine JE analog. MCP-1 is produced from a 99 amino acid precursor to yield a 76 amino acid mature form. The MCP-1 receptor (CCR2A, CCR2B) is a seven transmembrane-spanning G protein-coupled molecule of 39 kDa with homology to other chemokine recep-

Table 1 Sources of MCP-1

Peripheral blood mononuclear cells
B lymphocytes
Fibroblasts
Vascular endothelial cells
Keratinocytes
Melanocytes
Smooth muscle cells
Mesangial cells
Synovial cells
Lipocytes
Pulmonary type II epithelial cells
Osteoblast cells
Liver fat-storing cells
Myelomonocytic THP-1 cell line, osteosarcoma-, glioblastoma-, rhabdomyosarcoma-cell
U373MG, osteoblastic cell line MC3T3-E1
T cell hybridoma clone D6-18

Table 2 Inducers and suppressors of MCP-1*Upregulation*

The production of MCP-1 can be induced by the following diverse agents:

MCP-1
IL-1
IL-4
TNF
PDGF (platelet-derived growth factor)
GM-CSF (granulocyte-macrophage colony-stimulating factor)
ECGF (endothelial cell growth factor)
TGF β 1 (transforming growth factor β 1)
IFN γ (interferon γ)
LIF (leukemia inhibitory factor)
PHA, Con A (phytohemagglutinin, concanavalin A)
LPS (lipopolysaccharide)
PSK (protein-bound polysaccharide/krestin)
TPA (12-*o*-tetradecanoyl-phorbol-13-acetate)
Hydroxyurea
Silica
Polyethylene particles
Minimally modified low-density lipoprotein
IgG complexes
Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine
 α -Thrombin
Double-stranded RNA poly(rI): poly(rC)
Viruses
Phagocytosis of *M. tuberculosis*, zymosan and latex by macrophages

Downregulation

The production of MCP-1 is suppressed by the following agents:

Glucocorticoids
17 β -Estradiol (E₂)
IL-10

tors. The CCR2B is also an HIV coreceptor, allowing fusion between the cell membrane and the HIV virus. A number of different cell types, both normal and malignant, can produce MCP-1 (Table 1). MCP-1 mRNA expression is induced within 30 min by IL-1 and tumor necrosis factor α (TNF α). A number of other factors may also induce MCP-1 production (Table 2), including MCP-1 itself, other proinflammatory cytokines, growth factors, mitogens, bacterial products, viruses and immunoglobulin G (IgG) complexes. MCP-1 production is inhibited by steroids and IL-10.

MCP-1 was initially identified because of its chemotactic effect towards monocytes. Apart from chemotaxis it also induces other events in monocytes such as enzyme release, increased expression of

Table 3 Biological effects of MCP-1*In vitro activities*

Monocytes:

Chemotaxis (human, guinea pig)
 $\uparrow$ Superoxide anion release
 $\uparrow$ *N*-acetyl β -glucuronaminidase release
 $\uparrow$ Cytostatic augmenting activity
 $\uparrow$ Cytosolic free Ca²⁺
 $\uparrow$ Expression of CD11b/CD18, CD11c/CD18
 $\uparrow$ Binding to endothelial cells
Respiratory burst
 $\downarrow$ Colony formation of immature subsets of myeloid progenitor cells

Basophils:

$\uparrow$ Histamine release
 $\uparrow$ Cytosolic free Ca²⁺
 $\uparrow$ Leukotriene release

T lymphocytes

Chemotaxis

NK cells:

Chemotaxis

In vivo activities

$\uparrow$ Monocyte infiltration (rat)
Degranulation of mast cells (mouse)

Table 4 Diseases in which MCP-1 has been detected

Rheumatoid arthritis	Pulmonary sarcoidosis
Giant cell arteritis	Bronchial asthma
Granuloma	Hypersensitivity pneumonitis
Psoriasis	Seasonal allergic rhinitis
Septic shock	Peritonitis
Meningitis	Inflammatory bowel disease
HPV-infection	Endometriosis
AIDS-associated CMV-infection	Glomerulonephritis
Leishmaniasis	Lupus nephritis
Cerebral ischaemia	Ovarian cancer
Atherosclerosis	Melanoma
Idiopathic pulmonary fibrosis	Prostate adenocarcinoma

β_2 -integrin cell adhesion molecules, as well as augmentation of monocyte cytostatic activity against several malignant cell lines (Table 3). Injection of MCP-1 subcutaneously into the ears of rats induces considerable monocyte infiltration, beginning at 6 h and peaking by 16 h. Additionally, MCP-1 degranulates dermal mast cells upon injection into mice. The related molecules MCP-2 and MCP-3 are also monocyte chemotactic.

It has been shown that murine tumors producing JE contain a greater number of tumor-associated macrophages and that the animals have a better

prognosis than animals with tumors not producing MCP-1. This, along with its capacity to activate monocytes, suggests a role for MCP-1 in tumor growth control. Evidence for an inflammatory role of MCP-1 is mainly circumstantial. Thus the detection of MCP-1 in several disease states indicates a possible role in infectious, autoimmune and malignant conditions (Table 4). MCP-1 is also assumed to play a significant pathophysiologic role in atherosclerosis, where the chemokine is produced by endothelial cells stimulated with oxidized low-density lipoprotein (LDL).

See also: **Chemokines; Chemotaxis; Cytokines; Cytokine receptors; Interleukin 8 and its receptor; Chemotaxis of lymphocytes; Chemotaxis of macrophages and monocytes.**

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MONOCYTES

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Monocytes are phagocytic cells that belong to the mononuclear phagocyte system and reside in the circulation before they enter the tissues where they differentiate into macrophages. Human monocytes in circulation have a diameter varying from about 10 to 18 μm ; the surface possesses microvilli and an undulating membrane. The heterogeneity in size may be explained by differences in maturation stage and/or activation state. The cytoplasm of monocytes contains a small number of granules, designated as primary lysosomes, a Golgi complex, rough endoplasmic reticulum, and mitochondria. The eccentrically placed nucleus is indented or reniform and generally contains one or two small nucleoli. The main contents of the lysosomes are acid phosphatase, peroxidase, lysozyme, neutral proteases, elastase and collagenase. Monocytes may play a central role in

host defense because of their capacity to process and present antigen to T lymphocytes, their regulatory function through the secretion of biologically active molecules, their tumoricidal and antimicrobial activities.

Origin and kinetics

Monocytes originate in the bone marrow from pluripotent stem cells; their direct precursor cell is the promonocyte that derives from the monoblast. After monocytes are formed by division of promonocytes, they remain only a very short time (less than a day) in the bone marrow compartment. When monocytes enter the circulation they are divided over a circulating pool and a marginating pool, each comprising about 50% of the monocytes present in the blood

vessels. Monocytes stay for a relatively long time in the circulation, the half-life being 17 h (mice) to 71 h (humans). Then they leave this compartment randomly by a process called diapedesis (see below) and migrate to tissues and body cavities where they differentiate into macrophages. The production of monocytes in the bone marrow is governed by growth-stimulating cytokines such as interleukin 3 (IL-3), stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF), and during inflammation also by factor increasing monocytopoiesis (FIM). As a consequence, the number of monocytes in the peripheral blood increases by 1½–2 times, the half-life of monocytes in the circulation is temporarily reduced to about 12 h, and the majority of this increased number of monocytes migrate to the site of inflammation.

Treatment with glucocorticosteroids decreases the number of circulating monocytes and high doses result in complete monocytopenia. When this occurs during inflammation the number of monocytes available to migrate to the site of the lesion is reduced and consequently the number of (exudate) macrophages in the inflammatory exudate is rather small. The decrease in monocytes in the peripheral blood is caused by decreased production in the bone marrow, by delayed migration of monocytes from the bone marrow into the circulation and by temporary margination of these cells in the circulation, but not by destruction of monocytes.

Functions

Monocytes should be considered to be in transport from their site of origin to the site of function, i.e. the tissues and body cavities. In the tissues, monocytes differentiate first into exudate macrophages, which still have many characteristics of monocytes, and then into resident macrophages. It is not known what functions monocytes exert when they reside in the circulation. The following summary concerns the functional abilities of monocytes and monocyte-

derived (exudate) macrophages when these cells are studied *in vitro*.

Monocytes are well equipped to ingest opsonized microorganisms, red blood cells and other materials. The process of phagocytosis can be divided into a series of stages. First, the (opsonized) particles attach to the surface of the phagocyte. The opsonins serve as ligands for the binding of the particles to specific receptors on the surface of the monocytes; nonopsonized microorganisms and other cells can also bind by sugars in their cell surface to lectin-like receptors on monocytes. After ligation of the particle to the cell surface, a signal is generated that promotes the internalization process. Pseudopods of the cell advance by assembly of actin filaments that reach the membrane and form a network in the pseudopods. The pseudopods adhere closely to the particle by interaction of the ligand with the receptors on the cell surface. This process is called the zipper mechanism (Figure 1). When the engulfment is completed the particle resides in the cytoplasm in a vacuole, which is formed by the inverted cell membrane, and called a phagosome. Next lysosomes fuse with the phagosome and lysosomal enzymes are released into the phagosome and come in contact with the phagocytosed particle. The following step in this process is that microbicidal mechanisms and digestive processes come into operation.

The antimicrobial functions of monocytes are mediated by oxygen-dependent and oxygen-independent mechanisms. The respiratory burst that accompanies phagocytosis leads to the formation of reactive oxygen metabolites including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). After fusion of the phagosome with granules that contain peroxidase, this enzyme together with H_2O_2 and a halide, such as chloride, forms HOCl, a hypochlorite with microbicidal activities. In mice, but not in humans, (exudate) macrophages form nitric oxide, which may be involved in antimicrobial activities of these cells. The oxygen-independent antimicrobial mechanisms of monocytes and (exudate)

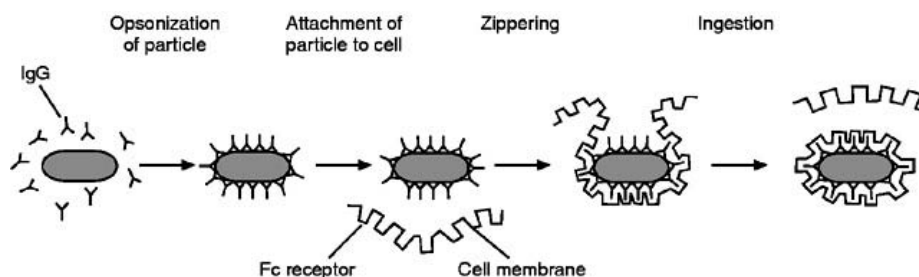


Figure 1 Various stages of the phagocytosis process.

macrophages include vacuolar acidification, nutrient deprivation and the action of antimicrobial polypeptides. Monocytes and macrophages contain granule-associated polypeptides such as lysozyme, defensins (only in rabbit alveolar macrophages), serprocidins (i.e. cathepsin G, elastase, proteinase 3 and azurocidin), and histone-related murine microbicidal proteins (MUMPs). The cytosol of macrophages contains a small, cationic, antimicrobial protein, called ubiquicidin. During differentiation of monocytes into resident macrophages, the monocytes lose their peroxidase activity, which is accompanied by a decrease in antimicrobial activity. However, resident macrophages can become activated by various kinds of cytokine, for example, interferon γ (IFN γ) formed by CD4⁺ T lymphocytes and natural killer (NK) cells, and then regain microbicidal activities and acquire tumoristatic or tumoricidal characteristics.

Another important function of mononuclear phagocytes, especially of (exudate) macrophages and to a smaller degree of monocytes, is the secretion of biologically active substances. Some substances are formed and secreted constitutively and others only after activation of the cells. These secretory products can be divided into enzymes (e.g. elastase, collagenase, proteoglycan degrading enzyme, plasminogen activator, lysosomal acid hydrolases, angiotensin-converting enzyme, arginase), enzyme inhibitors (e.g. α_2 -macroglobulin, plasminogen activator inhibitor, α_1 -antitrypsin), arachidonic acid derivatives (e.g. prostaglandins, thromboxane), plasma proteins (e.g. complement components, coagulation factors, fibronectin, apoprotein E), and many cytokines regulating functions or growth of other cells (e.g. IL-1, IL-6, IL-8, IL-10 and IL-12, tumor necrosis factor α (TNF α), GM-CSF, angiogenesis factor, IFN γ). The repertoire of secretory products may depend on the degree of maturation and/or the state of activation of the cell, and/or its localization in the body. The existence of subsets of monocytes and macrophages that are specialized to form only one or a small number of secretory products is rather unlikely.

Membrane characteristics

A large number of biological activities of monocytes and macrophages are determined by specific molecules present on the surface membrane of these cells. Some of these molecules are receptors for immunoglobulins (i.e. Fc receptors), cytokines, growth factors or plasmaproteins (e.g. complement or coagulation factors); others serve as adhesion molecules and regulate the interaction with subcellular matrix components or with other cells. During differen-

tiation or activation of monocytes the expression pattern of these receptors changes constantly.

Monocyte receptors for immunoglobulins

Human monocytes express receptors for the Fc region of immunoglobulin G (IgG), namely Fc γ receptor I (Fc γ RI; CD64), Fc γ receptor II (Fc γ RII; CD32) and, after *in vitro* culture, Fc γ receptor III (Fc γ RIII; CD16). Fc γ RI binds monomeric human IgG with high affinity, but also cross-reacts with murine IgG. The number of Fc γ RI molecules expressed on the plasma membrane of human monocytes is about $2-4 \times 10^4$ and increases about 2 to 4-fold upon differentiation into macrophages or stimulation with IFN γ . Fc γ RII on monocytes expresses polymorphism and binds human IgG only in its polymeric or complexed form with relatively low affinity. The number of Fc γ RII on monocytes is generally higher than that of Fc γ RI. There are two forms of Fc γ RIII: the transmembrane form, Fc γ RIIIA on monocytes, macrophages and NK cells, which binds human IgG with medium affinity, and the glycosylphosphatidylinositol (GPI)-linked form, Fc γ RIIIB, which is only expressed on neutrophils and interacts only with immune-complexed IgG. Only after *in vitro* culture monocytes express moderate quantities of Fc γ RIIIA.

IL-4-stimulated monocytes may express the low-affinity receptor for IgE, Fc ϵ RIIB (CD23). Furthermore, monocytes express a receptor for IgA, Fc α RI (CD89), which is a transmembrane glycoprotein that binds both isotypes of IgA. Fc α receptors on phagocytes exert their function mainly at the mucosal membranes where IgA antibodies are predominant. Fc γ receptors on monocytes play an important role in host defense against microbial infections. The attachment of the Fc region of IgG to the surface of monocytes via Fc γ receptors triggers a variety of cellular processes such as the internalization of small immune complexes (endocytosis), phagocytosis and intracellular killing of Ig-opsonized microorganisms and the secretion of inflammatory mediators.

Monocyte receptors for complement components

Components of the complement system enhance the antibacterial activity of monocytes and macrophages. This function is exerted by two important surface receptors for C3b, namely complement receptor type 1 (CR1; CD35) and complement receptor type 3 (CR3; CD11b/CD18). CR1 binds to the C3c region of C3b. CR3 is a heterodimeric protein that belongs to the β_2 subfamily of integrins, and is a receptor for C3bi. Both CR1 and CR3 are mediators in the phagocytosis of C3b- or C3bi-opsonized particles.

Monocyte receptors for adhesion

Monocytes express molecules which play a dominant or accessory role in the adhesion to platelets, lymphocytes and other cells, e.g. endothelial cells, or to a subcellular matrix. Based on functional and structural similarities, adhesion molecules on monocytes or macrophages are categorized in three distinct families: the immunoglobulin (Ig) superfamily, the selectins and the integrin molecules (see **Table 1**). Monocytes display several Ig superfamily molecules on their surface, namely the intercellular adhesion molecules (ICAM), platelet-endothelial cell adhesion molecule (PECAM), Fc γ receptors, leukocyte function antigen (LFA-3) and class I antigens, and after stimulation, class II major histocompatibility complex (MHC) antigens. These Ig superfamily glycoproteins play an important role in immunological processes including phagocytosis, antigen presentation and transendothelial migration.

L-selectin is the only selectin expressed on monocytes and to a much lesser extent on macrophages. The N₂-terminal Ca²⁺-dependent lectin-like domain of L-selectin is of importance in the initial attachment of monocytes to the vascular endothelium at inflammatory sites, which is an obligatory event before transendothelial migration can occur. Integrins on monocytes or macrophages belong to four different subfamilies, the β_1 -, β_2 -, β_3 - and β_5 -integrin subfamily, that can be distinguished based on the type of β chain that associates noncovalently with an α chain to form the functional heterodimeric molecule. The functional state of β_1 - and β_2 -integrins, in terms of their affinity for their ligands, is determined by the activation state of the cell. The β_1 -, β_3 - and β_5 -integrins on monocytes have been shown to interact with extracellular matrix components such as fibronectin, laminin or vitronectin, whereas β_1 - and β_2 -integrins mediate the interaction of monocytes or

Table 1 Major adhesion molecules expressed on human monocytes or macrophages

	Name	Subunits	Ligands
Integrins			
β_1 -integrins	VLA-2 (CD49b/CD29)	$\alpha_2\beta_1$	Collagen
	VLA-4 (CD49d/CD29)	$\alpha_4\beta_1$	VCAM-1, fibronectin
	VLA-5 (CD49e/CD29)	$\alpha_5\beta_1$	Fibronectin, <i>B. pertussis</i>
	VLA-6 (CD49f/CD29)	$\alpha_6\beta_1$	Laminin
β_2 -integrins	LFA-1 (CD11a/CD18)	$\alpha_L\beta_2$	ICAM-1, -2, -3
	CR3 (CD11b/CD18)	$\alpha_M\beta_2$	ICAM-1, C3bi, fibrinogen, Factor X
	p150,95 (CD11c/CD18)	$\alpha_X\beta_2$	C3bi, fibrinogen
	CD-/CD18	α_d/β_2	ICAM-3
β_3 -integrins	Vitronectin receptor (CD51/CD61)	$\alpha_V\beta_3$	Vitronectin, fibronectin, von Willebrand factor, Thrombospondin
β_5 -integrins	CD51/CD-	$\alpha_V\beta_5$	Vitronectin
Selectins	L-selectin (CD62L)		E-, P-Selectin, sialyl-Lewis ^x , CD34, MadCAM-1, GlyCAM-1
Ig supergene family	ICAM-1 (CD54)		LFA-1, CR3, rhinovirus, <i>P. falciparum</i>
	ICAM-2 (CD102)		LFA-1, CR3
	ICAM-3 (CD50)		LFA-1
	PECAM-1 (CD31)		PECAM-1, heparan sulfate, Proteoglycans, $\alpha_V\beta_3$ -integrin
	Fc γ RI, II and III (CD64, CD32 and CD16)		Fc region of IgG
	LFA-3 (CD58)		CD2
	MHC class I and II molecules		Processed foreign antigen
Noncategorized molecules	Leucosialin/sialophorin (CD43)		ICAM-1
	Sialoadhesin		Sialic acid-glycoproteins
	Hermes-1/Pgp-1 (CD44)		Hyaluronic acid, others
	Sialyl-Lewis ^x , sLe ^x (CD15s)		P-, E-, L-Selectin
	CD14		LPS/LPB, activated endothelium

macrophages with other cells, including lymphocytes and vascular endothelial cells. In the latter process members of the Ig supergene family may serve as excellent ligands for integrins.

Adhesion molecules in transendothelial emigration of monocytes

Under normal, noninflammatory conditions, a limited number of circulating monocytes adhere to and traverse the endothelial lining of the vessel wall. Under these conditions the efflux of monocytes from the circulation to the tissues ensures maintenance of the macrophage population in a given tissue compartment. During an inflammatory reaction, a variety of locally released inflammatory mediators, such as IL-1 α , TNF α , IFN γ , chemoattractants (e.g. anaphylatoxin C5a, leukotriene B₄), formylmethionyl peptides (e.g. fMLP), or chemokines (e.g. IL-8 or monocyte chemotactic protein-1 (MCP-1)), can modulate the adhesion of monocytes to endothelial cells. The increased adhesion of monocytes to the vascular endothelium and subsequent migration to the inflamed tissue satisfies the demand for macrophages at that site.

Intravital microscopy studies revealed that extravasation of monocytes, and of leukocytes in general, is preceded by a coordinated sequence of adhesion events that enables these cells to withstand the hemodynamic forces generated by the flow of blood and to make firm contact with the vascular endothelial cells. Three separate consecutive events can be distinguished: monocytes first attach to the vessel wall in a rolling interaction, next the cells are arrested and stick firmly to the surface of endothelial cells and thereafter transendothelial migration by diapedesis occurs.

Rolling of monocytes along the endothelial lining of small venules is observed exclusively at inflammatory sites or sites of vascular injury and *in vitro* under conditions of flow. Rolling involves short-lived attachment of monocytes via L-selectin to ligands such as P-selectin (CD62P), E-selectin (CD62E), or sialomucins (e.g. CD34, GlyCAM-1) on activated endothelial cells which is followed by detachment from the endothelium. Consequently the flow of monocytes in the main vasculature slows down. During rolling of the monocytes they become activated and enter the next phase of adhesion in which monocytes are arrested at a particular site of the vascular endothelium and migrate over the luminal surface of the endothelial cells.

The firm adhesion of monocytes to endothelium

relies primarily on the activation of β_2 -integrin molecules, resulting in an increase in affinity for their endothelial ligands (e.g. ICAM-1). However, also the β_1 -integrin VLA-4 and CD14 mediate firm adhesion of monocytes to activated endothelium. Activation of β_2 -integrins may be triggered by the chemokine MCP-1, which is produced by endothelial cells or elicited by binding of monocytes to the endothelial cell surface.

Monocytes finally cross the vessel wall by squeezing between tightly apposed endothelial cells in an ameboid fashion, called diapedesis, a process in which β_2 -integrin molecules are involved. This is concluded from the lack of neutrophil and monocyte diapedesis observed in patients with the leukocyte adhesion deficiency (LAD) syndrome, who are genetically deficient in the functional expression of leukocyte β_2 -integrin molecules. However besides β_2 -integrins, homophilic interaction of PECAM-1 on monocytes with PECAM-1 on the endothelial cells is also involved in transendothelial migration of monocytes.

Following diapedesis, monocytes attach to and subsequently traverse the subendothelial matrix, or basement membrane, consisting of fibronectin, laminin, collagen and sulfated proteoglycans, and enter the extravascular tissues.

See also: Diapedesis; Macrophage activation; Microbicidal mechanisms, oxygen-dependent; Microbicidal mechanisms, oxygen-independent; Mononuclear phagocyte system; Opsonization; Phagocytosis.

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MONONUCLEAR PHAGOCYTE SYSTEM

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The similarities between the morphological, cytochemical, and functional characteristics of monoblasts, promonocytes, monocytes and macrophages, together called mononuclear phagocytes, combined with knowledge about the origin and kinetics of these cells, led to the concept of the mononuclear phagocyte system (MPS).

The origin of mononuclear phagocytes

During ontogeny, the hematopoietic stem cell – a cell of mesenchymal origin – arises in the yolk sac. In a later stage of gestation, these cells migrate to the fetal liver, where immature mononuclear phagocytes develop; hematopoiesis in the liver does not cease until term or the second week after birth, depending on the species. Soon after hematopoiesis begins in the fetal liver, monocytes appear in the circulation; only after that does hematopoiesis commence in the bone marrow. Before hematopoiesis commences in the bone marrow, osteoclasts formed by fusion of circulating mononuclear phagocytes (e.g. monocytes or younger cells of this cell line) are found in embryonic bone. The approximate time during gestation, when mononuclear phagocytes appear in hematopoietic organs and in the circulation of mouse and human, is shown in Table 1.

In adult life, mononuclear phagocytes are present in all tissues, but under normal conditions proliferating mononuclear phagocytes occur only in the bone marrow. The most immature cell of the mononuclear phagocyte cell line present in the bone marrow is the monoblast. This cell is derived from a granulocyte-monocyte progenitor cell which can be considered to be a committed stem cell that is derived from the multipotent hematopoietic stem cell. Division of the monoblast gives rise to two promonocytes, and the two daughter cells of a promonocyte are monocytes.

Thus, in the bone marrow from monoblast to monocytes, there is a cell amplification of four times. Monocytes remain in the bone marrow for only a short time (less than 24 h) and then enter the circulation, where they are present in a circulating or marginating pool. The marginating monocytes migrate through the endothelium of the vessel wall to the various tissues and body cavities. Adhesion molecules on monocytes and on endothelial cells, together with chemotatic factors, are involved in the migration of monocytes from the blood vessels into the tissues or body cavities.

In the extravascular tissues, monocytes transform into (exudate) macrophages. In the lungs, for example, monocytes differentiate directly into alveolar macrophages without maturation in the interstitium; in the sinusoids of the liver, monocytes become the Kupffer cells; and in the peritoneal cavity, monocytes arrive directly from the capillaries or via the milky spots in the omentum. In normal tissues, only a very small percentage of macrophages divide, these macrophages also having recently originated from the bone marrow. Little is known about the ultimate fate of the macrophages. Since there is no evidence that macrophages recirculate via the lymph vessels to the circulation, it seems likely that they die in the tissues where they reside or migrate to local lymph nodes.

Mononuclear phagocytes and inflammation

During an acute or chronic inflammation, the number of macrophages may increase and both the degree of the increase and the time at which it occurs are dependent on the nature of the stimulus and the type of tissue in which the inflammation occurs. Circulating monocytes are mainly responsible for the

Table 1 Ontogeny of mononuclear phagocytes

Site of formation of mononuclear phagocytes	Localization of mononuclear phagocytes at other sites	Time of gestation	
		Mouse	Human
Yolk sac		Days 7–12	Days 21–63
Fetal liver		Day 11 to 2nd week postnatal	5th week to term
	Circulating monocytes	After days 11–12	After weeks 7–8
	Osteoclasts	After day 15	After weeks 8–9
Bone marrow		At birth	After week 11

increase in the number of macrophages in the inflammatory exudate, and transitional forms between monocytes and resident macrophages are present in such exudates and are called exudate macrophages. There is also an increase of the number of dividing mononuclear phagocytes in an inflammatory exudate and in granulomas, due to the recruitment of immature mononuclear phagocytes from the bone marrow.

Alterations in the functional state of the (exudate) macrophages include their locomotion, chemotactic response, endocytic, microbicidal, cytostatic or cytotoxic activity, as well as their secretory state, their adherence to and spreading on a surface, and their cooperation with T and B lymphocytes. The inflammatory stimulus may act directly upon the macrophages and change their functional state, and such a change can also be mediated by cytokines secreted by lymphocytes or macrophages (i.e. an autocrine regulation), or by serum proteins such as immunoglobulins or complement.

Two other types of cell often present in chronic inflammatory lesions are the epithelioid cell and the multinucleated giant cell, both of which also derive from monocytes. Epithelioid cells in granulomas have characteristics that resemble those of macrophages. Multinucleated giant cells are formed by fusion of (exudate) macrophages, and not by nuclear division without cytoplasmic division. There are two types of multinucleated giant cell: the Langerhans type with relatively few nuclei arranged peripherally in the cytoplasm, and the foreign-body type with many nuclei dispersed throughout the cytoplasm.

Cell systems

Cells are usually identified primarily on the basis of their morphological characteristics, but such criteria alone are often insufficient for the identification of cells, and therefore other characteristics must be taken into account such as the presence of nonspecific esterases or other cytoplasmic enzymes, or ectoenzymes, the binding of monoclonal antibodies specific for antigens on mononuclear phagocytes, the presence of membrane receptors, or the capacity of immune phagocytosis. Since the specificity and the sensitivity of the available markers and techniques are not yet optimal, it is not possible to classify a cell as a mononuclear phagocyte with the use of only one marker. Classification of a cell population as a mononuclear phagocyte must therefore be based on a combination of properties.

Cells sharing a common morphology, origin, function or other properties may be considered collectively as forming a system. Many attempts have been

made in the past to define the system formed by phagocytic cells (Table 2). Metchnikoff was the first to use the term 'macrophages' (i.e. big eaters), because these cells are capable of ingesting large particles such as erythrocytes, spermatozoa, protozoa, etc., as opposed to the polymorphonuclear leukocytes or granulocytes, which he termed 'microphages', since these cells are only able to take up smaller particles (e.g. bacteria). Metchnikoff already recognized the close relationship between the phagocytic cells in the spleen, lymph nodes and bone marrow, and the macrophages outside these organs (e.g. in the connective tissue), which led him to introduce the term 'macrophage system' (Table 2). Kiyono introduced the term 'histiocytic cell system'. Aschoff developed this concept further and grouped several kinds of cell together in the 'reticulo-endothelial system' in which he included endothelial cells, fibrocytes, reticular cells, reticuloendothelial cells, Kupffer cells, histiocytes, splenocytes and monocytes. The concept of the reticuloendothelial system has been widely criticized (see Further reading). Maximow considered the term reticuloendothelial system inappropriate, because the vascular-endothelial cell is very different from the histiocyte and even more so from the reticular cell. Thomas criticized the term reticuloendothelial system and reintroduced the term 'reticulo-histiocyte system', which had been proposed by Volterra (Table 2).

Mononuclear phagocyte system

Since neither the concept of the reticuloendothelial system nor that of the reticulohistiocyte system provides a fully satisfactory framework into which the current knowledge of phagocytic mononuclear cells can be fitted, the concept of the mononuclear phagocyte system (MPS) was introduced in 1969. This concept is based on what is known about the morphology, function, origin and kinetics of the mononuclear phagocytes. The cells now assigned to the MPS are shown in Figure 1.

Candidate cells for the MPS

Dendritic cells and Langerhans cells are considered as candidates for the MPS because of their bone-

Table 2 Names given to phagocytic cell system

Macrophage system	E. Metchnikoff, 1892
Histiocytic cell system	K. Kiyono, 1918
Reticulo-endothelial system	L. Aschoff, 1924
Reticulo-histiocyte system	M. Volterra, 1927
Mononuclear phagocyte system	R. van Furth, 1969

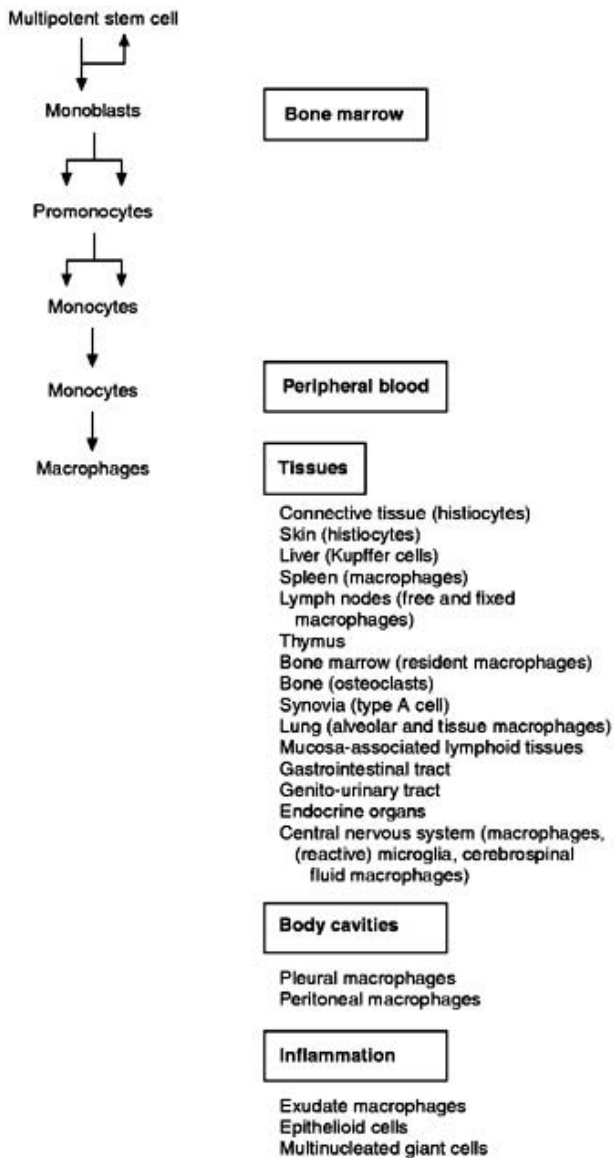


Figure 1 Mononuclear phagocyte system.

marrow origin, and their morphological and/or functional characteristics *Dendritic cells* occur in lymphoid organs, e.g., lymph nodes, thymus, and spleen, but also in organs such as lungs, skin, liver, small intestine, etc., originate from precursor cells in the bone marrow. These precursor cells are not yet characterized nor are the mononuclear cells in the circulation that differentiate in the tissues into dendritic cells. In cultures of bone marrow and blood mononuclear cells, in the presence of GM-CSF and IL-4, dendritic cells develop, which supports the bone-marrow origin of these cells. Stem cell factor (*e-kit* ligand) increases the yield of dendritic cells, and maturation of dendritic cell is promoted by TNF, LPS and other agents. Furthermore, glucocortico-

steroid, which induce a rapid decline of circulating mononuclear cells, including monocytes, cause also a rapid disappearance of dendritic cells from the tissues. Together, these findings support the view that dendritic cells derive from precursors in the bone marrow, but whether monocytes can differentiate in dendritic cells is still uncertain. Although dendritic cells have also many features in common with macrophages, such as cell-surface markers, functions like endocytosis, antigen processing and presentation, these cells can still not yet be considered as bona fide mononuclear phagocytes, because their derivation from monocytes has not yet been firmly established.

Langerhans cells have the same life history and functions as dendritic cells. They derive also from precursors in the bone marrow cells, and circulating mononuclear cells that migrate to the skin differentiate into Langerhans cells. The sojourn time of Langerhans cells in the skin is short and next the cells are transported, via the lymph draining the skin, as veiled cells, which are cells with long cytoplasmic viels, to the lymph nodes where they reside as interdigitating cells.

Cells not belonging to the MPS

Reticulum cells and fibroblasts, present in connective and lymphoid tissues and in various organs, and mesothelial cells of serous cavities, have morphological characteristics giving them some resemblance to macrophages, with which they are often confused. However, these cells are not regarded as mononuclear phagocytes, because they lack other characteristics of macrophages. Furthermore, these cells do not originate in the bone marrow, but renew themselves by division locally in the tissues. Confusion has been caused by adjectives applied to the reticulum cells, histiocytic reticulum cells (syn. histiocytes) and interdigitating reticulum cells are macrophages, and dendritic reticulum cells, reticular cells, and metalophil cells are all reticulum cells.

Terminology of mononuclear phagocytes under various conditions

In many reports, the mononuclear phagocytes participating in an inflammatory reaction (whether induced experimentally or occurring spontaneously) are not defined carefully, and various terms are used interchangeably. The characteristics of the mononuclear phagocytes in an inflammatory exudate depend on the inducing agent, the method of isolation, the developmental stage and the functional state of the cells. The mononuclear phagocytes in an

inflammatory exudate may be in different developmental stages, including resident macrophages already residing in the tissues or body cavity before the onset of the inflammation, monocytes recruited from the circulation that differentiate into exudate macrophages, and monocyte precursors (i.e. dividing mononuclear phagocytes derived from the bone marrow).

Since the cells in an inflammatory exudate can be quite heterogeneous, they should be characterized cautiously. To indicate the degree of heterogeneity of the population of cells in the exudate, it is essential to report not only qualitative but also quantitative data for each characteristic.

The terminology applied to the cells in an inflammatory exudate is generally unclear. For example, terms such as 'stimulated', 'activated', 'elicited' and 'induced' are used interchangeably. To deal with this problem, the following definitions can be used:

1. *Resident macrophages*. Macrophages present at any given site in the absence of exogenous or endogenous inflammatory stimulus. These cells may occur in an inflammatory exudate as a subpopulation because they were already present before the stimulus was applied. They are also called 'normal macrophages'.
2. *Exudate macrophages*. Macrophages occurring in an inflammatory exudate.
3. *Exudate-resident macrophages*. These cells are a transitional form between resident and exudate macrophages.
4. *Activated macrophages*. Macrophages with increased functional activity induced by a given stimulus *in vivo* or *in vitro*. Activation thus implies a new functional activity of a cell or an increase in one or more of these activities. Before activation, these cells may have been resident or exudate macrophages. Explicit mention should be made of how the macrophages were stimulated and how activation was measured.
5. *Elicited macrophages*. Macrophages attracted to a given site by a given substance. This term only indicates the accumulation of (exudate) macrophages at a particular site, and does not refer to the developmental stage of the cells or their functional state. An elicited population of cells is usually heterogeneous in these respects. Since 'elicited' and 'evoked' mean the same thing, use of the latter term is acceptable.

The term *stimulated macrophage* is imprecise, because stimulation means the application of a stimulus that may result in the elicitation and/or acti-

vation of cells. This adjective should not be used. The term *induced macrophage* is also inexact and should not be used, since induced can imply either elicited or activated.

Other terms used in relation to mononuclear macrophages

The terms *accumulation* and *proliferation* of cells are often used erroneously. Proliferation should be reserved for cases in which the increase in the number of cells is known to be due to division of cells already present at or recruited to a site. The term accumulation should be used for the increase of the number of cells caused by migration of (nondividing) cells from other sites.

A distinction should also be made between *differentiation* and *specialization*: the former refers to a change in the morphological development in the direction of another morphological stage, whereas the latter means a change in the functional state of a cell in a special direction.

See also: Adhesion molecules; Bone marrow and hematopoiesis; Chemotaxis; Diapedesis; Endothelium; Macrophage activation; Motility of immune cells; Ontogeny of the immune response; Phagocytosis; Chemotaxis of macrophages and monocytes.

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MOTILITY OF IMMUNE CELLS

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Immune cell migration is a primary host defense mechanism for the mobilization of leukocytes to inflammatory sites and the development of cell-mediated immune responses. Initiation of migratory responses occurs when signal molecules interact with plasma membrane receptors, triggering cytoskeletal reorganization and cell shape change. Motile responses may be random, chemokinetic, chemotactic or haptotactic, and are classified by *in vitro* assays which quantify the directional responses of cells. Random migration of unstimulated motility and chemokinetic migration, i.e. stimulated random movement of cells in the absence of a stimulus gradient, are motile responses which do not have consistent directionality. In contrast, chemotactic and haptotactic responses are directional; they occur when cells are exposed to a signal gradient and the cells migrate toward an increasing concentration of the stimulus. Chemotactic responses occur when the signal is a fluid phase gradient, whereas haptotactic responses occur when the stimulus gradient is attached to a substratum. The relative importance of random, chemokinetic, chemotactic and haptotactic motility *in vivo* has not been determined. All of these motile responses may be involved in the mobilization of immune cells to inflammatory sites.

Leukocyte chemoattractants

A variety of molecules (chemoattractants) stimulate the directed migration of immune cells. Leukocyte chemoattractants may be generated at inflammatory sites or as a consequence of immune cell interactions and may serve to regulate the directed recruitment of specific leukocyte subsets to inflammatory foci *in vivo*. Some of the most potent leukocyte chemoattractants are summarized in Table 1. Neutrophils and monocytes share chemotactic responsiveness to several chemoattractants, including a cleavage product of the fifth component of complement (C5a), synthetic *N*-formyl-methionyl peptides which are structurally similar to *N*-formyl peptides generated by bacteria, arachidonic acid lipoxygenation metabolites such as leukotriene B₄ and platelet-activating factor.

Numerous secreted proteins of approximately 70–90 amino acids in length have been identified as chemokines. Some chemokines are generated constitutively; however, most chemokines act as second-

Table 1 Leukocyte chemoattractants

Chemoattractant	Responding cell type
<i>N</i> -formyl peptides	Neutrophil, eosinophil, basophil, monocyte
C5a	Neutrophil, eosinophil, basophil, monocyte
Leukotriene B ₄	Neutrophil, monocyte
Platelet-activating factor	Neutrophil, eosinophil, monocyte
Chemokines	
C chemokines	
Lymphotactin	T lymphocyte
C-C chemokines	
MCP-1	Monocyte, basophil
MIP-1 α,β	Neutrophil, eosinophil, basophil, monocyte, T lymphocyte
RANTES	Monocyte, eosinophil, T lymphocyte
I-309	Monocyte
C-X-C chemokines	
IL-8/NAP-1	Neutrophil, basophil
CTAP-III/NAP-2	Neutrophil, basophil
gro/MSGA	Neutrophil
ENA-78	Neutrophil
SDF-1	Neutrophil, monocyte, T lymphocyte

ary cell activation signals during inflammatory responses. Chemokines have been divided into three groups (C, C-C and C-X-C) based on the number and arrangement of cysteines. The C chemokines have two cysteines. The C-C and C-X-C chemokines have four cysteines and are distinguished by the presence (C-X-C) or absence (C-C) of a single amino acid between the first and second cysteines. Cysteines 1 and 3 and cysteines 2 and 4 form disulfide bonds in C-C and C-X-C chemokines. The chemokines exhibit 20–30% amino acid sequence identity across groups and can be substantially more conserved within groups.

Examples of selected chemokines and their responding cell types are provided in Table 1. Only one C chemokine, lymphotactin, has been identified, whereas more than a dozen each of C-C and C-X-C chemokines have been discovered. The chemokines exhibit specificity for leukocyte types. The C-X-C chemokines predominantly attract neutrophils; however, lymphotactin and most C-C chemokines do

not. Most C-C chemokines attract monocytes, whereas lymphotactin and C-X-C chemokines do not. One C-C chemokine, MIP-1 α , β , attracts neutrophils, eosinophils, basophils, monocytes and T lymphocytes. The other C-C chemokines do not stimulate neutrophil migration. A chemokine distantly related to C-X-C and C-C chemokines, SDF-1, exhibits a broad spectrum of activity, attracting neutrophils, monocytes and T lymphocytes. SDF-1 is the most active T lymphocyte chemoattractant thus far identified and is also a potent stromal cell-derived preB cell growth-stimulating factor.

Chemoattractant receptors

Leukocyte chemoattractant receptor primary structures have been deduced from cloned cDNAs for *N*-formyl peptides, C5a, platelet-activating factor and eight of the chemokines. All of the receptors are seven-transmembrane domain rhodopsin-like G protein-coupled receptors. Their sequences are all approximately 350 amino acids in length and they share greater than 20% amino acid identity with each other. A general structural model of these receptors has been developed from a statistical analysis of sequences found in the hydrophobic domains and the known structures of rhodopsin and bacteriorhodopsin. The primary structural features include 1) an extracellular amino-terminus, 2) an intracellular carboxy-terminus, 3) seven α -helical transmembrane domains that are oriented perpendicularly to the plasma membrane, with kinks occurring in transmembrane domains II, IV, V, VI and VII by intrahelical prolines, and 4) three intracellular and three extracellular connecting loops comprised of hydrophilic amino acids. The amino-terminal segments of the receptors are usually acidic and contain potential sites for asparagine-linked glycosylation. The carboxy-termini are rich in serine and threonine residues, which may serve as phosphorylation sites for receptor desensitization, as occurs for rhodopsin and β_2 -adrenergic receptors.

Structural changes in chemoattractant-stimulated leukocytes

Within seconds after adherent leukocytes are exposed to a chemoattractant, plasma membrane ruffling occurs and the cells elongate with a broad lamellipodium formed anteriorly and a thin uropod formed posteriorly. Cytoplasmic actin filaments are assembled (polymerized) near the plasma membrane and appear in increased concentrations in the lamellipodium and uropod, whereas intermediate filaments are found in the core of the uropod and microtubules

are concentrated in the body of the cell extending toward, but not into, both poles of the oriented leukocyte. The kinetics of actin polymerization and depolymerization correlate closely with leukocyte shape change and are believed to be crucial events in chemotaxis. During leukocyte polarization, chemoattractant receptors are concentrated near the lamellipodium, whereas coated pits, clathrin-containing structures that mediate pinocytic uptake, are clustered at the uropod. Chemoattractant ligand is internalized under physiologic temperature conditions of 37°C and the ligand has been found to transiently associate with cytoskeletal proteins in neutrophils. Adherent, chemoattractant-stimulated neutrophils also exocytose specific (secondary) granules which contain an intracellular pool of *N*-formyl peptide chemoattractant binding sites. It has been proposed that chemoattractant receptor replenishment occurs at the lamellipodia of migrating neutrophils by either the recycling of previously internalized receptors or by the translocation of a receptor pool associated with neutrophil-specific granules or other organelles which are released during chemotaxis. Neutrophil receptors appear to move in a consistent pattern from the lamellipodium toward the uropod as the neutrophils exhibit amoeboid-like migration in a relatively straight line up the concentration gradient. Neutrophil and monocyte exposure to relatively high concentrations of chemoattractants also activates the extrusion of lysosomal enzyme-containing granules (degranulation) and increased oxygen consumption resulting in oxygen radical generation. Although degranulation and oxygen radical production do not appear to be involved in the initiation of chemotaxis, these events may modulate the chemotactic response and enhance the microbicidal activity of phagocytic leukocytes mobilized to critical sites *in vivo*. The dramatic alterations in leukocyte plasma membranes as well as the rapid changes in cytoskeletal architecture which accompany leukocyte exocytosis, pinocytosis and polarization all appear to be essential morphologic rearrangements that occur during chemotaxis. The precise sequence of structural and biochemical changes required for leukocyte chemotaxis remain undefined.

Biochemical changes in chemoattractant-stimulated leukocytes

Transient elevations in intracellular Ca²⁺, Na²⁺ and cyclic AMP levels, as well as membrane potential changes, occur within seconds after leukocyte exposure to chemoattractants. Chemoattractant binding to receptor activates phospholipase C via guanine nucleotide binding-proteins (G proteins).

The G_{i2} and G_{i3} proteins have been found to be the primary G proteins associated with chemoattractant receptors. Upon activation by chemoattractant binding to the receptor, the Gi dissociates into the GTP-bound $G\alpha$ subunit and $\beta\gamma$ subunit complex. The $G\beta\gamma$ complex regulates phospholipase C β isoforms in leukocytes. Phospholipase C β activation results in the breakdown of phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-triphosphate (IP3) and 1,2 *sn*-diacylglycerol (DAG). The two products of this reaction, IP3 and DAG, activate calcium mobilization (via IP3) and protein kinase C (via DAG). Calcium and protein kinase C appear to be involved in leukocyte cytoskeletal protein modifications. Intracellular free Ca^{2+} is increased in the lamellipodia of migrating neutrophils and affects actin polymerization in chemoattractant-stimulated neutrophils. Other cytoskeletal protein reorganizations may occur via DAG which stimulates protein kinase C to phosphorylate cytoskeletal proteins. Inhibitors of protein kinase C, kinase A and tyrosine kinases inhibit neutrophil chemotaxis to several chemoattractants. These findings and others demonstrating the phosphorylation of cytoskeletal proteins in chemoattractant-stimulated neutrophils suggest that cytoskeletal protein phosphorylation is involved in neutrophil chemotaxis.

The coupling and uncoupling of G protein with a chemoattractant receptor may occur via modifications to the G protein and/or the receptor protein. Protein carboxyl methylation may be involved in the modifications of these proteins. Several macrophage membrane proteins are carboxyl methylated by S-adenosylmethionine in an enzymatic reaction that is greatly stimulated by the presence of guanine nucleotides. Macrophage chemotaxis is suppressed by bacterial toxin inhibitors of G proteins and by methylation inhibitors, strongly suggesting that carboxyl methylations may regulate the function of some membrane G proteins.

The receptors for several different chemoattractants have been reported to exist in two affinity states and guanine nucleotides regulate the receptor affinity. One model, which assumes a static population of high- and low-affinity receptors, has proposed that the high-affinity receptors transduce chemotaxis, while the low-affinity receptors transduce oxygen radical production. A second model, which takes into account interconverting receptor states rather than static populations of receptors, postulates that all chemoattractant receptors may participate in all leukocyte responses coupled via G proteins; the responses with low 50% effective doses (ED_{50} s) require only a few occupied receptors, whereas the

responses with high ED_{50} s require many receptors to initiate and sustain the response.

Chemoattractant binding to neutrophils and monocytes activates phospholipase A_2 and results in arachidonic acid release. Free arachidonic acid is converted by cyclo-oxygenase to thromboxanes and prostaglandins and by lipoxygenation to a variety of hydroxyeicosatetraenoic acids, including leukotrienes. Peak intracellular concentrations of these metabolites occur within minutes after chemoattractant stimulation. The precise roles of these products are unknown; however, they may regulate leukocyte responsiveness to chemoattractants and participate in alterations of leukocyte plasma membranes during activation.

Migratory properties of immune cells

Lymphocytes differ from neutrophils and monocytes in their *in vitro* and *in vivo* migratory properties. Neutrophils are the most rapidly motile immune cells and are generally the first leukocytes to appear at an inflammatory site. Monocytes usually appear after neutrophils and precede lymphocytes at an inflammatory locus.

The emigration of neutrophils and monocytes from the vasculature appears to be regulated by three distinct molecular signals, which include interactions between selectin-carbohydrate, chemoattractant-receptor and integrin-immunoglobulin family interactions which occur in sequence. Selectins mediate the initial tethering of neutrophils and monocytes to the endothelial cells and their rolling on the vessel wall. Chemoattractant-receptor interaction, which stimulates the events reviewed in the preceding sections, also alters the number of integrin molecules expressed on neutrophil and monocyte surfaces and provokes conformational changes in integrins which modulate the adhesiveness of these leukocytes to their endothelial substrata. The endothelial cells express immunoglobulin superfamily molecule members which serve as integrin ligands for leukocytes. This complex series of events is coordinated such that these cells may attach, migrate and detach from endothelial substrata in response to inflammatory stimuli.

Lymphocyte recirculation involves distinct traffic patterns for naive and memory lymphocytes. Separate streams appear to exist for lymphocytes that recirculate through the skin, gut and lung and drain into their associated lymphoid tissues. Naive and memory lymphocytes prefer different recirculation pathways. Upon exposure to antigen, naive lymphocytes with receptors specific for the antigen are stimulated to clonally expand and are converted to

memory lymphocytes that have altered expression of adhesion receptors and circulatory patterns. For peripheral tissues and lymph nodes, memory lymphocytes emigrate preferentially through tissue endothelium, whereas naive lymphocytes enter the lymph node through high endothelial venules (HEVs). The emigration of lymphocytes through peripheral node HEVs appears to require sequential signals that are analogous to those required for neutrophil and monocyte emigration from the vasculature. The putative lymphocyte chemoattractant secreted by peripheral lymph node HEVs and the chemoattractant receptor that is selectively expressed on naive lymphocytes that recirculate through peripheral lymph node HEVs remain to be determined.

See also: Anaphylatoxins; Arachidonic acid and the leukotrienes; Chemokines; Chemotaxis; High endothelial venules; Interferon β ; Interleukin 2; Lymphocyte trafficking; Lymphocytes; Macrophage migration inhibitory factor (MIF); Monocytes; Neuroendocrine regulation of immunity; Neutrophils; Platelet-activating factor (PAF); Recruitment; Transforming growth factor β (TGF β); Chemotaxis of macrophages and monocytes; Chemotaxis of neutrophils; Chemotaxis of lymphocytes.

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MOUSE INBRED STRAINS

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In mouse and human, the susceptibility and resistance to diseases, as well as the response to therapeutic agents, involves a complex interplay between multiple genetic and environmental factors. Here we focus on a few well-studied inbred strains of mice that display particular immunological characteristics. Response to infectious agents, including tumor-causing viruses and some of the opportunistic infections found in patients with the acquired immune deficiency syndrome (AIDS) are included in this brief review.

As the Human Genome Mapping Project has progressed, many genetic diseases with single gene etiologies have been mapped. However, much remains to be learned. Diseases defined by single gene mutations can show a wide spectrum of phenotypes influenced by modifier genes, genetic background and environment. Other diseases, such as cancers and diabetes, are polygenic and the genetic contributions to these diseases are not yet understood.

As a major model organism with extensive genetic tools, the mouse is providing important insights into human genetic diseases. The Mouse Genome Data-

base (MGD) developed at the Jackson Laboratory, is a critical link to providing researchers with mouse genetic and phenotypic data and cross-connecting these data with analogous information on human genes and phenotypes. MGD contains information on genetic markers, mammalian homology (>50 species), probes, polymerase chain reaction (PCR) primers, haplotype data from backcross mapping panels, gene expression data, strain distribution patterns from recombinant inbred and recombinant congenic strains, quantitative trait loci (QTL), MIT DNA marker maps, Chromosome Committee reports and phenotypic information in the Mouse Locus Catalog (MLC) and Dr MFW Festing's inbred strain listing. There are links with other genomic databases including the human Genome Database (GDB) and Mendelian Inheritance in Man (OMIM), as well as DNA sequence databases – GenBank, GSDB, EMBL, DDBJ.

Inclusion of inbred strain information collected over many years at the Jackson Laboratory is in MGD future plans. This will enable an investigator to search for data on the anatomy, biochemistry, morphology, physiology, disease susceptibility or resistance and response to therapeutic reagents, etc. of inbred strains of mice. Our World Wide Web URL is: <http://www.informatics.jax.org/mgd.html> and help is available through e-mail at mgi-help@informatics.jax.org.

A/J

Origin

Cross between a Cold Spring Harbor albino and a Bagg albino (Strong, 1936). Carries a Y chromosome of Asian *Mus musculus musculus* origin (Tucker *et al*, 1992).

Predominant characteristics

1. A four locus model (*Pas1*, -2, -3, -4) of pulmonary adenoma susceptibility (Malkinson *et al*, 1985).
2. Cranio-facial disorders (Juriloff *et al*, 1995).
3. Hyperreactive airway response to a variety of bronchoconstricting agents including acetylcholine (Ewart *et al*, 1995), 5-hydroxytryptamine (Levitt *et al*, 1989) and ozone (Takahashi *et al*, 1995). *Bhr1*, -2, -3, -4 bronchial hyperresponsiveness genes mapped by QTL analysis (De Sanctis *et al*, 1995).
4. Extreme sensitivity to carcinogens (Stoner *et al*, 1993).

Infectious agents

Susceptible to: Friend leukemia virus, herpesvirus type 1, Moloney virus, *Candida albicans*, *Giardia muris*, *Leishmania tropica*, *Schistosoma mansoni*, *Taenia taeniformis*, *Trypanosoma brucei*, *T. congolense*, *T. cruzi*, *T. musculi*, *T. rhodesiense*, *T. vivax*, *Corynebacterium kitchneri*, *Pseudomonas aeruginosa*, *Rickettsia tsutsugamushi*, *Ectromelia* (Nesbitt *et al*, 1984), *Plasmodium chabaudi* (Stevenson *et al*, 1992), *P. berghei* (Contreras *et al*, 1980), *Cryptococcus neoformans* (Rhodes *et al*, 1980).

Resistant to: mouse hepatitis virus 3; *Brugia malayi*, *Echinococcus multilocularis*, *Encephalitozoon cuniculi*, *Entamoeba histolytica*, *Fasciola hepatica*, *Trichomonas vaginalis*, *Nematospores dubius* reinfection, *Listeria monocytogenes*, *Mycobacterium bovis* BCG, *M. lepraemurium*, *M. tuberculosis*, *Salmonella typhimurium*, *Schistosoma japonicum*, *Eimeria ferrisi*, *Histoplasma capsulatum* (Nesbitt *et al*, 1984).

Immunological parameters

Deficient in: natural killer (NK) cell activity (Pollack *et al*, 1982), tumor necrosis factor alpha (TNF α) (Gosselin *et al*, 1995), hemolytic complement (Rhodes *et al*, 1980) and interferon gamma production (Lucchiari *et al*, 1992). Limited immunoglobulin G (IgG) response, sufficient IgM response (Miller *et al*, 1992). Resists amyloid formation (Nesbitt *et al*, 1984).

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AKR/J

Origin

Somewhat obscure early history. Bred as a high leukemia strain (Furth, 1946). There are striking differences between AKR substrains, particularly AKR/J and AKR/Cum (Acton *et al*, 1973). AKR/J carries a *Mus musculus domesticus* origin of Y chromosome (Tucker *et al*, 1992).

Predominant characteristics

1. High leukemia and high viremia (DiFronzo *et al*, 1993).
2. An 80–90% susceptibility to spontaneous T cell lymphomagenesis (Yamada *et al*, 1994; Haran-Ghera *et al*, 1995a; Michie *et al*, 1995) and a 5–10% susceptibility to spontaneous B cell lymphomagenesis (Haran-Ghera *et al*, 1995b).

Infectious agents

Susceptible to: lactate dehydrogenase-elevating virus-induced poliomyelitis (Anderson *et al*, 1995), MCF-247 recombinant virus (Peled *et al*, 1995), 3 Gross leukemia virus (Rich *et al*, 1995), murine herpes virus (Kapoor *et al*, 1992), Friend virus (Silver, 1986), *Ectromelia* (Wallace *et al*, 1985), *Trichuris muris* (Else *et al*, 1992), *Echinococcus multilocularis* (Gottstein *et al*, 1994), and *Neisseria meningitidis* infection (Woods *et al*, 1988). Resistant to: *Trichinella spiralis* infection (intermediate) (Kennedy *et al*, 1991). Low dental caries after infection with JC-2 *Streptococcus mutans* (Kurihara *et al*, 1991).

Immunological parameters

Spontaneous lymphocytic choriomeningitis (LCM) (Allan *et al*, 1989). High tumorigenesis with MNU (Angel *et al*, 1993) and ENU (Diwan *et al*, 1973). Low immunosuppression by ultraviolet B radiation (Noonan *et al*, 1994). Resistant to DMH-induced colon carcinogenesis (Rosenberg *et al*, 1995). Low susceptibility to the induction of autoimmune orchitis (Tokunga *et al*, 1993).

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BALB/c

Origin

Halsey J Bagg albino to LC Strong in 1920 (Strong, 1978). Differences in BALB/c substrains have been documented (Potter, 1985, Hilgers *et al*, 1985). Carries an Asian *Mus musculus musculus* Y chromosome (Tucker *et al*, 1992).

Predominant characteristics

1. Pre-neoplastic plasmacytomas easily induced using pristane (Potter *et al*, 1964) or silicone gels (Potter *et al*, 1994).

Infectious agents

Susceptible to: Theiler's murine encephalomyelitis virus (TMEV) (BALB/cAnNCr), mouse hepatitis virus (Godfraind *et al*, 1995) with BALB/cBy showing resistance (Nicholson *et al*, 1996), *Leishmania major* (Noben-Trauth *et al*, 1996), *Trypanosoma congolense* (Ogunremi *et al*, 1995), chronic Chagas'

cardiomyopathy in postacute *Trypanosoma cruzi* infection (Rowland *et al*, 1992), *Hymenolepis nana* (Asano *et al*, 1993), *Salmonella typhimurium* (Xu *et al*, 1995).

Resistant to: *Eimeria vermiformis* (Rose *et al*, 1996), *Helicobacter* infections (Mohammadi *et al*, 1996), *Plasmodium berghei* ANKA (Moumaris *et al*, 1995), *Candida albicans* (Costantino *et al*, 1995).

Immunological parameters

Susceptible to colon tumors (Moen *et al*, 1996). High IgE responder (Mori *et al*, 1990). Low or non-responder to tumor induction by SV40 large T Ag (Zerrahn *et al*, 1996).

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C3H

Origin

LC Strong originated C3H strain in 1920 from a cross of a female Bagg albino × DBA male with selection for high mammary tumor incidence (Strong, 1935). Significant differences among substrains. Carries a Y chromosome of *Mus musculus domesticus* origin (Tucker *et al*, 1992).

Predominant characteristics

1. The C3H/HeJ-*Ips*^d substrain carries a defective lipopolysaccharide response allele while most of the other C3H substrains carry the *Ips*⁺ allele for normal LPS response (Glode *et al*, 1976; Ding *et al*, 1995).
2. High incidence of spontaneous and induced mammary tumors (Outzen *et al*, 1985; Lukacher AE *et al*, 1995), although in some C3H substrains this characteristic may be reduced or lost in time (TJL Technical Support, 1996). MMTV characterized by Lee and Eicher (1990).

Infectious agents

Susceptible to: MuLV (Nouvel *et al*, 1993), lymphocytic choriomeningitis (LCM) virus (Stellrecht *et al*, 1992), encephalomyocarditis virus-induced myocarditis (Okada *et al*, 1992), measles virus (Finke *et al*, 1995), Junin virus (Scolaro *et al*, 1991), cardiovirulent coxsackievirus B3 (CVB3) (Tracy *et al*, 1992); *Mycobacterium tuberculosis* (Lecoeur *et al*, 1989), L-form *Salmonella typhimurium* (C3H/HeJ) (Nishikawa *et al*, 1994), Lyme borreliosis (*Borrelia burgdorferi*) (Fikrig *et al*, 1992), *Trypanosoma musculi* (Wechsler *et al*, 1988), *Cryptosporidium parvum* (Enriquez *et al*, 1991), *Candida albicans*, *Mycobacterium bovis* BCG and *Schistosoma mansoni* (Jeevan *et al*, 1992), leishmaniasis (Laskay *et al*, 1995), (*Treponema hyodysenteriae* Serpulina (Mysore *et al*, 1992).

Resistant to: mouse hepatitis virus (Decimo *et al*, 1993), Murray Valley encephalitis virus (Sangster *et al*, 1993); *Cryptococcus neoformans* (Rhodes *et al*, 1980), *Trypanosoma cruzi* (Tarleton, 1990).

Immunological parameters

Resistant radiation-induced pulmonary tumors (Johnston *et al*, 1995). Hyporeactive airway response to ozone, and resistant to silicosis (Ohtsuka *et al*, 1995). Females poor Hya (H-Y) antigen responders to syngeneic male skin grafts (Desquenue-Clark *et al*, 1992). High IgE responder (Mori *et al*, 1990). Females susceptible to cardiac calcinosis (Brunnert *et al*, 1990). Susceptible to experimental autoimmune

orchitis (Tokunaga *et al*, 1993) and spontaneous and induced liver tumors (Rumsby *et al*, 1994). Low responder to cholera toxin (Elson, 1992).

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C57BL/6J

Origin

CC Little, JAX, developed this strain in 1921 from a mating of female 57 with male 52 from Abbie Lathrop's stock. Substrains C57BL/6 and C57BL/10 were separated prior to 1937 (Russell, 1978). LINE-1 elements from *Mus spretus* detected in the C57BL/6J background suggest a maximum of 6.5% of the C57BL/6J genome may be *Mus spretus* derived (Rikke *et al*, 1995). Carries an Asian *Mus musculus musculus* Y chromosome (Tucker *et al*, 1992).

Predominant characteristics

1. Develops mouse acquired immune deficiency syndrome (MAIDS) after infection with LP-BM5 MuLV (Simard *et al*, 1994; Huang *et al*, 1995).
2. Extremely high preference for drugs of abuse, including alcohol (Chadha *et al*, 1991); Phillips *et al*, 1994), morphine (Berrettini *et al*, 1994; Belknap *et al*, 1995) and cocaine (Lopez *et al*, 1992; Watzl *et al*, 1992; Grahame *et al*, 1995).
3. Multi-locus model (*Ath-*) for atherosclerotic lesions induced by an atherogenic diet (Nishina *et al*, 1993).
4. Alopecia areata-like hair loss (Thornburg *et al*, 1973; Militzer *et al*, 1986).

Infectious agents

Contrast with the A strain listing in a review by Nesbitt and Skamene (1984). Susceptible to: rabies virus (Lodmell, 1982); induction of amyloidosis (Hebert *et al*, 1990), *Toxoplasma gondii* (McLeod *et al*, 1984), *Mycobacterium avium* (Hubbard *et al*, 1992). Resistant to MTV (Murray *et al*, 1967), polyoma virus-induced tumorigenesis (Freund *et al*, 1992), experimental encephalomyocarditis (Mendel *et al*, 1995), *Plasmodium chabaudi* (Yap *et al*, 1992), *Mycoplasma fermentans* (Gabridge *et al*, 1972), *Angiostrongylus costaricensis* (Ishii *et al*, 1989), *Histoplasma capsulatum* (Wu-Hsieh, 1989), *Lyme borreliosis*-induced carditis (Barthold *et al*, 1990), *Trypanosoma cruzi* (Reed *et al*, 1994), *Actinomyces viscosus*-induced bone loss (Gilbert *et al*, 1988), mousepox-1 (Brownstein *et al*, 1992).

Immunological parameters

Resist arthritis induction (Yong *et al*, 1988) and ovalbumin-induced anaphylactic shock (Cowdery *et al*

al, 1982). High responder to dextran (Fernandez, 1992). Intermediate IgE responder (Mori *et al*, 1990). Endotoxin-responsive (*Lps*ⁿ) to TCDD (Clark *et al*, 1994). High responder to cholera toxin (Elson, 1992).

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C57BL/10

Origin

Developed at JAX from a mating of a female 57 × male 52 from Abbie Lathrop (Russell, 1978). Carries the Asian *Mus musculus musculus* Y chromosome (Tucker *et al*, 1992).

Predominant characteristics

1. Predominant substrain used as the genetic background for the production of many histocompatibility congenic resistant lines (Snell *et al*, 1965).

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C57BLKS/J (previously C57BL/KsJ)

Origin

Derived from nonpedigreed mice bred at JAX and widely distributed. Returned to JAX after the 1947 Bar Harbor fire. Genomic analysis of the C57BLKS inbred strain shows that 84% of the alleles in this strain are shared with C57BL/6 and 16% are shared with DBA/2J indicating genetic contamination early in the strain's history (Naggert *et al*, 1995; Slingsby *et al*, 1995). The diabetes mutation, *Lepr*^{db} arose on this strain and the effects of the gene are particularly severe on this background (Hummel *et al*, 1966; Leiter, 1987). Carries an Asian *Mus musculus musculus* Y chromosome (Tucker *et al*, 1992).

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CBA/J**Origin**

Bred for low mammary tumor incidence from a cross of a Bagg albino female and a DBA male (Strong, 1936). Genetic contamination is thought to account for the differences between substrains. Carries a *Mus musculus domesticus* origin Y chromosome (Tucker *et al*, 1992).

Infectious agents

Susceptible to: Theiler's virus (Rodriguez *et al*, 1990), measles virus (Neighbour *et al*, 1978), *Ectromelia* virus strain C (Ermolaeva *et al*, 1972), *Bacillus piliformis* (Tyzzer's disease) (Waggie *et al*, 1981), *Onchocerca lienalis microfilariae* (Folkard *et al*, 1995), *Mycobacterium lepraemurium* (Roch *et al*, 1991), *Schistosomia mansoni* (Henderson *et al*, 1993).

Resistant to: murine herpesvirus (Kapoor *et al*, 1992), rabies virus (Lodmell *et al*, 1995); *Salmonella typhimurium* strain (Matsiota-Bernard *et al*, 1993), *Neisseria gonorrhoeae* (Johnson *et al*, 1989), *Plasmodium berghei* ANKA strain (Contreras *et al*, 1980), *Streptococcus mutans*-induced dental caries (Kurihara *et al*, 1991).

Immunological parameters

High gross tumor incidence (Storer, 1966; Smith *et al*, 1973). Susceptible to ozone toxicity (Goldstein *et al*, 1973) and streptozoin-induced renal epithelial tumors (Hard, 1985).

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Smith GS *et al* (1973) *Journal of the National Cancer Institute* 50: 1195–1213.

Storer JB (1966) *Journal of Gerontology* 21: 404–409.

Strong LC (1936) *British Journal of Experimental Pathology* 17: 60–63.

Tucker PK *et al* (1992) *Mammalian Genome* 3: 254–261.

Waggie KS *et al* (1981) *Laboratory Animal Science* 31: 139–142.

DBA/1**Origin**

Developed in 1909 by CC Little, while in Castle's laboratory at Harvard University. Carries a Y chromosome of *Mus musculus domesticus* origin (Tucker *et al*, 1992). DBA/1 and DBA/2 have identical endogenous viral genotypes (Atchley *et al*, 1991).

Predominant characteristics

1. Spontaneous inflammatory and erosive joint disease (Nordling *et al*, 1992), and collagen-induced arthritis (Hansson *et al*, 1993).

Infectious agents

Susceptible to: *Mycoplasma fermentans* (Gabridge *et al*, 1972), *Trypanosoma cruzi* (Rowland *et al*, 1992), *Coxiella burnetii* infection (Scott *et al*, 1978), *Ectromelia* (Briody, 1966). Resistant to: *Plasmodium berghei* infection (Most *et al*, 1966).

Immunological parameters

Age-dependent contact photosensitivity (Brown *et al*, 1989), low gross tumor incidence in males (Storer, 1967), DBA/1 complement sufficient, DBA/2 complement deficient (Nilsson *et al*, 1966). DBA/1 carries the H2^u haplotype, DBA/2 the H2^d haplotype (Klein *et al*, 1983). DBA/1 is resistant to most DBA/2 tumors, with nonfostered substrains carrying the mammary tumor virus (Hoag, 1963). Resistant to pristane-induced plasmacytoma development (Potter *et al*, 1992; Muller *et al*, 1996).

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DBA/2

Origin

This dilute brown strain was developed by CC Little, while in Castle's laboratory at Harvard University. There are a large number of differences between the DBA/1 and DBA/2 strains. Carries a Y chromosome of *Mus musculus domesticus* origin (Tucker *et al*, 1992).

Predominant characteristics

1. Extreme aversion to drugs of abuse, including alcohol (Phillips *et al*, 1994), morphine (Berrettini *et al*, 1994), and cocaine (Tolliver *et al*, 1995).
2. Highly susceptible to audiogenic and chemically induced seizures (Desarro *et al*, 1995; Ferraro *et al*, 1995).

Infectious agents

Susceptible to: Friend virus (Bartman *et al*, 1994); *Trypanosoma cruzi* (Reed *et al*, 1994), *Candida albicans* (Ashman *et al*, 1993).

Resistant to: *Yersinia enterocolitica*-induced arthritis (Yong *et al*, 1988), allergic encephalomyelitis (Takeda *et al*, 1993), *Salmonella typhimurium* (Kita *et al*, 1992), *Mycobacterium tuberculosis* (Flory *et al*, 1992), females resistant, males susceptible to *Leishmania mexicana* (Alexander 1988), *Plasmodium chabaudi* (Stevenson *et al*, 1982).

Immunological parameters

Hemolytic complement sufficient (Rhodes *et al*, 1980). High IgE responder (Mori *et al*, 1990). DBA/2 carries the H2^d haplotype, DBA/1 carries H2^a (Klein *et al*, 1983).

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SJL

Origin

Originated from Swiss Webster stock of three sources brought to JAX between 1938 and 1943. Pen-bred until 1955 when inbreeding was started by Jim Lambert (SJL = Swiss Jim Lambert). Carries a *Mus musculus domesticus* Y chromosome (Tucker *et al*, 1992).

Predominant characteristics

1. Develop spontaneous T cell proliferation-driven class II B cell lymphomas resembling the large cell lymphoma found in the human (Stavnezer *et al*, 1989; Tsiagbe *et al*, 1993).
2. High gross tumor incidence (Storer, 1966), with pre-B-cell lymphoma being the predominant tumor type (Mucenski *et al*, 1988).
3. Low IgE responder (Mori *et al*, 1990).
4. Mouse model for encephalomyelitis and encephalomyocarditis (Lindsley *et al*, 1991; Kang *et al*, 1993; Tuohy *et al*, 1995).

Infectious agents

Susceptible to: Friend virus-induced and DMBA-

induced leukemia (Dietz *et al*, 1972; Crispens, 1973); *Mesocostoides corti* (Lammas *et al*, 1990).

Resistant to: measles virus (Rammohan *et al*, 1980), P3193 and P2184 Sendai virus (Parker *et al*, 1978), street rabies virus (Perry *et al*, 1991), herpes simplex encephalitis (HSE) (Hudson *et al*, 1991); *Borrelia burgdorferi*-induced carditis (Barthold *et al*, 1990), *Cryptosporidium parvum* infection (Enriquez *et al*, 1991), B3502 *Cryptococcus neoformans* (Rhodes *et al*, 1980), fungal toxin sporidesmin-induced biliary tract injury (Bhathal *et al*, 1990).

Immunological parameters

Low NK activity (Lin *et al*, 1992). Susceptible to mercury-induced systemic autoimmunity (Kouri *et al*, 1973). Low susceptibility to ultraviolet B-induced contact hypersensitivity (Noonan *et al*, 1994). High level of catalytic antibodies after immunization with a *p*-nitrobenzyl phosphonate hapten (Tawfik *et al*, 1995) and high incidence of spontaneous amyloidosis (Taylor *et al*, 1984).

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SWR/J

Origin

'Swiss' mice from Lausanne, inbred at the Rockefeller Institute from about 1926 (Lynch, 1969). Now widely used in research as a general-purpose strain. Carries a *Mus musculus domesticus*-origin Y chromosome (Tucker *et al*, 1992).

Predominant characteristics

1. High level of cholelithiasis (gallstones) (Khanuja *et al*, 1995).
2. Carries no detectable endogenous ectropic MuLV DNA sequences (Jenkins *et al*, 1982).
3. Resistant to diet-induced obesity (West *et al*, 1994).
4. Susceptible to spontaneous and induced ovarian granulosa cell tumors (Beamer *et al*, 1985, 1993) and susceptible to DMH-induced colon carcinogenesis (Rosenberg *et al*, 1995).

Immunological parameters

Hemolytic complement-deficient (Rhodes *et al*, 1980). Pertussis HSF responsive (Wardlaw, 1970).

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See also: **Congenic mice; Inbred strains; Mouse inbred strains, origins of.**

MOUSE INBRED STRAINS, ORIGINS OF

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The genus *mus* is composed of a dozen different species spread practically over the whole world with the exception of cold and arid areas, and its taxonomy has remained a matter of controversy for many decades. Modern systematics, owing much to molecular techniques, has, however, successfully characterized otherwise morphologically quite similar taxa and yielded a now reliable classification. Besides ancient and widespread classical laboratory strains, most of these taxa are now established as laboratory colonies or even inbred strains and are available to investigators.

The complex species *Mus musculus*

The polytypic species *Mus musculus* finds its origins in Asia, like many murid rodents. It can be resolved into several subspecies as described below:

Peripheral species

Mus musculus domesticus (Rutti, 1772) Mice of this species are found in western Europe, around the Mediterranean basin, in Africa, in Arabia and in the Middle East. This subspecies includes geographical races sometimes improperly designated with other Latin trinomens, such as *Mus m. brevisrostris*, *Mus m. poschiavinus*, *Mus m. praetextus* etc., but these races are biochemically and genetically identical with *Mus m. domesticus*.

Although most of the animals belonging to *Mus m. domesticus* exhibit a karyotype with 40 acrocentric chromosomes, several subpopulations have been reported with varying numbers of Robertsonian translocations in different combinations.

Mus musculus musculus (Linnaeus, 1758) Individuals of this species occupy the region from eastern Europe to Japan across Russia and northern China. Like the former subspecies, individuals of this group are either commensal or wild whereas other groups are almost exclusively wild.

Mus musculus castaneus (Waterhouse, 1843) This species is found from Sri Lanka to South-East Asia including the Indo-Malayan archipelago.

The central populations

Mus musculus bactrianus (Blyth, 1846) is found

from Iran to Pakistan. This trinomen has long been attributed to populations ranging from Iran to Pakistan. According to recent work by Din and colleagues, this is a rather heterogeneous ensemble, and the *bactrianus* species *sensu stricto* are probably limited to the Afghan valleys. An accurate taxonomical description of these central populations is still needed.

Origins of the species

None of the above-mentioned subspecies are completely genetically isolated from the other three and, in those locations where they meet, there is evidence of exchanges ranging from limited introgression to complete blending. Studying the genetic relationships between the above-mentioned subspecies, Din and colleagues have proposed that the northern part of the Indian subcontinent was the cradle of the species *Mus musculus*. According to the pattern of genetic divergence observed, they proposed that the extant peripheral subspecies resulted from a radiation proceeding from the central populations in several directions outwards (Figure 1). This would have taken place between 0.5 and 1 million years ago, according to the analysis of mitochondrial DNA by Boursot and coworkers, and would account for most of the genetic variability presently available in the species.

These taxa would have secondarily and much more recently colonized the rest of the world because

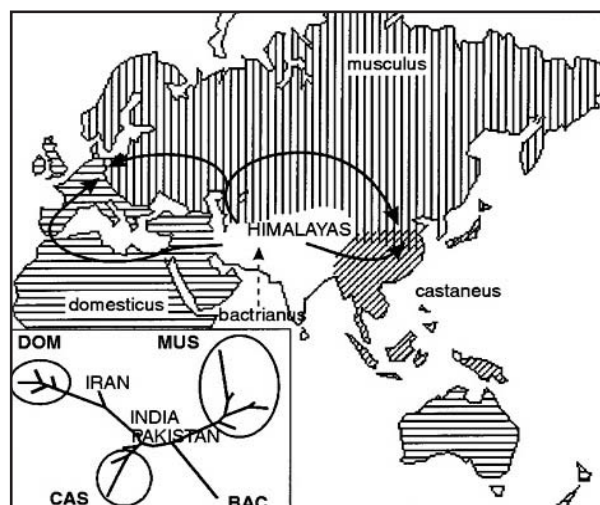


Figure 1 Map showing the proposed radiation of subspecies from a central population of *Mus musculus*.

of their capacity to interact with humans (commensalism). These exchanges justify the use of subspecific names (Latin trinomens) for their designation. The best documented cases of such gene exchanges are those occurring between *Mus m. musculus* and *Mus m. domesticus* in Europe, and between *Mus m. musculus* and *Mus m. castaneus* in Japan. In this archipelago, the two subspecies have hybridized extensively, giving rise to a unique population often referred to as *Mus m. molossinus*.

For a more exhaustive review of the genetical and ecological interactions between the various species, see Boursot *et al* (1993) and Sage *et al* (1993).

Other species of the genus *Mus sensu stricto*

Other species of the genus *Mus* are found in western Europe and in Asia.

European species

The European species are the following:

- The western Mediterranean short-tailed mouse *Mus spretus* (Lataste, 1883), found in the south of France, in Spain, Portugal and in North Africa.
- The mound-builder mouse *Mus spicilegus* (Petenyi, 1882) found in central Europe and also called *Mus hortulanus* or *Mus 4b* in previous reviews.
- The eastern Mediterranean short-tailed mouse *Mus macedonicus* (Petrov and Ruzic, 1983), also known as *Mus abbotti*, *Mus spretoideus* or *Mus 4a* and found from the former Yugoslavia to Israel and Iran. Biochemical criteria discriminate easily between these different species which, although they occur sympatrically with representatives of the complex species *Mus musculus*, never produce hybrids with them in nature. For a discussion concerning the nomenclature and systematics of these species see Auffray *et al* (1990).

Asian species

The main Asian species are the following:

- *Mus caroli*
- *Mus cervicolor*
- *Mus cookii*.

Finally, the most distantly related forms still belonging to the genus *Mus sensu stricto* (with a 40 acrocentric chromosomes complement) are the Indian pigmy species *Mus booduga* and *Mus dunni* also called *Mus terricolor*, see Sharma (1996) for details on this group.

Figure 2 represents a synthetic phylogeny of the taxa described here based on an analysis of molecular markers by She and colleagues. The multifurcations presented are points where alternative branching orders are possible due to the proximity of the splitting events and the limited resolving power of the technique. The species set described here corresponds to the group of species falling within the genus *Mus*, *sensu stricto*, which is sometimes also considered as a subgenus relatively to other more distantly related groups *Nannomys*, *Coelomys* and *Pyromys*.

Wild mice in the laboratory

Over the last 10 years, a variety of strains derived from ancestors trapped in known geographical locations and having well-defined taxonomical origins have been created in various laboratories. A list of those that are completely inbred or nearly so is given in Lyon *et al* (1996). Moreover many useful random bred stocks of wild mice are maintained in various laboratories, and a complete description of these stocks has been given in Potter *et al* (1986).

Mus m. domesticus is by far the most widely represented taxon amongst these stocks, followed by *Mus m. musculus*, *M. m. molossinus* and *M. m. castaneus*. Beside these strains, belonging to the *Mus musculus* species complex, representatives of *Mus macedonicus*, *Mus spicilegus*, *Mus spretus* and even *Mus caroli* are now maintained in the laboratory and some of them have already undergone 20 generations of sib matings.

Viable F1 offspring have been obtained between laboratory female mice and each of the European species by natural fertilization. Using artificial insemination West and colleagues recovered embryos sired by male *Mus dunni* and *Mus caroli* sperm, but

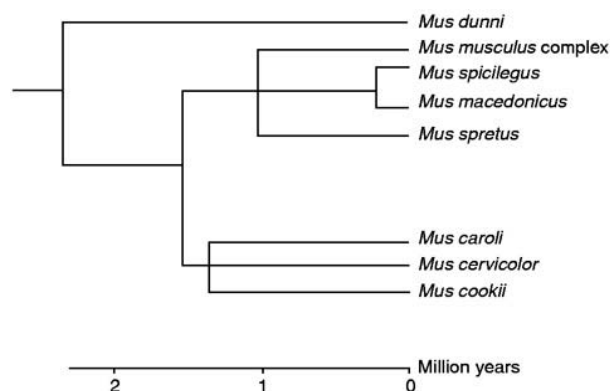


Figure 2 Phylogenetic relationships between the different species of the *Mus* genus.

failed to recover any when *Mus cervicolor* sperm was used.

Concerning interspecific hybrids, special mention must be made of *Mus spretus* because of the very large number of allelic differences involved when intercrosses are set up with any of the standard laboratory strains. Whatever the cloned DNA probe used, experience shows that it is relatively easy to find suitable restriction fragment length polymorphism (RFLPs) segregating between *Mus spretus* and laboratory strains such as C57BL/6 or BALB/c so that checking the progeny of an interspecific backcross makes gene mapping not only easier but sometimes the only way. The technique used for the first time by Robert and coworkers has been summarized in Guénet (1986). Similar crosses could be done with either *Mus macedonicus* or *Mus spicilegus*. However, they show a lesser degree of divergence from *Mus musculus* than *Mus spretus*.

The relationships between laboratory mice and wild species

Started at the beginning of this century, the establishment of genetically standardized laboratory strains owes much to the existence of a pet mouse tradition. The known pedigrees of the classical inbred strains show that many intercrosses occurred in the early part of their development, together with introductions of unknown parentage, either by purchase from pet dealers or by trapping wild animals. These laboratory lines were classically taken as being archetypes of *Mus m. domesticus* and their closeness has been demonstrated for instance by the fact that almost all of them possess the same *Mus m. domesticus* type of mtDNA. Further studies on the characterization of the Y chromosome and other genetic markers, however, have shown unambiguously that the bulk of classical inbred strains are in fact a mosaic of various genetic components derived from more than one taxon of the complex species *Mus musculus*. In particular, they almost all show a *Mus m. musculus* component (the Y chromosome in particular) that could be of Asian origin and linked to the early introduction of Japanese fancy mice (waltzing mice for instance) into the European pedigrees. Irrespective of their origin, however, it is clear that the allelic diversity they show cannot be interpreted as resulting from high mutation rates during their hundred year history. In a sense, today's classical inbred laboratory strains may be considered as interspecific recombinant inbred strains between *Mus m. domesticus* and (at least) *Mus m. musculus* and for this reason it would be wise to refer to them simply as *Mus musculus* or even *Mus 'laboratorius'*.

The approximate divergence time of *Mus musculus* from the other European species is probably around 1–2 million years as estimated by scnDNA hybridization data and it is up to 2–3 million years for the divergence between the Asian group *caroli/cooki/cervicolor* and the European species. The same techniques define *Rattus/Mus* divergence at around 8–11 million years.

See also: Inbred strains; Mouse inbred strains.

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MUCOSA-ASSOCIATED LYMPHOID TISSUE (MALT)

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The mammalian mucosal immune system is an integrated network of tissues, lymphoid and constitutive cells and effector molecules which protect the host from infection of the mucous membrane surfaces. This signifies a major difference from the peripheral immune system, where lymphoid cells and effector molecules are confined to lymph nodes and spleen, and communication occurs by cell trafficking through the lymphatic and blood circulation. The mucosal immune system is anatomically and functionally divided into sites where foreign antigens are encountered and are selectively taken up for initiation of immune responses, and sites where a more diffuse collection of B and T lymphocytes, differentiated plasma cells, macrophages and other antigen-presenting cells (APCs), as well as mast cells constitute effector tissues for mucosal immunity.

The term mucosa-associated lymphoid tissue (MALT) grew out of a knowledge base which suggested that ingested antigens were taken up into organized lymphoid tissues in the gastrointestinal tract, i.e. the gut-associated lymphoid tissue (GALT). This knowledge was extended by the realization that bronchus-associated lymphoid tissue (BALT) of experimental animals also contained precursors of immunoglobulin A (IgA) plasma cells. Thus, approximately 20 years ago the experimental evidence suggested that BALT and GALT, which collectively represent MALT, were major sites for provision of precursors of effector T and B cells for a common mucosal immunity in all effector sites. We now realize that this mucosal immune network is even more highly integrated and is finely regulated. The outcome of mucosal tissue encounters with foreign antigens and pathogens can range from mucosal secretory IgA (S-IgA), serum antibody responses and T cell-mediated immunity, to systemic anergy to orally or intranasally administered

antigen, a response commonly termed mucosal tolerance.

Organization of the mucosal immune system

In order to understand how mucosal immunity occurs, it is important to appreciate that foreign antigens, allergens and microbial pathogens are encountered either through ingestion or by inhalation. The mammalian host has thus evolved organized secondary lymphoid tissues in these regions, which facilitate antigen uptake for processing and presentation for ultimate induction of mucosal immune responses. In mice, rats and rabbits (among others), a distinct BALT occurs which appears to take up and respond to intranasal/inhaled antigens; however in humans, BALT does not develop and the major inductive tissues appear to be the palatine tonsils and adenoids (nasopharyngeal tonsils), which together form a physical barrier of lymphoid tissues termed Waldeyer's ring. Thus, we now more frequently describe tonsils and adenoids as nasal-associated lymphoid tissue (NALT) and assume that NALT are major inductive sites for intranasal/inhaled antigens. Likewise, we assume that GALT and especially the Peyer's patches are major inductive sites in the gastrointestinal tract so that collectively, MALT consists of NALT and GALT in humans and of NALT, BALT and GALT in experimental mammalian systems. These MALT inductive sites contain B and T lymphocytes which respond, in the presence of appropriate APCs, to the encountered antigen by developing into effector and memory B and T cells. These antigen-specific B and T cell populations then migrate from the inductive environment via lymphatic drainage, circulate through the bloodstream and home to mucosal effector regions. Thus, mucosal effector sites

include the more diffuse tissues where antigen-specific T and B lymphocytes ultimately reside and perform their respective functions (i.e. cytotoxicity, cytokine production or antibody synthesis) to protect mucosal surfaces.

Functional anatomy of NALT and GALT

The human tonsils and NALT

The human tonsils are the most accessible secondary lymphoid tissue for study, and as such, we know a great deal about the component cells. However, the palatine and nasopharyngeal (adenoids) tonsils are largely covered by a squamous epithelium and thus they are often not appreciated as mucosal tissues. Nevertheless, the palatine tonsils usually contain 10–20 crypts which increase their surface area. The deeper regions of these crypts contain microfold (M) cells which appear to take up encountered antigens (Figure 1). In addition, evidence for antigen presentation by epithelial cells which are class II positive (HLA-DR⁺) has been put forth; however their precise role in antigen presentation has not been documented to date. The tonsils contain all major classes of APCs including dendritic cell types, Langerhans cells, macrophages, class II⁺ B cells and antigen-retaining, follicular dendritic cells in germinal centers (Figure 1). A significant number of studies have shown that tonsillar APCs induce T cell proliferative and cytokine responses following *in vitro* stimulation with appropriate vaccines such as tetanus and diphtheria toxoid and purified protein derivative (PPD).

Approximately 50% of tonsillar cells are B lymphocytes which mainly occur in follicles containing germinal centers. Most tonsillar B cells are surface IgG positive (sIgG⁺); although significant numbers of sIgM⁺ and sIgA⁺ B cells are also found. Nevertheless, it is difficult to describe the tonsils as only a mucosal IgA inductive site, due to the presence of B

cells committed to other isotypes, especially for IgG subclasses. Approximately 40% of tonsillar cells are T cells and >98% of these express the $\alpha\beta$ T cell receptors (TCR). Somewhat higher ratios of CD4:CD8 cells are found in tonsils (3:1) when compared with peripheral blood (~1.5:1). In summary, the tonsils clearly have features of mucosal inductive sites; however their role in host mucosal immunity following intranasal immunization is not fully established.

Peyer's patches and GALT

The GALT is well-developed in most mammalian species and has been especially well-studied in rabbits and mice. GALT includes: 1) the Peyer's patches (PP), which are distinct nodules found mainly in the small intestine (10–12 in mice), and 2) the appendix, which is most pronounced in rabbits and humans. Mammals also contain solitary lymphoid nodules (SLN), smaller follicles throughout the small intestine and colon which, because of their morphologic similarities to PP, may represent more microscopic IgA inductive sites. Although the PPs, appendix and SLNs share similarities such as a dome region containing a specialized epithelium, important differences exist. The appendix, for example, has a less developed parafollicular region (T cell zone, Figure 2) than PPs, and the relative proportions of B cell subsets isolated from the appendix differ in numbers of sIgA⁺, sIgG⁺ and sIgM⁺ cells from B cell subsets obtained from PPs. Generally, most studies with GALT cells have been done with PPs, because it is assumed that the PPs are the major component of GALT. More studies are required to verify that this assumption is correct.

Discovered by Peyer in 1677 in human intestines, PPs were shown by Cornes in 1965 to develop during gestation, reach maximum numbers at puberty (>200), and decline slowly with age. PPs, present in

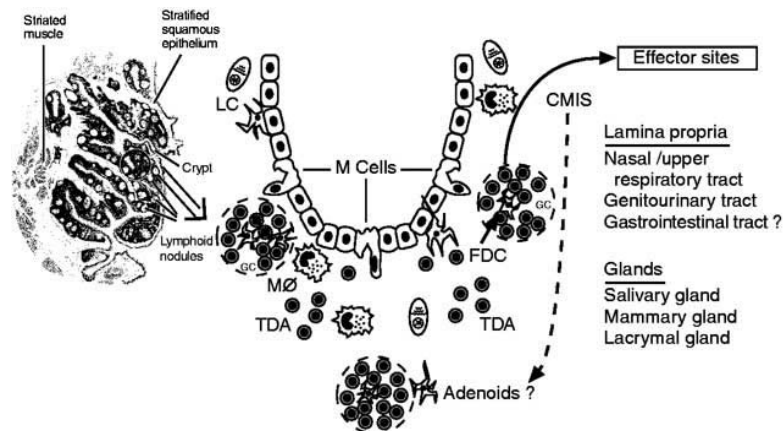


Figure 1 The potential role of NALT for development of mucosal immunity. A palatine tonsil with deep crypts illustrates the microanatomy. The cartoon portion shows the major APCs (LC, Langerhans cells; MØ, macrophages; FDC, follicular dendritic cells), B cell germinal centers and parafollicular T cell-dependent areas (TDA). Effector/memory lymphocyte homing occurs via the common mucosal immune system (CMIS).

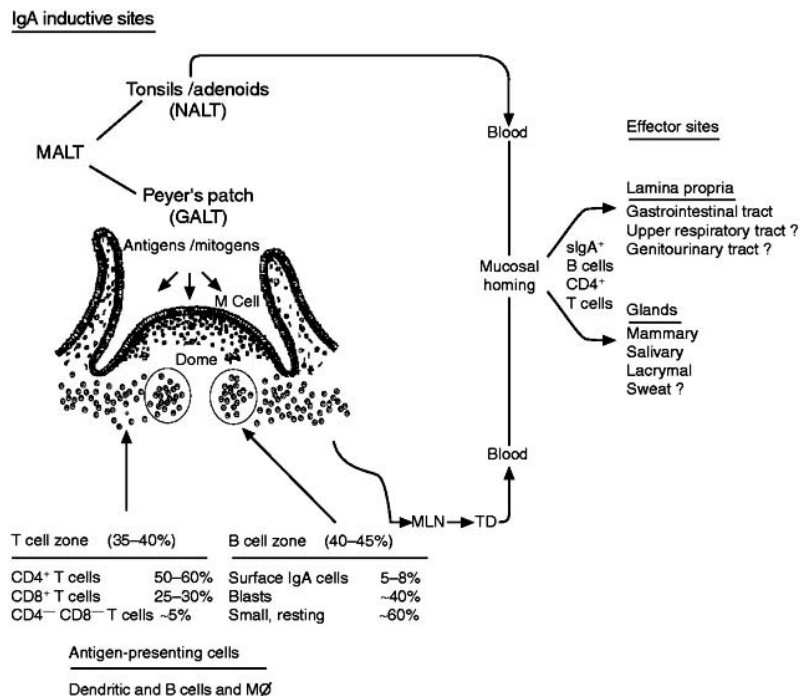


Figure 2 The role of MALT in the induction of mucosal IgA responses. Antigen uptake by M cells occurs in GALT and NALT (MALT) and results in the initial induction of the immune response. Antigen-sensitized, precursor sIgA⁺ B cells and CD4⁺ T helper cells leave via efferent lymphatics and migrate to mesenteric lymph nodes (MLN) and then into the thoracic duct (TD) to reach the bloodstream. These migrating cells enter the IgA effector sites where terminal differentiation, synthesis and transport of S-IgA occurs. This induction in MALT and exodus of cells to effector sites is termed the common mucosal immune system.

all experimental animals, have been described in some detail in rabbits, mice and rats and to a lesser degree in swine, calves, dogs, goats and in subhuman primates. Not only is it likely that all mammalian species have PPs as IgA inductive sites, some studies have also suggested that avian species exhibit PP-like structures, which may also function as IgA inductive sites.

An important feature of PPs is the epithelium covering the dome region. This epithelium, which consists of cuboidal (not columnar) epithelial cells and a few goblet cells, contains little mucus to impede antigen uptake. Lymphocytes lie next to the epithelium, leading to the term follicle-associated epithelium (FAE). Specialized antigen-sampling cells, the M cells, are found in this FAE and also occur in appendix, SLNs and NALT (Figure 1). M cells exhibit short microvilli, small cytoplasmic vesicles, and thin extensions around the lymphoid cells in the lymphoepithelium. They can actively pinocytose proteins or phagocytose particular antigens, including virions, bacteria, and even small parasites. Because uptake of antigen does not result in degradation in lysosomes, the intact antigen can reach underlying lymphoreticular cells, including those which present antigen (Figure 2). In some instances, pathogenic viruses and bacteria enter the host by passing through M cells to reach systemic tissues, a process termed translocation. Thus, the M cell, in the absence of a mucus layer, serves as an antigen-sampling mechanism for uptake from the gut lumen. It is unlikely that M cells

actually present antigen in a class I or class II restricted sense, since they usually do not express major histocompatibility complex (MHC) class II determinants; however, they are important for antigen delivery to underlying APCs for induction of immune responses. More studies are required to determine the cellular origin of M cells and to understand the regulation of their function by cytokines and microbial cell products.

The PPs are divisible into three regions: 1) the dome, 2) the follicles with germinal centers (B cell zone), and 3) the parafoallicular or T cell-dependent area (T cell zone) (Figure 2). Mucosal lymphocytes enter the PPs through high endothelial venules (HEV) in the parafoallicular region (see mucosal lymphocyte homing below) and all cells exit the tissue via efferent lymphatics which drain into the mesenteric lymph nodes (MLN) (Figure 2). Each PP region contains a unique array of cells. The dome contains B and T cells with significant numbers of macrophages and small numbers of plasma cells. The B cell follicles normally exhibit 1–2 germinal centers, which are enriched in sIgA⁺ B cells and in which there is continual B cell class switching to IgA. The T cell zone possesses MHC class II⁺ antigen-presenting, dendritic cells. The PPs, which are less well-developed at birth, are first populated by T cells from the thymus and only later do distinct B cell zones develop. These B cell zones are dependent upon the gut microflora, since germ-free animals exhibit poorly developed PPs with no germinal centers.

Disassociated PP cells

The use of enzymatic procedures has allowed the isolation of presumably all major lymphoreticular cells from murine PPs. This analysis has shown that ~40% (or more) of isolated cells are of B lineage and ~35% are mature T cells of various subsets (Figure 2). Most isolated B cells are sIgM⁺; however, higher numbers of sIgA⁺ B cells occur (5–8%) than are seen in other secondary lymphoid tissues. Two major cytokines, interleukin 4 (IL-4) and tumor growth factor β (TGF β), have been shown to induce activated sIgM⁺ B cells to switch to downstream isotypes and TGF β has been most effective for switches to IgA. This cytokine induces $\mu \rightarrow \alpha$ switches in mice and to $\alpha 1$ and $\alpha 2$ in humans; the actual switches are preceded by induction of sterile α transcripts. Elegant studies have also provided direct evidence that somatic mutations occur during germinal center responses under control of T helper cells, a time when antigen-specific B cells form high-affinity Ig receptors. Presumably, isotype switches to IgA take place in NALT and GALT; however *in vivo* evidence that T helper cells/cytokines play a role in germinal center B cell switches to IgA have yet to be demonstrated. A significant number of PP B cells are dividing, which is consistent with the prominent germinal centers and continuous influx of environmental antigens into the PPs. Further, this explains why the PPs are major IgA inductive sites for response to locally encountered antigen, but does not explain why the dividing B cells fail to differentiate terminally into IgA (and other isotype)-producing plasma cells in the PPs themselves.

The IgA response is T cell-dependent and the PPs contain significant numbers of T cells. The majority of isolated CD3⁺ TCR⁺ T cells are CD4⁺ and exhibit the characteristics of T helper cells; however, CD3⁺, CD8⁺ T cells also occur with cytotoxic functions. For example, immunization of gut segments with reovirus results in the induction of CD8⁺ cytotoxic T lymphocytes (CTLs). Two major types of CD4⁺ T cells have been isolated and cloned from PPs. One type induced sIgM⁺ to sIgA⁺ B cell switches whilst the other preferentially supported IgA synthesis.

T_H2 cytokines, e.g. IL-5, IL-6 and IL-10, are of particular importance for inducing sIgA⁺ B cells to differentiate into IgA-producing plasma cells. In this regard, IL-6-induced high IgA responses *in vitro* in both mouse and human systems, and mice with targeted disruption of the IL-6 gene showed greatly reduced numbers of IgA plasma cells. One would predict from these observations that higher frequencies of T_H2 cytokine-producing T cells may occur in mucosal effector sites; however, as discussed

below it has been difficult to activate isolated lamina propria CD4⁺ T cells.

All major types of APCs also occur in significant numbers in the various PP regions. Macrophages, including both classical and tingible body types associated with uptake of apoptotic cells, are found in the dome region and germinal centers, respectively. The T cell zones are enriched in dendritic cells (DC), which have been shown in functional assays to form clusters with CD4⁺ T cells that are adept in supporting IgA synthesis. No studies have addressed the role of APCs in the three major PP regions, i.e. the dome, T or B zones after M cell uptake, and especially whether the B cells (which express MHC class II) can function in this capacity. The role of persistent antigen could be important for the generation of sIgA⁺B and CD4⁺ T helper cell responses in Peyer's patches. In this regard, it is well-established that follicular dendritic cells (FDC) in B cell areas of the tonsils can retain native antigen, usually via antigen-antibody complexes. The slow release of these complexes may be important for stimulation and expansion of antigen-specific B cell clones. The Peyer's patch contains FDCs, and these cells may serve a similar role in this IgA inductive site; however, more studies will be required to determine the possible role for persistent antigen and IgA immune complexes in these mucosal inductive sites. Antigen trapped by FDCs (as immune complexes) is released in the form of immune complex coated bodies (iccosomes). Specific germinal center (GC) B cells find iccosomes remarkably palatable and endocytose them. Iccosomal antigen is then processed with great efficiency and is presented to T cells. The antigen, or possibly IgA immune complexes, may associate with FDCs in the GC and release iccosomes for prolonged periods. This slow release may be sufficient to allow selective induction of antigen-specific CD4⁺ T helper and sIgA⁺ B cells in the Peyer's patches. A second encounter with antigen would result in expansion of CD4⁺ T helper cells and increased IgA responses in mucosal effector tissues.

Mucosal lymphocyte homing

Lymphocytes of B and T lineages recirculate via the bloodstream and re-enter secondary lymphoid tissues. This lymphocyte homing of naive cells insures that a diverse repertoire exists in mucosal and peripheral lymphoid tissues, while homing of memory and effector cells is responsible for keeping lymphocytes compartmentalized, i.e. mucosal in the mucosa and peripheral in the periphery. It is now clear that lymphocytes leave the blood and enter lymphoid compartments (usually via HEVs) in a

multistep extravasation process. Homing is dependent upon the expression of adhesion molecules such as selectins and integrins, collectively termed 'homing receptors' on lymphocytes, with specific ligands on HEV and venule endothelial cells which are collectively termed 'addressins'.

Some generalizations can be made to this rapidly changing area. First, naive T and B lymphocytes can express homing receptors for addressins on either peripheral or mucosal inductive tissues, i.e. peripheral lymph nodes versus Peyer's patches. It is generally accepted that naive lymphocytes can recirculate between secondary lymphoid tissues; however only memory or effector lymphocytes have receptor-ligands for movement into certain microenvironments such as lamina propria or the epithelium of the intestinal mucosa. Peripheral lymphocyte homing is mainly controlled by expression of L-selectin and the ligand peripheral lymph node addressin (PNAd); however, other receptors and ligands are also involved. Naive mucosal lymphocytes express β_7 -integrin family members, particularly $\alpha_4\beta_7$. Peyer's patch HEVs and gastrointestinal tract lamina propria express the mucosal addressin cell adhesion molecule (MAdCAM-1) which serves as the specific ligand for $\alpha_4\beta_7$. Interestingly, mucosa-derived memory lymphocytes express higher levels of $\alpha_4\beta_7$, which presumably allows for binding to MAdCAM-1 on PP HEVs as well as to the low level expression of this addressin on lamina propria venules in the gastrointestinal tract. Although $\alpha_4\beta_7$ -MAdCAM-1 are the major receptor-ligand for mucosal homing in the gastrointestinal tract, other receptor-ligand combinations are also involved.

A second β_7 -integrin, $\alpha_E\beta_7$ is expressed on >98% of human intraepithelial lymphocytes (IELs, see below) and the ligand for this homing receptor is E-cadherin expressed on the basolateral portion of epithelial cells. It has been shown that TGF β markedly enhances the expression of $\alpha_E\beta_7$ on IELs, and thus promotes their increased adherence to epithelial cells.

Perhaps the most intriguing aspect of mucosal lymphocyte homing is that no lung-specific integrins have yet been identified. Further, the homing receptors present on BALT (in rabbits) and NALT (in humans) which directs mucosal lymphocytes to other regions, e.g. the lungs and the genitourinary tract, remain to be fully defined. Nevertheless, it is known that intranasal immunization is quite effective for induction of mucosal IgA responses in the upper respiratory and the female reproductive tracts. It is tempting to suggest that NALT lymphocytes bear homing receptors for addressins expressed in these mucosal effector sites. Clearly other integrins and addressins may be involved and may ultimately

explain how MALT cells from NALT redistribute more frequently to certain effector sites, while GALT repopulate common as well as different mucosal effector sites.

Mucosal effector sites

It is known that local application of antigen on to mucosal tissues or direct injection into exocrine glands can result in the production of S-IgA antibodies at that site. This local response often requires the use of adjuvants, results in an influx of inflammatory cells, and subsequently leads to the production of antibodies (of IgG, IgM and IgA isotypes) in the serum. It is likely that this local stimulation results in an accumulation of clonal B and T helper cells (from MALT ?) and that cognate interactions induce the terminal differentiation of IgA plasma cells and local S-IgA responses. The leakage of antigen-adjuvant from local mucosal sites and their entrapment in systemic lymphoid tissues probably accounts for the serum antibody response. However, it is important to emphasize that in humans the serum IgA (largely monomeric IgA1) is derived from the bone marrow, spleen and peripheral lymph nodes. Further, >98% of S-IgA antibodies present in human external secretions (principally as dimeric IgA1 and IgA2 subclasses) are locally produced by plasma cells in mucosal tissues. The polymeric IgA (with J chain) is selectively transported by the polymeric Ig receptor secretory component (SC) that has been produced by epithelial and ductal cells and is released at the luminal surface as a polymeric IgA molecule containing J chain and SC.

The best-studied effector sites are the lamina propria (LP) and the intraepithelial lymphocyte (IEL) compartments in the gastrointestinal tract. Interestingly, the LP of the gastrointestinal tract is the only tissue where large numbers of plasma cells occur under normal physiological conditions. Seventy to ninety per cent of these plasma cells are of the IgA isotype and daily IgA production in the human adult is estimated at 3–4 g per day. In the mouse, the average lifespan of these cells is approximately 5–9 days, and thus these cells must be continuously replaced, presumably by GALT-derived precursors. It is generally assumed that migrating, sIgA⁺ B cells rapidly differentiate into IgA plasma cells in the presence of T cell and cytokine help.

Estimates of the percentage of T cells amongst LP lymphocytes range from 40 to 90% and this wide range is most likely due to the different techniques used to identify cells (*in situ* immunohistochemical staining versus analysis of isolated cells prepared by enzymatic digestion). The majority of CD3⁺ T cells

in the LP (65–80%) are CD4⁺, again consistent with the observation that the LP is the major site of T helper cell-regulated IgA synthesis. The LP T cells demonstrate lower proliferative responses to T cell mitogens when compared to T cells from lymphoid tissues or the peripheral blood; however, these T cells proliferate spontaneously when cultured in the presence of IL-2, and LP T cells spontaneously secrete cytokines. These observations are consistent with the hypothesis that LP T cells are activated *in vivo*. More studies are needed to fully characterize the lymphoid cells present in the LP of the gastrointestinal tract. The role of MHC class II⁺ B cells and macrophages in the activation of CD4⁺ T cells in this region should also receive more emphasis. Further, the mechanisms whereby T cells provide help for the terminal differentiation events for B cells is another area for study. In spite of the difficulties in enzymatic isolation of functional cells and in triggering T cells, this will no doubt be a major focus of future studies in the mucosal immune system.

Intraepithelial lymphocytes

Intraepithelial lymphocytes (IELs) reside between intestinal epithelial cells and are the first cells to contact luminal antigen that crosses the mucosa in an M cell-independent manner. This heterogeneous population of mainly T cells comprises a very large number of cells (more than half the number of T cells in peripheral organs) but their function remains poorly understood. The majority of murine IELs (80%) are CD3⁺, and over 75% of these also express CD8. In mice and chickens, significant numbers of $\gamma\delta$ TCR⁺ IELs are found (40–60%) in addition to $\alpha\beta$ TCR⁺ cells; however, this number varies with mouse strain, age and housing conditions. The $\gamma\delta$ TCR⁺ IELs represent a minor component of human IELs (~15%) under normal conditions, but significantly increase under certain conditions, such as celiac disease.

The physiological role of IELs is poorly understood; however, several functions for these cells have been described and this diversity is consistent with their phenotypic heterogeneity. IELs have been shown to mediate antigen-specific delayed-type hypersensitivity (DTH) responses, to exhibit virus-specific CTL function, to express natural killer (NK)-like activity and to produce a local graft-versus-host reaction (GVHR) when transferred to semiallogeneic hosts. IELs respond poorly to conventional T cell mitogens when compared to splenic T cells; however, there is evidence that human IELs are induced to proliferate via a CD2 pathway rather than through stimulation of the CD3–TCR complex. With regard to regulation of humoral immunity, $\gamma\delta$ TCR⁺ IEL T

cells have been shown to abrogate the induction of mucosal tolerance, while $\alpha\beta$ TCR⁺ IEL T cells provide help for production of IgA. Interestingly, the targeted disruption of the δ chain of TCR in mice results in a lack of $\gamma\delta$ T cells among IELs and in a mucosal IgA deficiency, suggesting that $\gamma\delta$ T cells may upregulate CD4⁺ T helper cells for mucosal immunity. IELs can be induced to produce a variety of cytokines which are characteristically produced by T_H1- and T_H2-type cells and $\alpha\beta$ TCR⁺ IELs can also provide help for B cell responses.

The common mucosal immune system

Studies in humans have also shown that orally administered antigen results in the presence of S-IgA antibodies in remote external secretions. For example, studies have shown that ingestion of killed bacterial vaccines, i.e. *Streptococcus mutans* or *Vibrio cholerae* plus cholera toxin B subunit (CT-B), induce specific S-IgA antibodies in external secretions but not in serum. Mitogen stimulation of human peripheral blood lymphocytes results in the synthesis of polymeric IgA (serum IgA is mostly monomeric) and in the induction of both IgA1 and IgA2 plasma cells. Further, the peripheral blood B cells of human volunteers ingesting bacterial vaccines produced specific IgA antibodies, suggesting that they were in a mucosal homing pathway. In fact, studies with several viral and other bacterial vaccines have clearly shown the subsequent presence of S-IgA antibodies in various human external secretions.

Equally compelling work in experimental animals is providing evidence that intranasal immunization is a particularly effective way to induce mucosal S-IgA as well as serum antibody responses. In these studies, less vaccine antigen and mucosal adjuvant is required for induction of effective mucosal immunity. Further, intranasal immunization appears to be particularly effective for induction of S-IgA antibodies in nasal washes, saliva, tears and in general nongastrointestinal external secretions. It is intriguing that this route of immunization is particularly effective in priming for genitourinary tract responses, and offers promise for development of vaccines to prevent sexually transmitted diseases.

In summary, mammals possess a common mucosal immune system, in which antigen stimulation of MALT induces an exodus of specific lymphocytes which home to the various mucosal effector sites (Figures 1 and 2). These responses are finely regulated and T cells and cytokines are of central importance for ultimate plasma cell differentiation and production of S-IgA antibodies in external secretions. The current need for vaccines, including the universal

efforts to develop immunity to human immunodeficiency virus (HIV), compels us to increase our understanding of how we can use the common mucosal immune system to advantage for the ultimate prevention of infectious diseases at mucosal sites.

See also: B lymphocyte activation; Cytokines; Gastrointestinal tract infections; Helper T lymphocytes; IgA; Immunoglobulin class switching; Joining J chain; Lymphocyte trafficking; Oral immunity; Oral tolerance; Secretory component (the polymeric Ig receptor); Vaccines.

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MUCOSAL IMMUNITY

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The mucosal immune system is comprised of specialized structures and sites, including Peyer's patches, bronchus-associated lymphoid tissue (BALT), local draining lymph nodes, and the lamina propria and epithelium, that contain a complex mixture of cells that undergo continuous activation, migration and homing, and terminal differentiation and effector functions including release of antibodies and numerous mediators. This complicated system has evolved under two apposing evolutionary pressures, on the one hand to provide the host with protective mechanisms against invasion by potential pathogens, such as bacteria, viruses and protozoans, across mucosal surfaces, and on the other hand the equally necessary function of permitting immunological tolerance against the wide array of harmless environmental antigens and products of normal digestion and the normal intestinal flora.

Nonimmunological host defense factors

Nonspecific and specific immune mechanisms in the gastrointestinal tract function in concert with other nonimmunological mechanisms to protect the host. The main effect of all of these factors is to limit the growth of organisms in the gastrointestinal tract and prevent entry of pathogens across the mucosa. The normal motor propulsive activity of the gastrointestinal tract is also crucial in limiting the growth of organisms in the small intestine. In addition, the release of mediators by mast cells that increase intestinal contractility is thought to be a primary mechanism of expulsion of pathogens in intestinal parasitic infections. Mucin is thought to be protective for the mucosa. The effect of mucus may be to bind and trap bacteria, thus limiting their interaction with epithelial cells. In the colon, the presence of a normal flora is thought to limit, through multiple mechanisms, the overgrowth of pathogens. Lactoperoxidase is released into the intestinal lumen, and it may act in concert with immune factors such

as immunoglobulin A (IgA) to limit the growth of pathogens. Recently, gastrointestinal Paneth cells have been shown to produce a class of defensin molecules named cryptdins. These molecules have a unique structure and have antibacterial properties, among others. All of these mechanisms are to some extent redundant, so that an alteration or absence of one of them, such as gastric acid, may not lead to any obvious pathological consequences if other mechanisms remain intact.

Structural organization of the mucosal immune system

Lymphocytes associated with mucosal sites are located in three major compartments: 1) organized tissue such as the Peyer's patches, BALT, tonsils, mesenteric lymph nodes and appendix; 2) the nonorganized lymphoid cells present diffusely throughout the lamina propria; and 3) the lymphocytes present within the mucosal epithelium, the intraepithelial lymphocytes (IELs). Lymphoid cells in organized structures such as the Peyer's patches are thought to be primarily less mature cells that play an important role in initiation of immune responses, i.e. they carry out functions of the 'afferent' limb of the immune response due to their interaction with antigens traversing the specialized M cell epithelium of these sites. On the other hand, lymphocytes in the diffuse lamina propria compartment are more mature cells, many of which are activated, that are thought to carry out effector functions, such as IgA production. The function of intraepithelial lymphocytes *in vivo* is uncertain, although their phenotypic and functional characteristics *in vitro* suggest that they also carry out host defense effector functions and may also have important interactions with enterocytes.

Organized mucosal lymphoid tissue

It has been estimated that about 10% of lymphoid cells in humans are associated with the organized lymphoid structures of the gastrointestinal tract. Peyer's patches are located throughout the jejunum and ileum of humans, being most concentrated in the terminal ileum. Lymphoid aggregates are also found in the colon, particularly in the rectum, however they differ from the typical small bowel aggregates in having relatively few activated B cell germinal centers. Peyer's patches and lymphoid aggregates are well developed in fetal life, but germinal centers do not appear until after birth, presumably due to the initiation of stimulation from food antigens and bacteria. This is supported by the observation that germ-free animals do not develop germinal centers. The number of Peyer's patches increases to about 250

during adolescence; their numbers decline rapidly initially after puberty, and then gradually, numbering about 100 in elderly individuals. The Peyer's patches are characterized by the presence of a specialized epithelium which contains M cells. M Cells appear during fetal life along with subepithelial lymphocytes. These cells are interspersed among absorptive epithelial cells covering the dome of the Peyer's patch on the luminal side. In contrast to the microvilli present on absorptive cells, M cells have only occasional short microvilli on their surface and short ridge-like folds. M Cells are actively pinocytotic and it appears that they transport much of what they take up to the underlying subepithelial lymphoid cells. This has led to the concept that they provide a critical sampling process of the contents of the intestinal lumen that allows the mucosal immune system to continuously sample the luminal contents. Many different types of macromolecules, particles and intact pathogens, including bacteria, viruses and protozoans, have been shown to be taken up by M cells. Small molecules may also cross the M cell by fluid phase endocytosis.

The nodules and germinal centers of Peyer's patches are composed primarily of B cells. The germinal centers contain central activated B cells and antigen-presenting cells and dendritic cells, and there is a surrounding dense layer of resting B cells. Adjacent to the germinal centers are interfollicular zones that contain predominantly CD4 T cells. The T cells of the Peyer's patch are thymic dependent, as demonstrated by the fact that thymectomy depletes cells from the interfollicular zone of the Peyer's patch. B Cells in the germinal center are enriched in surface IgA-positive cells. However, IgA-positive B cells are only a minority of B cells present in this site, and there is evidence that Peyer's patch germinal center B cells undergo immunoglobulin class switching to the IgA phenotype. Cells activated in the Peyer's patch do not mature into immunoglobulin secreting cells in this site; rather, once activated, they migrate to mesenteric lymph nodes, undergo further division, and home back to the lamina propria where terminal differentiation into IgA-secreting plasma cells occurs.

Functional antigen-presenting cells are present in the Peyer's patches. Dendritic cells and macrophages are present directly beneath the epithelium of the Peyer's patch where they are likely to be the first cells to encounter antigens transported by M cells. Antigen-presenting cells are also present in the germinal centers and interfollicular areas. Both cell types are capable of antigen presentation, and both may also carry out other roles via cytokine production. In particular, dendritic cells may play an important role in class switch of B cells towards IgA commitment.

Diffusely distributed mucosal lymphoid cells

The nonorganized lymphoid cell compartment located diffusely within and beneath the mucosal epithelium contains a large, complex mixture of cells that are thought to play an essential role in immune effector functions in the gastrointestinal tract. The lamina propria contains a large number of T cells, B cells and plasma cells, macrophages, mast cells and smaller numbers of eosinophils, neutrophils and dendritic cells. About 80–90% of the B cells and plasma cells in the normal gastrointestinal tract are IgA positive. Most of the non-IgA committed cells are IgM-positive, with about 1–2% each of IgG- and IgE-positive cells. About one-third of the cells in the lamina propria are T cells, and while representing a heterogeneous group of cells, they also have features that distinguish them from the systemic immune system.

Mucosal T cells

The effector functions mediated by lymphoid cells in the lamina propria are probably essential for effective host defense against pathogens. T cells are found in the epithelial layer (intraepithelial lymphocytes, see below) and in the loose connective tissue compartment of the lamina propria. Due to the large size of the intestine, the intestinal lamina propria represents the largest T cell site in mammals, and particularly in humans. A significant number of T cells in the intestinal mucosa are activated. The phenotypic distribution of CD4 and CD8 T cells is similar to that of peripheral blood. T cells in the intestinal lamina propria have almost exclusively the CD45RA low (in humans and primates) and CD45RB low (in rodents) phenotypes that are typically found on 'memory' lymphocytes. Although lamina propria T cells appear to only infrequently undergo proliferation, their activation can lead to high levels of cytokine gene expression. Most T cells in the human lamina propria express either CD4 or CD8 and $\alpha\beta$ T cell receptors (TCRs), suggesting that they have arisen from the bone marrow and undergone prior thymic education. The TCR repertoire of lamina propria T cells is very diverse, and is not limited as it is in the epithelium and certain other sites.

Intraepithelial lymphocytes

The intestinal mucosa contains a specialized subset of lymphocytes that are interspersed within the epithelial layer and referred to as intraepithelial lymphocytes (IELs). While the major physiological functions of these cells is still uncertain, a number of possibilities are under active study, including their role in host defense as cytolytic cells and their

response to antigens presented on the surface of adjacent epithelial cells. It is possible that IELs and epithelial cells may interact in ways that modify each other's functions. The IEL subpopulation of cells in both mice and humans is phenotypically pleomorphic, but certain features characterize this subset: in humans the majority of cells are CD8⁺, CD3⁺ and have a large granular lymphocyte morphology. In addition, while the majority in humans have $\alpha\beta$ T cell receptors, there is an increased proportion of cells having $\gamma\delta$ receptors that are rare in the peripheral circulation. In mice, many of the cells are Thy-1 negative, and a substantially greater proportion of IELs have $\gamma\delta$ T cell receptors, although this proportion seems to be highly variable, depending on environmental factors such as the presence or absence of pathogens in the animal colonies. In humans, an increased proportion of IELs express activation markers such as CD25 compared to peripheral blood, and a high proportion express the $\alpha_E\beta_7$ -integrin adhesion molecule. Interestingly, it is difficult to maximally activate IEL proliferation *in vitro* with mitogens or anti-CD3 alone; however, these cells can exhibit high proliferation when triggered through CD2 by using sheep erythrocytes or combinations of anti-CD2 monoclonal antibodies. These results clearly indicate that these lymphoid cells may require special types of cell–cell interactions for triggering.

B cells in mucosal immunity

The gastrointestinal mucosa is characterized by a great preponderance of IgA B cells and IgA-secreting plasma cells. There is evidence that exogenous factors, including T cell cytokines such as transforming growth factor β (TGF β), play a role in class switching to IgA. However, the understanding of this event is still incomplete. Furthermore, the site at which class switching of these cells occurs is still unknown. Clearly, the bone marrow is a major site of IgA production and this event can occur in that site. Although the Peyer's patches are enriched in IgA-bearing cells, it has also been thought that IgA switch may be occurring in this site. The events that control the maturation of IgA B cells into IgA-secreting plasma cells is better understood. Although B cells can respond to soluble antigens in the absence of antigen-presenting cells, for many antigens the process of terminal differentiation into plasma cells requires T cell 'help'. The factors provided by T cells include cytokines that act in synergy, including interleukin 2 (IL-2), IL-4, IL-5, IL-6 and interferon γ (IFN γ). In addition, it seems likely, although not proved, that cell contact between T and B cells may

provide additional 'cognate' interactions that augment IgA B cell differentiation.

Mucosal mast cells

Mast cells are derived from the bone marrow and migrate to tissue sites throughout the body. There is evidence in animal models that connective tissue mast cells differ in a number of ways from mast cells found in mucosal sites, such as in having relatively little heparin and the serine protease chymase. Mast cells are numerous in the gastrointestinal submucosa, often being found in close proximity to blood vessels, although they are often not recognized unless special stains are used for histological examination. Mast cells contain numerous preformed mediators, such as histamine, proteoglycans and serine proteases, and have the ability to synthesize numerous other inflammatory mediators such as prostaglandins, leukotrienes, platelet-activating factor (PAF) and cytokines, including IL-3, IL-4, IL-5, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Mast cells have high-affinity IgE receptors, so that in tissue sites they may be 'armed' by the presence of preformed IgE with specific antigen specificity. Upon encounter with antigen, the mast cell rapidly releases its preformed mediators and may synthesize others. The mediators produced by mast cells have a wide range of physiological and pathophysiological effects both on the immune system and elsewhere. Thus, for example, during intestinal parasite infection mast cell products may contribute to

increased intestinal motility and fluid and electrolyte transport that is important in host defense.

Regulation of lymphocyte recirculation to the mucosa (homing)

An important characteristic of the immune system and the mucosal immune system in particular is the mobility of its cellular components. B cells and T cells originate in the bone marrow and thymus and migrate to the Peyer's patches (Figure 1). Upon stimulation by antigens or pathogens, cells either become tolerized or become activated, upon which they migrate to mesenteric lymph nodes, whereupon they enter the systemic immune system to recirculate. Cells that have been stimulated in the mucosal immune system have a tendency to recirculate to the sites where they were stimulated (homing), which allows the mucosal immune system to be repopulated with cells having memory for gut antigens and with cells that can mediate effector functions against pathogens on rechallenge. Because cells that are stimulated in the gastrointestinal tract can also recirculate to other mucosal sites, such as those of the lung, lactating breast and genitourinary tract, the concept of a 'common mucosal immune' system has been postulated. Although there is only partial cross-over of lymphocytes from the pulmonary and genitourinary system, this notion is important because it suggests the possibility that general protective immunity at mucosal sites could possibly be gener-

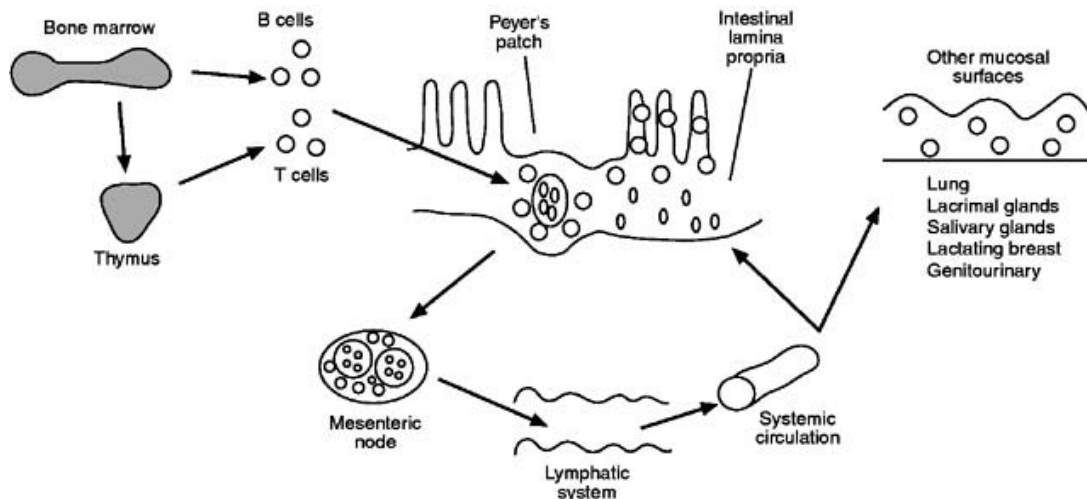


Figure 1 Origin and recirculation of mucosal lymphocytes. Most if not all lymphoid cells in the gastrointestinal tract originate in the bone marrow. B cells migrate to Peyer's patches, where they tend to undergo further commitment to the IgA lineage. T cells undergo thymic education and then migrate to Peyer's patches and may also undergo specialized differentiation. A small subpopulation of T cells, those bearing $\gamma\delta$ T cell receptors, have a propensity to migrate to the intestinal epithelium. Cells that are activated in the Peyer's patch probably leave the patch soon after activation and undergo further rounds of proliferation in mesenteric lymph nodes. Cells that have been activated in the Peyer's patches have a tendency to migrate back to or 'home' to mucosal surfaces, particularly of the gastrointestinal tract.

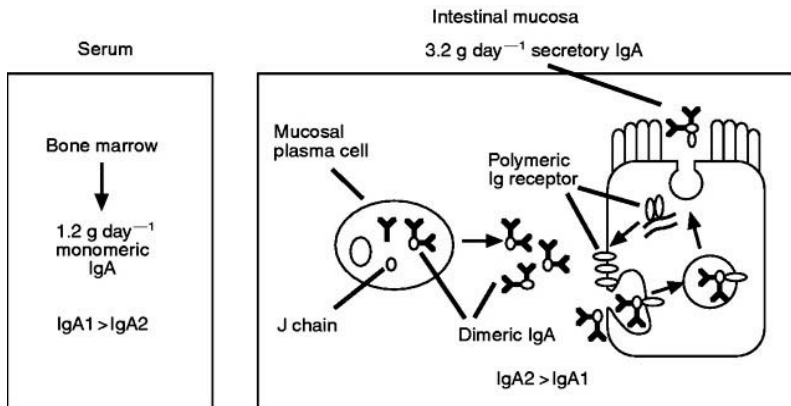


Figure 2 IgA in serum and secretions are different. IgA in serum is predominantly the product of bone marrow cells, there is more IgA1 than IgA2, it is produced at a rate of about 1.2 g day⁻¹ and is monomeric. In contrast, secretory IgA is produced primarily by intestinal plasma cells and contains J chain, also produced by plasma cells. There is more IgA2 than IgA1 and it is produced at a rate of about 3.2 g day⁻¹. Finally, IgA in secretions contains a portion of the receptor used for translocation across the epithelial cell, the polymeric immunoglobulin receptor (secretory component).

ated by use of an immunogenic oral vaccine. The best evidence for selective migration is for IgA B cells, but there are also some data to show that T cells may also undergo selective migration.

A number of cell surface adhesins appear to be important in homing. In particular the $\alpha_4\beta_7$ -integrin expressed on some activated lymphocytes seems to be a ligand for MAdCAM-1 (mucosal addressin cell adhesion molecule 1) expressed on mucosal endothelial cells. There is some evidence that L-selectin and $\alpha_4\beta_7$ -integrin may be synergistic for migration of lymphocytes into Peyer's patches. A second ligand, $\alpha_E\beta_7$ -integrin is highly expressed on intraepithelial lymphocytes and appears to be a receptor that is important for retention of lymphocytes in the gastrointestinal epithelium. Other relevant structures may include the fibronectin, vitronectin and hyaluronic acid receptors (CD44) on lymphoid cells, which allow the cell to adhere to components of the extracellular matrix. It is clear that these ligands on lymphocytes and endothelial cells are not static but are dynamically regulated by factors such as cytokines and inflammatory mediators.

IgA in mucosal host defense

IgA is quantitatively the most important of the immunoglobulins, having a synthetic rate exceeding that of all other immunoglobulins combined when secretory as well as circulating IgA is taken into account. In humans it is encoded by two genes within the immunoglobulin gene locus on chromosome 14. The 5' IgA gene encodes IgA1, the predominant circulating IgA and a major component of the IgA in mucosal secretions. The more 3' IgA gene encodes IgA2, the IgA that is particularly abundant in mucosal secretions. IgA1 differs from IgA2 in being susceptible to cleavage in its hinge region by proteases secreted by a number of different bacteria. Cleavage can lead to markedly reduced function.

Another difference between IgA1 and IgA2 is that the latter occurs in two allotypic forms, IgA2(m1) and IgA2(m2), which are distinguished from one another by the fact that IgA2(m1) lacks interchain (H-L) disulfide bonds. The IgA heavy chain, in common with the IgM heavy chain, has an extra cysteine residue in the C-terminal segment. This permits IgA to interact J (joining) chain to form IgA dimers and trimers. IgA polymerization is important to IgA function because polymerized IgA (pIgA) has an increased capacity to bind to and agglutinate antigens.

Polymeric immunoglobulin receptor (secretory component)

Only dimerized IgA and IgM can react with the polymeric immunoglobulin receptor (pIgR, secretory component, SC) produced by epithelial cells. pIgR acts as a transport receptor for IgA and becomes part of the secreted IgA molecule. It renders the IgA molecule less susceptible to proteolytic digestion and more mucophilic, thus enhancing the ability of the IgA molecule to interact with potential pathogens and to prevent their attachment to the epithelial surface. The sequence of events occurring during IgA transport involves first the binding of polymeric IgA to pIgR on the basolateral surface of the epithelial cell, followed by endocytosis of IgA into vesicles, movement of IgA-containing vesicles to the apical surface of the cell, and finally, release of IgA pIgR complexes into the mucosal lumen (Figure 2). This final step requires cleavage of the pIgR receptor molecule so that the receptor for IgA becomes part of the secreted IgA molecule. Cellular synthesis and translocation of pIgR is independent of the presence of IgA and usually exceeds the amount necessary for transport, leading to the secretion of free pIgR.

IgA transport mediated by pIgR occurs in the epithelium of the digestive tract, the salivary glands, the bronchial mucosa and the lactating mammary

glands. It also occurs in the uterine epithelium, where it is regulated by the effects of estrogen on pIgR synthesis by uterine epithelial cells. IgA transport mediated by pIgR also takes place in the liver, in which case it results in secretion of IgA into the bile. This involves the biliary epithelial cells, and in rodents, hepatocytes. Hepatic uptake of IgA may be one mechanism by which IgA secreted into the circulation is redirected back to the mucosa in species with well-developed pIgR-mediated hepatic transport capabilities, possibly to clear the circulation of potentially damaging antigenic material that has penetrated the mucosal barrier and become bound to circulating IgA.

Neuroimmune interactions with the mucosal immune system

There is substantial evidence indicating that the nervous system and immune system can interact in a variety of ways that modifies the function of each. Anatomically, the gut has a rich plexus of nerves that play a critical role in organization of gut motility, and are likely to be important in other functions such as regulation of epithelial cell function. Nerve fibers can be observed in close proximity of lymphoid cells in Peyer's patches and in the lamina propria. The major neuropeptide mediators of the enteric nervous system are substance P, vasoactive intestinal peptide (VIP) and somatostatin, and lymphoid cells have receptors for each of these transmitters. *In vitro*, each of these neurotransmitters has been shown to have the potential for modifying the response of lymphoid cells. The effects of these transmitters are complex, but in general, somatostatin and VIP inhibit proliferation and differentiation of lymphoid cells, whereas substance P is stimulatory. In addition, VIP has been shown to be chemotactic for monocytes and it may have an effect on the recirculation of lymphoid cells. These observations have obvious implications for the possible ability of the nervous system to modify immune responses. This possibility has been demonstrated *in vivo* in animal studies in which repeated conditioning with a simultaneous visual and antigenic stimulus eventually led to the ability to release mast cell products on stimulation with the visual signal alone. Conversely, leukocytes, including lymphocytes, monocytes and mast cells, have the ability to synthesize neuropeptides, and potentially during an immunological response it is possible that there may be direct feedback from the immune system to the nervous system.

Oral tolerance

When antigens are fed to adult animals or humans, for the most part there is little or no detectable response of the systemic immune system, and, usually, in the mucosal immune system. The mechanisms by which this occurs are uncertain, but undoubtedly it serves to prevent a massive response of the immune system to the large numbers of different potentially immunogenic food and bacterial antigens present in the intestinal lumen that would be deleterious to the host. The lack of response is variable and depends on the nature of the antigen. The lack of response is nearly complete for most food antigens, but there may be responses to complex antigens, particularly when present on live organisms such as bacterial pathogens and viruses. The exact mechanisms by which the immune system is able to distinguish a pathogen from commensal bacteria is unknown, but probably involves the ability of pathogens to attach to or invade the mucosa and induce inflammatory responses that lead to activation of the immune system.

There appear to be multiple different mechanisms that result in tolerance, including T cell deletion, both in the thymus and peripherally, and B cell deletion. In addition, there is good evidence in animal models that the mucosal immune system may generate suppressor cells, which may be either antigen specific or antigen nonspecific, that inhibit the generation of antibody responses in the systemic immune system. Interestingly, this mechanism sometimes permits the development of a local IgA response while inhibiting potentially inflammatory IgG responses. In addition to these mechanisms, T cells can be anergized by exposure to antigens in the absence of costimulatory molecules, which appears to result in a relatively permanent change in the signal transduction pathways in these cells.

See also: Adhesion molecules; Mast cells; IgA; Joining J chain; Lymphocyte trafficking; Mucosa-associated lymphoid tissue (MALT); Neuroendocrine regulation of immunity; Oral immunity; Oral tolerance; Secretory component (the polymeric Ig receptor).

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MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is the major human demyelinating disease of the central nervous system, affecting most commonly young adults. The disease is characterized by episodes of neurologic symptoms occurring over a period of days to weeks, followed by complete or incomplete remissions of various durations. Early symptoms of MS include fatigue, gait and limb ataxia, spasticity, dizziness, double vision, acute optic neuritis and numbness and weakness in one or more limbs. Depending on the progression of the disease, MS can be classified into several categories. Although these categories are not entirely distinct, for practical purposes MS can be classified as follows:

- Relapsing–remitting disease, defined as the occurrence of exacerbations with complete remission, leading to mild disability only with no overall change in daily functioning.
- Chronic relapsing disease (= relapsing progressive MS) characterized by exacerbations with incomplete remissions and greater disability with each attack.
- Chronic progressive disease, characterized by lack of remission accompanied by continued progressive disability.

Sixty-five per cent of MS patients have the relapsing–remitting form at onset, but more than 40% of these develop progressive MS later on. MS starts most commonly between 20 and 50 years of age and affects women more frequently than men.

The multiple sclerosis lesion

MS is characterized by multiple areas of myelin destruction in the central nervous system (CNS), known as plaques. CNS myelin is made by local oligodendrocytes that are gradually lost along the progression of the disease. Microscopic examination of the lesions or plaques indicates that demyelination is often accompanied by an active immune response. Inflammatory infiltrates are located perivenularly and the cellular composition of the infiltrates varies with the age of the lesions. Acute lesions consist of macrophages, lymphocytes, plasma cells and nonmyelinated axons, while the inflammatory infiltrates are less pronounced in the chronic plaques. Reactive gliosis and inefficient attempts at remyelination can also be observed, suggesting that the immunological mechanisms in more chronic lesions are different from those in acute lesions.

Studies of the experimental animal model for MS, experimental autoimmune encephalomyelitis (EAE), have demonstrated that myelin basic protein (MBP)-specific, proteolipid protein (PLP)-specific or myelin oligodendroglia glycoprotein (MOG)-specific T cells mediate the autoimmune pathogenesis. The encephalitogenic epitopes on myelin antigens differ between species and strains and are influenced by the major histocompatibility complex (MHC) make-up of the animal. Furthermore, in some strains the encephalitogenic T cells use a very limited set of T cell receptor (TCR) chains in the recognition of the com-

plex formed by disease-associated MHC class II molecules and encephalitogenic peptides. This has led to innovative therapeutic approaches targeting each of the components specifically. In the chronic-relapsing form of EAE in the SJL/J mouse, the disease exacerbates and remits as in MS, and the murine pathology strongly resembles that seen in humans. It is possible that MS, like EAE, is caused by T lymphocytes recognizing antigens found in the CNS. However, whether the therapeutic approaches successfully used in EAE will be effective in MS is not clear, since the relationship between a specific TCR and disease is not evident. Furthermore, to date no specific antigens for the inflammatory processes in MS have been described.

Oligoclonal bands in cerebrospinal fluid

The synthesis of immunoglobulins in the CNS of MS patients is increased. About 90% of MS patients show a limited number of distinct bands (= immunoglobulins), as observed by electrophoresis or isoelectric focusing. These so-called oligoclonal bands are used as a diagnostic aid for MS but are not specific for the disease. In contrast to infectious diseases such as syphilis, in which immunoglobulin production in the CNS is antigen-driven, the specificity of the increased CNS immunoglobulin synthesis is not known in MS. The increased production of immunoglobulins in MS may be caused by an as yet unidentified (infectious) agent or may be related to a defect in immune regulation. Indeed, evidence for an impaired immune regulation is provided by the increased reactivity of B cells which could be due to impaired suppression.

Autoantigen-specific T cell responses

Although the responsible autoantigens for the disease are unknown, it is widely accepted that MS is caused by disturbances in the regulation of autoantigen-specific T cells. It is also possible that more than one antigen is involved. Some of the most prominent candidate brain antigens are shown in Table 1.

MBP-specific CD4⁺ α/β T cell lines and clones can be generated from peripheral blood lymphocytes (PBLs) of MS patients and healthy individuals. The frequencies, fine specificities, restrictive HLA determinants and TCR usage are similar in both populations, but in MS patients a significantly higher proportion of these cells is in an activated state, which gives them a higher potential to cross the blood-brain barrier. In contrast to EAE, in MS a TCR heterogeneity can be seen among MBP-specific T cell clones from different individuals although there is a limited TCR-V β gene usage among MBP-specific T cell clones of the same individual. Furthermore, the predominant V β gene used by the studied MBP-specific T cell clones does not correlate with the epitope specificity of T cells nor with their restricting HLA molecule. The heterogeneity of MBP-specific T cell clones in MS patients is less pronounced than in healthy individuals and is highly suggestive of a clonal expansion of a limited set of antigen-specific T lymphocytes.

The natural stimuli that lead to activation of myelin-activated T lymphocytes in MS are as yet poorly understood, but viruses or bacterial superantigens could be responsible for the activation of autoantigen-specific T cells. In addition to the activation of autoantigen-specific T cells, interactions between cell adhesion molecules (CAMs) and selectins expressed on endothelial cells and their ligands on encephalitogenic T lymphocytes could also play an important role in the disease process. By the same token it may be that cytokines released by encephalitogenic T lymphocytes (tumor necrosis factor α (TNF α) and interferon γ) are involved in the upregulation of MHC antigens and adhesion molecules on endothelial cells and antigen-presenting cells (APCs) in the CNS, and the recruitment and activation of other T cells and macrophages. These cytokines can also mediate direct toxicity to the myelin membranes. There is also some evidence supporting a possible role of γ/δ T cells in the secondary inflammatory processes in MS.

Table 1 Myelin antigens

<i>Protein</i>	<i>Size (kDa)</i>	<i>Localization</i>
Myelin basic protein (MBP; 30%)	18.5	Cytoplasm of oligodendroglial cells
Proteolipid protein (PLP)	30	Myelin membrane
Myelin-associated glycoprotein (MAG; 1%)	100	Myelin sheath membrane
Myelin oligodendroglia glycoprotein (MOG)	54	Myelin membrane at the surface of oligodendrocytes

Etiology

The etiology of MS is not known but there is ample evidence that MS has an immunopathogenic basis and that both genetic and environmental factors contribute to the pathogenesis of the disease.

Genetic factors

Most direct evidence to support the influence of genetic factors comes from family studies and from the comparison of concordance in monozygotic (MZ) and dizygotic (DZ) twins. A higher concordance rate (30%) has been found consistently in MZ twins. Furthermore, magnetic resonance imaging (MRI) studies showed lesions in 40% of 'clinically normal' monozygotic twins of individuals with MS. Although the disease is not genetically determined, genetic factors such as the HLA class II alleles within the MHC consistently seem to influence the susceptibility to develop MS. The development of the disorder is believed to be the consequence of multiple genes plus an environmental factor. Different HLA-DR specificities are associated with MS in different ethnic groups and the MHC association with MS appears to be less important than that seen in other autoimmune diseases such as rheumatoid arthritis and type I diabetes, suggesting that other loci contribute to the genetic susceptibility.

Environmental factors

Evidence for environmental factors is largely derived from epidemiological studies. The epidemiological data indicate that MS is related to an infectious agent encountered during childhood. Over the years, more than ten different viruses have been implicated in the etiology of MS, but thus far, no single agent related to the disease has been confirmed. MBP-specific lymphocytes belong to the 'normal' T cell repertoire and it is possible that these cells are expanded by myelin release during MS or virus infection. It is also possible that self-reactive T cells are activated by the recognition of epitopes shared by virus and autoantigen (molecular mimicry).

Diagnosis

Diagnostic criteria have been developed which now have been almost universally adopted and found reliable. Evoked potential studies, MRI and abnormalities of the cerebrospinal fluid (oligoclonal bands and elevated IgG levels) are nonspecific and therefore, by themselves, not diagnostic.

Clinical evaluation

The Expanded Disability Status Scale (EDSS) is the most common clinical scoring system used to evaluate the condition of MS patients. The overall disability score ranges from 0 (normal) to 10 (death due to MS) in half-point increments. The clinical course is quite variable and can be manifested by either relapsing or gradually progressive neurologic deficits. Neuropsychological studies document that 43–65% of MS patients suffer from cognitive dysfunction. Cognitive decline is more frequent at the more advanced stage of the disease and in chronic progressive MS. MRI indicates that considerable subclinical disease occurs and that, early in lesion development, there is a breakdown in the blood–brain barrier.

Magnetic resonance imaging

MRI, a computerized method of depicting the morphology of anatomic structures in cross-section, has been used as one of the parameters to establish the diagnosis of MS and to monitor disease activity. MRI has shown continuous disease activity in clinically stable disease, making it even more difficult to place MS patients in a distinct category.

Therapeutic interventions

Until recently, most attempts to treat MS have focused on general immunosuppressive drugs since the disease was presumed to be immunologically mediated. However, clinical trials were often inconclusive and discouraging. A reliable specific treatment for MS is not yet available. Corticosteroids and corticotropin (ACTH) are commonly used for the treatment of exacerbations and interferon β and copolymer 1 appear to be effective in decreasing the number and severity of relapses. There are, however, many ways of relieving or diminishing the severity of the symptoms of the disease.

More specific treatments have been proposed based on the hypothesis that autoantigen-specific T cells are involved in the pathogenesis of the disease. Studies in EAE have shown that encephalitogenic T cells recognize short peptides of the myelin antigens in the context of MHC molecules, express a restricted number of TCR molecules and secrete interferon γ and tumor necrosis factor α/β (TNF α/β). Although the variability in specificity in the TCR usage in MS has raised questions regarding the relevance of these cells in MS patients, the importance of these cells can be established by abrogating their activity through specific therapies. T Cell vaccination with attenuated MBP-specific T cells in animals and humans has

shown that MBP-specific T cells can be downregulated by an upregulation of anticolonotypic T lymphocytes. Clinical evaluation of a limited number of vaccinated MS patients showed evidence of stabilization of the disease, but awaits further confirmation by means of multicenter clinical trials. Similar experiments with TCR-specific peptides and oral treatment with MBP are promising but also await further evaluation.

See also: Autoantigens; Autoimmune diseases; Autoimmunity; Experimental autoimmune encephalomyelitis (EAE); HLA class II; Immunopathology; Immunosuppression; Interferon β ; Interferon γ ; MHC restriction; Molecular mimicry; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T cell vaccination; Tumor necrosis factor α .

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MURAMYL DIPEPTIDE

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Heat-killed mycobacterial cells suspended in mineral oil (so-called Freund's complete adjuvant, FCA) provide one of the most potent immunoadjuvant for the induction of cell-mediated immunity and humoral antibody formation. In 1971, Azuma showed that the cell wall fraction was the major active component for the immunopotentiating activity of mycobacterial cells. The peptidoglycan portion was shown to be the most important for the immunoadjuvant activity of mycobacterial cell wall. In 1974, the minimum essential structure required for the immunoadjuvant activity in the peptidoglycan was defined independently as an *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP, muramyl dipeptide; Figure 1) by the groups of E. Lederer and S. Kotani. Several hundred derivatives and related compounds of MDP have been synthesized chemically and their immunopharmacological activities and structure–activity relationships have been investigated (Figure 2). MDP is established as the adjuvant-active component not only of mycobacterial cells but also of other bacterial cell walls.

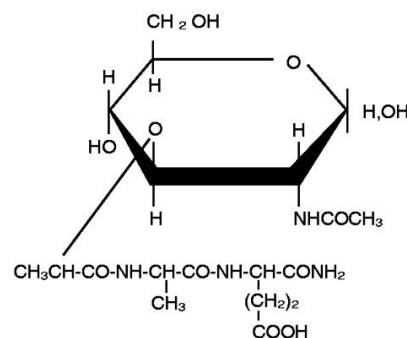


Figure 1 *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP).

Immunopharmacology and toxicology

MDP has variety of biological activities including:

- adjuvant activity on immune responses including the activation of immune cells, especially macrophages and monocytes,
- induction of cytokines,
- potentiation of nonspecific host resistance against bacterial, fungal and viral infections,
- pyrogenicity,

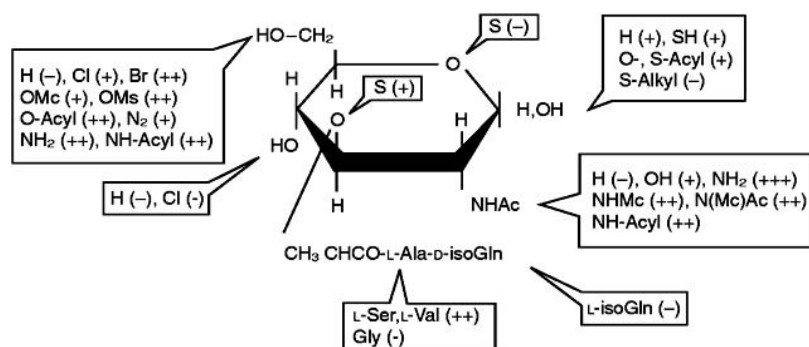


Figure 2 Adjuvant activity-structure relationship of MDP derivatives. Guinea pigs were immunized with MDP derivatives and antigen (azobenzene arsonate *N*-acetyltyrosine) in water-in-oil emulsion and adjuvant activity was determined by the induction of delayed-type hypersensitivity to antigen (azobenzene arsonate-bovine serum albumin).

- increasing the toxicity of endotoxin (lipopolysaccharide),
- adjuvant arthritogenicity,
- transient leukopenia followed by leukocytosis including monocytosis,
- somnogenic effect and analgesic activity.

These activities suggest that MDP has effects not only on immune responses but also on the neuro-endocrine system. Although extensive data suggests that MDP acts on macrophages/monocytes and induces several cytokines which stimulate immune networks, the MDP receptors on macrophages and other mononuclear cells have not yet been defined.

Pharmacokinetic studies indicate that MDP is rapidly excluded from the blood circulation following intravenous injection of MDP in saline. Within 2 h, 95% of the injected dose of MDP can be recovered unaltered in the urine. Several approaches have been utilized including to obtain more useful long-acting immunopotentiating agents for clinical application, the development of delivery systems such as liposomes and the chemical synthesis of lipophilic MDP derivatives.

Immunopharmacological activities and clinical development of MDP derivatives

The discovery of MDP led to immunopharmacological studies aimed at clinical application of MDP as a chemically defined, fully active immunoadjuvant. These expectations were soon frustrated by the realization that MDP itself is not suitable for clinical use, mainly because of its toxicity and poor pharmacokinetic profile, i.e. the rapid clearance of MDP from the body. This was, however, the beginning of a search for molecules with desirable properties without serious side-effects. Several hundred derivatives were synthesized in order to clarify the interrelationships between structure, activity and toxicity. In 1991, 17 years after the discovery of MDP, romur-

tide (MDP-Lys(L18)) (Figure 3), a lipophilic MDP derivative, was licensed in Japan and employed to induce bone marrow recovery in cancer patients. Following romurtide, other MDP derivatives and analogs, such as MDP (Thr), B30-MDP, MTP-PE and murabutide (Figure 3), are in clinical trials for infectious diseases and cancer.

Immunopharmacological activities of lipophilic MDP derivatives

The lipophilic derivatives show enhanced interaction with macrophages and improved pharmacokinetics, and thus exhibit higher biological activities than MDP. Four such derivatives, MDP-Lys(L18) (romurtide), B30-MDP, MTP-PE and murabutide, have been studied extensively for their immunopotentiating effects and toxicity. Romurtide is effective against experimental infections not only when injected before challenge (immunoprophylaxis), but also when injected simultaneously with or after challenge (immunotherapy). One of the underlying mechanisms of the stimulating effect of romurtide on host resistance is an extremely strong hematopoietic activity for granulocytes and monocytes. Romurtide became the first clinically licensed MDP derivative employed to induce bone marrow recovery following radiation therapy in cancer patients. In patients treated with romurtide, the duration of granulocytopenia is shortened and the number of episodes of infection reduced, allowing completion of cancer therapy. Additionally, romurtide augments platelet production. This effect is of importance because thrombocytopenia caused by cancer chemotherapy is one of the most serious concerns in the management of cancer patients. Clinical trials investigate therapeutic efficacy of romurtide on certain refractory infections, including atypical mycobacterial infection and tuberculosis with multidrug-resistant pathogens, are under progress.

In contrast, B30-MDP is a derivative that exhibits

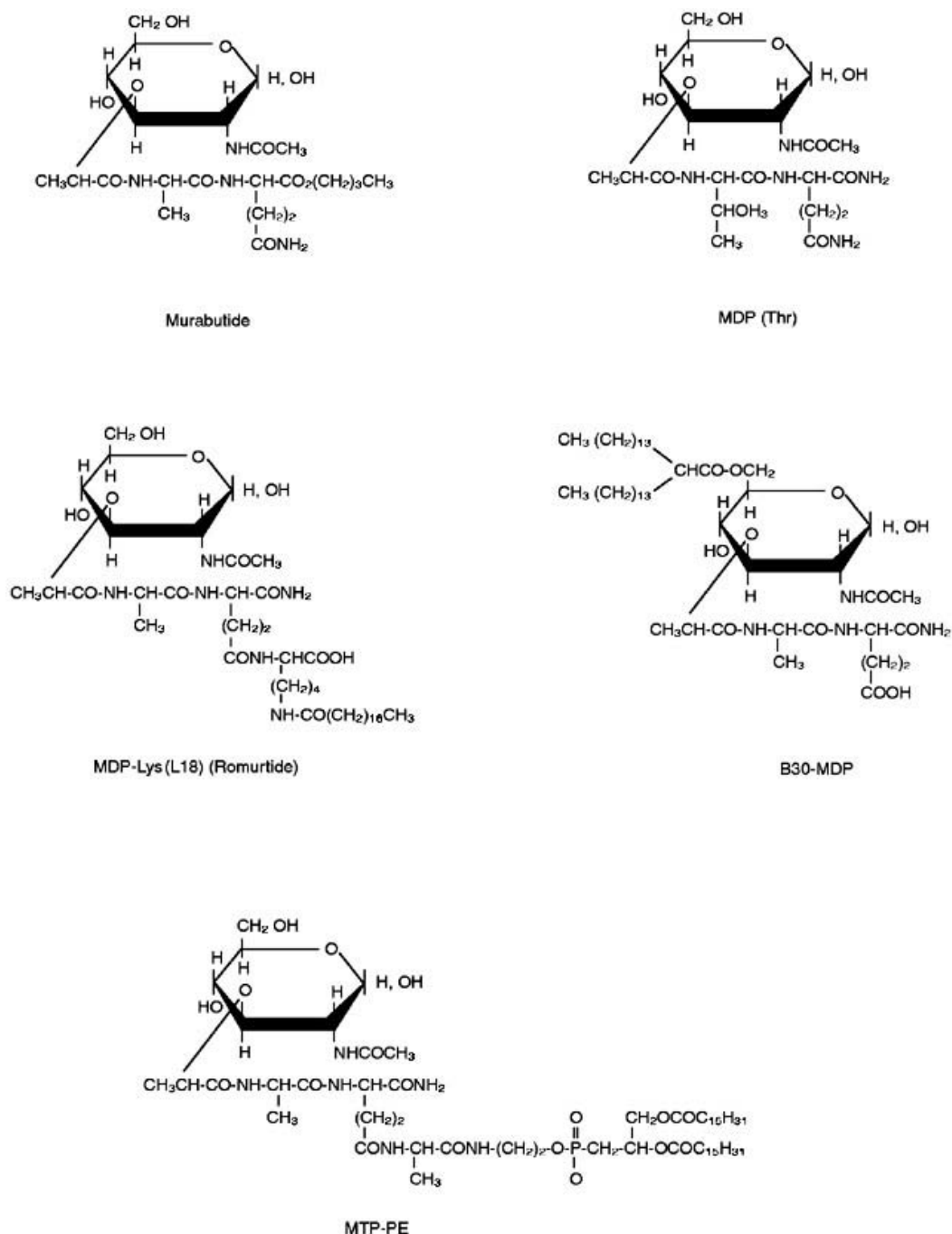


Figure 3 Chemical structures of various MDP derivatives applied to clinical trials.

adjuvanticity but shows no substantial activity regarding nonspecific resistance to infection. Clinical trials using B30-MDP as an adjuvant effect in an influenza virus vaccine demonstrate that it is able to induce both humoral and cellular immunity. MTP-PE has been shown to be highly effective in generating tumoricidal properties of macrophages. Liposome-encapsulated MTP-PE (L-MTP-PE) has been

designed for *in vivo* targeting to macrophages and decreasing side-effects, and is in clinical trials for cancer. A series of derivatives replacing the D-isoglutaminyl residue with D-glutamine alkyl esters of various sizes was shown to be active but much less pyrogenic than MDP. The representative derivative of this series is murabutide, a compound which does not elicit any febrile response in animal experiments.

Clinical trials of murabutide as a vaccine adjuvant and as a potentiator of nonspecific resistance to infection are currently in progress.

Allison and coworkers found that a MDP analog, *N*-acetylmuramyl-L-threonyl-D-isoglutamine (MDP (Thr)) retained potent adjuvant activity but was less pyrogenic in rabbits and rats. MDP(Thr) has been applied as an immunoadjuvant with the vehicle 'Syn-tex Adjuvant Formulation, SAF', which consists of pleuronic L121, squalane, Tween 80 and PBS.

MDP derivatives as a multicytokine inducer

Differences have been observed in the immunopotentiating activity of each MDP derivative. This may be attributable to the difference in spectrum and/or amount of cytokines induced by each derivative. For example, romurtide has been found to induce stem cell factor (SCF), erythropoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF), granulocyte CSF (G-CSF), interleukin 1 (IL-1), IL-6, IL-8, tumor necrosis factor α (TNF α) and interferon γ (IFN γ). For stimulation of hematopoiesis by romurtide, SCF, GM-CSF, M-CSF, G-CSF, IL-1 and IL-8 may be involved in monocyte and granulocyte production and their recruitment to the periphery. SCF, GM-CSF and IL-6 and erythropoietin, may be involved as megakaryocyte CSF and megakaryocyte potentiator, respectively, in platelet production. For the stimulation of nonspecific resistance to infection by the derivative, TNF α and IFN γ may play an important role in addition to other cytokines. In the case of L-MTP-PE, human monocytes/macrophages that phagocytose L-MTP-PE produce IL-1, IL-6, IL-8, TNF α and monocyte chemotactic and activating factor (MCAF). Following intravenous infusion of L-MTP-PE into cancer patients, induction of circulating TNF α , IL-6, and neopterin was demonstrated. The major source of cytokines induced by MDP derivatives is thought to be monocytes/macrophages. Although the receptors for MDP have not been defined, the direct interaction of MDP and romurtide with macrophages has been reported. It is noteworthy that an intracellular receptor for MDP has been suggested, which would explain the stimulating effect on macrophage functions of liposome-encapsulated MDP phagocytosed by macrophages. Lymphocytes may be involved as a secondary source of cytokines, following MDP-induced cytokines secretion by macrophages. For example, murine T lymphocytes produce CSFs when cocultured with macrophages stimulated by romurtide: such macrophages can be substituted by exogenous IL-1.

Clinical toxicology of MDP derivatives

No serious adverse effect of romurtide has been reported since its clinical application with the standard regimen of subcutaneous injection of 200 μ g once a day for 10 days. In phase II and III clinical studies, 704 cancer patients were enrolled to analyze the effectiveness and safety of romurtide. Side-effects caused by romurtide administration were observed in 275 patients (39.1%), mostly fever and a transient skin reaction at the injection site. Other signs and symptoms in 3.6% included skin rash, loss of appetite and nausea. The febrile responses could be controlled by antipyretics without intervention on its effectiveness. In phase II clinical trials of L-MTP-PE for osteosarcoma, the recommended dose was 6 mg m⁻². Despite such a high dose, the side-effects were moderate, including chills (80% of the patients), fever (70%), malaise (60%) and nausea (55%), and 24 week therapy was accomplished. It seems likely that the serious side-effects observed in animals, such as arthritis in rats, uveitis in rabbits and necrosis at the injection site in guinea pigs, are strictly species dependent and humans may not be a high responder to the potency of MDP and its derivatives for inducing such reactions.

Future prospects

Romurtide and other MDP derivatives such as B30-MDP MTP-PE and SAF including MDP(Thr), have been shown to stimulate circulating antibody formation to several viral and malaria vaccines. Romurtide and other MDP derivatives have been reported to enhance host resistance to microbial infections via stimulation of mucosal immune systems after intranasal, oral or intrarectal administration. Romurtide and other MDP derivatives have also been shown to prevent cancer metastasis in experimental models.

The cytokine-inducing properties of MDP derivatives may have an important role to play in cancer therapeutics, especially as a supporting therapy for patients treated with chemotherapy and/or radiation therapy. This approach is expected to improve the quality of life of such patients.

See also: Adjuvants; Cytokines; Immunopotential; Macrophage activation.

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MYCOBACTERIA, INFECTION AND IMMUNITY

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Mycobacteria is a genus of numerous, mostly saprophytic species, many of which have not yet been characterized. They can be divided into two distinct subgenera: the fast growers and the slow growers. *Mycobacteria* differ in structure from other bacteria in that the peptidoglycan layer, present in all bacteria, is covalently linked to a layer of arabinogalactan, which is in turn linked to an outer layer of mycolic acids. These are waxy substances, 60–90 carbon atoms long. Further high molecular weight lipids and glycolipids are noncovalently associated with this outer layer. This structure is resistant to mammalian enzyme systems. Several of its components have striking immunostimulatory properties and *mycobacteria* are routinely included in Freund's complete adjuvant.

The pathogenic mycobacteria

Most *mycobacteria* are environmental saprophytes but the two major pathogenic species, *M. tuberculosis* and *M. leprae*, are amongst the most important pathogens of humans. There are 8–10 million new cases of tuberculosis in the world each year, of which about 3 million die, and this figure is growing with the increased incidence of human immunodeficiency virus (HIV) infection. Similarly, there are between 10 and 15 million cases of leprosy. The major pathogens in the slow-growing subgenus are *M. tuberculosis* (and the related organisms *M. africanum*, *M. bovis* and *M. microti*, which infects rodents), *M. avium* (and the related *M. lepraemurium* which causes a leprosy-like disease in rodents, and *M. paratuberculosis* which infects the

bowels of cattle), *M. intracellulare*, *M. scrofulaceum*, *M. kansasii* and *M. ulcerans* (the cause of Buruli ulcer). Several members of the fast-growing subgenus can cause infections but these are usually opportunistic or abscesses. Relevant species include *M. chelonae* and *M. fortuitum*.

The relevance of environmental mycobacteria

Saprophytic *mycobacteria* are extraordinarily common in the environment, and can be detected in soil samples or scrapings of waterpipes by direct staining and microscopy. Their persistence in the environment is partly attributable to the massive cell wall structure described above. *Mycobacteria* appear not to be part of the normal commensal microbial flora, but they are ubiquitous, resistant to degradation, and very immunogenic, so skin-test surveys reveal that individuals are sensitized to the species characteristic of their own environment. It is now clear that this is relevant to the variable efficacy (0–80% protection in different trials) of vaccination against mycobacterial diseases with BCG, because preimmunization by environmental saprophytes that prime a T_H1 pattern of response is protective, but experimentally, immunization with such saprophytes using doses and routes that prime a T_H2 cytokine pattern, actually increases susceptibility to the disease. It may be that variable exposure to environmental *mycobacteria* can also predetermine patterns of response to heat shock proteins which have been implicated in immunoregulation and autoimmune disease.

Diagnosis of mycobacterial disease

This relies mainly on clinical assessment, microscopy of stained sputum (tuberculosis) or skin smears (leprosy), and culture of the organisms, except for *M. leprae*, which has not been cultivated. Diagnosis of leprosy and opportunist infections is usually backed up by histological examination of biopsies. The skin test using the soluble components of sonicates ('new tuberculin') can be used to distinguish between infections with the various opportunist species. For tuberculosis, skin test with purified protein derivative (PPD) (derived from a crude culture supernatant of *M. tuberculosis*) can be helpful. The delayed hypersensitivity response to PPD can be positive because of present or past tuberculosis, previous BCG vaccination, or heavy exposure to cross-reactive environmental mycobacteria. Therefore, interpretation of the test depends on the environment from which the patient comes. In past or present cases of tuberculosis, the tuberculin test site is frequently necrotic (discussed in detail below). Serodiagnosis remains unsatisfactory for three reasons. First, these assays do not distinguish easily between past and present infection. Secondly, all individuals are exposed to highly immunogenic cross-reactive antigens from environmental species. Thus antibody levels in tuberculosis patients are often no higher than in normal donors, particularly while the patient is acutely ill, though the titer may rise after treatment. Thirdly, although the problem of cross-reactive environmental species would theoretically be avoided if species-specific epitopes could be discovered, such epitopes are either poorly immunogenic, or immunogenic in only a percentage of individuals. Alternative approaches receiving intensive study include the detection of mycobacterial antigens such as persistent carbohydrates in patients' serum and urine, or the use of the polymerase chain reaction to detect mycobacterial DNA sequences.

Host factors and susceptibility to mycobacterial disease

There are weak associations between mycobacterial disease and major histocompatibility complex (MHC) haplotypes. In leprosy, these seem to determine the type of disease which develops rather than overall susceptibility. In rodents, susceptibility is also linked to the *bcg/Ity/Lsh* gene, which affects aspects of the baseline state of macrophage activation, and may be involved with control or transport of nitric oxide. Susceptibility to *M. tuberculosis* is particularly sensitive to factors that decrease T cell function, such as severe stress leading to activation of the cytokine

hypothalamo-pituitary-adrenal axis, protein malnutrition and immunosuppression. Tuberculosis occurs early during infection with HIV. Infection with the *M. avium/intracellulare/scrofulaceum* (MAIS) complex is seen when greater degrees of T cell dysfunction occur.

The antigens of mycobacteria

The genes for numerous mycobacterial antigens have been cloned and sequenced, and epitopes recognized by T cells and B cells have been identified. This has not led to the definition of 'protective' antigens or epitopes that seem particularly suitable for use in subunit vaccines. The 65 kDa heat shock protein (hsp) and several secreted proteins have been shown to be capable of eliciting protective responses in animal models (mice and guinea pigs), but at present it seems that the important factor is the nature of the response elicited rather than its fine specificity. For instance the 65 kDa hsp is not protective against tuberculosis when given in incomplete Freund's adjuvant, which elicits a major T_H2 component, but it is very protective when administered as macrophages transfected with the hsp65 gene. The transfected cells evoke a T_H1 response and cytotoxic T cells. Other functionally important antigens include the cytokine-triggering glycolipids lipoarabinomannan and trehalose dimycolate (see below).

Tuberculosis

Immunity to *M. tuberculosis*

Immunity to mycobacteria depends upon cell-mediated pathways described below. The critical problem in both leprosy and tuberculosis is that after prolonged treatment with antibacterial drugs, 'persisters' remain which can lead to relapse unless treatment is prolonged. Optimal so-called 'short course' chemotherapy for tuberculosis therefore takes 6 months. In other bacterial infections, it is sufficient to kill the majority of the bacteria with drugs, because the host response rapidly eliminates the remainder. This does not occur in tuberculosis because the host's response is inappropriate and causes tissue damage (immunopathology) rather than effective bacterial killing.

Immunity in the mouse Experiments with neutralizing antibodies to cytokines and with gene knockout mice have shown that immunity requires MHC class II, a T_H1 cytokine pattern, and tumor necrosis factor α (TNF α). This is supported by evidence that when murine peritoneal macrophages are

activated by interferon γ (IFN γ) they develop the ability to inhibit the proliferation of *M. tuberculosis* and *M. avium*, and MHC class II-restricted T cell lines cultured with infected murine macrophages will exert this effect via release of IFN γ . The ability of murine macrophages to inhibit mycobacteria may be related to the ability of these cells to make nitric oxide. IFN γ activates this pathway, whilst TNF α , released in response to several mycobacterial components triggers the production of large amounts of nitric oxide by the IFN γ -activated cell.

Gene knockout has also revealed a requirement for β_2 -microglobulin in immunity to mycobacteria. This may indicate a role for CD8⁺ MHC Class I-restricted cytotoxic T cells, or for the $\alpha\beta$ T cell receptor-bearing CD1-restricted CD4⁺CD8⁻ T cells that have been defined recently. These cells can recognize mycobacterial lipids and glycolipids. Similarly, many of the lymphocytes that bear $\gamma\delta$ T cell receptors recognize mycobacterial antigens, including heat shock proteins, compounds containing 5' triphosphorylated thymidine, and prenyl pyrophosphate derivatives. Nevertheless, these various cell types all secrete IFN γ , and are cytotoxic. They therefore fit within the concept of 'type 1' response. Presumably the cytotoxicity releases bacteria from effete macrophages so that they can be taken up by fresh cells.

In contrast, if a type 2 response is primed, mice become more susceptible to tuberculosis than are nonprimed controls, and in progressive disease the response in mice switches from T_H1-dominated to a mixed T_H1 plus T_H2, or perhaps T_H0, pattern.

Immunity to tuberculosis in humans It is probable that in humans, as in mice, immunity is associated with a T_H1 pattern of response leading to macrophage activation and to cytotoxic removal of effete infected cells. Studies of cells in tuberculosis pleural effusions support this concept, since this is a high resistance form of the disease, and the T_H1 pattern dominates. However data are less complete than in the mouse because there is still no unequivocal proof that human macrophages can kill *M. tuberculosis*. This may change now that protocols able to induce nitric oxide production by human macrophages have been devised.

The immune response in tuberculosis patients Studies of peripheral blood mononuclear cells from tuberculosis patients precultured with mycobacterial antigen *in vitro* reveal both T_H1 and T_H2 cells. If peripheral blood lymphocytes from patients are examined without preculture it becomes apparent that the interleukin 4 (IL-4) gene is expressed, while there is a deficit in IL-2 expression. Similarly tuberculosis patients

have specific immunoglobulin E (IgE) and IgG4 antibody. Both of these are IL-4-dependent in humans. Many patients' sera also contain high levels of IL-10. When tuberculin test sites were studied using a laser Doppler velocimeter it was found that the extent to which blood flow was reduced in the centre of the site at both 6 h and 48 h, was related to the level of specific IgE antibody to *M. tuberculosis*. This appears to represent incipient necrosis. Thus as in mice, progressive disease in humans is associated with the presence of an inappropriate T_H2 component.

The role of TNF α in tuberculosis The alveolar macrophages and monocytes of tuberculosis patients are primed for release of TNF α . The alveolar lavage cells release TNF α apparently spontaneously in large quantities *in vitro*. Circulating free TNF α is rarely detectable in serum, but there are high levels of soluble TNF α receptors. *M. tuberculosis* is itself a potent trigger of cytokine release from primed macrophages. The major triggering molecule in *M. tuberculosis* is a phosphatidylinositol mannoside known as lipoarabinomannan (LAM), but other components such as trehalose dimycolate (cord factor) may also have this property. This cytokine appears to have a dual role in tuberculosis, being needed for protection, but also playing a role in immunopathology. The drug thalidomide, which reduces the half-life of the mRNA encoding TNF α , causes weight gain and symptomatic relief in tuberculosis patients.

The immunopathology of tuberculosis

The lesions are necrotic in tuberculosis patients, and the disease is characterized by fever and weight loss. Moreover, as first described by Koch in tuberculosis guinea pigs (the 'Koch phenomenon'), a tuberculin test in past or present tuberculosis patients evokes a necrotic response. Tuberculin itself is nontoxic, and it does not evoke necrotic skin-test responses in past recipients of BCG or in tuberculoid leprosy patients, even when there is vigorous induration. Therefore necrosis is not an inevitable component of a positive cell-mediated response to mycobacterial antigen, but reflects a particular mechanism, the likely nature of which is described below. It is possible that the necrosis helps to wall off established infectious foci, but it is ineffective in the elimination of persistent bacilli, and fails to prevent relapse if chemotherapy is curtailed.

The mechanism of the tissue damage in tuberculosis The necrosis (Koch phenomenon) may be explained by the recent observation that the toxicity

of TNF α in a mycobacterial lesion depends upon the nature of the T cell response involved. Thus although TNF α fails to cause necrosis if injected into T_H1-mediated inflammatory sites, it provokes striking tissue damage when injected into sites mediated by T cells with a mixed T_H1/T_H2 cytokine profile. This can be shown in deliberately immunized mice, primed so that they have T_H1 or mixed T_H1/T_H2 responses to mycobacterial antigen, or it can be shown in tuberculosis mice in which injected TNF α becomes tissue-damaging once the response has shifted from T_H1 to mixed T_H1/T_H2, as occurs in the progressive phase of the disease.

Immunotherapy of tuberculosis

Conventional treatment relies on antibiotics and chemotherapeutic agents, and must be continued for at least 6 months for the reasons explained above. However, recent clinical trials suggest that the tissue-damaging pattern of response to *M. tuberculosis* outlined above is potentially subject to immunoregulatory restraints which can be activated by an intradermal injection of autoclaved *M. vaccae*. This is thought to operate by restoring a T_H1 pattern of response to shared epitopes such as those of heat shock proteins.

Leprosy

The spectrum of leprosy

M. leprae cannot yet be cultivated *in vitro*. It will infect primates and armadillos, and give limited proliferation in mice after infection with small numbers of organisms. It enters numerous different cell types *in vivo*, and is found inside Schwann cells, neurons, endothelial cells, muscles and fibroblasts. It proliferates within the cytoplasm, rather than within phagosomes, and this may shield it from protective pathways. It is the predilection for nerves that is responsible for most of the pathology.

Leprosy patients can be classified on a 'spectrum' according to the apparent efficacy of the cell-mediated response. The problems that require explanation are quite different at the two extremes of this spectrum.

Tuberculoid leprosy In tuberculoid disease, the *in vivo* and *in vitro* correlates of T cell-mediated responsiveness, such as skin-test responsiveness and lymphoproliferation in response to antigens of *M. leprae*, appear to be intact, bacilli are few and histologically the lesions are classical T lymphocyte-dependent granulomata containing CD4⁺ T cells, activated macrophages, and epithelioid cells, with a

few CD8⁺ cells round the periphery. The lesions are largely due to nerve damage secondary to expansion of the granulomata and inflammation within nerve sheaths. There is evidence of T_H1 cytokine production in these lesions. Without treatment, many of these individuals will downgrade towards the lepromatous pole, where the T_H1 response is compromised (see below). Since the T cell-mediated responses appear to be functioning, it is not clear why this type of leprosy frequently fails to be self-limiting.

Theoretically, two types of explanation seem possible. First, the T cells responding may be the wrong type. This cannot be resolved at present because we do not know what effector mechanisms are required for the elimination of *M. leprae*, though as for tuberculosis, immunity is likely to depend upon macrophage activation and cytotoxic T cells mobilized by a T_H1 response. There is a T_H1 response in tuberculoid leprosy so why do the patients have a disease?

An alternative hypothesis is that the response in tuberculoid patients is adequate in mechanism, but is directed towards the wrong antigens. Again this cannot be resolved until protective antigens can be identified, but one possibility is that the critical antigens are the secreted ones. These would presumably be the first to be available for presentation to T cells, and would therefore allow a rapid response to infection. They would also allow recognition of live organisms. Thus it is possible that tuberculoid leprosy patients have a chronic granulomatous response to persistent cell wall fragments derived from dead organisms which are difficult to metabolize, while their lymphocytes fail to recognize the live organisms proliferating elsewhere.

Lepromatous leprosy In contrast, lepromatous patients show minimal evidence of T cell-mediated immunity either *in vivo* or *in vitro*, and their lesions consist of large numbers of macrophages filled with bacilli, showing no signs of activation. Often these patients remain unresponsive to the organisms even after years of treatment when the antigen load has been drastically reduced. Thus in this form of leprosy we need to explain the persistent absence of a response, whereas in tuberculoid leprosy the problem is the paradoxical failure of a superficially intact one. The meaningful study of this type of leprosy is hampered by the fact that the organism is nontoxic, and in the absence of a strong inflammatory response it can proliferate within the patient's tissues for many years before its presence becomes apparent. Thus much of what has been revealed by immunologists may be the consequence of prolonged heavy antigen

load, rather than the actual basis for the failure to eliminate the organisms in the first place. It is now clear that the lack of an apparent T cell-mediated response to *M. leprae* in lepromatous leprosy is not due to a lack of T cells recognizing this organism. However the T cells present tend to express T_H2 cytokines. Moreover peripheral blood leukocytes from some (but not all) patients give a proliferative response to *M. leprae* if the cultures are supplemented with IL-2 or other lymphokines.

Several groups have been able to demonstrate T cells that suppress lymphoproliferation in the presence of *M. leprae*. These may represent some type of 'suppressor' cell, or the effects seen may be attributable to T_H2 cytokine production. It remains possible that these phenomena are secondary to the presence of a large antigen load for many years before diagnosis. It may be significant that once the organism has become established within nerves (which do not have lymphatics) and endothelial cells, antigens will be released directly into the circulation which is likely to evoke suppression of cell-mediated responses, and high antibody levels.

Links between mycobacteria and autoimmunity

There are several aspects of the mycobacteria which lead to the possibility that these organisms, or antigens cross-reactive with them, are involved in autoimmune disease.

1. Tuberculosis is one of a group of diseases in which there is a rise in the percentage of circulating IgG molecules that lack terminal galactose from the oligosaccharide which is always present on the CH₂ domain of the Fc. This 'glycoform' of IgG is known as agalactosyl IgG. There is also a transient rise during episodes of erythema nodosum leprosum (ENL) in leprosy. The other conditions where agalactosyl IgG rises strikingly are Crohn's disease, rheumatoid arthritis and rodent models of autoimmune arthritis.
2. The mycobacterial 65 kDa heat shock protein (or autoantigens cross-reactive with it) is implicated in the pathogenesis of the rodent models of arthritis, and of the human disease.
3. Induction of arthritis in the rodent models can be blocked by pretreatment with the mycobacterial 65 kDa hsp.

In view of the fact that mycobacteria are ubiquitous in soil and water, but absent from the commensal microbial flora, there is the tantalizing possibility that variable exposure to mycobacteria in different geographical environments plays a role in the regulation of susceptibility to certain autoimmune diseases.

See also: Autoimmunity; Bacteria, immunity to; Bacterial cell walls; BCG; Delayed-type hypersensitivity; Glycosylation of immune system molecules; Granuloma; Hypersensitivity reactions; Stress proteins; T cell vaccination.

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MYCOPLASMA, INFECTION AND IMMUNITY

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Mycoplasmas and ureaplasmas are bacteria of the class Mollicutes (soft-skinned), the order Mycoplasmatales and the family Mycoplasmataceae. They have no cell wall, the cytoplasm being bordered by a membrane only, and their morphology is varied, from filamentous to flask-shaped, and in older cultures coccoid (Figure 1). They are considered to be

among the smallest self-replicating organisms; in fact they are so small and pliable that they can pass through bacterial filters. In the history of mycoplasmaology this has caused confusion, and the first described mycoplasma, the agent of bovine pleuropneumonia, was mistaken for a virus. Later, because of the absence of a cell wall, mycoplasmas were considered to be L-form bacteria and only in the early 1960s, based on determinations of the guanine–cytosine content and on DNA–DNA hybridization data, were they recognized as a class of their own.

Except for *M. penetrans* which is an intracellular organism, mycoplasmas and ureaplasmas colonize cell surfaces, usually those of epithelial cells of the upper respiratory and urogenital tracts. In the human they are mostly harmless commensals. However, mycoplasmas can also cause serious disease in humans and animals, from farmed crocodiles to poultry and cattle. Other mollicutes can infect insects and plants. Moreover, mycoplasmas are contaminants of about 30–50% of cultured animal cell lines.

Characteristics of the organism

The genome sizes of mycoplasmas range from 580–1380 kilobase pairs. Characteristic for mycoplasma DNA is its low guanine plus cytosine content. The genomes of two species, *M. genitalium* and *M. pneumoniae*, were recently completely sequenced. Because of their parasitic growth in a nutrient-rich environment, mycoplasmas can survive without the enzymatic equipment to synthesize vitamins, amino acids, fatty acids, cholesterol and nucleotides. This implies that cultivation of mycoplasmas requires rich media with appropriate additions of these precursors for macromolecules and lipids. It also means that mycoplasmas are notoriously fastidious and especially difficult to cultivate from clinical specimens.

Host–pathogen interactions

In order to survive in the host, mycoplasmas have to adhere to the host tissue, mostly the epithelium, to have access to the appropriate nutrients, and to avoid the defense system. Studies of mycoplasma–host cell adhesion have centered around *M. pneumoniae* and *M. genitalium*. Both organisms

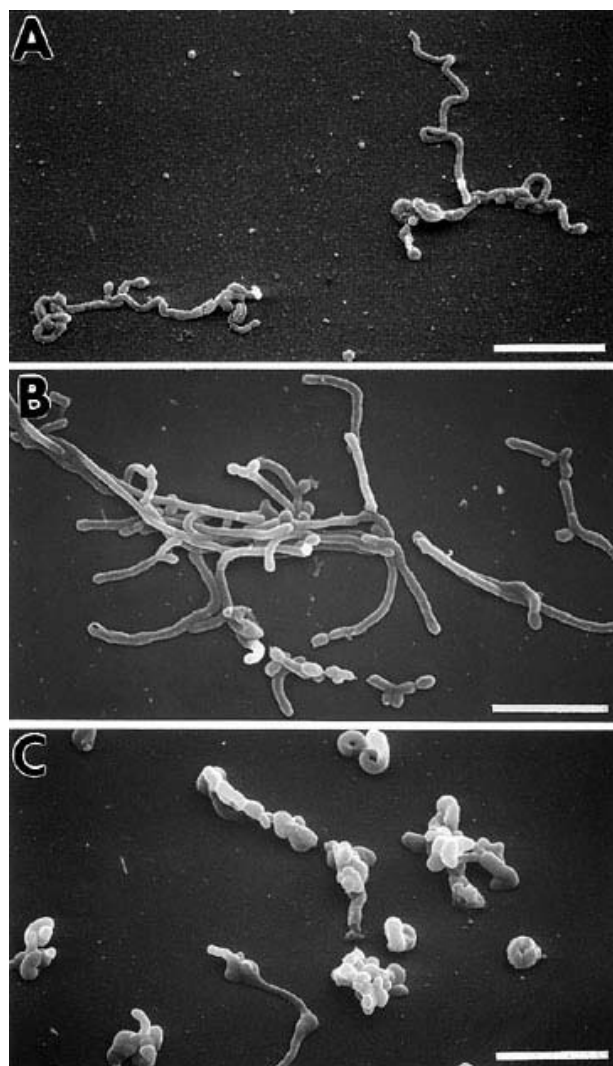


Figure 1 Scanning electron micrograph of critical point-dried mycoplasmas from a liquid culture. (A) *M. pneumoniae*; (B) *M. fermentans*; (C) *M. hyorhinis*. Bars represent 1 μ m (Courtesy of M Rohde, Gesellschaft für Biotechnologische Forschung mbH.)

are of a characteristic flask-shape, with several proteins, so-called adhesins, which are involved in the adhesion process, clustered at specific tip organelles. These proteins have been identified in studies involving trypsin treatments, specific antibodies, and mutants and revertants which lost and regained the ability to cytoadhere. The cellular receptor molecules for *M. pneumoniae* adhesins were identified as long-chain sialo-oligosaccharides of the Ii antigen type. This may contribute to the fact that high-titer cold agglutinin-type autoantibodies against this antigen are occasionally found after *M. pneumoniae* infections. According to findings in a mouse model, colonization of epithelia is related to hormonal status: estradiol promotes attachment to cervical and uterine epithelia, possibly by receptor induction.

As shown in animal models of mycoplasma infections, phagocytes which amass in infected tissue comprise the first line of defense against these organisms. However, in the absence of specific antibodies, mycoplasmas can survive neutrophil phagocytosis. This may be one of the mechanisms by which dissemination into unusual sites and persistence occurs in hypogammaglobulinemic patients. Interestingly, *in vitro* mycoplasmas can modulate class II major histocompatibility complex (MHC) expression by macrophages. Whether this influences the antigen-presenting function of these cells during an actual infection is still unclear. The fact that patients with hypogammaglobulinemia are more frequently colonized with mycoplasmas and ureaplasmas than persons with an intact immune system, and the finding that a significant fraction of those patients also suffer from mycoplasmal septic arthritis strongly suggest that antibodies protect the host from these bacteria. Secretory immunoglobulin A (IgA) appears to be important in preventing localized colonization, whereas systemic antibodies may protect from primary infection and secondary spread from localized colonization. Unlike other mycoplasma species, *U. urealyticum* possesses a host-specific IgA protease as a potential virulence factor. Moreover, ureaplasmas and many mycoplasmas, e.g. *M. fermentans*, *M. hominis*, *M. hyorhinis* and several others, have the facility to escape the humoral immune response by undergoing high-frequency antigenic variation of some of their surface antigens. The genetic mechanisms for this 'phase variation' were recently elucidated. There have been surprisingly few recent investigations into the involvement of T cells in a specific response to mycoplasmas, although early studies had shown that patients develop specifically proliferating T cells a few weeks after *M. pneumoniae* infection, and that this response may persist for several years. The importance of cellular immunity also became

evident from studies in an animal model with nude mice. These animals, which are devoid of a functional T cell repertoire, could not be protected, as control animals, against vaginal colonization by *M. pulmonis* by prior oropharyngeal infection with the same organism.

In vitro effects of mycoplasmas and their products on various cells of the immune system are manifold. Occasionally an apparently mycoplasma-mediated inhibition of lymphocyte proliferation was recorded in experiments in which proliferation was measured by uptake of radioactive thymidine. However, this effect was found to be due to scavenging of thymidine by living mycoplasmas. On the other hand, stimulation of B or T cell proliferation by heat-killed mycoplasmas has been noted, as well as mycoplasma-mediated generation of cytotoxic T cells, and activation of macrophages to become cytolytic and to release proinflammatory cytokines, nitric oxide and arachidonate metabolites. Some of the effects on lymphocytes are indirect, and are due to a macrophage activator that is present to various extents in the membranes of many if not all mycoplasmas and ureaplasmas. This macrophage activator also modulates class II MHC expression. Recent biochemical data indicate that this material consists of mycoplasmal lipoproteins and lipopeptides derived from the former. *M. arthritidis*, a strain arthritogenic in mice and rats, is the only mycoplasma known so far which produces a superantigen. This protein was recently sequenced, and has been the subject of many studies that have elucidated its specific interaction with particular T cell receptor and class II MHC species.

Mycoplasmas and ureaplasmas in human disease

This section focuses on the role of mollicutes in the human. While most mycoplasma species are harmless commensals in the human and, where pathogenic, are life-threatening only under exceptional circumstances, one should be aware of the fact that some mycoplasmas exist in animals that cause 100% morbidity and 50% mortality, as e.g. *M. mycoides* subspecies *mycoides*, the agent of pleuropneumonia in cattle.

As with other bacteria, mycoplasmas may be considered normal flora in one site, e.g. *Ureaplasma* colonizing the human vagina, and abnormal when present at other sites, e.g. when found in joint fluid. Such findings may, in fact, be a first sign of immunosuppression in hypogammaglobulinemia. The induction of chronic disease states in patients with normal defense systems is an intriguing feature of mycoplasma infections. Besides the above-mentioned

evasive strategies which are common to most mycoplasmas, each pathogenic mycoplasma may present specialized mechanisms of evasion of the immune system.

Primarily respiratory pathogens

Although *M. pneumoniae* resides primarily in the oropharynx and can be isolated from secretions during lower respiratory disease, this organism has occasionally been found in the urethra or cervix of patients attending clinics for sexually transmitted diseases. Aside from causing classical 'atypical pneumonia' in school-age children, younger age groups may exhibit bronchitis, bronchiolitis, pharyngitis and croup. *M. pneumoniae* and concomitant host reactions can induce hemolytic anemia, Stevens-Johnson syndrome, myo- and pericarditis (9% of cases) and adult respiratory distress syndrome. *M. pneumoniae*-associated neurologic diseases (encephalitis, meningitis, myelitis, polyradiculitis) are being recognized with increasing frequency (following at least 1/1000 respiratory infections), some with debilitating or fatal outcome. Direct invasion, a neurotoxin, autoimmune complex formation with gangliosides or vasculopathy are all being discussed as pathomechanisms. Neuropathy, including Guillain-Barré syndrome following *M. pneumoniae* infection, has been associated with the induction of autoantibodies to galactocerebroside.

Primarily urogenital pathogens

U. urealyticum was first isolated from male patients with nongonococcal urethritis (50% of cases). It sometimes causes prostatitis, but may otherwise be found in the male urethra without any symptoms. *U. urealyticum* is normal flora of the female genital tract after onset of sexual activity (in some populations up to 80% of pregnant women are positive), but in conjunction with anaerobic gram-negative rods, *Gardnerella vaginalis* and a concomitant depression of protective lactobacilli, it is part of the pathologic bacterial vaginosis and is then present in large numbers. Bacterial vaginosis predisposes to upper genital tract infections (especially endometritis) and an adverse pregnancy outcome, and it doubles the risk of spontaneous preterm delivery. Like other organisms associated with bacterial vaginosis, *U. urealyticum* and *M. hominis* are suspected to be involved in intrauterine growth retardation of the fetus. Isolation of *U. urealyticum* from the chorioamnion with signs of histological chorioamnionitis rather than colonization of the lower genital tract is associated with prematurity and low birthweight. Mid-pregnancy antimicrobial treatment directed against anaerobes and *U. urealyticum*

reduces the rate of premature delivery in high-risk women with bacterial vaginosis.

The prevalence of *U. urealyticum* depends on the population studied. In Europe, about one-tenth of mature newborns carry *U. urealyticum* at birth, whereas one-third of extremely low birthweight infants (prematures of less than 1000 g birthweight) are colonized. In mature newborns, congenital *Ureaplasma* pneumonia has occasionally been described. Extremely low birthweight infants with *Ureaplasma* colonization of the lower respiratory tract, however, have a twofold increase in chronic lung disease characterized by the prolonged need for ventilation and/or supplemental oxygen.

M. hominis can be found in male urethritis in 19% of cases, and in the female genital tract as a commensal and as a member of the bacterial vaginosis family. Pelvic inflammatory disease, pyelonephritis, postpartum fever, posthysterectomy wound infection and fetal scalp abscesses may ensue from there. *M. hominis* has also been isolated from respiratory secretions of premature infants with pneumonia and in adult pharyngitis. In patients with reduced immunity, dissemination of genital mycoplasmas to other sites is possible, e.g. to the cerebrospinal fluid in premature infants, to the joints of hypogammaglobulinemic persons or nosocomially to operative wounds. *M. hominis* and *U. urealyticum* have recently been demonstrated in adenotonsillitis in children.

M. genitalium has been found associated with male urethritis (in 14–17% of cases), female urethritis, salpingitis, pelvic inflammatory disease, and arthritis in the immunocompromised state. *M. genitalium* has also been detected in the respiratory tract, but care must be taken in distinguishing this organism from *M. pneumoniae*, as both behave similarly in culture and share genetic and antigenic traits.

Mycoplasmas/ureaplasmas and arthritis

Association of mycoplasmas or ureaplasmas with arthritis in animals is an accepted fact and has been thoroughly studied in animal models such as, e.g. that with *M. arthritidis* in the mouse and rat. Although *M. arthritidis* may be a special case, as this strain which does not typically infect humans produces a superantigen, an association of mollicutes with human arthritis has long been under debate, and solid evidence in favor of an involvement of mycoplasmas and ureaplasmas in arthritis is increasing. In general, these organisms remain undiscovered unless specifically sought. Thus, mollicutes are taken to be a rare cause of septic inflammatory arthritides. However, mollicutes are responsible for about 40%

Table 1 Antibiotic susceptibilities of mycoplasmas causing human disease

	Antibiotic minimal inhibitory concentration (MIC) ($\mu\text{g ml}^{-1}$)		
	<i>M. pneumoniae</i>	<i>M. hominis</i>	<i>U. urealyticum</i>
Tetracycline	0.06–1	0.06–128 ^a	0.5–256 ^a
Doxycycline	0.25–0.5	0.03–16	0.06–256 ^a
Erythromycin	≤ 0.06 –8	R	0.25–>128
Clarithromycin	≤ 0.04 –0.125	R	0.03–0.5
Roxithromycin, azithromycin	<0.01–0.03	R	0.5–4
Chloramphenicol	1.6–6.4	<0.2–0.8	0.4–16
Ciprofloxacin	0.5–8	0.25–8	2–16
Clindamycin	0.5–4	0.03–2	1–>128

R, resistant. The large spread in MIC is due to the Tet M determinant in some strains, causing high-level tetracycline resistance. Wherever a wide range is given, susceptibility testing is recommended.

of the septic arthritides in patients with hypogammaglobulinemia or other immune deficiencies. *M. hominis*, *M. genitalium*, *M. fermentans* and *U. urealyticum* have all been isolated from the joint fluid of such patients. Diagnosis of mollicutes is tedious and time-consuming when done by culture but will eventually become easier and more sensitive using the polymerase chain reaction (PCR). This may be the reason why mycoplasmas only recently were also detected in the joint fluid of immunologically normal patients with different forms of inflammatory rheumatic diseases but not in control persons.

In the case of reactive arthritis, defined as a seronegative oligoarthritis triggered by a preceding extra-articular infection, involvement of mollicutes is also likely. However, since the urogenital tract of a great percentage of sexually active persons is colonized with mollicutes without causing any symptoms, the establishment of a causal relationship between colonization with mollicutes and a reactive arthritis has to rely on statistics. These are in favor of such a correlation but may need confirmation.

Mycoplasmas and HIV

At about the same time as the first effects of mycoplasmas on human immunodeficiency virus (HIV) replication in cell culture were reported, a discussion about the role of mycoplasmas as cofactors in the acquired immune deficiency syndrome (AIDS) was started, and it continues today. While a seeming inhibition of reverse transcriptase by mycoplasmas was found to be due to mycoplasmal nuclease, and stimulation of virus replication in some *in vitro* systems could be explained by proinflammatory cytokines, involvement of mycoplasmas in the progress of HIV-mediated disease is still under debate. Depending on the methodology used, various or no mycoplasma species were found associated with HIV infection.

Several independent studies agree on the association of *M. penetrans*, a strain which is transmitted by homosexual practices, with HIV. The situation with *M. fermentans* is less clear: some studies report an association of this mycoplasma with HIV, whereas others find no such correlation.

Diagnosis

Routine diagnosis is limited to the relatively few mollicutes involved in human disease. Mycoplasmas can be diagnosed 1) indirectly by testing patient's sera for specific antibodies, 2) directly by cultivation and identification, and 3) by methods of molecular biology. Ureaplasmas are usually not diagnosed by serological means but by cultivation or PCR. Sero-diagnosis (IgM and IgG) can be achieved by complement fixation assays or enzyme-linked immunosorbent assays. For *M. pneumoniae* a commercial glycolipid antigen preparation is available which is suitable for a preliminary diagnosis only, as this antigen is not entirely specific. Metabolic inhibition tests or (hem)agglutination assays are now less frequently in use. Cold agglutinins, though not specific, may be an indication of an *M. pneumoniae* infection. Successful cultivation and isolation requires careful collection of samples from the appropriate sites, short transportation times or preservation, and the use of different formulations of broth and agar media in a specialized laboratory. All these parameters are critical, and cultivation may require 2 weeks or longer. A great number of poly- and monoclonal antibodies specific for several mycoplasma species or different serovars of *U. urealyticum* are available. These serve in the identification of individual colonies on agar blots or in western blot analysis. Also DNA restriction or protein separation patterns may help to identify less frequently occurring species. As myco-

plasmas quickly become nonviable during transport of clinical material and in body fluids containing antibiotic substances, strain-specific DNA probes for *in situ* detection and PCR have been developed for all pathogenic mycoplasmas. The sensitivity and specificity of PCR comes close to or surpasses that of cultivation methods. For all that, culture confirmation may be essential because PCR is notorious for possible false-positivity, and antimicrobial sensitivity testing and future reference cultures may be needed for clinical purposes. However, used judiciously, molecular tools will certainly change epidemiological data on mycoplasmas, as the true incidence and prevalence of these organisms becomes apparent.

Treatment

Many mycoplasma infections do not require treatment. Vaccines against pathogenic mycoplasmas of humans are presently not available, a major difficulty being the antigenic variability of most mycoplasmas. For animal mycoplasmal diseases, inactivated and live vaccines have been developed against bovine, porcine, avian and caprine pneumonia.

Since mycoplasmas lack a cell wall, they are not susceptible to antibiotics such as β -lactams which inhibit cell wall synthesis. Aminoglycosides are merely inhibitory. Treatment options with *in vitro* patterns of resistance (minimal inhibitory concentrations) are listed in Table 1. Among other caveats, care must be taken to adapt antibiotic therapies to age (e.g. tetracyclines are toxic for the fetus and infants), to the causative organism (e.g. *M. hominis* is universally resistant to erythromycin) and to the site of infection (e.g. erythromycin penetrates inadequately into body fluid spaces).

See also: Adhesion molecules; Antigenic variation; Complement fixation test; Human immunodeficiency viruses; Macrophage activation; Rheumatoid arthritis, animal models; Rheumatoid arthritis, human; Sex hormones and immunity; Western blotting.

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MYELOID ANTIGENS

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Hematopoietic cells express a range of antigens, which in some cases are restricted to a particular myeloid lineage or stage of differentiation. The International Workshops and Conferences on Human Leukocyte Differentiation Antigens, have classified, a number of antigens as myeloid antigens (Table 1). This is a somewhat arbitrary distinction and signifies lack of reactivity of the antibodies in that particular cluster of differentiation (CD) with the mature lymphoid elements of the blood as much as myeloid specificity. Antibodies reactive with the myeloid CD13 antigen for instance, also react with the connective tissue of lymph nodes, thymus and spleen, bile canaliculi and sinus lining cells in the liver, glomeruli and proximal tubules in the kidney, and some skin cells, pancreatic cells, and cerebellar perineural cells. One of the most myeloid-specific antigens appears to be CD66b, which is a member of the carcinoembryonic antigen family.

Differentiation-associated expression of myeloid antigens

The changing expression of some of the myeloid antigens during granulocytic and monocytic differentiation is illustrated in Figure 1. Use of antibodies reactive with these antigens has assisted in the identification and purification of cell populations at specific stages of differentiation. Primitive myeloid cells express the CD34 antigen as do the primitive lymphoid progenitor cells. Successful hematopoietic stem cell transplants can be carried out, using purified CD34⁺ bone marrow or mobilized peripheral blood cells, although it has been suggested recently that a small population of the most primitive stem cells are CD34 negative. Considerable attention has been focused on the phenotypic and functional subdivision of the CD34⁺ hematopoietic cells. The more primitive stem cells, that give rise to long-term bone marrow cultures, are CD33⁻, CD38⁻, CD45RA^{-/low}, CD71^{-/low}, *c-kit* (SCF receptor CD117)^{low}. There is controversy as to the major histocompatibility complex (MHC) class II status of primitive stem cells. In fetal marrow those cells with the highest plating and replating efficiency are CD34⁺, CD38⁻, HLA-DR⁺, whereas in adult marrow those cells capable of giving rise to long-term cultures are HLA-DR⁻. The vast majority of colony-forming cells are HLA-DR⁺, but the expression of this antigen then ceases between

the myeloblast and promyelocyte stage. The CD13 antigen is expressed on a proportion of progenitor cells, and is most strongly expressed on those cells which are actively cycling. Antibodies to the CD33 and CD13 antigens have proved valuable in the diagnosis of myeloid leukemias.

A number of antigens appear in the identifiable granulocytic and monocytic precursor pool including CD14 and CD15. CD14 was initially thought to be monocyte specific but is expressed weakly on at least a proportion of granulocytes. CD15 is expressed on all neutrophils, most monocyte precursor cells, but only a small proportion of circulating monocytes. Of particular interest is the CD10 antigen (CALLA), originally thought to be restricted within the hematopoietic system to early B lymphoid cells and now known to be expressed as a late event in neutrophil differentiation and also to be present on bone marrow stromal cells.

The phenotype of eosinophils and basophils has some similarities with neutrophils but there are also distinct differences. Both eosinophils and basophils express the leukocyte integrins (CD11a, CD11b, CD11c, CD18), CD13, CD32, (FcγRII), CD35, CD43, CD44, and CD45, as do neutrophils. In contrast to neutrophils, eosinophils express CD9 and lack CD10, CD16 (FcγRIII) and CDw17. Basophils, unlike neutrophils, express CD25 (IL-2R), CD38, CD40 and CD54 (ICAM-1), but lack CD10, CD16 (FcγRIII), CDw65, CD66a and CD66b. CD15 expression is weak.

The phenotype of early erythroid progenitor cells is similar to that of early granulocytic and monocytic progenitor cells, although expression of the transferrin receptor (CD71) may be higher on the erythroid cells. With erythroid differentiation into the recognizable precursor pool, there is acquisition of a large number of red cell-specific antigens, including many blood group antigens, 'band III' and glycoporphin. Similarly, megakaryocytes and platelets acquire a number of differentiation-associated antigens, such as gpIIb (CD41), gpIX (CD42a), gpIbα (CD42b), gpIbβ (CD42c), gpV (CD42d), the vitronectin receptor (CD51), the integrin β₃ subunit (CD61) which can associate with CD41 or CD51, and P-selectin or PADGEM (CD62P). Some platelet lysosomal proteins only become expressed on the cell surface after platelet activation (e.g. CD63, CD107a, CD107b) and antibodies to these antigens are being increasingly used in the assessment of platelet status and function.

Table 1 Selected antigens expressed on myeloid cells

<i>CD no.</i>	<i>Mol. wt (kDa)</i>	<i>Biochemical nature</i>	<i>Reactivity with mature hematopoietic cells</i>	<i>Reactivity with other tissue cells</i>
10	100	Neutral endopeptidase	Neutrophils	Lymphoid progenitor cells. Renal glomeruli and tubular epithelium. Myoepithelial cells of breast and salivary glands. Some smooth muscle cells
13	150	Aminopeptidase N	Monocytes, macrophages, granulocytes	Some myeloid progenitor cells; lymphoid organ connective tissue; renal glomeruli and proximal tubules; bile canaliculi; liver sinus lining cells; cerebellar perineural cells around skin sweat glands; pancreatic ducts and secretory cells
11a	180 α chain	α/β Heterodimeric glycoprotein (integrin family)	All leukocytes	Neuroectodermal cells
11b	165 α chain	α/β Heterodimeric glycoprotein (integrin family) CR3	Granulocytes, monocytes, NK cells	
11c	150 α chain	α/β Heterodimeric glycoprotein CR4	Monocytes, granulocytes, NK cells, some T cells, some B cells	
18	95 β chain	α/β Heterodimeric glycoprotein; CD18 is linked to CD11a, CD11b, or CD11c	All leukocytes	
14	55	PI-linked	Monocytes, macrophages, some granulocytes	Dendritic reticulum cells in lymphoid tissues; some glandular epithelial cells of skin
15	Multiple bands	Lacto-N-fucopentose III (Lewis ^x)	Neutrophils, eosinophils, some monocytes	Some glandular epithelial cells; pituitary astrocytes; Reed Sternberg cells
65		Ceramide dodecasaccharide	Granulocytes	Hepatocytes; epidermis and sweat glands of skin; pancreatic secretory cells, bronchial epithelium; lung alveolar cells; renal tubular cells; colonic epithelial glands
66a	160–180	PI-linked glycoprotein Biliary glycoprotein 7	Neutrophils	Bile canaliculi; sweat glands, renal endothelium and some tubular cells; colonic epithelium
66b	95–100	PI-linked glycoprotein CGM-6	Granulocytes	
66c	90	PI-linked glycoprotein NCA	Neutrophils	Colon carcinoma
66d	30	PI-linked glycoprotein CEA	Neutrophils	
33	67	Transmembrane glycoprotein	Monocytes, some granulocytes	Myeloid progenitor cells; Langerhans cells
34	105–120	Transmembrane glycoprotein	None	Lymphohematopoietic stem cells; capillary endothelial cells
38	45		B cells and plasma cells; monocytes; activated T cells	Most lymphohematopoietic progenitor cells
16	50–70	Fc γ RIII	Neutrophils, macrophages, NK cells	Lymph node endothelium
32	40	Fc γ RII	Granulocytes, monocytes, B cells, some T cells platelets	Myeloid progenitor cells; some endothelial cells; some epithelial cells
64	70	Fc γ RI	Monocytes, some macrophages, neutrophils following interferon exposure	

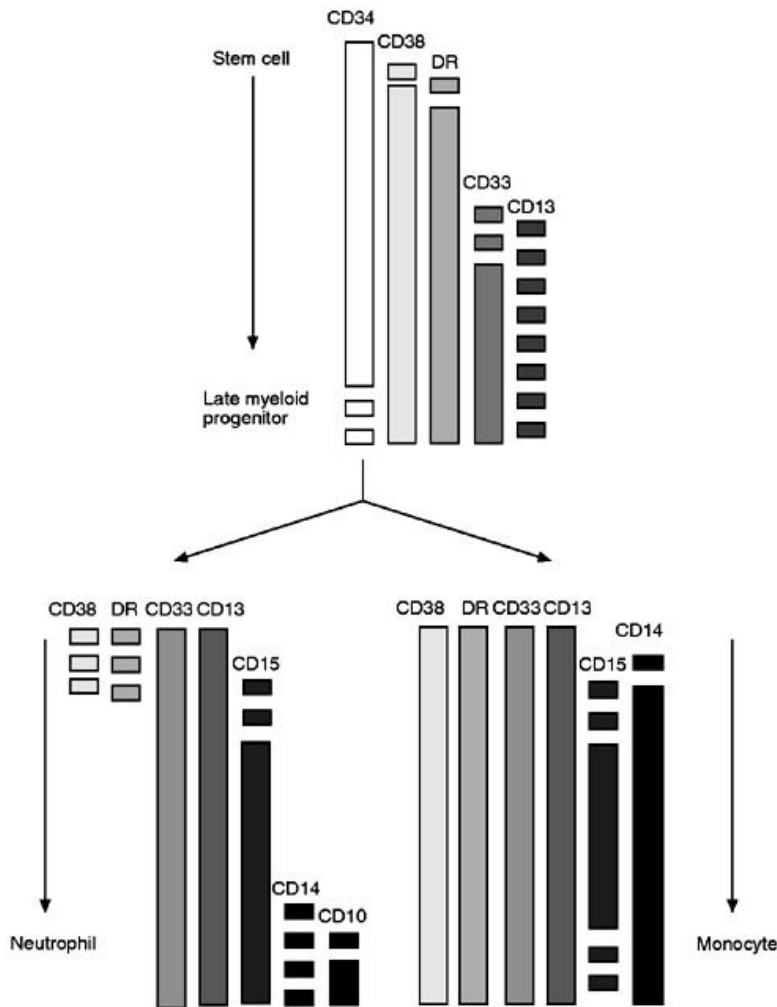


Figure 1 Changing antigen expression during myelo-monocytic differentiation.

Other leukocyte antigens expressed by myeloid cells

Myeloid cells express many other antigens which are not usually considered as myeloid antigens. These include the HLA class I antigens expressed on all myeloid cells except mature red cells and the common leukocyte antigen (CD45) expressed on all leukocytes and early erythroblasts, but not intermediate or late normoblasts. The leukocyte cellular adhesion molecules (Leu-CAMs) or integrins are expressed on granulocytes and monocytes, the CD11b/CD18 complex being heavily expressed on granulocytes and monocytes, and the CD11c/CD18 complex being preferentially expressed on monocytic cells. The phagocyte integrins, in common with CD35 (CR1), CD15, CD10 and CD13, are stored in intracellular granules and are rapidly unregulated several-fold by a range of stimuli, including C5a, formyl-Met-Leu-Phe, tumor necrosis factor α (TNF α) and granulocyte-macrophage colony-stimulating factor (GM-CSF). All phagocytic cells express the low-affinity

Fc γ RII (CD32), as do many other cell types, including B cells, some T cells, and some endothelial and epithelial cells. The low-affinity receptor Fc γ RIII (CD16) is expressed on neutrophils and natural killer (NK) cells, and the high-affinity receptor Fc γ RI (CD64) is expressed on monocytes. Fc γ RI expression on monocytes is upregulated many-fold by exposure to interferon γ (IFN γ), and also becomes detectable on neutrophils following interferon treatment.

Functional aspects of myeloid antigens

The function of the leukocyte integrin molecules (CD11a, CD11b, CD11c, CD18) and the Fc receptors (CD64, CD32, CD16) are at least partly understood. As well as being involved in intracellular adhesion, CD11b and CD11c function as complement receptors (CR3 and CR4 respectively) and CD35 is the CR1 receptor.

Surprisingly little is known about the function of many of the other myeloid antigens. CD13 is an aminopeptidase N, which catalyzes the removal of

N-terminal amino acids from peptides with a preference for neutral residues. CD10 is a neutral endopeptidase (enkephalinase). It is thought that these surface molecules may cause hydrolytic inactivation of regulatory peptides and growth factors. Both molecules appear to modulate the phagocyte response to the bacterial cell wall peptide formyl-Met-Leu-Phe.

Various claims have been made for antibodies to many myeloid antigens either causing activation or inhibition of phagocyte functions. These must be viewed with caution, however, as most studies have been performed with intact antibodies which can simultaneously bind via their Fc portion to the various Fc receptors and effect signal transduction through these molecules.

See also: Adhesion molecules; CD antigens; Complement receptors; Integrins; Monocytes; Mononuclear phagocyte system; Neutrophils.

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NATURAL ANTIBODIES

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That the sera of normal human beings and animals contain substances able to neutralize toxins or to agglutinate or lyse bacteria and erythrocytes was already well known at the end of the last century. It soon became evident that these substances corresponded to antibodies, and it was also rapidly established that sometimes these natural antibodies were able to react with constituents of the organism that produced them. Thus, natural antibodies toxic to spermatozoa or antibodies inhibiting the hemolytic action of erythrocyte-specific antibodies were found in the sera of various animal species. The results of Lansteiner and his group on the presence in normal human sera of antibodies specifically reacting with blood group substances, which led to the classification of human erythrocytes, were the beginning of a long series of studies on natural antibodies. With the passing years, it was shown that natural antibodies to bacteria, viruses, protozoa, parasites, heterologous and isologous erythrocytes, tissues and their extracts, tumors, toxins and spermatozoa were present in mammals, birds, reptiles, fishes and amphibians.

On the basis of the results obtained in these early studies, several conclusions were drawn. Two kinds of natural antibodies are present in sera. The first are specific and represent antigen-induced antibodies. Their formation depends on external antigenic stimuli and corresponds therefore to acquired specificities. The second most often, but not always, expresses a broad specificity, is genetically determined and does not depend on a given antigenic stimulation. Both kind of antibodies require permanent external antigenic stimulation for their maintenance. This stimulation is provided by related cross-reactive antigens. Natural antibodies to bacteria and viruses being widespread and present in all individuals, possess direct bactericidal and virus-neutralizing activities or, by binding to these pathogens, enhance their phagocytosis by macrophages and polymorphonuclear cells. Similarly antierythrocyte natural antibodies, by binding to effete erythrocytes, increase their opsonization and participate in their removal, while antitissue natural antibodies facilitate the elimination of tissue breakdown products. Based on this information, Grabar proposed that natural

antibodies, acting as carriers or transporters of self constituents, are part of the mechanism involved in the handling of metabolic and catabolic byproducts. Boyden advanced the concept that natural antibodies are the main means by which vertebrate and possibly invertebrate phagocytes discriminate between foreign and nonforeign matter.

Systematic studies on natural antibodies have been in progress since the beginning of the 1980s. They have benefited enormously from the recent intensive progress in immunobiotechnology. The main message of this new information is that the normal or preimmune repertoire is constituted principally of polyreactive antibodies able to recognize both self and nonself antigens equally well.

Occurrence and general characteristics of natural antibodies

A series of studies have shown that natural antibodies of the immunoglobulin M (IgM), IgG and IgA isotopes directed against an immense variety of antigens are present in normal sera of humans and other animal species including various fish of very distant phylogenetic orders (e.g. shark, torpedo, sturgeon and salmon). Internal, cell surface and circulating proteins, carbohydrates, nucleic acids, lipids and haptens are recognized by these natural antibodies. These constituents represent ubiquitous, self or highly conserved antigens and also environmental foreign antigens. IgM and IgG natural antibodies reacting with cell surface molecules of the immunoglobulin superfamily have been repeatedly detected in normal sera. Often IgG natural antibody activity was partially or completely masked in whole serum but it was unambiguously revealed after appropriate treatment of the serum. Depending on the antigen used, 5–100% of the normal sera examined were found to contain antibodies directed against a given antigen.

Human and mouse sera analyzed by means of ELISA or immunoblotting using homologous tissue extracts as the antigen source exhibited distinct IgM and IgG autoreactivity profiles. Two types of antibodies were found and isolated from normal sera: those that react with more than two, apparently unrelated antigens and those specific to a single antigen. Polyreactive antibodies are by far the most abundantly found in normal sera and usually possess low affinities. Monospecific antibodies behaved like the experimentally induced antibodies and usually possessed high affinities.

Similarly, B cell clones prepared from various human and mouse lymphoid organs during fetal and adult life secreted monoclonal natural antibodies

reacting with a wide variety of self and nonself antigens. Depending on the stage of development and the lymphoid organ, 2–100% of all the clones secreting immunoglobulins synthesized natural autoantibodies and, among them 2.5–100% reacted with two or more self and nonself antigens. The natural antibodies are produced by conventional B lymphocytes, although in some instances, it has been reported that Ly-1⁺ B cells were preferentially involved in their synthesis. Natural IgM autoantibody-producing B cell clones have also been obtained from nude, germ-free and antigen-free mice. Recently antibodies able to recognize self antigens with high specificity were isolated from a phage display library composed of the rearranged V genes of peripheral blood lymphocytes from a normal individual.

Properties and biological functions of natural antibodies

A series of studies has provided a better definition of several of the properties of natural antibodies. Polyreactive but also monoreactive natural antibodies are encoded by nonmutated or minimally mutated germ-line genes. Usually, but not always, natural antibodies possess low affinities but high avidities, and each one of them corresponds to the product of a single clone. Expression of natural antibody reactivity seems to be under the control of T cells.

Polyreactive natural antibodies carry common idiotypes and establish a dense idiotypic network among themselves early in ontogeny.

Natural antibodies have been reported to effect various biological functions (Table 1). Depending on the situation, they can play either a physiological, i.e. beneficial, or a pathological, i.e. harmful, role. Treatment of mice with idiotypically interconnected monoclonal antibodies or anti-idiotypic antibodies to natural antibodies results in an increase or decrease

Table 1 Biological functions of natural antibodies

Immunoregulation through idiotypic interactions
Immunoregulation by interacting with cytokines
Immunoregulation by interacting with B and T cells
Antigen processing and presentation by B lymphocytes
Rejection or protection of cellular engraftments
Interference with the development of autoimmune states
Induction of autoimmune-like syndromes
Induction of glomerulonephritis
Tumor inhibition or enhancement
Clearance of altered self constituents
Elimination or protection of pathogens
Expression of catalytic or inhibitory activities

of the immune response according to the idiotypes involved. In normal humans and mice, a natural antibody idiotype-like network exists that regulates the binding of IgG autoantibodies to self antigens, and this network is deficient in autoimmune states. Moreover, natural antibodies able to interact with molecules present on the surface of B and T cells have been shown to inhibit B cell growth or to suppress activation of T cell or autologous mixed lymphocyte reactions. Similarly, natural antibodies to various cytokines possess immunoregulatory functions.

B cells that bear natural antibodies as receptors have been shown to play an important role in the processing and presentation of antigens. Natural antibodies able to enhance tissue rejection but also, in contrast, to increase graft survival have been detected. By binding to altered self constituents, natural antibodies contribute to the clearance of catabolic products from the organism. Similarly, by reacting with pathogenic agents, they increase the opsonization of the latter by the reticuloendothelium system and contribute in their elimination from the organism. In some instances, natural antibodies can also inhibit the pathogenic effect by interacting with the host components rather than with the pathogen itself.

On the other hand, by binding to pathogens, natural antibodies focus the complement attack on non-lethal sites and protect the pathogen from destruction. Furthermore, they can even promote pathogenesis by mediating the internalization of the pathogens to gain access to its cellular niche. Likewise, depending on the situation, natural antibodies reacting with malignant cells enhance, retard or have no effect on tumor development.

Natural antibodies and pathological states

Recent studies have shown that there are extensive similarities between natural antibodies and the 'pathogenic' antibodies found in various immunopathological situations. Thus, in human and murine lupus, a substantial number of the autoantibodies against DNA which can be eluted from renal biopsies are able to react with many other antigens. Polyreactive IgM and IgG autoantibodies increase during the development of the murine lupus syndrome and form glomerular immune deposits. Furthermore, monoclonal polyreactive antibodies against DNA derived from lupus mice when injected into normal mice are able to induce nephritis. Many antibodies against DNA are encoded by nonmutated or minimally mutated genes but some show clear evidence of somatic mutations. These results suggest that in

lupus, high-affinity mutated 'pathogenic' DNA autoantibodies may arise from germ-line encoded low-affinity autoantibodies through an antigen-driven selection. However, germ-line encoded polyreactive natural antibodies without mutations may be pathogenic under certain circumstances.

Similar results were obtained and conclusions reached by analyzing the rheumatoid factor antibodies appearing in rheumatoid arthritis.

Recent studies have shown that antibodies produced after infections are often polyreactive and encoded by nonmutated germ-line genes. Thus, for example, analysis of the expressed V genes for IgG antibodies against herpes simplex virus and varicella-zoster virus showed that these antibodies use the same germ-line V_H genes used by autoantibodies. Similarly murine monoclonal antibodies reacting with group A streptococcal antigen were found to react with various autoantigens and to be encoded by unmutated germ-line genes. Furthermore, monoclonal antibodies obtained from *Trypanosoma cruzi*-infected mice reacting with this parasite were found to be encoded by the germ line and recognized antigens present on the membrane of activated T cells. It would appear, therefore, that infection by viruses, bacteria and parasites stimulates the natural antibody-producing cells, resulting in an increased synthesis of these antibodies.

See also: Affinity; Autoimmune diseases; Autoimmunity; CD5; Gammaglobulin; Idiotypic network; Monoclonal antibodies (mAbs); Specificity.

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NATURAL KILLER (NK) CELLS

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Since their discovery in the early 1970s, natural killer (NK) cells have been recognized as a distinct subset of lymphocytes. Morphologically, they are large granular lymphocytes (LGLs) and functionally, they have the ability to kill a variety of cellular targets, including tumor cells and virus-infected cells, without damaging normal cells. The name 'NK cells' reflects the capability to kill susceptible targets spontaneously, i.e. without prior sensitization. For over two decades, NK cells have been considered to function as nonmajor histocompatibility complex (MHC)-restricted effector cells. Today, this functional definition of NK cells has to be modified. The family of recently discovered surface receptors on NK cells appears to regulate killing mediated by NK cells. The receptors recognize polymorphic class I MHC molecules expressed on target cells. The level of expression of HLA-A, -B or -C molecules on susceptible targets regulates their death or survival. The HLA-specific receptors are either inhibitory (killer cell inhibitory receptors, KIRs) or stimulatory (NK activating receptors, NKARs). The mechanisms responsible for this type of recognition and the molecular pathways mediating positive or negative signals generated upon interaction of the NK cell with its potential target are currently under intense investigations.

NK cells have important biological functions: in addition to selective elimination of abnormal targets (immune surveillance), they participate either directly or indirectly in multiple networks involved in development, regulation and communication within the immune system. They are found in the peripheral circulation as well as various lymphoid and nonlymphoid tissues. NK cells are remarkably

responsive to exogenous or endogenous activation signals by the rapid upregulation of cytolytic and proliferative functions as well as the production of numerous cytokines, including interferons, interleukin 1 β (IL-1 β), IL-10, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF α) and lymphotoxin (TNF β).

Activated NK cells express high levels of cellular adhesion and costimulatory molecules on the cell surface and acquire the ability to enter into solid tissues and to participate in interactions with tissue cells and extracellular matrix (ECM) components. These characteristics enable the NK cell to function as a highly efficient and aggressive effector cell, which is capable of modulating its own micro-environment and shaping the fate of neighboring cells. Understanding of the biology of NK cells is essential for defining their role in human disease.

Phenotype characteristics of NK cells

NK cells which are found in the human peripheral blood account for 5–15% of circulating lymphocytes and represent a minority of total body NK cells. In rodents, NK cells are more numerous in the spleen (10%) than in the peripheral circulation but can also be found under physiologic conditions in several nonlymphoid tissues, e.g. lung or intestinal mucosa. In some organs, e.g. the liver, NK cells represent up to 45% of tissue-associated lymphocytes. NK cells can be recognized and enumerated in blood or tissues by the use of monoclonal antibodies which recognize distinctive surface receptors on lymphocytes. In human peripheral blood, three subsets of NK cells can be thus identified: CD3⁺CD56⁺CD16⁺ (80–

90%); CD3⁻CD56⁺CD16⁻ (5–10%); and CD3⁻CD56⁻CD16⁺ (5–10%). However, both CD56 and CD16 markers are also found on the surface of other hematopoietic cells, and no membrane receptor unique for NK cells has been identified so far. To distinguish NK cells from other hematopoietic cells, it is, therefore, necessary to employ a panel of antibodies and to discriminate between the presence as well as the absence of certain surface markers. Thus, mature circulating human NK cells express the following phenotype: CD3⁻TCR⁻CD56⁺CD16⁺CD2^{dim}CD8^{dim}CD14⁻CD19⁻. They are distinguishable from T cells by the lack of T cell receptor (TCR) or of rearranged TCR genes, which retain the germline configuration in mature NK cells; from B cells by the lack of immunoglobulin (Ig) gene expression; and from monocytes by the absence of CD14 expression. Although NK cells do not produce Ig, they may bear surface Ig bound to FcR, which are expressed on most NK cells.

Activating receptors on NK cells

Similar to other lymphocytes, NK cells express a variety of surface receptors (R), which are responsible for interactions of the cells with soluble ligands, ECM components, and molecules expressed on the surface of surrounding cell types (Table 1). The majority of circulating human NK cells constitutively express IL-2R β and γ in the absence of IL-2R α (CD25) and bind IL-2 with intermediate affinity. Upon activation, NK cells produce CD25 *de novo* and rapidly upregulate expression of high-affinity IL-2R. While NK cells can proliferate in the presence of IL-2 alone, they require costimulatory signals in addition to IL-2 for optimal and sustained proliferation. Such costimulatory signals are delivered by other receptors, including cellular adhesion molecules (CAMs) and FcRs. The Fc γ RIII (CD16) is an activation receptor on NK cells, and its ligation by CD16-specific antibodies, immune complexes or tar-

Table 1 Activating receptors on NK cells^a

Receptor	Surface structure	Ligand	Effect on NK cell functions
NKR-P1	Type II lectin-like	Carbohydrate (?)	Recognition and lysis
Ly-49	Type II lectin-like	Class I MHC alleles (murine)	Recognition and lysis
CD94/p43	Type II lectin-like	Class I MHC alleles	Recognition and lysis
CD94/p39 (NKG2-C)	Type II lectin-like	? Molecules on some tumor targets	Recognition and lysis
p50	Type I IgSF	HLA-C alleles	Recognition and lysis
CD69	Type II lectin	Carbohydrate	Activation and lysis
CD2	Type I IgSF	CD58	Costimulation
CD16 (Fc γ RIII)	Type I IgSF	IgG, IgG complexes	ADCC, costimulation
CD32 (Fc γ RII)	Type I IgSF	IgG	ADCC, costimulation
CD49d/CD29	$\beta_1\alpha_4$ -integrin	VCAM-1, FN	Adhesion, costimulation
CD49e/CD29	$\beta_1\alpha_5$ -integrin	FN	Adhesion, costimulation
CD49f/CD29	$\beta_1\alpha_6$ -integrin	Laminin	Adhesion, costimulation
CD11a/CD18	$\beta_2\alpha_L$ -integrin	ICAM-3	Costimulation
CD11b/CD18	$\beta_2\alpha_M$ -integrin	ICAM-1, CR3	Costimulation
CD11c/CD18	$\beta_2\alpha_X$ -integrin	CR3	Costimulation
CD51/CD61	$\beta_3\alpha_V$ -integrin	VTR	Adhesion, costimulation
CD54 (ICAM-1)	Type I IgSF	β_2 -integrin	Costimulation
CD102 (ICAM-2)	Type I IgSF	β_2 -integrin	Costimulation
CD50 (ICAM-3)	Type I IgSF	β_2 -integrin	Costimulation
CD62L	L-Selectin	MAcCAM	Adhesion
<i>Cytokine receptors</i>			
IL-2R	Type I hematopoietic family	IL-2, IL-15	Activation, proliferation
TNFR1	Type I TNFR family	TNF α , lymphotoxin	Activation, apoptosis
CD95 (Fas)	Type I TNFR family	CD95L (FasL)	Activation, apoptosis

IgSF, Immunoglobulin superfamily; VCAM, vascular cellular adhesion molecule; ICAM, intercellular adhesion molecule; CR, complement receptor; FN, fibronectin; VTR, vitronectin.

^aThe list of activating or cytokine receptors expressed on NK cells is not all inclusive.

get cells bearing Ig results in antibody-dependent cellular cytotoxicity (ADCC), proliferation and cytokine production by NK cells. Engagement of the CD16 complex, consisting of CD16 and associated dimers of CD3 ζ -CD3 ζ ; Fc ϵ RI γ -CD3 ζ ; Fc ϵ RI γ -Fc ϵ RI γ on NK cells leads to activation of the intracellular signaling pathway similar to that induced by TCR-CD3 ζ activation in T cells. Importantly, the participation of antibody in this type of signaling confers specificity upon those NK cells that express Fc γ RIII. Some human NK cells also express Fc γ RIIc (CD32, isoform c), and it appears to be a signaling molecule on NK cells. The third type of FcR recently described, Fc μ R (receptor for IgM), is a triggering molecule expressed on the majority of NK cells. However, its cross-linking with IgM at physiological concentrations results in negative regulation of several NK cell functions, including cytotoxicity. A broad range of CAMs are detectable on NK cells (Table 1) and participate in signal transduction and activation of NK cells. For example, VLA-4 and VLA-5 (receptors for fibronectin) and VLA-6 (receptor for laminin) facilitate binding of NK cells to the relevant substrates and induce NK cell activation. Costimulatory molecules, such as CD2, CD69 or NKR-P1 may also be involved in triggering

of cytolytic function in NK cells. Monoclonal antibodies to these various surface molecules, acting as agonists, can initiate antibody redirected lysis of FcR-bearing targets (Figure 1d). Overall, it appears that cooperative engagement of several activating or costimulatory receptors interacting with their respective ligands on target cells is sufficient to induce a lytic pathway in NK cells.

Inhibitory receptors on NK cells

A novel class of receptors recognizing class I MHC molecules and capable of inhibiting lysis has been recently described on NK cells (Table 2). In rodents, the Ly-49 family of receptors expressed on NK cells includes at least eight distinct type II disulfide-linked homodimers of C-type lectins which bind H-2 class I ligands on target cells and inhibit NK cell-mediated lysis. In humans, a distinct family of killer cell inhibitory receptors (KIRs), which are type I transmembrane proteins of the Ig superfamily, has been identified. These receptors bind class I HLA molecules on target cells, and their ligation induces a negative signal and abrogation of the lytic process (Figure 1a). This function is mediated by phosphorylation of cytoplasmic tyrosines, which recruit the protein tyrosine phosphatase SHP-1. The absence

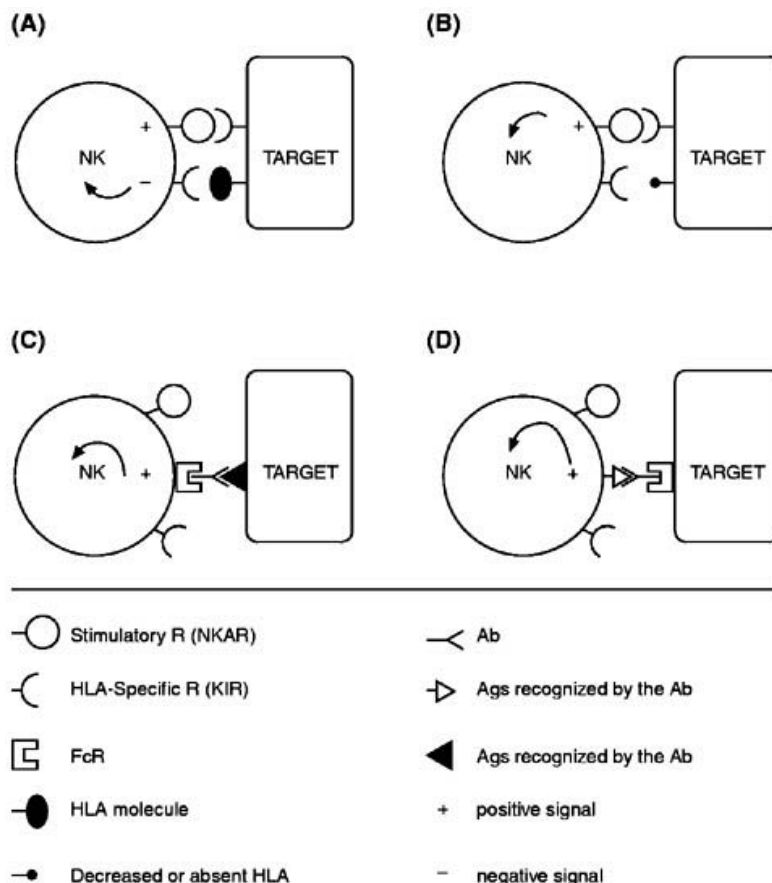


Figure 1 Interactions of NK cells with cellular targets do not result in lysis if the target cell expresses class I HLA molecules recognized by the HLA-specific receptor on the NK cell. Positive signals induced by binding of NKARs to their ligands on the target are superseded by negative signals transduced via KIRs, which bind to HLA alleles recognized on the target (A). If, however, expression of the HLA-alleles recognized by KIRs on the target is decreased or the target lacks these alleles, negative signals cannot be generated, and the target cell is lysed by the NK cells receiving positive signals via NKARs (B). In the presence of any antibody decorating the NK cell through Fc γ RIII and recognizing an antigen on the target, antibody-dependent cellular cytotoxicity (ADCC) leads to target cell lysis (C). If, however, the antibody decorates the target cell and also recognizes a signaling molecule on the NK cell, Ab-mediated redirected lysis occurs (D).

Table 2 Inhibitory receptors of NK cells (KIRs)^a

Receptor	Surface antigen structure	Ligand	Effect on NK killing
Ly-40 family	Type II lectin-like	Class I MHC alleles (murine)	Inhibition
p58	Type I IgSF (2D)	HLA-C alleles	Inhibition
p70 (NKBI)	Type I IgSF (3D)	HLA-BW4 alleles	Inhibition
p140	Type I IgSF (3D) Ig-like protein (3D)	HLA-A alleles	Inhibition
CD94/kp43 (NKG2-A)	Type II lectin-like	Class I alleles	Inhibition

D, Ig domain; IgSF, immunoglobulin superfamily.

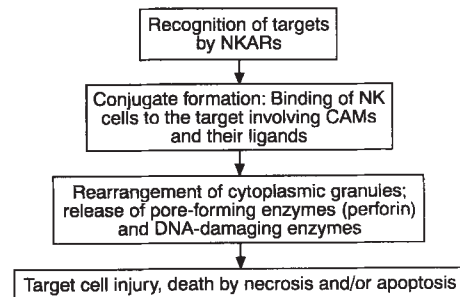
^aInhibitory receptors on NK cells contain xY/xL sequences in the cytoplasmic tail, which are termed immunoreceptor tyrosine-based inhibiting motifs (ITIM) and which are necessary for inhibitory function.

of class I HLA molecules or their decreased expression on target cells leads to NK cell-mediated lysis of the target (**Figure 1b**). KIRs belong to a large and diverse family of receptors, which appear to be distributed in different combinations on distinct NK cell clones and NK cell subsets. This family also contains MHC receptors which activate rather than inhibit NK cytotoxicity, e.g. the p50.3 receptor involved in HLA-C recognition in humans (**Table 1**). Furthermore, MHC receptors have been detected not only on NK cells but also on subsets of T cells. The presence of both inhibitory and stimulatory MHC receptors on the same NK or T cell indicates that immune responses are regulated by maintaining a delicate balance between positive and negative signals induced upon recognition of MHC alleles on target cells.

Functional Characteristics of NK cells

Lysis of target cells

The ability to recognize and lyse a broad range of targets with altered surface expression of MHC molecules, such as virally infected or transformed cells or certain allogeneic targets, is the fundamental functional attribute of NK cells. While the molecular events involved in the process of target recognition and signaling remain to be clarified, cellular alterations which result from effector (E)–target (T) cell interactions are known. Several distinct and sequential steps are involved in the process of lysis, as indicated in **Figure 2**. Human NK cells can utilize two different and independent mechanisms of killing: secretory (necrosis) and nonsecretory (surface receptor-mediated apoptosis). NK-sensitive targets, such as K562, are predominantly killed by necrosis. In contrast, NK-resistant targets, such as fresh tumor or solid tissue cells, are killed only by apoptosis in the absence of demonstrable necrosis. Activated NK cells can rapidly kill a broad range of targets by a combination of these two mechanisms. The NK cell

**Figure 2** Schematic representation of the mechanism of lysis of target cells by NK cells.

which survives a lytic event is able to recirculate, that is, it can bind to another susceptible target and kill it. The efficiency of NK cell-mediated lysis is dependent on the ratio of E:T present, state of NK cell activation, and sensitivity of the target cells to lysis.

Response of NK cells to cytokines

Among lymphocytes, NK cells are the first to respond to activation by IL-2, because they constitutively express the β chain of IL-2R. Consequently, incubation of peripheral blood lymphocytes with IL-2 induces selective activation of NK cells and results in rapid upregulation of NK cell activity and, later, generation of LAK cell activity and proliferation. In the presence of IL-2, a small subset of NK cells (10–30%) rapidly (within minutes) acquires the ability to adhere to plastic or other solid surfaces *in vitro*. These cells are almost exclusively CD3⁺CD56^{dim}CD16^{dim} or CD16⁺, and they respond to relatively high levels of IL-2 by rapid upregulation of the IL-2R β chain, β_2 -integrins and activation antigens on the cell surface and by *de novo* expression of the IL-2R α chain. These NK cells also upregulate expression of mRNA for a variety of cytokines. These cells have been named 'A-NK' cells, 'A' standing for the adherence and activation that characterize their early response to IL-2. The property of adherence to plastic can be reliably used to isolate and

culture A-NK cells from human peripheral blood or spleens of rodents.

All NK cells undergo activation and expansion in the presence of IL-2 and/or other cytokines (e.g. IL-6, IL-12 and IL-15). NK cells which are derived from bone marrow precursors, can be generated *in vitro* by culture of CD34⁺ bone marrow cells with IL-2. Activated NK cells, and especially the subset of A-NK cells, become highly mobile and develop membrane structures called podosomes, which facilitate movement along solid surfaces and binding to tissue cells or to ECM. A-NK cells show significantly increased integrin-mediated adhesion to fibronectin- or laminin-coated plates and increased binding to human umbilical vein-derived endothelial cells (ECs) cultured on type I collagen. Associated with this increased adhesion and mobility of A-NK cells are changes in expression of activation markers and CAMs on the cell surface, upregulation of mRNA levels for cytokines, increased adhesiveness to ECs and transendothelial migration as well as highly augmented cytotoxicity.

NK cells in intercellular communication

A variety of soluble factors produced by activated NK cells are responsible for the importance of NK cells as mediators or effectors of the intercellular communication network. Activated NK cells produce a spectrum of cytokines: hematopoietic cell growth factors, interferons, interleukins, TNF α , lymphotoxin, TGF β and other growth factors. Enzymes produced by NK cells, especially those associated with the cell membrane, play an important role in cell-to-cell interactions and NK cell migration within tissues. A-NK cells also express, and upregulate upon activation, the receptors for a variety of chemotactic factors, cytokines, growth factors and hormones, including neuropeptides. This allows them to respond to signals generated not only within the immune system but also elsewhere in the body.

Regulation of NK cell functions

The functions of NK cells have to be carefully regulated, because of the potential for tissue damage by inappropriate cytokine release and/or lysis. While the ability to swiftly respond to antigen-independent stimuli is obviously advantageous for the effector cell, it also requires a regulatory 'check and balance' system. This is in part achieved by controls operative at the receptor level and resulting in positive or negative signals received and processed by the cell, presumably depending on its external environment. Also, NK cell functions are controlled at the level of development, differentiation and availability of effec-

tor cells at a particular site. The number of NK cells present at the site of tissue injury, for example, is a function of their ability to migrate and proliferate. Cytokines and chemokines generated by tissue and/or hematopoietic cells modulate NK cell availability and their activity *in vivo*. The development of NK cells from the lymphoid cell precursor in the bone marrow depends on soluble factors produced by stromal cells in the marrow and on cytokines produced by T and B lymphocytes. NK cell maturation from their precursors present in the blood, spleen or other lymphoid organs is also dependent on a mix of cytokines produced by other hematopoietic cells. In addition, subsets of T cells may be able to down-regulate NK cell functions.

The biological importance of NK cells

Table 3 indicates that NK cells may participate either directly or indirectly in a variety of essential biological processes, ranging from reproduction to immunoregulation. While there is evidence for the involvement of NK cells in various physiological pathways, the mechanisms responsible for many of the NK cell-mediated effects are not entirely clear. For example, NK cells have been shown to mediate antiviral, antifungal and antibacterial activity *in vitro*. Their *in vivo* importance in control of infections is supported by studies of experimental viral infections in mice and by reports correlating low NK activity with increased sensitivity to frequent and severe disseminating viral infections in humans, including herpes simplex virus (HSV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) infections. *In vivo* responses of NK cells to a viral challenge involve upregulation of cytotoxicity, proliferation, migration of NK cells to infected tissues and production of IFN γ .

IL-12 is a potent inducer of IFN γ production by NK cells. Virus-induced interferons (IFN α/β) upregulate NK cell functions and also modulate IL-12 expression, contributing to the development of an antiviral milieu early on during infection and prior

Table 3 Biological significance of NK cells

Elimination of intracellular pathogens: viruses, fungi, bacteria
Surveillance against cancer and elimination of metastases
Immunoregulation
Involvement in pregnancy and fertility
Regulation of liver growth and regeneration
Regulation of hematopoiesis
Interactions with the neuroendocrine system

to the appearance of virus-specific T cells. Activated NK cells may reduce viral replication by killing infected host cells, and they are able to produce a variety of cytokines which can inhibit viral replication as well as facilitate antigen processing and presentation by increasing expression of HLA molecules. NK cells have been long recognized as effectors responsible for elimination of bloodborne metastases. Newer evidence obtained in experimental studies in rodents suggests that activated NK cells play an important role in both limiting local tumor growth and prevention of metastasis development. In particular, A-NK cells, which are able to enter solid tissues, migrate to sites of metastasis and eliminate malignant targets but spare normal tissue cells, appear to have all the attributes of ideal anti-tumor surveillance as well as effector cells.

The observation that NK cells accumulate in substantial numbers at the maternal-fetal interface has led to the speculation that uterine NK cells play an important role in pregnancy. More recent data confirm that NK cells have both a trophic and regulatory role in the growth of the fetal-placental unit. Through cytokine production, uterine NK cells apparently influence trophoblast behavior at the implantation site early in pregnancy, and through release of cytolytic and hydrolytic enzymes, they may contribute to separation of the placenta at parturition. On the other hand, protection of the trophoblast from lysis by uterine NK cells may be regulated at the level of expression on trophoblast cells of HLA-G and HLA-C molecules, which engage KIRs on NK cells and prevent lysis.

The involvement of NK cells in the regulation of hematopoiesis is due to their ability to produce a spectrum of cytokines, including colony-stimulating factors (CSF) and other growth factors, necessary for the differentiation of hematopoietic cells. In addition, subsets of NK cells have been described in mice, which can positively or negatively regulate hematopoiesis. Expression by these subsets of different Ly-49 alleles may determine their hematopoietic growth-promoting or -inhibitory functions. NK cells participate in immunoregulation by virtue of cytokine secretion in response to stimulation by endogenous or exogenous stimuli. NK cells can secrete immunoinhibitory cytokines, such as IL-10 or TGF β and through TNF α , lymphotoxin or FasL-mediated interactions with activated T cells can induce apoptosis of those immune cell subsets that are responsible for immunoregulation.

Finally, interactions of NK cells with the neuroendocrine system have achieved considerable attention recently. NK cells express receptors for neuropeptides, opioids, prolactin and other hormones

regulating interactions between the brain and the immune system. Therefore, NK cells are highly responsive to changes in the serum levels of these hormones. Effects of stress on the immune response can be monitored by serially following changes in NK cell activity. For example, inability to deal with stressful life events in otherwise healthy individuals is associated with low NK cell activity. Also, a possibility exists for homotypic interactions between NK and brain cells via neural cellular adhesion molecules (NCAMs, e.g. CD56 or CD57) expressed on both.

With the NK cell involvement in a number of biologically important interactions, it is essential that NK cell activity remains under stringent and finely tuned control. The danger of unsolicited lysis or release of inappropriate cytokines by A-NK cells is regulated at several levels. First, the immune system itself is capable of downregulating NK cell functions by engaging regulatory T cell subsets. Second, a system of inhibitory and stimulatory receptors present on NK cells allows for environmental control of NK cell responses, including cytotoxicity and cytokine production. Third, the availability of NK cell precursors at a relevant site and their ability to differentiate and proliferate *in situ* is likely to be dependent on the chemokine and cytokine milieu, representing a sensitive and effective control mechanism. Since NK cells may be called to action early in the course of an immune response, their rapid mobilization is essential, but the subsequent termination of their functions, which may be dangerous to other cells, has to be rigorously implemented. When one or more of the redundant control mechanisms fail, disease may ensue.

NK cells in disease

Human diseases associated with NK cell abnormalities can be categorized as NK cell deficiencies or excessive NK cell activity. In either case, only chronic abnormality in NK cell activity is considered pathologic, because transient changes accompany a variety of physiologically normal events (e.g. circadian variations, daily stress, exercise or common cold). In every individual, NK activity fluctuates over time, but it can be quite precisely defined as a baseline level of NK cell activity $\pm$ SD by performing serial NK cell assays. The baseline level of NK cell activity is a characteristic trait for every individual. On the other hand, persistently low or high levels of NK cell activity are likely to be associated with disease. Chronically low levels of NK cell activity are seen in cancer, acquired or congenital immunodeficiency diseases (e.g. acquired immune deficiency syndrome, AIDS), severe life-threatening viral infections (HSV.

EBV, CMV), a subset of patients with chronic fatigue syndrome (CFS), certain autoimmune diseases (e.g. connective tissue diseases) and behavioral disorders (e.g. depression).

Persistently elevated NK cell activity is relatively rare, being reported in autoimmune liver diseases, including viral hepatitis, and in NK cell lymphoproliferations. The latter present as elevations in the number of activity of NK cells or both and are characterized by a benign course, lymphocytosis of LGLs, cytopenias and splenomegaly. Rare acute proliferations of LGL have been reported and are consistent with aggressive leukemia/lymphoma.

Persistently low levels of the NK cell number or activity in patients with advanced metastatic or other diseases may be associated with more severe symptoms or increased risk of disease progression. Therefore, it may be advantageous for patients with chronically low NK cell activity to receive therapy designed to augment NK cell functions. Such therapies are available today and consist of: 1) administration of agents known to upregulate endogenous NK activity (e.g. interferons, IL-2 or IL-12) or 2) adoptive transfer of autologous A-NK cells either systemically or locoregionally.

Measurement of NK cells

NK cell activity and the number of NK cells are measured in the peripheral blood in humans and in the spleen in rodents. A short-term (4 h) ^{51}Cr -release assay is used to determine NK cell activity, with a standard number (5×10^3) of cultured K562 cells as targets (T) and peripheral blood mononuclear cells (PBMCs) as effectors (E). The latter are adjusted in number to give at least four different E:T ratios, thus allowing for generation of a lytic curve by plotting the per cent specific lysis measured at each E:T ratio. The results of NK cell assay are usually expressed as lytic units (LU) calculated from the lytic curve and defined as the number of effector cells required to kill 5×10^3 targets in a batch of tested cells. Computer programs are available to facilitate transformation of the per cent specific lysis into LU.

Using variations of this cytotoxicity assay by the addition of antibody or by changing the target, it is possible to measure ADCC or spontaneous LAK activity, respectively. The measurement of the NK cell number should be performed at the same time as the NK cell assay, because both are necessary for an adequate evaluation of natural immunity or for following its changes during disease or therapy. To determine the number of NK cells, PBMCs are stained with labeled monoclonal antibodies specific for NK cell-associated markers (e.g. CD56 or CD16

in humans; NKR-P1 in rats and NK1.1 in mice) and the percentage of positive cells in a population is determined by flow cytometry. This percentage value is then converted into the absolute number of NK cells, based on a simultaneously obtained differential lymphocyte count. A variety of assays are available for assessment of NK cell functions other than lysis. For example, generation of LAK activity *in vitro*, proliferation, cytokine production, migration, binding to substrates, ability to induce apoptosis or to transduce signals can be accurately measured provided purified NK cells are available. Negative selection with immunobeads is generally used to obtain highly enriched preparations of NK cells for these assays.

See also: Adhesion molecules; Antibody-dependent cellular cytotoxicity; Cytokines; Cytotoxicity, assays for; Cytotoxicity, mechanisms of; Fc receptors; Immune surveillance; Innate immunity; Large granular lymphocytes; Lymphokine-activated killer (LAK) cells; Perforin; Tumors, immune response to; Viruses, immunity to.

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NEISSERIA, INFECTION AND IMMUNITY

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The genus *Neisseria* belongs to the family Neisseriaceae and contains 12 species, of which only *N. gonorrhoeae* and *N. meningitidis* are considered primary pathogens. Although these two species share 80% DNA homology they cause diverse types of infections. *N. gonorrhoeae* causes primarily a mucosal infection of the urogenital tract known as uncomplicated gonococcal infection (UGI). In a few patients, complicated gonococcal infection presents as pelvic inflammatory disease (PID) in women and as epididymitis and prostatitis in men. Disseminated gonococcal infection is rare and presents as septicemia, rash or arthritis. It is more common in women and is associated with strains that are highly susceptible to penicillin and require arginine, hypoxanthine and uracil for growth. *N. meningitidis* colonizes the mucosa of the upper respiratory tract as a commensal and encapsulated strains can cause systemic infection resulting in meningitis. *N. gonorrhoeae* and

N. meningitidis are obligate human pathogens and have no other natural reservoir.

Neisseria spp. are gram-negative diplococci, produce cytochrome c (oxidase) and utilize carbohydrates by the oxidative pathway. *N. gonorrhoeae* utilizes only glucose whereas *N. meningitidis* utilizes both glucose and maltose. They require complex media for growth and are enhanced by 5–7% carbon dioxide.

The neisserial cell envelope consists of a cytoplasmic membrane, a thin peptidoglycan layer and an outer membrane. The main outer membrane antigens of *N. gonorrhoeae* and *N. meningitidis* are listed in Table 1.

N. gonorrhoeae can be divided into serovars dependent on the antigenic specificity of Por which occurs in two types, PIA and PIB. Gonococci belonging to PIA serovars are more susceptible to antibiotics and PIB more resistant. Encapsulated strains

Table 1 Cell wall antigens of *Neisseria gonorrhoeae* and *N. meningitidis*

Current symbol	Previous symbol		Description	Function
	<i>N. gonorrhoeae</i>	<i>N. meningitidis</i>		
Por, PorA	Protein I (PI)	Class 1	Major OMP	Porin
PorB		Class 2 and 3	Major OMP	Porin
Rmp	Protein III (PIII)	Class 4	Reduction modified protein	Site for blocking antibody
Opa	Protein II (PII)	Class 5	Heat-modifiable protein	Attachment
Pilin		Class I pilin	Pili	Attachment
LPS, LOS	Lipopolysaccharide/lipooligosaccharide		LPS/LOS	Toxic to epithelial cells
Cps	—	Capsule	Capsular polysaccharide	Associated with invasive disease
Lip	H8	H8	Lipoprotein	Not known
Opc	—	—	Variable OMP	Attachment
Mtr	—	—	Variable OMP	Efflux pump
Tbp, Lbp, HmBP, Hpu, FrpB	Iron-binding proteins		Various OMPs	Iron acquisition

OMP, outer membrane protein.

of *N. meningitidis* have been subdivided into 13 serogroups by the difference in the composition of the capsular polysaccharide. The most prevalent serogroups in disease are A, B, C, W-135 and Y. Meningococci can be further divided into serotypes based on the antigenic specificity of the outer membrane proteins, which is independent of the serogroup. Commensal meningococci are invariably nonencapsulated and piliated, whereas invasive meningococci are encapsulated and nonpiliated; phase variation of these antigens occurs, and may allow switching between phenotypes.

Immune response of the host

Patients with gonococcal infection show increased levels of serum and mucosal gonococcal-specific antibody. Both immunoglobulin G (IgG) and IgA are directed at surface exposed antigens described in Table 1. Protective immunity is not acquired by natural infection and patients presenting with multiple episodes of UGI are common. This may be due to the antigenic heterogeneity of the surface antigens or because the mucosal antibody response is short-lived and serum antibody has no function in the mucosal infection. The ability of gonococci to attach and colonize the mucosa and their interactions with polymorphonuclear neutrophils may be more important than the immune response in UGI. Repeated episodes of PID occur, but women are not reinfected with strains of the same Por serotype. In contrast, protective immunity to meningococcal infection is known to be correlated with the presence of complement-dependent, bactericidal antibodies in the serum directed against the capsular polysaccharide.

Children under 2 years of age do not reliably produce antibodies either to natural infection or to immunization with meningococcal polysaccharides. In addition, patients with an inherited deficiency of one of the terminal components of the complement pathway are also susceptible. There is little evidence for a cell-mediated immunity.

Evasive strategies by the organism

Surface antigens of *N. gonorrhoeae* such as pilin, Opa and LPS undergo phase and antigenic variation. The range of molecules expressed determines a number of crucial bacterial phenotypes related to infection. Antigenic variation *per se* promotes evasion of host immune defenses, facilitating repeated infection. Tissue tropism depends on the combination of Opa and pilin molecules expressed. *In vivo* gonococci expressing particular sialylated lipopolysaccharide

(LPS) molecules are serum resistant, but unable to evade epithelial cells. Antigenic variation can also produce an LPS that cannot be sialylated – such organisms are invasive. Sialylated LPS molecules mimic eukaryotic cell surface markers, compromising recognition by the host immune system. *In vitro* most strains become serum sensitive due to the absence of the sialic acid donor, although some gonococci do remain resistant, suggesting an additional unidentified mechanism. Serum-resistant gonococci show reduced development of C5 convertases in normal human serum. Naturally occurring IgG and antibodies to Rmp can block complement-mediated killing.

Repeated infection with *N. meningitidis* is rare because specific immunity is protective and re-exposure to a strain expressing a different polysaccharide capsule is uncommon. Phase and antigenic variation is exhibited in a similar manner to gonococci for many surface molecules common to both species. In addition, phase variation of the capsule occurs, both by the insertion of a transposable element in a sialic acid biosynthesis gene, and by slipped-strand mispairing in the polysialyltransferase gene.

Both *N. gonorrhoeae* and *N. meningitidis* produce a protease that cleaves human IgA1. It is presumed to be a virulence factor but its role in pathogenesis is still unclear.

Vaccines

No successful gonococcal vaccine has been produced. This is due to the antigenic heterogeneity of the antigens chosen as candidate vaccines and because the type or amount of antibody required to produce protection is unclear. There is also no animal model that correlates with human infection. Pilin vaccines have proved unsuccessful. Por- and iron-binding proteins are still considered potential vaccine candidates, particularly for preventing complicated infection in women. Polysaccharide vaccines to *N. meningitidis* of serogroup A and C have been shown to produce bactericidal antibodies and to be protective, although this response is age-dependent.

A vaccine incorporating polysaccharide from serogroups A, C, Y and W-135 is now available and should stimulate bactericidal antibodies in 90% of adults. In order to increase the immunogenicity in young children the polysaccharide A and C vaccine has been conjugated to protein carriers such as tetanus toxoid. Many cases of meningococcal disease are caused by serogroup B strains and polysaccharide vaccines do not elicit protection. This has been attributed to its poor immunogenicity or the similarity

between the polysaccharide and sialic acid moieties in human tissue.

Protection against serogroup B strains may be associated with antibody to outer membrane proteins (OMPs). OMPs have been used either alone or in combination with polysaccharide in attempts to produce immunity. Vaccines that have been used or are in clinical trials include LPS-depleted OMs of serotype 15 subtype P1.16 (Norwegian), LPS-depleted outer membrane (OM) vesicles from serotype 4, subtype P1.15 together with serogroup C polysaccharide and a high molecular weight protein complex (Cuban) and a hexavalent class 1 OMP vaccine (Dutch). Their efficacy in young children still needs to be clarified.

See also: Bacteria, immunity to; Bacterial cell walls; Carbohydrate antigens; Complement, classical pathway.

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NEONATAL IMMUNE RESPONSE

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Both humoral and cellular immune responses of neonatal individuals differ in quality and quantity from those of adults. Early in ontogeny, these differences may result from the lower incidences and/or decreased functions of immunocompetent cells (e.g. B cells, T cells, antigen-presenting cells).

B lymphocyte development and function in the neonate

Pre-B cells express cytoplasmic μ chain and lack surface immunoglobulin (Ig). In humans, pre-B cells are detected in fetal liver and omentum as early as 7–8 weeks of gestation. One to two weeks later, B lymphocytes (surface IgM⁺) are found in fetal liver and the development of B lymphocytes expressing other surface Ig isotypes follows. In the mouse, the earliest B lineage precursor cells can be identified as CD45R (B220)⁺ cells in fetal liver at ~12–13 days gestation. B lineage cell development also involves the surrogate light chains, (e.g. $\lambda 5$, VpreB) which are

expressed both in pro-B cells and, as a complex with μ chain, in pre-B cells. Surrogate light chain ($\lambda 5$) transcription is seen by day 13 in fetal liver and pre-B cells are evident by day 14. Pre-B cells exhibit heterogeneity in surface antigen expression (e.g. CD43) and in expression of other molecules (e.g. RAG-1, RAG-2) indicative of their activity and precise stage of maturation. B lineage precursors from adult mice differ from those seen early in ontogeny. For example, murine fetal pre-B cells do not express major histocompatibility complex (MHC) class II antigens, in contrast to adult pre-B cells. B cells are clearly demonstrable in murine fetal liver by days 16–17 of gestation; these fetal B cells are generally restricted to expression of surface IgM. Eventually, the bone marrow becomes the main organ of B lymphopoiesis in both mouse and human throughout adult life.

IgM synthesis has been detected as early as 10–12 weeks of gestation in the human fetus; however, serum concentrations of IgM in the newborn are

approximately 10% of those observed in adults and do not reach adult levels until 1–2 years of age. Serum concentrations of nonmaternal IgG are also abnormally low in the neonate and do not approach adult concentrations until 4–6 years of age.

Developmentally, immunocompetence does not extend to all antigens and, early in ontogeny, humans are constrained in their capacity to mount humoral responses to carbohydrate antigens. Children less than 2 years of age are generally deficient in response to unmodified bacterial polysaccharide vaccines. This, in turn, may contribute to the susceptibility of infants to certain bacterial infections (e.g. *Streptococcus pneumoniae*).

Fetal/neonatal B cells exhibit a restricted antibody repertoire

It has been shown that the fetal/neonatal repertoire of available antibody specificities is restricted. The development of antibody specificities during ontogeny proceeds in a temporally dependent and programmed manner. BALB/c mice possess B cells capable of responding to the hapten dinitrophenyl and to the carbohydrate antigen β 2,6-fructosan by day 1 after birth; however, reproducibly, those B cells bearing the T15 idiotype and responsive to the antigen phosphorylcholine are only apparent at ~1 week after birth. As a consequence of this pattern of antibody repertoire acquisition, the developing neonate has a more limited spectrum of available B cell specificities which diversifies in a predictable fashion.

Studies in both human and mouse indicate that fetal and neonatal pre-B and B cells exhibit biased recombination and expression of those immunoglobulin heavy-chain variable region (V_H) gene families located most proximal to the D_H and J_H gene segment loci. Early in ontogeny, the enzyme terminal deoxynucleotidyl transferase (TdT) is limited. Lack of TdT, by restricting junctional diversity, also contributes to decreased Ig repertoire complexity. Although the decreased diversity present in the emerging B cell repertoire could contribute to the failure to respond to particular antigens as discussed above, this need not be the case. For example, as discussed by Schroeder and colleagues, the V3–23 immunoglobulin heavy-chain variable region gene in humans is a major contributor to the fetal repertoire and yet encodes specificities to antigens some of which (e.g. polysaccharides) are not responded against until ~2 years of age. This suggests that the more limited capacity to respond to the spectrum of antigens early in ontogeny and the programmed readout of the functional immune repertoire is due

to complex mechanisms of which V_H gene usage is only one component.

The composition of B cells within the developing neonate may also contribute to constraints on immune function. B cells are divisible into subpopulations based on the expression of surface antigens. In both human and mouse, a subset of B cells (B1 cells), many of which express the CD5 surface antigen, is more prominent early in ontogeny. It has been shown that the CD5 B cells represent a separate B cell lineage with precursors distinct from those which yield the conventional (CD5⁻) B2 cell population which dominates the peripheral B cell compartment in the adult. Notably, precursors of CD5 B cells are present early in fetal/neonatal life, but are not derived from adult bone marrow. In both human and mouse, CD5 B cells may be biased toward recognition of self and environmental antigens and also exhibit degenerate specificity. Differences in the composition of the B cell compartment between neonates and adults may also contribute to differences in their humoral immune capabilities.

Antibodies generated from murine neonatal spleen demonstrate an unusual capacity for recognizing idiotypic determinants on each other ('connectivity'). The neonatal antibody repertoire, although restricted in diversity, is skewed toward reactivity with self molecules, including idiotypic determinants present on immunoglobulins. This has led to the hypothesis that neonatal B cell responses may be of importance in establishing an immune network.

T cell development and function in the neonate

In humans, at approximately 7 weeks gestation, bloodborne stem cells enter the developing thymus. Similarly, in the mouse, hematopoietic cells enter the embryonic thymus by day 11 of gestation. In both mouse and human, the initial thymocyte populations are comprised of lymphocytes lacking expression of either the CD4 or CD8 molecules as well as T cell antigen receptors (TCRs). Rearrangement of TCR gene segment loci rapidly ensues with subsequent appearance of surface TCR-bearing T cells. Notably, early in development at the double-negative phase, expression of a functionally rearranged V_β chain together with the pre- T_α chain and CD3 provides a complex which favors further development and expansion. Initially, the majority of thymic T cells express TCR of the γ/δ type at ~14–15 days gestation in the mouse and ~9.5 weeks gestation in humans; however, by 20 days of murine and ~10 weeks of human gestation, T cells bearing α/β TCRs gain predominance as is typically observed in the

Table 1 B cell properties during ontogeny

	Fetal/neonatal B cells	Adult B cells
Ig combining site repertoire	Restricted V _H , V _L usage Restricted N region diversity	Random V _H , V _L usage Extensive N region diversity
B cell subsets	B1 and B2 B cells	Mainly B2 B cells; Few B1 B cells
Isotypes produced during an immune response	IgM>IgG	IgG>IgM
Tolerance induction	Sensitive	Resistant
Self-reactivity/connectivity	High	Low

adult. Although some studies suggest that the T cell repertoire may be less diverse in the early human fetus and in fetal/neonatal mice, the murine splenic cytotoxic T cell precursors specific for particular MHC class I alloantigens in 8-day-old neonatal mice are similar in diversity to those of adults.

The capacity of T cells in both neonatal mice and humans to provide help for immunoglobulin secretion is limited mainly to IgM production. T cell helper function in the neonate is often difficult to quantitate due to the activation of suppressor T cells. Furthermore, both the rejection of MHC allogeneic cells as well as responses in mixed lymphocyte cultures and cytotoxic activity may be diminished in neonatal mice as compared to adults. In contrast to these reduced T cell functions, neonatal spleens are enriched in T cells capable of vigorously suppressing a variety of immune responses *in vitro* and *in vivo*. Both lymphocyte proliferation and immunoglobulin production are affected by neonatal suppressor T cells. The subset of T cells responsible for neonatal suppression is not clear and both CD4⁺ and CD8⁺ T cells have been implicated. In humans, the capacity to mount suppressive responses is prevalent throughout early life (e.g. in 8 weeks gestation liver) and begins to wane rapidly by ~1 year of age.

CD4⁺ T cells provide help to B cells and promote inflammatory reactions (delayed-type hypersensitivity). These functions are mediated largely by cytokines released upon T cell activation. Adult T cells produce a diversity of cytokines, including interleukin 2 (IL-2), IL-4, and interferon γ (IFN γ); however, T cells from neonates produce decreased levels of IL-2 (e.g. upon stimulation with anti-CD3 antibodies) as well as substantially lower levels of both IL-4 and IFN γ . The reduced production of these cytokines may contribute to diminished activation and/or maturation of macrophages, natural killer (NK) cells, and cytotoxic T cells as well as reduced help to B cells. Decreased help for B cell responses may also be reflected in the paucity of CD40 ligand on the surfaces of activated neonatal T cells; CD40 ligand, interacting with B cell CD40, functions in

promoting B cell help. The impaired activation of T cells may result from abnormalities in antigen uptake, processing, and/or signaling by appropriate antigen-presenting cells. With regard to the latter possibility, it is of interest that in early development, macrophage/monocytic cells exhibit decreased surface densities of class II MHC antigens which may impair appropriate antigen presentation to T lymphocytes. It has also been suggested that reduced incidence of memory T cells in neonates contributes to their decreased function, presumably a consequence of the antigenically naive status of the neonate.

Although the above studies indicate that fetal/neonatal T cell immunity is deficient, recent studies by Sarzotti and coworkers suggest that neonatal T cells can generate protective cytotoxic T cell responses dependent upon the dose of antigenic virus employed. Ineffective cytotoxic T lymphocyte (CTL) responses to high doses of virus reflected establishment of T_H2 (type 2) immune responses in the neonates. Therefore, immune 'deficits' in the neonate may actually represent deviations of the immune response.

Tolerance induction among neonatal lymphocytes

Neonatal lymphocytes, in contrast to their adult counterparts, are exquisitely sensitive to inactivation following occupation of their antigen-specific surface receptors. The inactivation of neonatal lymphocytes may occur via clonal abortion or deletion or, alternatively, by clonal anergy. The sensitivity of neonatal T and B lymphocytes to tolerance induction is probably of considerable importance in purging high-affinity autoreactive lymphocytes. However, these results have recently been challenged by Ridge and colleagues and Forsthuber and coworkers, wherein neonatal T cell tolerance versus activation was shown to be malleable and is likely to be more dependent upon the characteristics of antigen-presenting cells rather than on the T cells themselves.

Table 2 T cell properties during ontogeny

	Fetal/neonatal	Adult
TCR repertoire	Limited (?)	Extensive
T cell subsets	$\gamma\delta$ and $\alpha\beta$ T cells	$\gamma\delta$ and $\alpha\beta$ T cells
T cell suppression	High	Low
CD4 ⁺ T cells:		
Cytokines produced upon activation	Low levels of IL-2, IL-4, and IFN γ	High levels of IL-2, IL-4, and IFN γ
Expression of CD40 ligand	Low	High

Summary

Both T and B cell functions may be more limited early in ontogeny than in adulthood (Tables 1 and 2). In part, this may reflect restrictions imposed on the diversity of the antigen-specific repertoires; however, differences in responses to various modes of activation by neonatal and adult lymphocytes also contribute to differences in immune outcome. The differences seen in immune responses to antigens early in ontogeny as opposed to that in adult life is of consequence in both host immunity to microorganisms and in development of appropriate vaccine strategies.

See also: Antigen, T dependent and independent; B lymphocytes; CD5; CD40 and its ligand; Cytokines; Effector lymphocytes; Idiotype network; Ontogeny of the immune response; T lymphocytes; Tolerance, peripheral.

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NEPHELOMETRY

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Nephelometry (Gr. *nephele*, cloud), also called immunonephelometry, is a method suitable for the quantitative determination of proteins and other biological substances. It is based on the precipitin reaction in fluid medium, whereby the reaction between an antigen and its corresponding antibody results in the formation of small antigen-antibody aggregates, which successively develop into floccules or precipitates. Detection of the antigen-antibody complexes formed is accomplished by measurement of the amount of scattered light at an angle to an incident beam passed through the solution. This principle differs from that of turbidimetry in which residual transmitted light is measured. Devices for the quantitative measurement of light scattered by particles in a fluid, nephelometers, were introduced on a more commercial basis in the early 1970s.

Characteristics of the precipitin reaction

The interpretation of results obtained by nephelometry requires an understanding of the course of the precipitin curve. The antigen-antibody reaction in a fluid medium may be considered as consisting of two phases, one rapid initial reaction with the formation of small molecular complexes, followed by a slower reaction in which nonspecific aggregation of the primary antigen-antibody complexes occurs. One of the characteristics of the precipitin curve is the direct relationship between the amount of antigen added and precipitate formed, providing that the reaction is performed with antibody in excess. With further addition of antigen, antibodies successively become saturated and the amount of precipitate formed levels off or eventually decreases (see Figure 1). The course of the precipitin curve in the antibody excess region varies for different types of antisera and is largely dependent on the animal species used for immunization. The precipitin reaction is fairly independent of temperature as well as variations of pH in the range pH 6–8.

Increasing ionic concentration generally decreases the strength of the binding between antigen and antibody and thereby the amount of precipitate formed, although with immune sera produced in chickens for example, immunoprecipitation is enhanced when performed at a high ionic strength. The finding that the addition of polymers, such as polyethylene glycol, enhanced the precipitin reaction by decreasing

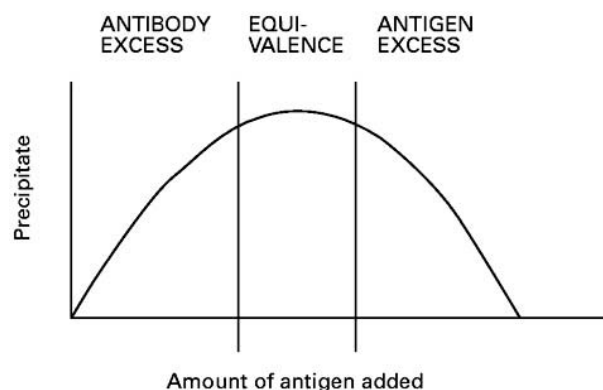


Figure 1 Schematic representation of the precipitation curve. The curve represents the amount of precipitate formed when increasing amounts of an antigen are added to a constant amount of the corresponding antibody.

the solubility of immune complexes has been of decisive significance in the commercial development of nephelometric techniques.

Light scattering by immune complexes

When the precipitin reaction is performed under proper conditions, the amount of light scattered will be directly related to the reactant concentration. A prerequisite for this is that the reaction is performed with one of the reactants, usually the antibody, in excess. A given antigen concentration will then result in a given amount of immune complexes and consequently a given amount of scattered light. At high antigen concentrations, however, when the precipitin curve approaches the equivalence zone or eventually proceeds into the antigen excess zone, the scatter response is no longer linear.

The light-scattering properties vary in different patients' sera, and in order to circumvent this problem, subtraction of individual serum blank values is required for each separate analysis. High blank values may be due to lipemic sera, or to the existence of endogenous immune complexes, for example. Aggregated immunoglobulins, particularly after repeated freezing and thawing, may also contribute to an increased background value. The problem of varying blank values is one reason for the greater sensitivity of protein quantitation in other body fluids such as urine, for example, as compared to serum. From the practical point of view it is usually

the problem of high blank values for serum samples which restricts the sensitivity in nephelometric analyses.

Detection of light scattering

Immunonephelometric techniques are suitable for automation and the methodological development within this field has, to a large extent, been dependent on the design of detection devices for scattered light. The initial instruments employed measurement of light scattering at an angle of 90° to the incident light beam, whereas later instruments took advantage of enhanced light scattering at lower angles (31° and less). In some instruments, the laser nephelometers, a helium-neon laser with an emitted wavelength of 632.8 nm is employed. In this device a high-intensity light is obtained at a reasonable cost. Furthermore, determinations at this wavelength will minimize potential autofluorescence from aromatic amino acids. Forward scatter is also measured at an angle of 31° or lower in laser nephelometers, which minimizes the problem of high blank values.

Further progress has been the development of rate nephelometry. In this modification the scatter is measured at two or more time points and the rate of scatter increase during the corresponding interval registered. This approach is based on the fact that the scatter is directly related to the size of the immune complexes, which in turn is dependent on the time of the antigen-antibody reaction. Plotting scatter against time gives a sigmoidal curve, the derivatives of which show a Gaussian-type shape. The rate of complex formation increases with increasing antigen concentration. In antigen excess, however, the rate levels off and decreases. High blank values are also of less importance in rate nephelometry, since the procedure implies registration of scatter over the sample background level.

Advantages and disadvantages

Immunonephelometry obviates various methodological difficulties. It is characterized by being simple to use and requiring relatively small sample volumes of unmodified body fluids for protein determination. The technique is sensitive, rapid and relatively inexpensive. Of particular interest are instruments designed during later years which use reagent volumes around 1–10 μl per analysis, thus reducing reagent costs. However, the high initial purchase cost restricts the use of nephelometric techniques to laboratories with a certain minimal body of analyses. Another advantage is that a large selection of monospecific commercial antisera are available which

makes the technique versatile with regard to the number of possible applications. Monoclonal antibodies have also been used in nephelometric estimations of proteins. The increased control of the specificity of the reaction has to be balanced against the possible disadvantage that saturation of antibodies in the solution occurs quicker than when conventional polyclonal antisera are employed. When saturation of antibodies occurs in antigen excess, as mentioned above, the direct relationship between antigen dose and scatter response is no longer valid and the response may even attain values within the normal operating range. This problem may be solved in several ways, e.g. by adding a second dose of antigen. If this results in an increased scatter value the initial value should be accepted whereas if no increase occurs or if decrease is registered, this indicates that the first reading was done at antigen excess and therefore should be discarded. The sample must then be reanalyzed at a higher dilution. In automated procedures, it is important to introduce control safeguards for the detection of values obtained in antigen excess.

Applications

Nephelometry has been applied to the quantitative determination of various protein and other antigens in blood serum, urine or cerebrospinal fluid such as lipoproteins, immunoglobulins, complement factors, rheumatoid factors and immune complexes. Measurement of light scattering has also been used for the determination of cell size, e.g. in the fluorescence-activated cell sorter (FACS). A recent development is the so-called microparticle-enhanced nephelometric immunoassay where antigen or antibody is conjugated to spherical, hydrophilic microparticles in the size range of 50–300 nm. This approach seems to provide higher sensitivity and lower reagent consumption as compared to conventional immunonephelometry and, if designed as an inhibition assay, haptens may also be quantified.

See also: Antibodies, detection of; Autoantibodies, tests for; Immune complexes; Precipitation reaction.

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NEUROENDOCRINE REGULATION OF IMMUNITY

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It is now clear that both the nervous system and the immune system have internal homeostatic mechanisms that control and regulate the functions of these systems and their responses to various stimuli. In recent years it has become apparent that some of these regulatory mechanisms act in an endocrine or paracrine manner, that is, neurotransmitters, neuropeptides and cytokines made in the nervous system either potentiate or inhibit immunologic responses and, correspondingly, mediators made by cells of the immune system act on cells of the nervous system (Figure 1).

The proposal that the immune system acts as a sensory organ, the so-called sixth sense, is finding increasing support and validation from studies in experimental animals and in clinical observations.

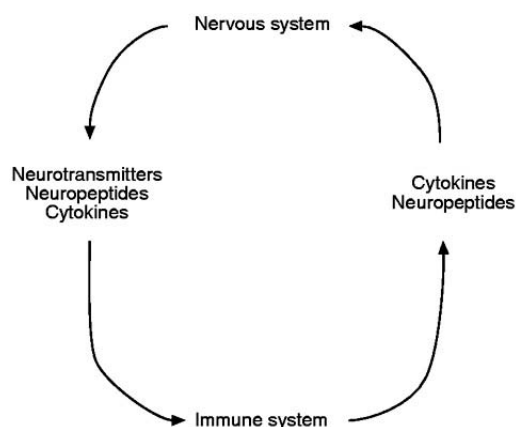


Figure 1 General schematic of the interactions between the nervous system and the immune system mediated primarily by soluble, secreted products of cells in each system. The prevailing view is that these two complex systems interact and share in the effort of promoting organismic homeostasis by the use of mediators produced in each system.

Indeed, the growing subdiscipline of psychoneuroimmunology is based on the study of the interactions between the nervous system and the immune system, and observations that immune responses to infectious agents and tumors may be modulated by mood, thought and behavior. In such a scenario, the immune system receives a noncognitive (antigenic) stimulus, responds, and sends signals (cytokines) to the nervous system which, in turn, reciprocates with immunoregulatory signals (neuropeptides, cytokines), some of which are the result of behavioral responses (Figure 2).

Neuromediators

Neurotransmitters

Anatomists and neuroscientists have long known that the neural innervation of lymphoid organs, such as the spleen, thymus, and lymph nodes, forms an important part of the homeostatic mechanism that regulates the size of, cell density in, and blood flow through these organs. A number of years ago it was noted that neurotransmission in nerve fibers innervating the spleens of animals undergoing immunization is increased, compared with that of spleens in animals not stimulated with a foreign antigen. Subsequently, the secretion of various neurotransmitters at the nerve terminals in lymphoid organs was found to be significantly increased following antigenic stimulation of the lymphoid organ and increased neural activity was correlated with lymphocyte activation and the initiation of an immune response (Figure 3). Such observations provided a basis for further investigation of the immunomodulatory role of hard wiring of lymphoid tissues by nerve fibers.

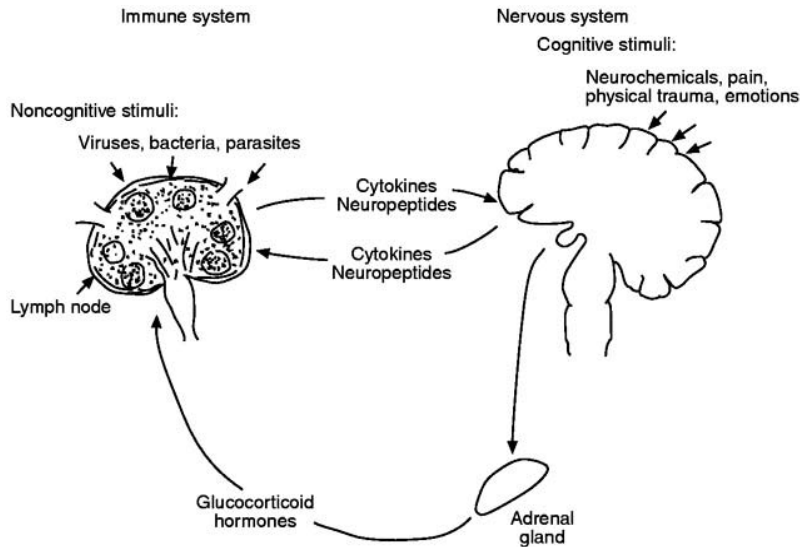


Figure 2 The immune system, typified by the schematic lymph node on the left, responds to antigenic stimuli and produces mediators that impact neural activity and neurosecretion of peptides and cytokines, which modulate the immune response to the antigen. This circuit is viewed as a bidirectional response system in which the immune system responds to noncognitive stimuli, such as infectious agents, and signals the nervous system, which responds to cognitive stimuli such as neurochemicals, cytokines, and physical and emotional stimuli and, in turn, signals the immune system.

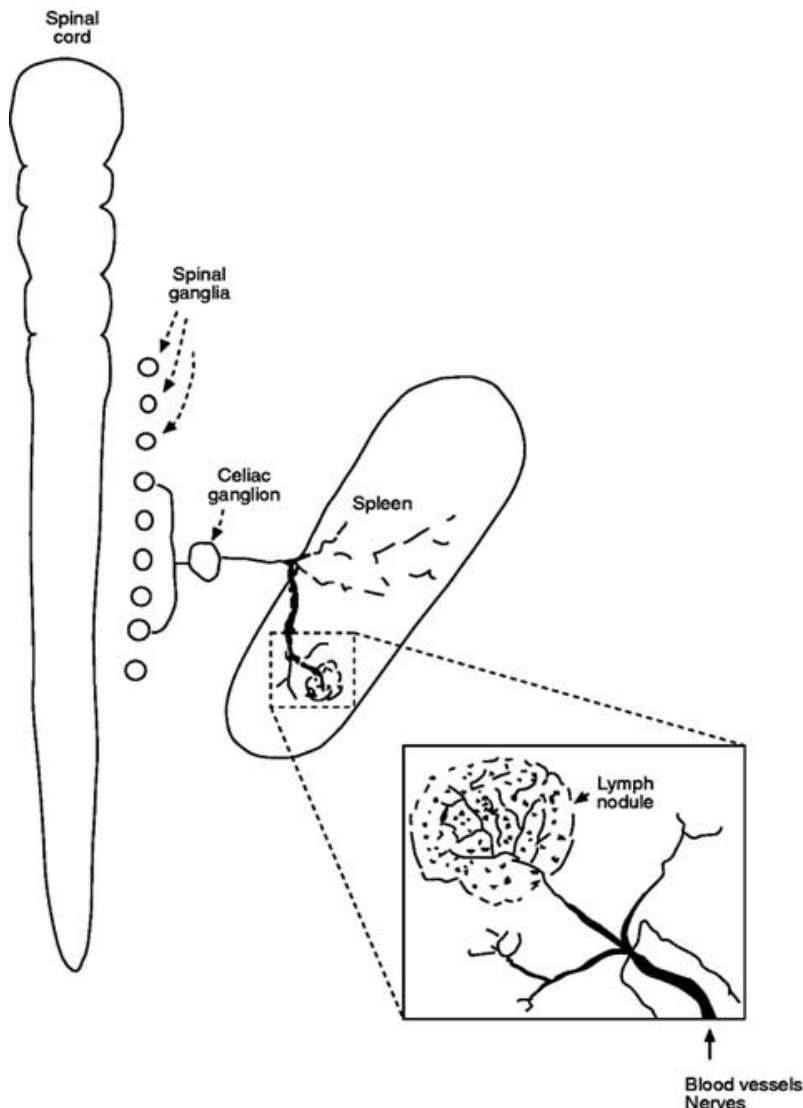


Figure 3 Noradrenergic fibers from the spinal ganglia enter the spleen through the trabecular meshwork, distribute around and innervate areas of accumulated lymphocytes called the white pulp and the periarteriolar lymphatic sheaths, which consist of dense aggregates of lymphocytes. During antigenic stimulation, there is increased production of norepinephrine, substance P, somatostatin, and vasoactive intestinal peptide, which regulates blood flow and lymphatic activation.

In cell culture experiments, pharmacologic concentrations of neurosecretory substances such as epinephrine, norepinephrine, and 6-hydroxydopamine were found to suppress T lymphocyte mitogenesis and the differentiation of B cells into antibody-producing cells. The immunomodulatory effects of the neurosecretory substances observed *in vitro* may be a correlate of the increased neurosecretion that follows antigenic stimulation *in vivo*. It may be that neurosecretory mediators act on professional antigen-presenting cells such as macrophages and dendritic cells, reducing the secretion of interleukin 1 and ultimately reducing the intensity of the T cell response. It is also possible that neurosecretory substances act directly on antigen-stimulated lymphocytes (Figure 4). Several studies have shown that epinephrine and norepinephrine modulate lymphocyte responses in cell culture and it is thought that such effects may be mediated *in vivo* as well.

Neuropeptides

Vasoactive intestinal peptide (VIP) and substance P have been investigated extensively for their actions on the immunologic functions and responses of lymphoid cells. These peptides and others, including mediators of the hypothalamic-pituitary-adrenal (HPA) axis such as growth hormone, prolactin, adrenocorticotrophic hormone (ACTH), and various opioid peptides, have been shown to modulate immune responses *in vitro*. Indirectly, using antagonists and agonists of these mediators, *in vivo* modulation of immunity has also been observed.

VIP is secreted by cells in the digestive system; the functions of this peptide are vaso- and bronchodilation, glycogenolysis, and stimulation of the secretion of other neuropeptide mediators such as prolactin, growth hormone and luteinizing hormone. VIP enhances or dampens lymphocyte-mediated

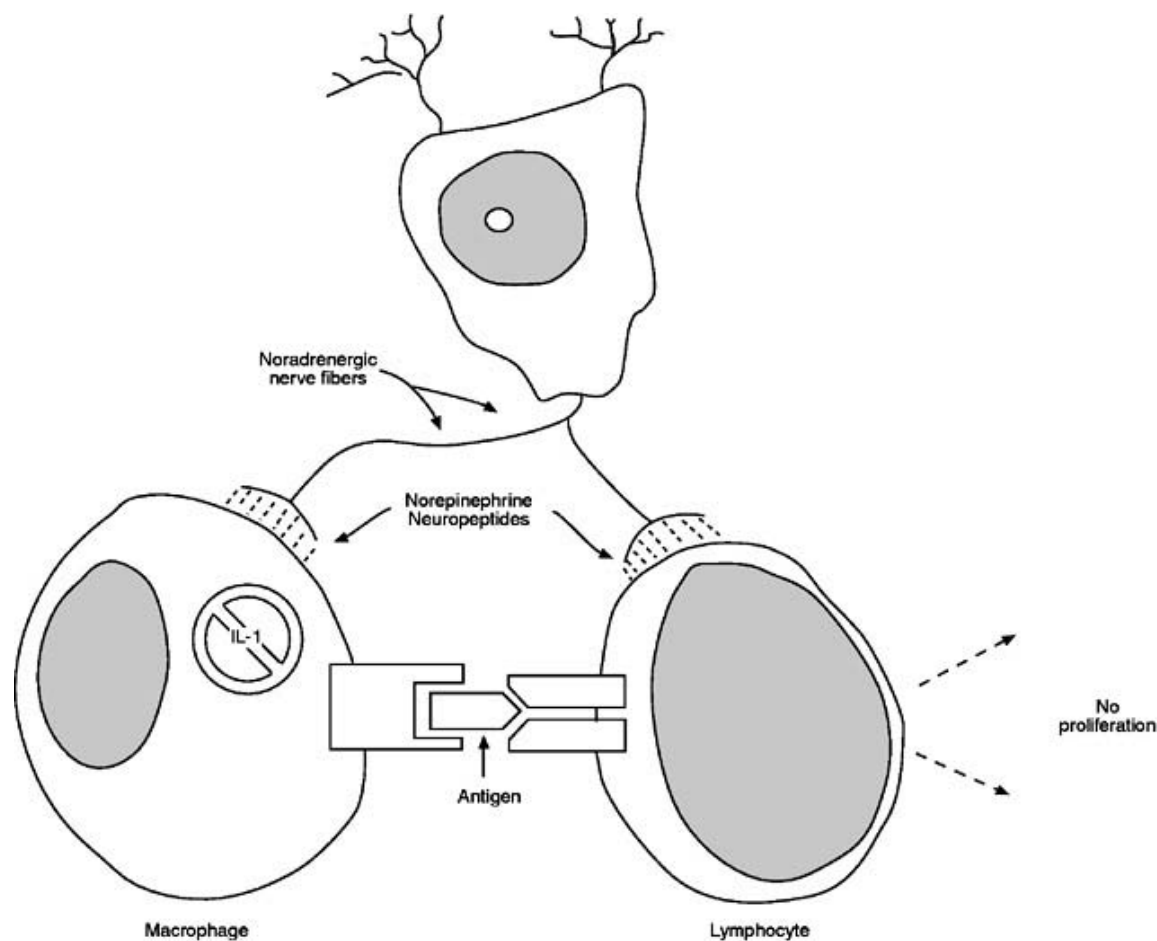


Figure 4 Antigen presentation by macrophages and the lymphocyte response are modulated by noradrenergic activity. Norepinephrine and neuropeptides may act on the antigen-presenting macrophage, suppressing interleukin 1 (IL-1) production and thereby preventing lymphocyte activation and cell division. Alternatively, noradrenergic nerve activity may act directly on the lymphocyte, suppressing activation and mitosis.

responses; the particular response depends, in part, on the concentration of the peptide. T and B lymphocytes express membrane coreceptors of the integrin family of molecules and use these coreceptors in cell-cell recognition, cell migration, and binding to intercellular adhesion molecules on tissue cell membranes. VIP modulates the expression of integrin molecules, thus altering the migration and localization of lymphoid cells in the tissues (Figure 5). Overall, VIP seems to affect T lymphocyte responses more strongly than B lymphocyte responses.

Substance P is produced by various cells in the body, including neurons, macrophages and perhaps other antigen-presenting cells such as dendritic cells and Langerhans cells in the epidermis. Acting in an autocrine fashion, substance P significantly enhances the phagocytic capacity of macrophages. In other contexts, substance P stimulates DNA and protein synthesis in T cells, an effect that is antagonized by the substance P antagonist somatostatin.

A variety of other neuropeptides, including calcitonin gene-related peptide, galanin, neuropeptide Y and others, have been less well-studied in terms of their actions on the immune system and immune responses. Clearly, this is an area for further investigation.

In general, the majority of observations of the immunomodulatory effects of neuropeptides are obtained in cell culture systems in which the neuropeptide is added in various concentrations to lymphocytes in the presence of mitogens or antigens. The various inhibitory and/or stimulatory effects of the neuropeptides have been well-documented *in vitro* but have not been adequately translated to the *in vivo* situation. This is another area in which further investigation holds great promise for the expansion of our understanding of neuroimmunology.

Mediators of the hypothalamic-pituitary-adrenal axis

Hypothalamic dysfunction, hypophysectomy (removal of the pituitary gland) and adrenalectomy, separately or in combination, have dramatic and long-lasting effects on circulating immunocompetent cells. The absence of the pituitary gland eliminates the major source of various neuroendocrine hormones and neuropeptides, including luteinizing hormone, follicle-stimulating hormone, ACTH, prolactin, and growth hormone. The effect of hypophysectomy on the immune response is to alter the numbers of circulating leukocytes, reduce the capacity of both monocytes and macrophages to present antigens, and inhibit cytokine production by immunologically competent lymphocytes. Because the HPA axis is interrupted by removal of either the pituitary or the adrenal gland, glucocorticoids are no longer released into the circulation, thus eliminating a major regulatory factor that controls both the numbers of circulating leukocytes and the ability of these cells to

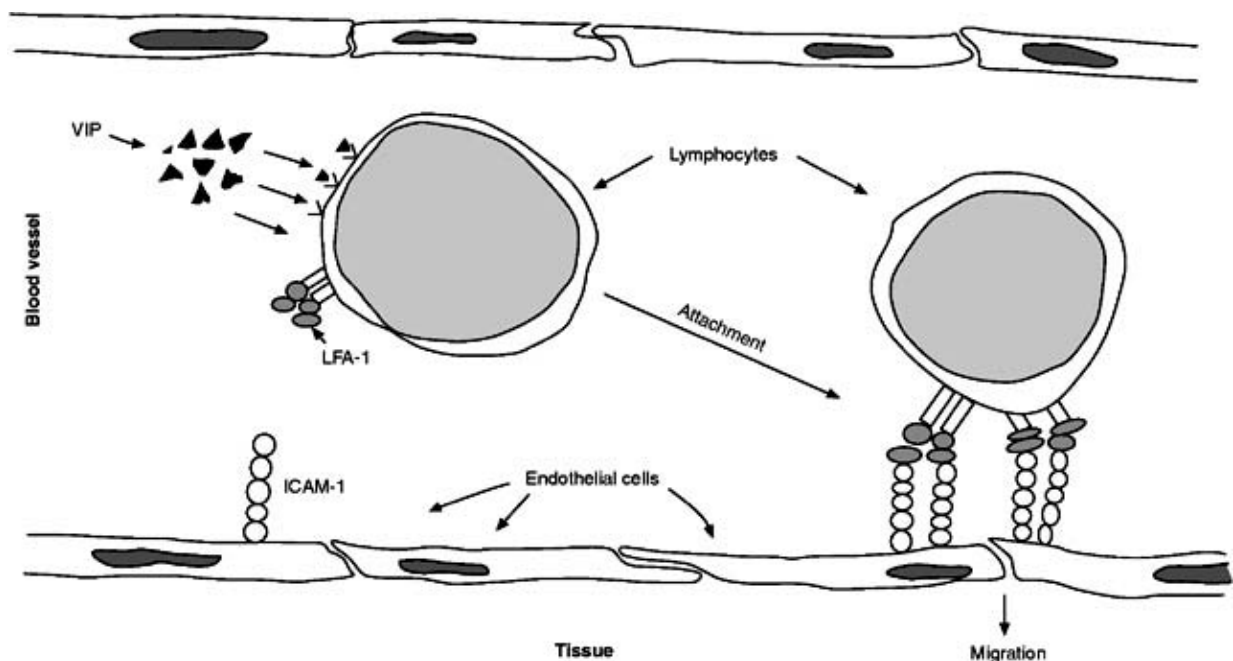


Figure 5 Vasoactive intestinal peptide (VIP) can alter lymphocyte movement and localization by stimulating the expression of intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 1 (LFA-1) on vascular endothelial cells and lymphocytes, respectively. Once receptor-ligand binding occurs, the lymphocytes can migrate from the blood vessel into the tissues.

participate in immune responses. In particular, the balance of immunoregulatory cytokine production is altered (Figure 6).

We and others have confirmed that certain white blood cells, especially mononuclear leukocytes (including lymphocytes), are the source of several of the neuropeptide hormones such as ACTH, luteinizing hormone, growth hormone and others (Figure 6). Recent studies using both cellular and molecular techniques revealed both the biological and molecular authenticity of leukocyte-derived neurohormones, including ACTH. Whether these leukocyte-derived neuropeptides can substitute for their HPA-derived counterparts *in vivo*, however, is still subject to some debate.

Much is known regarding the family of pro-opiomelanocortin (POMC) peptides, which includes the endorphins, enkephalins and ACTH. It has long been known that mononuclear leukocytes have receptors for ACTH, but it was not until recently that these receptors were shown to be functional. When both structural and functional correlates for ACTH receptors were demonstrated on cells of the adrenal cortex and on lymphocytes in a patient with the ACTH-

insensitivity syndrome, it became apparent that the presence of these receptors on the lymphocytes was not a happenstance. It is evident that these receptors enable lymphocytes to respond to, and to be regulated by, a neuropeptide hormone such as ACTH.

In vitro, ACTH inhibits the maturation of antibody-producing cells responding to the thymus-dependent antigen SRBC (sheep red blood cells); the maximal inhibitory effect occurs when the neuropeptide is present at the time of antigen exposure. However, the exact mechanism whereby ACTH exerts this inhibitory effect is not yet known, although it is tempting to speculate that receptor-mediated events either prevent T helper cells from making cytokines necessary for antibody-producing cell maturation or stimulate T helper cell production of an inhibitory cytokine that suppresses this maturation process (Figure 7). For example, activation of the ACTH receptors on T lymphocytes *in vitro* may inhibit production of a paracrine cytokine such as interleukin 4 (IL-4) or IL-5. These interleukins, among others, are required for the differentiation of B lymphocytes and the expression of isotype-specific antibody production. It is now possible to further study such sys-

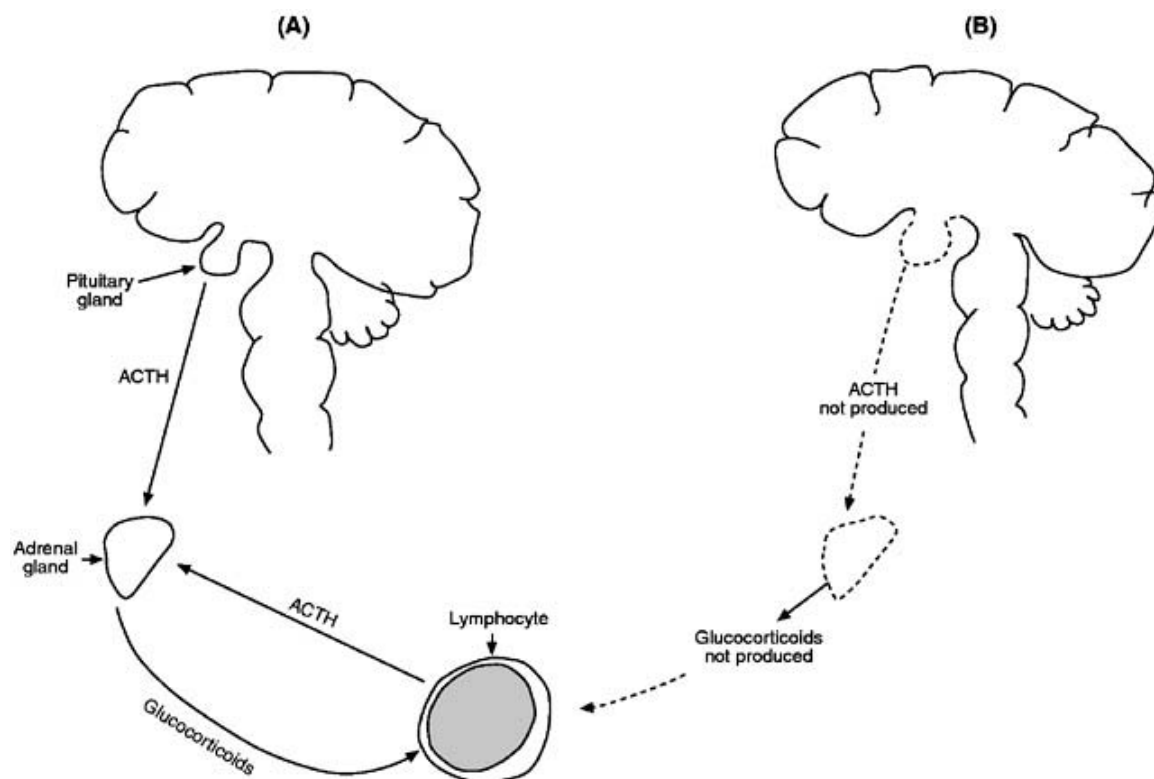


Figure 6 Adrenocorticotrophic hormone (ACTH) produced in the pituitary gland stimulates the production of glucocorticoids by the adrenal glands (A). These naturally occurring steroids alter lymphocyte recirculation and cytokine production. Removal of the pituitary or adrenal gland interrupts the pathway involved in the production of ACTH and glucocorticoids (B). There is accumulating evidence that lymphocytes can produce biologically active neurohormones such as ACTH and thus may serve as an alternative source of such mediators.

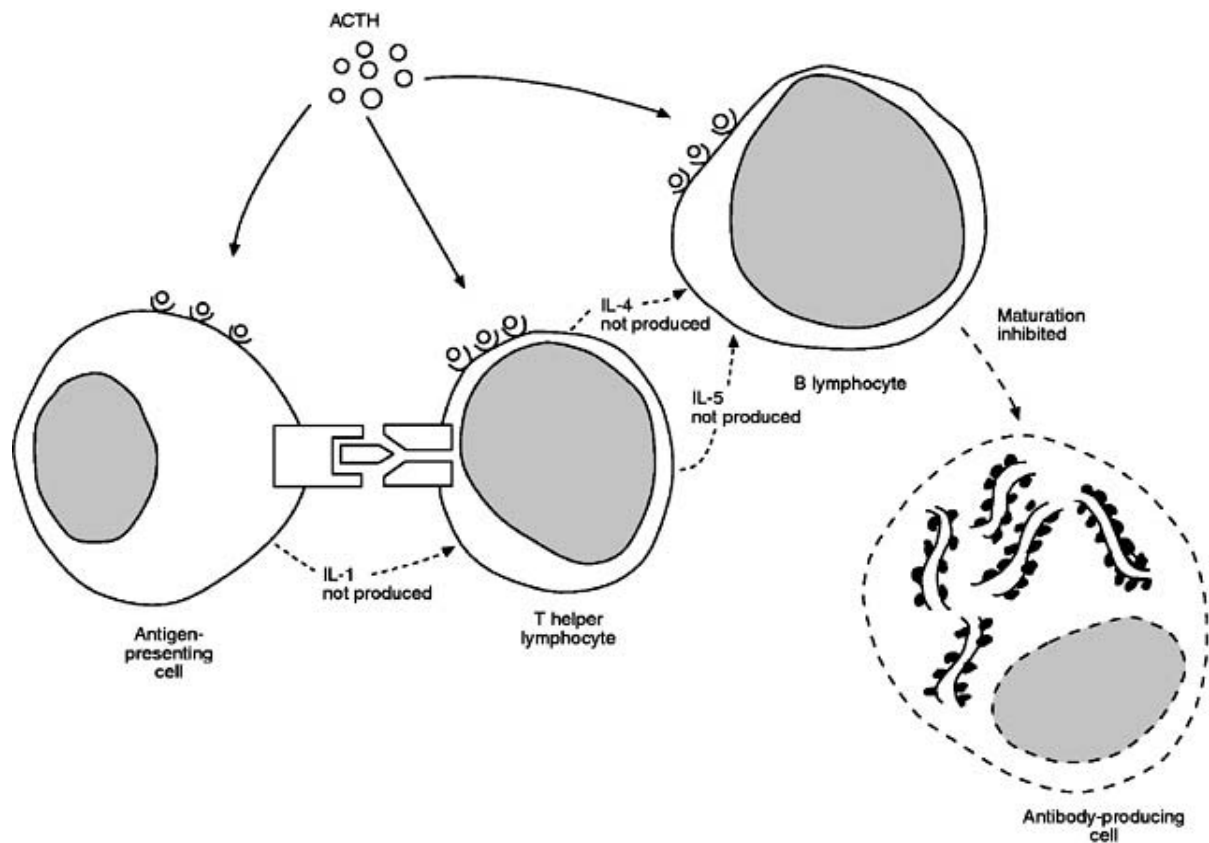


Figure 7 ACTH suppresses antibody production *in vitro*. The hormone may act on the antigen presenting cell, the T helper cell, or the B cell, or all three, inhibiting cytokine-mediated growth and maturation of the antibody-producing cells.

tems using purified cell populations and to perform molecular analyses of gene expression in antigen-presenting cells, B cells, and T cells in these culture systems in order to determine more precisely the mechanisms of action of various neuropeptides.

Enkephalins and endorphins, which are peptides also derived from the POMC gene complex, also affect lymphocyte responses in cell culture. β -Endorphin enhances the proliferative response of rat lymphocytes to T cell mitogens and α -Endorphin suppresses the responses of human peripheral blood T lymphocytes to concanavalin A. These findings, which appear to be contradictory, only serve to highlight the importance of neuropeptide hormone concentration and the complexity of the cell culture systems used to study such interactions. Receptors for the naturally occurring opioids have been characterized on human and mouse lymphocytes. Both μ - and δ -class opioid receptor agonists have been shown to modulate immunoglobulin production and natural killer (NK) cell activity. The recent cloning and sequencing of an orphan opioid receptor on mouse lymphocytes confirms the existence of these receptors on the cells of the immune system and provides a

basis for future studies of the regulation of the immune system by naturally occurring opioids.

Growth hormone is a pituitary-derived neuropeptide that has important physiologic and homeostatic actions in the growth and differentiation of vertebrate organisms. Interestingly, in addition to overall regulation of organismic homeostasis, this neuropeptide has also been shown to modulate immune responses both *in vivo* and *in vitro*. *In vitro*, growth hormone promotes the differentiation of cytotoxic T lymphocytes in cell culture and enhances the proliferation of T lymphocytes in mixed lymphocyte culture in the presence of insulin. *In vivo*, growth hormone boosts immune responses in immunologically deficient animals, and has been shown to augment immunity and immune responses in normal and aged animals. In particular, this molecule is an important mediator in the proliferation of lymphocytes and the differentiation and enhancement of NK cell activity.

Prolactin is another pituitary-derived neuropeptide that has significant effects on lymphocyte responses *in vitro*. In high concentrations prolactin suppresses T cell proliferation, whereas in low concentrations it enhances this process. Lowering prolactin

concentration by hypophysectomy has an inhibitory effect on the antibody response and can inhibit the development of graft-versus-host disease in experimental animals. Table 1 is a compilation of neuromediators and their effects on immune responses.

Cytokines: a bridge between the nervous system and the immune system

Cytokines are cell-derived peptides that may modify the functions of the cells that secrete them, as well as the functions of other cells that have receptors for them. Table 2 lists a number of cytokines that are produced and act in both the nervous system and the immune system.

Some of our initial knowledge of cytokines that are produced by cells outside the nervous system but that have an apparent effect within the nervous system came from clinical observations. One early study demonstrated that during an acute phase response to a systemic bacterial infection, the associated fever and transient accumulation of inflammatory cells in the central nervous system (CNS) are mediated by IL-1 and IL-6. In addition, numerous anecdotal clinical observations pointed to effects of IL-2, interferon γ (IFN γ), tumor necrosis factor α (TNF α), and transforming growth factor β (TGF β) on the nervous system, even though these cytokines may be synthesized by cells outside of the nervous system.

Initially, many investigators sought pathways by

which exogenously-produced cytokines could be transported into the CNS. Although such pathways have now been identified and the circumstances under which the blood-brain barrier is sufficiently compromised to allow cytokines to pass from the blood into the CNS are known, it has also been shown that these pathways are not the only means by which these mediators come to be present in the brain. A number of years ago, it was demonstrated that IL-1 and IL-6 are produced by cells in the pituitary gland and that IL-1 may be the regulator of IL-6 production by these cells. Given the knowledge that these two cytokines are mediators of inflammatory reactions and that one or both can stimulate the production of glucocorticoids by the adrenal gland, it became obvious that nervous system-derived cytokines, including the interleukins, could be a direct link between the nervous system and the immune system.

Since these early observations were made, the literature regarding cytokine production and cytokine-producing cells in the nervous system has burgeoned. It is now known that astrocytes and microglial cells are sources of IL-1, IL-6, TNF α , colony-stimulating factor and TGF β , as well as a host of other chemokines whose structures and functions are now being clarified. More recently it has been shown that oligodendrocytes can also produce IL-1 and TGF β . The fact that astrocytes and microglial cells produce these cytokines in response to a variety of stimuli has caused some investigators to speculate that these cells represent an arm of the immune system within the nervous system. Certainly, the production of IL-1 and IL-6, as well as TNF α , by astrocytes and microglial cells makes a strong case for the role of these mediators within the nervous system in the regulation of proliferation, differentiation and the cellular response to infectious agents and injury as part of the effort to promote survival of the organism (Figure 8).

The link between the nervous system and the immune system is evident from the fact that nervous system-derived cytokines and chemokines act on immune cells outside the system. Thus, IL-1 and IL-6 produced by microglial cells and astrocytes may act on immune cells outside the nervous system and certainly can act on lymphocytes and macrophages that enter the nervous system during infectious processes. There is evidence that lymphocytes and antigen-presenting cells accumulate at sites of herpes virus infections in both the peripheral and the central nervous system; it seems likely that the mediators necessary both to promote the attraction of these immune system cells and to promote and enhance their immune function within the nervous system are

Table 1 Neuromediators and their effects on immunity^a

<i>Neuromediator</i>	<i>Effect on humoral immunity</i>	<i>Effect on cellular immunity</i>
Neurotransmitters		
Epinephrine	↓	↓
Norepinephrine	↓	↓
Dopamine	↓	↓
Serotonin	↓	↓
Acetylcholine	↑	↑
Histamine	↓	↓
Neuropeptides		
Enkephalins	↓	↑
Adrenocorticotrophic hormone (ACTH)	↓	↓
Growth hormone	↑	↑
Prolactin	↑	↑
Somatostatin	↑	↓
Vasoactive intestinal peptide (VIP)	↑	↓
Substance P	↑	↓
Calcitonin gene-related peptide	↑	↓
Nerve growth factor	↑	↑

^a Much of what is known about these effects is derived from *in vitro* systems; a number of the neuromediators exhibit concentration-dependent effects on immunity and thus characterization of enhancement (↑) or suppression (↓) of a response depends on the conditions of the experiment.

Table 2 Cytokines that act in both the nervous system and the immune system

Cytokine	Nervous system		Immune system	
	Source	Action	Source	Action ^a
IL-1	Microglia, astrocytes, anterior pituitary cells, neurons	Glial growth, differentiation, stimulation of somatostatin and corticotropin-releasing factor (CRF) production	Macrophages, fibroblasts	T cell activation, leukocyte chemotaxis
IL-2	Microglia	Oligodendroblast proliferation, neurite outgrowth, stimulation of β -endorphin and ACTH secretion	T cells	T cell growth factor
IL-6	Microglia, astrocytes, anterior pituitary cells	Astrocyte growth, nerve growth factor production, ACTH and growth hormone production	T and B cells, macrophages, endothelial cells	T and B cell growth, differentiation, induction of acute phase protein production in liver
TNF α	Microglia, astrocytes	Astrocyte proliferation, stimulation of CRF production	Lymphocytes, macrophages	Mediation of inflammatory reactions, leukocyte chemotaxis
IFN				
α	Microglia, astrocytes	Inhibition of astrocyte proliferation, upregulation of MHC gene expression	T cells, fibroblasts, macrophages	B cell maturation, macrophage activation
β	Microglia, astrocytes			
γ	Neurons			
TGF α,β	Glial cells, neurons	Glial cell proliferation, neuron survival and neurotropism	Macrophages, T cells	Mediation of inflammatory reactions, control of cell differentiation

the cytokines made by glial cells within this system. It is known that astrocytes and microglial cells produce TNF α , which may modulate the expression of class I and class II major histocompatibility complex (MHC) molecules on some cell types in the CNS. Although there is no compelling evidence that neurons can be induced to express MHC class II molecules and the density of expression of MHC class I molecules by these cells is so low as to be difficult to measure, microglial cells, astrocytes, oligodendrocytes and perhaps other glial cells in the nervous system can express both MHC class I and class II molecules and, therefore, have the potential to act as antigen-presenting cells to lymphocytes attracted into the CNS by cytokines and chemokines (Figure 9).

In a recent series of studies, investigators have demonstrated the production of biologically active IFN γ by neurons in both the peripheral and the central nervous system. If confirmed, the production of so-called immune interferon by neurons provides yet another definitive link between the nervous system and the immune system and adds a new aspect to the bidirectional communication between these systems.

Psychoneuroimmunology

The mind-body connection

The recent resurgence of interest in studying our ability to ensure good health and to resist infections and diseases has been prompted in part by our increased understanding of regulatory mechanisms that affect the immune system and in part by our human desire to control and regulate our external and internal environments.

Psychoneuroimmunology is a subdiscipline of neuroimmunology that, to date, has attracted only a few mechanistically inclined immunologists. For the most part, the existing studies of the impact of emotional and environmental conditions, stress, and emotional disturbances on specific types of immunological responses have been initiated by psychiatrists, psychologists and behavioral biologists. Nevertheless, there are numerous published accounts of the effects of factors such as environmental stress, task/reward paradigms, crowding and overpopulation, and physical trauma on immunologic functions, including suppression of NK cell activity, antibody responses, and cell-mediated immune responses, as well as a

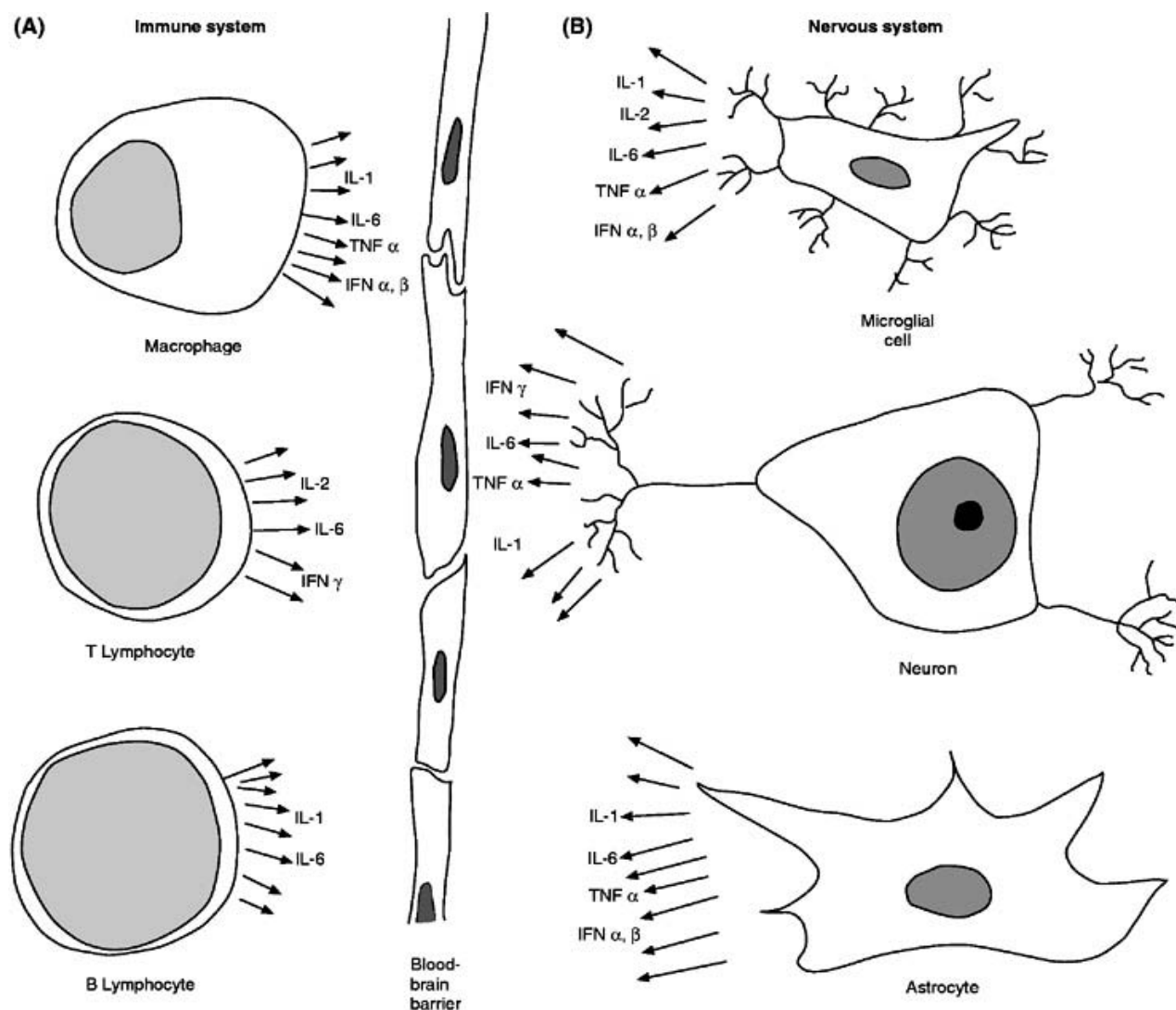


Figure 8 Production of cytokines by cells in the immune system (A) and nervous system (B). Some of the cytokines produced in each system act on cells within that system, but can also act on cells of the other system. Some of these cytokines cross the blood-brain barrier. In general, the cytokines involved mediate cell growth, cell differentiation, and serve as tropic factors in each system.

generalized reduction in the functioning of the immune system, relative to psychological well-being. Thus, evidence for such a link is growing.

The darker side of this area is represented by those who, in concert with the lay press, have promulgated the notion that the individual is capable of emotional control over the body's immune functions. In this context, it has been suggested that behavioral modification and one's emotional outlook can be used to defeat life-threatening diseases such as cancer, intractable pain and chronic debilitating viral infections including the acquired immune deficiency syndrome (AIDS). To date, there is no scientifically sound evidence that the nervous system, positive thoughts, neural activity and/or neuropeptides can separately or together defeat or reverse terminal conditions in

human beings. It is evident that a positive attitude and a good mental outlook undoubtedly help an individual's passage through a terminal disease, but it is not evident that such an outlook can prevent or cure such conditions. Unfortunately, this area of psychoneuroimmunology has been used as a basis on which to sell the public a variety of nostrums – putatively chemo- and/or psychotherapeutic – that may do more harm than good.

Summary

The science of neuroimmunology has become a bona fide division of immunologic thought and study in recent years. The observation that the nervous and endocrine systems exert regulatory actions on the

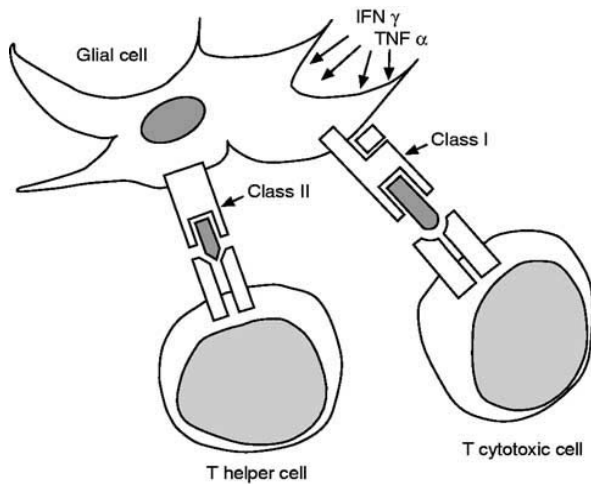


Figure 9 Induction of expression of MHC class I and class II molecules on glial cells allows these cells to present antigens to lymphocytes. Glial cells (microglia, astrocytes and oligodendrocytes) respond to such cytokines as $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$, express class I and/or class II molecules, and present antigens to lymphocytes.

immune system, and on lymphocytes in particular, has provided a fertile area for investigation and holds great promise for the future, in terms of modulating immune responses in human beings. It is now clear that bidirectional communication between the nervous system and the immune system is mediated by shared neuropeptides and cytokines. Lymphocytes communicate with cells in the nervous system by producing neuroendocrine mediators and cytokines, and cells in the nervous system communicate with lymphocytes by producing neuroendocrine mediators and cytokines. The immune system can perceive non-cognitive stimuli such as infectious agents, foreign antigens, allergens, and perhaps tumors; signal the nervous system concerning the presence of the threat; and elicit a CNS response which, in turn, promotes and regulates the immunologic response to the invader. This balanced regulatory loop can be perceived as functioning in the normal organism as part

of the physiological goal of achieving homeostasis and resistance to disease processes and infections (Figure 10).

Acknowledgements

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See also: **Acquired immune response; Adrenal autoimmunity; Autoimmune diseases; Behavioural regulation of immunity; Cell-mediated immunity; Cooperation, mechanisms of cellular; Cytokines; Glucocorticoids; Histamine; Immune response; Lymphocyte trafficking; Serotonin; Sex hormones and immunity; Stress and the immune system.**

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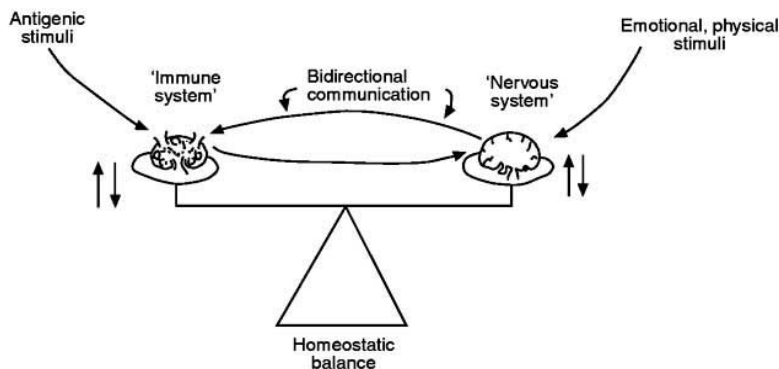


Figure 10 The homeostatic balance maintained by the responses of the immune system and nervous system. These systems interact following the receipt of stimuli. Cytokines and neuromediators elicit and modulate the responses of cells in both systems.

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NEUROLOGICAL AUTOIMMUNE DISEASES

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Autoimmune diseases that affect the nervous system can be divided into three categories: those that alter function of 1) the nerve–muscle junction, 2) the peripheral nerves, and 3) the central nervous system.

Nerve–muscle junction

Myasthenia gravis

This disease is a T cell-dependent antibody-mediated process. The antibody is directed against the acetylcholine receptor situated on the muscle side of the motor nerve–muscle synapse. The acetylcholine receptor exists in two forms: extrajunctional (or fetal), found along the length of immature or denervated myotubes, and junctional, confined to synapses. The extrajunctional form has two α chains, a β chain and a γ chain. The junctional form has two α chains, a β chain and an ϵ chain. In myasthenia gravis the antibody response is chiefly directed against an epitope on the α chain known as the main immunogenic region but also to some extent against β and γ chains. Presence of antibodies directed against γ chain indicates that the initial response in myasthenia gravis is to the extrajunctional form of the receptor even though the disease is directed at the junctional form. The T cell component of the immune response is directed against numerous epitopes simultaneously, many of them on the α chain. The epitopes recognized by T cells differ from those recognized by the B cells that make the antibody.

When a motor nerve impulse reaches the terminal axon the neurotransmitter molecule acetylcholine is released into the synaptic cleft. Released acetylcho-

line binds to the acetylcholine receptor. This binding is followed by the opening of ion channels, sodium enters the muscle fiber, and this entry transduces a signal which leads to muscle contraction. In myasthenia gravis antibody cross-links the acetylcholine receptors which are then shed or pinocytosed. The end-result is that the muscle self-strips itself of its receptors. Some damage to the muscle end-plate by a complement-dependent mechanism also occurs. With loss of receptors comes weakness, the cardinal clinical feature of myasthenia gravis since nerve impulses, even though they lead to release of acetylcholine in normal amount, are not followed by muscle contraction. The muscle is not destroyed in myasthenia gravis and is fully capable of synthesizing new acetylcholine receptors. For this reason if the antibody can be eliminated, complete recovery can occur. The antibody can be eliminated either by downregulating the T cells that provide help to the B cells, by downregulating the B cells that actually make the antibody, or by removing it by means of plasmapheresis.

Thymic abnormalities are usual in myasthenia gravis. Germinal centers, which bespeak local antibody production, are found within the thymus in 70–80% of cases. Germinal centers are particularly frequent in young adult female myasthenics who carry the HLA-B8-DR3 haplotype. A thymic epithelioma (thymoma) is found in 10% of cases. Thymoma patients tend to be older than those without a thymoma and show no increase in any HLA haplotype. A third group of cases are elderly. Here males predominate, and the thymus is atrophic. Thymic myoid cells, a minor cell type within the thymus found at

the cortico-medullary junction and within the medulla, carry extrajunctional-type acetylcholine receptors. The disease may begin as an autoimmune response within the thymus directed initially against the extrajunctional acetylcholine receptor on thymic myoid cells and misdirected subsequently against the junctional acetylcholine receptor of muscle. Thymomas do not express acetylcholine receptors but do express a 153 kDa cytoplasmic protein that shares an epitope with the α chain of the acetylcholine receptor.

Antibodies to the acetylcholine receptor can be detected in the serum of 90% of cases of myasthenia gravis and they are quasi-specific for the disease. Serum from antibody-negative myasthenics sometimes will adoptively transfer disease to experimental animals, suggesting that in them an antibody directed against some other protein causes the disease. Antibody titer does not correlate with disease severity, indicating that factors other than antibody titer alone determine severity. It has been suggested that antibody directed against certain epitopes and in particular against the site on the acetylcholine receptor to which acetylcholine binds may be more potent than antibody directed against other epitopes. Myasthenics with such antibodies might have severe disease despite low titer. Alternatively, antibodies directed against the neuronal side of the myoneural junction could contribute to symptomatology, as might products released by macrophages and/or cytokines released by T cells. Macrophages are found at the myoneural junction at times of acute worsening of myasthenic symptomatology.

An experimental model for myasthenia gravis has been developed. Animals immunized with acetylcholine receptor develop antibodies to it and a myasthenic syndrome. At the onset of this experimental illness there is a brisk influx of macrophages into the myoneural junction region. At this time antibody titer is low. The macrophages disappear after a few days and the animal recovers clinically even while antibody titer continues to rise. A second wave of disease without a macrophage influx follows some weeks later at a time when the antibody titer has risen to a high level. In acute myasthenic crises in humans a similar macrophage influx is seen, and it seems likely that macrophage activity can contribute to the severity of disease. When serum from animals with experimental myasthenia gravis is injected into naive recipient animals myasthenia follows. Female recipients get more severe disease than male recipients and young recipients more severe disease than older ones, pointing to host factors independent of antibody as determinants of disease severity. Inhibi-

tors of macrophage function lessen the severity of adoptively transferred disease.

Immunotherapy with glucocorticoids, immunosuppressive drugs such as azathioprine or cyclosporine and, in acute emergencies, plasmapheresis provide the mainstays of treatment of human myasthenia gravis. Intravenous immunoglobulin G (IgG) treatment has also been reported to be beneficial. Early thymectomy is advocated by many, including this author. Cases associated with thymoma should be viewed as a remote effector of cancer. In them, the tumor should be removed though remission of disease following thymectomy is less common in patients with thymomas than in other cases.

Lambert-Eaton syndrome

This disease is a T cell-dependent antibody-mediated process in which the antibody is directed against voltage-gated calcium channels of the motor axon terminal. The response is directed chiefly against P-type channels, the major calcium channel type that mediates cholinergic transmitter release at the neuromuscular junction. Antibody cross-links the channels which are shed or pinocytosed, and hence stripped. Calcium entry into the terminal axon is critical for the release of acetylcholine and when calcium channels are lost, calcium entry is precluded. For this reason acetylcholine release fails, and weakness, a characteristic clinical feature of the Lambert-Eaton syndrome, ensues. There is a pathognomonic electromyographic abnormality in the Lambert-Eaton syndrome. Muscle contraction is weak when the motor nerves are stimulated at low frequencies. At higher stimulation frequencies the failure of calcium entry is in part overcome and the force of contraction increases. This is known as facilitation.

Fifty per cent of cases of the Lambert-Eaton syndrome occur on a background of cancer, most commonly small cell cancer of the lung. All small cell cancer cells carry several types of calcium channels including the P-type discussed above. In the Lambert-Eaton syndrome an antitumor response (which may to some extent hold the tumor in check) is misdirected against the motor axon terminal because both share identical calcium channels. Why only a small proportion of patients with small cell cancer develop antibody to the calcium channel is not known. In the other half of cases of the Lambert-Eaton syndrome (which are not associated with cancer) there is an over-representation of the histocompatibility alleles HLA-B8 and -DR3, indicating a genetically determined propensity to develop the disease. Many of these cases have other autoimmune diseases in addition to the Lambert-Eaton syndrome. Favorable response to plasmapheresis or intravenous

immunoglobulin in conjunction with glucocorticoids and immunosuppressive drugs is frequently observed.

Peripheral nerves

Guillain-Barré syndrome

The Guillain-Barré syndrome is characterized by acutely evolving weakness, loss of reflexes, and elevated spinal fluid protein. Weakness can be mild or so severe that total paralysis and respiratory failure ensue. The illness evolves over from 1 to 4 weeks, followed by a plateau period, and then by recovery over weeks to a year or more. Recovery is usually complete or nearly so, but 15% of cases are left with residual disability.

Approximately two-thirds of patients recall an antecedent acute infectious illness that has usually abated by the time neuropathic symptoms begin. The interval between the prodromal infectious episode, usually an acute respiratory illness or a dysenteric episode, is most often from 1 to 3 weeks, occasionally longer. Twenty per cent of cases follow *Campylobacter jejuni* enteritis. Ten per cent of cases follow the glandular fever-like syndrome caused by cytomegalovirus (CMV). At times, IgM antibody to CMV, presence of which argues strongly for infection with CMV in the immediate past, may be detected even when no recent illness can be recalled. It follows that the severity of the antecedent infection in no way correlates with the occurrence of a complicating polyneuritis. Five per cent of cases follow a surgical procedure. Usually blood transfusions have been given in such cases, and it is distinctly possible that CMV was introduced in the transfused blood. Five per cent of cases are associated with infectious mononucleosis, a disease caused by the Epstein-Barr virus (EBV). Two per cent of cases follow varicella (chickenpox) or zoster (shingles). Note that CMV, EBV, and varicella-zoster virus are all herpesviruses, suggesting some common thread. Marek's virus is an oncogenic herpesvirus of the chicken. An acute demyelinating polyneuritis which faithfully mimics the Guillain-Barré syndrome in its clinical, electrophysiologic and pathologic features follows infection of chickens with Marek's virus. Marek's virus also causes lymphomas, and it is noteworthy that in humans as well the Guillain-Barré syndrome may complicate lymphomatous disease, particularly Hodgkin's disease. Five per cent of Guillain-Barré cases have serologic evidence of active or recent mycoplasmal infection. A few cases have followed parenteral ganglioside administration.

Polyneuritis in association with HIV infection is being noted with increasing frequency. Onset occurs

most often shortly after seroconversion. HIV-positive patients frequently harbor other organisms (including CMV), and it is not clear whether the occurrence of the Guillain-Barré syndrome on a background of HIV infection is triggered by HIV itself or by some other organism.

A substantial proportion of Guillain-Barré syndrome cases can be accounted for by infection with a limited number of organisms. Why should these infections cause the disease or predispose toward it when other infections do not? In this regard there has been considerable interest in the concept of molecular mimicry. It is hypothesized that there are close sequence similarities between small regions of proteins of infectious agents and small regions of host proteins. The infection triggers an immune response to the sequence on the infectious organism which then cross-reacts with the analogous region of the host protein. In this way tolerance to self is broken and a tissue-destructive autoimmune response ensues. Molecular mimicry has been demonstrated in several autoimmune diseases so that the concept is an attractive one in the Guillain-Barré syndrome; unfortunately, firm evidence to support it is lacking.

Acute inflammatory demyelinating polyneuropathy (AIDP) Four subtypes of the Guillain-Barré syndrome are recognized. Of these, by far the commonest in North America and Europe is acute inflammatory demyelinating polyneuropathy (AIDP). AIDP is characterized by weakness, sensory loss, cranial nerve involvement, and sympathetic nervous system compromise. The characteristic pathologic feature is one of scattered inflammatory cellular infiltrates within nerves and roots composed primarily of T cells and macrophages. At sites of inflammation there is destruction of peripheral nerve myelin by the invading macrophages. Destruction begins on the abaxonal surface, i.e. furthest from the axon. The macrophages secrete proteases, reactive oxygen and nitrogen species, and complement components, all of which contribute to myelin damage. Acute demyelination blocks nerve impulse conduction, hence the paralysis. When the inflammatory response is particularly intense there may be axonal destruction as well. Activated T cells and activated macrophages are found in the blood at the onset of AIDP.

After a few weeks the nerves remyelinate, a relatively rapid repair process. When there is superimposed axonal destruction, recovery may be delayed over months to a year or more since regrowth of axons is a slow process.

A model for AIDP known as experimental allergic neuritis (EAN) has been developed. Animals immunized with the P₀ and/or P₂ proteins of peripheral

nerve myelin develop an acute demyelinating disease which faithfully mimics the human one clinically, pathologically, and electrophysiologically. EAN is mediated by CD4⁺ T cells acting in conjunction with macrophages, but only a minority of AIDP patients demonstrate T cell or antibody responses to the P₀ or P₂ proteins, so the model is imperfect.

While EAN is a purely T cell-mediated process, the situation in AIDP may be more complex. It has been established that plasmapheresis exerts a beneficial effect on the course of the human illness. Since plasmapheresis removes circulating immunoglobulin, among other plasma components, the possibility that circulating antibody may have a role in the pathogenesis of the Guillain-Barré syndrome is attractive. Complement-fixing myelin-specific IgM antibodies (most cases) and IgG antibodies that bind to GM1 ganglioside (20% of cases) have been reported in the serum in AIDP, particularly early in the course of the illness, a finding which lends credence to this suggestion. Demonstration of the terminal attack complex of complement both in the blood and the spinal fluid in AIDP cases reinforces the contention that antibody has a role in the pathogenesis of AIDP. At the same time the process is unlikely to be purely and simply antibody-mediated since this will not account for the inflammatory cellular infiltrates that are the pathologic hallmark of the disease. A synergism between an antibody-mediated process and a T cell-mediated one is distinctly possible. The blood-nerve barrier is ordinarily impervious to immunoglobulin but access into the nerves may be provided when a cellular immune response opens the blood-nerve barrier. EAN can be adoptively transferred with T cells, but the disease is more severe if recipients are also given myelin-specific antibody.

The clinical diagnosis of AIDP usually presents little difficulty. Onset of symptoms is subacute – evolution is complete after 2 weeks in 50% of cases, after 3 weeks in over 80%, and after 4 weeks in over 90%. A stable period, which is usually brief, is followed by a recovery period that proceeds to completion within a period of weeks to a few months. Fifteen per cent of cases are left with a permanent handicap. In most instances the disease is monophasic; about 5% of cases have one or more recurrences.

The major complaint is of weakness, which covers a wide spectrum from mild ataxia to total paralysis of every motor and cranial nerve. In most instances, weakness begins in the legs, more commonly distally at first and then proximally, but the arms may be involved before the legs and proximal involvement may precede distal. The process is characterized by relative but seldom absolute symmetry. Facial weak-

ness, usually bilateral, is noted in half the cases. In 20% of cases weakness becomes so profound that respiratory assistance is required. Tendon reflexes are generally abolished. Ataxia may be prominent early in the disease and can cause confusion as to the diagnosis. Sensory symptoms are in general less prominent than motor ones, although almost all patients complain of subjective tingling or numbness, usually in a distal symmetric glove- and stocking-like distribution. Objectively demonstrable sensory loss is less extensive than the subjective complaints would lead one to anticipate. Significant muscular or neuropathic pain occurs in 30% of cases. Autonomic function is frequently abnormal and may cause wide fluctuations in blood pressure, pulse, abnormalities of sweating, and gastric atony.

The cerebrospinal fluid (CSF) protein is normal during the first few days of the illness, but then rises, reaching sometimes very high values after 3–4 weeks. Cells in the CSF are not prominent, but a few cases may show 20–30 cells mm⁻³ and in those cases associated with HIV infection a pleocytosis is the norm rather than the exception. Conduction of nerve impulses through areas of segmental demyelination is severely compromised. Conduction velocity is characteristically slowed, though not invariably so, and prolonged distal latencies are frequent. Nerve conduction studies, if carefully performed, reveal abnormalities in 90% of cases.

In severe cases, the major effort in terms of clinical management must be devoted to the prevention of complications. Seriously ill patients are best cared for in intensive care or respirator units. A course of vigorous plasmapheresis treatment has been shown to lessen time of hospital confinement, and in severe cases, to lessen time spent on a respirator. Response to intravenous immunoglobulin is sometimes seen. Immunosuppressive and glucocorticoid treatments are not recommended.

Acute motor axonal neuropathy (AMAN) This is the commonest form of the Guillain-Barré syndrome in China and Mexico. Most cases follow *Campylobacter jejuni* infection. AMAN is primarily a disease of children and young adults from rural areas. Incidence peaks in the summer whereas AIDP occurs throughout the year. The clinical presentation is purely motor, often with total paralysis. Electrical studies indicate axonal damage rather than demyelination. Pathologic study of fatal cases has revealed axonal interruption confined to motor roots in some cases but no axonal interruption in others. The absence of axonal interruption in some cases is not surprising since recovery is usually rapid, at a tempo in keeping with axonal functional compromise

but inconsonant with frank axonal interruption. Immunoglobulin and complement are deposited on axons at nodes of Ranvier. Macrophages are found interdigitated between axons and overlying intact myelin, a location totally different from that seen in AIDP. T Cells are absent. Many cases have had antibodies to GM1 ganglioside. The disease is thought to be antibody-mediated. From 5 to 20% of Guillain-Barré cases in the United States and Europe have this form of the disease. Response to intravenous immunoglobulin treatment has been reported.

Acute sensori-motor axonal neuropathy (ASMAN) This form of the Guillain-Barré syndrome has the same pathologic features as AMAN except that both motor and sensory roots are involved. The process is purely axonal and likely to be severe. Recovery is delayed relative to AMAN, pointing to more extensive axonal interruption. ASMAN is relatively rare in the USA and Europe.

The Miller Fisher syndrome (MFS) This form of the Guillain-Barré syndrome constitutes not more than 5% of cases. The clinical picture is one of ophthalmoplegia, ataxia and areflexia. Most cases follow *Campylobacter jejuni* infection. The process is usually self-limited and response to plasmapheresis is often gratifying. Almost all MFS cases have antibody to GQb1 ganglioside, a minor ganglioside of peripheral nerves but one that is enriched at nodes of Ranvier of the extraocular nerves and in dorsal root ganglia. The former location is relevant to the ophthalmoplegia and the latter to the ataxia and areflexia. It seems likely that the antibody to GQb1 ganglioside causes the disease.

Chronic inflammatory demyelinating polyneuropathy

This illness is generally viewed as a variant of AIDP. The process is indolently progressive, however, rather than explosive in onset. A history of an antecedent infection is uncommon. The clinical presentation is one of weakness with absence of deep tendon reflexes and distal sensory loss. Nerve conduction studies reveal slowing, a finding that points to a demyelinating process. The pathologic hallmark is T cell and macrophage infiltrates with accompanying demyelination, though the inflammatory infiltrates are less florid than in AIDP. Some cases have responded to plasmapheresis treatment or intravenous immunoglobulin and a favorable clinical response to glucocorticoids is sometimes observed.

Dorsal root ganglion neuronitis

This is a rare disease. Most cases have had an associated neoplasm, most commonly small cell carcinoma of the lung, though sporadic cases are encountered. The pathologic feature is one of a lymphocytic and macrophagic infiltrate of dorsal root ganglia with destruction of dorsal root neurons. The clinical presentation is one of progressive sensory loss and areflexia with preservation of muscle strength. The sensory loss may advance to the point that the patient becomes bedridden. Antibodies to a protein called Hu, found in dorsal root and other neurons, can be detected in most cases associated with lung cancer. Hu-specific antibodies recognize a family of 35–37 kDa RNA-binding proteins that are encoded by at least three discrete genes. Hu proteins bind AU-rich regions of mRNAs that regulate cell proliferation. The disease is thought to be autoimmune, but formal proof for this contention is lacking. A similar neuropathy with like pathology sometimes complicates Sjögren's syndrome.

Acute pandysautonomia

Fewer than 50 cases of this illness have been described. The disease is characterized by an acute loss of sympathetic and parasympathetic function with severe orthostatic hypotension, anhidrosis, dry eyes and mouth, disturbed bladder and bowel function, fixed heart rate, and fixed pupils. A few cases have had an associated small cell cancer of the lung but most have been sporadic. The illness is monophasic and sometimes remits, but most cases are left with at least some residual disability.

Demyelinating neuropathy associated with antimyelin-associated glycoprotein (MAG) antibodies

This illness presents as a slowly progressive, symmetric mixed (motor and sensory) neuropathy with slowed nerve conduction. Most often it has been associated with an IgMκ gammopathy and the IgMκ binds to a 100 kDa myelin protein known as myelin-associated glycoprotein (MAG), to carbohydrate moieties on several other myelin-associated glycoproteins of lower molecular weight, and to acidic sulfoglucuronyl glycolipids of myelin. IgMκ and complement deposits along myelin sheaths in nerve can be detected and by electron microscopy a widening of myelin lamellae at the minor dense line can be shown. There are no inflammatory cells. Injection of the monoclonal antibody into experimental animals reproduces the disease. Some cases have responded to plasmapheresis or to treatment of their underlying gammopathy with cytotoxic drugs.

Multifocal motor neuropathy with conduction block

This entity is characterized by weakness that is asymmetric, usually distal, and affecting the arms more than the legs. There may be wasting, fasciculations and cramps. Reflexes are paradoxically preserved. The clinical features may mimic those of amyotrophic lateral sclerosis. Electrical study reveals denervation and motor nerve conduction block. These patients have a monoclonal IgM antibody that binds to GM1 and GD_{1b} gangliosides as well as to motor end-plates at the neuromuscular junction. Experimental animals immunized with GM1 ganglioside develop a similar disease and injection of patient antibody into animals reproduces it. It seems likely, therefore, that the monoclonal antibody causes the disease. Some patients have improved when antibody titer was lowered or in response to cytotoxic agents.

Neuropathy of sclerosing myeloma

Most myelomas are osteolytic but from 1 to 3% cause bone sclerosis. Fifty per cent of sclerosing myelomas are associated with a neuropathy that is slowly progressive but nonetheless invariably fatal unless the sclerosing myeloma is discovered and treated. Pathologically, the process is characterized by destruction of both myelin and axons. Sclerosing myelomas produce monoclonal IgM or IgA, but the quantity may be small and a spike may not be detected in the blood. Monoclonal IgG or IgA is not deposited in the damaged peripheral nerves. In addition to the neuropathy there may ultimately be widespread failure of other organs plus bony overgrowth at a distance from the myeloma as, for example, in lumbar vertebrae. The condition is often called the POEMS syndrome because of associated features including polyneuropathy, organomegaly, endocrinology, an M-spot, and skin changes.

The cause of this neuropathy is not known, but it is likely that products other than immunoglobulin that are toxic to peripheral nerves are released in excess by sclerosing myelomas or by macrophages, induced to do so by some product released by the myeloma cells. Candidates include calcitonin, interleukin 1, interleukin 6, tumor necrosis factor α , vascular endothelial growth factor or some combination of them. All of the above are found in increased amount in the circulation of patients with this neuropathy. Radiation of an isolated myeloma can be followed by remission. When disease is more widespread, response to chemotherapy is sometimes obtained.

Central nervous system

Postinfectious and postrabies vaccination encephalomyelitis

This is an acute monophasic illness in which the brain and spinal cord are peppered with innumerable minute perivenular foci of demyelination. T Lymphocytes and macrophages, the final vectors of myelin destruction, are found at sites of myelin destruction. T Cells from patients proliferate *in vitro* when exposed to a protein of central nervous system myelin known as myelin basic protein (MBP). Antibodies to MBP are also detectable in most instances. In former times the majority of cases came on within a few days of the development of a measles rash. Such cases are now rare except in those less developed countries where measles remains endemic. An identical syndrome is seen as a complication of rabies vaccination with vaccines prepared from brain tissue. Animals immunized with MBP develop the same disease. The animal model, which is mediated by a delayed-type hypersensitivity response, is called experimental autoimmune encephalomyelitis (EAE).

Clinically postinfectious encephalomyelitis begins abruptly with symptoms and signs that point to damage chiefly of the white matter of the brain and spinal cord. Headache and delirium at onset may give way to lethargy and coma. Seizures at onset are frequent. There may be neck stiffness and fever. Focal signs may be superimposed, spinal cord involvement with limb paralysis being the most frequent. The spinal fluid shows an increased protein content and sometimes a lymphocytic pleocytosis. Mortality is 20%, and half the survivors have residual deficits. The illness does not recur. Administration of high doses of glucocorticoids every 4–6 h is the treatment of choice.

Multiple sclerosis

Multiple sclerosis (MS) is characterized by areas of myelin destruction in the central nervous system known as plaques. Central nervous system myelin is made by oligodendrocytes and as the illness progresses oligodendrocytes come to be lost. In addition, axons coursing through areas of demyelination may be destroyed, particularly in the later stages of the disease.

MS usually begins in early adult life though some patients do not manifest clinical symptoms until the 5th or 6th decades. Two-thirds of cases are in women. MS is characterized by attacks which last for days to weeks followed by remissions which may last from months to years. Recovery from early attacks may be complete or incomplete, but as one attack follows another residual disability

accumulates. A proportion of patients present with a slowly but nonetheless inexorably progressive illness rather than with the more frequent pattern of attacks followed by remissions. Others may, after a time, sometimes many years, switch from a relapsing–remitting course to a progressive one. The basis for the switch from flaring–remitting to progressive disease is not known.

The clinical features of the disease are protean. This reflects the fact that any site within the CNS white matter can be attacked by invading inflammatory cells. Nonetheless, there are certain sites of predilection. These include the optic nerves, the periventricular and deep white matter of the cortex, cerebellar inflow and outflow tracts, and the spinal cord. As a consequence visual loss, spasticity, weakness, incoordination, diplopia, and sensory loss, particularly of vibration and joint sense, are frequent features.

Multiple sclerosis is an inflammatory disease. B cells, macrophages, and CD4⁺ and CD8⁺ T cells are found within and at the margins of active plaques. All these cell types are activated; the B cells synthesize IgG which exhibits oligoclonal restrictions, the macrophages release interleukin 1 (IL-1), tumor necrosis factor α (TNF α), and IL-6. The CD4⁺ T cells are chiefly of the T_H1 cell subset. They secrete IL-2, IFN γ and lymphotoxin (LT), all of which are found in active MS plaques.

MS is most commonly viewed as an autoimmune disease, but no antigen against which the immune response is thought to be directed has been identified. Periodically, interest in a viral causation for MS resurfaces, but to the present no virus has been identified that links to the disease despite diligent search. Nonetheless, there is reason to believe that an MS-relevant antigen does exist.

Propensity to develop MS is at least in part genetically determined. Concordance for MS between siblings is 3%, a value some 25 times that for the population at large. For identical twins, concordance is 20%, based on clinical features, but if paraclinical features (abnormal MRI scan or spinal fluid) are taken into account concordance rises to 40%. Inheritance of susceptibility is surely polygenic since the steep drop in concordance from monozygotic twins, who share all their genes, to siblings who share half their genes cannot be explained by any single gene model.

Genes alone do not determine who will get MS since even in identical twins concordance is only 40%. This observation indicates that some environmental factor is also in play. Identical twins, both with MS, can have disease of quite different severity so that disease course is determined by something

other than genetic endowment. One gene marker has been identified that is overrepresented in MS populations though it is not necessarily present in any individual MS patient. This is the class II histocompatibility allele HLA-DR2/DQW1 on chromosome 6.

Most attacks of MS occur without any obvious triggering event. It is known, however, that viral infections in MS patients are commonly followed within a few weeks by an MS attack at a frequency 3–5 times background. Administration of IFN γ also triggers attacks. Since IFN γ is one of the products of activated T_H1-type T cells and since infectious illness activates these same cells it is possible that both triggering events are in fact the same. Many MS patients relate a period of stress as an antecedent to their attack. Their contention may have merit but must be viewed as conjectural at this time. Pregnancy protects in MS; attack frequency falls well below baseline levels during the second and third trimesters.

Several abnormalities have been detected in the blood at the onset of and during MS attacks. IL-2 and IFN γ levels rise, whereas release by macrophages of prostaglandin, an inhibitor of immune responses, falls at onset of attacks. An increased proportion of activated CD4⁺ T cells has also been noted. Non-specific suppressor function is reduced during MS attacks and recovers with remission. The defect in suppressor function lies primarily in CD8⁺ T cells.

Considerable effort has gone into a search for agents that will correct these abnormalities with the hope that such agents once identified will prove efficacious therapeutically. IFN β corrects deficient non-specific suppressor function and lowers IFN γ production by activated T_H1-type T cells. Interleukin 10 is an inhibitory cytokine for T_H1-type T cells. IFN β increases IL-10 production. IFN β lessens MS attack frequency and is widely used to treat the disease.

B cells in MS plaques make immunoglobulin but whether any of this is directed against an MS-relevant antigen is not known. What is known is that antibodies to numerous viruses as well as to epitopes that clearly have nothing to do with MS, for example tetanus toxoid, are synthesized within the CNS. It is thus evident that some of the antibodies being made are irrelevant antibodies insofar as the pathogenesis of MS is concerned. Possibly all are irrelevant antibodies.

TNF α is synthesized by activated macrophages and microglia. TNF α causes oligodendrocytes to retract their processes and to close their K⁺ channels, at least *in vitro*. When TNF α is added to organotypic cultures demyelination ensues and death of oligodendrocytes has also been reported. The notion that TNF α may be a major vector of myelin destruction in MS is attractive since IFN γ , a product of activated

T_H1 -type T cells, is known to induce both $TNF\alpha$ synthesis and secretion by macrophages and as mentioned $IFN\gamma$ provokes attacks of MS. Activated T_H1 -type T cells release lymphotoxin ($TNF\beta$) in addition to $IFN\gamma$. Lymphotoxin binds to the same cell receptors as $TNF\alpha$ and duplicates many of its actions. Accordingly, a delayed-type hypersensitivity response mediated by T_H1 -type T cells acting in conjunction with macrophages is thought to have a major role in the genesis of MS lesions. $TNF\alpha$ and LT have totally different effects on astrocytes than on oligodendrocytes. $TNF\alpha$ and LT promote astrogliosis as does IL-1, another product of activated macrophages. It should be noted that astrogliosis is a characteristic feature of established MS lesions.

$CD8^+$ T cells are also present in MS plaques. $CD8^+$ T cells have two established functions. The first is cytolysis mediated by perforin and other molecules secreted by killer cells once they have made contact with antigen presented within the epitope-bearing cleft on the class I major histocompatibility complex (MHC) molecules of the target cell. Perforins are capable of damaging oligodendrocytes, and though oligodendrocytes in health do not express class I MHC, they can be induced to do so by $IFN\gamma$. Thus the anatomical substratum for cytolysis exists within the brain but whether $CD8^+$ T cell-mediated cytolysis occurs in fact is not known.

The second major role for $CD8^+$ cells is suppression. In most strains of rats or mice, experimental autoimmune encephalomyelitis (EAE) is a monophasic disease; animals that have recovered from an EAE attack rarely relapse. $CD8$ knockout mice lack $CD8$ cells. In them, EAE is a relapsing disease. The data suggest a role for $CD8$ suppressor cells as a preventor of relapses. The loss of $CD8$ suppressor cell function observed at the time of MS attacks may be relevant in this regard. $CD8$ knockout mice recover from their first attack at the same tempo as controls. Relapses are also self-limited. Though $CD8$ cells are found in the brain in MS it is by no means clear that they have any role in the ending of an attack.

Secondarily, progressive MS poses a problem. The reason why the disease should change its course is not understood. Progressive MS patients are almost invariably spastic. It has been proposed that a strategic hit of the disease itself interferes with normal communications between the nervous and immune systems and that this unfavorably alters immune homeostasis. There is increasing evidence that the nervous system does exert some control over the immune system. Pituitary hormones may either up- or downregulate the immune system. Prolactin, for example, is stimulatory to the immune system

whereas corticotropin is inhibitory. The lymphoid organs also receive a substantial intraparenchymal sympathetic innervation. Ablation of the sympathetic nerves in experimental animals is followed by augmented immune responses. The severity of EAE, for example, is augmented in sympathetic nervous system (SNS)-ablated rats whereas treatment with the β -adrenergic agonist drug isoproterenol lessens the severity of EAE. Lymphocytes carry β_2 -adrenergic receptors and the number of these receptors rises following SNS ablation.

In progressive MS patients it is often possible to show that SNS function is defective, presumably because MS lesions have interrupted the normal flow of signals down the spinal cord to the neurons in the lateral columns that relay to the sympathetic ganglia and to the adrenal medulla. β -Adrenergic receptor density on $CD8^+$ T cells is 2–3-fold upregulated in progressive MS, suggesting that the progressive MS patient may be a mimic, albeit an imperfect one, for the SNS-ablated rat. Alternatively increased β_2 -adrenergic receptor on $CD8^+$ T cells may simply reflect immune system activation.

The mainstay of treatment of multiple sclerosis attacks is glucocorticoid therapy. One popular treatment regimen is intravenous solumedrol at a dose of 250 mg every 6 h for from 3 to 5 days.

Necrotizing myelopathy

In this rare disease the central core of the spinal cord is destroyed. Macrophages are prominent in areas of necrosis; lymphocytes are rare. Spinal cord destruction begins most often in the thoracic region and extends rostrally and caudally over time. The illness occurs sporadically but is also seen on a background of systemic lupus erythematosus or of pulmonary tuberculosis, in heroin addicts and as a remote effect of cancer particularly small cell carcinoma of the lung and lymphoma. There is some evidence to suggest that the process is antibody-mediated.

Acute necrotizing hemorrhagic leukoencephalitis

This devastating affliction comes on with brutal suddenness, usually after an ill-defined upper respiratory illness. It resembles postinfectious encephalomyelitis in the sense that perivenular inflammatory foci are found but in addition there is fibrinoid necrosis of blood vessels, multiple foci of petechial hemorrhage and frank necrosis of brain substance. A model for it has been developed by superimposing a Sanarelli–Shwartzman reaction on to the standard immunization protocol for induction of EAE.

Paraneoplastic cerebellar degeneration (PCD)

This is the most frequently encountered paraneoplastic syndrome affecting the CNS. PCD occurs on a background of one of the following tumors: 1) small cell cancer of the lung, 2) Hodgkin's disease, 3) breast cancer, 4) ovarian cancer, and 5) cancer of the female genital tract. The neurologic syndrome may antedate detection of the underlying tumor by months or even years. Onset is subacute with escalating difficulties in coordination of the limbs and trunk, disturbed speech and chaotic eye movements. Ultimately the patient may be rendered bedfast. The spinal fluid protein often shows a mild elevation of protein and a modest lymphocytic pleocytosis. Magnetic resonance imaging is usually normal early in the course but after a time diffuse cerebellar atrophy may become apparent. Pathologically there is selective loss of cerebellar Purkinje cells and of the neurons of the deep cerebellar nuclei. Perivascular mononuclear cell cuffing in the cerebellum, brainstem and leptomeninges may or may not be present.

In cases with breast cancer, ovarian cancer or cancer of the female genital tract, antibodies that react with the cytoplasm of Purkinje cells, so-called Yo-specific antibodies, can be demonstrated. Yo-specific antibodies react against two Purkinje cell proteins. The first has a molecular weight of 34 kDa and a remarkable structure with inexact tandem repeats of a six amino acid unit extending over 90% of its sequence. No function for this protein is known. The second protein has a molecular weight of 62 kDa and is a leucine zipper RNA-binding protein. Both proteins are found in the tumors of patients with subacute cerebellar degeneration and in 20% of tumors of the same organs from other patients. Yo-specific antibodies are found in the spinal fluid; they are synthesized locally within the CNS. Cases of PCD on a background of small cell lung cancer frequently have Hu-specific antibodies. Some cases occur in the absence of an underlying tumor.

The role that antibody against Purkinje cell cytoplasm plays in the disease is uncertain. Most patients with Hodgkin's disease and subacute cerebellar degeneration do not have antibodies to neuronal components, yet their clinical picture is indistinguishable from those who do.

Response to glucocorticoids and plasma exchange has been disappointing.

Paraneoplastic encephalomyelitis

A small proportion of patients with small cell cancer of the lung have circulating Hu-specific antibodies that react with the nuclei of all neurons of the human brain, spinal cord and dorsal root ganglia in a diffuse

homogeneous pattern with sparing of the nucleoli. Hu antigens are found in all small cell cancers. Antibodies to Hu antigens are present in the spinal fluid; much of this antibody is synthesized intrathecally.

Patients with Hu-specific antibodies may develop 'encephalomyelitis'. The clinical picture has varied widely. Cases have been described showing: 1) relatively selective involvement of the cerebellum as described above; 2) brainstem encephalitis; 3) anterior horn cell degeneration; 4) necrotizing myelopathy; 5) dorsal root ganglion neuronitis; 6) the Lambert-Eaton syndrome (which clearly is not caused by Hu-specific antibodies); and 7) various combinations of the above. These various entities have already been discussed. In addition, some cases with antibodies against Hu have presented with limbic encephalitis, an entity in which the mesial portions of the temporal lobes are selectively destroyed. Profound memory loss is the predominant clinical feature of limbic encephalitis. Partial complex seizures are frequent as well. It is unclear why individuals with Hu-specific antibodies should develop such varied clinical manifestations. What is clear is that when a paraneoplastic syndrome is suspected, the presence of anti-Hu provides a marker for the presence of an underlying small cell cancer. A finding of Hu-specific antibodies is important since the tumor is likely to be occult and/or resectable.

Stiff man syndrome

This rare disease is characterized by axial and limb rigidity and painful spasms. More cases occur in men than in women. Stiff man syndrome occurs both sporadically and as a remote effect of cancer. Most sporadic cases, but not all, have high titer circulating antibodies to glutamic acid decarboxylase (GAD). The enzyme catalyzes the formation of gamma amino butyric acid (GABA) from glutamic acid. GABA is a major inhibitory neurotransmitter and GABA agonists ameliorate the symptoms of stiff man syndrome. Ten per cent of cases have epilepsy. GABA agonists are antiepileptic. Antibodies to GAD are a marker for insulin-dependent diabetes mellitus though the titers are much lower than in stiff man syndrome. One-third of cases of stiff man syndrome have diabetes. There is a high incidence of autoimmune diseases in sporadic cases. It seems likely that GAD-specific antibodies cause the disease.

Stiff man syndrome also occurs on a background of breast cancer and very rarely other tumors. Breast cancer patients do not have antibodies to GAD. Rather, they have antibodies to amphiphysin, a 128 kDa nonmembrane protein but one that is concentrated at presynaptic vesicles. The topography of amphiphysin largely overlaps that of GAD. Its func-

tion is unknown but a role in neurotransmitter exocytosis seems likely. Response to immunotherapy has been seen in some cases.

Rasmussen's encephalitis

This is a rare disease of childhood. The clinical features are intractable seizures, progressive hemiparesis, and intellectual deterioration. The pathologic features are progressive unilateral cortical atrophy with lymphocytic infiltrates in the involved hemisphere. Several cases have had circulating antibodies to the GluR3 glutamate receptor.

Anti-phospholipid antibodies, strokes, epilepsy and migraine

Antibodies to phospholipid are seen in 25% of systemic lupus erythematosus cases. They also occur sporadically. Phospholipid-specific antibodies bind to and activate platelets. This leads to vascular thromboses which, when they occur in the CNS, cause strokes. Areas of infarction provide a nidus for focal seizures. Platelets are activated during migraine, and platelet activation by phospholipid-specific antibodies is one cause, albeit rare, of migrainous symptomatology.

Opsoclonus-myoclonus

Opsoclonus is characterized by erratic, rapid eye movements. Myoclonus is characterized by jerking involuntary movements of other muscles. Two per cent of children with neuroblastoma develop opsoclonus-myoclonus. The condition also occurs in the absence of tumor, often as a sequelum of an infectious illness.

Opsoclonus also occurs as a remote effect of breast cancer. Such patients have a circulating antibody, designated anti-Ri, that binds to proteins of molecular weights of 55 and 80 kDa. The Ri antigen is found in the nuclei of CNS neurons and in the tumor.

Both childhood and breast cancer-associated cases may wax and wane spontaneously and both respond to glucocorticoid treatment. The cause is unknown, but an antibody-mediated process is plausible.

See also: Autoantigens; Autoimmune diseases; Autoimmunity; Cytokines; Experimental autoimmune encephalomyelitis (EAE); Forssman antigen; Glucocorticoids; Immunosuppression; Interferon γ ; Molecular mimicry; Multiple sclerosis; Neuromuscular junction autoimmunity; Neuroendocrine regulation of immunity; Neurological infections; Perforin; Plasmapheresis; Tumor necrosis factor α ; TNF receptors.

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NEUROLOGICAL INFECTIONS

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Infections of the central nervous system (CNS) occur in two fundamentally different tissue compartments: the leptomeninges (meningitis) and the brain parenchyma (encephalitis). While the blood–brain barrier (BBB) ordinarily deters the invasion of microorganisms into both sites, it also excludes most circulating leukocytes and antibodies, thus making the CNS uniquely susceptible to infection when invasion does occur. Intrinsic cells of the CNS have tightly regulated immunological function, and substances present normally within the brain can have immunomodulatory effects. In general, successful immune responses to neurological infections must overcome these structural and functional restrictions without causing damage to the nonrenewable cells of the CNS.

Unique anatomical features of the CNS

The structures that comprise the BBB prevent the entry of infectious pathogens, inflammatory cells and circulating proteins such as antibodies and cytokines into the CNS. Cerebrovascular endothelial cells maintain tight intercellular junctions and very low rates of vesicular transport that differentiate them from other endothelium. A dense basement membrane ensheaths these cells which is then surrounded by a network of pericytes and astrocytic foot processes that actively maintains the integrity of the BBB (Figure 1). In only a few specific locations within the CNS is the BBB ‘fenestrated’ and easier to cross.

The Virchow–Robin spaces immediately surrounding blood vessels that penetrate the surface of the brain are actually extensions of the subpial space (Figure 2). These spaces contain macrophage-like cells with phagocytic and antigen-presenting capacities beyond those found within the brain parenchyma itself. Foreign antigens and lymphocytes pass through these spaces into the lymphatic channels of the head and neck. As a result, the Virchow–Robin spaces form well-defined sites, distinct from the brain itself, where important immunological reactions can take place. They typically become engorged with inflammatory cells during a variety of neurological infections (Figure 3).

The cerebrospinal fluid (CSF) is produced by cells of the choroid plexus which is contained within the ventricles of the brain. Its protein content, including antibodies and complement, are derived from the

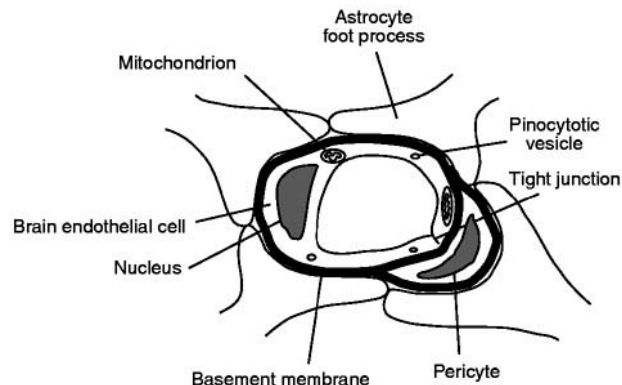


Figure 1 Ultrastructural features of the blood–brain barrier. Within the CNS, capillary endothelial cells contain many fewer pinocytotic vesicles, greatly increased numbers of mitochondria, and have intercellular tight junctions. This is surrounded by a dense basement membrane, pericytes, and astrocytic foot processes. In contrast, general capillaries have intercellular clefts, fenestrae, and prominent pinocytotic vesicles (not shown).

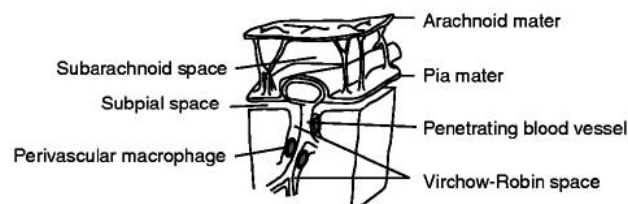


Figure 2 The Virchow–Robin space. This extension of the subpial space contains perivascular macrophages and can serve as a conduit through which foreign antigens and other leukocytes leave the CNS. (Adapted with permission from Esiri and Gay (1990) *Journal of the Neurological Sciences* 100: 3–8.)

blood and are normally present at less than 1% of circulating levels. Conversely, the CSF contains abundant nutrients (glucose, amino acids, etc.) that many infectious pathogens can utilize. Furthermore, recirculation of the CSF throughout the subarachnoid space may disseminate microorganisms throughout the neuraxis. During a variety of infections within the leptomeninges or the brain parenchyma, the CSF contains inflammatory cells, antibodies and immunoregulatory cytokines.

Immune capabilities of intrinsic cells of the CNS

In general, cells of the CNS lack expression of class I and class II major histocompatibility complex

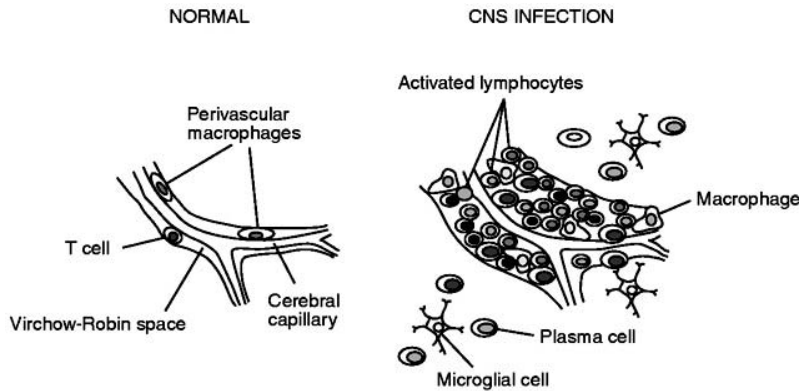


Figure 3 Perivascular inflammation within the Virchow–Robin space. Infection within the CNS recruits many types of circulating leukocytes across the blood–brain barrier into the Virchow–Robin space. These cells can often extend into the brain parenchyma itself; this allows for direct interactions with infectious organisms and infected neural cells. (Adapted with permission from Esiri and Gay (1990) *Journal of the Neurological Sciences* 100: 3–8.)

(MHC) antigens. While glial cells such as astrocytes can be induced to express these antigens *in vitro*, only microglia and perivascular macrophages reliably express MHC antigens during CNS infections *in vivo*. Neither of these cells appear to have the antigen-presenting capacity of dendritic cells that are found in other tissues.

In a murine model of viral encephalitis, many different cytokines are induced in the brain before inflammation develops, suggesting that intrinsic cells of the CNS produce these immunological mediators *in vivo*. Likewise, proinflammatory cytokines can be identified within microglial cells by immunocytochemistry using brain tissue specimens from autopsy cases of both acute and chronic human neurological infections. Despite often being the target of infection, neurons have not been reliably shown to express MHC antigens or release cytokines.

Entry of circulating inflammatory cells into the CNS

At baseline, cerebrovascular endothelial cells express few adhesion molecules and nonactivated lymphocytes and monocyte/macrophages do not enter the CNS with any efficiency. With infection, however, a variety of adhesion molecules including ICAM-1 and VCAM-1 are upregulated on endothelial cell surfaces. Activated lymphocytes and monocytes can then extravasate across the BBB without regard to their antigenic specificity. The entry of T cells in particular is dependent on the expression of LFA-1. Lymphocytes that identify their antigen are selectively retained within the CNS while the remaining cells depart. Although multiple cell types within the CNS can probably present antigen to activated T cells, it is controversial whether any serve as primary activators of naive T cells.

Regulation of lymphocyte function within the CNS during infection

Once T cells have entered the brain, they produce cytokines but there is little evidence that they proliferate locally in response to antigenic stimulation. The pattern of cytokines produced depends on the type of infection and on the genetic background of the host. Nevertheless, data from several systems suggest that skewing of T cell responses towards the production of type 2 cytokines is more likely in the CNS than in other organs. Both the lack of proliferation and the preferential release of interleukin 4 (IL-4) and IL-10 may be due to the effects that brain-derived gangliosides exert on T cells.

Features of the immune response to specific types of pathogens within the CNS

Viruses

Viruses invade the CNS through hematogenous dissemination or by retrograde transport up neurons that have processes extending into sites of infection in the periphery. As intracellular pathogens, their tropism for different neural cells and effect on host cell function determine the severity of disease. The paucity of MHC antigen expression within the CNS can make the cytotoxic T lymphocyte (CTL)-mediated clearance of viruses difficult and may predispose to chronic or relapsing infections. Their capacity to establish latency also allows viruses to avoid detection by the immune system. In other circumstances, antiviral antibodies can shut off virus production by infected neurons in a noncytolytic manner and promote recovery from disease. During chronic CNS infection caused by human immunodeficiency virus (HIV), the long-term production of

inflammatory mediators such as tumor necrosis factor α (TNF α) may promote the development of neurological symptoms.

Bacteria

Bacteria require specific virulence factors to allow invasion of the CNS and production of disease. Organisms that cause acute meningitis are usually encapsulated; this inhibits phagocytosis and activation of complement through the classical pathway. As a result, these bacteria survive in the bloodstream longer and are more likely to invade the CNS. Once in the subarachnoid space, favorable conditions (i.e. limited intrinsic host defense, abundant nutrients) allow for rapid bacterial multiplication. Cytokines, particularly IL-1 and TNF α , are secreted in response to bacterial products and induce endothelial expression of adhesion molecules important for neutrophil egress. The production of other chemotactic stimuli including IL-8 also recruits neutrophils, but low levels of opsonins, antibody and complement in the CSF limit bacterial phagocytosis by these infiltrating cells. These responses combine to produce BBB dysfunction, leakage of protein and cerebral edema. Slow-growing bacteria adapted to survive inside macrophages, such as mycobacteria, induce mononuclear inflammatory responses and cause sub-acute or chronic disease.

Fungi

Fungi are generally avirulent pathogens; most individuals with fungal infections of the CNS have some underlying immune deficiency. The greater frequency of these disorders in patients with impaired cell-mediated rather than humoral immunity implies that the former is the primary means of host defense against fungal pathogens that invade the CNS. In the setting of deficient cellular immunity, fungi can grow unchecked in the CSF without eliciting an inflammatory response. As a result, these infections are difficult to eradicate from the CNS completely and require long-term antifungal therapy. Mortality in general is high.

Parasites

Only a few parasites frequently invade the nervous system. Free-living amoebae that cause acute meningitis enter the CNS by crossing the olfactory mucosa. All other parasites are assumed to enter from the bloodstream. Intracellular pathogens such as *Toxoplasma* may establish latent infections which reactivate in the face of acquired deficiencies of cellular immunity. The cysticercus of *Tania solium* induces an intense inflammatory reaction which may induce

seizures. The organisms are eventually killed, leaving behind calcified nodules. Malaria, on the other hand, induces a toxic encephalopathy associated with ICAM-1-mediated adhesion of parasitized erythrocytes to cerebral capillary endothelial cells and induction of local cytokine production.

See also: Acquired immune deficiency syndrome (AIDS); Acute inflammatory reaction; Adhesion molecules; Antigen-presenting cells; *Bacillus*, infection and immunity; *Borrelia*, infection and immunity; *Bunyavirus*, infection and immunity; Chemotaxis; Coronavirus, infection and immunity; *Cryptococcus*, infection and immunity; Cysticercosis; Cytokines; Cytomegalovirus, infection and immunity; Endothelium; Fungi, immunity to; *Haemophilus*, infection and immunity; Herpes simplex virus, infection and immunity; Herpesvirus-6, infection and immunity; *Histoplasma*, infection and immunity; Human immunodeficiency viruses; Immune surveillance; Transmissible spongiform encephalopathies, infection and immunity; *Listeria*, infection and immunity; Lymphocyte trafficking; Malaria; Monocytes; *Mycobacteria*, infection and immunity; *Neisseria*, infection and immunity; *Nocardia*, infection and immunity; Opportunistic infections; Opsonization; Picornavirus, infection and immunity; Rhabdovirus, infection and immunity; *Rickettsia*, infection and immunity; *Streptococcus*, infection and immunity; Togavirus, infection and immunity; Toxoplasmosis; *Treponema*, infection and immunity; Trypanosomiasis, African; Viruses, infection of immune cells by.

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NEUROMUSCULAR JUNCTION AUTOIMMUNITY

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There are three antibody-mediated disorders of the neuromuscular junction. In myasthenia gravis (MG) antibodies cause loss of the muscle acetylcholine receptor (AChR). It meets in full Witebsky's criteria for antibody-mediated autoimmune disease: 1) the presence of antibodies against a defined antigen (AChR); 2) transfer of the disorder to naive experimental animals by injection of MG immunoglobulins; 3) induction of the disorder in experimental animals by specific immunization against AChR; and 4) transfer of disease from immunized to naive animals by their lymphocytes. The Lambert-Eaton myasthenic syndrome (LEMS) is an antibody-mediated presynaptic disorder where the target is the nerve terminal voltage-gated calcium channel, and in which the first two of the above criteria are met. It is of additional interest as the best example currently available of an autoantibody-mediated paraneoplastic neurological disease. Acquired neuromyotonia (Isaacs' syndrome) has recently been shown to be due to autoantibodies to voltage-gated potassium channels in peripheral motor nerves, leading to their increased excitability and consequent continuous muscle fibre activity. This disorder also meets the first two criteria listed above.

Myasthenia gravis

The dominant clinical feature is fatiguable weakness of skeletal muscle, typically affecting the ocular muscles early in the disease, and bulbar, limb, trunk

and respiratory muscles in severe cases. MG appears to affect all races, the prevalence in the Western world being about 5-9 per 100 000 with an annual incidence of about 4 per million. It may occur from early childhood to extreme old age. Other autoimmune diseases occur at increased frequency, especially thyroid disease, and other autoantibodies are common. About 8% of babies born to myasthenic mothers have a transient neonatal myasthenia due to placental transfer of AChR-specific antibody, and in a small proportion there may be limitation of intrauterine movement with development of arthrogryposis multiplex congenita.

Weakness in MG is due to loss of the number and function of AChRs that are transmembrane proteins (mol. wt 250 kDa) located on the peaks of the postsynaptic folds at the neuromuscular junction, and consist of five subunits (α_2 , β , γ/ϵ , δ) grouped around a central ion channel. The α subunits bind acetylcholine and also a snake toxin, α -bungarotoxin. Loss of AChRs in MG, as shown by reduced [125 I] α -bungarotoxin (BuTx) binding to the neuromuscular junction, results in a decrease in the acetylcholine sensitivity of the postsynaptic membrane. Thus the electrical potentials that result from release of acetylcholine from the motor nerve terminal are reduced, resulting in decreased probability of triggering a muscle action potential and of muscle contraction.

Loss of functional AChRs is due to AChR autoantibodies that are detectable in 85% of patients with generalized MG and in 50-60% of patients

with restricted ocular MG. In the 15% of patients with generalized weakness who are 'seronegative' for anti-AChR there are thought to be antibodies to other determinants at the neuromuscular junction. As in anti-AChR positive MG, the role of antibodies in seronegative MG is clearly demonstrated by the response to plasma exchange and immunosuppressive drug treatment, the occurrence of neonatal MG and the passive transfer of experimental disease to animals.

Antibodies against AChR are measured by a radioimmunoassay using AChRs obtained from human amputated leg muscle (cross-reactivity with other species of AChR is incomplete), labeled with [125 I] α -BuTx. Antibodies (absent in controls) are detected in some patients who are clinically in remission, and very occasionally in those with autoimmune predisposition without signs of MG. In general the titer correlates only weakly with disease severity between individuals. However, within an individual the titer shows an inverse correlation with muscle strength.

Antibodies against AChR are heterogeneous and all subclasses are usually represented. Most antibodies appear to be directed to the conformational epitopes on the extracellular domain of the AChR; some of these are to the 'main immunogenic region' on the α subunits, to which the sequence α 67-76 makes an important contribution. In some patients a proportion of antibodies are directed to the γ (fetal) AChR, but in others antibodies bind preferentially to the ϵ (adult) AChR. There is no substantial idiotypic sharing.

Several antibody-mediated mechanisms contribute to loss of functional AChRs. These include complement-mediated lysis of the postsynaptic membrane, downregulation of AChRs secondary to cross-linking and, less frequently, blockade of AChR function. There may be marked simplification of the postsynaptic folds in severe cases, and immunoglobulin G (IgG) and complement components (C3 and C9) are found both on the membrane itself and on debris in the synaptic cleft.

Clinical, immunological and immunogenetic characteristics allow at least three subgroups of patients to be identified in the AChR antibody-positive Caucasian MG population. The largest group is predominantly female, of young onset (<40 years) with high AChR antibody titers, medullary hyperplasia of the thymus, and a strong association with HLA-B8 (80%) and HLA-DR3 (70%). The older-onset group (>40 years) is more often male with relatively low anti-AChR titers and thymic atrophy, and associated weakly with HLA-B7 and HLA-DR2; it is further distinguished from the young onset

group by a strong association with Ig heavy chain loci, as shown by restriction fragment length polymorphism (RFLP) typing using a probe for the C μ switch region. The third group comprises those with thymoma; mean anti-AChR titers are intermediate and no HLA association has been consistently identified in Caucasians. In Oriental patients, young-onset, low anti-AChR titer cases predominate and the late-onset group is not evident. Different immunogenic associations also occur, notably with HLA-DR9 and Gm1,2,21.

The thymus is implicated in many MG cases. It is a site of specific antibody production, especially in those showing changes of medullary hyperplasia, i.e. lymphoid follicles with germinal centers. Other sites of antibody synthesis include lymph node, peripheral blood and bone marrow. Rates of AChR antibody production by thymic cells in culture correlate with the serum antibody titer; and antibody fine specificities, defined by competition with monoclonal antibodies to the AChR, in culture supernatants and in serum are closely matched. The thymus in MG is also enriched for AChR-reactive T cells, and AChR has been demonstrated *in situ* in thymic myoid cells by staining with monoclonal antibodies against the AChR. Myoid cells are found, in both MG patients and in controls, mainly in the medullary epithelium and septal regions and appear to be HLA class II negative. However, rare nonmyoid cells that are both AChR positive and HLA class II positive can be identified in MG thymus and may be antigen-presenting cells.

In the case of thymoma, few or no antibodies to the AChR are synthesized in culture by the thymoma-derived lymphocytes, but the adjacent thymus may show change of hyperplasia and some anti-AChR production. Immunohistochemical studies indicate that thymomas are epithelial tumors of unusual phenotype. They may arise from rare cells in the cortex that express markers for both cortical epithelium and subcapsular/medullary epithelium. They do not appear to express AChR, but nevertheless contain a 155 kDa protein that cross-reacts with a cytoplasmic AChR epitope. The role of this epitope and other related neuronal or muscle antigens in inducing MG in thymoma cases is unclear.

Class II-restricted T cell epitopes have been identified in MG patients (and also in controls) using *Torpedo* (electric fish) AChR α subunit, mouse α subunit fusion proteins, and synthetic peptides or recombinant polypeptides of the human AChR. The epitopes and restricting class II haplotypes appear to be diverse. Some T cells recognize synthetic peptide epitopes on the extracellular domain of the AChR sub-

units, but their functional significance is not yet clear.

Anticholinesterase medication provides some symptomatic improvement. Thymectomy is usually recommended for those with thymoma and in young-onset patients with generalized symptoms. In those with restricted ocular disease, low-dose alternate-day prednisolone treatment is often effective. Late-onset generalized cases and those who fail to respond to thymectomy often respond to treatment with prednisolone and/or azathioprine. Combined treatment is more effective and better tolerated than prednisolone alone. Severe disease can be temporarily controlled by plasmapheresis or intravenous immunoglobulin.

Experimental autoimmune myasthenia gravis

The discovery by Patrick and Lindstrom that rabbits immunized with AChR, purified from the electric organ of electric eel *Electrophorus*, developed a myasthenia-like illness (experimental autoimmune myasthenia gravis, EAMG) provided an animal model for the human disease. The clinical features of EAMG bear a striking resemblance to MG, especially when induced in primates, and include fatigue, flaccid limb paralysis, drooping of eyelids, paralysis of eye movements and respiratory muscle weakness.

EAMG is classically induced by repeated immunization with xenogeneic or syngeneic native AChR in Freund's complete adjuvant. It can also be induced without adjuvant, however, or by immunizing with particular synthetic peptides of the α subunit. Additionally, it can be passively transferred to recipients by immunoglobulins or purified IgG from immunized animals, or by experimentally raised AChR-specific monoclonal antibodies.

As in the human disease, AChRs are decreased in number and miniature end-plate potentials are small. In mice immunized with AChR, Freund's adjuvant and additional *Pertussis*, the end-plates show a prominent mononuclear cell infiltration typically 7–10 days after a single immunization. If the animal survives, this acute phase, which has no known counterpart in MG, is followed by a chronic phase in which there is simplification of the postsynaptic folds and loss of AChR. Complement-depleted (cobra venom-treated) rats are resistant to EAMG induced either by active immunization or passive transfer. Modulation of AChR numbers by antibody cross-linking also contributes to AChR loss, but blocking antibodies are not prominent.

Murine susceptibility to EAMG is linked to particular H-2 haplotypes that control the T cell response to the AChR, and to particular Ig heavy chain genes. However, the correlation between AChR antibody levels and clinical disease in mice is poor, and other

strain-specific factors may play a part. In rabbits there is a better correlation between AChR antibody titers, clinical weakness and electrophysiological abnormalities. Antibodies are of the IgG class, and many are directed against the main immunogenic region on the immunizing AChR and cross-react with autologous AChR (see above).

Antibodies to the AChR are T cell dependent. The T cell *in vitro* proliferation response to AChR correlates with the level of anti-AChR, and also with disease severity as judged by the muscle AChR content. A number of T cell epitopes on the α subunit have been identified in the Lewis rat, and CD4⁺ AChR-specific T cell clones can help anti-AChR production by AChR primed B cells.

Several different preimmunization treatment regimens have been shown to interfere with the development of EAMG, including corticosteroids, azathioprine, cyclosporine and oral treatment with AChR (oral tolerance). High-dose cyclophosphamide with or without irradiation followed by bone marrow transplantation diminishes the autoimmune response in established EAMG, and cyclosporine partially inhibits the response to secondary immunization. Anti-idiotypic antibodies can alter the induction of EAMG passively induced by monoclonal antibodies to the AChR, but have variable effects on the actively immunized disease. Anti-I-A monoclonal antibody therapy can be protective.

Lambert-Eaton myasthenic syndrome

This disorder is much less common than myasthenia gravis. It is characterized by proximal muscle weakness, depressed tendon reflexes, post-tetanic potentiation and autonomic changes, including dry mouth and constipation. Onset can be in adolescence, but is usually after 40 years of age. Other autoimmune diseases may associate, notably thyroid disease and vitiligo, and other autoantibodies occur at increased frequency.

About 60% of cases have associated small cell lung cancer (SCLC). The neurological disorder typically precedes radiological evidence of the tumor by up to 2 years, and exceptionally 4–5 years. Many of the remainder have been followed for 10 years or more, making an underlying tumor very unlikely.

The physiological abnormality is presynaptic, in contrast to myasthenia gravis. Quantal release of acetylcholine by the nerve impulse depends on the opening of voltage-gated Ca^{2+} channels (VGCCs) and the consequent local influx of Ca^{2+} into the nerve terminal; in LEMS, the calcium-dependent release of transmitter is greatly reduced, and freeze-fracture electron microscopy shows loss of active

zone particles that are thought to represent VGCCs. Postsynaptic AChRs are normal.

The disorder is caused by IgG autoantibodies that bind to VGCCs (or to structures closely associated with them). There are at least four subtypes of neuronal VGCCs (T-, L-, N-, P/Q-). Antibodies to P/Q-type VGCCs dominate the immune response, and can be detected by radioimmunoprecipitation assay using a cone snail neurotoxin ($[^{125}\text{I}]\omega$ -conotoxin MVIIC) to label the VGCCs. Serum titers broadly correlate with disease severity in individual patients. LEMS IgG injected into mice transfers the physiological changes, i.e. reduced quantal release of acetylcholine and morphological changes such as clustering and reduction in the number of active zone particles. These effects appear to be dependent on antibody divalency, as Fab fragments are without effect, suggesting that loss of VGCCs may be brought about by cross-linking. Recent passive transfer studies to mice have shown that the autonomic changes may similarly be mediated by autoantibodies to P/Q-type and occasionally N-type VGCCs.

HLA-B8 and (less strongly) HLA-DR3 and Ig heavy chain markers associate with LEMS, both in its SCLC-associated and noncancer forms. SCLC patients without LEMS do not show these associations, implying that susceptibility to LEMS in SCLC cases is partly conferred by immune response genes.

SCLC is thought to be of neural crest origin. SCLC cell lines express VGCCs, as shown by depolarization-induced $^{45}\text{Ca}^{2+}$ influx. LEMS IgG, both from SCLC and non-SCLC cases, causes a highly significant reduction of K^{+} -stimulated $^{45}\text{Ca}^{2+}$ flux into these cells compared with control IgG, and inhibition of flux correlates with an electromyographic measure of disease severity. These results suggest that the antibodies against VGCCs in SCLC cases are triggered by tumor VGCCs, and that the cross-reactivity with nerve terminal VGCCs leads to the neurological disorder. The immune response in these patients may be generated within the tumors as macrophage infiltration in LEMS-associated SCLC is greatly increased compared with non-LEMS cases.

Thus, in its cancer-associated form, LEMS provides the first clear evidence that a paraneoplastic neurological syndrome can be caused by an antibody-mediated autoimmune process. Consistent with this, treatment of the neoplasm by surgery, chemotherapy or deep X-ray is usually followed by improvement or remission of the neurological syndrome in those who survive the effects of the tumor. Many of the noncancer cases respond to immunosuppressive treatment (prednisolone and azathioprine), and short-term improvement may follow plasmapheresis or intravenous immunoglobulin.

Acquired neuromyotonia (Isaacs' syndrome)

Acquired neuromyotonia is a rare disorder characterized by widespread muscle twitching (myokymia) and cramps, sometimes associated with muscle hypertrophy, stiffness, weakness, increased sweating and central effects. Onset can be from adolescence to old age. There is an association with thymoma, with myasthenia gravis, and probably also with lung cancer.

The physiological abnormality is an increased excitability of peripheral motor nerves that can discharge spontaneously or fail to shut off after voluntary activation. The abnormal activity is principally generated at motor nerve terminals (i.e. it continues distal to local anesthetic block), and it continues during sleep and general anesthesia. The abnormality is consistent with a decrease in the number of functional voltage-gated potassium channels (VGKCs) that normally control nerve excitability.

The disorder appears to be due to IgG antibodies against VGKCs. The neuromyotonic discharges decrease following plasma exchange, and patients' IgG injected into mice produces changes consistent with the downregulation of VGKCs. Antibodies to VGKCs can be detected by immunoprecipitation assays using $[^{125}\text{I}]\alpha$ -dendrotoxin that labels some (but not all) VGKCs, and in a majority of patients by a molecular immunohistochemical assay in which the relevant human VGKC is expressed in *Xenopus* oocytes.

Neuromyotonia can be controlled in some patients by carbamazepine or phenytoin (which both reduce nerve excitability), and in others by immunosuppressive drugs, as used in MG.

See also: Autoantigens; Autoimmune diseases; Autoimmunity; Idiotypic network; Immunosuppression; Neurological autoimmune diseases; Plasmapheresis; Thymus.

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NEUTRALIZATION OF BIOLOGICAL REACTIONS BY ANTIBODIES

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The immunological manipulation of endocrine and allied reproductive regulatory systems provides us with an example of how both our basic knowledge and its applied dimensions have progressed through the interdisciplinary catalysis of immunology and reproductive biology. Over the first 70 years of the twentieth century, the complex feedback interactions between gonadotropins and steroids were described, resulting, for example, in new approaches to infertility (gonadotropin releasing hormone, GnRH), fertility control (the contraceptive pill) and the management of domesticated farm stock (progesterone sponges, prostaglandins). By the early 1970s, pure preparations of most of the generic hormones were available and their mechanisms of action had been tested through immunological neutralization. The discovery by Canfield and Pierce in 1971 of the sub-unit structures of the gonadotropins explained why previous attempts to immunize against the entire hormones – luteinizing hormone (LH), follicle-stimulating hormone (FSH), chorionic gonadotropin (CG) – resulted in cross-reactions between these and thyroid-stimulating hormone. It also allowed progression from crude immunizations to the development of specific epitope, anti-idiotypic and sequenced subfraction antibodies, now progressing to the sequencing of the key genes and prohormones. The discovery that potential ‘vaccines’ against self antigens could be achieved, through conjugation with nonself antigens, such as tetanus toxoid, opened new avenues for the development of immunological

fertility regulation. In addition, a range of new immunological probes to study gamete maturation, receptor function, sperm and egg surface antigens, embryonic cell signal transduction, trophoblast and placental antigens, and the embryo–maternal dialog at implantation have become available, together with some advances in adjuvant technology that reduce the trauma of immunization with Freund’s adjuvant. Species differences in the sequences and antigenicity of gonadotropins, or the differing emphasis in biological actions between related groups of progestagens, androgens or estrogens in various species, still hinders the easy extrapolation of results between species. However, similarities between the endocrine and immunological characteristics of the fetal allograft with those of certain carcinomas has allowed some shared approaches to diagnosis and monitoring, for example with CG in pregnancy, choriocarcinoma or teratocarcinoma.

Rationale

An immunological approach to reproductive physiology, asking fundamental questions, is paying off at the basic, strategic, and applied levels. Perturbation of the closely interrelated feedback systems, shown in **Figure 1** and **Table 1**, is giving us a better understanding of the efficacy, specificity, and practicality of intervention, together with the priorities, benefits, and risks of the sites now available. In theory, active or passive neutralization has many advantages, as a

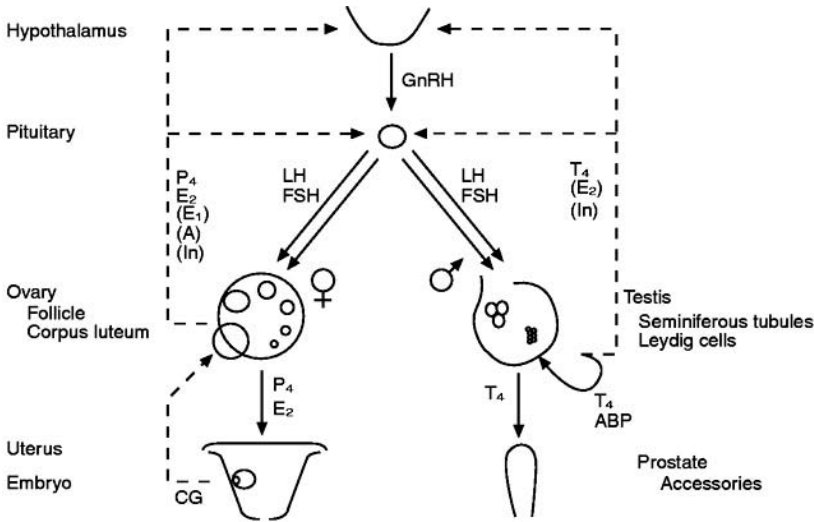


Figure 1 Reproductive endocrine feed-back in primates. GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; P₄, progesterone; E₂, estradiol 17-β; E₁, estrone; T₄, testosterone; In, inhibin; A, androstenedione; ABP, androgen-binding protein.

Table 1 Neutralization effects with antibodies

	Hormone neutralized	Effect
<i>Female</i>		
Hypothalamus	GnRH	Reproductive shutdown
Pituitary	LH/FSH	Prevents ovulation, disrupts feedback
Ovary	Progesterone/estradiol 17-β	Broad effects on LH increase
	Estradiol	FSH increase and behavior
	Inhibin	FSH increase
	Estrone/androstenedione	Raises ovulation rate
Ovum	Zona pellucida	Blocks fertilization
Embryo	Platelet activating factor	Inhibit fertility; unconfirmed
	Early pregnancy factor	
	Chorionic gonadotropin	Prevents implantation
<i>Male</i>		
Sperm	LDH-CD4 isoenzyme	Reduces fertility
	Sperm surface antigens	Reduce fertility

controlled response can be short or long term, potentially reversible, relatively noninvasive, and well targeted either to a system or to a specific site. In practice, caveats remain concerning species specificity, induced autoimmunity or its consequences when continually secreted antigens are inhibited, or unpredictable side-effects through the neutralization of as yet poorly understood genes, proteins, enzymes or steroids that may have multiple roles or feedback links.

Consequently, neutralization with antibodies may be utilized to: 1) advance understanding of the mechanisms of action of factors affecting reproductive processes; 2) suppress fertility in the management of human and animal reproduction; 3) enhance fertility

in certain cases; 4) understand the etiology of disorders such as precocious puberty, polycystic ovarian disease and premature menopause; or 5) develop generic methodologies such as those of radioimmunoassay, ELISA and other immunoassays that have transformed the field.

Experimental manipulation

The endocrine and developmental processes regulating reproduction at the molecular, cellular and systemic levels are open to immunological perturbation by neutralization of hormones, enzymes or cell surface antigens. From the mid-1970s, Fraser, Jeffcoate and others immunized rodents, sheep and

primates against GnRH, producing a shutdown of the reproductive system through a deprivation of gonadotropins. This physiological castration, while with some potential for management of domesticated farm stock, pets or pest species, has little general applicability to the human. Similarly immunization against LH or FSH produced general suppression of the reproductive endocrine system, although Moudgal reported more specific effects with heterologous FSH preparations and Baird, DeKretser, Knight and others pursued the inhibin-FSH relationship. The enigmatic role of prolactin in seasonal breeding and in lactation was studied by McNeilly, while the relationships of oxytocin from pituitary and ovarian sources were explored in parturition and in the support of the corpus luteum.

The potential of chorionic gonadotropins, as a specific signal in early primate pregnancy, to yield a long-term, reversible contraceptive for the human is being investigated by several groups. Active or passive immunization against the whole β subunit, or the C-terminal specific peptide which differs from LH, conjugated with heterologous ovine LH α subunit in one case, was shown by Hearn, Stevens, Talwar, Thau and their groups to be effective in suppressing primate pregnancy and is now being tested in phase II clinical trials on humans by Jones, Stevens, Talwar and others. The Talwar heterospecies dimer comprising human CG β subunit and ovine LH α subunit has successfully completed phase II efficacy trials. Canfield and colleagues, Nial and Tregear and others are exposing the structural-physiological function relationships of this hormone, together with its effects on embryo-maternal signaling at the start of pregnancy. Allen has profitably explored the interspecies regulation of pregnancy in equids by equine CG, using embryo transfer techniques.

Neutralization of reproductive steroids has also provided a great deal of information on sites of action, feedback mechanisms, endocrine regulation of puberty, and of sexual behavior, but the widespread effects of steroids throughout the body precludes any permanent, nontherapeutic intervention in humans. An interesting agricultural dimension is Scaramuzzi's work in increasing the ovulation rate and productivity in sheep or cattle by immunizing against androstenedione, testosterone and estrone, while the role of prostaglandins in corpus luteum function and in early pregnancy is being studied in many laboratories. In addition, Heap, Wang and others are examining the requirements for progesterone in early pregnancy using monoclonals, idiotypes and anti-idiotypes to probe embryo and endometrial interactions.

Looking to more local interactions, cell surface antigens have been studied as markers to sperm maturation and as possible targets for immunological neutralization. Alexander, Anderson, Moore, Voisin and others are testing a range of potential antigens. The vast numbers of sperm produced make an immunocontraceptive approach questionable, but the significance of naturally circulating sperm antibodies, or those produced as a consequence of vasectomy, is being clarified. The LDH-C₄ sperm-specific isoenzyme, now characterized, partially sequenced, and shown by Goldberg to be immunogenic and to suppress fertility in a variety of species, may have practical applications in immunocontraception. Potentially more specific targets are zona pellucida antigens and perhaps sperm receptor molecules that might inhibit fertilization. Aitken, Dunbar and others have identified a range of potential candidates.

A great deal of effort is being expended in defining the roles of the multiplicity of trophoblast antigens, where Beer, Billingham, Johnson and others are making progress in this difficult field, while Redman is exploring the importance of fetal alloantigenic signaling in the human and its relationship with recurrent abortion or pre-eclampsia. There appears to be a greater incidence of such disorders in partners with similar HLA allotypes.

Although only a few examples are provided here, it is clear that the interface of immunology and reproductive biology is advancing our fundamental and strategic knowledge in virtually all of the areas open to study. The greater availability of carefully cloned genes, specific immunological probes, defined reagents for immunocytochemistry, polymerase chain reaction (PCR) and *in situ* hybridization should allow us to define, far more precisely, the ontogeny of expression, mechanisms of action and complex interrelationships of reproductive events in the future.

See also: Contraception, immunological; Embryonic antigens; Fetus as allograft; Immunoassays; Infertility, immunological causes of; Tumor antigens.

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NEUTROPHILS

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Neutrophils, the major type of polymorphonuclear leukocytes, are mobile cells that can ingest and kill invading microorganisms. For this purpose, neutrophils use both newly generated, highly reactive oxygen compounds, and microbicidal or microbistatic proteins stored in their granule compartments. The biological significance of this line of defense is well illustrated by the overwhelming infections in patients with severe neutropenia or neutrophil dysfunctions. However, neutrophils may also cause serious tissue injury, for instance during persistent infections or chronic inflammation. Therapeutic intervention is then indicated.

Neutrophils are often called 'granulocytes'. Strictly speaking, however, 'granulocytes' is a collective noun for the polymorphonuclear leukocytes, which include neutrophilic granulocytes (neutrophils), eosinophilic granulocytes (eosinophils) and basophilic granulocytes (basophils).

Neutrophil maturation and morphology

Neutrophils mature in the bone marrow in about 2 weeks. In the first half of this period, the neutrophil precursor cells undergo six divisions and differentiate from myeloblasts through promyelocytes to neutrophil myelocytes (**Figure 1**). Myeloblasts are cells with a large nucleus relative to the surrounding cytoplasm, which contains no or few granules. In the promyelocyte stage, the azurophil granules (black dots in **Figure 1**) are formed, whereas the specific granules (open organelles in **Figure 1**) are formed in the myelocytic stage. Because cell divisions still occur after azurophil granule formation has stopped, these granules are distributed over the daughter cells and are complemented with specific granules actively formed during this stage. The final ratio of azurophil to specific granules in human neutrophils is about 1:2. Later stages of neutrophil differentiation comprise metamyelocytes, band forms and segmented cells. As the names indicate, these stages are characterized by

the typical appearance of the neutrophil nucleus. Divisions do not take place during this period.

About 60% of all nucleated cells in the bone marrow belong to the myeloid series. Indeed, the bone marrow comprises a reserve pool of mature neutrophils that contains about 20 times the number of neutrophils in the circulation. Under normal conditions these cells are released into the blood 2 days after (apparent) completion of their maturation. During infections these cells are released sooner, together with band forms (up to 10^{12} per day in adults instead of the usual 10^{11} per day). The appearance of immature neutrophils in the blood is reflected by a decreased chemotactic responsiveness of these cells. In the circulation about half of the neutrophils are in a margined pool of cells sequestered in postcapillary venules. This pool is released when epinephrine is generated by exercise or injected intravenously. Neutrophils circulate for only 6–10 h; thereafter, they move to the tissues, where they remain active for 2–6 days. The site of neutrophil apoptosis and destruction by tissue macrophages is not known.

Neutrophil movement

Egress of neutrophils from the bone marrow to the circulation, and diapedesis from the circulation to the tissues, involves squeezing of the neutrophils through the blood vessel wall, in between the endothelial cells and through the basement membrane. For this purpose, neutrophils create gaps between endothelial cells of about $1\ \mu\text{m}$, without disturbing the endothelial cell layer. Thanks to its lobulated nucleus the neutrophil is very flexible, which greatly facilitates its passage through these pores (a rounded neutrophil has a diameter of $12\text{--}15\ \mu\text{m}$). It is not known which humoral factors cause egress from the bone marrow in healthy individuals.

Adherence of neutrophils to endothelial cells in postcapillary venules is facilitated by passive move-

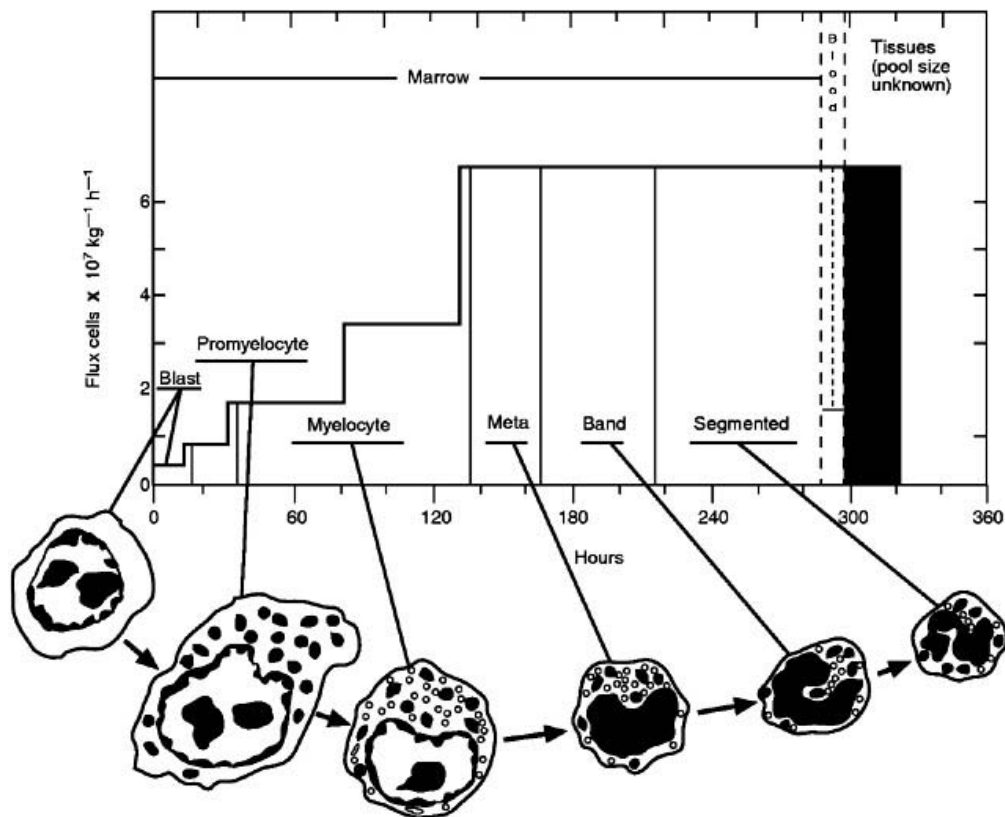


Figure 1 Neutrophil life span and stages of maturation. The ordinate shows the flux through each compartment and the abscissa shows the time in each compartment. The area of each of the compartments gives the number of cells in each compartment. The stepwise increase in cell numbers through the first three compartments represents serial divisions of the cells in these compartments. Note that no divisions occur after the myelocyte stage. (Reproduced with permission from Bainton DF (1980) The cells of inflammation: a general view. In Weissmann G (ed) *The Cell Biology of Inflammation*, vol 2, pp 1–25. Amsterdam: Elsevier/North-Holland.)

ment of the neutrophils to the vessel wall, which causes reversible interaction with selectin molecules on the endothelial cells, influenced by the fluid flow (neutrophil rolling). This is followed by a more stable interaction with accessory molecules, such as ICAM-1 (neutrophil spreading), which leads to diapedesis and movement into the tissues. During inflammation these processes are strongly increased as a result of the formation of chemotactic agents in the inflamed area, such as the complement fragment C5a, the tripeptide formyl-Met-Leu-Phe (fMLP), interleukin 8 (IL-8) and leukotriene B₄. These agents increase the surface expression of certain adhesion molecules on the neutrophils, such as the CD18 integrin β_2 family, and also activate these molecules for enhanced binding to endothelial cell structures, probably by changing their configuration. Moreover, these chemotactic agents also induce the neutrophils to move to areas of higher concentration of these agents. This is the process of chemotaxis, which directs the cells toward the infected or inflamed area. On the other hand, endotoxin, released by microorganisms, induces macrophages to generate IL-1

and tumor necrosis factor (TNF). These agents, including endotoxin, affect the endothelial cells by upregulating the adhesion proteins ICAM-1, VCAM-1 and E-selectin, thus promoting neutrophil adherence. Finally, endothelial cells also generate platelet-activating factor and IL-8 under these conditions, which promotes neutrophil migration.

The biological significance of adherence and migration is clearly demonstrated by the clinical symptoms of patients with leukocyte adhesion deficiency, i.e. severe, recurrent bacterial infections, impaired wound healing, persistent leukocytosis and severe deficiency in mounting an inflammatory response. Neutrophils from these patients lack all three CD18 adherence proteins, are strongly depressed in their ability to adhere to endothelial cells or other neutrophils (aggregation) and are chemotactically inactive.

Phagocytosis and bacterial killing

Neutrophils are specialized in ingestion and intracellular killing of a large array of different bacteria,

yeasts, fungi, mycoplasmas, and perhaps also viruses. Most microorganisms can only be phagocytosed after opsonization with specific antibodies and/or complement fragments. The neutrophil carries specific receptors on its surface for immunoglobulin G (IgG) (Fc γ receptors, Fc γ R) and for the complement components C3b (complement receptor type 1, CR1) and iC3b (CR3). Within certain size limitations, any material covered by these opsonins will be ingested – not only microorganisms but also simple immune complexes containing only one particular antigen.

Binding of opsonized material to neutrophil surface receptors leads to concentration of such receptors around the area of contact. Subsequently, the cell extends pseudopods that engulf the opsonized particle (Figure 2). By consecutive receptor binding, these pseudopods fit tightly around the particle and finally fuse with each other to form a closed membrane vesicle (phagosome) around the particle, within the neutrophil. Neutrophils may 'overeat' themselves in infected areas, i.e. they rupture. Macroscopically, this is manifested as pus formation.

Receptor binding also starts two other processes, i.e. the generation of reactive oxygen compounds and the release of granule contents (degranulation). Both reactions are localized events in that they are restricted to the release of microbicidal products into the phagosome (Figure 2); however, the secretion of these products begins before the phagosome is closed, and some of the oxygen compounds and granule enzymes may thus escape into the extracellular environment of the neutrophils. Moreover, neutrophils adhering to opsonized material that is too large to be ingested (e.g. immune complexes deposited along basement membranes) may secrete these products in large quantities into the extracellular space.

Generation of reactive oxygen products is initiated

by the activation of NADPH oxidase. This enzyme is located in the plasma membrane of neutrophils (and eosinophils, monocytes and macrophages), and thus also in the phagosomal membrane (Figure 2). It accepts electrons from NADPH on the cytosolic side of the membrane and transmits these electrons to oxygen on the other side of the membrane (in the phagosome or in the extracellular space). This reaction yields superoxide ($O_2^{\cdot -}$), a univalently reduced oxygen radical. NADPH oxidase is a complicated enzyme. It consists of at least five different proteins, two of which are incorporated in the plasma membrane and three that are, in resting cells, located in the cytosol. In this configuration the enzyme is not active. Upon neutrophil activation (e.g. by binding of opsonized microorganisms to cell surface receptors), the three cytosolic NADPH oxidase components are translocated from the cytosol to the plasma membrane and then form an active enzyme complex with the two membrane components of the oxidase. In this configuration the enzyme generates superoxide.

Superoxide spontaneously dismutates into hydrogen peroxide (H_2O_2), which may then react with chloride ions to form hypochlorous acid (HOCl), in a reaction catalyzed by myeloperoxidase. This enzyme is an abundant constituent of neutrophil azurophilic granules, and is released upon cell activation into the phagosome and into the extracellular space. HOCl is very toxic for a broad range of microorganisms but is rather short lived. However, it can react with primary and secondary amines, and thus give rise to *N*-chloramines, some of which are very stable, microbicidal agents. Under normal, phagocytosing conditions, neutrophils convert more than 75% of their superoxide into hypochlorous acid and *N*-chloramines, and thus create a highly toxic environment within the phagosomes and in the cell surroundings.

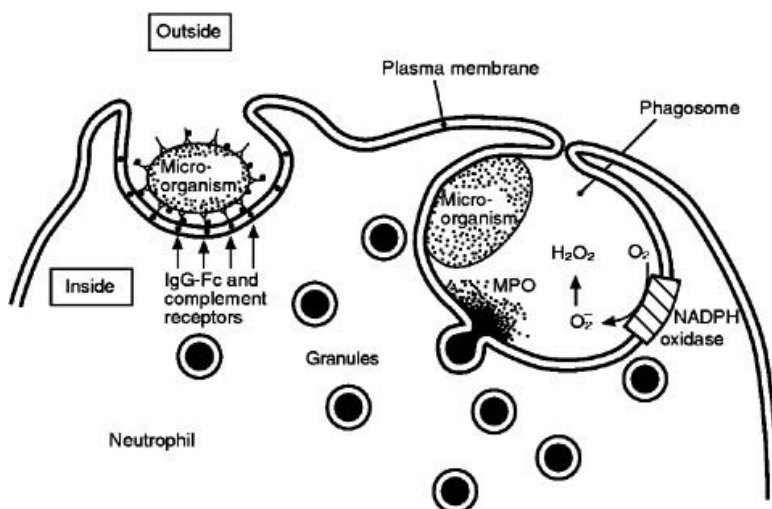


Figure 2 Neutrophil activation. Contact of opsonized microorganisms with neutrophil surface receptors (IgG-Fc and complement receptors) starts phagocytosis, NADPH oxidase activation and degranulation. Within the phagosome, oxidative products may interact with granule proteins for effective killing of ingested microorganisms. MPO, myeloperoxidase. (Reproduced with permission from Roos D (1991) The respiratory burst of phagocytic leucocytes. *Drug Investigations* 3: 48–53.)

Speculation as to the formation by neutrophils of oxygen radicals other than superoxide lack experimental evidence. For the formation of hydroxyl radical ($\text{OH}^\bullet$), iron is needed as a catalyst, and this is not available in the proper configuration within neutrophils. In fact neutrophils prevent $\text{OH}^\bullet$ formation by binding iron to lactoferrin, a protein stored in the specific granules and released upon activation. Only within target cells (e.g. within ingested bacteria) may iron be available in a form that catalyzes $\text{OH}^\bullet$ formation, and this may help in the destruction of these targets. Also, singlet oxygen, an energetically activated type of oxygen, is not generated by neutrophils.

Degranulation does not occur in resting neutrophils. During phagocytosis or adherence of neutrophils to large substrates, changes in the cytosolic free Ca^{2+} concentration, in addition to other signals, induce the fusion of granules with the plasma membrane. Neutrophils contain at least two different types of granule (Table 1). The azurophil granules resemble the lysosomes in other cell types in that they contain acid hydrolases, with a low pH optimum. Moreover, these granules also contain the aforementioned myeloperoxidase, as well as a number of serine proteinases. Further, the azurophil granules also contain large amounts of defensins, small peptides with a broad range of bactericidal activity, and bactericidal permeability-increasing protein (BPI), a very potent antibiotic against gram-negative bacteria. Lysozyme, an enzyme that hydrolyzes certain peptidoglycans of gram-positive bacteria, is present in

the azurophil as well as in the specific granules of neutrophils. Other proteins found in the specific granules are lactoferrin, an iron-binding and therefore bacteriostatic protein, and vitamin B_{12} -binding protein. The metalloproteinases collagenase and gelatinase are also present in the specific granules. Finally, neutrophils also contain so-called secretory vesicles, which actively exchange their membrane-bound receptors and enzymes with the plasma membrane. The distinction between the different types of granule is sometimes difficult. Myeloperoxidase and lactoferrin are considered good markers for azurophil and specific granules, respectively. However, granules without either marker do exist, possibly due to 'immaturity' of these granules.

The release of oxygen products and granule contents is not a simple all-or-nothing phenomenon. Both reactions can be potentiated by so-called priming agents, which by themselves do not induce secretion but only enhance the reactions initiated by activating agents. Physiological priming agents include TNF, platelet-activating factor and endotoxin. Thus, the same agents that cause upregulation and activation of surface receptors, and chemotaxis, also prime the neutrophils for enhanced microbicidal activity. However, neutrophils adhering to a surface, i.e. with ligands bound to their β_2 integrins, degranulate in response to these otherwise priming agents.

The biological significance of the neutrophil's microbicidal activity is illustrated by patients lacking either NADPH oxidase activity or proper granule contents. Patients with chronic granulomatous disease (CGD), unable to generate superoxide, suffer early in life from serious infections of the lungs, skin, soft tissues, lymph nodes, bones and liver with catalase-positive bacteria (catalase-negative bacteria secrete some hydrogen peroxide, which aids in killing these organisms in the phagosomes of CGD neutrophils). Patients with specific-granule deficiency (a rare disorder) suffer from recurrent infections with bacteria, without increased susceptibility to a particular pathogen. Patients with Chédiak-Higashi syndrome are characterized by partial oculocutaneous albinism, neutropenia and recurrent pyogenic infections. The neutrophils (and many other cells) of these patients contain giant granules composed (in the neutrophils) of aggregated azurophil granules. As a result, these neutrophils are less mobile and do not release azurophil granule contents upon phagocytosis.

Involvement in inflammatory reactions

Neutrophils play an important role in inflammatory reactions, both in the removal of microorganisms

Table 1 Contents of neutrophil granules

<i>Azurophil granules</i>	<i>Specific granules</i>
<i>Serine proteinases</i>	
Elastase	
Cathepsin G	
Proteinase 3	
p29b (= AGP7)	
<i>Acid hydrolases</i>	
β -Glucuronidase	
β -Glycerophosphatase	
N-acetyl- β -glucosaminidase	
α -Mannosidase	
Cathepsin B	
Cathepsin D	
<i>Metalloproteinases</i>	
	Collagenase
<i>Other proteins</i>	
Myeloperoxidase	
Lysozyme	Lysozyme
Azurocidin	Histaminase
Defensins	Lactoferrin
Bactericidal permeability-increasing protein (BPI)	Vitamin B_{12} -binding protein

and aged and damaged cells, as well as in the generation of inflammatory agents (chemotactic peptides, cytokines, leukotrienes and other lipid mediators). Moreover, neutrophils also mediate tissue injury. This last reaction is a necessary evil, meant to remove alien material from the tissues. Under normal conditions, when the causative agent has been removed and chemotactic factors are no longer produced, neutrophil (and macrophage) invasion stops and tissue repair commences; however, when neutrophil infiltration does not stop, as in chronically inflamed areas, the tissues may be permanently damaged.

Plasma and tissue fluids contain large amounts of a series of proteinase inhibitors, such as serine proteinase inhibitors (serpins, e.g. α_1 -proteinase inhibitor, α_1 -antichymotrypsin, plasminogen-activator inhibitor) and α_2 -macroglobulin. These inhibitors are very efficient in blocking serine proteinase activity. However, during neutrophil activation, reactive oxygen compounds are released and inactivate these inhibitors. Additionally, elastase, if not blocked by α_1 -proteinase inhibitor, is able to inactivate many of these tissue-protecting inhibitors. Moreover, the metalloproteinases collagenase and gelatinase, which are secreted by neutrophils as inactive precursors, are activated by the reactive oxygen compounds from these cells.

Thus, the following situation exists. Oxidative products of neutrophils are either short lived (HOCl) or – even more importantly – they act very non-specifically by oxidizing methionine residues in all proteins; therefore, these oxidative agents will quickly be scavenged by albumin or other abundant proteins surrounding the neutrophil. On the other hand, the proteinases released by neutrophils are also harmless because they are either secreted as inactive precursors (metalloproteinases) or will be quickly inactivated by serpins in the extracellular fluid. In combination, however, the neutrophil products act synergistically and become highly toxic for the surrounding tissues.

Well-known noninfectious conditions accompanied by neutrophil infiltrations are gout, rheumatoid arthritis, autoimmune vasculitis and some forms of glomerulonephritis. Tissue injury in these inflammatory conditions is at least in part due to products from neutrophils. Neutrophils are also involved in some inflammatory reactions in the lung. Adult respiratory distress syndrome (ARDS) may develop in combination with, or as a consequence of,

pulmonary aspiration, diffuse intravascular coagulation, severe pneumonia, hypertransfusion or sepsis. Inflammatory mediators generated under these conditions may cause intravascular aggregation of neutrophils, leading to obstruction of pulmonary capillaries and localized release of toxic mediators from these cells. The effects of TNF on lung cells may also play a role in ARDS and endotoxin shock. Lung emphysema is supposed to be caused at least in part by elastase released from neutrophils. Also, in the second phase of acute allergic asthma, infiltrating neutrophils (and eosinophils) probably contribute to the tissue damage.

Thus, although neutrophils form an indispensable element in our host-defense system, conditions exist in which the powerful mediators from these cells are harmful to the host. Therapeutic amelioration of these reactions is then necessary, but care must be taken to preserve the antimicrobial potential of the neutrophils.

See also: Acute inflammatory reaction; Chemotaxis; Chemotaxis of neutrophils; Complement receptors; Diapedesis; Fc receptors; Immunodeficiency, primary; Microbicidal mechanisms, oxygen-dependent; Microbicidal mechanisms, oxygen-independent; Platelet-activating factor (PAF).

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NITRIC OXIDE

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Since the initial demonstration in 1987 that nitric oxide (NO) is produced by mammalian cells and regulates blood flow, several reports have described a number of diverse functions that can be traced to the production of this gaseous molecule. The production of NO is now known to play an integral part in the maintenance of several physiological functions, being involved in the control of cardiovascular and nervous systems as well as in the regulation of the immune system.

The induction of NO production is observed under conditions of immune activation where it can be both beneficial, in the eradication of microorganisms and tumor cells, and harmful, in the generation of a number of pathologic conditions. In both cases, upregulation of NO is implicated in the terminal stage of the inflammatory process: killing of the invading organism or sensitive host tissues. The induction of NO by the immune system during this process has been extensively studied and has allowed insight into the microbicidal mechanisms used by the host to expel invading pathogens and the factors contributing to the development of pathology.

Induction of nitric oxide production

Nitric oxide production has been demonstrated to occur in a number of cells of the immune system including neutrophils, and some transformed B cells, although most research has focused on the induction of NO in cells of the macrophage/monocyte lineage. The induction of macrophage NO production begins in response to an inflammatory stimulus such as that provided by the cytokines interferon γ (IFN γ), tumor necrosis factor α (TNF α), interleukin 1 β (IL-1 β) or bacterial lipopolysaccharide (LPS). IFN γ acts synergistically to upregulate NO production in the presence of the other mediators mentioned and is therefore particularly important in the generation of large amounts of NO. Two to four hours after receiving this stimulus, newly synthesized inducible nitric oxide synthase (iNOS; type II NOS) is produced in the macrophage cytoplasm. This enzyme is one isoform of at least three distinct NOS isoforms, all of which catalyze the production of NO and L-citrulline from L-arginine and molecular oxygen (Figure 1). The other two NOS isoforms are constitutive proteins, termed neuronal cNOS (type I NOS) and endothelial eNOS (type III NOS), both of which are

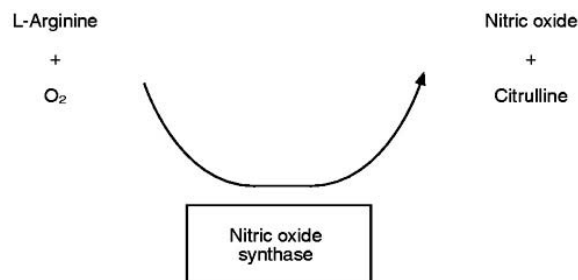


Figure 1 The generation of NO via nitric oxide synthase.

expressed in a number of tissues and catalyze the production of small quantities of NO. All three proteins contain four consensus sequences important for the binding of the cofactors NADPH, FAD, FMN and calmodulin. While all three proteins require calmodulin binding for enzymatic activity, the iNOS isoform is constitutively bound to calmodulin and therefore is not dependent on calcium flux for activity. The continuous production of large amounts of NO is catalyzed by iNOS while the other isoforms allow the production of small amounts of NO in response to calcium flux. It is this NO production by iNOS which is toxic to many microorganisms and certain host tissues, although under conditions of sustained calcium elevation within a cell the constitutive enzymes can produce appreciable quantities of NO.

Regulation of NO production

A number of immunosuppressive cytokines can inhibit macrophage expression of iNOS, including transforming growth factor β (TGF β), interleukin 4 (IL-4), and interleukin 10 (IL-10). Both IL-4 and IL-10 are produced by the T_H2 subset of CD4⁺ T cells, whereas T_H1 cells produce IFN γ and induce NO production. Thus the regulation of NO production by macrophages can be directly controlled by the activation of opposing T cell subsets (Figure 2). This functional polarization of T_H1 and T_H2 cells in the ability to induce NO reflects a greater requirement for host NO production in defense against intracellular pathogens. In addition to cytokines controlling NO production, the production of NO itself can also serve to inhibit further NO induction by inactivating NOS in a feedback manner. Thus, perhaps due to its toxic properties when in contact with self tissues, the

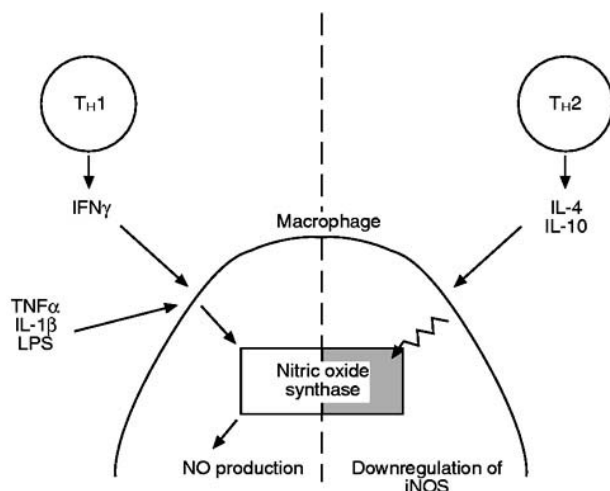


Figure 2 T cell subset regulation of NO production.

expression of iNOS and subsequent production of NO is under tight-regulation.

Microbicidal activity of nitric oxide

The production of NO, catalyzed by iNOS, is important in the destruction of a number of pathogenic organisms by the host. This role is most clearly observed in the control of infection with intracellular organisms such as *Leishmania* and *Listeria* which infect macrophages, but NO is also cytotoxic for tumor cells and extracellular pathogens and thus is not restricted to the effector mechanisms involved in destruction of intracellular pathogens. While the direct measurement of NO is difficult due to its short half-life, the dependence of the immune system on NO production in resolving infections can be clearly demonstrated.

The ability of activated macrophages to kill the protozoan parasite *Leishmania major* clearly correlates with the production of NO *in vitro* and the expression of iNOS *in vivo*. Furthermore, production of NO by macrophages can be inhibited using arginine analogs such as L-N^G-monomethylarginine (L-NMMA) which effectively blocks the ability of macrophages to kill *Leishmania* *in vitro* and *in vivo*. The use of gene-targeted disruption has also allowed the generation of mice which have no productive iNOS enzyme and while these mice are otherwise healthy, they succumb to infection with intracellular bacteria and parasites, demonstrating conclusively the importance of NO in the control of such pathogens.

In addition to the role that NO plays in curing an initial infection with these and other organisms, there is also evidence that the continual production of NO after an apparent disease cure is required to prevent

recurrence of infection in some chronic disease models. The treatment of mice which have 'cured' *L. major* infection with L-NMMA allows the resurgence of an otherwise unobserved infection. It is not clear whether this is true for other chronic diseases but it does underline the importance of NO in the maintenance of health as well as in the initial resolution of infection in conditions where the organism persists.

The actual mechanism by which NO mediates destruction of a microorganism is unclear but may involve a disruption of electron transport in the mitochondria by NO itself or alternatively may involve the interaction of NO with the oxygen radical $O_2^{\cdot-}$, a reaction which produces the peroxynitrite anion ($ONOO^-$).

Interference in the process of NO production by products of invading organisms may explain why some organisms can evade the efficient killing process outlined above. The microorganism may induce suppressive cytokines in the host to aid survival, or alternatively inhibit NO production via products of its own such as bacterial LPS or glycoinositol-phospholipids of the *Leishmania* parasite which can both mediate inhibition of iNOS induction.

Pathology caused by nitric oxide

The production of elevated levels of NO can have pathological consequences for the host as NO is toxic to host cells as well as microorganisms. Disregulated production of NO during an inflammatory response to infection or allergens may cause severe damage to host tissues. In addition, under conditions where there is an autoimmune recognition of host tissues NO may mediate the actual tissue damage observed.

The induction of toxic shock in mice after injection with LPS can be prevented by treatment with L-NMMA and, similarly, iNOS 'knockout' mice recover from LPS-induced shock while normal littermates do not, indicating that a pathogenic role can be attributed to NO production in response to bacterial products. In addition to toxic shock, the disregulated production of NO has been demonstrated to contribute to the pathology associated with neurodegenerative and inflammatory disorders.

Self-recognition and the induction of autoimmune aggression may cause the production of NO which causes damage to cells within the inflammatory focus. NO has been implicated in the tissue damage observed in several models of autoimmune disease, including autoimmune diabetes and rheumatoid arthritis. In addition, intestinal enteropathy associated with graft-versus-host disease, which involves

recognition of host tissues by grafted immune cells, also depends on the induction of NO, as L-NMMA treatment in a mouse model of this disease reduces gut damage.

Human NO production

There has been considerable uncertainty about the ability of human macrophages to produce NO. Human peripheral blood monocytes and some human macrophage cell lines failed to produce appreciable amounts of NO in response to immunological stimuli which readily induce NO synthesis in rodent macrophages. Evidence is accumulating that human macrophages, especially those derived from patients with chronic infection or inflammatory conditions such as rheumatoid arthritis, can express significant levels of iNOS and produce relatively large amounts of NO *in vitro*. However, the conditions for the induction of NO synthesis by human macrophages are likely to be different from those studied in murine models. The optimal stimulatory components required for NO synthesis by human macrophages remains to be determined.

See also: **B lymphocyte activation; Interferon γ**

Leishmaniasis; *Listeria*, infection and immunity; Microbicidal mechanisms, oxygen-dependent; Parasites, immunity to.

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NOCARDIA, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Members of the genus *Nocardia* are aerobic, gram-positive, filamentous bacteria that belong to the Actinomycetes (Figure 1). They share several biological properties with the closely related mycolic acid-containing genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Rhodococcus* and *Tsukamurella*. Most species of *Nocardia* are environmental organisms that reside primarily in the soils of all regions of the world. Many of the nocardiae may cause disease in a variety of animals and some plants. The *N. asteroides* complex (composed of *N. asteroides* sensu stricto, *N. farcinica* and *N. nova*), as well as *N. brasiliensis*, *N. pseudobrasiliensis* and *N. otitidiscaviarum*, cause disease in humans.

The *N. asteroides* complex is recognized most frequently as the etiology of nocardiosis in both immunocompromised and 'normal' individuals.

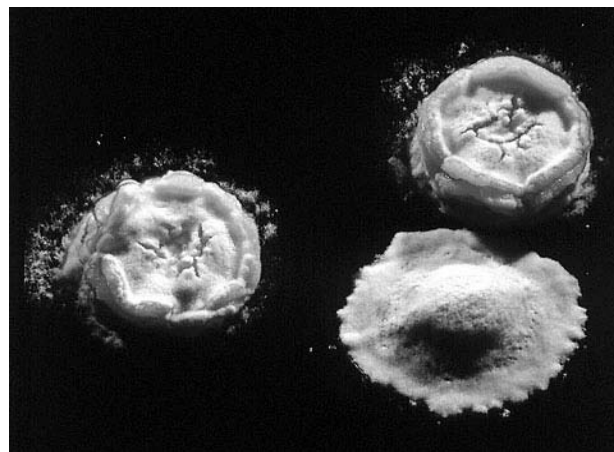


Figure 1 Different colonial morphotypes of *Nocardia asteroides* grown for 14 days at 37°C on glucose yeast extract agar. The photograph was made on Ectachrome film using a laser scanning camera with a macro focusing 100 mm lens. These colonies are approximately 0.5 cm in diameter.

Nocardiosis usually begins as a pulmonary infection that may remain localized, or it may become disseminated via the bloodstream to any organ; however, the brain is the usual secondary target, followed by the skin, kidneys, adrenals, eyes, heart, etc. The disease may range from a self-limited, subclinical infection to a progressive, aggressive disseminated infection that fails to respond to therapy resulting in death. *N. brasiliensis* and *N. otitidiscavarium* (*N. caviae*) may also cause nocardiosis; however, these organisms usually cause skin infections following trauma. The skin lesions may range from a benign, self-limited condition to a chronic, progressive disease that extends from the skin into the deeper tissues including the bone. This latter process is referred to as an actinomycetoma. Deep-seated, systemic infections previously attributed to *N. brasiliensis* are usually caused by the closely related *N. pseudobrasiliensis*.

The antigenic structure of *Nocardia* is complex and numerous antigenic components have been recognized. Several of the antigens of *Mycobacterium*, *Rhodococcus* and *Corynebacterium* share epitopes with *Nocardia*. Thus, a major obstacle for developing specific antigens that have diagnostic value is cross-reactivity (nonspecificity). Because of the antigenic heterogeneity of *Nocardia*, it has been difficult to identify antigens that are broadly specific for *Nocardia*, yet not so selective as to exclude different strains of *Nocardia*. Currently, the 55 kDa antigen identified by Angeles and Sugar (probably the same as the diagnostic 54 kDa antigen described by Boiron and colleagues) appears to be most promising for recognition of patients infected with *Nocardia* spp. Unfortunately not all strains of *Nocardia* infecting humans express the 55/54 kDa antigen. Skin test antigens (purified protein derivatives, PPDs) have been prepared; however, both the sensitivity and specificity for using these antigens to identify individuals infected with *Nocardia* have not been determined adequately.

Serodiagnosis of disease caused by nocardiae is problematic. It was reported in the USA that 20% of a relatively young, healthy population of medical students in California (>100 individuals) had antibody titers of $\geq 1:100$ to all of the diagnostic antigens of nocardiae using western blot immunoanalysis. The same study reported that 50% of an elderly population (>160 people) in Kansas were seropositive for nocardiae using these same western blot assays, whereas only 85% of individuals with culturally proven nocardiosis were seropositive. These observations indicate that there is significant exposure to nocardiae with probable subclinical infection developing in the absence of overt disease,

the incidence of which appears to increase with age. Furthermore, a significant number of individuals do not express detectable antibody against nocardial antigens during progressive disease (even in 'normal', nonimmunocompromised patients).

Immune responses of the host

Naturally acquired, progressive nocardial infections (excluding mycetomas) are recognized more frequently in humans that are immunocompromised. In general, persons with a deficiency in cell-mediated immunity appear to be more susceptible to infection than those that are deficient in complement or immunoglobulin. Furthermore, defects in polymorphonuclear function (i.e. chronic granulomatous disease) or in macrophage function enhance host susceptibility.

In vitro studies have shown that virulent strains of *N. asteroides* are not killed by either human polymorphonuclear leukocytes (PMNs), monocytes or macrophages. Indeed, these organisms readily grow in monocytes and macrophages. The PMNs were demonstrated to inhibit the growth of *Nocardia in vitro*, but this inhibition is only temporary. Activated macrophages, but not monocytes, are also inhibitory to *Nocardia*, but the ability of these cells to kill *Nocardia* is minimal. Studies demonstrated that pre-activation of either murine macrophages or human monocytes with recombinant interferon γ (IFN γ) and tumor necrosis factor α (TNF α) enhanced nocardial growth. Furthermore, the addition of a secondary signal such as lipopolysaccharide (LPS) enhanced nocardial filamentation even more. In contrast, coincubation of *Nocardia* spp. with either recombinant IFN γ or TNF α and normal human monocytes resulted in inhibition of intracellular nocardial growth.

Host responses to *Nocardia* have been studied using a variety of experimental animals. There have been numerous reports on nocardial interactions with *in vitro* maintained rabbit and murine macrophages. Virulent strains of *Nocardia* are readily phagocytosed by macrophages in the absence of serum or complement. Once the nocardiae are intracellular, they readily initiate growth that leads to macrophage destruction. Methods to prevent nocardial growth of macrophage monolayers have been explored. Macrophages killed all of the cells of virulent *Nocardia* only when activated macrophages were combined with specific immune serum and T lymphocytes. Physical interaction between the T lymphocyte and the macrophage appears to be essential. Furthermore, it was discovered that mice immunized against whole cells of *N. asteroides* have T cells that

demonstrate immunologic specificity for binding directly to *Nocardia*. These T cells kill nocardiae directly.

In vivo studies in mice, rabbits and guinea pigs support the *in vitro* observations. Virulent cells of *Nocardia* are not killed by the 'normal' naive animal. At both 3 and 6 h following intravenous injection of virulent nocardiae, one can readily recover 100% of the inoculum dose. The lag phase for nocardiae is about 6–8 h, thus no increase in colony-forming units occurs during this time period. Furthermore, no bacteria are killed during this same period. After 6 h, the nocardial numbers increase, especially in the brain and kidneys. Only 48–72 h after i.v. injection, do the number of nocardiae within the host decrease.

The use of germ-free mice demonstrates that activation of the host by the presence of a normal microflora is important for innate resistance of the animal to nocardiae. *N. asteroides* grow unimpeded in germ-free mice, whereas the 'normal' control animals are able to delay *in vivo* growth. Athymic, nude mice, which are T cell deficient, are more susceptible to nocardiae than their littermate controls, while in contrast, B cell-deficient mice are not. In addition, adoptive transfer of purified T cells from immunized mice to nonimmunized athymic mice, confers resistance. Adoptive transfers of B cells do not have the same effect. These observations indicate that T cells, but not B cells, are essential for host immunity to nocardial infection.

Evasive strategies by *Nocardia*

The relative virulence of strains of *Nocardia* for mice can be determined, and the *Nocardia* can be grouped into three general categories: 1) high virulence, 2) moderate virulence, and 3) low virulence. A large variety of factors affect the relative virulence of *Nocardia* in these models. These include the following: stage of growth; growth temperature; composition of the growth media; conditions of inoculum preparation; strain differences; route of exposure to the host; method of exposure to the host; and the type, age, weight and condition of the host.

By comparing differences in properties between highly virulent isolates and those with low virulence, correlations emerge that relate certain capabilities to virulence. By selecting specific mutants of these strains, the precise role of each factor in pathogenesis can be measured.

Cells of virulent strains of *Nocardia* 1) inhibit phagosome-lysosome fusion in macrophages and monocytes, 2) block or neutralize phagosomal acidification, 3) reduce the lysosomal enzyme acid phosphatase, 4) resist oxidative microbicidal activities of

phagocytes, and 5) grow within monocytes, macrophages and PMNs. In contrast, low-virulence strains of *Nocardia* do not have any of these capabilities, whereas moderately virulent strains are intermediate. It was shown that the highly virulent organisms secrete a unique superoxide dismutase (SOD) into the environment and this SOD becomes cell surface associated. In addition, these cells have high levels of intracytoplasmic catalase. *In vitro*, these highly virulent strains are very resistant to superoxide, hydroxyl ion, hydrogen peroxide, and to halides combined with these metabolites. In contrast, low-virulence *Nocardia* do not secrete SOD, and they have low levels of catalase. These organisms are killed by the above-listed components of oxidative metabolism. In addition to SOD and catalase, other cell wall components such as glycolipids appear to be involved in nocardial pathogenesis. *Nocardia* that contain the glycolipid trehalose-dimycolate (cord factor) are more virulent for mice and affect phagocyte function more completely than low-virulence strains that lack cord factor. Furthermore, the size and degree of unsaturation of the mycolic acids in the cord factor appear to be critical in modulating host cell function.

Vaccines

There are currently no effective vaccines developed against *Nocardia*. However, studies have suggested that immunization with certain nocardial components enhances host resistance, one of the most promising of these utilizing nocardial SOD as the protective antigen. In addition, *Nocardia* have cell wall components that enhance or augment the immune response. These components (peptidoglycolipids) can completely replace mycobacterial products in Freund's adjuvant. Also, the nocardial cell wall contains a potent T-independent B cell mitogen. Therefore, nocardial components are being explored as powerful immunomodulators.

See also: Adjuvants; Bacteria, immunity to; Bacterial cell walls; Coryneform bacteria, infection and immunity; *Mycobacteria*, infection and immunity; Polyclonal activators; Respiratory and cardiac infections.

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NORTHERN BLOTTING

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Cells in different states express different sets of genes. Some of these genes are specifically expressed in particular types of cells, and the specific expression of such genes is apt to determine the phenotypes of the cells. Although the expression of genes may be regulated at multiple steps in the pathway leading from DNA to protein, it has been proven to be regulated mainly at the level of transcription. Various techniques in molecular biology have been developed to examine transcripts qualitatively and quantitatively. Among them, northern blotting is an important and commonly used technique to determine both the size and the relative amount of the transcript of the gene of interest. The procedure of this technique is similar to that of Southern blotting (DNA blotting), and was named by analogy. The northern blotting technique is composed of three experimental steps: 1) agarose gel electrophoresis under a denaturing condition to separate RNA; 2) transfer of the separated RNA to a membrane; and 3) hybridization with a radiolabeled probe to detect a specific sequence. There are several variations in each step, and a brief summary of each step in the protocol is described here.

1. Separation of RNA by denaturing gel electrophoresis

For northern blotting, either total or poly(A)⁺ RNA can be used. Most cellular RNAs are single-stranded polynucleotides and generally have secondary structures formed by intramolecular base-pairing. The secondary structures prevent RNA from being separated according to size by electrophoresis through an agarose gel. To disrupt the secondary structure of RNA, either formaldehyde or glyoxal/DMSO (dimethyl sulfoxide) is commonly used as a denaturing reagent. Formaldehyde is simply added to the samples and gels, so that the method using formaldehyde is easier to run the gels than that using glyoxal/DMSO. In the glyoxal/DMSO method, RNA is denatured by association with glyoxal, and the association is kept under a condition where the pH is neutral or acidic. Therefore, the gels must be run slowly and the buffer must be circulated to avoid the formation of an H⁺ gradient in the buffer during electrophoresis. Despite this inconvenience, the resolution of RNA is better in the glyoxal/DMSO system because a specific RNA is detected as a sharper band by hybridization. The size of the RNA fragment can be determined by comparing the migration distance

of the RNA with those of molecular weight markers, which should be run in parallel, or those of the 28S (4.7 kb) and 18S (1.9 kb) rRNAs contained in the samples of the total RNA. The molecular markers and rRNAs can be detected by staining gels with ethidium bromide after electrophoresis.

2. Transfer of RNA to a membrane

After electrophoresis is completed, the separated RNA is transferred from the gel to a membrane. Two types of membrane, nitrocellulose and nylon, are generally used. Nylon membranes have advantages in that they are more durable than nitrocellulose membranes, allowing repeated hybridization without significant loss of signal. However, a higher level of background hybridization tends to be detected when the nylon membranes are used. Using an increased amount of salmon sperm DNA as a blocking reagent contained in the hybridization solution may decrease the level of background. RNA is transferred to membranes by capillary transfer or vacuum transfer. RNA is carried from the gel in the flow of the transfer buffer in both methods. Vacuum transfer requires a special transfer apparatus and is more efficient than capillary transfer. In addition, the electrophoretic transfer method is available only for nylon membranes. This method cannot be recommended for use with nitrocellulose membranes because a high concentration of salts is required for the binding of RNA to nitrocellulose membranes but not to nylon membranes. The high ionic strengths of the transfer buffers permit efficient conduction of electric current, leading to depletion of the buffering power by electrolysis and causing overheating. The use of large volumes of buffers and continuous external cooling are therefore required to avoid these problems. The transferred RNA is fixed to either membrane by baking at 80°C for 2 hours in an oven. Alternatively, the RNA transferred to a nylon membrane can be immobilized by ultraviolet cross-linking or alkaline fixation.

3. Hybridization with a radiolabeled probe

To detect a specific RNA immobilized on the membrane, hybridization is performed using a radiolabeled probe prepared from the gene of interest. Several types of probe can be used, including double-stranded DNA, single-stranded DNA, RNA and end-labeled oligonucleotides. The double-stranded and single-stranded DNA probes are prepared by nick translation, random oligonucleotide priming method or polymerase chain reaction (PCR). A fragment purified from the vector by restriction digestion and a single-stranded plasmid containing the sense strand of cDNA are used as templates for the double-stranded and single-stranded (an antisense strand) DNA probes, respectively. On the other hand, the

RNA probe, which should also be antisense, is made by *in vitro* RNA synthesis using RNA polymerases from bacteriophages such as SP6, T3 and T7. In this case, the template is constructed by cloning the DNA fragments which will be transcribed to RNA into a double-stranded vector incorporating promoters for these RNA polymerases. The specific activity of these probes should be $>10^8$ c.p.m. μg^{-1} . The RNA-RNA hybrid is more stable than the RNA-DNA hybrid, so that RNA probes provide increased sensitivity but require more stringent hybridization and washing conditions. The hybridization solution should contain 50% formamide, permitting hybridization at a lower temperature and minimizing the degradation of RNA. After hybridization, the membrane is washed in the buffer containing a lower concentration of salts. The washing conditions become more stringent by decreasing the concentration of salts or by increasing the temperature of the buffer. The specific transcript is subsequently detected by autoradiography. The northern blotting is a sensitive technique by which at least 1 pg of a specific transcript can be detected.

Recently, various types of hybridization systems using DNA or RNA probes labeled with nonradioactive reagents including digoxigenin (DIG), biotin and fluorescein have been developed. In these systems, the probes are labeled by incorporating nonradioactive reagent-conjugated nucleotides. Although the standard methods described above can be applied for probe labeling, hybridization and washing, additional experimental procedures similar to those used in western blotting are necessary to detect the hybridized probes. After washing, the hybridized probe is bound with an enzyme-linked antibody for the nonradioactive reagent (DIG or fluorescein) or enzyme-linked streptavidin (biotin). Alkaline phosphatase or peroxidase is usually used as the enzymes of these conjugates. The membrane is subsequently exposed to the detection reagent containing a substrate for the enzyme, and a specific transcript is detected by chromogenic or chemiluminescent visualization. The sensitivities of these methods are comparable to those using a radioisotope.

See also: Fluorochrome labeling; Polymerase chain reaction (PCR) amplification; Radiolabeling; Southern blotting; Western blotting.

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NUDE (ATHYMIC) MICE

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The congenitally mutant mice characterized by hairlessness were discovered in 1963 and were subsequently demonstrated to carry the autosomal recessive mutant gene 'nu' located on chromosome 11. The 'nu' gene has recently been found to encode a new member of the winged helix domain family of transcription factors that is expressed specifically in skin and thymus. Since the finding, in 1968, that hairless nu/nu (nude) mice were congenitally athymic and had severely reduced numbers of T cells, nude mice have been extensively used as a T cell-deficient system for studies on the role of T cells in immunological responses and as an *in vivo* experimental tool for propagation and expansion of tumor cells in oncology research. Recent experimental advances reinforce the notion that nude mice do have some lymphocytes which are comparable to the T cells of normal mice. Therefore, nude mice also provide us with an interesting experimental system for the study of T cell differentiation along an extrathymic pathway. However, it is likely that such studies in the future will rely more heavily on the use of targeted gene knockout mice.

Breeding and maintenance of nude mice

Good animal husbandry and strict precautionary monitoring for microbiological control are mandatory if meaningful experimental results are to be obtained from nude mice. Housing systems for maintaining nude mice are as follows:

1. A system of plastic cages with sealed air filters is usually used for successfully housing and conducting experiments with nude mice.
2. The best system from the point of view of isolation from the environment for maintenance of pathogen-free nude mice is the use of isolator units.
3. Another secure but more expensive system is provided by laminar air flow cage units and laminar air flow rooms.

The development of congenic lines of nude mice with a definite genetic background is also essential for immunological research. Female nude mice lactate poorly and are often infertile. Thus, mating schemes generally involve mating heterozygous (nu/+) females with homozygous (nu/nu) males. Three breeding systems are used: outbreeding, inbreeding

and cross-breeding. Inbreeding (generally using brother × sister pairs set up at the time of weaning) is used to obtain mice of specific and uniform genetic background. The available strains of nude mice are as follows: B6CByF1/J-nu, CByB6F1/J-nu. NU/J nu, BALB/cByJ-nu, C57BL/6J-nu, AKR/J-nu^{str}+, C57BL/6-nu^{str}.

An additional mutation at the nu locus, called streaker (nu^{str}) was found to occur on the AKR/J strain backgrounds. Homozygotes (nu^{str}/nu^{str}) do not express leukemogenic retroviruses and fail to show leukemic changes in the lymphocytes. However, when grafted with thymic epithelial tissue nu^{str}/nu^{str} mice develop the high incidence of T cell lymphoma.

Immunological characteristics of nude mice

Phagocytes and natural killer (NK) cells

The activities of phagocytes including phagocytic uptake, FcR expression and killing of tumor cells and bacteria are greater in nude mice than in euthymic (nu/+) littermates. These alterations are considered as a result of stimulation with lipopolysaccharide (LPS) derived from gut microflora. However, the fact that increased activities of phagocytes are also detected in nude fetuses suggests that the athymic mutation influences the state of professional phagocytes. The NK activity is also higher than that in nu/+ mice even bred under specific pathogen-free conditions. The increased NK cell activity appears to be due to elevated levels of interferon in nude mice. High levels of NK activity may lead to increased immunosurveillance, and the occurrence of spontaneous tumors is not greater than in euthymic mice.

T lymphocytes

The presence of the thymus is essential for the full development of the immune system. The thymic primordium, which is derived from pharyngeal epithelium, is first colonized by bloodborne lymphoid stem cells at the 11th day of gestation in the mouse embryo. These stem cells proliferate and give rise to thymocytes, some of which become capable of immune responsiveness shortly before birth. Stem cells for the T cell lineage are clearly present in nude mice and T cell precursors have been demonstrated in bone marrow. However, they cannot differentiate

to a substantial number of immunocompetent T cells in the absence of thymic influence. Anatomically, all the T cell compartments of the lymphoid system in nude mice appear empty with very few, if any, functional T cells. A thymic rudiment exists at 12 days' gestation in nude mice. Major histocompatibility complex (MHC) molecules on thymic epithelial cells play an important role in T cell development in the thymus. Normal thymic epithelial cells express MHC class II by day 13 of gestation, whereas such expression is not detected in the 13-day thymic rudiment of nude mice. The thymic rudiment degenerates by birth. Electron microscopic surveys of the early embryonic thymus reveal no lymphoblasts within the thymic lobes.

There are, nevertheless, several lines of convincing evidence that some cells sharing phenotypic and functional properties of T cells do develop in nude mice. Limiting-dilution studies have shown that antigen-specific precursors of both cytolytic and helper T cells exist in such animals, albeit at a significantly (5- to 10-fold) lower frequency than in *nu/+* mice. Since the thymus rudiment is not settled by lymphocytes at any stage of development, the possibility that a few T cells do mature in the early thymus of nude mice can be excluded. An appreciable number of T cells are also detected in nude mice born to *nu/nu* homozygous mothers, suggesting that maternal T cells are not the origin of the T cells in such animals. Systemic study of the age-associated increase in lymphocytes expressing the T cell markers Thy-1, CD4 and CD8 in nude mice suggests that T cell precursors can differentiate along a thymus-independent pathway.

Although only a minor population of peripheral T cells express TCR $\gamma\delta$, this population is relatively abundant in intraepithelial lymphocytes in intestine, skin and uterus in euthymic mice. Nude mice contain intraepithelial lymphocytes expressing V γ 1 in intestine and epidermis, suggesting that this subset of $\gamma\delta$ T cells can develop extrathymically.

$\alpha\beta$ T cells also can develop outside the thymus in nude mice. The cell surface density of the TCR complex, however, is lower on the T cells in nude mice than on those in euthymic mice although it is higher than on the majority of immature thymocytes. The $\alpha\beta$ T cells with intermediate levels of TCR, which are preferentially found in the liver of euthymic mice, express NK1.1 (a member of NKR-P1 family) and interleukin 2 (IL-2) receptor β chain, both of which are NK markers. The T cells with NK markers consist of CD4⁻8⁻ double-negative T cells and CD4⁺ T cells and have a restricted TCR V α /V β repertoire such as V α 14 and V β 2,7,8. The development of these T cells is dependent on MHC class Ib or CD1 mol-

ecules which are abundantly expressed by epithelial cells in intestine and liver. During T cell differentiation in the thymus, T cells with intermediate affinity for self-MHC gene products are selected (positive selection) and T cells reactive to self antigen are eliminated (negative selection). T cells differentiating outside the thymus have not undergone negative selection and seem to be capable of generating the full range of TCRs randomly. However, functional, serological and molecular analyses of the TCR repertoire in nude mice have revealed that the nude T cell repertoire is more limited than the normal one. It can be speculated that nude T cell precursors can differentiate at epithelia in liver, intestine or skin, which express preferentially MHC class Ib including TL, Qa and CD1 molecules exhibiting only minimal polymorphism. Restricted antigen presentation by these molecules could be involved in positive and negative selection of the T cells outside thymus, resulting in the generation of a limited TCR repertoire in nude mice. It is also possible that environmental antigens are responsible for expansion of such T cell clones dominating the T cell repertoire.

It is not clear at present whether the reduced TCR density on nude T cells is just a curious abnormality or may be informative with regard to pathological T cell development. TCRs, either $\alpha\beta$ or $\gamma\delta$ chains, are associated with CD3 γ , δ , ϵ and $\zeta\zeta$ chains. Some subsets of T cells, especially epithelia-associated T cells use the common γ chain of FcR instead of ζ chain of TCR complex. In nude mice, the epithelia-associated T cells have CD3 γ , and ϵ chains but not δ or ζ chains. T cells in nude mice may therefore be activated via a different pathway from those in euthymic mice.

B lymphocytes

Although nude mice do have an appreciable level of T cells, most of the lymphocytes in the peripheral lymphoid tissues are B cells. The absolute number of B cells is much the same as that in *nu/+* littermates. B cells can be divided into three lineages: B1-a cells expressing CD5, B1-b cells expressing Mac-1 and conventional B cells. B1-a and B1-b cells, both of which express IL-5R, are present in the peritoneal cavity at an early stage of ontogeny and secrete natural antibodies including anti-phosphatidylcholine antibody. The nude mutation does not result in a lack of B1 cells but there are slightly reduced levels of such B cells, which might imply a relative independence from thymic influence in the generation of B1 cells but some T cell influence on the maintenance of such cells.

As compared with euthymic controls, nude B cell responses are considerably lower, very deficient in

IgG1, and somewhat limited in IgG2, indicating a significant part of these responses is induced and/or amplified by helper T cells. T cell-independent (TI) and T cell-dependent (TD) antigens were originally defined as ones that could, or could not, stimulate antibody production in nude mice, respectively. TI antigen induces essentially IgM and IgG3, whereas TD antigen induces mainly IgG2a and IgG2b. The amount of IgM in the serum of nude mice is much the same as that in nu/+ mice, while the level of IgA, IgG1 and IgG2 are reduced. The antigen-specificity repertoire of immunoglobulins in nude mice is much less well-defined. This is in part due to the fact that age, strain and environmental stimulation affect the repertoire of immunoglobulins in the blood of the nude mice.

Use of nude mice in immunology and oncology

Studies of T cell differentiation and host defense mechanisms

The lack of a thymus has rendered the nude mouse a good model for the study of T cell differentiation. Analysis of T cells present in nude mice reveals the characteristics of T cells differentiating along extrathymic pathways. Thymus-grafted nude mice are useful for studying the roles of thymic components in T cell differentiation. The nude mouse also provides a model for studying host defense mechanisms against microbial infection and tumors. Inoculation of *Mycobacterium leprae* into the paws of nude mice has produced a disease very similar to human leprosy in many of its symptoms. Treatment with chemotherapeutic agents of nude mice affected by leprosy has given essentially the same results obtained with such agents in humans. Accordingly, the nude mouse has proved to be a valuable model for the study of experimental chemotherapy of leprosy in humans.

Growth of transplants of normal and neoplastic tissues

Although an appreciable level of functional T cells

and an increased level of NK activity are detected in nude mice, these mice show an inability to reject allo- and xenografts. Therefore, nude mice have been used as recipients of normal tissues and for the expansion of various tumor cell lines, as well as for the maintenance of hybridomas for monoclonal antibody production. Skin from other species has been grafted successfully and remained viable indefinitely. All skin grafts survived and continued to perform their normal functions such as hair and feather growth, production of sebum, and renewal of epithelium. Tumors developing in other species, especially humans, have been inoculated into nude mice. Anatomical structure at both the macro- and microscopic levels is well preserved and the grafted tumor is generally indistinguishable from the structure of the tumor in the patients. Secretion of hormones in secretory tumors and production of other biological substances is maintained. Human tumors growing in nude mice produce metastases, albeit not frequently. Therefore, nude mice have been an invaluable tool for characterizing tumors and establishing the effectiveness or lack of effectiveness of a drug on a specific tumor.

See also: Thymic epithelium: potential role in regulatory T cell tolerance; Hu-SCID mice; Immunodeficiency, animal models; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; Thymus; T lymphocyte differentiation; T lymphocytes; Tolerance, peripheral.

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NUTRITION AND THE IMMUNE SYSTEM

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Malnutrition is the most common cause of immunodeficiency worldwide. The frequent presence of nutritional deficiencies in developing countries is well known. It has now been revealed that mild to moderate undernutrition is not uncommon even in industrialized countries. For example, iron deficiency is seen in at least 9% of the North American population. In hospitalized patients, obvious malnutrition is frequent. The combination of malnutrition and infection is responsible for much of the morbidity and mortality in all age groups. In patients with a variety of primary diseases, such as cancer and Crohn's inflammatory bowel disease, nutritional deficiencies further complicate the picture and increase the risk of infectious complications. Although much of the initial work on nutrition and immunity was done on young children in developing countries, the general principle that nutrition is a critical determinant of immunocompetence is applicable universally.

Protein-energy malnutrition

Epidemiological studies have documented the adverse effect of protein-energy malnutrition (PEM) on morbidity and mortality. Pathological examination of tissues from children dying of PEM showed the frequent presence of several opportunistic microorganisms including *Pneumocystis carinii*. Morbidity due to diarrheal disease, respiratory infection, measles, tuberculosis, and perhaps even to human immunodeficiency virus is increased.

Lymphoid tissues show a significant atrophy. For instance, the size of the thymus is small. Histologically, there is a loss of corticomedullary differentiation, there are fewer lymphoid cells, and the Hassall's corpuscles are enlarged, degenerated and, occasionally, calcified. In the spleen, there is a loss of lymphoid cells around small blood vessels. In the lymph node, the thymus-dependent areas show depletion of lymphoid cells.

Delayed cutaneous hypersensitivity responses both to recall and new antigens are markedly depressed. There is a significant positive correlation between the size of skin response and visceral protein synthesis as judged by serum albumin concentration. It is not uncommon to have complete anergy to a battery of different antigens. These changes are observed in moderate deficiencies as well. Findings in patients with kwashiorkor were more striking compared with

those in patients with marasmus. One plausible reason for reduced cell-mediated immunity in PEM is the reduction in the number and function of mature T lymphocytes particularly CD4⁺ helper T cells. The reduction in serum thymic factor activity observed in PEM may underlie the impaired maturation of T lymphocytes. There is an increase in the amount of deoxynucleotidyl transferase activity in leukocytes, a feature of immaturity. The number of CD4⁺ cells is decreased markedly, often to values less than 50% of controls. The change in number of CD8⁺ T cells is less marked. Thus the 'helper/suppressor' ratio is significantly decreased. Lymphocyte proliferation and synthesis of DNA are reduced, especially when autologous patient plasma is used in cell cultures. This may be the result of inhibitory factors as well as a deficiency of essential nutrients lacking in patients' plasma.

Serum antibody responses are generally intact in PEM, particularly when antigens in adjuvant are administered or in the case of T cell-independent antigens. Rarely, the antibody response to organisms such as *Salmonella typhi* may be decreased. Before impaired antibody response can be attributed to nutritional deficiency, one must carefully rule out infection as a confounding factor. Antibody affinity is decreased in patients who are malnourished. This may provide an explanation for a higher frequency of antigen-antibody complexes found in such patients. As opposed to serum antibody responses, secretory immunoglobulin A (IgA) antibody levels after immunization with viral vaccines are decreased; there is a selective reduction in secretory IgA levels. This may have several clinical implications, including an increased frequency of septicemia in undernourished children.

The process of phagocytosis is also affected in PEM. Complement is an essential opsonin and the levels and activity of most complement components are decreased. The best documented is a reduction in complement C3, factor B, and total hemolytic activity. Although the ingestion of particles by phagocytes is intact, subsequent metabolic activation and destruction of bacteria and fungi is reduced. Finally, recent work in humans and animals has demonstrated that the production of several cytokines, e.g. interleukin 2 and interferon γ , is decreased in PEM.

Selected nutrient deficiencies

Clinical malnutrition is usually a complex syndrome of multiple nutrient deficiencies. However, observations in laboratory animals deprived of one dietary element and findings in rare patients with a single nutrient deficiency have confirmed the crucial role of several vitamins and trace elements in immunocompetence. Deficiencies of pyridoxin, folic acid, vitamin A, vitamin C and vitamin E result in impaired cell-mediated immunity and reduced antibody responses. Vitamin B₆ deficiency results in a decreased lymphocyte stimulation response to mitogens such as phytohemagglutinin. A moderate increase in vitamin A intake enhances immune response and affords partial protection against the development of certain tumors in animals. Zinc deficiency, both acquired and inherited, is associated with lymphoid atrophy, decreased cutaneous delayed hypersensitivity responses and homograft rejection, and lower thymic hormone activity. In laboratory animal models, these findings can be confirmed and in addition one can demonstrate a reduced number of antibody-forming cells in the spleen and impaired T killer cell activity. Wound healing is impaired. Excess zinc also depresses neutrophil function and lymphocyte responses and is associated with increased morbidity in the elderly.

A deficiency of iron is the most common nutritional problem worldwide, even in industrialized countries. On the one hand, free iron is necessary for bacterial growth (removal of iron with the help of lactoferrin or other chelating agents reduces bacterial multiplication, particularly in the presence of specific antibody) and, on the other, iron is needed by neutrophils and lymphocytes for optimal function. The lymphocyte proliferation response to mitogens and antigens is impaired: response to tetanus toxoid and herpes simplex antigens is low in iron-deficient subjects and iron therapy results in a significant improvement in their response. There are many molecular explanations for impaired lymphocyte and neutrophil function in iron deficiency, including the deficiency of myeloperoxidase and ribonucleotidyl reductase.

Copper-deficient animals show a reduction in the number of antibody-producing cells compared to healthy and pair-fed controls. Dietary deficiencies of selected amino acids decrease antibody responses; in other states of amino acid imbalance, the overall response may be enhanced, indicating that changes in suppressor cells may occur. The phagocytic clearance of macromolecules from the blood is reduced, as is antibody affinity in inbred animals. A modest increase in the intake of arginine and glutamine,

especially orally, improves immune responses and enhances resistance to infection.

Nutritional regulation of immune function in low birth weight infants and in the elderly

There are many striking similarities between the two ends of the age spectrum. Both neonates and the elderly have suboptimal immune responses and are susceptible to infection. When nutritional deficiency complicates the picture, impairment of immunocompetence is more marked and longer-lasting.

The immune system develops during fetal life and the first few months after birth. If an infant is born preterm or if he or she exhibits growth retardation as a result of a number of environmental factors, including maternal malnutrition or infection, immunocompetence is reduced. The impact on T lymphocyte numbers and cell-mediated immunity is most discernible. The preterm infant of low birth weight generally recovers its ability to mount immune responses by the age of 3 months. However, the small-for-gestational-age (SGA) infant may continue to show reduced cell-mediated immunity for several months and years. There is a significant difference in the immunocompetence of SGA infants who exhibit a higher level of morbidity and that of infants with lower morbidity. In laboratory animal models of intrauterine malnutrition, immune responses are impaired both in first- and second-generation offspring.

At the other extreme of life, many but not all elderly individuals show a progressive loss of immune function. Cell-mediated immunity is impaired. This may be due in part to a marked reduction in the putative thymic hormone(s). It is interesting that about 35% of subjects above 65 years retain immunocompetence at levels seen in young adult life. Other studies have documented alterations in nutritional status and body composition, including decreased lean body mass, loss of visceral protein, and increase in the relative proportion of body fat. These changes in body constituents may result from a variety of pathogenic factors, such as altered taste acuity, reduced food intake, malabsorption, and the metabolic consequences of concurrent disease. Deficiencies of vitamins and trace elements are observed in almost one-third of all elderly. To date, there are only a few studies that have looked concurrently at both nutrition and immunity in the elderly.

The age-related decline in immunity is similar to that seen in protein-calorie malnutrition. Since the elderly are known to be among the most poorly nourished in industrialized countries and since

changes in food intake, body composition and protein metabolism are known to occur with advancing age, the possibility arises that altered immune status in the elderly could be ascribed in part to nutritional deficiency. A number of recent studies have shown the frequent occurrence of nutritional deficiencies in the elderly. When these are corrected even partially, there is a significant improvement in a variety of cell-mediated immune responses and in natural killer (NK) cell activity and cytokine production. Even morbidity may be reduced when such nutritional support is provided. The causal contribution of altered nutritional status in the elderly to deficits in their immune responses needs further study. Recent data indicate that a causal relationship does exist between undernutrition and impaired immunity in many elderly individuals and that this is a correctable abnormality in the majority of them. It is not clear whether maintenance of good nutrition and improved immunocompetence will alter morbidity and longevity. Obviously, long-term prospective studies are required to answer these questions.

Practical applications

The many observations on the interactions between nutrition and immunity have led to several practical applications:

1. The outcome of surgery can be predicted on the basis of preoperative assessment of nutritional status and of immunocompetence.
2. Tests of immunocompetence can be used as sensitive functional indices of nutritional status and for determination of the upper and lower safe limits of nutrient intake.
3. Response to prophylactic immunization can be improved if nutritional support is provided before, and even after, the administration of the vaccine.
4. The incidence of opportunistic infections can be reduced when nutritional care is provided at the same time as medical treatment in many chronic diseases, such as inflammatory bowel disease, HIV infection, cancer, etc.
5. Feeding formulas have been developed that selectively enhance immune responses and resistance to infection.
6. Megadoses of certain nutrients can impair immune responses.

See also: Antioxidant micronutrients and the immune system; Arginine and immunity; Copper and the immune system; Fatty acids (dietary) and the immune system; Iron and the immune system; Vitamin A and the immune system; Vitamin B group and the immune system; Vitamin C and the immune system; Vitamin D and the immune system; Zinc and the immune system.

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ONCHOCERCIASIS

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Onchocerciasis or river blindness, a filarial parasitic disease caused by infection of humans with *Onchocerca volvulus*, is a severe public health problem in different parts of Africa, Central and South America and Yemen. The major pathology of the disease is debilitating dermal and ocular complications; in fact onchocerciasis is the second leading cause of infective blindness. An estimated 120 million people live in endemic areas, of whom 18 million are infected. Approximately 2% of the infected individuals eventually become blind, although this may be 30% or higher in hyperendemic areas. The life cycle and antigens of the parasite, the complex immune responses of the host and its evasion by the parasite, and different means of controlling onchocerciasis will be discussed with particular reference to the human disease, although many other species of *Onchocerca* can infect cattle (*O. gibsoni*, *O. gutturosa*, *O. lienalis*, *O. ochengi*), horses (*O. cervicalis*, *O. reticulata*) and a variety of wildlife, particularly ungulate hosts.

Characteristics of the organism and its antigens

O. volvulus is a parasitic filarial nematode worm which undergoes a complex set of developmental stages involving two different hosts. Infection is transmitted when the insect vector, female black flies of the genus *Simulium*, bites the human host and introduces infective larvae which subsequently molt twice and mature to male and female adult worms over a period of 3–18 months. The adult worms are encapsulated in subcutaneous nodules of host collagenous tissue called onchocercomata. The adult worms are unable to replicate within the human host, where they can survive up to 10–15 years. Following reproduction within the onchocercomata, the females release microfilariae which migrate out of the nodules to invade the host tissue and concentrate in

the dermis. Approximately 7–34 months (prepatent period) pass before microfilariae are detected in the skin (patent infection). Completion of the life cycle is initiated when infected individuals are bitten by a female black fly which ingests microfilariae along with host tissues from the skin. A small portion of the ingested microfilariae migrate from the gut to the thoracic muscles of the fly, develop into infective larvae over a period of 6–8 days and then migrate to the head of the fly. When this fly bites another individual the infection is transmitted. **Figure 1** illustrates the life cycle of *O. volvulus*.

Major impediments for characterization of *O. volvulus* antigens have been its complex life cycle and limited availability in the absence of a suitable *in vitro* culture system. Since only adult worms

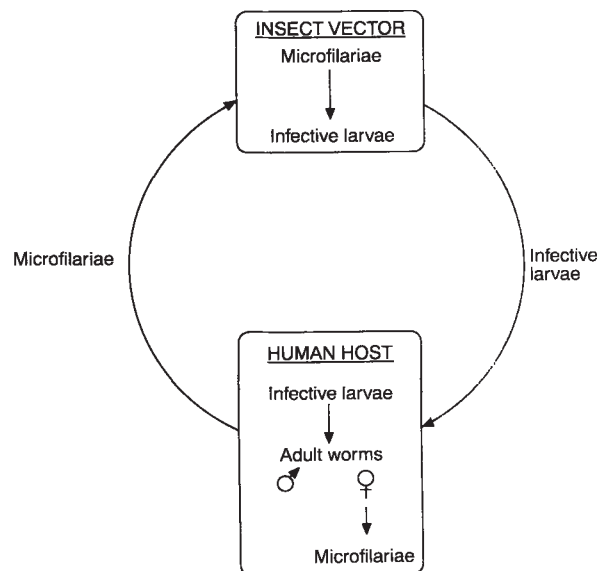


Figure 1 Life cycle of *Onchocerca volvulus*. The sizes of different forms of the parasite are: infective larvae 440–700 μm long, 19–28 μm wide; male adult worms 3–6 cm long, 130–150 μm wide; female adult worms 23–70 cm long, 275–325 μm wide; microfilariae 300–320 μm long, 6–9 μm wide.

obtained by nodulectomy of infected individuals offer reasonable amounts of material, initial studies on the characterization of onchocercal antigens focused mainly on adult worms. However, advances in recombinant DNA technology have led to characterization of a number of antigens of microfilariae, adult worms and infective larvae at the molecular level. The antigens of *O. volvulus* have a wide range of biochemical and physical properties and specificity with respect to their surface-bound, secretory or intracellular nature as well as their characteristic occurrence in particular stages of the life cycle. Antigenic differences have also been observed between different geographical isolates of *O. volvulus*, e.g. savannah and rain forest areas of West Africa. Cross-reactivities between onchocercal antigens with other filarial as well as host antigens have been documented, suggesting structural similarities. Many of the parasite antigens are potent immunogens, as they stimulate production of antibodies in infected individuals.

Immune responses of the host

O. volvulus infection leads to the development of parasite antigen-specific immunoglobulin G (IgG), IgM, IgE and IgA antibodies as well as polyclonal B cell activation. Although the exact time of onset of immune reactions in humans is not known, experimental onchocerciasis in chimpanzees (permissive host for *O. volvulus*) indicates the onset of humoral and cell-mediated immune responses as early as 1 month following infection. The precise role of antibodies in protection or pathogenesis is not well defined; however, antibodies directed against microfilariae can mediate *in vitro* adherence of granulocytes, and eosinophils and neutrophils can mediate antibody-dependent killing. In fact eosinophils may be the major effector cells in antibody-dependent killing of microfilariae, as evidenced by degranulation of eosinophils *in vivo* in areas of the skin surrounding microfilariae, suggesting local and even distant tissue damage from repeated and massive eosinophil degranulation.

Many *O. volvulus*-infected individuals have elevated IgE levels which possibly contribute to acute inflammatory complications, including some ocular lesions. Interestingly, compared with IgG, a higher proportion of IgE antibody is *O. volvulus* antigen specific. Antigen-antibody complexes are also present in the sera of infected persons. Immune complex deposition may cause acute inflammation, tissue damage and downregulation of the host immune response.

Both *in vivo* (skin testing) and *in vitro* (lymphocyte

proliferation) evidence suggests that cell-mediated immune responses are downregulated to *O. volvulus*-specific as well as non-specific antigens in infected individuals. Diminished reactivity is more prominent in those with microfilariae distributed throughout the skin but with little cutaneous reaction compared with those with highly active skin disease and few demonstrable microfilariae. The lymphocyte reactivity of infected young persons (12–16 years) is greater than that in older infected individuals, indicating acquired downregulation of their cellular immune response. Studies on immunoregulation in onchocerciasis show a predominant T_H2-type cytokine response to *O. volvulus* antigens in individuals with demonstrable ocular pathology (both in human and murine models), whereas a T_H1-type response is observed in exposed but infection-free 'putatively immune' persons. The roles of interleukin 4 (IL-4) and IL-5 in killing of infective larvae and clearance of microfilariae have been demonstrated in murine models. While for experimental onchocercal keratitis induced in mice there is predominant infiltration of CD4⁺ T cells, both CD4⁺ and CD8⁺ T cells have been demonstrated to have roles in protective immunity against microfilariae in murine models. The widely used microfilaricidal drug ivermectin (a semisynthetic macrocyclic lactone) improves delayed-type hypersensitivity responses and cellular proliferation and modifies antibody levels as well as the cytokine pattern in onchocerciasis patients, at least temporarily.

The host immune response appears to play a role in both pathology and protection in onchocerciasis, onchocercal pathology being associated with the host immune response to dying or degenerating (but not intact) microfilariae. Based on clinical evaluation of dermal pathology, endemic populations can be broadly divided into three categories: 1) generalized onchocerciasis – common among infected individuals with microfilariae but few clinical symptoms and hyporesponsiveness to parasite antigens; 2) localized onchocerciasis (sowda) – infected individuals with few microfilariae but intense dermal pathology and hyperresponsiveness to parasite antigens; and 3) putatively immune individuals – without any detectable microfilariae or clinical symptoms. The course of *O. volvulus* infection has been shown to be influenced by HLA-D alleles. Ocular pathology is the most deleterious effect of onchocerciasis and can affect both anterior (punctate keratitis, sclerosing keratitis and iridocyclitis) or posterior (optic neuritis, optic atrophy and chorioretinitis) segments of the eye. Host inflammatory responses against dead microfilariae possibly lead to pathological changes in the anterior segment, while migrating microfilariae,

circulating immune complexes, direct interaction of immune cells with microfilariae as well as auto-immune mechanisms may be involved with the posterior segment pathology.

Evasive strategies by the organism

How the parasites survive within the human host is not clear; however, it is obvious that they have evolved ways to avoid or downregulate the host inflammatory response which is detrimental not only to the parasite but also to the host, as onchocercal pathology is associated with dying or degenerating and not intact microfilariae. Immunological hyporeactivity is more prominent in patients with skin microfilariae showing mild clinical symptoms, in contrast to those with few skin microfilariae who manifest severe clinical symptoms and relatively strong cellular and humoral immune responses. Successful transmission of the parasite is thus facilitated. Although the exact mechanism of the observed immunological hyporeactivity is not clear, it may exist at different stages of T cell activation and possibly involves immune tolerance, parasite-derived immunosuppressive antigens, etc. In addition, the onchocercomata may be an effective means by which the adult worms can shield themselves from host defense mechanisms.

Vaccines

Prevention of onchocerciasis based on vector control by spraying larvicides has achieved partial success in some areas. The drug ivermectin, widely used to control onchocerciasis, is effective only against microfilariae and needs repeated dosage, thus requiring a sophisticated primary health care infrastructure, which is lacking in most endemic areas. Development of a vaccine that confers immunity to infection over a prolonged period is considered a more realistic solution.

Epidemiological, clinical and animal model data suggest the existence of protective immunity to onchocercal infection. For example, the level of skin microfilariae in infected individuals attains a plateau level after the fourth decade of their lives; in hyperendemic areas, in spite of repeated infection, a percentage of the exposed population remains infection free throughout their lives, even in the absence of a chemotherapeutic agent; residents in many areas are exposed to thousands of infective larvae per year but only a small percentage of these develop into adult worms; and it has been possible to induce protective immunity in mice immunized with radiation-attenuated infective larvae. Larval antigens seem to be the

best targets for developing a prophylactic vaccine. Heterologous protection by antigens conserved among different filarial parasites remains a possibility. Availability of suitable animal models (*O. volvulus*/*O. lienalis* – mouse, *Acanthocheilonema vitae* – jird, *O. ochengi*/*O. lienalis*/*O. gibsoni* – cattle) and sufficient quantities of recombinant parasite antigens have made it possible to study the efficacy of vaccine candidates. Although no vaccine is yet available to control onchocerciasis, the recent demonstration of the ability of some onchocercal antigens, e.g. OvB20 and tropomyosin, to induce partial protection in a rodent model of onchocerciasis is encouraging.

See also: Antigen; Cell-mediated immunity; Cytokines; Delayed-type hypersensitivity; Dermatological infections; Eosinophils; Eye infections; Filariasis, lymphatic; Humoral immunity; Immune complexes; Immune response; Immunopathology; Immunosuppression; Lymphocytes; Parasites, immunity to; Vaccines.

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ONTOGENY OF THE IMMUNE RESPONSE

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The acquisition by lymphocytes of the ability to mount immune responses is related to a series of complex cellular and molecular processes which take place during ontogeny. Lymphocytes are divided into two major functionally distinct but morphologically indistinguishable subsets: B and T cells. These are distinguished by different functions, but in the course of ontogeny their precursors can be distinguished by other phenotypic traits and molecular features.

Lymphocytes are derived from multipotent stem cells harbored in the fetal hematopoietic organs such as the fetal liver. During the differentiation process, multipotent progenitors give rise to the progenitors of B and T lymphocytes (Figure 1). In adult life, B cells differentiate in the bursa of Fabricius in birds and in the bone marrow in mammals, whereas T cells differentiate within the thymus.

Phenotypic changes during ontogeny

Various receptors specific for biologically active ligands together with cell differentiation antigens are associated with the membrane of all cell types, including the lymphocytes. Immunoglobulins are the specific receptors of B cells, whereas T cell receptors (TCR) are the specific antigen receptors of T cells.

A number of cell differentiation antigens are associated with mature B and T cells, and others are expressed at certain stages of ontogeny of the immune system. Thus, multipotent progenitors express a low density of Thy-1 antigen present on mature T cells, Gr-1 (an antigen characteristic of granulocytic lineage), Mac-1 of monocytic lineage, B220 of B cell lineage, and Sca-1 antigen. During the differentiation of multipotent progenitors into B cell progenitors, they lose the Gr-1, Mac-1 and Sca-1

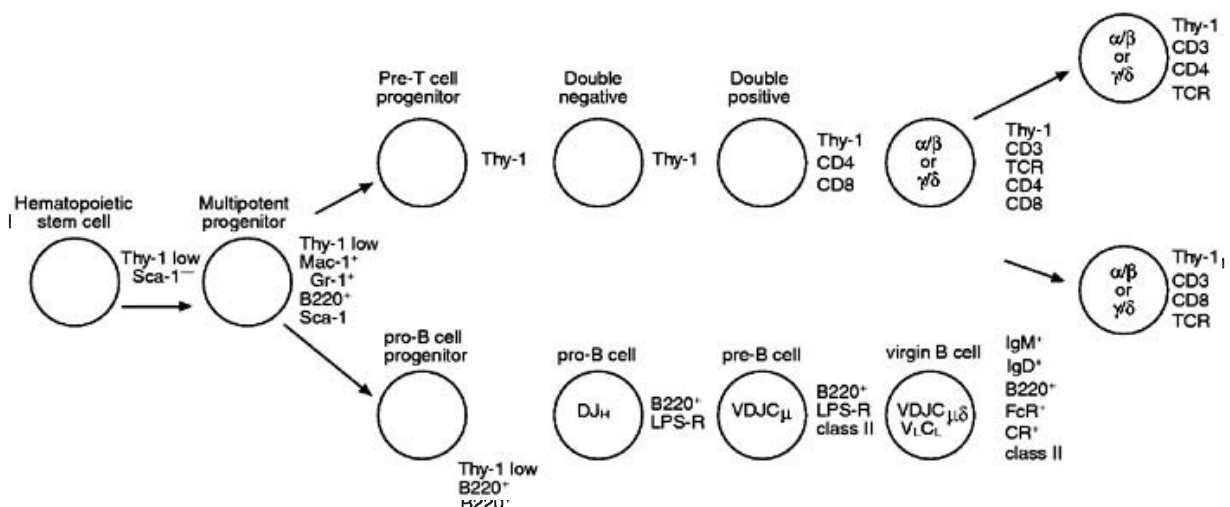


Figure 1 Different pathways of T and B lymphocyte.

antigens, and their phenotype can be defined as Thy-1⁺, Ig⁻, B220⁺. There are no good markers to distinguish the progenitors of B cells from pro-B cells, which are the most primitive B cells giving rise to the B lineage.

Stroma cells in fetal liver and bone marrow constitute the microenvironment in which lymphocytes of the B lineage differentiate. Stroma cells are considered to be nonhematopoietic and are a very heterogeneous population, but it is not clear whether or not stroma cell subsets have different effects on the differentiation process of various lineages. Stroma cells produce biologically active substances which can play an important role in the differentiation and proliferation processes. For example, there is good evidence that interleukin 7 (IL-7), which promotes the proliferation of pro-B cells as well as of the double negative (CD4⁻ CD8⁻) T cells, is a product of stromal cells.

The pro-B cell (pre-B I cell) is the most primitive cell of the B lineage. It expresses leukosialin (CD43), CD19, *c-kit* and surrogate receptor λ encoded by *VpreB* and $\lambda 5$ genes associated with a glycoprotein complex gp35–gp130. Pro-B cells differentiate into pre-B cells (pre-B II cells). The hallmark feature of this heterogeneous set of cells is the absence of surface immunoglobulin (Ig) and the presence of cytoplasmic μ chain. They also express major histocompatibility complex (MHC) class II antigens. Two discrete stages of differentiation have been distinguished in pre-B II cells: 1) cycling large pre-B cells which have lost *c-kit* expression and partially lost expression of CD43, express $V\lambda 5$ associated with μH and express CD25; 2) resting small pre-B cells in which V_L (κ and λ) genes are rearranged and in which CD40 is expressed.

Pre-B cells differentiate into immature B cells expressing surface IgM, CD19, CD25, CD40 and Fc and complement receptors. These cells differentiate into mature virgin B cells which lose CD25 but co-express IgM and IgD.

T cell lineage development is divided into three stages: the prethymic phase, the thymic phase, and the mature phase. The prethymic phase, during which multipotent stem cells develop into prethymic cells, begins with hematopoiesis in the fetal liver and then in bone marrow. These cells lack TCR as well as the CD4 and CD8 antigens, and are called double negative. Prethymic cells reach the thymus via the bloodstream, and enter by passing between endothelial cells in venules and then lodge in the medulla and at the corticomedullary junction. At the beginning of the thymic phase, cells divide rapidly and have the appearance of lymphoblasts and express a pre-T α receptor. Initially, these cells differentiate

into lymphocytes which acquire both the CD4 and CD8 antigens ('double-positive' cells) and then TCR and CD3. These cells give rise to CD4⁻ CD8⁻ and CD4⁺ CD8⁺ mature lymphocytes.

Molecular changes associated with ontogeny

There are complex molecular events associated with the development of the B cell lineage consisting of activation or repression of genes encoding cytodifferentiation antigens, or activation of genes encoding various receptors associated with the lymphocytic membrane. However, the most important molecular events are related to the expression of immune receptor; namely, the Ig of B cells and the TCR of T cells. The surface Ig of B cells is the product of rearranged V, D, J and C gene segments of heavy chain and V, J, C segments of κ and λ light chains.

The earliest B cell-specific rearrangement of Ig genes occurs in pro-B cells and consists of $D \rightarrow J_H$ rearrangement. This process is associated with the activation of recombination activating genes RAG-1 and RAG-2 as well as of terminal deoxynucleotidyl transferase (TdT) which plays a role in augmentation of diversity by addition of nucleotides during the gene rearrangement process. The activation of these genes leads to the rearrangement of the heavy chain locus and expression of μ chain. At this stage, the pro-B cells begin to multiply and differentiate into large pre-B II cells. At this point the RAG genes are downregulated, perhaps in order to achieve allelic exclusion. They are reactivated at the small pre-B II stage to permit the rearrangement of the Ig light chain loci and are then irreversibly shut down when pre-B cells differentiate into immature small lymphocytes.

Following the successful assembly of a complete Ig light chain and its combination with the heavy chain, a complete IgM antibody molecule appears on the surface of mature B lymphocytes. κ light chain loci rearrange before λ loci. Successful κ gene rearrangement seems to prevent further V gene rearrangement. If κ rearrangement is unsuccessful in both alleles, λ genes will then recombine.

Mature B cells are characterized by coexpression of surface IgM and IgD. This phenomenon is possible since a single VDJ, associated with two different isotypes, is enabled by differential splicing of mRNA.

In contrast to B cells which have a single type of immune receptor – namely, immunoglobulin molecules – T cells can express one of two types of receptor: α/β or γ/δ . Both of these receptors are associated with the CD3 molecules, a cytodifferentiation antigen present only on the T cell lineage and which

plays a role in the activation of T cells. The α , β , γ , and δ chains of heterodimeric TCR molecules are encoded by V, J, and C segments similar to Ig molecules, with the β and δ chains additionally containing D segments as found in Ig heavy chains.

The $\gamma\delta$ bearing T cells are the first to appear in murine fetal thymus, before day 14 of gestation. At this early stage, T cells are Thy-1⁺, CD4⁻ and CD8⁻, and about 5–10% express $\gamma\delta$ TCR. The proportion of these cells decreases dramatically after day 16 of gestation to 0.2% in adult thymus. The appearance of $\gamma\delta$ cells in thymus is followed by their migration to spleen, epidermis, and intestinal epithelium. There is strong evidence that $\gamma\delta$ T cells do not differentiate from $\alpha\beta$ T cells. This is supported in particular by data that show that δ genes are deleted in $\alpha\beta$ T cells; the location of δ genes in the α locus results in the deletion of δ genes during V α -J α rearrangement.

In ontogeny, the rearrangement of V β , DJ β genes is delayed by 1–2 days and rearrangement of the α locus occurs at a later date. The expression of $\gamma\delta$ or $\alpha\beta$ receptor is associated with the synthesis of CD3 antigen; the CD3 gene is probably expressed in prothymocytes before the rearrangement of the $\gamma\delta$ or $\alpha\beta$ genes and before the appearance of the CD4 or CD8 cytodifferentiation antigens.

At the double-negative stage, the cells express RAG genes which generate a functional β chain. This event promotes the differentiation of the double negative to the double positive stage, which express low levels of CD3. At the double-positive stage β chain is expressed on the surface with pre- α chain. The expression of this pre-TCR signals the progression to the rearrangement of the TCR α locus.

Targeted gene deletion technology allowed the determination of the precise role of several genes in the development of B and T cells. One transcription factor that governs the early commitment of stem cell precursors to the lymphoid lineage is Ikaros. Knock-out of the Ikaros gene prevents the appearance of pro-B cells, double-negative T cells as well as natural killer (NK) cells, indicating that the Ikaros gene controls the differentiation of lymphoid lineage precursors from stem cells. The deletion of RAG genes arrests the differentiation of B cells at the pre-B I stage, since no pre-B cells containing μ chain are detected in RAG knockout mice. Three other targeted mutations arrest the development of B cells at the same stage. They are inactivation of transcription factors such as Pax-5/BsAP, of $\lambda 5$ and of the Ig β signal molecules associated with cell surface Ig.

Collectively, these observations show that, in particular, molecular events that concern the genes which encode the immune receptor are associated

with various stages of the differentiation of T and B lineages.

Ordered expression of various V gene families and acquisition of antigen specificities during ontogeny

In pioneer studies, Silverstein demonstrated that responses of lambs to certain antigens, such as viruses, ferritin, azoproteins, ovalbumin and hemocyanin, could be obtained during fetal life, whereas responses to diphtheria toxoid, O antigen, and BCG occurred only after 40 days of postnatal life. Studies on antibody responses and of idiotype expression in young mice have also helped to show that there is a sequential or ordered activation of clones during postnatal life. There are some polysaccharides such as $\beta 2$ –6 fructosan, *S. tranaroe* lipopolysaccharide (LPS) bearing the α -methyl-D-galactoside immunodominant sugar, or $\beta 1$ –6 galactan, which can elicit an immune response in 1-day-old mice. Immunization with $\beta 2$ –6 fructosan is not paralleled by an increased expression of A48 and UPC10 cross-reactive idiotype (IdX). In contrast, about 25% of LPS-specific antibodies express MOPC387 IdX of a myeloma protein specific for *Salmonella tranaroe* LPS. Similarly, 1-day-old mice immunized with gum ghatti develop a significant antigalactan plaque-forming cell (PFC) response, of which 30% expressed the X24 idiotype. Studies on the ontogeny of antiphosphocholine (PC), phenylarsonate (Ars), and trinitrophenyl (TNP) antibody responses have shown that the production of antibodies against these antigens, which share the IdX of myeloma proteins with the same specificity, can be induced in 1-week-old mice. Thus the majority of the PC specific precursors bearing T15 IdX can be detected 4–5 days after birth, while cells able to produce IdX⁺ Ars-specific antibodies are present before day 7 in neonatal mice. Immunization of 1-day-old mice with TNP-LPS and of 1-week-old mice with TNP-Ficoll elicited a significant anti-TNP response. However, the 460Id expressed on the DNP-binding myeloma protein MOPC460 was detected only in 1-week-old mice immunized with TNP-LPS and in 3-week-old mice immunized with TNP-Ficoll. The activation of 460Id⁺ anti-TNP clones in 7-day-old mice coincided with the age when diversification of the anti-TNP response occurs. In the case of $\alpha 1$ –3 dextran, although the precursors expressing MOPC104Id can be detected during the first week of life, J558IdX precursors appear only 15–22 days after birth and then rapidly become dominant. We have further observed a substantial ontogenic delay in the case of anti- $\beta 2$ –1 fructosan (inulin) responses. A substantial increase

in the anti-inulin response bearing IdX has been observed only in 28-day-old mice. This delayed response is associated with a lack of precursors in young animals. A delayed ontogenic response was also observed in the case of α 1-6 dextran and is also related to the absence of precursors. Fernandez and Moller actually showed that the precursors of anti- α 1-6 dextran can only be detected in 1-month-old mice.

The majority of the data discussed above concern T independent antigens and, therefore, the delayed ontogenic responses are not due to the immaturity of T cells. The sequential activation of some antibody responses cannot be ascribed to a lack of V gene rearrangements in young mice. It seems unlikely that the late maturation of some responses can be explained by the tolerance of the precursors in young animals. In the case of late anti- β 2-1 fructosan responses, the tolerogen would have to be inulin rather than levan. The lack of responsiveness is limited to β 2-1 fructosan and not to β 2-1 fructosan linkage, and both epitopes are carried by bacterial levan which is an environmental antigen. A more plausible explanation is that the sequential activation is acquired by antigen-independent generative mechanisms or T cell influences. Certain unknown mechanisms may determine the time of particular $V_H:V_K$ pairing that might be critical for the specificity of the combining sites recognizing particular epitopes.

Finally, the sequential activation of clones expressing a particular set of V genes can be driven by internal forces such as anti-idiotypic antibodies. This idea is strongly supported by the data of Vakil and Kearney who, while analyzing the binding specificity for some major IdX of antibodies produced by hybridomas originating from fetal liver or neonates, observed that a high number (7%) of them exhibited anti-Id activity.

There are numerous V_H and V_K germ-line genes both in mice and humans. Based on protein sequence homology, the V_H and V_K germ-line genes were classified into several subgroups and now, based on DNA homology, they have been classified into various families. The germ-line genes belonging to a family are grouped in clusters, rarely interspersed, and provide an order on chromosome 12 for heavy chain or on chromosome 6 for V_K light chain.

Analysis of V gene family expression in Abelson-transformed pre-B cell lines has shown a preferential usage of 3' families. In fact, V_H 81X, the most D-proximal member of this family of genes, has been observed in hybridomas of pre-B cell type as well as those prepared from fetal liver. In contrast, different results have been obtained in studies in which the usage of gene family was investigated in nontransfor-

med pre-B cell lines prepared from 6- to 8-week-old nude BALB/c mice. In this study, it was shown that all probes hybridized with detectable intensity to RNA from resting pre-B cells. These data indicate that all V_H gene families were transcriptionally active in resting pre-B cells from adult mice. Incubation for 7 days with dendritic cells and mitogen-stimulated T lymphocytes caused a higher expression of the V_H 7183 family suggesting that the expression of this family is regulated by factors other than ontogeny and, perhaps, is related to specific stages of the differentiation of the B cell lineage. The functional significance of the nonrandom rearrangement of V_H genes is of considerable importance in terms of the emerging functional B cell repertoire of the fetus.

Yancopoulos and Alt have hypothesized that the position-dependent preference of V_H family utilization in pre-B cells and neonatal liver is most likely related to a one-dimensional tracking mechanism, mediated by recombinases, during VDJ joining rather than to a dissociative joining related to collisions during three-dimensional diffusion. There are data which indicate that V_H genes from one family can be replaced by V_H genes from another family in young lymphocytes. It appears that V gene replacement is a rather rare event; however, it may contribute to the establishment of the pre-B cell repertoire. A highly restricted set of V_H gene segment usage has also been observed in human fetal B cells. Analysis of human V_H repertoire at 130 days of gestation has shown a biased usage of certain J_H genes (J_H 3, J_H 4 and J_H 5) and, also a V_H gene designated 56P1 which shows a high homology with murine V_H 81X, a member of the V_H 7183 family preferentially rearranged in murine pre-B cells.

Similarly, certain V_K families are preferentially expressed during ontogeny. Kaushik and colleagues analyzed the usage of V_K families by neonatal murine B cells using an LPS-induced B cell colony assay. The results show that a group of V_K families such as V_K 1, V_K 9 and V_K 8, located in the middle of the V_K locus, are highly used by neonatal C57BL/6 mice. Interestingly, the expression of V_K 21, the most J_K proximal family, was not observed among neonatal B cell colonies. These data suggest that V_K usage in neonatal mice does not reflect positional bias for the expression of 3' families, rather a preferential usage of V_K 1 and V_K 9 family, located in the center of the V_K locus. The differences imply that the mechanisms for V_K gene rearrangement and expression differ from those controlling the V_H locus.

See also: B lymphocyte differentiation; Bone marrow and hematopoiesis; Bursa of Fabricius; Thymic epithelium: potential role in regulatory T cell tolerance;

Immune response; Thymus; T lymphocyte differentiation.

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OPISTHORCHIASIS-CLONORCHIASIS

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Opisthorchiasis, caused by *Opisthorchis viverrini* and *O. felineus*, and clonorchiasis, caused by *Clonorchis sinensis*, are the two most important liver fluke infections in humans. Juvenile flukes, excysted from infective metacercariae present in poorly cooked fishmeal, migrate from the duodenum into the bile duct where they develop into mature worms and survive for several years. The most obvious pathological change is the proliferation of the biliary epithelium thought to be the result of worm movement, toxic metabolites or the immunological response of the host. The most serious complications are biliary obstruction with or without cholangiocarcinoma. Diagnosis is currently based on the demonstration of eggs in stool, duodenal fluid or bile. However, serodiagnosis for antibody detection in the patient's serum has been reported to give satisfactory results. A monoclonal antibody-based ELISA has been recently reported for the detection of soluble antigen secreted in the feces of infected individuals. Specific DNA probes are also useful for the demonstration of parasite egg DNA. Praziquantel is highly effective and is currently the drug of choice for treatment. Vaccination is neither available nor practical.

Characteristics of the liver fluke antigens

Both somatic extract and metabolic or excretory-secretory products (ES antigens) of *Opisthorchis* and *Clonorchis* are highly immunogenic in humans and

the antibodies produced cross-react extensively. Polyacrylamide gel electrophoretic analysis of the somatic extracts from both parasites shows similar protein patterns.

Detailed analysis of adult *Opisthorchis* somatic extract shows the presence of a large number of antigens ranging from 16 to >116 kDa. The predominant components of 16–17 kDa are associated with the parasite tegument and the 16 kDa protein reacts strongly with opisthorchiasis serum. The ES antigens obtained by *in vitro* maintenance of the parasites contain predominantly an 89 kDa glycoprotein that reacts strongly with sera from patients with opisthorchiasis. A number of sera from patients with clonorchiasis also react. Monoclonal antibodies (mAb) to the secreted 89 kDa and the somatic 16 kDa proteins give intense immunofluorescent staining of the gut secretion and surface tegument, respectively. In addition, mAb against another somatic component of around 90 kDa reacts most strongly with muscle fibers underneath the surface tegument as well as those lining both oral and ventral suckers, pharynx and cecum.

Immune response of the host

Patients infected with *Opisthorchis* or *Clonorchis* have antibody responses that can be detected by a number of immunological techniques including ELISA, immunoelectrophoresis, passive agglutination and immunofluorescence. Although a high

degree of sensitivity and specificity has been achieved using crude somatic antigen, the availability of a more refined antigen should enhance the potential of these techniques for the diagnosis of the infection.

Antibodies in the serum of patients and of experimentally infected animals react with all developmental stages of the parasites. Both stage-specific and cross-reactive common antigens have been detected and identified. Although the predominating classes of antibodies in the serum are immunoglobulin G (IgG) and, to a lesser extent, IgE, on rare occasions small amounts of IgA and IgM have also been detected. On the other hand, a predominant class of antibodies in the bile is secretory IgA. However, IgG, IgE and rarely IgM antibodies have also been detected. It should be mentioned that parasite antigens also have IgE-potentiating activity, as is evident from elevated total IgE levels in the serum.

Although high antibody titers have been reported in the serum of patients and experimental animals, the protective capacity of these antibodies is still questionable. It was previously reported that these liver flukes were killed in the presence of fresh serum from infected humans or experimentally infected animals, thus suggesting the protective role of complement-fixing antibody. However, there was also a report that *Opisthorchis* could activate complement via the alternative pathway, resulting in tegumental damage and parasite killing. Cell-mediated immune responses also develop following natural infection or in animals immunized with the parasite antigen. The role of such T cell responses have yet to be established. However, different lines of evidence suggest that following a primary infection there is little, if any, protection against reinfection by the same parasite. Passive transfer of either serum or lymphoid cells or both from infected donor animals fails to confer protection in recipients against a subsequent challenge. Epidemiological data and clinical observations of patients residing in endemic areas are consistent with a lack of protective acquired immunity.

Evasion of the host defenses

Several parasite and host factors may contribute to the longevity and survival of these flukes within the biliary system, which may be considered to be an immunologically privileged site. Both IgG antibody and complement previously reported to kill these parasites are present in low quantities in bile. However, overwhelming evidence points to the conclusion that the antibody produced, regardless of its isotype, seems to have very little protective power. Moreover, a suppressive serum factor that interferes with macrophage function has been reported in patients

with opisthorchiasis. One other liver fluke (*Fasciola hepatica*) is also known to secrete lymphotoxic substance(s). It is possible, therefore, that these factors may be responsible, at least in part, for the immunosuppression noted during *O. viverrini* infection in experimentally infected animals, since following praziquantel treatment immune responses return to normal. Lastly, these parasites can readily shed their surface tegument following damage by immune mechanisms, thus providing them with another strategy to evade the host's defenses.

Vaccines

Although vaccines may be neither essential nor practical for the control of liver fluke infection, for academic reasons several attempts have been made to induce immunity in experimental animals using a number of parasite preparations. However, the results have been largely unsatisfactory. This is not unexpected in view of the fact that natural infection itself is known to confer little, if any, immunity to reinfection. For instance, a large proportion of individuals in an endemic area of infection can be readily reinfected following praziquantel treatment. Moreover, even if an effective vaccine becomes available, it would have to be given during the first few years of life, as young children in endemic areas are known to acquire infection very early. Furthermore, if the infection induces immunosuppression in humans, as it does in experimental animals, the vaccine will need to overcome such effects.

See also: Fascioliasis; Gastrointestinal tract infections; Parasites, immunity to.

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OPPORTUNISTIC INFECTIONS

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Opportunistic infections occur when host defenses are compromised by disease or its treatment. The pattern of infection that may occur in a compromised host can be predicted to some extent from an understanding of the nature of the underlying defect and the normal mechanisms that protect against infection with particular microorganisms. Table 1 illustrates the effect of various disease states on host defenses, and the kinds of infection typically encountered. By far the commonest conditions associated with opportunistic infection are malignant disease,

organ transplantation, and human immunodeficiency virus (HIV) infection. Immune defects in these patients may be selective, but frequently affect more than one aspect of the immune response. In general, the risk of infection is influenced more by the duration of immune suppression than its degree.

Infections occur readily with organisms that are recognized pathogens, but are typically caused by commensal or other normally nonpathogenic agents. Thus community-acquired infections may be caused by ubiquitous saprophytic microorganisms such as

Table 1 Pattern of infection in disease states associated with impaired host defenses

Disease state	Main host immune defects	Typical pattern of infection
Hospitalization, various conditions	Damage to mechanical barriers from i.v. cannulation, malignancy or cytotoxic therapy, obstruction or perforation	Bacteremias, phlebitis, pneumonias, empyemas, peritonitis, etc.
Acute leukemias, aplastic anemia	Neutropenia, phagocytic deficiency	Bacterial infections (bacteremias, pneumonias, soft-tissue infections); invasive candidal and fungal infections
Primary hypogammaglobulinemia, common variable immunodeficiency	Impaired antibody production	Bacterial infections, esp. pneumonias with <i>H. influenzae</i> , <i>S. pneumoniae</i> , <i>S. aureus</i>
B cell lymphomas, chronic lymphocytic leukemia, myeloma	Secondary hypogammaglobulinemia	As primary hypogammaglobulinemia
Transplantation, immunosuppressive and cytotoxic therapy	Impaired T cell responses; neutropenia and phagocytic impairment	Bacteremias; pneumonitis; herpesvirus and mycotic infections; EBV-induced lymphoma
HIV infection (AIDS)	CD4 and monocyte/macrophage impairment	Pneumocystis, toxoplasma, cryptosporidium, atypical mycobacteria, CMV, HSV, candidiasis; Kaposi's sarcoma, EBV-induced lymphoma

Aspergillus spp., *Nocardia* and *Cryptococcus*, or by exposure to geographically localized mycoses such as histoplasmosis, as well as by more common pathogens such as the pneumococcus, respiratory viruses and – in some communities – tuberculosis and common parasitic infections such as strongyloidiasis. Immunosuppressed patients are also prone to more general public health hazards such as the presence of cryptosporidium in water supplies, *Listeria monocytogenes* in unpasteurized milk products, and salmonella food-poisoning.

Hospital-acquired (nosocomial) infections are particularly significant, as hospitals may harbor organisms that are resistant to standard antimicrobial therapy, as well as more serious pathogens such as *Pseudomonas* spp. Hospitalized patients are more likely to undergo invasive procedures, and may acquire infection from blood transfusion. Furthermore, some hospital buildings are potential sources of *Aspergillus* and *Legionella* spp.

The successful management of infection in the compromised host requires a high degree of suspicion, a knowledge of the underlying disorder, and a willingness to investigate rapidly and aggressively. The usual signs of infection may be absent: for example, fever may be reduced or suppressed in patients taking corticosteroids, and local inflammation and abscess formation may be impaired in patients with neutropenia. Diagnostic tests may also require different interpretation: antibody responses may be impaired in patients with humoral immunodeficiency, and skin tests may be similarly unreliable in those with cellular immunodeficiency. Standard microbiological culture techniques may require modification to detect opportunistic invaders, and tissue biopsy may be required to characterize some invasive infections.

The pattern of infection in the compromised host has been altered somewhat by the availability of effective prophylaxis for some of the commoner opportunistic infections. Cotrimoxazole effectively prevents pneumocystis pneumonia in patients with hematologic malignancy and in transplant patients. It is less useful in HIV infection as hypersensitivity reactions to the sulfonamide component are common. Inhaled pentamidine is a useful alternative. Herpes simplex virus (HSV) reactivation can be prevented by acyclovir; some protection may also extend to other herpesviruses including cytomegalovirus (CMV). Some of the newer quinolone antibiotics such as ciprofloxacin and norfloxacin can reduce the incidence of bacteremia in neutropenic patients.

Infections in patients with malignant disease

Epithelial malignancy

Patients with epithelial malignancy do not typically have any intrinsic immune defect, although impaired responses to infection may result from treatment with radiation or cytotoxic chemotherapy. On the other hand, the tumor itself may promote local infection through obstruction (for example of the bronchus leading to pneumonia), ulceration or perforation of a viscus. Hospitalization, with its attendant risk of instrumentation may predispose to infection through damage to mechanical barriers (e.g. skin or mucous membranes).

Hematologic malignancy

Hematologic malignancies are typically associated with profound defects in one or more aspects of host defense, with the result that infection is a major cause of morbidity and mortality in this group of patients.

Neutropenia and impaired phagocytic capacity due to bone marrow failure is an intrinsic feature of the acute leukemias but can occur from marrow infiltration in the later stages of other malignancies, and is an almost inevitable if temporary side-effect of curative cytotoxic chemotherapy for these and other conditions. Neutropenic patients are particularly susceptible to bacteremia from Gram-negative bowel commensals, although the increasing use of long-term central venous catheters for vascular access has been associated with a rise in the incidence of staphylococcal and other Gram-positive bacteremias and local tissue infections.

Focal sepsis in neutropenic patients commonly affects the lungs, oropharyngeal mucosa, perineum and skin. Invasive mycotic infections with *Candida* spp. or *Aspergillus* spp. commonly occur in patients with prolonged neutropenia. These are usually associated with intensive cytotoxic therapy – which also impairs cell-mediated immunity, and prolonged antibiotic therapy – which reduces the so-called ‘colonization resistance’ afforded by normal commensals.

Fever in severely neutropenic patients must be treated promptly. Blood and other appropriate cultures must be taken and the patient given intravenous antibiotics without waiting for the results. The choice of suitable antibiotics is normally determined by local policy in the light of knowledge of typical pathogens and patterns of antibiotic resistance. A typical approach is to use ceftazidime or a combination of an aminoglycoside with a semisynthetic

penicillin such as piperacillin. If cultures are negative and the fever persists for more than 48 h, empiric antimycotic therapy with amphotericin should be considered.

Impaired humoral immunity with hypogammaglobulinemia is a feature of low-grade B cell malignancies including some types of non-Hodgkin lymphoma, chronic lymphocytic leukemia, and myeloma. The pattern of infection closely resembles that seen in primary humoral immunodeficiency – predominantly respiratory tract infections with *Streptococcus pneumoniae* or *Haemophilus influenzae*. The incidence of such infections can be reduced by regular infusions of normal immunoglobulin.

Cellular immunodeficiency is common in patients with Hodgkin's disease, but is also an undesirable effect of cytotoxic and corticosteroid therapy and thus affects a much wider range of patients. The commonest associated infections are due to reactivation of latent herpesviruses, described below. *Pneumocystis carinii* infection may occur in patients who have received particularly prolonged or aggressive chemotherapy, but can now be largely prevented in this population by co-trimoxazole. Meningitis and other infections with listeria or cryptococci are seen occasionally. Tuberculosis is classically associated with Hodgkin's disease but is now uncommon, although previously infected patients undergoing cytotoxic therapy are at high risk of reactivation and should receive prophylactic medication such as isoniazid.

Corticosteroid therapy

Corticosteroids are commonly used in the treatment of lymphoid malignancies, but are also used as an anti-inflammatory agent in nonmalignant conditions such as autoimmune diseases. They act in these conditions in part by suppressing cell-mediated immunity (T cell responses), but their beneficial effects are at the expense of a risk of infection, particularly reactivation of latent herpes virus. Particular care must be taken in patients who have not previously had varicella (chickenpox). Primary chickenpox infection in patients with impaired cell-mediated immunity – including those on steroids – is frequently severe and potentially fatal. Early visceral dissemination is common and may be the presenting feature. Special care should therefore be taken in children, who may not previously have been exposed to chickenpox.

Infection in transplant recipients

Successful organ transplantation requires a profound modification of the recipient's immune system to prevent graft rejection. Not surprisingly, graft recipients

are susceptible to infection. As graft rejection is predominantly a cell-mediated process, immunosuppression is directed primarily at T cell responses, and the pattern of infection is again predictable. Agents commonly used are prednisolone, cyclosporine and azathioprine; antilymphocyte globulin is used occasionally. The duration of immunosuppression required is variable: marrow graft recipients typically require only a few months' immunosuppression to prevent graft-versus-host disease (GVHD), whilst renal graft recipients generally require life-long immunosuppression.

Bone marrow transplantation (BMT)

Although allogeneic BMT is occasionally performed for primary marrow failure (aplastic anemia), it is most widely used to allow very high doses of cytotoxic chemotherapy or radiation to be administered to patients with hematologic malignancy without fear of fatal marrow toxicity. Following such therapy, rejection of histocompatible grafts is rare, but immune competent cells derived from the graft may react against the host, producing damage to skin, gastrointestinal tract and liver. This GVHD can be prevented by selectively eliminating the cytotoxic T cell population from the donor marrow prior to transplantation, or modified by short-term immunosuppression with cyclosporine and/or other agents such as methotrexate, cyclophosphamide or prednisolone.

Following successful BMT, the recipient's immune system is replaced by cells of donor origin. Immune reconstitution takes about a year, but is delayed or prevented in patients with GVHD. Responses to infection are impaired until this process is complete, and may be severely compromised by GVHD.

The pattern of infection in BMT recipients changes with time after transplant. In the first 3–4 weeks patients experience severe mucosal ulceration, are profoundly neutropenic, and are prone to bacteremia and reactivation of herpes simplex infections. During the second and third months a major and often fatal problem is interstitial pneumonitis. Total body irradiation is an important factor in the development of pneumonitis, but about one-half are associated with CMV infection or reactivation. Pneumocystis pneumonia is uncommon. Primary CMV infection can be avoided in CMV-negative patients provided their donor is also CMV-negative and only CMV-negative blood products are used. Prevention of reactivation in CMV-positive recipients is difficult, but early detection and therapy with ganciclovir may be effective. Fatal CMV infections are less common following recovery of endogenous antibody production about 2 months after transplantation.

Life-threatening infections are uncommon after the first 3 months unless GVHD persists. Zoster reactivation occurs in about one-half of BMT recipients, but later than HSV and CMV infection. It may be typically segmented or more disseminated. Primary chickenpox is uncommon in adults, but less so in children. Presentation may be atypical with symptoms or signs of visceral rather than skin involvement.

Infections in other transplant recipients

Although long-term immunosuppression is usual in recipients of kidney, liver and heart transplants, the major risk of infection occurs in the first few months after transplantation. Immunosuppressive therapy is at its most intense as rejection is most likely to occur during this period. Patients are hospitalized for much of this time and therefore nosocomial infections are common. In addition, patients may develop bacterial infections associated with surgery or other invasive interventions, may experience exacerbations of pre-existing infections, or may even acquire infection transmitted via the grafted organ itself.

In the first month after transplantation bacterial infections and HSV reactivation are common. CMV and pneumocystis pneumonia are a hazard especially in the second and third months, though routine co-trimoxazole prophylaxis has reduced the latter. Herpes zoster occurs in around 25% but generally at a later time. Other opportunistic infections are seen occasionally.

Epstein-Barr virus (EBV) primary infection or reactivation is not uncommon following transplantation. It generally produces little or no distinctive clinical illness and may only be recognized retrospectively by serology. Its major importance is the increased incidence of EBV-associated B cell malignant lymphoma in transplant recipients.

Acquired immune deficiency syndrome (AIDS)

Human immunodeficiency virus (HIV) infects helper (CD4⁺) lymphocytes and cells of the monocyte/macrophage series. This produces a slowly progressive, profound immune defect which may be further exacerbated by opportunistic infection with, for example, CMV, EBV and other viruses. Several months or years may intervene between initial HIV infection and the development of opportunistic infection. A febrile prodrome associated with general malaise, intermittent diarrhea, and severe weight loss may precede the first identifiable opportunistic infection.

The pattern and timing of opportunistic infections seen in HIV patients depends upon their geographical location and the local prevalence of infectious dis-

eases. In Africa, for example, where the general prevalence of infection is high, opportunistic infections tend to occur early in the course of HIV illness and with similar organisms to those encountered commonly in the general population such as tuberculosis, pyogenic infections and salmonellosis. In contrast, in Western countries infections occur later, with pneumocystis pneumonia, toxoplasma encephalitis, cryptococcal meningitis, cryptosporidial diarrhea and local candidal infections, being the most commonly encountered. As the disease progresses, multiple simultaneous infections may occur with any of the above and in addition atypical mycobacterial infections and CMV infections of the retina and gastrointestinal tract.

Two patterns of malignant disease occur in HIV-infected patients. Kaposi's sarcoma (KS) has been particularly associated with the homosexual population and is seen rarely in infected hemophiliacs. Recent evidence has linked Kaposi's sarcoma in HIV-infected individuals to concurrent infection with human herpesvirus 8 (HHV8). EBV-associated non-Hodgkin lymphoma and also Hodgkin's disease have an increased incidence in all HIV-infected groups.

See also: Acquired immune deficiency syndrome (AIDS); Bone marrow and hematopoiesis; *Candida*, infection and immunity; Cryptosporidiosis; Cytomegalovirus, infection and immunity; Epstein-Barr virus, infection and immunity; Graft-versus-host reaction; *Haemophilus*, infection and immunity; Herpes simplex virus, infection and immunity; Immunodeficiency, primary; Immunodeficiency, secondary; Immunosuppression; *Legionella*, infection and immunity; *Listeria*, infection and immunity; Lymphoma; *Mycobacteria*, infection and immunity; *Nocardia*, infection and immunity; *Pneumocystis carinii*, infection and immunity; *Pseudomonas aeruginosa*, infection and immunity; *Staphylococcus*, infection and immunity; *Streptococcus*, infection and immunity; Toxoplasmosis; Transplantation; Varicella-Zoster virus, infection and immunity.

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OPSONIZATION

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Phagocytes (granulocytes, monocytes and macrophages) can kill a wide variety of prokaryotic and eukaryotic cells, including microorganisms and tumor cells. Killing can occur intracellularly after endocytosis (e.g. after phagocytosis of bacteria) or extracellularly by secretion of factors from the phagocytes that are harmful to the foreign cell. In both cases, recognition of the particle by the phagocyte is essential. It has been known for more than 90 years that recognition and ingestion of microorganisms by phagocytes is facilitated by the binding of serum factors (opsonins) to these microorganisms.

In 1903, Wright and Douglas discovered that the uptake of staphylococci by human granulocytes is enhanced by serum. They concluded that 'the body fluids modify the bacteria in a manner which renders them a ready prey to the phagocytes'. This opsonic effect of serum (*L. opsono*, 'I cater for'; 'I prepare victuals for') was reduced, but not completely lost, when the serum was heated. Nowadays it is known that the major heat-stable opsonin is immunoglobulin G (IgG), whereas the major heat-labile component in the opsonization process is composed of components of the complement system.

Receptors for IgG and complement are the best characterized receptors involved in recognition and phagocytosis, but receptors for IgE and IgA may also serve as opsonin receptors. Antibodies and complement components usually act synergistically in the opsonization of particles (Figure 1). In addition to these opsonins, other factors may stimulate the ingestion of particles, including acute phase proteins, collectins, defensins, extracellular matrix components and tuftsin. Microorganisms that are not opsonized can also be recognized by phagocytes because of, for example, the presence of carbohydrate residues

(mannose, L-fucose, galactose, glucose and *N*-acetylneuraminic acid) on the microorganisms that bind to sugar-specific receptors on macrophages. Also, receptors belonging to the CD11/CD18 integrin family may mediate the binding of unopsonized microorganisms to phagocytes, indicating that these receptors are involved in the phagocytosis of both opsonized and nonopsonized microorganisms.

Immunoglobulins

Immunoglobulin G

The capacity of antibodies of the IgG class to promote phagocytosis is well established. IgG increases the uptake of particles by an interaction with receptors for IgG (Fcγ receptors) that are present on phagocytes. In humans, immunoglobulins of the IgG1 and IgG3 subclasses are the most effective heat-stable opsonins, and are the subclasses that are bound with the highest affinity to Fcγ receptors. Binding to antigenic determinants on the particles is mediated by the Fab fragment of the IgG molecule, whereas the Fc part of IgG interacts with Fcγ receptors on the phagocyte cell membrane. Human phagocytes can express three classes of Fcγ receptors (FcγRI, FcγRII and FcγRIII) that have different and partly overlapping functions. The low-affinity FcγRII is probably the receptor that is most important in mediating phagocytosis of IgG-coated particles. For the interaction between IgG and its receptor, it is essential that IgG is located on the outer surface of the particle (e.g. the bacterial capsule), where its Fc part is accessible to the Fcγ receptor.

In addition to forming a bridge between the particle and the phagocyte, IgG enhances the uptake of bacteria by increasing their hydrophobicity. This mechanism is probably less important than the aforementioned specific interaction between the IgG-coated particle and the phagocytic Fcγ receptor.

Immunoglobulin M

Human phagocytes do not bear a receptor for IgM on their surface. Therefore IgM itself cannot act as an opsonin. However, since IgM is a very effective activator of the classical pathway of the complement system, IgM-coated particles can be opsonized with complement components, and thus be recognized by phagocytes.

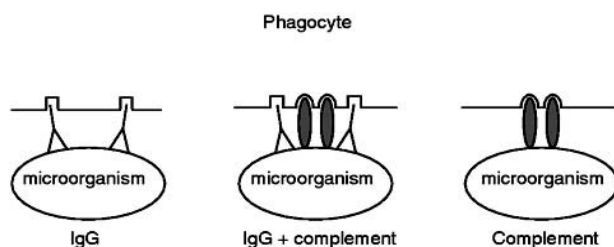


Figure 1 Opsonization by IgG alone, IgG and complement, and complement alone, and interaction of these opsonins with specific membrane receptors present on phagocytes.

Immunoglobulin A

Two types of receptors that may mediate binding and ingestion of IgA-coated particles are present on the cell surface of phagocytes: receptors for the constant part of IgA (FcR α) and the asialoglycoprotein receptor (ASR). IgA was originally thought to lack opsonic activity, or even to act as a dysopsonin (a molecule that inhibits phagocytosis). Recent reports demonstrate the capacity of IgA to promote phagocytosis of microorganisms and erythrocytes. Since IgA activates the alternative pathway of complement, opsonization of particles by IgA may be enhanced by the binding of complement components to the particle.

Immunoglobulin D

No opsonic activity for IgD has been reported.

Immunoglobulin E

High-affinity receptors for IgE (Fc ϵ RI) are present on mast cells and basophils, and low-affinity receptors (Fc ϵ RII) on macrophages, eosinophils and platelets. The expression of Fc ϵ RII on monocytes can be induced by interleukin 4 (IL-4). The main function of the phagocytic Fc ϵ RII receptor resides in the defense against parasites. Since most parasites are too large to be ingested, IgE usually does not act as an opsonin.

Complement components

Optimal phagocytosis requires the presence of both antibodies and complement components on the particle. Particles opsonized with IgG or IgM antibodies can activate complement through the classical pathway, resulting in binding of fragments of complement components to the particle. These particles will be ingested to a higher extent than particles opsonized with antibodies alone because of the presence of receptors for complement components on the surface of the phagocytes. Fc γ receptors and complement receptors have been shown to act synergistically in mediating the phagocytosis of particles opsonized with IgG and complement components. Many microorganisms do not require the presence of specific antibodies for activation of the complement system. Gram-negative bacteria can activate the alternative pathway of complement due to the presence of lipopolysaccharide in their outer membrane. Other microorganisms bear surface structures that activate complement in the absence of antibodies. These microorganisms can be opsonized with complement components in nonimmune serum or serum deficient in immunoglobulins.

The complement components known to be involved in opsonization are C1q, C4b, C3b, iC3b, C3dg and C3d. Bound C3-derived fragments are the most important opsonins generated upon complement activation by either the classical or the alternative pathway. Activation of either pathway results in the formation of a C3 convertase that cleaves C3. The major cleavage product of C3, C3b, binds covalently to the particle surface. Subsequently C3b can be degraded by a number of inactivators of complement to iC3b, C3dg and C3d. Because C3b is rapidly degraded to iC3b (especially on particles that activate the classical pathway of complement), and C3dg and C3d are poor opsonins, iC3b is probably the most important opsonin generated upon cleavage of C3.

Phagocytes bear three types of receptors that may mediate the binding and uptake of C3-coated particles: CR1, CR3 and CR4 (p150/95). The role of CR1 and CR3 in enhancing phagocytosis of IgG-coated particles by neutrophils, monocytes and macrophages is well established. However stimulation of either CR1 or CR3 alone by, respectively, C3b- or iC3b-coated particles does not result in their ingestion, although the particles are bound to the receptors. Ingestion requires the presence of very small amounts of IgG on the particle or an additional stimulus that activates CR1 or CR3. Stimuli that can induce phagocytosis *in vitro* through CR1 or CR3 alone are extracellular matrix proteins (fibronectin, serum amyloid P and laminin) or soluble components such as C5a and the tumor-promoting phorbol ester phorbol myristate acetate (PMA).

The site of binding of the C3 fragments to the activator is essential for its capacity to function as an opsonin. Studies with encapsulated pneumococci have demonstrated that the capsule forms a physical barrier between the C3b bound to the cell wall of the bacteria and C3 receptors on the phagocyte.

Other opsonins or phagocytosis-stimulating factors

Acute phase proteins

The serum concentration of C reactive protein (CRP) increases many fold during inflammation, tissue injury and bacterial infection. CRP binds to *Streptococcus pneumoniae* and mediates the phagocytosis of these bacteria. Its opsonic activity is dependent on its ability to activate the classical pathway of complement. Since it binds to *S. pneumoniae* and possibly to a few other bacteria, its importance as an opsonin in the defense against bacterial infections is limited. It has been suggested that CRP may be more important in the clearance of damaged or dead cells

from sites of tissue injury. The opsonic activity of another acute phase protein, α 2-HS glycoprotein, is a result of its ability to increase the hydrophobicity of the bacterial surface, thereby promoting ingestion of these bacteria. Since during inflammatory reactions the serum concentration of α 2-HS in contrast to that of CRP decreases, its opsonic role *in vivo* is not clear.

Collectins

Recently the collectin family of humoral lectins has been described. It includes pulmonary surfactant proteins SP-A and SP-D, and mannose-binding protein (MBP). Collectins contribute to host defense against infection through their opsonic activity and their ability to activate the complement system. Their lectin domain mediates the binding to carbohydrate structures on microorganisms, whereas collagenous regions are involved in binding to the collectin receptor on, for example, phagocytes. The collectin receptor also functions as a receptor for the complement component C1q.

Fibronectin and vitronectin

Fibronectin is a multifunctional molecule that is found in extracellular matrix and plasma. Although it primarily plays a role in cell adhesion, it is involved in many parts of the inflammatory reaction. It binds to a variety of structures, including some bacteria and immune complexes. Two mechanisms are involved in the enhancement of phagocytosis by fibronectin. Fibronectin-coated particles are recognized by fibronectin receptors on the phagocyte and thereby fibronectin promotes the attachment of these particles. However, binding of fibronectin-coated particles does not result in their ingestion and therefore fibronectin is not regarded as an opsonin. In addition to promoting attachment, binding of fibronectin to its receptor on monocytes and macrophages results in the enhanced ingestion of IgG- and iC3b-coated particles. Furthermore, fibronectin activates CR1 and CR3 receptors, resulting in phagocytosis of particles opsonized with C3b or iC3b without IgG.

Receptors for IgG and iC3b can also be activated by vitronectin (also named S protein), a glycoprotein that shares many functional properties with fibronectin. An arginine-glycine-aspartic acid sequence (RGD) present in both fibronectin and vitronectin is recognized by the receptors for these molecules. These receptors belong to the same integrin family. Since vitronectin binds to some bacteria, it may also promote attachment of these bacteria to phagocytes. Its role as an opsonin is however not yet established.

Defensins

Defensins are small cationic proteins with microbicidal activity against a wide variety of microorganisms. They are present in the granules of phagocytes and are involved in nonoxidative killing of microorganisms by these phagocytes. Certain defensins, macrophage cationic peptide-1 (MCP-1) and MCP-2 (present in neutrophils and alveolar macrophages of rabbits), can enhance the ingestion of certain bacteria and fungi by rabbit alveolar macrophages. These defensins bind to both the microorganisms and to the alveolar macrophages.

Tuftsins

The tetrapeptide tuftsins (Thr-Lys-Pro-Arg), which is derived from the CH₂ domain of IgG, has been reported to affect a wide variety of biological responses in neutrophils and mononuclear phagocytes, including phagocytosis. The stimulation of phagocytosis by tuftsins appears to be mediated by binding of the peptide to phagocytes. Therefore tuftsins can not be regarded as an opsonin itself.

Influence of antimicrobial drugs on opsonization of microorganisms

Antimicrobial drugs can change the opsonic requirements of bacteria by inducing changes on the surface of the bacteria. Drugs that inhibit protein synthesis are especially effective in this respect. For example, treatment of *Streptococcus pyogenes* with clindamycin or lincomycin decreases the expression of M protein on the bacteria. Since M protein inhibits complement activation, decreased expression of M protein results in an increased deposition of C3 molecules on the surface of the bacteria. This explains the enhanced phagocytosis of clindamycin- or lincomycin-treated *S. pyogenes* by granulocytes and monocytes. Clindamycin has also been shown to increase the phagocytosis of *Staphylococcus aureus* by granulocytes and macrophages. This has been attributed to a decreased protein A synthesis by clindamycin-treated *S. aureus*. This may result in more effective opsonization since protein A binds to the Fc part of IgG.

See also: Bacteria, immunity to; Chemotaxis; Complement, alternative pathway; Complement, classical pathway; Complement receptors; Fc receptors; Immunoglobulin, functions; Monocytes; Mononuclear phagocyte system; Neutrophils; Phagocytosis.

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ORAL IMMUNOLOGY

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The oral cavity is a unique anatomical environment consisting of specialized tissues and fluids required for the initial food intake and processing, speech, taste and other sensory perceptions. The uniqueness of the oral cavity lies in the complex association of the hardest tissue in the body, enamel on teeth, with epithelium. The teeth and oral epithelium as well as the junction between them are continuously under considerable physical, chemical and microbial pressures. Soon after birth the human oral cavity becomes colonized with a unique microbial flora which undergoes compositional alterations and additions as the oral cavity undergoes developmental changes associated with the maturation of the child. The eruption of teeth provides a unique surface for the colonization of specifically adapted microorganisms. Bacteria attached to teeth are a unique problem for the body's protective immune system since they are both difficult to reach by conventional antimicrobial cells or agents (e.g. leukocytes, antibodies and complement) and they can remain colonized for an indeterminate period of time. The shallow crevice between the tooth and gum contains the second unique problem for the host's defenses within the oral cavity. Bacterial plaques accumulate on the surface of the teeth down to the point of attachment to the tooth surface by a specialized epithelium, called the junctional epithelium. The immediate proximity of the plaque to the gum tissue in the crevicular area results in the bacterial stimulation of the host's inflammatory and immune systems. Some of these responses are seemingly protective but are not able to effectively remove the total plaque and a chronic inflammatory response to the resident bacterial plaque results. This chronic inflammatory response causes tissue destruction, resulting in loss of the epithelial attachment to the tooth and a deepening of the crevice between the tooth and gum, which then is able to harbor more and different plaque bacteria (i.e. subgingival plaque) some of which are considered pathogens.

Dental caries and saliva

Teeth are constantly exposed to saliva and bacteria in the oral cavity. Through a complex series of events the teeth are coated with salivary proteins, resulting in an acquired pellicle, which both protects the surface of the tooth and facilitates the binding of specific oral bacteria to the tooth. Attachment of free bacteria to adherent bacteria and their proliferation result in the growth and maturation of deposits of bacterial plaques on the exposed surfaces of teeth. The development of bacterial plaque deposits on teeth is a dynamic event with net results being a continuous increase in size and complexity of the plaques. Unlike the epithelium, which sloughs off the outer cell layer or the mucus-bacterial aggregates, mechanical removal is required to clean teeth of plaque deposits and within hours after cleaning the colonization process repeats itself. Consequently, there is a chronic source of microbial challenges to the teeth and to the connective tissues abutting the teeth, resulting in oral diseases.

To protect teeth, the body uses a combination of immune mechanisms contained in saliva. Caries, a major oral infectious disease, is due to the accumulation of acid-producing bacteria, *Streptococcus mutans*, in plaque and a high sucrose diet, which result in acid demineralization of localized areas of the enamel tooth surface (i.e. dental caries) under the plaque deposit. Saliva originates from the submandibular, sublingual, parotid and other minor salivary glands. It contains components common to other mucosal secretions, which vary as to the specific functions of the epithelium to be serviced. Saliva in the oral cavity is referred to as whole saliva since it contains the glandular secreted materials plus serum proteins from the gingival crevicular fluid. In addition to the rinsing effect of saliva, salivary mucins (MG1 and MG2), parotid agglutinin, lysozyme, and β_2 -microglobulin agglutinate bacteria into large aggregates which are easily cleared. Secretory

immunoglobulin A (sIgA), the adaptive immune component, is generally derived from sIgA-secreting plasma cells in the salivary glands which were stimulated through the common mucosal immune response system, although local stimulation and synthesis in the salivary gland can occur. sIgA is antigen-specific and can prevent the initial binding of bacteria, virus or fungi to epithelial cells or the acquired pellicle on teeth. sIgA can also specifically agglutinate bacteria and neutralize viruses. IgA consists of two isotypic forms, IgA1 and IgA2. IgA1 is predominant in serum as a monomer and IgA2 in saliva and other secretions in a dimeric form held together by a J chain and also having an attached secretory peptide. IgA1 is susceptible to degradation to Fab and Fc fragments by an IgA protease produced by a number of microbial pathogens. The role of sIgA specific for *Strept. mutans* in the control of dental caries remains controversial. Serum-derived IgG is also found in whole saliva, but its role in host defense in the oral environment is not defined. Other salivary proteins with antimicrobial activities are lysozyme, histatins, lactoferrin and proline-rich proteins. Histatins and acidic proline-rich proteins are only found in saliva. Interestingly, histatins appear to be tailor-made for the protection of the oral cavity, as they are potent candidacidal agents and neutralize lipopolysaccharides, as well as having antimicrobial activities against strains of *Strept. mutans* and *Porphyromonas gingivalis*, two oral pathogens. Acidic proline-rich proteins are antimicrobial as well as being active in mineral homeostasis and toxin neutralization. Lactoferrin binds iron, which is required for microbial proliferation, and lysozyme either lyses the bacterial wall or induces autolysis by the bacterium. In parallel to the antigen-specific and antigen-nonspecific antimicrobial activities, salivary proteins also help maintain the integrity of the fragile oral epithelium, buffer pH fluctuations in plaque thereby slowing acid induced tooth demineralization, and help maintain a calcium balance to counteract demineralization of the teeth.

Gingivitis/periodontitis

Gingivitis and periodontitis (G/P) make up the second major oral infectious disease. They are inflammatory conditions of the connective tissues that support the teeth. Gingivitis differs from periodontitis in that no permanent damage has occurred to the connective tissues. Loss of both normal gingival structures and alveolar bone are characteristic of periodontitis. G/P illustrate both the protective and destructive natures of the immune response in the oral cavity and closely resemble immune responses to chronic antigen deposition in the lungs,

kidneys and liver. The accumulation of bacterial plaques on teeth at the interface of the tooth and gum initiates an inflammatory response to bacterial and immune products generated from the plaque. Classical acute inflammation is the initial host response to plaque accumulation in the gingival tissues. Clinical indicators are redness and swelling of the local gingival tissue, an increase in gingival crevicular fluid (GCF) flow rate, and an increase in the concentration of polymorphonuclear leukocytes (PMNs) in the GCF. Mechanical removal of the plaque usually results in the arrest of the inflammatory disease and, in the case of gingivitis, a return of the gingival tissue to normal, with no tissue destruction and loss of attachment of the junctional epithelium to the tooth.

Polymorphonuclear leukocytes

The initial acute inflammatory response to plaque accumulation near the gum line provides the first evidence for the importance of the PMN in the protection of the gingival tissues and ultimately the periodontium. In healthy gums PMNs constantly migrate from the vascular system, through the gingival tissue, and into the gingival crevice (GC) in response to chemoattractants released from the bacteria or to immune factors (e.g. C5a, interleukin 8 (IL-8)) triggered by bacterial products. The bacterial accumulation on the teeth and in the GC directly affects the numbers of PMNs migrating into the GC and the extent to which those cells are activated. In the GC the PMNs interact with plaque bacteria or aggregates of bacteria usually coated with complement and Igs. PMNs phagocytose the bacteria or bacterial components, degranulate and synthesize and secrete cytokines. A margin of PMNs is often observed between the plaque deposits and the junctional epithelium. PMNs from healthy individuals have been shown to be bactericidal for suspected periodontal pathogenic bacteria only if the bacteria is phagocytosed after being opsonized by IgG antibodies and complement.

Strong evidence for the protective role of PMNs in the dentogingival area comes from the increased severity of disease in the periodontium of patients with neutropenia, genetically determined deficiencies in leukocyte functions (e.g. leukocyte adhesion deficiency, chronic granulomatous disease), chronic disease with an associated leukocyte functional suppression (e.g. diabetes), or drug-induced suppression of PMN activities. In contrast, immunodeficiencies of either T cell or B cell origin, including human immunodeficiency virus 1 (HIV-1) infection, are usually not associated with an accelerated loss of

tooth support tissues. The dentogingival margin may be the area in the body most sensitive to the loss of PMN function. Localized juvenile (LJP) and prepubertal periodontitis are two specific forms of periodontal disease found in young adults and children, respectively – populations not commonly afflicted by periodontal diseases. The majority of patients with these conditions have PMN functional deficiencies. In most of the LJP patients there are no other symptoms of a leukocyte functional problem. Identified defects in PMNs from these patients include genetic defects in the maximum expression of chemoattractant receptors on the cell membrane, an intracellular signal transduction defect, or an inability to kill *Actinobacillus actinomycetemcomitans*, a plaque bacterium thought to be the etiological agent in LJP. Clinical and *in vitro* experimental data strongly indicate that the PMN is the protective immune cell for the dentogingival area.

Humoral immunity

In addition to sIgA the adaptive systemic vascular humoral immune response is involved in oral immunity; however, its exact role is not clearly defined. Elevated levels of IgG and often IgA antibodies specific for subgingival plaque bacteria are usually detected in serum of patients with periodontal diseases compared with normal age-matched controls. In the majority of cases the bacterium to which the elevated serum antibodies specifically bind are the putative etiological bacteria for the type of periodontal disease identified clinically in the patient. For example, LJP patients almost always have elevated levels of polyclonal IgG antibodies specific for *A. actinomycetemcomitans* antigens and adults with chronic adult periodontitis have elevated levels of serum polyclonal IgG antibodies specific for antigens of *P. gingivalis*, a putative etiologic bacteria for that form of periodontal disease. Moderately elevated levels of antibodies to other plaque bacteria are sometimes found, but they are not definitive. The route of immunization is thought to be through the adjacent gingival tissues utilizing the tissue macrophages or Langerhans cells to process and carry the antigens to the regional lymph nodes. Tonsils and adenoids have recently become possible candidates for oral induction of immune responses. In general, plaque bacteria do not invade. *A. actinomycetemcomitans* and *P. gingivalis* are the only plaque bacteria shown to invade gingival tissues. Bacterial products such as endotoxins can be found in gingival tissues and a bacteremia is often produced during teeth cleaning, but elevated antibody responses to multiple plaque bacteria are not normally found in patients.

Local synthesis of Igs by resident plasma cells is a major feature of advanced periodontitis lesions. IgM, IgG and IgA synthesis has been identified in the inflamed gingival tissues and locally produced antibodies specific for plaque bacterial antigens have been identified. Antibodies specific for certain plaque bacteria have also been identified in GCF. Whether or not they were synthesized locally or derived from the vasculature is difficult to determine. In GCF from periodontitis sites the levels of IgG4 are found to be significantly greater than in serum, suggesting local synthesis of this IgG subclass. Further support for a role of local Ig synthesis contributing to the Ig in the GCF is indicated by the predominance of plasma cells producing IgG3 and IgG4 subclasses.

The significance of antibodies in serum or GCF specific for certain plaque microorganisms is thought to be generally protective, especially early in the disease process when colonization can be affected by a combination of PMNs, opsonic antibodies, protective antibodies such as antileukotoxin, and complement. A protective role for anti-*A. actinomycetemcomitans* antibodies is suggested by the observation that LJP patients have significantly higher levels of anti-*A. actinomycetemcomitans* than do patients with the generalized version of the disease. High levels of specific serum antibodies in immunized monkeys are associated with a decrease in oral colonization by potential periodontal pathogenic bacteria and a decrease in alveolar bone loss in infected animals compared to unimmunized controls. Immunization of gnotobiotic rats with fimbriae from *P. gingivalis* prevented periodontal disease, as indicated by alveolar bone loss in *P. gingivalis*-infected animals. However, high levels of specific antibodies in serum are more accurately indicative of disease and the presence of alveolar bone loss than identification of the suspected etiologic bacterium in the subgingival plaque, suggesting that the antibody response may be a consequence of infection rather than a controlling factor.

T cells

The relative contribution of T_H1 cells and T_H2 cells in the immune response to plaque bacterial pathogens has not been defined. Clearly the immune response to the periodontal pathogens mentioned above involves T_H2 cells, as significant polyclonal IgG antibodies are generated against specific immunogens from those microorganisms. Interestingly, in a significant percentage of HIV-1-infected individuals with very low $CD4^+$ T cell counts, rampant periodontal disease is not apparent, even in the presence of high plaque loads. In addition SCID mice are more

resistant to the invasion and distant abscess formation by the periodontal pathogen, *P. gingivalis* than normal immune competent mice, suggesting that active T cell-dependent immunity may not be important in the prevention of adult periodontitis. However, T cells and macrophages are a major feature of the histology of periodontitis lesions, especially relatively early in the progression of the disease. Early reports identified both CD4⁺ and CD8⁺ T cells in periodontitis lesions with the ratio of CD4/CD8 being less than in the peripheral blood. As would be expected, a majority of both subsets of T cells were CD45RO⁺, activated T cells. The antigen specificities of the T cells in the diseased gingival tissues have not been identified, but plaque bacterial-specific T cell clones have been generated from mononuclear cells isolated from diseased gingival tissue. mRNAs for interferon γ (IFN γ), IL-6 and IL-13 but not IL-2, IL-4 and IL-5 were identified in CD4⁺ T cells isolated from periodontal lesions. However, the significance of these findings is dependent on a precise status of the disease progression at the time of sampling. The general progression of the disease lesions to ones with significant numbers of plasma cells might suggest that the effective TH cell response is of the T_H2 type. The relatively high proportion of B cells synthesizing IgG3 and IgG4 in the lesions would also indicate that the B cell response is being locally driven by type 2 cytokines. Levels of prostaglandin E₂ (PGE₂) and the cytokines, IL-1 and IL-6, have been reported to be increased in periodontal disease lesions, with IL-2 levels decreased. IL-1 and PGE₂ are both potent stimulators of bone resorption and have been proposed as a potential cause of the alveolar bone loss in advanced disease lesions.

Immunodeficiencies and oral health

The oral cavity can be considered a window to the health and immune status of an individual. Although the oral cavity is anatomically unique, it enjoys both the benefits and liabilities of systemic immunity. Perturbations of the T cell compartment of the immune system are often indicated by oral mucosal infections by opportunistic microorganisms, resulting in oral ulcerations. Children with severe combined immune deficiency (SCID) have rampant growth of *Candida albicans* on their oral mucosal surfaces. Most individuals with T cell deficiencies, as in major histocompatibility complex (MHC) class II deficiency, DiGeorge anomaly, ataxia telangiectasia, Wiskott-Aldrich syndrome and HIV-1 infections, develop oral opportunistic infections most often candidiasis. Infants before the complete development of the T cell

response are prone to develop oral thrush, *C. albicans* infection of the oral mucosa. Boys with a deficiency of the CD40 ligand are unable to develop leukocytosis in response to a bacterial infection, and many develop neutropenia, resulting in severe local gingival infections by oral bacteria. Passive administration of granulocyte-macrophage cell-stimulating factor reverses the neutropenia and alleviates the dentogingival infections. Hereditary angioneurotic edema due to a lack of the complement component, C1 inhibitor, manifest oral symptoms characterized by the swelling of oral tissues, especially the lips. As described above, deficiencies in the number or function of PMNs are associated with oral bacterial infection of the gingival crevicular area and rapid loss of tooth supporting tissues. The loss or significant decrease in saliva flow in Sjögren's syndrome or after glandular damage from radiotherapy results in rampant dental caries, increased severity of periodontal disease, recurrent bacterial and fungal infections of the oral epithelium, difficulties in chewing and swallowing food as well as in speech, and loss of the taste sensation. Regardless of the cause, a deficiency in one or more of the innate or adaptive immune mechanisms can seriously jeopardize oral health.

Immune-mediated oral disease

Although PMNs are essential for oral health, periodontitis does occur in individuals with normal PMN function, indicating that there are numerous immune mechanisms involved in the protection or destruction of the oral tissues. A significant number of middle-aged adults develop adult chronic periodontitis, a chronic inflammation of the gingival tissues that varies from site to site in its progression and can eventually cause extensive loss of tooth-support tissues. All the components required to initiate and maintain the immunopathologic processes involved in hypersensitivities types II, III and IV are present at some time during the progression of this disease. During the entire inflammatory response, PMNs are migrating from the vascular system into the gingival fluid and eventually into the oral cavity. Consequently, both PMNs and mononuclear immune cells have the opportunity to contribute biologically active agents which promote inflammation or tissue destruction at the disease sites. The loss of collagen fibers, junctional epithelium, periodontal ligament and alveolar bone adjacent to the tooth are the hallmark signs of progressing disease resulting in the loss of the teeth. Current dogma suggests that products from both the acute and chronic inflammatory cells in the lesions contribute to the observed tissue destruction. Metalloproteinases, elastase and

other proteolytic enzymes, prostaglandins and cytokines are examples of the host-derived tissue modulating agents possibly responsible for the loss of bone and other connective tissue. In the majority of cases removal of plaque deposits and/or antibiotic therapy will arrest the disease. A very low percentage of periodontitis patients do not respond to conventional therapies and continue to lose tooth-supporting bone in spite of having subclinical levels of plaque. The reason for the continued loss of tissue is not understood but a hypersensitive immune mechanism is one possibility. A significant problem in the treatment of periodontitis is predicting which patients will develop progressive disease. Recently a variation in the IL-1 β gene has been identified, resulting in an identifiable genotype for the proinflammatory cytokine IL-1 which regulates extracellular matrix metabolism and bone resorption. This genotype is associated with increased levels of IL-1 synthesis which promotes bone resorption and it is a strong statistical indicator for susceptibility to advanced periodontitis.

Vaccines

Actively enlisting the immune response to prevent oral infectious diseases is under investigation. Work with experimental animals suggests that immunization may be useful in the prevention or control of dental caries and periodontal diseases. Oral (gut-induced) and/or intranasal routes of vaccine administration may potentially be very useful in regard to oral infections because both mucosal and systemic humoral responses are stimulated by those routes. Induction of protective immunity to oral viral and fungal infections may profit from the recent finding in mice that the oral administration of recombinant IL-12, a type 1 cytokine, in liposomes along with the vaccine antigen result in stimulation of type 1 responses as well as sIgA. Other approaches to immunologically modulate oral infections by *Strept. mutans* and *P. gingivalis* have generated provocative results. In both experimental animals and humans, exposure of freshly cleaned teeth to a solution of a murine monoclonal IgG antibody specific for an epitope on *Strept. mutans* several times during the first week after cleaning is associated with the absence of recolonization of the supragingival plaque with *Strept. mutans* for many months longer than in controls. In a similar experimental design, passive transfer orally of murine monoclonal antibody to an epitope specific for *P. gingivalis* also was associated with a major delay in recolonization of the subgingival plaque by *P. gingivalis*. The mechanism by which short-term passive immunization results in relatively

long-term prevention of colonization by oral pathogenic microorganisms is not currently understood. However, if the initial findings hold up after further independent experimentation, the two most prevalent oral infectious diseases could be prevented by self-administration of passive antibodies in a mouth rinse or toothpaste or by annual or semiannual treatments during routine dentist visits.

As the understanding of the immune response and how to use it to promote health rapidly moves forward, that knowledge as well as the delineation of the subtleties of oral immunology will ultimately result in the improvement of the overall health of individuals.

See also: Bacteria, immunity to; Fungi, immunity to; Hypersensitivity reactions; IgA; Immunodeficiency, primary; Innate immunity; Mucosa-associated lymphoid tissue (MALT); Opportunistic infections; Skin, autoimmune diseases; Skin, contribution to immunity; Viruses, immunity to.

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ORAL TOLERANCE

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Oral tolerance refers to the observation that if one administers a protein orally and then immunizes with the same protein, a state of systemic hyporesponsiveness to that protein exists. It was first described in 1911 when Wells fed hen's egg proteins to guinea pigs and found them resistant to anaphylaxis when challenged. In 1946, Chase fed guinea pigs the contact sensitizing agent, dinitrochlorobenzene (DCNB), and observed that animals had decreased skin reactivity to DCNB. Subsequently, numerous investigators have found that animals fed proteins such as ovalbumin or sheep red blood cells do not respond as well to these antigens when subsequently immunized, but do respond normally to other antigens. The phenomenon of oral tolerance has also been observed in humans fed and immunized with keyhole limpet hemocyanin (KLH).

Immunologic tolerance is a basic property of the immune system that provides for self/nonself discrimination so that the immune system can protect the host from external pathogens without reacting against self. When the immune system reacts against itself, autoimmune disease may result. For a time it was thought that self/nonself discrimination was a simple matter of deleting autoreactive cells in the thymus, but it is now clear that the maintenance of immunologic tolerance is a much more complicated process. Autoreactive cells, such as those reacting with brain, are not deleted and can be found in normal individuals. Why these cells become activated and cause disease in some individuals whereas in others they remain harmless is a major question in basic immunology. How to control the autoimmune process once it has been initiated is a major problem in clinical medicine.

These two areas have come together in recent years as oral tolerance has been successfully used to treat autoimmune diseases in animal models and is now being applied to the treatment of human disease states. Furthermore, an understanding of the basic mechanisms by which orally administered antigens induce immune tolerance is beginning to emerge. As

with immunologic tolerance in general, oral tolerance involves multiple mechanisms. Thus the term 'oral tolerance' is misleading as it implies that there is one unique mechanism of tolerance induction when antigens are administered orally. This is not the case. Although the gut has unique properties that favor tolerance induction, oral tolerance is not a single immunologic event.

Gut-associated lymphoid tissue

Orally administered antigen encounters the gut-associated lymphoid tissue or GALT (**Figure 1**), a well-developed immune network that evolved not only to protect the host from ingested pathogens, but also developed the inherent property of preventing the host from reacting to ingested proteins. The GALT consists of villi which contain epithelial cells capable of antigen presentation, intraepithelial lymphocytes, and lamina propria lymphocytes. In addition, there are Peyer's patches, lymphoid nodules interspersed among the villi, which are one of the primary areas in the GALT where specific immune responses are generated. Investigators have attempted to use the GALT as a route for vaccination but have been hampered by the systemic hyporesponsiveness or oral tolerance that is naturally generated. Nonetheless, as described below, active induction of selected immune responses in the GALT is one of the primary mechanisms by which oral antigen suppresses systemic immunity.

In addition to inducing immune responses in the GALT, some oral antigen is absorbed. Although most dietary antigens are partially degraded by the time they reach the small intestine, studies in humans and rodents demonstrate that some undegraded or incompletely degraded antigen is absorbed into the systemic circulation. Absorbed or processed antigen may also be involved in inducing tolerance although the mechanism associated with this pathway has not yet been defined.

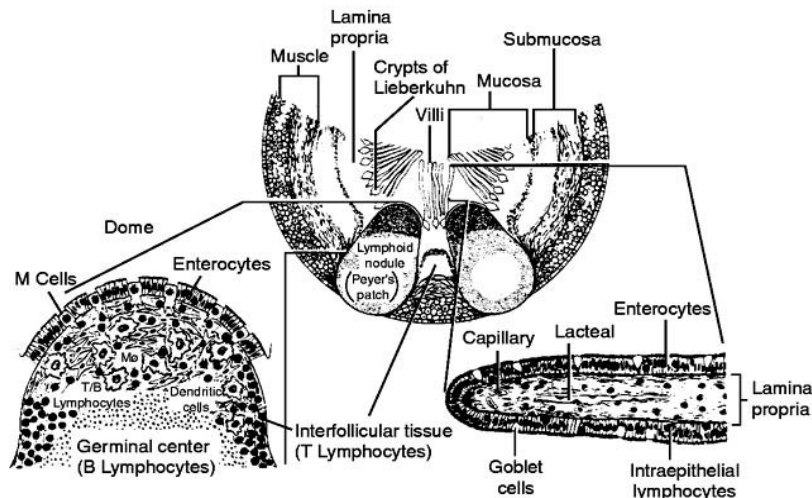


Figure 1 Histological structure of gut-associated lymphoid tissue (GALT). Cross-section of murine small intestine illustrates the main areas of gut lymphoid tissue: lamina propria, intraepithelial lymphocytes (IEL), and lymphoid nodules (Peyer's patches).

Mechanisms of oral tolerance

The primary mechanisms by which oral tolerance is mediated include deletion, anergy and active cellular suppression with the determining factor being the dose of antigen fed (Figure 2). Low doses favor active suppression mediated by the induction of regulatory T cells in the gut-associated lymphoid tissue, such as Peyer's patches, which then migrate to the systemic immune system. When higher doses of antigen are fed, clonal anergy or deletion results.

Active cellular suppression of immune responses has been studied extensively over the years and has remained ill-defined due to difficulties in cloning sup-

pressor cells and defining their mechanism of action. More recently, it appears that one of the primary mechanisms of active cellular suppression is via the secretion of suppressive cytokines such as tumor growth factor β (TGF β), interleukin 4 (IL-4) and IL-10 after antigen-specific triggering. In this sense the GALT is unique as it favors the induction of cells which secrete these cytokines, T_H2 as opposed to T_H1 cells and T cells which secrete TGF β , a potent immunosuppressive and antiinflammatory cytokine. T Cells in lymphoid organs drained by mucosal sites secrete IL-4 as a primary T cell growth factor, whereas those drained by nonmucosal sites secrete IL-2. Oral tolerance has often been demonstrated by a decreased delayed-type hypersensitivity (DTH) response to the fed antigen and it is known that DTH is a T_H1 response inhibited by IL-4-producing T_H2 cells. TGF β plays an important role in local function of the gut as it serves as a switch factor for IgA production in the mucosa and may also be involved in the homing mechanism of the cells to high endothelial venules. TGF β is produced by both CD4 $^{+}$ and CD8 $^{+}$ GALT-derived T cells and is an important mediator of the active component of oral tolerance. Mice that are TGF β -deficient have inflammation that affects multiple organ systems, demonstrating the importance of this cytokine in maintaining immune homeostasis. TGF β is also associated with natural recovery from experimental autoimmune encephalomyelitis (EAE), and regulatory cells which suppress certain forms of inflammatory colitis act via secretion of TGF β .

We cloned TGF β -secreting myelin basic protein (MBP)-specific CD4 $^{+}$ cells from the mesenteric lymph nodes of SJL mice. These clones were structurally identical to T_H1 disease-inducing clones in T cell receptor (TCR) usage, major histocompatibility com-

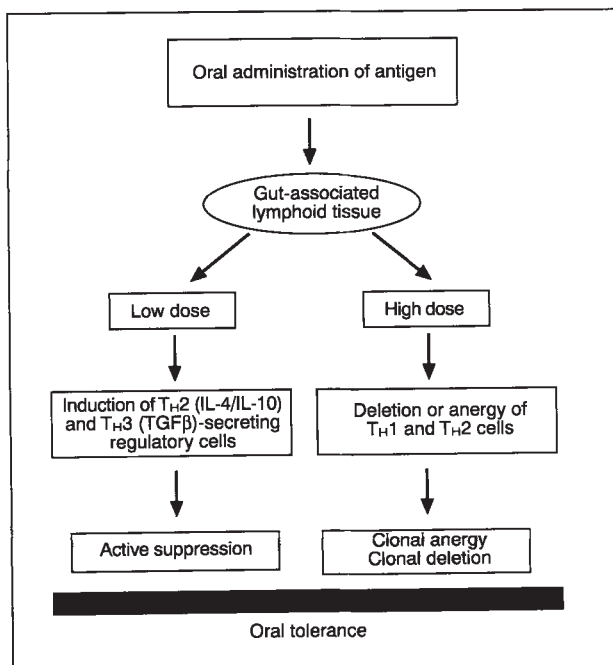


Figure 2 Mechanisms of oral tolerance.

Table 1 T cell subsets

	T_H1	T_H2	T_H3
Cytokine profile			
IFN γ	++++	—	±
IL-4	—	++++	±
TGF β	±	±	++++
Growth factors	IL-2	IL-2/IL-4	IL-4
Functions			
Help	DTH/IgG2a	IgG1/IgE	IgA
Suppression	T_H2	T_H1	T_H1/T_H2

plex (MHC) restriction and epitope recognition but suppressed rather than induced disease. We have also recently cloned TGF β -secreting CD4⁺ cells from orally tolerized (low dose fed) MBP TCR transgenic mice. These latter clones do not secrete IL-2, interferon γ (IFN γ), IL-4 or IL-10 and their primary growth factor is IL-4. Thus, CD4⁺ cells which primarily produce TGF β appear to be a unique T cell subset with both mucosal T helper function and downregulatory properties for T_H1 and other immune cells. In contrast to T_H1 and T_H2 cells, these cells provide help for immunoglobulin A (IgA) production. These TGF β -secreting T cells have been termed T_H3 cells (Table 1) and have also been observed in multiple sclerosis (MS) patients orally dosed with myelin. TGF β -secreting T_H3 clones grow poorly, which has thus far hampered rapid characterization of these cells. The fact that T_H3 cells do not proliferate well may also account for their relative resistance to deletion as compared to T_H1 and T_H2 cells following oral administration of high-dose antigen to ovalbumin (OVA) TCR receptor transgenic mice.

Bystander suppression

Bystander suppression was described during the investigation of regulatory cells induced by oral administration of low doses of MBP. It solves a major conceptual problem in designing antigen- or T cell-specific therapy for inflammatory autoimmune diseases such as multiple sclerosis, type 1 diabetes and rheumatoid arthritis, in which the autoantigen is unknown or where there are reactivities to multiple

autoantigens in the target tissue. During the course of chronic inflammatory autoimmune process in animals there is intra- and interantigenic spread of auto-reactivity at the target organ. Similarly, in human autoimmune diseases there are reactivities to multiple autoantigens in the target tissue. For example, in multiple sclerosis there is immune reactivity to at least three myelin antigens, myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). In type 1 diabetes, there are multiple islet cell antigens that could be the target of autoreactivity, including glutamate decarboxylase (GAD), insulin and heat shock proteins. Because regulatory cells induced by oral antigen secrete antigen-nonspecific cytokines after being triggered by the fed antigen, they suppress inflammation in the microenvironment where the orally administered antigen is localized. Thus, for a human organ-specific inflammatory disease, one need not know the specific antigen that is the target of an autoimmune response but only orally administer an antigen capable of producing regulatory cells which then migrate to the target tissue and suppress inflammation.

Bystander suppression was demonstrated *in vitro* when it was shown that cells from MBP-fed animals suppressed proliferation of an ovalbumin line across a transwell, but only when triggered by the fed antigen. The soluble factor was identified as TGF β . Bystander suppression has been demonstrated in a number of autoimmune disease models (Table 2). PLP peptide-induced EAE can be suppressed by oral administration of MBP, and MBP-specific T cell clones from orally tolerized animals which secrete TGF β also suppress PLP-induced disease. In the Lewis rat EAE model, MBP peptide 71–90-induced disease can be suppressed by oral administration of peptide 21–40. In arthritis models, adjuvant- and antigen-induced arthritis can be suppressed with oral type II collagen. A viral-induced model of diabetes was developed by expressing a lymphocytic choriomeningitis virus (LCMV) protein on the insulin promoter and then infecting animals with the LCMV virus. In this model, the viral-triggered diabetes can be suppressed by oral administration of insulin. Bystander suppression *in vitro* has recently been shown in humans using common dietary proteins

Table 2 Bystander suppression

Immunizing antigen	Oral antigen	Target organ
Proteolipid protein	Myelin basic protein	Brain
BSA, mycobacteria	Collagen type II	Joint
Lymphocytic choriomeningitis virus	Insulin	Pancreatic islets
MBP peptide 71–90	MBP peptide 21–40	Brain

(OVA, bovine gammaglobulin) to stimulate cells *in vitro* which then suppressed *in vitro* tetanus toxoid responses. Bystander suppression may be more difficult to induce in uveitis models and feeding OVA has been reported to induce bystander suppression in adult animals but anergy in young animals.

Bystander suppression theoretically could be applied for the treatment of organ-specific inflammatory conditions which are not classic autoimmune diseases, such as psoriasis, or to target anti-inflammatory cytokines to an organ where inflammation may play a role in disease pathogenesis even if the disease is not primarily inflammatory in nature. Although initially described in association with regulatory cells induced by oral antigen, bystander suppression could in principle be induced by any immune manipulation that induces T_H2- or T_H3-type regulatory cells.

Modulation of oral tolerance

If oral tolerance is broadly defined as the inhibition of T_H1 responses in the periphery either by large doses of antigen that induce anergy or deletion or by gut-induced T_H2- or T_H3-type regulatory cells, anything that favors T_H1 versus T_H2 responses would abrogate oral tolerance and the converse (Table 3). Thus, large doses of IFN γ abrogate oral tolerance and similar results would be expected with IL-12. Indeed investigators have found that anti-IL-12 enhances oral tolerance in OVA TCR transgenic animals and is associated with increased TGF β secretion. In the uveitis model, IL-2 potentiates oral tolerance and is associated with increased production of TGF β , IL-4 and IL-10 in Peyer's patches. We have recently found that IL-4 administration enhances low-dose oral tolerance to MBP in the EAE model. Lipopolysaccharide (LPS) enhances oral tolerance to MBP and is associated with increased expression of IL-4 in the brain, and IFN β synergizes with the induction of oral tolerance in SJL/J mice fed low doses of MBP.

Cholera toxin (CT) is one of the most potent

mucosal adjuvants and feeding CT abrogates oral tolerance when fed with an unrelated protein antigen. However, when a protein is coupled to recombinant cholera toxin B subunit (CTB) and given orally, there is enhancement of peripheral immune tolerance. It is not known how CTB enhances oral tolerance, though it may do so by the more efficient induction of regulatory cells due to the special binding properties of the CTB subunit. The abrogation of oral tolerance by CT may also be the result of an active immune process in the gut, as it has been shown that CT given orally with KLH primes both T_H1 and T_H2 responses, even though T_H2 responses may be preferentially generated. The priming of T_H1 responses via the gut may make it impossible to suppress systemic T_H1 responses following peripheral immunization. It has also been reported that antibodies to the chemokine MCP-1 abrogate oral tolerance, perhaps by interfering with chemotaxis of the T_H2- or T_H3-type regulatory cells generated in the gut or by acting as a factor required for the generation of such cells. Oral antigen delivery using a multiple emulsion system also enhances oral tolerance.

Nasal and aerosol mucosal tolerance

Investigators have also begun to investigate other forms of mucosal tolerance, especially the administration of antigen via the nasal route using aerosols. This route appears equally, and in some instances may be more, effective in suppressing autoimmune diseases in animal models. Smaller doses of antigen are administered, perhaps because there is less degradation of the proteins before they reach the mucosal surface. The degree to which the mechanisms of oral versus nasal tolerance are the same remains to be elucidated.

Treatment of autoimmune diseases in animals (Table 4)

Experimental autoimmune encephalomyelitis (EAE)

Several studies have demonstrated the effectiveness of orally administered myelin antigens (MBP, PLP) in both the rat and mouse models of EAE. In the Lewis rat high doses of MBP result in clonal anergy whereas lower doses induce transferable cellular suppression. In the nervous system of animals orally administered low doses of MBP there is the down regulation of inflammatory cytokines such as TNF and IFN γ and the upregulation of TGF β . Administration of myelin to sensitized animals does not exacerbate disease. EAE can also be suppressed in MBP TCR transgenic animals fed MBP. Nasal MBP peptides have also been reported to suppress EAE.

Table 3 Modulation of oral tolerance

Augments	Decreases
IL-4	IFN γ
Anti-IL-12	IL-12
CTB antigen	Cholera toxin
LPS	Anti-MCP-1
IFN β	
IL-2	
Multiple emulsions	

Table 4 Suppression of autoimmunity by oral tolerance

	<i>Oral antigen</i>
<i>Animal models</i>	
EAE	MBP, PLP
Arthritis	Type II collagen
Uveitis	S-Antigen, inter-photoreceptor retinoid binding protein (IRBP)
Diabetes (NOD mouse)	Insulin, glutamate decarboxylase
Myasthenia gravis	Acetylcholine receptor
Thyroiditis	Thyroglobulin
Transplantation	Alloantigen, MHC peptide
Colitis	Haptenized colonic proteins
<i>Human disease trials</i>	
Multiple sclerosis	Bovine myelin
Rheumatoid arthritis	Chicken type II collagen
Uveoretinitis	Bovine S-antigen
Type 1 diabetes	Human insulin

Arthritis

Oral administration of type II collagen (CII) suppresses several models of arthritis including collagen-induced arthritis (CIA), adjuvant arthritis and pristane-induced arthritis. One of the first studies to demonstrate that an orally administered autoantigen can suppress an autoimmune disease was the use of oral CII in CIA. Of note is that oral type I collagen has also been shown to suppress adjuvant arthritis. Oral administration of an immunodominant human collagen peptide modulates CIA in mice and CII peptides given nasally also suppress CIA in mice.

Uveitis

Oral administration of S-antigen (S-Ag), a retinal autoantigen that induces experimental autoimmune uveitis (EAU), or of S-Ag peptides prevents or markedly diminishes the clinical appearance of S-Ag-induced disease as measured by ocular inflammation. Oral administration of interphotoreceptor-binding protein (IRBP) suppresses IRBP-induced disease and is potentiated by IL-2.

Myasthenia gravis

Although myasthenia gravis is an antibody-mediated disease, either oral or nasal administration of the Torpedo acetylcholine receptor to Lewis rats prevented or delayed the onset of myasthenia gravis. Large doses of antigen were required.

Diabetes

Oral insulin has been shown to delay and in some instances prevent diabetes in the NOD (nonobese diabetic) mouse model. Such suppression is transferable and it has been transferred primarily with CD4⁺ cells. Immunohistochemistry of pancreatic islets of

insulin-fed animals demonstrates decreased insulinitis associated with decreased IFN γ and tumor necrosis factor α (TNF α) together with increased expression of IL-4, IL-10, TGF β , and prostaglandin E₂ (PGE₂). It has also been recently reported that nasal administration of the insulin B chain or GAD suppresses diabetes in the NOD mouse.

Transplantation and other models

Oral allogeneic cells prevents sensitization by skin grafts, and transforms accelerated rejection of vascularized cardiac allografts in primed animals to a time course typical of unsensitized recipients. Orally administered allopeptides in the Lewis rat reduces delayed-type hypersensitivity (DTH) responses to the peptide. Oral but not intravenous alloantigen was accompanied by elevation of intragraft IL-4. Oral thyroglobulin has been shown to suppress autoimmune thyroiditis and oral administration of peptides from the Der p I allergen suppressed responses to the whole allergen. Recently it has been shown that experimental granulomatous colitis in mice is abrogated by TGF β -mediated oral tolerance after administration of haptenized colonic proteins.

Treatment of autoimmune diseases in humans

Investigators have shown that exposure of a contact sensitizing agent via the mucosa prior to subsequent skin challenge led to unresponsiveness in a portion of patients studied. Orally administered KLH, 50 mg, given daily for 2 weeks over a 3-week period to human subjects has been reported to decrease subsequent cell-mediated immune responses although antibody responses were not affected. Nasal KLH has also been reported to induce tolerance in humans.

Based on the success of oral tolerance in animals models of diseases and the safety of the approach, human trials have been initiated in multiple sclerosis, rheumatoid arthritis, uveitis, and type I diabetes. Although these phase I/II trials have shown positive results, the final demonstration of clinical efficacy of oral antigen in these diseases must await the results of large-scale phase III trials that are in progress, or planned (Table 4). What can be concluded from the initial trials is that there has been no toxicity or exacerbation of disease and results in humans have paralleled in many respects what has been observed in animals. Specifically, in MS patients, MBP- and PLP-specific TGF β -secreting T_H3-type cells have been observed in the peripheral blood of oral myelin-treated but not control patients. These results demonstrate that it is possible to orally immunize via the

gut for autoantigen-specific TGF β -secreting cells in a human autoimmune disease simply by oral administration of the autoantigen. Furthermore, it also appears that continuous oral administration of oral antigen is required as MS patients had worsening of disability over a 3-year observation when they were not taking oral myelin.

In rheumatoid arthritis, a 280 patient double-blind phase II dosing trial of CII in doses ranging from 20 μ g to 2500 μ g demonstrated statistically significant positive effects in the group fed the lowest dose (20 μ g). Feeding larger doses of bovine CII (1–10 mg) did not show a significant difference between tested and placebo groups though a higher prevalence of responders was reported for the CII-treated groups. These results are consistent with animal studies of orally administered CII in which protection against adjuvant and antigen-induced arthritis, and the generation of bystander suppression, was observed only at the lower fed doses. An open label pilot study of oral collagen in juvenile rheumatoid arthritis found positive results with no toxicity. This lack of toxicity is an important feature for the clinical use of oral tolerance, especially in children, for whom the long-term effects of immunosuppressive drugs is unknown.

In multiple sclerosis, a recently completed 515 patient double-blind phase III trial of single-dose bovine myelin in relapsing-remitting MS did not show differences between placebo and treated groups in the number of relapses. Preliminary analysis of magnetic resonance imaging data showed significant changes favoring oral myelin in certain patient subgroups.

In uveitis, a pilot trial of S-antigen and an S-antigen mixture has also recently been completed and showed positive trends with oral bovine S-antigen. Finally, trials have been initiated in new-onset diabetes in which recombinant human insulin is administered orally and trials are planned in subjects at risk for diabetes or part of the diabetes prevention trial (DPT-1).

Future directions

Although it is clear that oral antigen can suppress autoimmunity in animals and results to date suggest that this approach may have utility in humans and is safe, much remains to be learned about the immunologic mechanisms associated with antigen-specific mucosally driven tolerance. Important areas of investigation include cytokine milieu, antigen presentation, costimulation requirements, routes of antigen processing, form of the antigen, role of the liver, the effect of oral antigens on antibody and CTL

responses and the role of $\gamma\delta$ T cells. As the mechanisms of oral tolerance are better understood, the ability to successfully apply mucosal tolerance for the treatment of human autoimmune and other diseases will be further enhanced.

See also: Autoimmune diseases; Cytokines; Immunotherapy of autoimmune diseases; Mucosal immunity; Mucosa-associated lymphoid tissue (MALT); Multiple sclerosis; Neurological autoimmune diseases; T lymphocytes; Transforming growth factor β (TGF β).

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ORGAN CULTURE OF LYMPHOID CELLS

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Techniques for culturing organs or tissues were developed in the beginning of the twentieth century. The classic studies of Harrison as well as Carrel first employed fetal tissues (e.g. neural tube or heart) explanted into a drop of 'homologous' lymph or plasma for subsequent study over a period of days or even months. The technique was used widely in the study of embryogenesis, especially in light of the landmark studies of Strangeways and Fell in the study of the developing fetal limb bud. Such experiments with organ culture systems provided an early demonstration of the utility of this approach and encouraged many investigators to employ similar protocols which are used today.

In the early 1960s, Auerbach was successful in using and popularizing techniques of organ culture for the study of lymphoid tissues. Organ cultures were initially used to define morphologically the ontogeny of the tissues but, in more recent studies, many investigators have begun to use such techniques in combination with sensitive cell selection and/or detection techniques (e.g. molecular biology, flow cytometry or immunohistochemistry) in an attempt to understand more detailed interactions of early lymphoid progenitor cells and their micro-environments.

Methods of organ culture

Several general methods exist for the organ culture of lymphoid tissue. The first of these is termed the 'plasma clot' method, and is very similar to the method described in the early 1900s. In the plasma clot method, crude embryo extract is added to a small amount of plasma and allowed to clot. The tissue or organ to be cultured, usually derived from fetal donors, is placed on top of the clot (**Figure 1A**) and cultured for various time periods to observe temporal changes in morphology and/or cell populations. Plasma clot techniques, like other organ culture methods to be described, allow the sequential analysis of development without the complexities of cell traffic (immigration and emigration), found *in vivo*. In addition, it frequently allows the maintenance of an organotypic three-dimensional structure with a complex cellular heterogeneity similar to the *in situ* organ. However, its utility in the study of the interactions between selected lymphoid populations or their precursors and their cellular micro-environment, and how that interaction may change with time, is limited by the heterogeneity of the initial population.

A similar technique for conducting these cultures without the plasma clot by using a supporting mem-

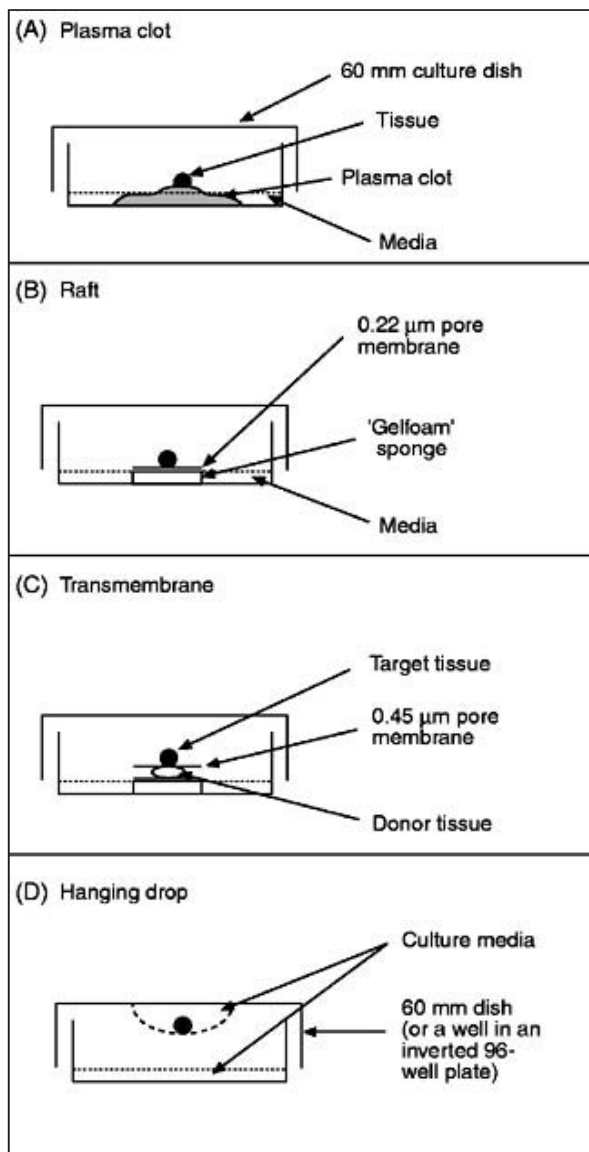


Figure 1 Approaches to the organ culture of lymphoid tissues. Fetal or adult tissues can be organ cultured in systems that employ: (A) plasma clot cultures; (B) raft cultures; (C) transmembrane cultures; or (D) hanging drop cultures. Raft cultures of fetal materials may be treated with deoxyguanosine or low temperature to first deplete the lymphoid populations, prior to being used as a 'target' tissue for a source of donor cells in the transmembrane system

brane raft in liquid culture (Figure 1B) is now employed by most investigators. Like the plasma clot technique, the raft systems allow the target tissue to be maintained at a gas:media interface in a relatively well-controlled *in vitro* environment. In addition, raft techniques provide for better control of soluble factors through the use of defined media and growth factors, if necessary. Clearly, one of the major drawbacks of the plasma clot method is its required use of an undefined embryo extract as a source of growth

promotion in the culture medium. Thus raft techniques allow controlled studies on the role of soluble factors on lymphocyte differentiation and function.

In many studies it would be useful to be able to separate the developing and/or functional lymphopoietic cells from their native stromal cell microenvironment and then selectively recombine them to better understand the nature of their interactions. Further modifications of raft organ culture techniques which employ temperature change or media supplements allow for the preferential depletion and/or enrichment of cell populations, with the potential for a purification of specific cell populations. Using these organ culture purification techniques, elements of the microenvironment have been isolated from the progenitor cell population and the two purified populations recombined under controlled conditions for the study of the influence of the microenvironment on differentiation of the progenitors. Such techniques have been utilized extensively in the study of lymphocyte differentiation in the thymus or B lymphocyte development in a marrow environment, as well as the ability of various tissues to serve as a potential source of lymphopoietic progenitor cells.

Application of organ culture techniques to the culture of lymphoid tissues

Organ culture techniques have been widely applied to examine normal development and the role of microenvironmental elements on hematopoietic differentiation in several different species. Many of these studies have also employed a transmembrane system (Figure 1C) that allows the initial separation and subsequent controlled recombination of purified progenitor and stromal cell populations. For example, since chick and quail cells can be distinguished on the basis of nuclear morphology, Le Douarin and colleagues employed the primitive thymic rudiment of the chick and the yolk sac, spleen or bone marrow cells of the quail in a transmembrane organ culture system to demonstrate that the thymic anlagen taken prior to colonization can become lymphoid if given a source of lymphopoietic progenitors at an appropriate time. In related studies, several waves of receptivity of the thymus anlagen could be demonstrated. The same group has used similar organ culture systems to investigate the relative lymphopoietic potential of the embryonic bursa.

In the murine system, the use of organ cultures to define the role of the thymic microenvironment has been widespread. Early studies of thymic epithelium and the thymic microenvironment utilized monolayer cultures derived from thymocyte cell suspen-

sions. Although many investigators reported the isolation of 'epithelial' cells, the techniques were generally unsatisfactory. A major problem in these systems was the presence of excessive numbers of macrophages and fibroblasts within the cultures, often overshadowing the role of the epithelium. In addition, since several epithelial cell populations found within the thymus *in vivo* may be required for normal lymphocyte development, one runs the risk of deleting an important cell population by preferential growth conditions or subcloning, both of which are common in monolayer systems.

Techniques which use treatment of thymic rudiment organ cultures with deoxyguanosine, low temperature or even a change in oxygen tension have all been shown to selectively enrich for the epithelial cell populations and thus facilitate the study of T cell differentiation, both *in vitro* and *in vivo*. Like the uncolonized thymic anlagen, purified thymic epithelium may become lymphoid when the appropriate progenitor cells are provided in transmembrane organ cultures where donor tissue explants are placed below a cell permeable filter upon which the lymphoid-free thymic explants are placed. These experiments have generally used bone marrow, fetal liver, or fetal thymus as a stem cell source. In situations where cell suspensions are used for the donor cells, often first highly purified by positive and/or negative selection techniques, the lymphoid-free thymic anlagen can be directly injected with a single putative stem cell or another appropriate source of progenitors. Alternatively, a hanging drop modification of the organ culture system (Figure 1D) may be used to facilitate cell interaction. Hanging drop systems also allow the selective continued separation of donor and target cell populations. In an elegant set of experiments utilizing deoxyguanosine-treated thymic rudiment organ cultures, combined with transmembrane cultures, hanging drop cultures or direct injections, Owen's group and others have demonstrated that single lymphopoietic progenitor cells were capable of giving rise to all of the subpopulations of thymocytes found within the developing thymus.

Organ culture-purified murine thymic epithelium of both fetal and young adult origin has been used in *in vivo* transplantation experiments to examine the ability of the grafts to recapitulate ontogeny and provide functional reconstitution. These studies have shown that although the grafts are colonized, they give rise to a variable number of peripheral T cells of possibly different subpopulation composition. Also *in vivo*, grafts of organ-cultured thymic epithelium have been used to investigate the role of epithelium on immune recognition and regulation. After grafting deoxyguanosine-treated thymic lobes into

allogeneic recipients, Jenkinson and colleagues were able to demonstrate that maturing host lymphoid progenitors were not rendered tolerant to the major histocompatibility complex (MHC) antigens expressed by the grafted thymic epithelial organ culture.

More recently, similar organ culture systems have been applied effectively to the study of human materials. Many of these procedures utilize 'tissue slices' and their utility in evaluating three-dimensional interactions of cell populations in assays with functional end-points is just emerging. Furthermore, the additional potential to use human fetal thymic fragment organ culture models to assess human immunodeficiency virus (HIV) pathogenesis may serve as an important functional model for that critical area of research.

Finally, in addition to the more traditional organ culture techniques already described, another system has been used which is often considered to be a method of organ culture. Utilizing a system similar to that described by Dexter and coworkers, Whitlock and Witte developed a monolayer culture system which supported the long-term production of B cells from an initial inoculum of mouse bone marrow cells. Once established, the cultures consist of a dense stromal cell adherent layer associated with a number of loosely adherent developing B cells. These cultures are capable of maintaining a population of early B cell progenitors in the presence of heterogeneous and poorly defined stromal cell population. Many stromal cell lines have been isolated from these cultures, a number of which are capable of inducing early events of B cell differentiation in B cell progenitors. Thus, like more classical organ culture techniques, the Whitlock-Witte culture system may allow for the purification and recombination of the stromal components of lymphoid microenvironments with early lymphoid progenitor cells.

See also: Aging and the immune system; Avian immune system; Bone marrow and hematopoiesis; Bursa of Fabricius; Chimerism, hematopoietic; Cytokines; Deoxyguanosine; Hematopoietic stem cell transplantation; T lymphocyte differentiation; Thymus; Tolerance, peripheral; Transplantation.

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OVINE IMMUNE SYSTEM

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The ovine immune system occupies a special place in immunology because the sheep is the best species for investigating the physiology of the immune system. The trend to reductionism in modern immunology, which is facilitated by the ease with which the cells of the immune system can be disaggregated from lymphoid tissue and studied *in vitro*, has meant that much of our knowledge of the immune system is based on the analysis of the cellular functions of *in vitro* immune systems divorced from any physiological limits. Although the *in vitro* analysis of the complex cell interactions of the immune system has considerably advanced knowledge, this needs to be integrated within the context of lymphoid tissue physiology at a whole-animal level.

The physiology of the immune system, especially in the context of the immunological compartments associated with different organ systems (e.g. lymph nodes, thymus, gut, mammary gland, respiratory system, urogenital system, central nervous system), can be studied separately in the sheep. These studies have gained much from the technique, first developed by Morris, Hall and Lascelles, of lymphatic cannulation of single lymph nodes in sheep, which provides a powerful system for analyzing immune cell popu-

lations within different immunological microenvironments. The lymph node is the heart of the immune system and cannulation of nodal afferent and efferent lymphatics provides a unique approach to the *in vivo* analysis of the immune system not possible in other species. The cannulated lymph node retains all its vascular and neurological connections. All the cells which enter the node, either from the blood or from the afferent lymphatics to the node leave the node in the efferent lymph. Cannulation of the efferent lymphatic therefore allows the monitoring of immunological events in a single lymph node. The system is currently being used to study the pathophysiology of the immune response to lymphotropic viruses such as the ruminant lentivirus, maedi-visna, the prototype lentivirus, which infects the accessory cells of organized lymphoid tissue. Intestinal immunity, especially to nematode parasites, can also be studied uniquely by cannulation of the lymphatic vessels draining the gut and mesenteric nodes.

In recent years, cellular and molecular markers for the ovine immune system have been produced by several laboratories. This has permitted the identification of lymphocyte populations, their subsets and receptors, major histocompatibility complex (MHC)

class I and class II genes and proteins, immunoglobulins and cytokines. Two international workshops have defined a large number of ovine CD molecules, T cell subset markers, MHC class I and II polymorphisms and cytokines including most of the cytokines associated with macrophages and lymphocyte subsets (e.g. IFNs, IL-1, IL-2, IL-4, IL-6, IL-7, IL-10 and IL-12, GM-CSF, TNF). These cytokine genes are cloned, sequenced and exist as recombinant proteins and several cytokine receptors are now defined (IL-2R etc). By using combinations of monoclonal antibodies and cytokine probes T_H1 and T_H2 subsets have been identified. Ovine equivalents of the integrins (CD11/CD18), L-selectins and lymphocyte homing receptors are also well characterised.

In terms of its overall organization, the ovine immune system is similar to that of humans but with some notable differences.

B cells

The ileal Peyer's patch in the gut functions as a primary lymphoid organ necessary for B cell lymphopoiesis. Judged by its cell content, architecture and surface phenotype of its constituent cells (98% sIgM⁺ B cells), this organ is analogous to the bursa of Fabricius in chickens. The entire B cell repertoire in the sheep is derived by hypermutation from a very limited set of V region germ-line sequences. Development of the B cell repertoire occurs in the ileal Peyer's patch. The immunoglobulin levels of IgG in serum are higher than in man (IgM, 2 mg ml⁻¹; IgG1, 17–20 mg ml⁻¹; IgG2, 8 mg ml⁻¹; IgA, 0.2 mg ml⁻¹) and no IgD has yet been described. IgG1, in addition to IgA, is a major immunoglobulin at mucosal surfaces necessary for antimicrobial immunity in the gut and mammary gland. High levels of IgG1 are present in the colostrum (IgG, 60 mg ml⁻¹; IgA, 2 mg ml⁻¹; IgM, 4 mg ml⁻¹) and all transfer of passive antibody to the fetus occurs via the colostrum and milk.

T cells

There are three major T cell subsets in sheep. The majority of cells are either CD4⁺ or CD8⁺ and express the $\alpha\beta$ T cell receptor structure along with the T3 molecule and other adhesion molecules. The third T cell subset is CD4⁻CD8⁻ (double negative), which predominantly contains the $\gamma\delta$ T cell subset. The $\gamma\delta$ T cell subset is a major subset in ruminants with very high levels (25–50% of PBMCs) being present in the blood and lymphoid tissues of young sheep (<6 months old). *In vivo* the $\gamma\delta$ subset is characteristically closely associated with epithelial surfaces and are migratory and recirculating cells.

Most of this subset expresses a marker unique to ruminants which is a 210–215 kDa molecule known as T19. The function of this molecule is unknown but serves as a useful marker for sheep $\gamma\delta$ T cells.

MHC

Sheep tissues express the classical class I and class II molecules. The ovine class II is at least as complex as the human, and sheep genes related to all the expressed human class II genes, with the exception of the DP α gene, have been found. The protein products of these genes have been characterized by immunoprecipitation, monoclonals and protein microsequencing, to reveal that the sheep α and β chains of the class II genes are similar to the DR and DQ- α and DQ- β chains in man. Sheep T cells, both resting and activated, constitutively express both DR and DQ, although the expression of the latter is less per cell than DQ, the more common isotype.

The non-classical class I molecules (CD1 family) are expressed both within the thymus and in the peripheral tissues of sheep. Five CD1 genes have been identified in the sheep and afferent lymph dendritic cells in peripheral lymph of sheep express high levels of cell surface CD1.

The core cells of the immune system involved in antigen processing and recognition are accessory cells (e.g. dendritic cells) and lymphocyte subsets. Their distribution and kinetics of flow through the tissues has been established through the analysis of these cells in afferent lymph, lymph node and efferent lymph.

Afferent lymph

The considerable phenotypic and functional differences between the cells in blood and afferent lymph suggests that there is selectivity in the extravasation of cells from the blood across the endothelium into the peripheral tissues (Table 1). Although the blood contains naive and memory T cells, afferent lymph T cells are mainly of memory cell phenotype. This implies that the endothelial cells in the periphery (e.g. skin) express adhesion molecules which only engage the receptors restricted to memory T cells. Although not blast cells, lymphocytes have an activated phenotype as judged by IL-2 responsiveness, class II expression, and mitotic activity. B cells are much reduced in afferent lymph (4–10%).

Sheep afferent lymph is a physiological source of antigen-presenting dendritic cells. In terms of their expression of CD1 and Fc receptor, there are at least four distinct phenotypic profiles for dendritic cells in afferent lymph, ranging from cells which either coexpress CD1 and FcR, or express neither of these two

Table 1 Ovine leukocyte markers: general characteristics and distribution in different lymphoid compartments^a

Marker ^b	Mol. wt (kDa)	Percentage of marker positive cells in:				Distribution on other cells
		Blood	Lymph node	Afferent lymph	Efferent lymph	
CD1b	44:12	<2	<2	6	<1	Also on cortical thymocytes and dendritic cells (DC)
CD1c	44:12	30	30	16	22	B cells and DC are CD1c ⁺
CD2 (T11)	55	50–60	50–60	70–75	50–60	Only expressed on $\alpha\beta$ TCR ⁺ cells; absent from $\gamma\delta$ T cells; low expression on naive T cells; adhesion and activation molecule; increased expression on memory T cells
CD3	19–25	NA ^c	NA ^c	NA ^c	NA ^c	Not yet defined by mAb; complex molecule involved in T cell activation
CD4	56	25–40	33	49–57	30–40	CD4:CD8 ratios in sheep can range from 2:1 to 5:1
CD5	67	55–60	55–60	84	60–75	On all T cells; low expression on B cells
CD8	36:32	12–16	13–16	13–16	14–18	CD8 appears to be expressed on $\alpha\beta$ and $\gamma\delta$ TCR ⁺ cells
CD11a (LFA-1)	180:95	100	100	100	100	All leukocytes; low expression on naive T cells; increased expression on memory T cells
CD44	95	100	100	100	100	Many tissues; low expression on naive T cells; homing receptor
CD45	220:200:190	100	100	100	100	Leukocyte common antigen; wide distribution
CD45R	220	35–50	35–50	8–15	15–30	B cells, NK cells and naive T cells; absent from memory T cells
CD58 (LFA-3)	42	100	100	100	100	All leukocytes; is ligand for CD2; low expression on naive T cells; increased expression on memory T cells
$\gamma\delta$ TCR	44:36	10–20	10	10–15	5	High numbers in blood of young sheep (30–60%); also identified by T19 mAb
sIgM	70:25	35–50	30–45	4–10	15–25	Expressed on B cells
MHC I	44:12	100	100	100	100	Widespread
MHC II	34:28	60	50–60	90–94	55	Also expressed on activated T cells and dendritic cells; T cell class II mainly DR

^aDrawn from authors' own data plus published data from other groups. In published data, there is considerable variation due to age, breed differences and exposure to pathogens. ^bParentheses refer to previous designation. NA^c, data not available.

molecules, or express only one. These phenotypic differences either identify functionally distinct dendritic cell subsets or represent different stages of differentiation of the one dendritic cell lineage. Dendritic cells in afferent lymph are functionally significant antigen-presenting cells involved in carriage of antigen in an immunogenic form from the skin to the draining node. Dendritic cells have a high level of IgM and IgG1 'cytophilic' immunoglobulin. This passively acquired immunoglobulin may be physiologically important in antigen processing, as it allows dendritic cells to specifically concentrate foreign antigen and localize it in the form of immunogenic complexes for internalization and presentation to T cells. Despite a high afferent input of dendritic cells into the node few, if any, appear in the efferent lymph, suggesting that in sheep there is a high turnover of dendritic cells within the node. This is consistent

with studies in other species that show that dendritic cells in afferent lymph are the precursors of the T antigen-presenting cells of the paracortical region of the lymph node which have a rapid turnover.

Emigration of T cells from the skin to afferent lymph across the peripheral vascular endothelium is profoundly influenced by the cytokines IFN- γ and TNF. Intradermal injection of each cytokine can induce significant lymphocyte migration across the vascular endothelium into afferent lymph. It is likely that these cytokines play an important role in the emigration of recirculating lymphocytes at the site of immune or inflammatory responses in the skin. The evaluation of the *in vivo* activities of cytokines is another good example of how the lymphatic cannulation model can be used to investigate cytokine physiology.

Lymph node

A total of 90% of lymphocytes within a lymph node are derived from the blood; the remainder come from lymphoproliferation within the node and afferent lymph. The route of entry is across a specialized part of the vascular endothelium known in other species as the high endothelial venule (HEV). There is no morphological equivalent structure in sheep, but functionally the postcapillary venules are the same as in rodents and humans. Lymphocyte-HEV interaction is now known to involve a complex family of adhesion molecules including homing receptors (or selectins) on lymphocytes and complementary ligands on endothelial (addressins) and cell matrix proteins. The nonrandom patterns of lymphocyte recirculation through different immunological compartments have been well defined in the sheep. Blast cells from intestinal lymph home to the lamina propria of gut, whereas blast cells from peripheral nodes home to spleen and lymph nodes. Within the small lymphocyte recirculating pool, there are subsets of lymphocytes that preferentially recirculate through the skin, peripheral nodes, or mucosal-associated lymphoid tissue such as the Peyer's patch and mesenteric node. These studies give physiological credence to more recent *in vitro* studies on lymphocyte-HEV which have clearly established the differential selectivity for lymphocyte subset adhesion to specialized vascular endothelial cells in peripheral or mucosal-associated tissues. CD4⁺ T cells are selectively enriched over CD8⁺ T cells in lymph nodes and B cells show a preferential traffic through gut-associated lymphoid tissue. Vascular endothelial cells in antigen-stimulated nodes in the sheep express MHC class II and can act as antigen-presenting cells to recruit selectively antigen-specific T cells into the node. In antigen-stimulated nodes, the rate of entry of lymphocytes is $2-4 \times 10^4$ cells s⁻¹. The immediate response to antigen challenge is a phenomenon known as cell shutdown, wherein the output during the first 24 h in response to secondary challenge with antigen is reduced by 90%. During this time, there is an increased input of cells into the node. This is followed 24-28 h later by a 5- to 6-fold increase in lymphocyte output in efferent lymph. These changes in cell kinetics reflect vascular changes within the node and inhibition of intranodal traffic by prostaglandins.

Efferent lymph

Immunological memory is disseminated from the node via the efferent lymph. Studies by Hall and Morris established that if all the cells leaving an antigen-stimulated lymph node were removed from the

sheep via the cannulated lymphatic, then no priming for a secondary response occurred. If the efferent lymph cells were returned intravenously, then immunological memory was established. Exit of T cell subsets from the node following antigen stimulation is nonrandom. Studies of secondary challenge with antigens show that there is a biphasic exit of CD4⁺ T cells followed by CD8⁺ T cells, presumably reflecting the sequential activation of CD4 and CD8 within the node. Efferent T cells contain a higher proportion of $\alpha\beta$ TCR⁺ T cells than $\gamma\delta$ and contain cells of both memory and naive phenotype. Efferent lymph T cells draining viral infected lymph nodes contain precursor cytotoxic T lymphocytes (CTLs) and the time course and kinetics of the CTL response to viral infection has shown that pCTLs can be detected as early as 5-7 days after infection.

This brief review of the ovine systems has outlined some of the more intriguing aspects of lymphocyte biology. The availability of cellular and molecular markers for the ovine immune system has confirmed the immense value of the sheep to fundamental immunology and provides for the future a central focus for the essential integration of the *in vitro* immune system(s) into a physiological framework.

See also: Adhesion molecules; High endothelial venules; Immune system, anatomy of; Lymphatic system; Lymph nodes; Lymphocyte trafficking; Lymphocytes; Maturation of immune responses; Ungulate immune systems.

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PAPILLOMAVIRUS, INFECTION AND IMMUNITY

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Papillomaviruses induce tumors of the skin and mucosa. In the 1920s, Shope purified and characterized a rabbit papillomavirus from warts. These extracts could generate new warts, confirming their infectious nature. Moreover, Rous observed that warts induced by the rabbit papillomavirus could progress to carcinomas. Since then many animal and human papillomaviruses (HPVs) have been isolated. Typically, papillomaviruses cause tumors that are benign. However, infection with certain HPV types, particularly HPV type 16 and HPV type 18, are associated with cancer, usually cervical cancer.

The papillomavirus particles are small (55 nm diameter), icosahedral in structure and are composed of capsomers assembled from the major (L1) and minor (L2) surface proteins. The double-stranded

circular DNA genome is about 8 kb. This DNA codes for a small number of proteins necessary for its maintenance and replication in addition to the capsid proteins. The genome organization of all the papillomaviruses is similar, as is the function of the viral proteins (**Figure 1** and **Table 1**). Papillomaviruses share a genus-specific antigen (capsid antigen) that can be detected by antibodies made to detergent-disrupted particles. However, classification of types is based on host range and relatedness of the viral genomes. A papillomavirus must have greater than 50% DNA homology to be considered the same type.

The typical lifecycle of the papillomaviruses involves infection of the keratinizing epithelium. The viral genome replicates as an episome. In the replicating basal cells of epithelium, a maintenance replication of the viral DNA occurs with very little viral transcription. Differentiation of epithelial cells is required for increased replication and transcription and virion production. In HPV-induced precancerous and cancerous lesions, only viral proteins E6 and E7 (**Table 1**) are expressed, and no virus is made. Therefore, an interest in immunotherapy has centered on the E6 and E7 proteins, oncoproteins or transforming proteins of the virus. Other vaccines based on the viral capsids could possibly prevent infection.

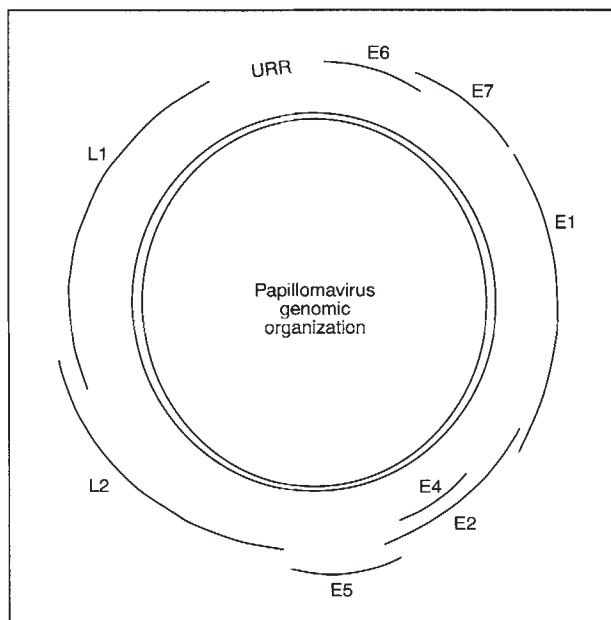


Figure 1 The papillomavirus circular DNA genome. The open reading frames are E1–E7, L1 and L2. The noncoding upstream regulatory region (URR) contains promoter/enhancer elements and origin of replication.

Table 1 Papillomavirus proteins

Viral protein	Function
E1	DNA replication
E2	Transcription regulation, DNA replication
E4	Alteration of cell skeleton
E5	Transformation
E6	Binding to p53, transformation
E7	Binding to pRb, transformation
L1	Major capsid protein
L2	Minor capsid protein

Immune response of the host

Humoral response

Sera from animals or humans with a history of infection generally react with papillomavirus capsid proteins. Antibodies to the E7 protein of HPV16 and HPV18 are frequently found in sera of patients with cervical cancer. Similarly, antibody titers against the E2, E6 and E7 proteins have been detected in patients' sera.

Cellular immune response

Infection with the common papillomaviruses of the skin and mucosa often results in a chronic infection. This chronic infection occurs in the immunocompetent host, suggesting that viral antigens are not recognized by the immune system. However, an increased incidence of clinically apparent HPV disease occurs in the immunosuppressed transplant recipient and also in patients with the acquired immune deficiency syndrome (AIDS). These observations indicate that cell-mediated immunity is important in controlling HPV infection. A decrease in the ratio of CD4⁺ (helper) to CD8⁺ (suppressor) T cells has been reported in patients with HPV infection or precancerous cervical intraepithelial neoplasia, which is usually a manifestation of HPV disease.

Other host responses

Natural killer (NK) cells may have a role in the cellular response to HPV infections. Patients with HPV-induced cancerous and precancerous lesions are reported to display decreased NK cell activity, and these NK cells have a restricted responsiveness to immunostimulatory cytokines.

Langerhans cells are specialized cells in epithelial tissues that are potent antigen-presenting cells. In most warts, there is a reduction of Langerhans cells resulting in a local immunodeficiency state in these lesions. Decreased numbers of Langerhans cells have been observed in the HPV precancerous lesions.

Class I MHC molecules present viral antigens to T cells. Downregulation of class I MHC and the TAP-1 transporter protein has been reported in HPV-induced cervical lesions and laryngeal lesions. Additionally, an association between certain class II MHC alleles (e.g. DQw3) and susceptibility to cervical cancer and laryngeal papillomatosis has been observed.

Evasive strategies by the organism

Examination of tissues reveals that papillomaviruses are ubiquitous, and much more epithelium contains

viral DNA than is manifested by lesions. The viral genome persists in apparently normal tissue with very little expression. Pathology of a papillomavirus infection is exacerbated by abrasion of infected epithelium, hormones and conditions of immunosuppression of the host. Moreover, differentiation of infected epithelium is required for increased expression and replication of the virus. It is clear that the virus has developed strategies to evade the immune system, as suggested by downregulation of MHC class I molecules and Langerhans cells and decreased susceptibility to NK cells.

Vaccines

Prophylactic vaccines appear to be possible with the structural virions and successful papillomavirus vaccines have been developed in animal models, most work involving cattle. Calves vaccinated with wart extract were protected when challenged with bovine papillomaviruses (BPVs). Protection is type specific. Animals inoculated with BPV4 are protected against infection with BPV4, but not BPV2, and vice versa. Subunit vaccines have been developed based on L1, L2 or E7 recombinant proteins and synthetic peptides. L1 vaccines conferred protection when animals were challenged with virus. L2 vaccines for BPV1 resulted in the production of neutralizing antibodies but not in the case of BPV4. However, the subunit vaccines of L2 for BPV2 or E7 of BPV4 did induce early regression of papillomas, even though these vaccines did not elicit neutralizing antibodies. Such lesions were characterized by infiltrating lymphocytes and macrophages, indicating a cell-mediated response.

Production of large quantities of virus has been problematic because of the difficulty of producing virus in tissue culture. However, production of recombinant structural proteins L1 and L2 and assembly into virus-like structures has led to promising early vaccine studies with a variety of papillomavirus types.

Therapeutic vaccines for persons already infected with papillomaviruses may be possible. The goal is to cause regression of tumors, particularly malignant tumors that have papillomaviruses as part of their etiology, using epitopes of viral oncoproteins. Regression of tumors should be possible if an immune response occurs. Trials in patients with advanced cervical cancer are in progress using E7 (Australia), E6-E7 (UK) and E7 peptides (The Netherlands).

See also: Tumors, immune response to; Viruses, immunity to.

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PARAMYXOVIRUSES, INFECTION AND IMMUNITY

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The family Paramyxoviridae is one of several families of viruses in the order Mononegavirales, a grouping which denotes enveloped, negative-stranded, nonsegmented RNA viruses. The Paramyxoviridae includes a variety of pathogens which infect many different hosts causing a diversity of diseases, ranging from more localized respiratory infections to systemic infections. Human paramyxoviruses are responsible for some common childhood diseases such as measles, mumps and respiratory tract infections. Members of this family are also responsible for distemper found in several animal species.

Paramyxoviruses encode six to ten proteins and initiate infection by attachment to and fusion with host cell plasma membranes. Viral transcription and replication occurs exclusively in the cell cytoplasm and virus assembly, budding and release takes place at the host cell membrane, thereby modifying the antigenicity of infected cells. A characteristic marker of these infections is the formation of syncytia or giant multinucleated cells. While infection with many of these viruses is lytic, some can result in persistent infections. Infections usually stimulate both soluble and cell-mediated immune responses.

Characteristics of the organisms and their antigens

Classification

Based on properties of the proteins, sequence analysis of individual genes, order and number of genes encoded by the genome, and sequences of intergenic regions and the 3' and 5' noncoding regions, this family of viruses is divided into two subfamilies encompassing four genera. One subfamily, Paramyxovirinae, includes the paramyxoviruses, rubulaviruses and morbilliviruses, while the other, Pneumovirinae, includes the single genus Pneumovirus. Individual members of each genus are listed in Table 1 as well as the proteins encoded by the viruses in each genus.

Virions

Paramyxoviridae virions are polymorphic structures composed of a helical nucleocapsid core surrounded by a lipid envelope with associated viral membrane proteins. Although filamentous forms can be seen, virions are usually roughly spherical (see Figure 1) with a diameter usually between 100 and 400 nm. Protruding 8–20 nm from the membrane are two spikes, one of which is a tetramer of the cell-attachment protein and the other a trimer or tetramer of the fusion protein. The lipid bilayer is derived from the host cell plasma membrane and,

Table 1 Family Paramyxoviridae

	<i>Paramyxovirinae</i>			<i>Pneumovirinae</i>
	<i>Paramyxoviruses</i>	<i>Rubulaviruses</i>	<i>Morbilliviruses</i>	<i>Pneumoviruses</i>
	Sendai virus (murine PIV1)	Newcastle disease virus	Measles virus	Human RSV
	HPIV1	Mumps virus	Rhinderpest virus	Bovine RSV
	HPIV3	HPIV2	Pest des petits ruminants virus	Pneumonia virus of mice
	Bovine PIV3	Simian virus 41	Canine distemper virus	Turkey rhinotracheitis virus
		HPIV4a and b	Porpoise distemper virus	Ovine RSV
		Simian virus 5 (canine PIV2)	Phocine distemper virus	Caprine RSV
		Avian PIV2-9	Dolphin distemper virus	
<i>Proteins</i>				
Transmembrane	HN, F	HN, F, (SH)	H, F	G, F, SH
Matrix	M	M	M	M, M2(22K)
Nucleocapsid-associated	NP, P, L	NP, P, L	N, P, L	N, P, L
Others (variously present in members of each genus)	C, W, V, D, X	V, I	V, C	NS1 (1C), NS2 (1B)

PIV, parainfluenza virus; HPIV, human parainfluenza virus; RSV, respiratory syncytial virus.

therefore, its composition usually approximates the lipid content of the host cell plasma membrane. Lining the inner surface of the lipid are multiple copies of a single protein, the M or matrix protein, a nonglycosylated peripheral membrane protein, although pneumoviruses have an additional matrix-

like protein (M2) of unknown function. The helical core of these viruses contains negative-stranded RNA ranging in size from 15.2 to 15.9 kb and approximately 2200–2600 copies of a tightly bound nucleocapsid protein (NP). Also associated with this ribonucleoprotein complex (RNP) are smaller amounts

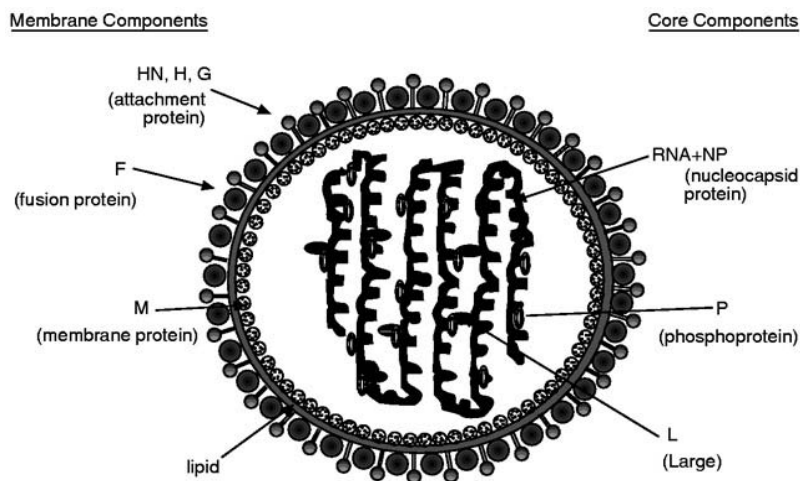


Figure 1 A paramyxovirus virion, showing the minimal structural components. The lipid bilayer is represented by a thick gray line surrounding the core of the virus. Spikes or peplomers, extending from the external surface of the lipid, are represented by two different shapes representing the oligomeric structure composed of the attachment proteins and the structure composed of the F proteins. The actual conformation of these structures is unknown. Some paramyxovirus virion membranes also contain SH proteins. Lining the inner surface of this lipid are matrix or membrane proteins. Precise details of M protein interactions with both the transmembrane proteins and the core are unknown. The virion core is composed of helical, negative-stranded, nonsegmented RNA with nucleocapsid protein bound along its entire length. Also shown are associated proteins, P and L, which form the virion-associated polymerase. C proteins are found in the interior of some viruses.

of two proteins, the L (large) and P (phosphoprotein), both of which form the virion-associated polymerase.

Paramyxovirus genomes contain minimally six genes, commonly in the order (from the 3' end): NP, P (V/C), M, F, attachment protein, and L. The P gene encodes several proteins in addition to P protein by novel use of open reading frames and insertion of nontemplated residues during transcription. Some rubulaviruses and the pneumoviruses contain extra genes, and the gene order of the pneumovirus attachment and F protein genes is reversed (Figure 2). In addition, all virus genomes contain extragenic 3' leader and 5' trailer sequences and intragenic sequences of various lengths.

Virus-encoded proteins

Attachment proteins

Attachment proteins initiate infection by binding to cell surface receptors and are a primary target for neutralizing antibody. All attachment proteins are type 2 glycoproteins with an uncleaved signal/anchor sequence near the N-terminus (Figure 3) and are modified with N-linked oligosaccharides, although the position and numbers of oligosaccharide chains in different proteins are variable. The pneumovirus-attachment proteins are also modified by O-linked carbohydrate. The attachment proteins of the paramyxoviruses and rubulaviruses, the HN (hemagglutinin-neuraminidase) glycoproteins, agglutinate red blood cells, an activity that may reflect cell binding activity. HN proteins also have a neuraminidase activity which may function in virus release from cell surfaces. The attachment proteins of morbilliviruses, H (hemagglutinin), have only red blood cell-binding activity. The pneumovirus-attachment protein, G (glycoprotein), has neither demonstrable hemagglutinating activity nor neuraminidase activity. Many of these proteins have an additional activity involved in

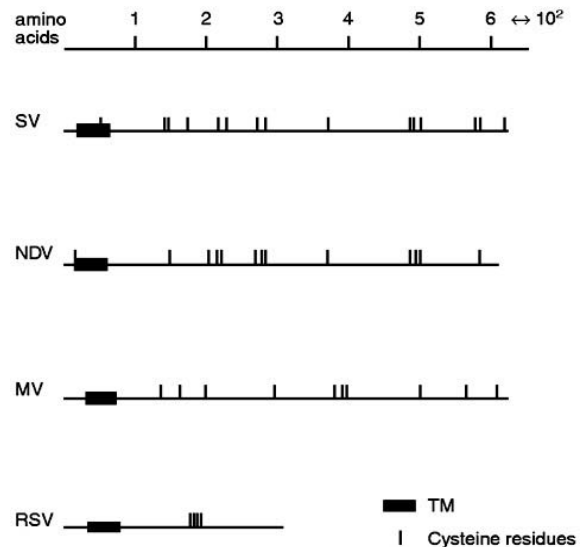


Figure 3 Paramyxovirus-attachment proteins: the linear sequence from the N-terminus to the C-terminus. Paramyxoviruses are represented by Sendai virus (SV), rubulaviruses by Newcastle disease virus (NDV), morbilliviruses by measles virus (MV), and pneumoviruses by human respiratory syncytial virus (RSV). While the positions of cysteine residues are relatively well-conserved within each genus, the positions and numbers (ranging from 3 to 10) of glycosylation addition sites (not shown) are not. Furthermore, studies of the simian virus 5, Newcastle disease virus, and measles virus attachment proteins have shown that not all addition sites are used. Thick lines correspond to the transmembrane domains (TM), which are approximately 20–25 amino acids. The cytoplasmic domains range in length from approximately 20 to 40 amino acids and the ectodomains range in size from 298 amino acids (RSV) to 617 amino acids. The structure of these proteins is unknown although it has been proposed that the HN proteins are folded like the influenza neuraminidase protein.

virus penetration termed fusion promotion. While the mechanism for fusion promotion is unclear, current data suggest that the attachment protein plays a role in activating the fusion protein activity.

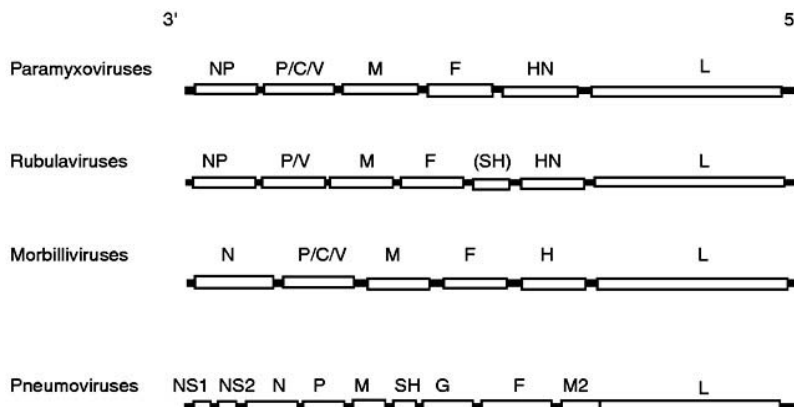


Figure 2 Gene order of paramyxoviruses. The order of genes (not drawn to scale) in each genus is shown from the 3' end of the genome. Leader sequences of 44–70 nucleotides form the 3' end of the genome followed by the first gene (nucleocapsid or NS1). After the L gene are trailer sequences of 44–155 nucleotides. Intergenic regions range from a single nucleotide to 52 nucleotides and, in one case, respiratory syncytial virus, the start of the L gene overlaps the end of the M2 gene. All genes within each genome begin with the same 10–15 nucleotides and end with the same sequence.

Fusion proteins

These glycoproteins, the most conserved in the family, are the other major target for neutralizing antiserum. They are responsible for the fusion of viral membranes and cell plasma membranes, an event required for virus penetration. The proteins are synthesized as a precursor, F_0 , which must be cleaved at a site 100–130 amino acids from the N-terminus, by host cell enzymes, into disulfide-linked F_2 and F_1 for fusion activity (see Figure 4). Some fusion proteins (exemplified by set 1, Figure 4B) have an amino acid sequence at the cleavage site which is a substrate for the furin family of proteases and are thus cleaved intracellularly in the trans-Golgi membranes. Other fusion proteins (Figure 4B, set 2) do not have this sequence motif and are cleaved by extracellular enzymes such as a protease released by Clara cells in the respiratory tract or a factor X-like activity in the allantoic fluid of embryonated eggs. As well-illustrated by different strains of Newcastle disease virus (NDV), the sequence at the cleavage site is a major determinant of the pathogenesis of a virus. NDV strains possessing a furin-recognition site result in systemic infections and are virulent viruses while those without this motif are avirulent and have a more restricted replication within the infected animal.

Fusion proteins have several domains which have

been shown by mutation to be important to their activity. The N-terminal 15–20 amino acids of F_1 , exposed after cleavage and termed the fusion peptide, is thought to directly insert into the target membrane. This sequence is conserved across the family and is composed of hydrophobic and short-chain amino acids. Also present in the F protein sequence are two heptad repeats, one just C-terminal to the fusion peptide and the other, a leucine zipper, just N-terminal to the transmembrane domain.

M proteins

Typical of most enveloped RNA viruses, the inner surface of the envelope is lined with an abundant, nonglycosylated membrane or matrix protein. These proteins, which vary from 256 to 375 amino acids in length, are basic with hydrophobic regions. Phosphorylated forms of some M proteins are also observed. Because of an affinity for lipid, for the nucleocapsid protein, and for itself as well as the cytoplasmic domains of either or both glycoproteins, these proteins serve as a link between the lipid bilayer of the virion and the inner core and may play a central role in virus assembly and budding. The protein also has a role in the regulation of RNA synthesis likely important during the budding of the virus.

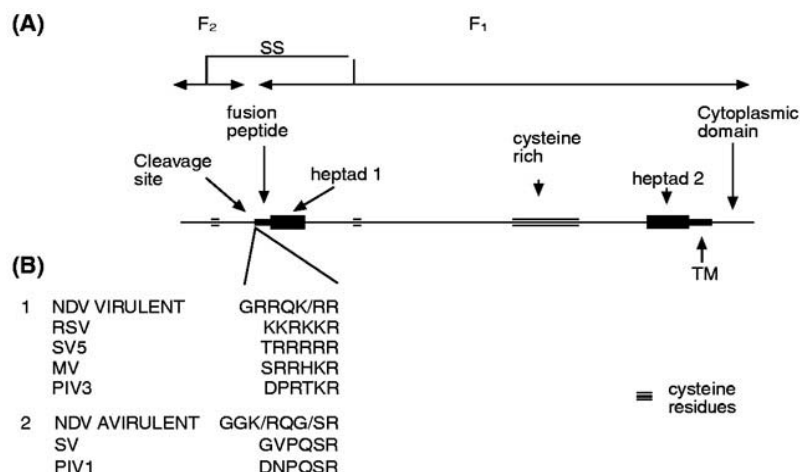


Figure 4 Paramyxovirus fusion proteins. (A) A diagram representing the linear sequence of fusion proteins (ranging in size from 529 to 564 amino acids) from the N-terminus to the C-terminus. Structural determinants, with the exception of glycosylation addition sites (not shown), are well conserved across all genera. The number and position of glycosylation addition sites varies within each genus and the actual usage of these sites has been defined for a very limited number of these viruses. The regions of the sequence corresponding to F_2 and F_1 are shown by arrows above the line. Also shown on these arrows are the positions of cysteine residues responsible for the disulfide linkage of F_1 and F_2 . Important structural domains within the sequences are shown by thick lines. The fusion peptide is approximately 15–20 amino acids; the heptad repeat one, 30–40 amino acids; heptad repeat 2, approximately 30 amino acids; transmembrane domain (TM), 20–30 amino acids; and the cytoplasmic domain ranges from approximately 20 to 30 amino acids. (B) Sequences at the cleavage site are shown for some representative viruses. Set 1 sequences have a furin recognition sequence (R, X, K/R, R) while set 2 do not and must be cleaved by extracellular proteases. TM, transmembrane domain; NDV, Newcastle disease virus; RSV, respiratory syncytial virus; SV5, simian virus 5; PIV, parainfluenza virus; SV, Sendai virus.

SH (small hydrophobic) proteins

Pneumoviruses and the rubulaviruses, SV5 and probably mumps have been shown to encode small (64 and 44 amino acids), hydrophobic, type 2 transmembrane proteins of unknown function. The pneumovirus protein has been found in cell and virion membranes while the SV5 protein only in plasma membranes. The respiratory syncytial virus SH protein has been shown to enhance membrane fusion.

Nucleocapsid proteins

These proteins, which range in size from 391 to 553 amino acids, tightly bind both genomic and antigenomic RNAs along their entire length, conferring RNAase resistance to the RNA. Binding of the RNA is essential for transcription mediated by the L and P proteins. Soluble nucleocapsid protein, associated with the P protein, is essential for replication of the genome. The nucleocapsid protein also associates with the M protein during assembly.

P proteins/P gene-encoded proteins

The P gene encodes the P protein as well as V and sometimes C, W, I, X and D proteins, utilizing different reading frames on the same mRNA or alternative transcripts that result from a novel transcription mechanism involving the insertion of one or two nontemplated G residues. The strategies for encoding the various products of this gene vary with the genus. The P protein, a phosphoprotein, is essential for RNA synthesis. The C proteins, present only in paramyxoviruses and morbilliviruses, are small basic proteins encoded in a different reading frame from P. Their role in infection is not well-characterized but it has been shown that they inhibit primary transcription. The role of the V proteins, found in most members of Paramyxovirinae, are also not well-characterized, but it has been shown that they can inhibit replication. These proteins share the same N-terminal sequence as the P protein, but because of a shift in reading frame, they contain a unique, cysteine-rich C-terminus. The roles of the other P gene-associated proteins are unknown.

L proteins

These are large, 2200 amino acid, poorly characterized proteins which are probably the actual polymerase, their sequences sharing some motifs common to many RNA polymerases. They are also responsible for the capping and methylation of mRNAs.

Infectious cycle

Infection begins when the virion-attachment proteins bind receptors on cell surfaces (Figure 5, step 1). The distribution of these receptors in an animal clearly represents one determinant of the host range of these viruses. The receptors for paramyxoviruses and rubulaviruses are sialic acid-containing molecules. Sendai virus binds to gangliosides. Several different molecules have been reported to be the measles virus receptor, but CD46 has most definitively been demonstrated as the receptor. The receptors for pneumoviruses are unknown.

Subsequent to attachment, the fusion protein, probably activated in some way by the attachment protein, mediates the fusion of viral membranes with the host cell plasma membrane (step 2). As a result of this fusion, the virion interior becomes contiguous with the cytoplasm of the cell and, therefore, accessible to precursors required for transcription. How the RNP becomes disassociated with the virion M protein, which serves to inhibit transcription, has not been characterized (step 3). The genomic RNA is fully transcribed by virion-associated L and P proteins to produce monocistronic mRNAs in a process termed primary transcription (step 4). After translation of this mRNA (steps 5 and 6), replication of the genomic RNA ensues. Replication is accomplished by producing a precise, full-length, plus-stranded copy of the genome (step 7), which, in turn, serves as template for generation of progeny genomes (step 8). These newly made genomes serve to amplify infection by functioning as template for further transcription in a process called secondary transcription (step 9) or as template for the synthesis of more antigenomic RNA (step 10).

The mechanisms responsible for the synthesis of subgenomic mRNA and full-length antigenomic RNAs have been the subject of investigation over many years both in this system and the closely related rhabdovirus system. Current models include the following observations: 1) The L-P protein complex begins synthesis of positive-stranded RNA at the 3' end of the genome, synthesizing the positive-stranded leader RNA. 2) In the absence of sufficient concentrations of soluble NP protein, the polymerase copies the genome by a start-stop mechanism generating monocistronic RNA. Poly-A sequences at the 3' end of each mRNA are generated by a slippage mechanism on a sequence of 5-6 U residues in the genomic RNA at the end of each gene. 3) Later in infection, when concentrations of nucleocapsid protein increase, soluble N protein plays a role in initiation of replication by serving as an antiterminator of RNA synthesis at the intragenic regions. The

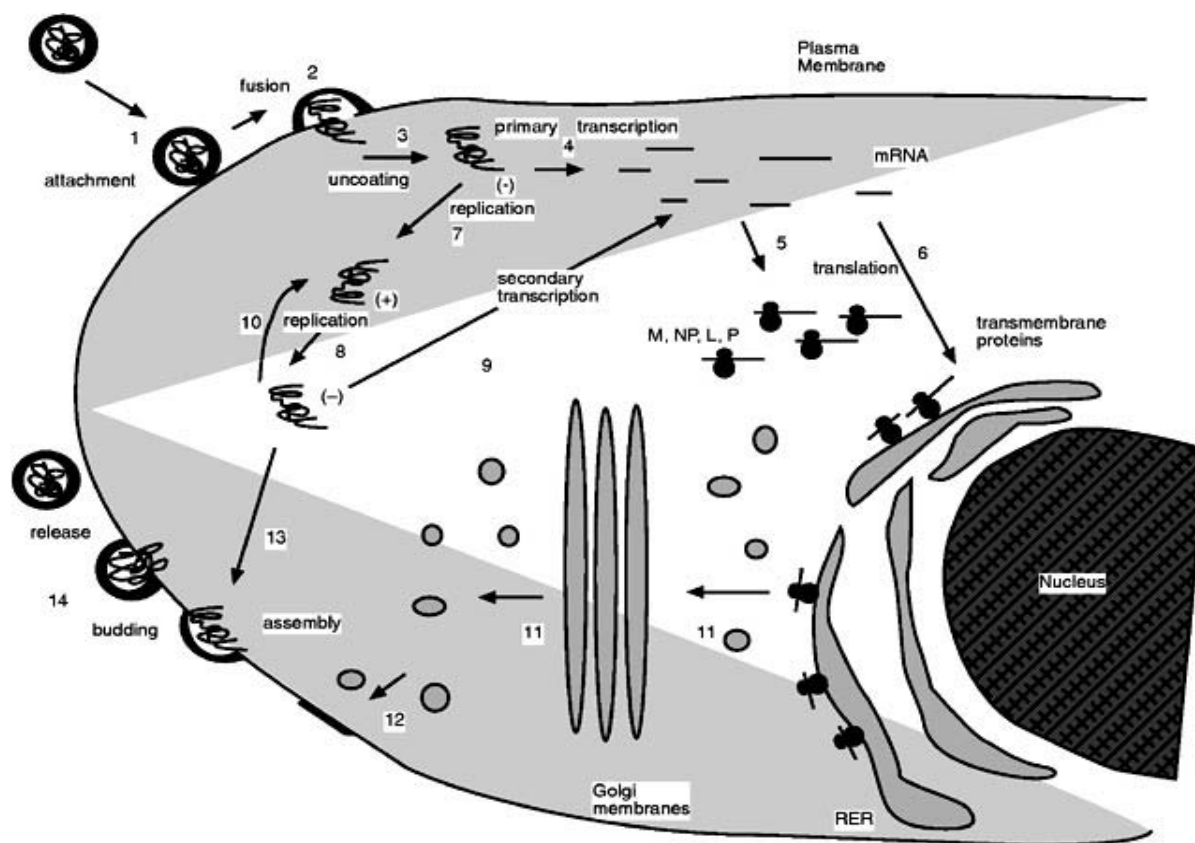


Figure 5 Paramyxovirus infectious cycle. Schematic representation of the steps in infection. Step 1, attachment; step 2, fusion of viral and host cell plasma membranes; step 3, penetration or uncoating (detachment of ribonucleocapsid from M protein); step 4, primary transcription; step 5, translation of nucleocapsid, M protein, P protein, and L protein on cytoplasmic ribosomes; step 6, translation of transmembrane proteins on membrane bound ribosomes; step 7, synthesis of antigenomic (+) full length RNA; step 8, synthesis of genomic (-) RNA; step 9, secondary transcription (transcription using progeny genomes as template); step 10, amplification of replication using progeny genomes as template; step 11, transport of transmembrane proteins through the membrane systems of the cell; step 12, insertion of transmembrane proteins into the plasma membrane; step 13, association of M protein and ribonucleocapsids with patches of plasma membrane containing glycoproteins; step 14, budding and release of progeny genomes.

V and C proteins (and perhaps other P gene-encoded proteins) may also play roles in regulating the levels of transcription and replication at various stages of infection.

Final assembly of progeny virions occurs at the host cell plasma membrane. The viral glycoproteins are inserted into the rough endoplasmic reticulum membrane during synthesis (step 6) and are processed through the membrane systems of cells (step 11) utilizing resident cellular chaperones and modifying enzymes. As are host cell plasma membrane proteins, the viral glycoproteins are inserted into the host cell plasma membrane (step 12). Thus viral glycoproteins serve to modify the antigenicity of the infected cell. The M protein associates with the cytoplasmic tails of the glycoproteins as well as the assembled RNP and the regions of plasma membrane containing all these components bud from the mem-

brane in poorly understood processes (steps 13 and 14). In polarized cells such as respiratory epithelium, paramyxoviruses bud from apical cell surfaces. This directed budding probably plays an important role in the pathogenesis of these viruses.

Effect of paramyxoviruses on host cells

Because of the presence of viral glycoproteins at the cell surface and because paramyxovirus fusion occurs at neutral pH, infected cells can fuse with adjacent uninfected cells, forming syncytia or multinucleated cells, a classic cytopathic effect of this family of viruses. The ability to form syncytia may also serve as an alternative mechanism to spread infection through an organ system.

Infection by many members of this family of viruses ultimately results in cell death. However,

the precise mechanisms involved remain poorly understood.

Immune responses of the host

In systems that have been studied, infection stimulates serum antibody as well as cell-mediated immune responses, both of which play roles in protection as well as recovery from disease although the relative importance of each varies with the virus. Secretory antibody production has also been characterized in many of the respiratory infections caused by some members of this family of viruses. Animal studies suggest that secretory antibody has an important role in protection in some infections although this antibody is only partially protective in some respiratory tract infections such as respiratory syncytial virus infections.

Serum antibody against all virion-encoded proteins can usually be detected after infection, although levels vary and antibodies against the M protein and F protein are often at low levels. While antinucleocapsid antibody is usually in the highest titers, only the antibody against the attachment protein and the fusion protein are neutralizing and, therefore, protective. Indeed, vaccination with vaccinia vectors expressing the F or HN proteins or both has shown that antibody against either protein is protective although expression of both antigens results in the most protective immune response.

Murine monoclonal antibodies have been raised against the attachment protein and the F protein in several systems. The attachment proteins of human parainfluenza virus 3 (HPIV3), Sendai virus, Newcastle disease virus, measles virus, and respiratory syncytial virus (RSV) have from 4 to 6 sites. Antibodies to some of the sites inhibit hemagglutination and, therefore, attachment, while antibodies to other sites are non-neutralizing. Similarly, F proteins have been shown to have 4–7 sites. Antibodies to some sites inhibit hemolysis, an assay generally thought to reflect the fusion activity of the protein, and are neutralizing.

The cell-mediated immune responses to these infections are generally less well characterized. Sendai virus infection of one strain of mice yielded CD8⁺ T cells with reactivity to a single sequence in the NP protein. After HPIV3 infections of humans, cytotoxic T lymphocytes (CTLs) with specificity to HN, P, and NP have been demonstrated. After RSV infection, CTLs with multiple reactivities have been demonstrated. CD4⁺ T cells have been demonstrated in some systems.

Evasive strategies by paramyxoviruses

Of the many ways viruses can evade host immune responses, there are two well-documented strategies employed by some members of the Paramyxoviridae family. First, best exemplified by measles virus, is modulation of the immune response of the host. It has been long recognized that measles virus infection results in a nonspecific suppression of cell-mediated immune responses during both acute and convalescent stages of the disease. Suppression may be related to measles infection of monocytes and macrophages. This suppression has also been recently linked to the binding of the measles virus H protein to the CD46 receptor. This binding signals a downregulation of the expression of interleukin 12 (IL-12) production which could account for a general suppression of cell-mediated immunity.

Sendai virus and bovine parainfluenza virus have been shown to infect alveolar macrophages, which may account for decreased resistance of infected animals to bacterial pulmonary infections.

A second evasive mechanism employed by some paramyxoviruses is the establishment of persistent infection. Again this phenomenon is perhaps best studied with measles virus since the disease SSPE (subacute sclerosing panencephalitis), a rare fatal neurological disease that is a sequela of acute measles infection, is the result of measles virus persistent infections. The establishment of persistent infections by these normally lytic viruses is probably due to incompletely understood cellular as well as viral factors as well as virus-specific antibody. Viruses present in persistently infected cells are usually mutants of the wild-type virus responsible for acute infections. Mutations most frequently associated with persistence seen in SSPE are in the attachment protein, the fusion protein, or the M protein, although alterations which result in decreased expression of these proteins are also associated with persistent viruses. Expression of these proteins at infected cell surfaces as well as virus budding are usually considerably reduced or absent. Infection in immunologically privileged sites may also account for the persistence of the virus.

Paramyxoviruses, typical of RNA viruses, are replicated by an RNA-dependent RNA polymerase which has no proofreading mechanism. Thus the frequency of mutation is high, allowing for variants in a population which may be less reactive to immune responses. However, for reasons that are not entirely clear, the antigenic drift observed in some other RNA viruses such as influenza viruses does not appear to play nearly as significant a role in the epidemiology of paramyxoviruses.

Vaccines

Vaccines against measles virus, mumps virus, Newcastle disease virus, canine distemper virus, and rhinderpest virus are currently in use. These vaccines are all live attenuated viruses. In most cases, attenuation was accomplished by growth of the virulent virus in tissue culture. In the case of Newcastle disease virus, naturally occurring avirulent strains of the virus are used as vaccine. Development of attenuated vaccine viruses for some other members of the family are currently underway.

Attempts to develop inactivated vaccines for measles virus and RSV have met with unfortunate results. Formalin-inactivated measles virus was tested as a vaccine and resulted in neutralizing antibody which rapidly declined in titer. More importantly, vaccinated individuals upon infection with live virus developed atypical measles, a much more severe form of the disease. The reason for the enhanced disease is unclear but has been attributed to alteration of F protein antigenicity by formalin and failure to develop antibodies to the F and NP proteins.

Similarly, formalin-inactivated respiratory syncytial virus was used in trials and also resulted in enhanced, life-threatening disease when the vaccinated individuals encountered the virulent virus. Animal studies suggest that enhanced disease was due to an imbalanced cell-mediated immune response to the inactivated virus and perhaps a failure of the vaccine to stimulate neutralizing antibodies to the F protein.

Inactivated virus vaccines against Newcastle disease virus and mumps virus have been used with some success and without reports of enhanced disease. Inactivated vaccines against parainfluenza 1, 2 and 3 administered intradermally have elicited an antibody response but no protection, a result thought to be due to the absence of stimulation of secretory antibody important for control of respiratory tract infections.

Conclusions

While the molecular characterization of the structure and function of the viral proteins is extensive, numerous questions remain concerning the mechanisms of virion entry, uncoating, replication, transcription and assembly. By comparison, there is less information about the immune response of hosts to infection, particularly the cell-mediated immune response. Further information in these areas will be important to the development of effective vaccines particularly for the respiratory tract infections.

See also: Influenza virus (*orthomyxovirus*), infection and immunity; Rhabdovirus, infection and immunity; Viruses, immunity to.

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PARASITES, IMMUNITY TO

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The term parasitism describes any relationship between two organisms in which one lives at the expense of the other and thus includes infections caused by prions, viruses, bacteria, fungi, protozoa and helminth worms. The word parasite, however,

is traditionally reserved for the eukaryotes, protozoa and helminths. Protozoa are small single-celled microparasites, mostly measuring 1–10 µm, which multiply within their definitive hosts in the same way as viruses and bacteria. Helminth worms, on the

other hand, are macroparasites; they are much larger multicellular organisms, measuring 100 μm to 1000 μm or more, and do not multiply within their definitive hosts. Parasites are found in every kind of invertebrate and vertebrate and individual hosts often harbor several different species, sometimes in massive numbers.

Humans, partly because of our lifestyles and habits, seem to be particularly susceptible to infections with parasites and over 50 species of protozoa belonging to 20 genera, and over 340 species of helminths belonging to about 50 genera have been recorded. These parasites of humans have attracted considerable attention because they cause some of the most widespread human diseases such as malaria, which affects 350–500 million people, schistosomiasis, which affects 200 million people, and ascariasis, which affects some 250 million people worldwide. In Africa, the presence of the parasitic protozoan responsible for trypanosomiasis prevents the use of over 10 million km^2 of land in the sub-Saharan region for cattle rearing. Parasites of domestic animals are also of increasing importance; coccidiosis, for example, is a major threat to battery fowl, particularly in industrialized countries.

Parasitic diseases are difficult to control because they often produce intractable conditions, largely as a result of the inability of the immune system to keep them in check.

Immune strategies in protozoal and helminth infections

The strategies required for a successful immune response are different for protozoa and helminths. Because protozoa multiply in their vertebrate host with a rapid but variable doubling time the host must mount an immune response against the rapidly fulminating infection which presents an immediate threat. In contrast, in helminth infections, in which the parasites do not normally multiply within their vertebrate host, the parasite load is limited by the number of exposures to infection so a rapid immune response to an initial infection is not essential. In spite of the differences between protozoa and helminths, the infections they cause have many common features, including being very long lasting, often several years or even lifetimes, and inducing immunopathological changes which may be more dangerous than the infection itself. This is because most parasites, having been recognized as foreign and having elicited an apparently appropriate immune response, are capable of escaping from the consequences of the response because they have life cycles involving sev-

eral discrete stages. These stages are often antigenically different, and occupy different sites.

All parasites have also evolved mechanisms for evading the immune response. Schistosomes, for example, disguise themselves with host antigens; the African trypanosomes undergo antigenic variation; *Leishmania* and *Toxoplasma* survive in macrophages; *Trypanosoma cruzi*, the protozoan that causes Chagas' disease, mimics host antigens; and many parasites express irrelevant antigens which either deflect the immune response from more vulnerable targets or are shed as they become cross-linked by antibodies.

The net result is partial control but not total elimination of the parasite so the host continues to mount increasingly complex and ineffective immune responses and, unless the host eventually kills the parasite, the parasite kills the host or there is an uneasy compromise which is the most common outcome. Parasitic infections, therefore, tend to be long and chronic; Chagas disease, African trypanosomiasis and some forms of leishmaniasis cause life-long infections, some malarias last for 60 years or more and the duration of most helminth infections can be measured in terms of 5–10 years – about the natural lifespans of the parasites. Whatever the outcome, there is usually some immunopathological damage, often caused by inappropriate immune responses which occur in the wrong place, at the wrong time or for the wrong duration.

Immune responses to parasites

Because of the long-lasting nature of parasitic infections it was widely believed until comparatively recently that there were very few effective immune responses against most parasites. However, it is now clear that immunity is the rule. The evidence for this comes from a number of sources. First, there is the epidemiological evidence: in endemic areas the majority of people develop clinical immunity to parasitic infections, the prevalence of infection falls with age while immunological parameters increase and some immunity to reinfection occurs. Second, there is the experimental evidence: in every animal model investigated some degree of immunity can be demonstrated and it can be argued that there is no reason why humans should be any different. However, immunity is seldom complete and tends to be unstable and individuals suffering from concurrent infections or undergoing immunosuppressive therapy often suffer recrudescences of their dormant infections.

The immune response to any parasite, as with other infectious agents, begins when the host

becomes infected and the parasite is recognized as foreign. In order for this to occur, the parasite must come into contact with the cells of the immune system and if it does not, then no immune response will be initiated. Intestinal parasites, such as the nematode worm *Enterobius vermicularis* and the non-pathogenic strains of the amoeba, *Entamoeba histolytica*, that do not breach the integrity of the gut wall cannot and do not initiate any immune response.

Most parasitic infections, however, do come in contact with the cells of the immune system and are recognized as foreign. The actual events involved in recognition are complex and much of what we know about them is concerned with protein antigens and, in the context of parasitic infections, little is known about what happens to carbohydrate antigens. In parasitic infections macrophages seem to be the cells usually involved as antigen-presenting cells and the effector cells of the immune system, as in all other infections, are cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, activated macrophages and B lymphocytes. Each of these has its own specific role and can act concurrently or sequentially to bring about the immobilization or destruction of the invading parasite.

Parasites, particularly helminths, represent large targets and are antigenically very complex but the immune response is normally only able to recognize a limited number of surface antigens, not all of which are involved in protection and some are even able to deflect the immune response away from more vulnerable targets. A great deal of research has been devoted to the identification and characterization of protective antigens in order to try to understand the mechanism of immunity to parasites and to target vaccines to relevant molecules. Much research has also been directed towards the identification of antigens that are irrelevant to protection, or are even counterprotective, those involved in the evasion of the immune response and those which may possibly contribute to pathology. The antigens that drive the cytokine response towards protection or pathology have only recently begun to receive any attention (see below).

What is known is that the totality of the immune responses to any particular parasite is complex but individual components are relatively simple and there is nothing unique or special about them. CTLs are frequently involved in viral infections but are seldom implicated in parasitic infections, the most important exception being *Theileria parva* in cattle in which CTLs predominate in protective immune responses, particularly in vaccinated animals. However, there is increasing experimental evidence for the involvement of CTLs against a number of intracellu-

lar protozoa, including malaria parasites. NK cells are also being increasingly implicated in protection against parasites. Activated macrophages are among the most important cells involved in protection against parasites and can kill parasites either intracellularly or extracellularly. If small, the target cell can be phagocytosed and destroyed in the phagolysosome via a number of pathways such as proteolytic enzymes and directly or indirectly by other toxic molecules, including reactive oxygen intermediates (ROI) and nitric oxide (NO), either on their own or together with other molecules. One of the key features of macrophage-mediated killing is that the toxic molecules produced are released not only into the phagolysosome but also extracellularly and are thus able to destroy cells in the close vicinity of the activated macrophage, including helminths, especially if the macrophage is closely bound to the target cell via an antibody bridge. These toxic molecules, however, can also damage host cells and their release in an inappropriate place or in excess is a major cause of immunopathology in parasitic infections.

Antibodies are effective against parasites in a number of ways including neutralization, agglutination, complement activation, opsonization and antibody-dependent cellular cytotoxicity (ADCC). The main function of immunoglobulin A (IgA) is the inhibition of invasion of the mucosa, either by agglutinating the parasites or blocking attachment to host cells, and this class of antibody is mainly concerned with defense against intestinal protozoa. IgG and IgM initiate complement-mediated lysis of parasites while both IgG and IgE bind to effector cells, activated macrophages or eosinophils respectively, and thus facilitate the destruction of the target cell via ADCC. Antibodies are also involved in ADCC by NK cells.

T cell subsets and immunity to parasites

The discovery of two subsets of CD4⁺ T helper cells (T_H1 and T_H2) by Mosmann and Coffman in 1986 has revolutionized our understanding of immunity to parasitic infections. These two subsets control the cell-mediated and antibody-mediated arms of the immune response respectively and this control is exerted by the cytokines produced. T_H1 cells characteristically produce interleukin 2 (IL-2) and interferon γ (IFN γ) and are responsible for the activation of cytotoxic T cells, NK cells and macrophages, while T_H2 cells produce IL-4 and IL-5 and are responsible for the induction of B cells to produce antibody and the activation of eosinophils. There is a considerable degree of cross-regulation between T_H1 and T_H2 cells; IFN γ inhibits antibody production

while IL-4 inhibits the production of IFN γ . These discoveries have enabled parasitologists to understand what is happening in parasitic infections and the paradigm of such studies is the protozoan parasite *Leishmania major* in mice. Briefly, what happens is that the *Leishmania* parasites live and divide in macrophages and the protective immune response involves IFN γ -activated macrophages which kill the intracellular parasites. However, this immune response is inhibited by IL-4 (and IL-10) which prevent the production of IFN γ . The net result is uncontrolled growth of the lesions and massive amounts of antibody that has no protective effect.

In many helminth infections the situation is different; T_H2 cells control the production of eosinophils and IgE and are protective whereas T_H1 cells are responsible for inflammation and pathology. By the early 1990s a general pattern had emerged, largely based on studies of parasites in mice, which suggested that in the majority of protozoal infections T_H1 cells were protective and T_H2 cells were counter-protective, whereas in helminth infections the reverse was the case. Since then the situation has changed dramatically and it is now known that CD8⁺ cells are also involved in cytokine production, NK cells are involved in the immune response, IL-12 plays an important role, the nature of the response is determined by the presence of particular cytokines and NO is probably the most important molecule involved in protection and pathology. It is now also known that human T cell subsets are basically similar to murine ones and, as far as immunity to parasitic infections is concerned, the significance of these discoveries is that the main features of the immune response can be analyzed against a background of the functional T cell dichotomy, which is similar in humans and mice and also in sheep and cattle.

An important point is that, whereas it had previously been assumed that CD8⁺ cells were essentially effector cells involved in major histocompatibility complex (MHC)-restricted target cell killing, we now know that they are also involved in the control of the immune response and are therefore presumably involved in immunity to parasitic infections.

Recent studies have also shown that NK cells are able to activate CD8⁺ T cells and are also important producers of IFN γ and thus they also strongly influence the outcome of the immune response. NK cells are activated by IL-12, which now occupies a central role in our understanding of immunity to parasitic infections. IL-12, produced by macrophages, is responsible for the activation of NK cells and is also involved in the regulation of IFN γ production and the growth and differentiation of T_H1 cells. IL-12 and NK cells, therefore, play a major role in T_H1-

type responses which determine the outcome of parasitic infections now thought to be determined by cross-regulation between T_H1 and T_H2 cells. Thus the immune response is controlled by a network of cytokines, some driving the immune response one way and some driving it the other.

It is only in the past few years that the role of NO in immunity to infection has been understood, largely as a result of studies on parasitic infections. NO is a powerful antimicrobial compound and is known to kill a range of organisms from bacteria and protozoa to helminth worms by acting on iron sulfur clusters in macromolecules, repressing DNA synthesis and inhibiting iron-containing enzymes, including those involved in respiration. NO activity is upregulated by IFN γ and tumor necrosis factor α (TNF α) and is downregulated by IL-4 and IL-10. Thus nitric oxide is a key molecule in the immune response against parasites and its production and activity are favored by T_H1 responses and inhibited by T_H2 responses.

The interrelationships between T cell subsets, cytokines and NO are now being actively investigated in many parasitic infections. There is, however, a major problem and this is that the protective cytokine profile can alter during the course of an infection, as appears to happen in experimental murine malaria and in a number of helminth infections, making it very difficult to draw any hard and fast conclusions about exactly what the pattern of cytokine activation actually is in any particular infection.

Immunopathology

The immune responses evoked by parasites are not unusual and are essentially the same as those involved in immunity to other infectious organisms but what is different is their complexity in response to diverse antigens expressed by different stages of complicated life cycles, often in different situations in the host. This leads to a plethora of antibody-independent and antibody-dependent responses which, over a period of time, can give rise to misdirected immune responses leading to pathological changes instead of protection. For example, during the course of many infections, parasite-derived molecules are deposited on host tissues and elicit autoimmune responses which contribute to the pathology of the infection. Some parasites, such as *T. cruzi*, evade the immune response by mimicking host molecules and, if effective, this is very successful but, if unsuccessful, can also initiate autoimmune reactions. Typically, parasitic infections are accompanied by periods of immunodepression as the host switches off what is becoming an ineffective or dangerous immune

response, rendering the host susceptible to concurrent infections as happens in malaria and African trypanosomiasis. During the course of long, chronic infections various aspects of the cytokine network are activated and the effector molecules produced are not necessarily beneficial. For example, in malaria the production of TNF α is beneficial but in excess is detrimental, and in schistosomiasis potentially protective cytokine-mediated immune responses lead to granulomas around nonthreatening eggs deposited in tissues. For these reasons, current interest in immunity to parasites is being concentrated on an understanding of the overall control of the immune response which is brought about by the interactions of the various cytokines involved in the cytokine network.

Immunity to specific parasitic infections

Our interpretations of the immune response to parasites as it appeared only a few years ago now seems very simplistic and the new discoveries have called for a reinterpretation of many of the facts observed. On the other hand, these new discoveries have enabled us to understand many of the phenomena observed much more clearly and it is easiest to illustrate these points by reference to a selected number of parasitic infections and to interpret these in terms of the human diseases.

In murine cutaneous leishmaniasis, the fundamental observations that protection is afforded by T_H1 cells and that T_H2 responses are counterprotective still stand; the effector molecule responsible for parasite killing is NO and the production of NO can be enhanced by the presence of IFN γ and other T_H1 cell-promoting cytokines. This information is being used as the basis for experimental vaccines. In humans, there is now abundant evidence that the pattern of cytokine activation is similar. Patients with localized cutaneous lesions tend to have high levels of IFN γ , whereas those with disseminated infections or mucocutaneous leishmaniasis tend to have high levels of IL-4. These cytokines are also predictive of the outcome of the cutaneous forms of leishmaniasis, patients with high levels of IFN γ tend to have a better prognosis than those with high levels of IL-4. However, the situation is not so simple in the case of visceral leishmaniasis where levels of IFN γ and IL-10, normally antagonistic, are both elevated. Overall, it seems as if the outcome of leishmaniasis infections in humans, as in mice, depends on a balance between those factors promoting a T_H1 response and those promoting a T_H2 response.

Toxoplasma gondii is also a parasite of macrophages and, like *Leishmania*, is killed by IFN γ -acti-

vated macrophages. Recently it has also been shown that TNF α synergizes with IFN γ in this killing. Interestingly, both CD4⁺ and CD8⁺ cells contribute to the production of IFN γ , but in mice without CD8⁺ cells, NK cells take over this role. *T. gondii* itself is able to stimulate the production of IL-12, which in turn stimulates NK cells which produce IFN γ and the actual parasite killing involves NO.

Malaria is a complex infection and immunity seems to involve both T_H1 and T_H2 responses and, in some murine models, there is a switch between early T_H1 and later T_H2 responses. Parasite killing involves NO but it is not at all clear how this gets into the infected red cell. One of the most interesting aspects of the role of NO in malaria is as a possible cause of the coma associated with cerebral malaria. This suggests that cerebral malaria might be prevented by inhibiting NO synthesis and there is evidence that this can be done by treating comatose children with anti-TNF α as TNF α is one of the costimulators of NO production. Malaria also demonstrates one of the fundamental problems in trying to control parasitic infections, getting the balance between protection and counterprotection right.

In schistosomiasis, protection involves ADCC comprising IgE and eosinophils, thus it would appear to be a T_H2-type response. However, parasite killing also seems to involve NO and there is now evidence that the initial protective response is a mixture of T_H1 and T_H2 responses with IFN γ being contributed by CD8⁺ cells and NK cells and IL-4 by basophils. The pathology associated with the granulomatous response to schistosome eggs has also had to be reinterpreted. These granulomas have long been considered to be due to a T_H1-mediated delayed-type hypersensitivity (DTH) response but it is now known that most of the inflammatory cells involved are eosinophils and that the development of granulomas is inhibited by the T_H1-activating cytokine IL-12.

Parasitic infections and human immunodeficiency virus (HIV)

The spread of HIV has had a major impact on parasitic infections and is responsible for more severe parasitic infections, the recrudescence of latent infections and even the occurrence of previously unknown parasites. The impact is greatest among the protozoan parasites and leishmaniasis, toxoplasmosis, Chagas disease, giardiasis, cryptosporidiosis, microsporidiosis and pneumocystosis are among the parasitic infections now associated with the acquired immune deficiency syndrome (AIDS). Of these, leishmaniasis-AIDS has emerged as a particularly formidable combination and pneumocystosis is a frequent

cause of death in AIDS patients. Several species of microsporidians have only been identified from AIDS patients. Virtually nothing is known about the immune responses to these more unusual parasites. There is no evidence of any interactions between HIV and malaria. Hyperinfections of the nematode worm *Strongyloides stercoralis* occurs in immunosuppressed patients but there is no evidence that this infection is a serious concomitant of AIDS.

Vaccination

There are no commercially available vaccines against any human parasitic diseases and only a few against parasites of veterinary importance. This is largely due to the fact that immune responses against parasites are so complex and that parasites themselves have evolved so many ways of evading the immune response. A number of vaccines based on living attenuated parasites have been developed. The most widely available commercially produced vaccine against any helminth worm is one based on the irradiated larvae of the lungworms *Dictyocaulus viviparus* in cattle and *D. filaria* in sheep, but a similar vaccine developed against the dog hookworm *Ancylostoma caninum* was abandoned in favor of chemotherapy. An attenuated vaccine based on infected blood has been in use for many years and is effective against cattle babesiosis; cultured attenuated parasites are widely used as vaccines against Mediterranean theileriosis caused by *Theileria annulata*; and a vaccine based on an avirulent strain of *Toxoplasma gondii* is being used in limited trials to control toxoplasmosis in sheep. Controlled low-level infections have also been used to immunize battery fowl against coccidiosis.

Immunization against East Coast fever (African theileriosis) caused by *Theileria parva* can be achieved by an infection-cure procedure which is very successful but time-consuming and expensive. A number of experimental vaccines based on attenuated larval stages of helminth worms, including *Haemonchus contortus* in sheep and *Schistosoma bovis* in cattle, have been encouraging but have not been developed commercially. A number of more defined vaccines are currently undergoing trials and these include recombinant vaccines against the tapeworm *Taenia ovis* and the nematode *H. contortus* in sheep.

Attenuated vaccines are unlikely to be widely acceptable for use in humans although there have been successes with such a vaccine against human cutaneous leishmaniasis both in the Middle East and in South America. A potentially useful synthetic vaccine against malaria, SPf66 developed in Colombia,

is currently undergoing clinical trials in several parts of the world but in spite of encouraging results in South America the vaccine has proved to be less effective in areas of endemic malaria in Africa. Other vaccines against human malaria, leishmaniasis and schistosomiasis are currently being developed under the auspices of the World Health Organization (WHO). Preliminary results have been encouraging and the hope is that vaccines may be able to produce better immunity than natural infections. In this context, there is considerable interest in the development of vaccines that manipulate the cytokine response so that the vaccine can work under the most favorable circumstances.

Summary and conclusions

This overview of immunity to parasites has emphasized the importance of the cytokine network in parasitic infections. We are gradually untangling this network and the hope is that by doing so it will be possible to devise new ways to combat these infections. There have already been successful attempts to drive the immune response in the desired direction by the incorporation of cytokines into a vaccine against leishmaniasis and similar experiments with IL-12 have prevented granuloma formation in schistosomiasis. However, the main problem will be to ensure protection while reducing the possibilities of counter-protection. There is also the need to discover exactly what is happening in humans before the theory based on experimental studies can be put into practice. Our interest now is not so much in determining whether or not an immune response occurs but instead is largely concerned with unravelling the complex interplay between invader and invaded which results in either protection or pathology or a combination of both. The practical aim on one hand is to determine what is actually responsible for protection and how this can be induced artificially and, on the other hand, to determine what causes pathology and how this can be ameliorated.

See also: Amebiasis; Ascariasis; Babesiosis; Chagas' disease; Cryptosporidiosis; Cysticercosis; Echinococcus, infection and immunity; Fascioliasis; Filariasis, lymphatic; Hookworm disease; Leishmaniasis; Malaria; Parasites, immunity to; *Pneumocystis carinii*, infection and immunity; Schistosomiasis; Strongyloidiasis; Theileriosis; Toxocariasis; Toxoplasmosis; Trichuriasis; Trypanosomiasis, African.

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PARVOVIRUS, INFECTION AND IMMUNITY

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Parvoviruses infect many animals including humans, and almost every species has its own parvovirus pathogen. Epidemics in dogs, swine, geese and mink are major practical concerns of veterinarians. Because of the requirement for actively proliferating cells, the developing fetus and the newborn are at highest risk of disease following parvovirus infection. Infection can lead to fetal death, congenital malformations or predominant damage to single organs that may not be manifest until weeks after birth, such as the cerebellar ataxia that follows *in utero* feline parvovirus infection. Parvovirus infection of young animals can be clinically severe, with manifestations of encephalopathy, hepatitis or myocarditis. Parvoviruses cause a variety of diseases in adult animals, including gastroenteritis in cats, dogs and mink, leukopenia in cats, and immune complex glomerulonephritis in mink persistently infected with Aleutian disease virus.

The human parvovirus B19 was discovered by Yvonne Cossart while investigating tests for hepatitis B. A serum labeled 'B19' gave anomalous results but ultimately was shown to contain 23 nm particles resembling parvovirus. Six years later, B19 was demonstrated to be associated with transient aplastic crises in sickle cell patients and only a decade after

its discovery the major manifestation of B19 infection, fifth disease, was described. Today, B19 is known as a virus that infects most humans early in life without any major clinical sequelae. Significant disease only develops in people who cannot tolerate even transient damage to the target tissue of B19, the erythroid compartment of the bone marrow, and in those with an inappropriate immune response to the virus.

Parvovirus B19 biology

B19 belongs to the Parvoviridae family, which has been divided into the subfamilies of insect parvoviruses (Densovirinae) and vertebrate parvoviruses (Parvovirinae). The Parvovirinae are further divided into three genera, of which one (*Dependovirus*) requires coinfection of target cells with a herpes virus, whereas the other two (*Parvovirus* and *Erythrovirus*) replicate autonomously. B19 and the recently discovered simian parvovirus (SPV) belong to the newly formed genus *Erythrovirus*.

B19 is a nonenveloped DNA virus with a single-stranded genome of 5.6 kb. The genomic organization is typical for all parvoviruses: two capsid proteins are encoded by the right side of the genome and

a large nonstructural protein is encoded by the left side. The capsid proteins, by convention denoted VP1 and VP2, are derived from overlapping reading frames. VP1 differs from VP2 only by an additional 227 amino acids at the N-terminus. More than 95% of the capsid of the mature virion is composed of VP2, the smaller protein, whereas only about 5% represent VP1, the larger protein. The nonstructural protein contains sequences which are highly conserved among parvoviruses, consistent with an essential role in the life cycle of the virus. The protein binds DNA and mediates apoptotic death of the host cell.

The target of parvovirus B19 infection is the erythroid progenitor cell of the bone marrow. *In vitro*, B19 productively infects erythroid cells in human bone marrow cultures, leading to depletion of differentiated erythroid progeny and to complete suppression of erythroid colony formation in progenitor assays. The cytopathic evidence of parvovirus B19 infection, the 'giant pronormoblast', is seen both after tissue culture inoculation and in the bone marrow of infected patients. Parvovirus B19 is extremely tropic for erythroid cells and has only been propagated in bone marrow, fetal liver (which is the site of red blood cell production *in utero*), and cells lines with erythroid features.

The molecular basis for the extreme tropism of B19 for erythroid cells was clarified by the identification of the receptor for B19 as globoside, a neutral glycosphingolipid predominantly expressed on erythroid cells, where it is known as blood group P antigen. The great majority of the population expresses P antigen and is thus susceptible to B19 infection. Amish individuals who genetically lack P antigen are not susceptible to B19 infection. None showed serologic evidence of previous infection and their bone marrow could not be infected with B19 *in vitro*.

P antigen is also found in endothelium, kidney cortex, myocardial cells of the fetus, and placenta. However, expression of the receptor is probably essential but not sufficient for productive infection. *In vitro*, viral replication depends on active erythropoiesis and erythropoietin, suggesting a requirement for erythroid-specific in addition to S-phase factors. Permissive cells allow full-length transcription of the viral genome, whereas there is an apparent block in mRNA synthesis in nonerythroid cells. Replication of the viral genome is not required for viral cytotoxicity. Transcription of the nonstructural protein without DNA replication can kill cells, which may explain why B19 can damage cells nonpermissive to productive infection such as megakaryocytes.

Natural infection and immune response to parvovirus B19

The natural course of parvovirus B19 infection has been evaluated by experimental infection of normal volunteers and in a prospective study of a major epidemic. After intranasal inoculation of normal persons, the virus infects and replicates in erythroid bone marrow cells. Viremia is first detected by day 6 and can reach peak levels $>10^{14}$ ml⁻¹ serum. Infection kills the host cells, so that at the peak of infection, almost no erythroid cells except for a few virtually pathognomonic giant pronormoblasts can be detected in a bone marrow aspirate. Specific immunoglobulin M (IgM) followed by IgG antibodies appear during the second week postinoculation and effectively terminate the infection.

Antibody to parvovirus can be detected using indirect capture immunoassays employing genetically engineered capsid preparations for antigen. Capture techniques to detect viral antigen in sera have been supplanted by DNA hybridization using either ³²P-labeled or biotinylated DNA or RNA probes. The most sensitive technique for detection of virus is DNA amplification by polymerase chain reaction. Small amounts of viral DNA can be detected for many weeks after acute infection; the biological significance of this finding is unknown.

IgM antibody is usually present in the first specimen obtained after the onset of illness: in one study, 78% of sera were positive 0–2 days and 93% were positive 3–7 days after the onset of symptoms. The peak in IgM titer occurs at 2–3 weeks and IgM has been detected in sera obtained months after the onset of symptoms. Specific IgG antibodies can be demonstrated in 42% of persons at 0–2 days and in 90% at 3–7 days.

VP2 is the primary target of antibodies induced by first exposure to the virus. As the antibody response matures, specificity against VP1 epitopes develops. Linear epitopes, clustered in the VP1-unique region, are essential for an effective, neutralizing immune response. Using standard dot blot analysis, virus is rarely detected in sera beyond day 15 after inoculation, but when evaluated by polymerase chain reaction, low level viremia can still be demonstrated in some patients for more than 2 months after natural infection.

IgG antibodies persist for life in most individuals, probably boosted with re-exposure. Anti-B19 IgG has been found in 36% of children by the age of 10, in 50–60% of adults aged 16–40 and more than 85% in individuals more than 70 years old. B19 infections occur worldwide and throughout the year, with a seasonal peak in spring. They frequently occur

in epidemics of about 3 year periodicity, but also as sporadic cases. B19 parvovirus is probably transmitted by the respiratory route. High levels of virus in saliva and heat stability of the virus combine to make parvovirus infection very contagious. B19 has also been transmitted by blood products and seems to resist pasteurization and solvent detergent treatment.

Acute parvovirus B19 infection in humans

Parvovirus B19 diseases arise either from direct viral toxicity for specific target cells or as a result of the host immune response (Table 1). Direct viral infection leads to destruction of marrow erythroid cells in normal individuals, but cessation of red cell production is short-lived due to rapid immune clearance of virus, and the long lifespan of erythrocytes precludes the development of anemia.

In many persons, B19 infection is asymptomatic. Fifth disease in children presents typically with a 'slapped cheek' appearance and a lacy, evanescent maculopapular cutaneous eruption. Adults more often present with a polyarthralgia/polyarthritis syndrome. Rash and arthropathy are usually transient but may recur for several weeks or months; some women have had a relapsing chronic rheumatic syndrome that resembled rheumatoid arthritis. Normal volunteer studies showed that both the rash and arthropathy develop concomitantly with the rise in antiviral antibodies, and in patients with community-acquired fifth disease who have circulating specific IgM antibody, parvovirus is difficult to recover. These results suggest that fifth disease is immune complex-mediated.

In contrast, direct viral toxicity dominates the clinical picture in individuals with a shortened red cell life span, usually as a consequence of inherited

hemolytic anemia such as sickle cell disease or hereditary spherocytosis. In these patients, cessation of red cell production causes an abrupt, occasionally fatal worsening of chronic anemia called transient aplastic crisis (TAC). The self-limited character of the illness is due to the development of neutralizing antibodies which terminate the infection. TAC is rarely followed by fifth disease symptoms, perhaps because the large amount of virus produced by the expanded erythroid bone marrow is unfavorable for antibody-antigen lattice formation and immune complex deposition. Variations in the antibody response may also account for the lack of symptoms in about 25% of normal persons with acute parvovirus infection.

Both the normal volunteer and epidemiologic investigations demonstrated that the same virus was responsible for different clinical syndromes. Virus variability is probably not important pathophysiologically, since multiple isolates have been shown to be very similar at the nucleotide level and as antigens, and minor differences on restriction enzyme mapping have not been correlated with different clinical syndromes.

Persistent disease in immunodeficient patients

Parvovirus B19 can persist in immunodeficient persons who are unable to mount an adequate antibody response. Persistent parvovirus infection has been documented in three patient groups:

1. people with congenital immunodeficiency;
2. children with acute lymphocytic leukemia in remission, and adults with lymphoma and Hodgkin's disease treated with chemotherapy (our unpublished cases) and patients after bone marrow transplantation; and
3. patients with the acquired immune deficiency

Table 1 Diseases caused by parvovirus B19

<i>Diseases</i>	<i>Patients</i>	<i>Mechanism</i>
<i>Immunocompetent host</i>		
Fifth disease	Hematologically normal children	Immune complex vasculitis and arthritis
Polyarthropathy syndrome acute and chronic	Hematologically normal adults	"
Transient aplastic crisis	Patients with shortened red cell life span, e.g. hemolysis	"
<i>Immunocompromised host</i>		
Chronic pure red cell aplasia $\pm$ neutropenia, $\pm$ thrombocytopenia	Patients with congenital and acquired immunodeficiency	Aplasia of erythroid progenitor cells due to viral cytotoxicity (induced autoimmunity in congenital anemia?)
Fetal disease	Fetuses of normal, nonimmune women	"

syndrome (AIDS) due to infection with the human immunodeficiency virus type 1 (HIV-1).

Persistent parvovirus B19 infection results in severe, transfusion-dependent anemia due to suppression of bone marrow erythropoiesis. The anemia of persistent parvovirus infection may be indistinguishable from the hematologic disease pure red cell aplasia. The anemia may occur in combination with some degree of neutropenia or thrombocytopenia. Serology is misleading in immunodeficient patients who do not produce antibodies; the diagnosis of B19 infection in those patients can only be established by the demonstration of viral antigen or DNA.

In most cases of persistent parvovirus infection, virus has been present in serial blood specimens for months and, in at least one case, for many years. Levels of viremia in persistent infection are often lower than in acute infection, 10^6 – 10^8 genome copies ml^{-1} , possibly due to depletion over time of the erythroid target cell pool. Virus has been demonstrated in the bone marrow of persistently infected patients by molecular and immunologic techniques, including Southern analysis to show replicative intermediate DNA forms. Viremia and anemia may spontaneously remit, possibly the result of temporary target cell exhaustion or transient antibody production. In the absence of antibody production, immune complex formation does not occur and patients do not experience fifth disease symptoms.

The fetus has to be considered an immunodeficient individual with ideal target tissue because of the high proliferative state. Intrauterine parvovirus B19 infection is usually asymptomatic but it can have serious adverse consequences. The virus has been demonstrated in hepatic erythroid precursor cells of aborted fetuses by immunofluorescence, *in situ* hybridization and electron microscopy. Death in nonimmune hydrops fetalis is secondary to congestive heart failure due to severe anemia, perhaps in combination with viral myocarditis.

The time during pregnancy in which maternal infection occurs is a critical variable in determining fetal outcome. Infection during the first trimester can result in spontaneous abortion which is not recognized as a viral sequela. However, fetal loss after B19 infection in the first trimester does not occur more often compared to noninfected women. During the last trimester, transplacental passage of protective maternal IgG antibody usually prevents development of disease of the almost mature fetus. The second trimester is the critical period with significantly increased fetal death compared to controls.

A peculiar syndrome of congenital anemia associa-

ted with parvovirus B19 has recently been described in three infants. All had been treated by blood transfusion *in utero* or at birth because of hydrops fetalis. After delivery the infants had hypogammaglobulinemia. In all three, sera lacked B19 but viral DNA was found in bone marrow. All were treated with immunoglobulin. One child died despite therapy and B19 was detected in bone marrow and at lower levels in other tissues. In the other two cases, virus could no longer be detected after therapy but the patients remain persistently anemic. These observations suggest a 'hit and run' mechanism, perhaps with virus-induced autoimmune-mediated suppression of erythropoiesis rather than direct viral cytotoxicity.

The immune deficit in persistent parvovirus infection and its treatment

In persistent human infections, two patterns of antibody production have been observed. In children receiving chemotherapy and in most patients with AIDS, no antibody to virus is detectable by either capture immunoassay or immunoblot. In patients with congenital immunodeficiency, antibody has been detected by capture assays, but reactivity on immunoblot was low or undetectable. Sera from these patients poorly neutralize virus infectivity *in vitro*. In one case, the pattern of antibody response (high IgM and anti-VP2 IgG alone) suggested a defect in class switching and confirmed the importance of VP1-specific antibodies for definitely controlling parvovirus B19 infection. Although CD4^+ T cells must be key players in the development of an effective IgG antibody response, only one group has reported a proliferative *in vitro* response of human lymphocytes to parvovirus antigen.

Commercial immunoglobulin preparations are a good source of neutralizing antibody to parvovirus B19. Commercial IgG has been successfully employed in the treatment of persistent parvovirus infection, its administration leading to an immediate reduction in the titer of circulating virus (about 10^6 -fold) followed by reticulocytosis and return of hemoglobin to preinfection values. Fifth disease symptoms may occur with the infusion of immunoglobulin.

In some patients with congenital immunodeficiency and acute leukemia during chemotherapy, IgG therapy seemed to be curative for parvovirus infection. In patients treated with chemotherapy, this can be explained by regained immunocompetence when treatment is discontinued. Modulation of the antibody network secondary to IgG has been hypothesized in patients with congenital immunodeficiency. In patients with irreversible immunodeficiency, as in HIV infection, relapse of parvovirus infection often

occurs 3–7 months after initial therapy and requires retreatment with immunoglobulin. In AIDS patients, low titers of parvovirus are detectable by polymerase chain reaction in remission of the infection, and circulating DNA rises prior to clinical relapse of parvovirus infection.

Summary and perspective

Parvovirus B19 infection is an excellent example of determination of the outcome of a viral infection by the immune system (Table 1). In immunocompetent individuals, timely antibody production prealters clinical symptoms due to direct viral cytotoxicity. Infected people are either asymptomatic or they experience mild and usually transient manifestations of immune complex-mediated symptoms such as rash or arthralgia. Rarely, the immune response is significantly pathogenic, as in the few patients with prolonged or relapsing arthropathy. In contrast, immunodeficient patients unable to produce neutralizing antibodies develop severe anemia due to suppression of erythropoiesis by the virus.

Viral diseases can be prevented and even eliminated by vaccination. Empty parvovirus capsids enriched for VP1 epitopes elicit effective antibody responses in animals. A recombinant formulation has entered clinical trials in normal volunteers, in whom it appears safe. Vaccination would be desirable for nonimmune women before pregnancy (similar to rubella vaccination), for seronegative patients before and during immunosuppressive treatment and in AIDS patients. A program of universal vaccination might be considered if B19 can be convincingly linked to other serious diseases such as myocarditis and vasculitis.

Vaccine development may be easier in the future due to the recent description of the first animal model for parvovirus B19. In 1992, simian parvovirus (SPV) was identified as the agent causing anemia in a group of cynomolgus monkeys of an animal facility. There is about 50% overall sequence homology of SPV with B19 but little homology with members of the genera *Parvovirus* and *Dependovirus*. *In vitro*, SPV selectively infects erythroid progenitor cells and the major clinical manifestation is anemia. Similar to B19, SPV infection is common and mostly subclinical. Significant anemia only develops in immunosuppressed monkeys such as those coinfecting with a retrovirus. All these observations suggest a close evolutionary relationship of SPV and B19.

See also: Immunodeficiency, primary; Immunodeficiency, secondary; Opportunistic infections; Viruses, immunity to.

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PASTEURELLA, INFECTION AND IMMUNITY

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Pasteurella multocida is primarily a veterinary pathogen that produces septicemic or respiratory diseases in both domesticated and wild mammals and birds. The organism can occur as a commensal in the nasopharyngeal region of apparently healthy animals. Uncomplicated diseases in which *P. multocida* is the primary etiologic agent are hemorrhagic septicemia of cattle and water buffalo, fowl cholera of poultry and free-flying birds, and rhinitis (snuffles) and pneumonia of rabbits. Diseases in which *P. multocida* is a complicating or secondary pathogen are atrophic rhinitis and pneumonia of swine, and shipping fever and pneumonia of cattle. In humans, most *P. multocida* infections arise from pet animal bites, particularly cats and dogs.

Characteristics of the organism and its antigens

P. multocida is a nonmotile gram-negative bacterium. Virulent strains are usually capsulated. When grown on transparent agar medium, colonies made up of capsulated cells appear iridescent in oblique transmitted light, whereas colonies made up of non-capsulated cells appear blue. *P. multocida* is a heterogeneous species consisting of different capsular serogroups and somatic serotypes. There are five distinct capsular serogroups lettered A, B, D, E and F. Mucopolysaccharides are associated with the capsules of serogroups A, D and F, rendering them inagglutinable in homologous, high-titered antisera. Specific mucopolysaccharidases which depolymerize the capsules can be used for presumptive identification of these serogroups (Table 1). Colonies of serogroup A, which contain hyaluronic acid, tend to be more mucoid and larger than those of the other serogroups. Fresh serogroup A isolates from the respiratory tract, particularly those from cattle, have a watery mucoid consistency similar to *Pseudomonas*

Table 1 Depolymerization of *Pasteurella multocida* capsules by specific enzymes

Enzyme	Serogroup				
	A	B	D	E	F
Hyaluronidase ^a	+	–	–	–	–
Heparinase III ^b	–	–	+	–	–
Chondroitinase AC ^b	+	–	+	–	+

^a From bovine testes.

^b From *Flavobacterium heparium*.

aeruginosa strains isolated from human cases of cystic fibrosis. The specific-polysaccharide capsule antigens are distinguished by indirect hemagglutination tests using antisera made in pasteurella-free rabbits. These heat-stable antigens readily adsorb to washed erythrocytes, are nontoxigenic, and can sometimes be demonstrated in cell-free tissue fluids of naturally and experimentally infected animals.

Sixteen somatic serotypes (numbered 1–16) have been distinguished. Serotyping is done by a gel diffusion precipitin test using heat extracts of formalinized bacteria versus antiserum made in chickens. Somatic serotype specificities are determined by lipopolysaccharide (LPS), structural components of the cell wall. The LPS of *P. multocida* has a chemical composition similar to that of typical members of the family Enterobacteriaceae. It contains lipid A, 2-keto-3-deoxyoctonate (KDO), heptose, and various neutral and amino sugars. Four LPS chemotypes have been described. The *P. multocida* LPS appears similar to R-type LPS of *Salmonella* sp. Biological reactions produced by *P. multocida* LPS are similar to those produced by endotoxins from typical gram-negative bacteria (i.e. pyrogenicity, Schwartzman reaction, endotoxic shock). Certain serotypes of the organism tend to be associated with specific diseases (Table 2) and virulence for certain animals.

Table 2 Association of *Pasteurella multocida* serogroups with specific diseases in domesticated animals

Disease	Serogroup ^a	Predominant somatic types ^b
Hemorrhagic septicemia	B , E ^c	2
Atrophic rhinitis	D , A	3, 12
Fowl cholera	A , F	1, 3, 4
Rhinitis (snuffles)	A, D	3, 12
Shipping fever	A , D	3
Pneumonia	A , D	3

^a Bold font indicates predominant serogroup associated with the disease.

^b Other somatic serotypes may occur.

^c Serogroup E has been isolated only from animals in Africa.

Some strains belonging to serogroups A and D produce a lethal protein toxin that can cause hemorrhage and necrosis when inoculated intradermally into experimental animals. Toxigenicity of a strain is not related to specific somatic serotype or animal of origin, and toxin from both serogroups is antigenically the same. Inoculation of purified toxin by a parenteral or nasal mucosal route will produce turbinate atrophy in swine.

A number of soluble and membrane-associated protein antigens are common to the various serotypes, and epitopes may be shared with closely related gram-negative bacteria. Also, common epitopes can occur between *P. multocida* and red blood cells of mammals and birds.

Immune responses of the host

Protection against *P. multocida* in mammals and birds appears to be primarily humoral mediated. However, cell-mediated immune responses have been demonstrated. Naturally acquired immunity to *P. multocida* can occur in unvaccinated mammalian species and is demonstrated by serum passive protection of mice. This immunity is particularly evident in cattle and water buffalo from geographic areas where hemorrhagic septicemia is endemic. Bactericidal reactions can occur with polyclonal antibodies or monoclonal antibodies made against *P. multocida* provided a complement source is available. Antibody-opsonized *P. multocida* are readily phagocytosed by monocytes, macrophages and polymorphonuclear neutrophils.

Most antigens in the bacterium probably exist as complexes (i.e. LPS-protein complexes). Protein antigens are presumed to be the major immunogens responsible for protection. However, except for the toxin found in some serogroup A and D strains, a protein purified to homogeneity has not been shown

to produce protective immunity. Antibodies against the protein toxin neutralize its toxic effects and protect mice and pigs against toxin-induced turbinate atrophy.

Cattle, buffalo and poultry vaccinated with bacterins of *P. multocida* produce antibody reactions to LPS. Also, antibodies to LPS are sometimes found in sera from animals with naturally acquired immunity. Adsorption of vaccinated-cattle antiserum with LPS will remove some protection in the mouse passive protection test. Purified LPS has produced little or no active immune protection of mice. In poultry, LPS from some serotypes will produce a moderate active immune protection. Complexes of LPS and basic protein or ribosomes from a heterogeneous source produce a better serological response and active immune protection. However, this protection is somatic serotype specific. The similarity of *P. multocida* LPS to the R-type LPS of enterobacteria, particularly with regard to molecular size, probably accounts for the poor immunogenicity of purified preparations.

Capsule antigens have been suspected to be responsible for immune protection, particularly in cattle and buffalo. Antibodies to these antigens have been found in sera from those animals vaccinated with bacterins as well as those with naturally acquired immunity. Adsorption of the sera with purified polysaccharide can remove some protection in the mouse passive protection test.

Evasive strategies by the organism

The presence of a capsule contributes significantly to virulence of *P. multocida* and its survival in a host. Little or no phagocytosis occurs with nonopsonized capsulated strains of any serogroup. In contrast, noncapsulated strains and strains whose capsules are enzymatically depolymerized are more readily phagocytosed. The nonantigenic mucopolysaccharides associated with capsules of serogroup A, D and F strains are chemically similar or identical to mucopolysaccharides produced by their hosts. The mucopolysaccharides may provide a mechanism for bacterial attachment to host cell receptors as well as protecting them from ingestion by phagocytic cells. Resistance to the bactericidal effect of normal serum has been associated with the capsule and virulent strains of the organism. Additionally, an antiphagocytic membrane protein has been described in an avian strain. The older literature describes aggressins produced by strains of *P. multocida* that cause hemorrhagic septicemia.

Vaccines

Bacterins and live-attenuated vaccines are used to control diseases caused by *P. multocida*. Bacterins usually consist of formalin-killed organisms in a water-in-oil emulsion; they produce a longer duration of protection than bacterins without adjuvant. Aluminum hydroxide is occasionally substituted as adjuvant. Protection produced by bacterins is largely serotype specific, whereas live-attenuated vaccines can protect against a variety of serotypes.

See also: Antibodies, detection of; Bacterial cell walls; Carbohydrate antigens; Endotoxin (lipopolysaccharide (LPS)); Humoral immunity; Phagocytosis; Septic shock; Toxins; Vaccines.

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PERFORIN

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The perforin model of lymphocyte-mediated cytotoxicity has its origin in the electron microscopic examination of target cells lysed by cytotoxic lymphocytes. In 1980, Dourmashkin detected circular lesions (designated polyperforins) of up to 16 nm inner diameter resembling the complement pores (Figure 1), which suggested a functional and mechanistic relationship between complement- and lymphocyte-mediated cytotoxicity. Of the two pathways implicated in target cell damage by cytotoxic T lymphocytes, perforin-dependent lysis seems to dominate in antiviral immunity, whereas the Fas pathway may regulate immune responses.

Molecular properties of perforin

The lymphocyte-generated pores are assembled from monomeric perforin (alternative names include pore-forming protein, cytolytic protein and C9-related protein)

which is stored in electron-dense cytoplasmic granules. These granules also harbor several serine proteases, designated granzymes, and proteoglycans of the chondroitin sulfate A type. Purified perforin has a potent lytic activity and lyses most tumor targets in a nonspecific manner.

Cytoplasmic granules are a common feature of both activated cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Upon T cell receptor-mediated binding of the target cell, granules selectively localize to the contact site, eventually fuse with the plasma membrane and release their contents, including perforin, into the microenvironment between the effector and target cells. It is generally accepted that perforin monomers, upon their release from granules, bind to phosphorylcholine moieties and insert into target cell membranes (Figure 2). This process is strictly Ca^{2+} -dependent. Individual proteins subsequently undergo a conformational change,

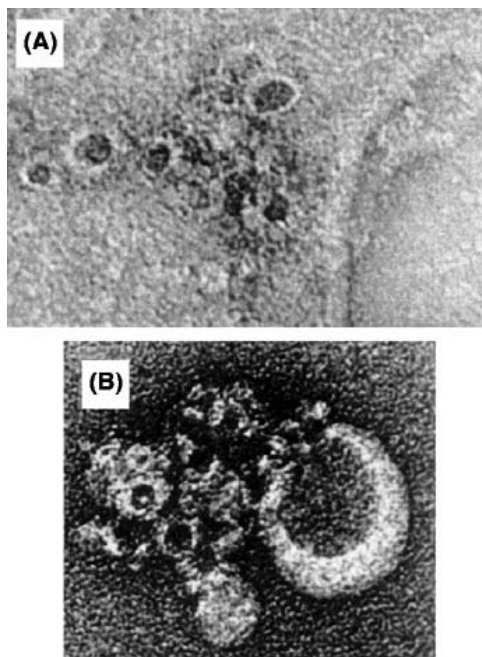


Figure 1 Pores formed by perforin (A) and complement C9 (B).

insert into the target membrane, and polymerize in the plane of the membrane, leading to transmembrane channel formation. The internal diameters of perforin pores vary from 5 to 20 nm depending on the number of oligomerized perforins (2–18).

The mature perforin exhibits a molecular weight of 65–75 kDa and has a pI of 6.44. The mouse, rat and human cDNAs coding for perforin have been sequenced, predicting a protein of 534 amino acids (mouse). The central portion of perforin, containing the membrane interaction site, shows sequence

homology with C9. This central segment is likely to be responsible for the structural and functional similarities between the two proteins, in particular the capacity to polymerize into similar tubules and to insert into the membrane. The C-terminal portion of perforin includes a C2 domain initially characterized in protein kinase C. C2 domains are thought to be involved in calcium-dependent phospholipid binding.

Perforin-mediated lysis

Initially, perforin was considered as the only lytic mediator required for target cell death. Significantly, mice with a defective perforin gene have severely impaired cytolytic activity of CTLs and NK cells. However, several lines of evidence implicate the serine proteases, granzymes, as an additional component in target cell damage. Purified perforin induces lysis of target cells without DNA fragmentation, whereas the addition of granzyme A or B to intact cells restores the apoptotic activity. Granzyme B cleaves after aspartate, a rare substrate specificity shared only with the cysteine proteinases of the interleukin 1 β -converting enzyme (ICE) family which are key molecules of apoptosis. Since ICE members require processing at aspartate, it has been speculated that during CTL attack, ICE precursors may be activated by granzyme B and accelerate cell death. Indeed, CTLs from granzyme B knockout mice were found to have impaired apoptotic activity.

See also: Antibody-dependent cellular cytotoxicity; Apoptosis; Cytotoxic T lymphocytes; Cytotoxicity, mechanisms of; Fas (CD95) and fas ligand; Granzymes; Natural killer (NK) cells.

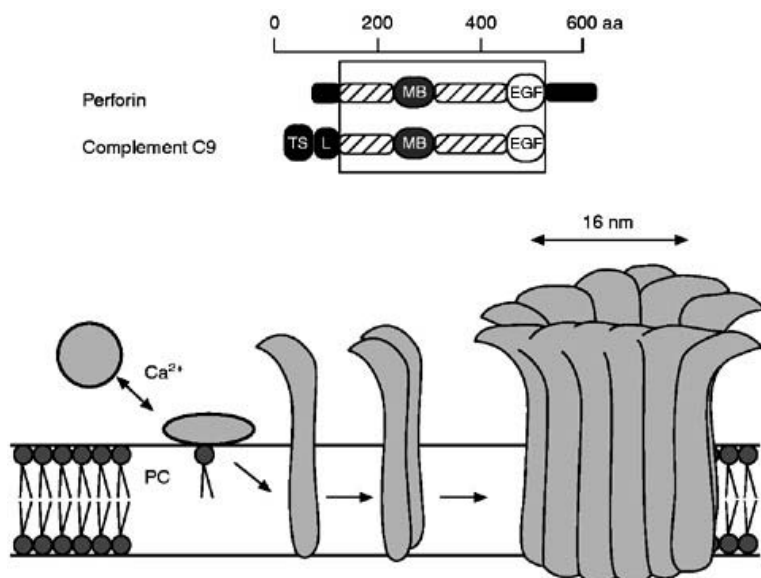


Figure 2 Structural organization of human perforin and the terminal complement component C9 and lipid insertion of perforin. Monomeric perforin binds to phosphatidylcholine (PC) headgroups of lipid membranes. This interaction is strictly Ca^{2+} -dependent and reversible. At physiological temperature, perforin undergoes a drastic conformational change and inserts into the lipid bilayer. Polymerization of the perforin protomers ensues. Cylindrical polymers of up to 18 monomers are formed. The largest tubules have an inner diameter of >10 nm. The inset above shows the arrangement of the various structural motifs present in perforin and C9: TS, thrombospondin module; L, LDL receptor class A module; EGF, EGF receptor module; MB, candidate lipid-binding domain.

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PHAGE DISPLAY OF ANTIBODIES

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Antigenic stimulation of the humoral arm of the immune system results in the production of specific antibodies with high affinity for the inciting antigen. This process involves a series of highly regulated genetic and cellular events, beginning with the generation of antibody diversity by somatic recombination of germline gene segments encoding immunoglobulin receptors in B lineage cells, and culminating in hypermutation of immunoglobulin receptors and clonal expansion and selection of B lymphocytes that express high-affinity receptors for antigen. It has recently been shown that the hallmarks of the humoral immune system may be mimicked entirely *in vitro* by phage display technology and antibody engineering. The construction of very large libraries of antibody fragments displayed on the surface of bacteriophage particles, the selection and multiplication of phages expressing desirable antibody specificities and the increase of antibody affinity by introduction and selection of favorable mutations in the genes encoding a selected antibody specificity represent the molecular biologists' solutions in an attempt to copy the immune system's impressive capacity to mount an immune response to a virtually unlimited number of antigens.

Antibody phage display technology

An important aspect of antibody phage display technology is the expression of fragments of antibody molecules on the surface of filamentous phage particles. The expressed antibody fragments may take various formats, including Fab fragments, single-chain (sc) Fv fragments and fragments composed of

disulfide bridged variable regions. A good example of a vector system that directs the production of phages bearing antibody fragments upon introduction in bacteria is a system based on phagemid vector pHEN1 (Figure 1). In phagemid pHEN1, an scFv antibody fragment is fused with the N-terminus of the M13 phage minor coat protein g3p. Constructs are placed under control of the lacZ promoter and directed to the periplasmic space of bacteria by a pelB leader peptide. A peptide tag for detection and purification is fused to the C-terminus of the scFv

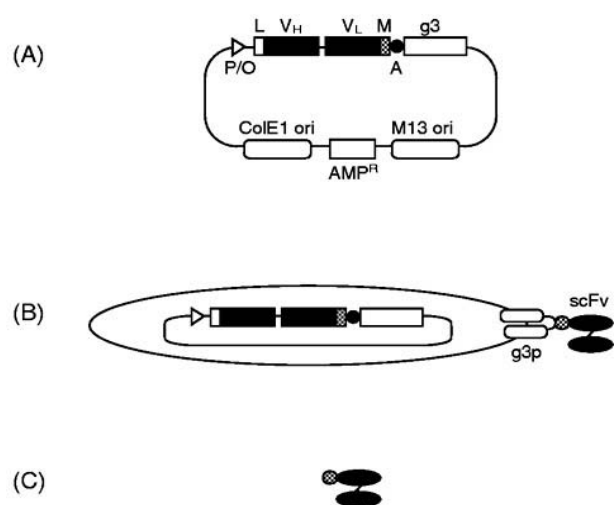


Figure 1 Phagemid pHEN1 (A), a phage antibody (B) and a soluble scFv fragment (C). P/O, lacZ promoter/operator; L, pelB leader; V_H + V_L, scFv encoding gene; M, Myc tag; A, amber codon; g3, gene3; colE1 ori, *E. coli* origin of replication; AMP^R, ampicillin resistance gene; M13 ori, phage intergenic region; g3p, coat protein 3.

fragment. The phagemid harbors an amber stop codon preceding the gene encoding g3p. As a consequence, in SupE suppressor strains of *Escherichia coli*, the amber codon is read as a glutamine and the antibody is displayed on the phage via a g3p anchor. In nonsuppressor strains, the amber codon is read as a stop codon, and soluble antibody fragments are secreted from the bacteria. Inclusion of the M13 phage intergenic region in the vector allows for replication and packaging of single-stranded phagemid DNA with the aid of helper phage. Because the filamentous particles contain a copy of the pHEN1 phagemid, selections based on the functional properties of the displayed antibody also result in selection of the genes encoding these antibodies. Phage displayed antibodies may be of any animal species including human, depending on the source of antibody variable (V) regions used for cloning.

Phage display libraries

A major goal of antibody phage display technology is to develop phage repertoires of large size and diversity to facilitate isolation of high-affinity antibodies of every conceivable specificity. Large libraries are constructed by molecular cloning of large numbers of immunoglobulin H and L chain genes and randomly combining H and L chain genes in the phage or phagemid vector. An important determinant of the composition and diversity of these *in vitro* immune systems is the source of antibody genes used as building blocks to construct the library. Libraries may be assembled from the V regions expressed by the B lymphocytes of an individual known to have mounted a particular immune response as a result of immunization, exposure to an infectious agent or as a result of an autoimmune response (Figure 2A). The pool of B lymphocytes in lymphoid organs of these individuals is enriched for antigen-specific B cells and plasma cells that have undergone clonal expansion and antigen affinity maturation. The likelihood of selecting high-affinity antibody fragments from the eventual 'immunized' phage library is increased as a consequence of the increased frequency of the desired immunoglobulin H and L chain genes used to construct the library. In principle, to further enrich for lymphocytes that bear the outward signs of antigenic contact, these libraries are constructed from the V genes expressed by B cells that have switched from expressing an immunoglobulin M (IgM) receptor to a receptor of one of the other immunoglobulin isotypes. Such libraries contain a high proportion of somatically mutated V regions.

Phage antibody display libraries may also be con-

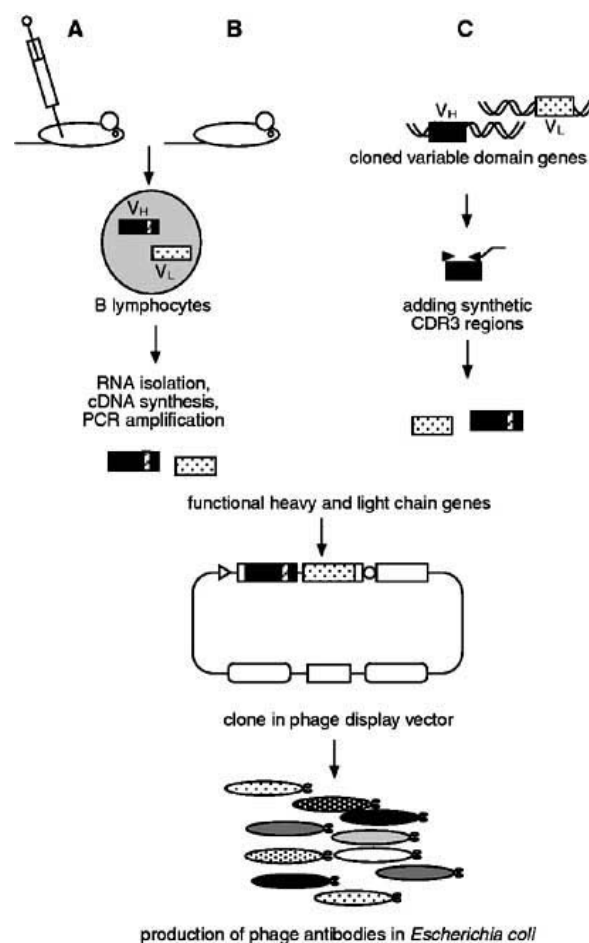


Figure 2 Constructing antibody phage display libraries. Immunized libraries are constructed from the B lymphocytes of an immunized individual (A) whereas nonimmunized repertoires are obtained from the B cells of an individual not undergoing an immune response (B). The lymphocytes are used to prepare RNA and cDNA from which the V_H and V_L genes are polymerase chain reaction (PCR) amplified using specific oligonucleotides. In a synthetic library (C), previously cloned V_H segments are used to construct functional V_H genes by adding randomized CDR3 sequences to the immunoglobulin genes by PCR. Completed scFv genes are cloned in phagemid vectors which are used to produce the phage antibody particles.

structed from the V regions expressed by the B cells of 'nonimmunized' individuals in an attempt to recruit all the diversity present in the pool of IgM-bearing B cells (Figure 2B). These so-called 'naive' antibody phage display libraries harbor a high proportion of germline V regions and are expected to encode a different collection of antibodies as compared to the immunized libraries. A third approach to create diverse libraries exploits the use of large collections of cloned V gene segments to which randomized CDR3 and J_H regions are fused *in vitro* by the polymerase chain reaction (Figure 2C). It may be anticipated that such semisynthetic libraries contain

'novel' specificities not present in the actual antibody repertoires of humans. Phage antibody display libraries of various sizes, constructed according to the above-mentioned strategies have been described. As a general rule, it appears that large-sized libraries ($>10^{10}$ clones) permit the direct selection of high-affinity antibodies, even from naive or semi-synthetic repertoires.

Phage selection strategies

Phages expressing antibody specificities of interest are selected from libraries in a four-step procedure (Figure 3): 1) binding of phages to the target antigen, 2) removal of nonbound phages by washing, 3) elu-

tion of bound phages and 4) infection and propagation of eluted phages in *E. coli* bacteria. Phage selections have been extensively performed on purified antigens coated to plastic surfaces. Although the antibodies obtained through this procedure perform well in ELISA, their effectiveness in other assays with antigens in their native conformation is usually limited. Presumably the coating procedure leads to the partial destruction of the conformational integrity of the antigen and the subsequent selection of phages against neo-epitopes.

Phage antibodies may also be selected using intact eukaryotic cells in suspension or cells growing in monolayers as targets. Selection procedures on eukaryotic target cells may be extended by an absorption step in which the library is incubated with absorber cells to remove undesired specificities. For example, selection of phages for binding to malignant melanoma cells followed by absorption of eluted phages on normal melanocytes yielded antibodies that exclusively recognized the melanoma cells. Along this line, the combination of flow cytometry and phage display library technology has proven to be a successful approach for isolating antibodies directed against surface markers present on subpopulations of cells in a heterogeneous mixture. In this procedure, the phage library is incubated with the heterogeneous population of cells, the subpopulation of interest is subsequently stained with fluorochrome-labeled monoclonal antibodies, and sorted from the heterogeneous mixture by fluorescence activated cell sorting. The nonselected cells in the mixture act as an absorber population.

Preparation of cells for phage selections may require treatment of tissues with proteolytic enzymes and/or cell culture, potentially resulting in the loss of phenotypic characteristics of the target cells. To circumvent this problem, intact fragments of tissues can be used as targets for phage selection. In this manner, scFv antibodies have been obtained binding to either membrane-bound or intracellular proteins.

Constructing antibody derivatives

The antibody fragments obtained from phage display libraries are monovalent, whereas in many *in vitro* and *in vivo* applications, multivalency is a desirable property. In addition, linking two or more binding sites efficiently increases the functional avidity of antibody molecules. Multivalent (and multispecific) scFv and Fab fragments have been successfully produced by association of antibody fragments through flexible linker polypeptides, chemical cross-linking and fusion to transcription factor-derived multimerization domains. For instance, scFv antibody frag-

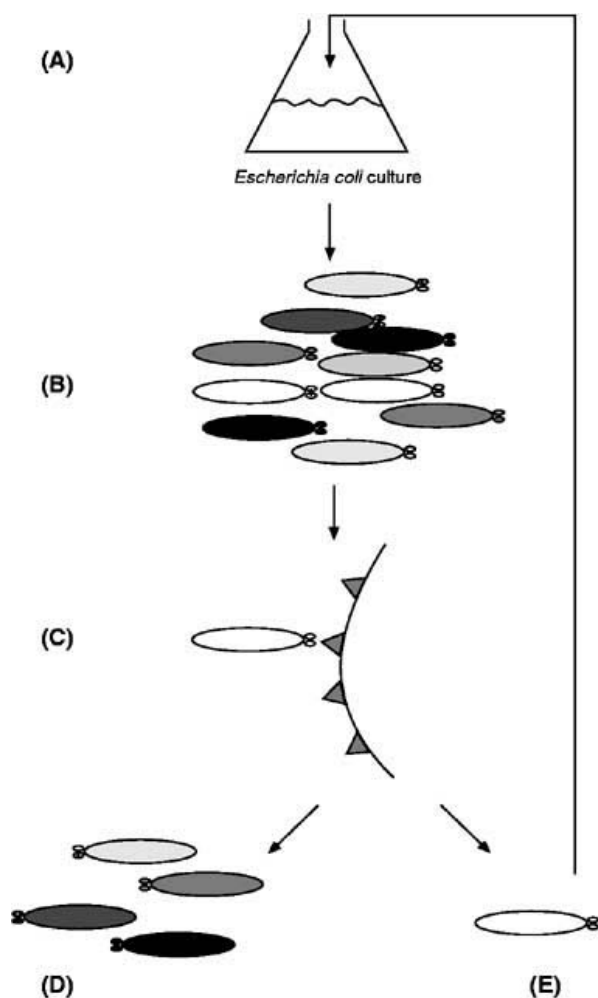


Figure 3 Schematic outline of the basic selection procedure. The antibody phage display repertoire is grown in *E. coli* bacteria (A) and purified from the medium (B). The phage antibodies are allowed to bind to the antigen, which may be either coated to a solid surface or expressed on a cell surface (C). Nonbinding phages are removed by washing (D) after which the remaining phage antibodies are eluted (E), propagated in bacteria and used for a next round of selection.

ments can be converted to dimers by genetic fusion of the scFv moiety to peptides derived from Fos and Jun or to tetramers by fusion to GCN4 protein interaction domains. Thus modified scFv fragments spontaneously multimerize to bivalent (scFv)₂ or tetravalent (scFv)₄ fragments in the bacterial periplasm. ScFv antibodies from phage display libraries have also been converted to complete immunoglobulin molecules by insertion of the variable region genes in eukaryotic expression constructs containing the constant region genes encoding each human immunoglobulin isotype. These 'natural' monoclonal antibodies can be produced in eukaryotic cells, retain the specificity of the original scFv and perform effector functions associated with the immunoglobulin Fc portion.

ScFv fragments may be incorporated into liposomes to create immunoliposomes with specific cellular or tissue-targeting properties. Addition of the *E. coli* lipoprotein signal sequence to scFv fragments results in the *in vivo* biosynthetic lipid modification of scFv, efficiently anchoring the scFv fragments to membrane encapsulated vesicles while retaining their specificity.

Conclusions

Phage display technology permits the selection of antibody fragments from large libraries constructed from the B lymphocytes of individuals or assembled *in vitro* from the genetic elements encoding antibodies. The procedure is rapid and independent of the immunogenicity of the target antigen, a drawback associated with conventional hybridoma technology. An array of selection procedures on whole eukaryotic cells and intact tissues facilitates the isolation of antibodies against membrane molecules and epitopes, even when the target cells are extremely rare or lose phenotypic characteristics upon preparation. These characteristics render this technology

a valuable research tool in searches for novel cell surface molecules, including tumor-specific antigens. Engineering steps allow the tailoring of antibody fragments to meet specific needs in terms of avidity, pharmacokinetic properties and biological effector functions. Monoclonal antibodies from phage display libraries constructed from human V regions represent a class of molecules particularly suitable for immunotherapy in humans.

See also: Affinity; Affinity maturation; B lymphocyte repertoire; Domains, immunoglobulin-type; Immunoglobulin class switching; Immunoglobulin genes; Somatic mutation.

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PHAGOCYTOSIS

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Phagocytosis by phagocytic cells is a crucial part of the host defense against invading microorganisms once the outer epithelial surface of the body has been breached (Figures 1 and 2). The most important cell types involved are polymorphonuclear leukocytes (PMNs) and mononuclear phagocytes (MNs; monocytes and macrophages), both of which are derived from precursor cells in the bone marrow. PMNs are released into the circulation in vast numbers in a fully differentiated state, with a half-life in the bloodstream of only about 6 h. The turnover rate of these cells is enormous; each day about 10^{11} PMNs disappear from the bloodstream to be replaced by equal numbers from the bone marrow. Monocytes are capable of further differentiation. After migration from the blood to tissues (diapedesis), they become macrophages of the mononuclear phagocytic system. These fixed macrophages are strategically placed to defend the body against invading microorganisms by lining blood sinusoids in the liver, spleen, bone marrow, adrenals, etc.

After invasion of the tissues by microorganisms, circulating PMNs become activated. This process is triggered by bacterial cell wall products (e.g. lipopolysaccharides, peptidoglycan, etc.), cytokines (e.g. interleukin 1, IL-1) and many other molecules (e.g. platelet-activating factor). Activated PMNs leave the bloodstream by adhering to endothelial cells and moving through the endothelial barrier to the site of the infection. This process of migration is called chemotaxis and is defined as cell movement in one direction in response to an agent (chemoattractant) (Figure 3).

Neutrophil chemotaxis requires the binding of chemoattractants to specific membrane receptors. Chemoattractants include bacterial products, activated complement factors (C5a), arachidonic acid metabolites (LTB₄), and cytokines such as IL-1, IL-8. These bind to G proteins of PMNs, leading to signal transmission to the interior of the cell which in turn leads to activation of the PMN and thus expression of adhesion molecules.

The first event during chemotaxis is the slowing or margination of the circulating leukocytes within the venule where the white cells are loosely tethered to the vessel wall and roll along the surface of the endothelium. After rolling, many neutrophils firmly adhere to the endothelial cell surface and become



Figure 1 Polymorphonuclear leukocyte in the process of phagocytosing *Escherichia coli*.

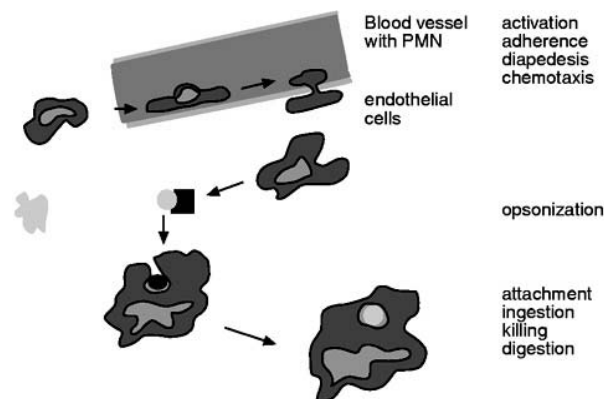


Figure 2 Processes involved in phagocytosis of bacteria by polymorphonuclear leukocytes. (Reproduced with permission from Verhoef and Visser (1993) In: Abramson and Wheeler (eds) *The Neutrophil* pp 109–137. Oxford: IRL Press.)

activated, changing from a spherical configuration to a flattened shape (Figure 3). During tethering, a family of three lectin-like carbohydrate-binding molecules called L-selectin, E-selectin and P-selectin are activated. L-selectins are expressed on most leukocytes, while E- and P-selectins are expressed by endothelial cells. Selectins promote leukocyte rolling and recognize specific carbohydrates on either leukocytes

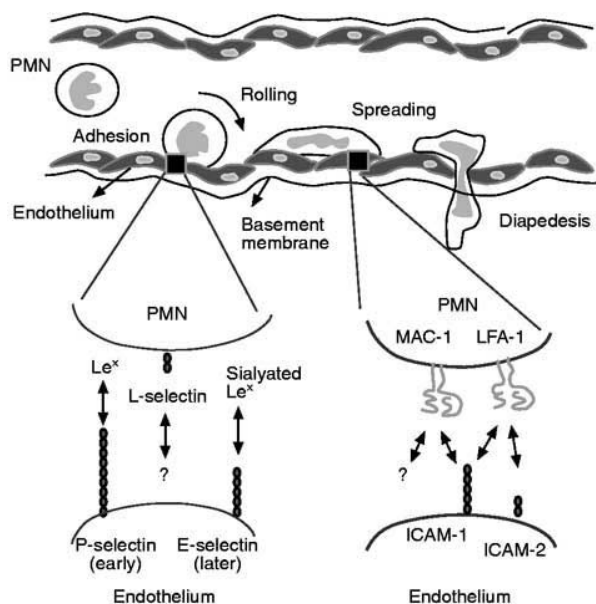


Figure 3 Process of chemotaxis. Neutrophils (PMN) are activated, adhere to the endothelial surface, roll over the surface, spread and move through the endothelial cell layer. (Reproduced with permission from Verhoef and Visser (1993) In: Abramson and Wheeler (eds) *The Neutrophil*, pp 109–137. Oxford: IRL Press.)

or the endothelium (L-selectin), which leads to tethering of the leukocytes. Adhesion by selectins is not strong enough to stop the flow of leukocytes completely. Adhesion molecules called integrins are responsible for the firm adhesion of leukocytes to endothelium. Activation of the integrins stops the flow of leukocytes.

Many of the activators (triggers) needed for the adhesion of PMNs to endothelial cells and subsequent chemotaxis are produced by the endothelial cells themselves, and by monocytes, bacteria, etc. There is growing evidence that the neutrophil itself is also able to produce chemotactic factors and activators.

Opsonization

Generally, microorganisms are only phagocytosed after they have been properly opsonized; that is, loaded with activated C3 and immunoglobulin (IgG) (Figure 4). PMNs have receptors specifically designed to bind to the Fc fragment of the IgG molecule present on the surface of the opsonized bacteria and other receptors designed to bind to the activated complement factors. Opsonization through activation of complement is primarily a function of C3b and iC3b. The PMN receptor that recognizes C3b is CR1, while the receptor that recognizes iC3b is CR3. For most opsonized particles the iC3b–CR3 interac-

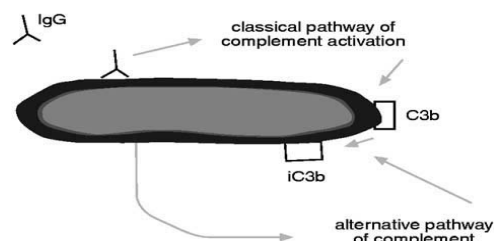


Figure 4 Opsonization of bacteria.

tion enhances attachment of the particle to PMN but not its ingestion. Ingestion only occurs in the presence of antibodies. Antibodies bind to specific antigens on the cell wall of bacteria; these antibody molecules also serve as ligands for the attachment of bacteria to the PMN.

Many bacteria have developed a defense against opsonophagocytosis and are thus able to escape phagocytosis by PMNs. The most important anti-phagocytic defense of bacteria is an enveloping capsule (Figure 5). These capsules protect the microbes against PMNs by interfering with opsonization. For example, *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and group B streptococci have polysaccharide capsules on their surface. Non-encapsulated derivatives of these organisms are less virulent.

Some other cell wall components that help microbes to evade the phagocytic defense of the host

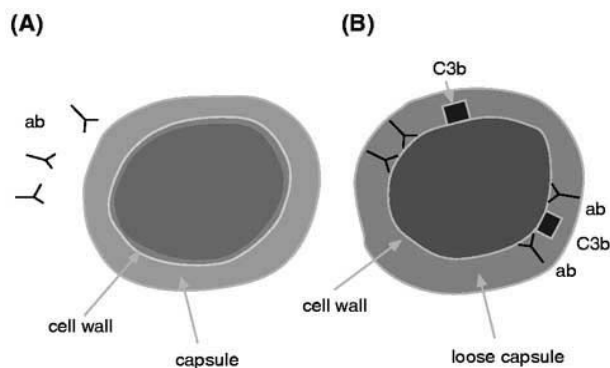


Figure 5 Encapsulated bacteria are resistant to phagocytosis because (A) opsonic antibodies cannot reach epitopes in the cell wall and therefore complement is not activated, and (B) antibodies and C3b are bound to cell wall epitopes but binding to PMNs is hindered by the capsule. (Reproduced with permission from Verhoef and Visser (1993) In: Abramson and Wheeler (eds) *The Neutrophil*, pp 109–137. Oxford: IRL Press.)

are peptidoglycans, protein A (*Staphylococcus aureus*), and protein M (group A streptococci). Some bacteria are able to adhere to PMNs in the absence of antibodies and/or complement. *E. coli* adhesins, for example, are important components that may mediate adherence of *E. coli* directly to PMNs without antibodies and complement.

In plasma there is a high molecular weight glycoprotein called fibronectin that aids the reticuloendothelial system in clearing the host of microorganisms and helps to maintain vascular stability. Several bacteria, for example, *S. aureus* and groups A, B, C and G streptococci, have receptors for fibronectin. Because PMNs can also bind fibronectin, it is possible that this glycoprotein acts as a bridge between PMNs and the bacteria and therefore facilitates phagocytosis in the absence of specific opsonins.

Phagocytosis

Phagocytosis is the process that leads to ingestion of the particle. It involves both attachment to receptors and subsequent engulfment. After engulfment, phagosomes are formed that surround the microbe, followed by fusion of secretory granules (lysosomes) with phagosomes to form phagolysosomes. Killing and digestion of the microorganisms take place within these phagolysosomes.

The process of receptor-mediated ingestion involves two steps: binding and ingestion. During binding, the receptor interacts with its ligand (opsonin) to bring the target particle in close contact with the phagocyte. For ingestion, the receptor has to deliver a signal to the cell to form pseudopodia around the target particle. The most important membrane receptors involved in the process of attachment are Fc receptors (FcγRs) and complement receptors (CRs). Human phagocytic cells bear at least three distinct types of FcR: FcγRI (CD64), FcγRIIa (CD32) and FcγRIIb (CD16). FcγRI is a 72 kDa glycoprotein with a high avidity for monomeric IgG. It is found mainly on monocytes and macrophages and contains three Ig domains in its extracytoplasmic portion and a short charged cytoplasmic tail. This receptor is upregulated by interferon γ. FcγRII is not a single receptor, but a family of closely related 40 kDa transmembrane glycoproteins with low avidity for monomeric IgG. FcγRIII is a glycoprotein that runs as a broad band between 50 and 80 kDa on SDS-PAGE gels. It is a receptor with low affinity for monomeric IgG that preferentially binds immune complexes. Human IgG1 and IgG3 subclasses interact with all FcγR classes. Human IgG2 is bound solely to FcγRII2a. Thus, uptake of bacteria by PMNs is determined by the subclass and allotype of

the opsonic IgG and the phenotype of the Fc receptor in the membrane of the PMN molecule.

Complement receptors

After opsonization, iC3b and C3b are recognized by different receptors: CR3 (CD11b/CD18) found on almost all phagocytes; and CR4 (CD11c/CD18) or gp150,95 found on some lymphocytes and phagocytes. CR1 (CD35), a glycoprotein with a molecular weight that varies between 160 and 250 kDa, only binds C3b with high affinity.

Resting PMNs have a low capacity to bind C3b-opsonized particles, but the binding capacity of PMN is rapidly upregulated during activation. The receptor-ligand interaction activates the engine of the ingestion phase: actin, myosin and actin-binding proteins. The actin microfilaments in the cytoplasm underlying the site of particle attachment polymerize and the membrane starts crawling around the particle. Pseudopodia are thus produced and surround the particle, producing a phagocytic vacuole (phagosome). Granules in the cytoplasm then fuse with the phagosome membrane to form a phagolysosome.

Uptake without opsonization

Phagocytic cell membranes contain various other receptors. One of these is the scavenger receptor, which was initially described as being involved in the removal of damaged erythrocytes and in the uptake by phagocytes of lipoprotein-proteoglycan complexes from human atherosclerotic lesions. It is now clear that these receptors also bind bacterial antigens and may be involved in binding and phagocytosis of unopsonized bacteria.

Killing

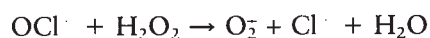
PMNs are able to kill microorganisms by two distinct mechanisms, one of which is oxygen dependent, while the other is oxygen-independent.

Oxygen-dependent events

The oxygen-dependent antimicrobial mechanisms are set in motion when PMNs undergo a 'respiratory burst'. How the interaction between PMNs and opsonized bacteria leads to a respiratory burst is not yet known. Much of our knowledge of the signal transduction that leads to enhanced oxidative metabolism after activation of the receptors for opsonins comes from studies with soluble chemoattractants. During signal transduction G proteins serve as intermediates between cell surface receptors and effector enzymes responsible for generating second

messengers. The interaction with G proteins leads to changes in conformation of the S protein and activation of adenylcyclase or phospholipase. Activation of phospholipase results in the activation of second messengers such as cAMP, inositol triphosphate, Ca^{2+} , arachidonic acid, etc. High levels of Ca^{2+} leads to activation of protein kinase A (PKA) and protein kinase C (PKC). It is likely that PKC and PKA and, possibly other kinases, play a role directly or indirectly in the activation of NADPH oxidase in the cell membrane, which reduces O_2 to superoxide (O_2^-).

Reduction of O_2 is the first step in a series of reactions that produce toxic oxygen species during the respiratory burst: $2\text{O}_2 + \text{NADPH} \rightarrow 2\text{O}_2^- + \text{NADP}^+ + \text{H}^+$. Next, O_2^- dismutates to hydrogen peroxide (H_2O_2) and the azurophil granule enzyme myeloperoxidase (MPO) catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid (HOCl):



H_2O_2 is a powerful oxidant; however, it reacts sluggishly with biological materials. H_2O_2 and O_2^- can give rise to more potent agents in the presence of ferric ion or Fe^{3+} chelates. O_2^- reduces Fe^{3+} while Fe^{3+} chelates to Fe^{2+} . The reaction of H_2O_2 with Fe^{2+} chelates yields iron-oxygen complexes that react directly with microbial components or that release hydroxyl radicals ($\text{OH}^\cdot$, Haber-Weiss reaction): $\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^\cdot$. Because $\text{OH}^\cdot$ reacts rapidly with chemical bonds of all kinds, it causes damage only in the direct vicinity of where it is produced.

HOCl is a potent microbial agent and it is unlikely that it acts directly in phagolysosomes. HOCl reacts rapidly with ammonium (NH_4^+) to yield monochloramine (NH_2Cl) and with amines to yield mono- and dichloramines (RNHCl and RNCl_2). Because the concentration of nitrogen compounds is very high *in vivo*, HOCl has little opportunity to react directly with target cells. It is likely that toxicity of the $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ system is mediated by chloramines. There is some (still controversial) evidence that human neutrophils produce $\text{NO}^\cdot$, although the effectiveness of this radical in killing staphylococci is unclear.

Oxygen-independent events

PMN cytoplasmic granules contain additional antimicrobial agents that are released into phagolysosomes and do not require the production of oxidants for activity. These agents include proteases, other

hydrolytic enzymes, such as phospholipases, glycosidases and lysozymes, and other proteins and peptides that disrupt microbial functions or structural components. It is likely that these agents must bind to the microbial cell surface to have antimicrobial activity. But activity of each agent is limited to certain microorganisms. Three well-characterized granule components, B/PI (bactericidal/permeability-increasing protein), CLCP (chymotrypsin-like cationic protein; cathepsin G) and defensins, have such microbicidal activity *in vitro*.

B/PI This 58 kDa protein of the primary (azurophil) granules may contribute to the ability of neutrophils to kill mainly gram-negative bacteria. Within 15 s of exposure to B/PI *Escherichia coli* no longer form colonies. Loss of viability is caused by an increase in outer membrane permeability to hydrophobic molecules and by activation of enzymes that are able to degrade peptidoglycan and outer membrane phospholipids. The specificity of B/PI for gram-negative bacteria is due to the interactions between B/PI and lipopolysaccharides. Both antibacterial and endotoxin-neutralizing activities reside in the N-terminal 23 kDa portion. A recombinant N-terminal 23 kDa fragment (rBPI₂₃) has been synthesized. Unexpectedly, this rBPI also exhibits gram-positive bactericidal activity and enhances the susceptibility of *S. aureus* to antibiotics.

CLCP Azurophil granules of human PMNs contain three immunologically cross-reactive cationic proteins that have both antibacterial and chymotrypsin-like neutral protease activity *in vitro*. Exposure of *S. aureus* or *E. coli* to CLCP causes inhibition of multiplication due to inhibition of macromolecular (protein, RNA, DNA) synthesis. The ability of CLCP to kill *S. aureus* and *E. coli* *in vitro* is markedly inhibited by altering the incubation conditions, e.g. by adding millimolar concentrations of calcium or magnesium ions or by increasing ionic strength or acidity. The antimicrobial activity of CLCP persists when protease activity is abolished by heat or by active-site inhibitors. Therefore, the microbicidal activity of CLCP does not depend on a primary proteolytic activity. CLCP has also been shown to act synergistically with PMN elastase against *E. coli* and *S. aureus*.

Defensins The cytoplasmic granules of PMNs contain a variety of antimicrobial cationic proteins with potent antimicrobial activity, called defensins. These peptides are small (3.5 kDa), cysteine-rich, only moderately cationic, and identical except in their N-terminal residues. They are also remarkably

abundant, comprising up to 5–7% of the total protein of human PMNs and 30–50% of the total protein of the primary (azurophil) granules. They have a broad spectrum of activity *in vitro* against gram-positive and gram-negative bacteria, fungi (including *Candida* species and *Cryptococcus neoformans*), and certain enveloped viruses. Human PMN defensins only kill metabolically active bacteria and induce loss of outer and inner membrane integrity.

Other granular agents that contribute to bactericidal activity of the PMN are, for example, elastase and lactoferrin. Lactoferrin binds iron and produces an iron-free milieu in the PMN. It is bacteriostatic because most bacteria do not multiply in the absence of iron. Lactoferrin also alters the cell wall of bacteria so that they become more accessible to lysozymes. Thus, lactoferrin and lysozyme act synergistically.

Lactoferrin has a high degree of homology with transferrin. At sites of inflammation, such as occurs at infected sites in the body, unsaturated lactoferrin is released by the specific granules of neutrophils into the extracellular environment. At the acidic pH often found at sites of inflammation, free iron is present because transferrin is unable to bind and hold iron under acidic conditions. Lactoferrin, on the other hand, has a high affinity for iron under acidic conditions and is thus able to bind this free iron instead, rendering it unavailable to pathogenic microorganisms. Iron–lactoferrin is then taken up by mononuclear phagocytes via receptors with a specificity for lactoferrin. By this mechanism, lactoferrin contributes to a localized hypoferrinemia at sites of infection.

The release of iron from lactoferrin within the mononuclear phagocyte appears to involve a different mechanism than that from transferrin. In the case of transferrin, endocytosed iron–transferrin enters a distinct, mildly acidic endosomal compartment within the cell where iron is released and then recycled back to the cell surface. Iron–lactoferrin also cycles through a mildly acidic endosomal compartment, but lactoferrin does not release iron under this condition. Iron is released from lactoferrin when a portion of the endocytosed lactoferrin enters the lysosomal compartment and is degraded. Thus, there is a potential indirect role for PMN in infections where mononuclear phagocytes play the major role, with the PMN providing lactoferrin and thereby enabling the monocyte macrophage to inhibit intracellular multiplication.

Digestion

Most of the microorganisms are digested after being killed and bacterial components are rapidly degraded by the numerous granule-associated degradative enzymes. The degree of digestion of the engulfed bacteria depends on the structure of the bacterial cell wall and on the presence of digestive enzymes in the phagocyte.

See also: Bacterial cell walls; Complement, alternative pathway; Chemotaxis; Chronic granulomatous disease; Complement, classical pathway; Complement receptors; Degranulation; Diapedesis; Endotoxin (lipopolysaccharide (LPS)); Fc receptors; Fibronectin; Granulocyte-macrophage colony stimulating factor (GM-CSF); *Legionella*, infection and immunity; Microbicidal mechanisms, oxygen-dependent; Microbicidal mechanisms, oxygen-independent; Mononuclear phagocyte system; Neutrophils; Opsonization; Tumor necrosis factor α .

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PHORBOL ESTERS

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Phorbol esters and protein kinase C

Phorbol esters are natural products derived from plants of the family Euphorbiaceae. Much interest in the phorbol esters arose from experimental results which demonstrated potent activity as skin tumor promoters in mice. Subsequently, phorbol esters were shown to have profound effects on a wide variety of biological systems *in vitro*. Some of these included effects on cellular differentiation, mimicry in normal cells of the transformed phenotype, stimulation of secretory responses and modulation of membrane activities.

Studies using radioactively labeled phorbol esters (Figure 1), PDBu (phorbol 12,13-dibutyrate) and PMA (phorbol 12-myristate, 13-acetate) identified specific phorbol ester receptors in a wide variety of cells and tissues. Interestingly, studies of these receptors and further characterization showed that they shared marked similarities with a serine/threonine protein kinase activity referred to as protein kinase C (PKC). Results from a number of laboratories confirmed that PKC was the major phorbol ester receptor. Nishizuka's group at Kobe University in Japan demonstrated, under appropriate conditions with limiting amounts of calcium and phospholipid, the required cofactors for PKC, that the phorbol esters were able to stimulate PKC activity. In addition, sev-

eral laboratories demonstrated that PKC enzymatic activity and phorbol ester binding activity copurified to homogeneity.

The identification of a kinase which is stimulated by phorbol ester provided a critical link in understanding the signal transduction pathways which are utilized by a number of hormones and growth factors. For a large class of these cellular effectors, the initial action, upon receptor occupancy, is the activation of phospholipase C. Activation of phospholipase C leads to the degradation of a specific phospholipid, phosphatidylinositol 4,5-bisphosphate, generating two products, inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate acts to elevate intracellular calcium resulting in the activation of calcium-dependent protein kinases. Nishizuka's group demonstrated that diacylglycerol (Figure 1), like the phorbol esters, is able to activate PKC, implying that PKC may be intimately involved in signal transduction for a large number of cellular stimuli, including hormones, neurotransmitters, antigens and growth factors.

The observation that diacylglycerol can substitute for phorbol ester in the *in vitro* activation of PKC suggested that it may be the natural analog mediating phorbol ester-like effects *in vivo*. A number of approaches were used to verify that assumption, including direct treatment of cells with synthetic diacylglycerols and bacterial phospholipase C to pro-

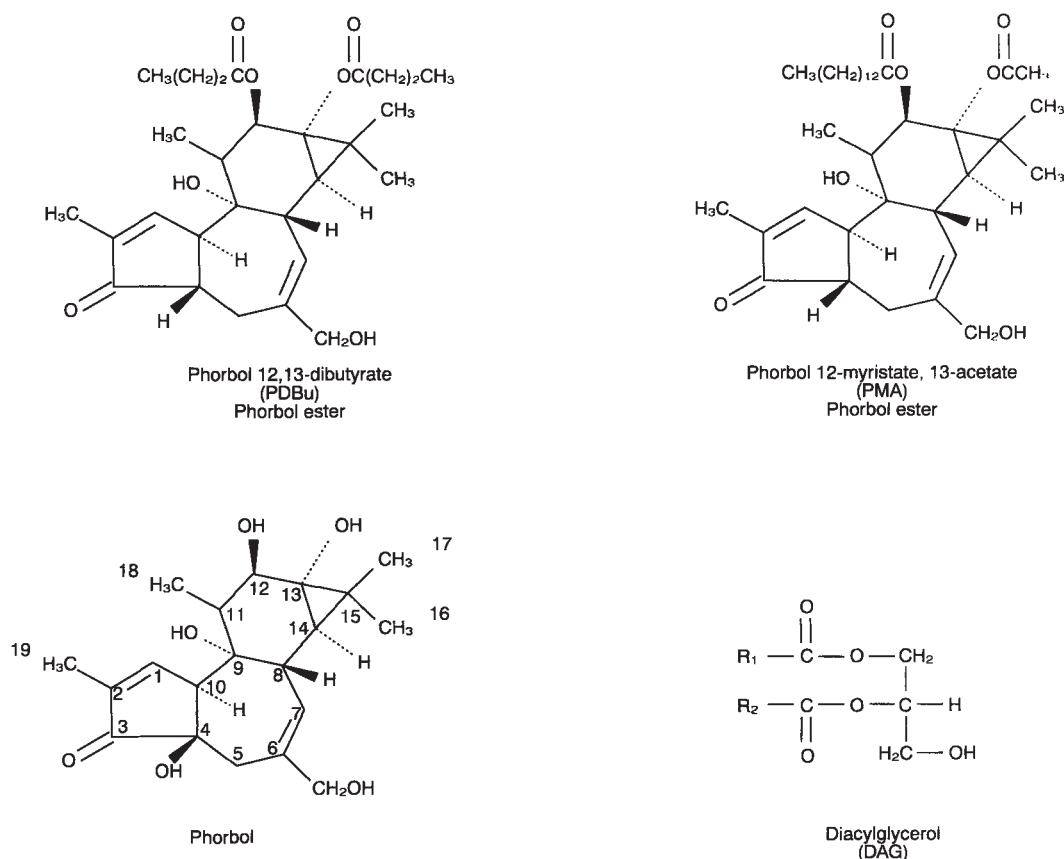


Figure 1 Structure of phorbol, phorbol esters and diacylglycerol. Phorbol, the nontumorigenic parent molecule to the phorbol esters, is shown with carbon atoms numbered in the standard format. The tumorigenic property of the phorbol esters is associated with the addition of hydrophobic moieties at carbons 12 and 13, thus forming molecules with a structure similar to diacylglycerol, a natural activator of protein kinase C.

mote cell surface phospholipid breakdown to diacylglycerol. Both these approaches showed that diacylglycerol can mimic some, but not all, of the effects associated with phorbol ester treatment. Furthermore, it was also observed that different phorbol esters could provoke different biological responses and under certain circumstances different concentrations of the same phorbol ester could produce a different effect. Although the evidence that PKC is the major phorbol ester receptor is very strong, it is clear that phorbol ester or diacylglycerol modulated effects are not exclusively mediated through a simple cellular pool of PKC.

The PKC isozyme family

Studies of phorbol ester effects mediated by PKC have revealed a number of mechanisms which may lead to the observed heterogeneity in the biological responses. Using oligonucleotide probes generated from the partial amino acid sequence of purified bov-

ine brain PKC, studies revealed that PKC exists as a family of closely related enzymes. Four subspecies (α , β I, β II and γ) emerged from the initial screening of a variety of cDNA libraries. Additionally, three other species (δ , ϵ , ζ) were identified from rat and rabbit cDNA libraries using a mixture of α , β II, and γ cDNAs as probes. In addition to certain structural differences, members of the PKC family differ in substrate specificity, ability to be activated by phospholipid, calcium, diacylglycerol and arachidonic acid. Members of this family also differ in tissue distribution and intracellular localization. While there is little evidence relating a specific function for each PKC family member, each subspecies may have a distinct role in the modulation of a variety of cellular responses, thus leading to the observed heterogeneity in PKC-mediated effects.

The primary structure of members of the PKC family consists of a single polypeptide chain (68–83 kDa) containing a regulatory and catalytic domain connected by a trypsin-sensitive hinge region

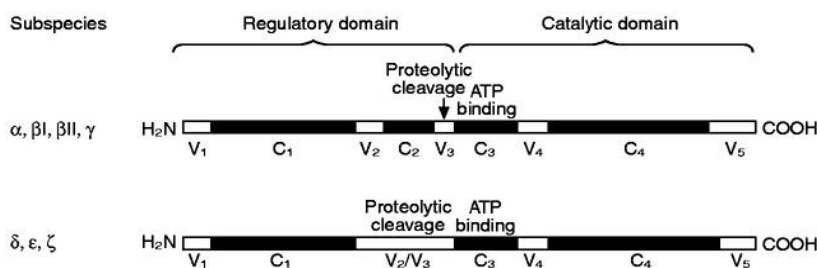


Figure 2 The structure of the PKC family. Regions which are relatively constant (C) among the subspecies are dark, while variable (V) regions are light. The major difference between the $\alpha, \beta I, \beta II, \gamma$ group and the group containing δ, ϵ and ζ is the loss of constant region 2 (C₂) in the latter. Regulatory and catalytic domains as well as the proteolytic cleavage and ATP-binding site are shown.

(Figure 2). It has been shown that limited proteolysis by calpain results in cleavage of PKC into a regulatory and fully catalytically active form (called protein kinase M). The *in vivo* implications of this observation are not completely understood; however, it appears that calpain activity is increased in the presence of phosphatidylserine plus diacylglycerol or PMA, implying that the activated form of PKC is the target for calpain action. Upon phorbol ester treatment, PKC is translocated from the cytosol to the membrane followed by subsequent depletion, termed downregulation of PKC. Thus, it appears that mechanisms exist which may lead to the rapid degradation of PKC upon activation.

Major questions regarding the role of PKC isozymes in biological signaling remain unanswered and are the focus of much research. Areas of investigation include: 1) specific isozyme coupling to recep-

tor activation; 2) alternative PKC activation pathways; and 3) identity and functional consequences of PKC substrate phosphorylation. Nevertheless, the PKC isozymes clearly appear to be an important kinase family involved in biological signal transduction.

See also: B lymphocyte activation; Protein kinases; Polyclonal activators; T lymphocyte activation.

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PHOTOIMMUNOLOGY

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Photoimmunology is the study of the effects of photons on the immune system. Although this field of study can be traced to ancient times, when sunlight was used to treat skin diseases, its origins as a scientific discipline stem largely from studies on ultraviolet (UV) radiation-induced skin cancers in experimental animals and from investigations of the cutaneous immune system. The skin is an important component of photoimmunology because all of the known effects of photons on the immune system are initiated in the skin, either from a direct interaction of UV radiation with immune cells in the skin or from indirect effects of UV radiation on other components of the skin that secondarily affect immune cells.

The photons of primary interest in photoimmunology are those with wavelengths in the UV region of

the solar spectrum, particularly the mid-UV or UVB (290–320 nm) region. UVB radiation is responsible for much of the biological activity associated with sunlight exposure, including tanning, sunburn, skin cancer and immune alterations. Wavelengths in the short UV or UVC (200–290 nm) region of the spectrum, are also immunologically active, but they receive much less attention because of their absence from natural sunlight. Long-wave UV, or UVA (320–400 nm) radiation is important because it triggers photoallergic reactions in humans and it is used in combination with photosensitizing drugs for the treatment of a variety of skin diseases, such as psoriasis and cutaneous T cell lymphoma. Recent findings have suggested that UVA may cooperate with UVB in the induction of skin cancers in mice,

cause skin aging, and contribute to immune suppression in humans.

In addition to its medical importance, interest in photoimmunology has been stimulated recently by environmental concerns. A decrease in the concentration of stratospheric ozone due to increasing concentrations of ozone-depleting chlorofluorocarbon compounds in the upper atmosphere has resulted in an increase in the amount of solar UVB radiation reaching the earth's surface. Thus, concerns about ozone depletion have focused attention on the potential health consequences of UVB exposure. The finding that UVB irradiation can suppress the immune responses of experimental animals to various antigens, including those of infectious microorganisms, has raised the question of whether increases in the amount of UVB radiation in sunlight might increase the incidence or severity of infectious diseases. UV radiation has also been a useful tool for understanding immune regulation, particularly with respect to the question of how the suppressor pathway of the immune system is activated, and for investigating the role of the immune system in cutaneous carcinogenesis.

The immunology of photocarcinogenesis

The first indication that UV radiation could produce systemic immunological alterations came from studies on UV-induced skin cancers in mice. These cancers are highly antigenic and are generally immunologically rejected upon transplantation into genetically identical mice. This unusual property of UV-induced tumors prompted studies to determine how such highly antigenic tumors could survive and grow progressively in the original host. Such studies demonstrated that the UV radiation-induced tumors were able to grow progressively when transplanted into mice that had been exposed to a short course of UV irradiation but had not yet developed primary skin cancers. Because the tumors would grow when transplanted into tissues not directly exposed to UV radiation, these studies demonstrated that UV exposure produced a systemic effect on the host that interfered with its ability to reject these highly antigenic skin cancers.

The systemic effect was immunological because it could be transferred to normal syngeneic mice by injecting them with T lymphocytes from UV-irradiated donors. Thus, repeated exposure of mice to UVB radiation induced ($CD3^+CD4^+CD8^-$) suppressor T cells, that inhibited immunological rejection of the UV radiation-induced skin cancers. These suppressor T lymphocytes also interfered with immune surveillance against the development of skin

cancers in the primary host. The conclusion drawn from these studies was that carcinogenesis by UV radiation involves at least three distinct effects of UV radiation: neoplastic transformation of cells within the skin, the formation of antigens in the UV-irradiated skin, and the induction of suppressor T cells directed against these antigens.

Alterations of immune responses by UV radiation

One of the most intriguing questions raised by studies of the immunology of photocarcinogenesis is how UVB irradiation brings about systemic alterations of immune function. It is not at all obvious how exposing the skin to UV rays brings about activation of the suppressor cell pathway in response to tumor-associated antigens. Clues for answering this question have come mainly from studies of the effect of UV irradiation on other immune reactions, particularly on the suppression of delayed and contact hypersensitivity reactions.

A crucial link between UV irradiation and the induction of suppressor T cells originated from studies on the role of epidermal Langerhans cells in the induction of contact hypersensitivity. Exposing mouse skin to suberythral doses of UV radiation brings about changes in the morphology and function of Langerhans cells. Application of a contact sensitizer to UV-irradiated skin fails to induce contact hypersensitivity, but rather induces hapten-specific tolerance and hapten-specific suppressor T lymphocytes in certain UV-susceptible strains of mice. The same phenomenon has been demonstrated in humans. The mechanism may involve an alteration in the ability of UV-irradiated Langerhans cells to stimulate different T cell subsets. Although normal Langerhans cells can present antigen to both T_H1 and T_H2 T cells *in vitro*, UV-irradiated Langerhans cells fail to activate T_H1 cells, but retain their ability to present antigen to T_H2 cells. Furthermore, the T_H1 cells are rendered tolerant by interaction with UV-irradiated Langerhans cells *in vitro*. Because the induction of tolerance was blocked by coculturing the UV-irradiated Langerhans cells with third-party allogeneic cells, it was suggested that UV may be inhibiting a costimulatory function of the antigen-presenting cells. The observation that UV exposure downregulates the expression of CD80 (B7.1) and CD86 (B7.2) on Langerhans cells supports this hypothesis. UV irradiation of antigen-presenting cells also decreases expression of class II major histocompatibility complex (MHC) molecules; however, loss of function can occur at UV doses where class II expression is unaffected, indicating that other mech-

anisms are probably responsible for their loss of function. Thus, one consequence of UV irradiation is to change the signal presented by Langerhans cells from one that activates T_{H1} cells, to one that tolerizes T_{H1} cell function.

Cytokines produced by UV-irradiated keratinocytes can also affect antigen presentation by Langerhans cells. Following UV irradiation, keratinocytes are activated to produce and secrete a wide variety of immunoregulatory cytokines. Among these, tumor necrosis factor α (TNF α) appears to contribute to the downregulation of contact hypersensitivity responses initiated within UV-irradiated skin. In addition, dendritic cells with altered antigen-presenting activity appear in both dermis and epidermis at the site of UV irradiation. Thus, alterations in Langerhans cells, local cytokine production, and a cellular infiltrate all appear to contribute to the modification of T cell-mediated immune responses induced at the site of irradiation.

In mice, UV irradiation also alters delayed and contact hypersensitivity responses initiated at sites distant from the irradiation and impairs the activity of splenic antigen-presenting cells. Generally, higher doses of UV are required than those needed to modify immune responses within the UV-irradiated site. This effect is thought to be due to the production of soluble mediators by UV-irradiated skin. Of particular importance in suppression of delayed hypersensitivity responses is interleukin 10 (IL-10), which is secreted by UV-irradiated keratinocytes and found in the serum of UV-irradiated animals. This cytokine blocks antigen presentation by macrophages and dendritic cells and suppresses the secretion of T_{H1} -associated cytokines (e.g. interferon γ), thereby blocking the induction of inflammatory immune reactions. Injecting UV-irradiated mice with monoclonal antibody to IL-10 blocks the UV-induced suppression of delayed-type hypersensitivity and reverses the impairment of splenic antigen presenting cell function. IL-10 also appears to alter antigen presentation to T_{H1} and T_{H2} cells. Whereas spleen cells from normal mice presented antigen equally well to T_{H1} and T_{H2} clones, spleen cells from UV-irradiated mice failed to present antigen to T_{H1} cells; injection of the UV-irradiated mice with IL-10-specific antibody reversed this effect. In contrast, antigen-presenting cells from UV-irradiated mice caused greater activation of T_{H2} cells than those from unirradiated mice, and injecting the UV-irradiated mice with anti-IL-10 blocked the enhanced activation of T_{H2} cells. These data suggest that one consequence of UV exposure is a shift from the activation of T_{H1} to T_{H2} cells, and induction of anergy in the T_{H1} cell population. Because IL-10 downregulates CD80

expression on antigen-presenting cells and coculturing the IL-10-treated Langerhans cells with allogeneic spleen cells overcomes the induction of tolerance, the mechanism may involve modulation of costimulatory molecules by IL-10.

In cell transfer experiments, antibodies to IL-4 and IL-10 were shown to interfere with the activity of the UV-induced suppressor cells, suggesting that these cells are indeed T_{H2} cells. Injection of IL-12 also reversed UV-induced immune suppression and blocked the induction of suppressor cells. Since this cytokine promotes the activation of T_{H1} cells, suppresses the activation and differentiation of T_{H2} cells, and interferes with the biological activities of IL-10, this finding also supports the concept that UV irradiation shifts the balance from a T_{H1} - to a T_{H2} -type of response.

One needs to keep in mind, however, that in addition to IL-10, UV-irradiated keratinocytes produce a wide variety of immunomodulatory cytokines and soluble factors, many of which have been implicated in the induction of systemic immune suppression. Prostaglandin E_2 (PGE $_2$), urocanic acid, IL-1, TNF α , and α -melanocyte-stimulating hormone are all produced following UV irradiation and all have been suggested to play a role in immune suppression. PGE $_2$ and IL-1 have been shown to inhibit the production of T_{H1} -like cytokines and favor a shift to T_{H2} cell activation. *cis*-Urocanic acid and TNF α have been shown to alter antigen-presenting cell function. Antibody against *cis*-urocanic acid prevents UV-induced suppression of certain delayed-type hypersensitivity responses, and anti-TNF α inhibits UV-induced suppression of contact hypersensitivity. Furthermore, *cis*-urocanic acid and α -melanocyte-stimulating hormone have been shown to upregulate serum prostaglandin E_2 levels, which in turn induces blood monocytes to secrete IL-10 and suppress IL-12 production. Thus, it is likely that several different mediators are involved in the induction of immune suppression following UV-irradiation. It is likely, however, that a key regulatory effect of many of these factors is an alteration of antigen-presenting cell function and upregulation of IL-10, which contribute to a shift toward an immune response dominated by T_{H2} cells.

Molecular targets of UV radiation within the skin

DNA absorbs UV radiation and is a primary molecular target of UV radiation in the skin. Mutagenesis, cellular transformation, and cell death all result from UV-induced DNA damage. DNA damage also appears to be an essential initiating event for many

forms of UV-induced immune suppression. Three experimental approaches were used to establish this finding. In the first, opossums (*Monodelphis domestica*) were exposed to UV radiation. Marsupial cells contain an enzyme that is activated by visible light and repairs UV-induced pyrimidine dimers. When the opossums were first exposed to UV and then to visible light, immune suppression was blocked. No inhibition was observed when the visible light was applied before UV, a situation that does not activate the enzyme to repair pyrimidine dimers. In the second series of experiments, a DNA repair enzyme (T4 endonuclease V) was encapsulated in liposomes and delivered to the skin of UV-irradiated mice. Here also, repair of pyrimidine dimer formation by the applied enzyme blocked the induction of immune suppression. No inhibition of immune suppression was observed when liposomes containing heat-inactivated enzyme were used. In addition, the production of IL-10 by keratinocytes was suppressed by applying liposomes containing the DNA repair enzymes to UV-irradiated skin or to keratinocytes *in vitro*. In the final approach, liposomes containing a restriction endonuclease (*HindIII*) were applied to mouse skin. Not only did applying the liposome-encapsulated enzyme damage epidermal DNA, as measured by the induction of DNA strand breaks, but it also upregulated IL-10 production and suppressed the induction of delayed and contact hypersensitivity. Thus, these experiments indicate that immune suppression can be initiated by DNA damage and reversed by repairing the pyrimidine dimers formed following UV irradiation of the skin. The implication of these findings is that DNA is a photoreceptor and that DNA damage is an essential initiating event for the induction of some forms of UV-induced immune suppression.

Other photoreceptors for UV radiation have been proposed. Most prominent is urocanic acid. *trans*-Urocanic acid is found naturally in the outermost layer of the skin and, upon exposure to UV radiation, isomerizes to the *cis*-form. Based on a comparison of the action spectra for the isomerization of urocanic acid and UV-induced immune suppression, urocanic acid was proposed to be the photoreceptor. Data from many laboratories showing that *cis*-urocanic acid has immunosuppressive effects support this hypothesis. It should be noted, however, that wavelengths in the UVA region of the solar spectrum, which are not immunosuppressive by themselves, are very efficient at forming *cis*-urocanic acid *in situ*, leading some to question the suggestion that urocanic acid is the sole photoreceptor for the induction of immune suppression. Recent studies in which

mice are treated with an antibody against *cis*-urocanic acid after UV irradiation suggest that this molecule is an essential mediator for suppression of some, but not all delayed hypersensitivity responses. Thus, some immunosuppressive effects of UV irradiation appear to be mediated by DNA damage and others by isomerization of urocanic acid.

Photoimmunology in humans

Although most of our information about photoimmunology comes from studies of laboratory animals, evidence of immunological effects of sunlight and UV exposure on humans is beginning to accumulate. Exposing human skin to sunlight or artificial sources of UV radiation causes the same types of alterations to human Langerhans cells as previously described in mice. Furthermore, human keratinocytes secrete a variety of immune modulatory factors following UV exposure, including IL-1, IL-6, IL-10, TNF α and PGE $_2$. Sensitization of humans with contact allergens through the UV-irradiated skin also suppresses the contact hypersensitivity reaction. Interestingly, although the UV-induced suppression of contact hypersensitivity was evident in approximately 40–50% of irradiated normal human volunteers, almost 100% of skin cancer patients were susceptible to the suppressive effects of UV radiation. These findings not only suggest that there is a genetic component that influences the sensitivity of humans to UV-induced immune suppression, similar to that in mice, but also that the UV-induced suppression of immune reactivity may be an additional risk factor in the induction of human skin cancer.

One question of considerable importance concerns the potential of UV-induced immune suppression to alter the immune response to pathogenic microorganisms. While animal studies have clearly shown that UV exposure can suppress the immune response to a variety of infectious agents and increase the incidence, severity and duration of infection, it is not clear whether photoimmunosuppression similarly affects the immune response to infectious agents in humans. Some evidence suggests that the ability of sunlight to trigger recrudescence of herpes simplex virus infection is due to the immunosuppressive activity of UV radiation. In addition, UV irradiation of the site of elicitation of a delayed hypersensitivity reaction to leprosy antigens in healthy, immune subjects reduced the granulomatous reaction and decreased the number of CD4 $^+$ T cells in the reaction site. Thus, UV radiation seems to have the ability to modify the immune responses of humans to infectious agents, but whether this translates into an

increase in the pathogenesis of infectious diseases remains unclear.

Conclusions

Photoimmunology is contributing to our understanding of basic immunological mechanisms, especially the role of antigen presentation in the activation of immunosuppressive T cells subsets and the identity of T cells with immunosuppressive activity. Its importance for cutaneous carcinogenesis has been clearly established in animal models, and data generated in a few studies with human skin cancer patients suggest that UV-induced immune suppression may play a similar role in human skin cancer induction. It is clear that exposure of mice to UV radiation can have systemic effects on the immune system and can suppress the immune reaction against infectious agents, but the role of photoimmunology in the pathogenesis of human diseases other than skin cancer remains to be elucidated.

See also: **Contact hypersensitivity; Skin, contribution**

to immunity; Suppressor T lymphocytes; Tumors, immunological escape of; Vitiligo.

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PHYLOGENY OF THE IMMUNE RESPONSE

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In comparison with vertebrates, the immune response of invertebrates is extremely limited. Specific antibody produced by interactive lymphocyte subpopulations together with extremely finely tuned discrimination of self from nonself and a high degree of memory are confined to the vertebrates, and yet, invertebrates comprise over 95% of all known species of animals. Clearly, the invertebrate immune system is not defective in any way so why have the vertebrates developed such sophisticated immune processes? Examination of the phylogeny of immunity and consideration of possible evolutionary and environmental pressures favoring its development may help to answer this question. As a preliminary, however, it is essential for a brief consideration to be made of the phylogeny of the main animal groups together with a basic description of the invertebrate and vertebrate immune systems.

Phylogeny of animals

Figure 1 presents the so-called monophyletic and polyphyletic origins of the main groups of animals. In the monophyletic scheme, the animal kingdom is divided into deuterostome and protostome groups according to whether the mouth and anus originate from a single opening (protostomes) or separately (deuterostomes). With the polyphyletic scheme, the major phyla of multicellular animals arose independently from ancestral metazoans. The implication of these two schemes, as far as evolution of the immune system is concerned, is that with a polyphyletic origin, the blood cells and humoral factors may have had a common ancestor and, with the exception of the vertebrates, evolved independently within each phylum. The branch leading to the vertebrates would have followed a sequential evolution passing from echinoderms, protochordates to the vertebrates, just as in the deuterostome branch of the monophyletic

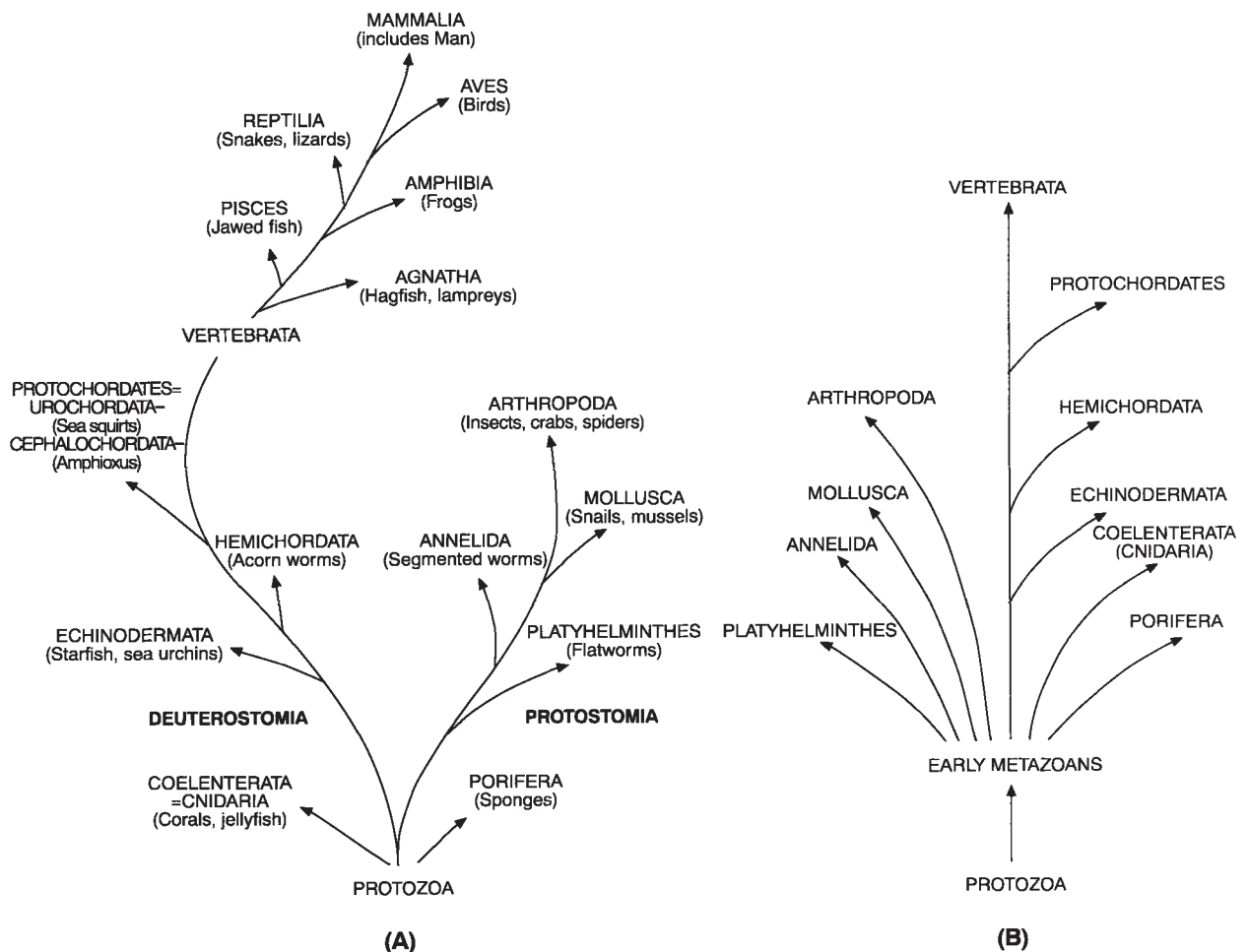


Figure 1 The phylogenetic relationships of animals. (A) monophyletic scheme; (B) polyphyletic scheme. (Reproduced with permission from Miller DA and Ratcliffe NA (1989). The evolution of blood cells: facts and enigmas. In: *Endeavour* 13: 72-77).

scheme. In this latter system, the animal kingdom bifurcates at the coelenterate level into the proto-stomes and deuterostomes with a separate evolution of the immune system in each of the groups.

In both of these evolutionary schemes, it is most probable that the ancestors of vertebrate immunity arose in the echinoderms, hemichordates and protochordates since these groups eventually led to the vertebrates. Similar structures in animals in these groups would be homologous, while any similarities with the protostomes would be purely analogous, having arisen by convergent evolution. The echinoderms and tunicates (urochordates) have been particularly studied (see below) in attempts to find the precursors of vertebrate immunity. A major barrier in tracing the evolutionary origin of the vertebrate immune system is that not only is there disagreement as to the exact origin of the vertebrates but the immediate ancestors of vertebrates are extinct. Thus, any conclusions drawn will be speculative and

assume that comparisons with living species are valid.

Invertebrate immunity

Like vertebrates, invertebrates have efficient physico-chemical barriers to infection. For example, the mucus surrounding the body of many coelenterates, annelids, molluscs and protochordates entraps and kills potential invaders. The tough exoskeleton of the coelenterates, molluscs, arthropods, echinoderms and protochordates also forms an effective barrier to infection.

Most higher invertebrates have a circulatory system containing white, but not usually red, blood cells which are termed hemocytes or coelomocytes, depending upon the nature of the body cavity. Due to the diversity of invertebrate species, and in contrast to most vertebrates, it is impossible to categorize the blood cells on the basis of staining or

morphology alone. Only a functional scheme is relevant to all the invertebrate groups and this recognizes five main types of cells: progenitors, phagocytes, hemostatic, nutritive and pigmented cells. The phagocytic and hemostatic cells are the main cells involved in defense reactions, in which they may function interactively.

Regarding cellular and humoral immunity, these too are not isolated processes but are interactive in nature. The cellular defenses include wound repair, clotting and coagulation responses, phagocytosis and encapsulation reactions and these all involve contributions from the plasma. Ingestion of small numbers of invading microorganisms is mediated by circulating phagocytes, while larger numbers of microbes and metazoan parasites are encapsulated by hemostatic and phagocytic-type cells. In many invertebrates, lectin-like molecules are probably responsible for nonself recognition processes involved in these reactions, although in most arthropod groups, the cuticle-tanning enzyme cascade, the prophenoloxidase system, may also be important. The lectin- and prophenoloxidase-based recognition systems have recently been shown to interact.

Apart from the recognition molecules, the humoral defences include a range of naturally-occurring, antimicrobial factors of which lysozyme is the most common. A few groups, such as the crustaceans, insects and molluscs, are also capable of producing inducible antimicrobial proteins but these are very different to vertebrate immunoglobulin. There are, however, recent reports of inducible proteins and antibody-like molecules in an insect and an echinoderm species but detailed biochemical analyses are wanting.

Most of the above-mentioned immune reactions are nonspecific (innate) with no accelerated or heightened response following secondary challenge and no memory component involved. Transplantation studies and mixed leukocyte cultures have shown, however, that in sponges, coelenterates, annelids, echinoderms and tunicates, allogeneic and xenogeneic recognition are present and that short-term memory may occur. The significance of these latter observations is discussed below.

Vertebrate immunity

Vertebrates also rely extensively on the physicochemical barriers such as the skin and the mucus covering the gut, respiratory and reproductive systems for protection from would-be infectious agents. The white blood cells arise from pluripotent cells in the bone marrow or their equivalent in lower vertebrates. These stem cells pass either through

the myeloid lineage to produce the phagocytic monocytes and polymorphonuclear granulocytes (polymorphs) or mature through the lymphoid lineage to form the lymphocytes. The relative lack of diversity of vertebrates allows the identification of these main cell types in simple blood smears taken from all the representative groups.

The monocytes and polymorphs undertake phagocytosis of invading microorganisms utilizing either nonspecific cell surface receptors or specific receptors for the Fc portion of antibody and the C3b fragment of complement. The monocytes mature into the tissue macrophages which form a phagocytic network, the reticuloendothelial system, in many organs of the body. The polymorphs are important cellular components of inflammation following wounding and rapidly arrive at the site of infection. Specific acquired immunity is most important in the vertebrates and is mediated by interactive events, governed by the major histocompatibility complex (MHC) system, between antigen-presenting cells, including macrophages, and particular populations of lymphocytes, termed T cells. One set of T cells (the CD4⁺ cells) then assist another population of lymphocytes, the B cells, to produce specific antibody, whilst a second set of CD4⁺ T cells induce other T cells (CD8⁺ cells) to function as cytotoxic cells, which form an immunosurveillance system in the body, eliminating virus-infected as well as mutated cells. All these lymphocyte subpopulations are identified by the nature of specific cell surface markers stained with monoclonal antibodies. In contrast to the invertebrates, which have a diverse array of hematopoietic organs, vertebrates have discrete accumulations of lymphocytes and antigen-presenting cells termed lymphoid organs. In these organs, the T and B cells undergo differentiation and development.

As with invertebrates, cellular and humoral immunity interact intimately, and in vertebrates, at least, these processes are finely controlled by a complex of molecules termed cytokines produced by activated leukocytes and other cells. In vertebrates, nonself recognition factors include the MHC antigens and receptors as well as complement components and highly specific antibody molecules. Nonspecific immunity in vertebrates also involves lectin molecules (cf. invertebrates) which activate the complement system. The hallmark of vertebrate immunity, however, is the presence of a specific acquired or adaptive immune response characterized by a high degree of specificity and a long-term memory component. Possible reasons for the development of this finely tuned adaptive response are discussed below.

Origins and comparisons of blood cells

Even the most primitive invertebrates, the protozoans, possess cell surface receptors, are capable of distinguishing self from nonself and contain lysosomes. They thus have the potential to give rise to immunocytes. With the appearance of primitive multicellular organisms or metazoans, well-organized cell layers with division of functions are formed. In sponges, for example, which lack a blood system, food is trapped by the choanocytes and carried around the body by phagocytic amoebocytes. Simple diffusion of nutrients, excretory products and respiratory gases through the body wall serves the needs of the primitive coelenterates and flatworms but, once the third body layer, the mesoderm, evolved, a circulatory system was required to transport trophic substances and respiratory gases around the body. This latter evolutionary step would have freed the blood cells from their nutritional role so that they migrated from the connective tissue into the circulatory system to take up their defensive role. This evolutionary step can be seen in some advanced platyhelminthes in which the phagocytes migrate from the parenchymatous connective tissue into the hemolymph. Once the blood cells took on a purely defensive role then differentiation into other cell types would have occurred because of various environmental and internal pressures detailed below.

The phagocytic cell type has been conserved throughout phylogeny. In invertebrates, it has a food-gathering role but in higher forms it also phagocytoses and encapsulates invading agents. In the vertebrates, the phagocytic cell evolved into polymorphs, monocytes and macrophages which retained their phagocytic role. The macrophage also took on the function of presenting antigen to the lymphocytes during specific acquired immunity. Vertebrate phagocytes also bear surface receptors for Fc and C3b which, with the exception of one echinoderm, appear to be absent on invertebrate phagocytic cells.

Much research has attempted to detect the origin of the vertebrate lymphocyte and indeed cells resembling lymphocytes in morphology are present in many invertebrates. In most cases, these are immature progenitor cells although some workers believe that invertebrates possess cells homologous to vertebrate lymphocytes. In annelids, the presence of allograft and xenograft rejection with specificity and short-term memory, adoptive transfer, graft infiltration by lymphocyte-like cells and blastogenic responses towards transplantation and T cell mitogens, indicate that the blood cells involved may be analogous to vertebrate lymphocytes and have arisen by convergent evolution. Similar properties are

reported for lymphocyte-like cells in tunicates, but the strongest case for true homology is provided by the echinoderms. In the starfish, *Asterias rubens*, lymphocyte-like cells secrete an antibody-like substance as well as a lymphokine-like factor. These axial organ cells can also be fractionated by a method used to separate mammalian B and T cells and respond like these cells towards mitogens. Another echinoderm is also reported to have C3b-like receptors on the surface of its blood cells. The case for true homology, however, awaits detailed molecular evidence of the sort recently produced in studies on the evolution of the vertebrate immunoglobulins.

Evolution of recognition molecules

In the animal kingdom there are a number of protein molecules which have been shown to function during immune recognition. These factors include the MHC system, immunoglobulins, lectins and components of the complement cascade. Much research has been undertaken to trace the phylogenetic origins of these molecules and to identify the main steps in the evolution of the vertebrate immune complex. To date, circulating immunoglobulins of the sort found in vertebrates have yet to be found in invertebrates. Possible exceptions to this statement are the inducible antibody-like response of the axial organ cells of the starfish, *Asterias rubens*, and the inducible protein involved in secondary reactions in the cockroach, *Periplaneta americana*, which shows both specificity and memory. However, until further detailed data to the contrary become available, it appears that functional, circulating immunoglobulins (Ig) are an invention of the vertebrates in which they have undergone rapid evolutionary change from the mainly single Ig class of fishes to the multiple Ig classes of mammals.

In contrast, the presence of allogeneic recognition of transplants in sponges, coelenterates, annelids, echinoderms and tunicates with a short-term memory component, indicates the presence of an ancestral MHC system. Furthermore, the alloimmune rejection process often involves lymphocyte-like cells with cytotoxic properties killing the allogeneic cells. On the basis of these results, Hildemann proposed that the histocompatibility system of cell-mediated immunity arose to deal with the threat of colony fusion in aquatic invertebrates and that the MHC complex is ancestral and entirely separate from the immunoglobulin systems of vertebrates. Immunoglobulins may have been added at the vertebrate level to provide a more extensive and sensitive immunorecognition ability in these more complex animals. The integration of the MHC and immunoglobulin

systems in the higher vertebrates resulted in an exquisitely controlled immune system involving interactive T and B lymphocytes together with antigen-presenting cells. Without the evolution of the MHC system, the various T cell subpopulations would not have developed since their activities are governed by surface receptors for MHC-encoded proteins.

The question arises as to whether the MHC and immunoglobulin systems could be interrelated in some way. One possible linking molecule is a low molecular weight (12 kDa) protein called β_2 -microglobulin which is not encoded for in the MHC region genes. β_2 -Microglobulin is closely associated with class I antigens at the cell surface and shows significant sequence homology with class I heavy chains, class II β chains and immunoglobulin constant region domains. It also shares homology with Thy-1, a differentiation antigen of thymocytes and T lymphocytes which is also found in the brain of an advanced mollusc, the squid, and is a member of the immunoglobulin superfamily. This family of molecules shares some structural and genetic homology with the immunoglobulins without necessarily having an immune function. Also of significance is the discovery of β_2 -microglobulin-like molecules in earthworms, crayfish, lobsters and *Drosophila*. Thus, some immunologists believe that the β_2 -microglobulin gene may have given rise to the immunoglobulin superfamily and the MHC antigens by gene duplication and adaptive selection. Marchalonis and his colleagues have, however, compared amino acid sequences of members of the immunoglobulin superfamily and question the idea that MHC antigens and Thy-1 share a common ancestry with immunoglobulins.

Other evolutionary ancient recognition molecules include the lectins, which identify nonself in at least the molluscs, insects, tunicates and vertebrates. Horseshoe crabs contain a lectin called limulin, which has been compared with immunoglobulin by Marchalonis and colleagues, although no sequence homology was found. Also of significance is the fact that limulin has considerable sequence homology with the pentraxins (C reactive protein family). This result was interpreted not as evidence of evolutionary homology but rather as convergent evolution of molecules binding the same ligand (phosphorylcholine), and having a similar geometry and charge distribution. Alternatively, 'mini gene insertion' of small segments of DNA ('diversity segments') occurred into molecules of diverse and unrelated genetic background. Thus, any similarity between invertebrate lectins, the pentraxins and immunoglobulin is probably analogous rather than homologous.

Another area of intense study by Clem, Du Pasquier, Litman, Marchalonis, Reynaud, Shambloott, Warr and many others has been of the immunoglobulin gene rearrangements to produce antibody diversity which occur in the various vertebrate groups. These studies have shown, amongst other things, that different patterns of gene organization and mechanisms of diversification occur in the immunoglobulin genes of the different vertebrate groups. In addition, since both light and heavy genes are organized similarly, then they probably arose by molecular coevolution. In vertebrates, there appears to have been a major change in organization of the immunoglobulin gene complex at a point in evolution when the cartilaginous and bony fish separated. Thus in teleosts, the V_H locus is similar to that of higher vertebrates, including mammals, in that gene clusters are no longer found but instead gene segments are arranged tandemly and different gene families exist in the V_H genes. Elasmobranchs are characterized by a cluster-type gene arrangement and a single family of V_H genes.

Yet another group of recognition molecules present in the animal kingdom are the complement components generated in the vertebrates. The C3 component, in particular, functions in immunorecognition with receptors for this molecule present on vertebrate phagocytes. Components of a complement-like system have been detected in insects, horseshoe crabs and echinoderms and may indicate an early evolution of this system as an immunorecognition and effector complex. Comparisons are also made of the arthropod prophenoloxidase and clotting systems and the complement cascade. Recently, however, the structural requirements for complement activation by lipid A (an active endotoxin component) have been shown to be quite different from those activating the clotting reaction in *Limulus*.

The role of evolutionary and environmental pressures

What possible pressures on the vertebrates necessitated the imposition of acquired or adaptive immunity on the very effective immune defenses of the invertebrates? After all, the invertebrates evolved successfully over several hundreds of millions of years to occupy every habitat and niche and to comprise of 95% of all animal species, without the need of a sophisticated vertebrate-type immune system. A possible explanation may involve the common occurrence of somatic mutations in vertebrates which, unless removed by a surveillance system, would develop into cancer. The increased size, longevity

Table 1 Evolutionary steps of possible significance in the phylogeny of blood cells and the immune system

<i>Evolutionary step/pressure</i>	<i>Immunological implications</i>
1. Protozoa	Recognition and discrimination
2. Metazoa including colonial forms	Histocompatibility system, allogeneic recognition, and short-term memory
3. Mesoderm and circulatory system; nutrition and defense as separate functions	Freely circulating and more diverse blood cell types; cellular immunity and erythrocytes
4. Cancer associated with increasing complexity and longevity	Immunosurveillance
5. Ancestral protovertebrate	Increased recognition and discriminatory powers?
6. Lower vertebrates with increased body size, longer lifespan, and reduced reproductive potential	True lymphocytes, lymphoid tissue, and antibody production (IgM); longer term memory
7. Emergence on to land, exposure to irradiation, and development of high-pressure blood vascular systems	Bone marrow, additional antibody classes, T and B lymphocytes, lymphoid organs with increased complexity, gut-associated lymphoid tissue (GALT)
8. Amniotes (reptiles, birds, mammals) with loss of free-living larval form	Advanced differentiation of immunocompetent cells allowing increased diversity and efficiency of immune system
9. Homoiothermy with more favorable environment for pathogens	Increased efficiency of immune system, integrated cellular and humoral responses, germinal centers in secondary lymphoid organs, lymph nodes
10. Viviparity with maternal-fetal interactions	Additional fine-tuning of immune system

Modified with permission from Ratcliffe NA and Miller DA (1988). Comparative aspects and possible phylogenetic affinities of vertebrate and invertebrate blood cells. In: Rowley AF and Ratcliffe NA (eds) *Vertebrate Blood Cells*, pp 1–17. Cambridge: Cambridge University Press.

and complexity of the vertebrates would increase the risks of somatic mutations occurring and developing into cancer. Such malignancies, if commonly occurring during the reproductive phase, would be disastrous. Adaptive immunity may thus have arisen to deal with threats developing from within the animal and would have been based on the recognition by patrolling effector cells of 'foreign' (i.e. mutated) antigens on the surface of malignant cells. This system would need to be extremely sensitive to distinguish normal self from abnormal self cells. Furthermore, since it involved circulating cytotoxic cells in surface recognition phenomena, then evolutionary pressure would favor the development and retention of the MHC system to present these antigens, and cellular rather than humoral immunity.

Recently, it has also been proposed that viral infection may impose evolutionary pressures to increase the efficiency of the MHC-based surveillance system of vertebrates. Cytotoxic T cells recognize not only mutated cells but also cells infected by viruses. In order to survive, pressure will be on the viruses to constantly modify their antigens to avoid inducing a vigorous response in association with the MHC antigens. Likewise, the immune system will be pressurized to produce MHC antigens which, in combination with the viral antigens, will be recognized by the cytotoxic T cells. This constant game of cat and mouse of the immune system in pursuit of the viral antigens will favor polymorphism of the MHC system in order to ensure that at least a few individuals can recognize the constantly changing patho-

gens. If viral infection imparts such important evolutionary pressure on the development of polymorphism in the MHC system in vertebrates then the question of why this has not also occurred in invertebrates is not easily answered.

Finally, Table 1, modified from Ratcliffe and Miller (1988), which has been compiled from the work of Burnet, Cone, Cooper, Hildemann, Manning, Marchalonis, Turner, Warr and colleagues, summarizes much of the above and also provides an outline of additional evolutionary pressures, which are self-explanatory, and which would have influenced the development of the vertebrate immune system.

See also: Amphibian immune system; Avian immune system; β_2 -microglobulin; Fish immune system; Immunoglobulin, evolution of; Invertebrate immune systems; Marsupial immune system; MHC, evolution of; Ovine immune system; Porcine immune system; Primate (nonhuman) immune system; Rabbit immune system; Reptilian immune system; T cell receptor, evolution of; Ungulate immune systems.

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PHYTOHEMAGGLUTININ (PHA)

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Phytohemagglutinin (PHA) is an extract from the red kidney bean (*Phaseolus vulgaris*) that contains potent cell-agglutinating and mitogenic properties. PHA played an important role in the development of cellular immunology. It was the first polyclonal mitogen discovered. Prior to the development of PHA, it was difficult to induce lymphocyte activation *in vitro*. The discovery of PHA allowed investigators to activate lymphocytes in *in vitro* cultures and to study the subsequent events such as growth and lymphokine production.

PHA is a mixture of five tetrameric glycoproteins. Each glycoprotein is composed of two different subunits, E and L, that have molecular weights of 33 kDa and 31.6 kDa, respectively. These subunits are combined into five possible tetrameric proteins termed E₄, E₃L₁, E₂L₂, E₁L₃ and L₄. The nomenclature E and L is derived from the observations that the E subunit appears to be the most effective subunit for inducing erythroagglutination, whereas the L subunit is a more effective lymphocyte mitogen and leukagglutinator. Thus, the E₄ protein is more than three orders of magnitude more potent an erythroagglutinator than L₄, whereas L₄ is approximately two orders of magnitude more potent a T cell mitogen than E₄. More recently studies have demonstrated that both E₄ and L₄ are effective lymphocyte mitogens, but that E₄ binds to a larger number of serum proteins. Therefore, the mitogenic capacity of E₄ is diminished more than the mitogenic capacity of L₄ under conditions in which serum is present. PHA-P is the protein form of PHA prior to separation into leucoagglutinin and erythroagglutinin.

Both E₄ and L₄ bind to glycoproteins. The carbohydrate specificity of L₄ lies within asparagine-linked oligosaccharides. The minimal structural unit required for binding appears to be GlcNAcβ1→2Man. Additions to this basic oligosaccharide greatly alters its affinity for PHA. E₄ has been shown to bind to a similar asparagine-linked oligosaccharide, although the minimal structure unit appears to be Gal1→4GlcNAc1→2Man.

PHA stimulates the proliferation of peripheral blood mononuclear cells. T cells are the responding cell within peripheral blood mononuclear cells, but responses are dependent on the presence of peripheral blood monocytes that function as accessory cells. B cells do not proliferate in response to PHA even in the presence of accessory cells. A variety of other cell types can function as accessory cells for PHA-induced T cell activation, including fibroblasts and endothelial cells.

PHA binds to a variety of glycoproteins on the surface of T cells. The specific glycoprotein(s) that is responsible for triggering T cell activation upon binding to PHA is not yet known. It is clear that PHA binds to the T cell antigen receptor. In addition, PHA binds to a member of the CD3 complex that is noncovalently associated with the T cell antigen receptor. Finally, PHA has been shown to bind to the sheep erythrocyte receptor termed CD2. Monoclonal antibodies to each of these determinants have the capacity to trigger T cell activation under certain circumstances. Thus, it appears likely that at least a portion of the activation signal is related to its ability to bind these determinants. Since PHA is a multi-

valent lectin, it is expected that PHA would not only bind T cell surface glycoproteins, but that it would cross-link these molecules as well. It has been demonstrated the CD3 must be cross-linked to generate an activation signal. Thus, the multivalent nature of PHA is likely to play an important role in its ability to trigger T cell activation. Recently, several studies have demonstrated that cross-linking CD3 together with a variety of other T cell surface molecules, including CD2, CD4, CD8, CD11a/CD18 (LFA-1), and class I major histocompatibility complex (MHC) molecules, generates an activation signal that is more potent than simply cross-linking CD3. The data suggest that the mechanism whereby PHA stimulates T cell activation is by engaging and cross-linking cell antigen receptor complex together with other T cell surface molecules.

The role of accessory cells in PHA-induced T cell activation is not clear. It has been suggested that one function of accessory cells is to bind PHA via surface glycoproteins and provide a matrix whereby PHA cross-links various T cell surface molecules. A similar function for accessory cells has been described for T cell activation induced by monoclonal antibodies to the T cell antigen receptor complex. This provision of a cellular matrix whereby PHA might cross-link various T cell surface molecules cannot completely explain the role of accessory cells, however, and other mechanisms have been proposed. These include a putative role for additional signals provided by direct contact between accessory cells and T cells and for soluble factors secreted by accessory cells such as interleukin 1 or tumor necrosis factor.

PHA remains an important investigative tool in immunology today. It is one of the most potent T cell mitogens. Moreover, it stimulates T cells in a manner that promotes their long-term growth. Thus, T cells can be cloned with nearly 100% cloning efficiency from the peripheral blood using PHA as the stimulus and these cell lines can be maintained for months with repeated stimulation. This technique

has recently been utilized to clone T cells from inflammatory tissue sites with a similar cloning efficiency. These techniques should facilitate the study of T cells that are involved in the pathogenesis of inflammatory diseases.

See also: Agglutination; CD2; CD3; Cytokines; Interleukin 2; Lectins; Polyclonal activators; T cell receptor, $\alpha\beta$; T lymphocyte activation.

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PICORNAVIRUS, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Picornaviruses are small icosahedral viruses with a diameter of 30 nm. They lack a lipid envelope and

comprise a 7–9 kb, single-stranded, monopartite RNA genome surrounded by a 35–70 Å thick protein shell. This capsid comprises 60 copies each of three major polypeptides (1B, 1C and 1D, alternatively named VP2, VP3 and VP1, respectively) and one

minor polypeptide (1A or VP4). The virus genome contains a single open reading frame coding for a polyprotein of approximately 240–250 kDa (Figure 1). The polyprotein is commonly described in an L-4-3-4 format in which an optional leader (L) polypeptide is followed by the four structural proteins (P1 region) and seven nonstructural proteins (P2 and P3 regions). Primary cleavages are between these units and are mediated by virus-encoded proteases. The polyprotein coding region is flanked on both sides by untranslated regions (UTRs). The 5' UTR is exceptionally long (approximately 600–1200 bases) and contains structures important for replication and cap-independent translation; in some members of the family a long poly-C tract separates these domains. The 5' end of the genome is uncapped; however, a small virus-encoded protein (VPg) is covalently attached. The 3' end of the genome is polyadenylated. Virion properties such as heat and acid stability can vary greatly.

The picornaviruses of vertebrates are divided into five genera: *Enterovirus*, *Rhinovirus*, *Cardiovirus*, *Aphthovirus* and *Hepatovirus*, based on their physicochemical and biological properties (Table 1). Recent analyses of complete genome sequences have shown that a number of picornaviruses require reclassification; 1) echovirus types 22 and 23 represent two species within a new genus, provisionally named *Parechovirus*; 2) equine rhinovirus type 1 is a possible new member of the *Aphthovirus* genus; and 3) equine rhinovirus type 2 should probably be classified as a new genus.

Within each genus there exist between two and >100 species which normally equate to serotypes. Generally, there is little cross-protection by viruses of different species, even within the same genus or subgroup.

The antigenic sites of picornaviruses are located on the exterior of the capsid and comprise a small number of amino acids derived either from a linear seg-

ment of polypeptide or, more generally, by the folding together of different regions of one or more of the three major structural proteins to form a discontinuous epitope. A number of epitopes have been defined and studied extensively in poliovirus (PV), human rhinovirus (HRV), mengovirus (a strain of encephalomyocarditis virus, EMCV), Theiler's murine encephalomyelitis virus (TMEV), and foot-and-mouth disease virus (FMDV) by the use of monoclonal antibody-resistant mutant viruses. Some picornaviruses show extreme antigenic variability (e.g. HRV and FMDV), whereas others are more stable (e.g. PV and HAV).

Picornavirus infections and vaccination using attenuated or chemically inactivated intact virus induce serological responses to some or all of the nonstructural proteins. The presence and specificity of serum antibody to nonstructural polypeptides is diagnostic for exposure to virus (anti-3D) and to distinguish responses elicited by infection from those of inactivated virus vaccination (anti-2C/3A).

Picornaviruses replicate in the cytoplasm of cells and entry is effected by receptor-mediated endocytosis. After replication, the progeny virus is released by lysis of the cell. The cellular receptors for HRV and PV have been cloned and sequenced (Table 2). The receptor for the major HRV group is ICAM-1 and for PV it is an unidentified member of the integrin/immunoglobulin supergene family. In the entero- and rhinoviruses a deep 'canyon' runs around the fivefold axis of the virus capsid. The cellular receptor is able to penetrate and bind to amino acids within this canyon; however, antibody molecules are too large to fit and thus cannot be directed against this conserved structure. Recently receptors for many other human enteroviruses (DAF, VLA-2, $\alpha_v\beta_3$ -integrin) and the minor HRV group (LDLR and $\alpha 2\text{MR/LRP}$) have been described. The cardioviruses appear to have a series of depressions

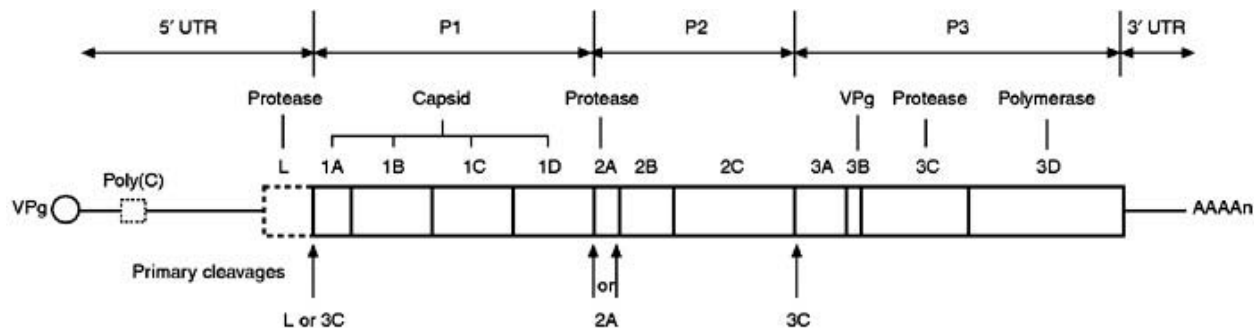


Figure 1 Generalized picornavirus genome map. The leader polypeptide is present in cardioviruses, aphthoviruses and equine rhinoviruses, however, it is a protease only in the latter two. A poly-C tract is present in aphthovirus, equine rhinoviruses and some cardioviruses. The P1/P2 cleavage occurs at the N-terminal end of 2A in entero- and rhinoviruses and at the C-terminal end in all other genera. Aphthoviruses have three VPg (3B) genes as tandem repeats.

Table 1 The picornaviruses

<i>Genus</i>	<i>Subgroup (serotype = species)</i>	<i>Examples of diseases associated with each group</i>
<i>Enterovirus</i>	Human poliovirus types 1–3	Acute anterior poliomyelitis
	Human coxsackievirus group A types 1–22, 24	Herpangina; paralytic disease; aseptic meningitis; encephalitis, ataxia; hand, foot and mouth disease; acute hemorrhagic conjunctivitis
	Human coxsackievirus group B types 1–6	Epidemic pleurodynia; aseptic meningitis; paralytic disease; encephalitis; myocarditis; hand, foot and mouth disease; swine vesicular disease
	Human echovirus types 1–7, 11–21, 24–27, 29–33	Aseptic meningitis; encephalitis; ataxia; paralytic disease; diarrhea
	Human enterovirus types 68–71	Hand, foot and mouth disease; acute hemorrhagic conjunctivitis; paralytic disease
	Simian enterovirus types 1–18	Neurological disease (?)
	Porcine enterovirus types 1–13+	Infectious porcine encephalomyelitis (Teschen disease); diarrhea; reproductive failure
<i>Rhinovirus</i>	Bovine enterovirus types 1–2	
	Human rhinovirus types 1–100	Common cold
<i>Cardiovirus</i>	Bovine rhinovirus types 1–3	Respiratory disease
	Encephalomyocarditis virus	Myocarditis; reproductive failure
	Theiler's murine encephalomyelitis virus	Paralysis; chronic demyelinating disease
<i>Aphthovirus</i> (proposed member)	Vilyuisk human encephalomyelitis virus	Encephalomyelitis
	Foot-and-mouth disease virus (7 types)	Food-and-mouth disease; myocarditis
<i>Hepatovirus</i>	Equine rhinovirus type 1	Upper respiratory tract illness
	Human hepatitis A virus	Infectious hepatitis
<i>Parechovirus</i> 'possible genus'	Simian hepatitis A virus	Infectious hepatitis
	Human echovirus types 22 and 23	Diarrhea
Unclassified/candidate picornaviruses	Equine rhinovirus type 2	Upper respiratory tract illness
	Equine rhinovirus type 3	Upper respiratory tract illness
	Acid-stable equine picornavirus	Upper respiratory tract illness
	Avian encephalomyelitis virus	Encephalomyelitis (epidemic tremors)
	Duck virus hepatitis types I and III	Hepatitis
	Picorna-like virus of harbor seals	
	Avian nephritis virus (3 types)	Nephritis
	Avian picorna-like viruses (~12)	Runting syndrome, hepatitis, pancreatitis, enteritis
	Fish picorna-like viruses	Encephalomyelitis
	Insect picorna-like viruses (at least 30 types)	Paralysis

or pits surrounding the fivefold axis and these may be involved in receptor binding. At least one receptor, VCAM-1, has been identified on susceptible cells. In the case of FMDV, which lacks the pit or canyon, a different mechanism for receptor binding has been postulated. This involves the binding of a flexible loop, containing an Arg-Gly-Asp (RGD) tripeptide, to the vitronectin receptor, $\alpha_v\beta_3$ -integrin. Other similar integrins are also thought to be candidate receptors for FMD viruses. Amino acid sequences surrounding the RGD tend to be variable, allowing immune evasion. The involvement of heparan sulfate in the initial binding of FMDV to cells has also been reported.

Most picornaviruses infect via the mouth or nose, although direct infection of target tissue such as skin (e.g. foot-and-mouth disease and swine vesicular disease) or the eye (e.g. acute hemorrhagic conjunctivitis) can also occur. Some picornaviruses replicate in the pharynx (rhinoviruses and aphthoviruses), whereas others traverse the alimentary tract to infect the gut (enteroviruses and cardioviruses). Viremia may occur, thus allowing transport of the virus to other target organs or tissues such as the central nervous system (e.g. polioviruses) or liver (hepatitis viruses).

Table 2 Cell surface receptors used by picornaviruses

Genus	Virus	Receptor
<i>Enterovirus</i>	PV-1, PV-2, PV-3	PVR (first domain) CD44 ^a
	CV-A13, CV-A15, CV-A18, CV-A21	ICAM-1 (first domain)
	CV-A2, CV-A5	Not known
	CV-A9	$\alpha_v\beta_3$ (VNR) and a second unknown ^b
	CV-B1 to B6	Not known (46–49 kDa)
	CV-B1, CV-B3, CV-B5	DAF (CD55) and $\alpha_v\beta_6$ ^c
	EV-1, EV-8	VLA-2 (α_2 chain)
	EV-3, EV-6, EV-7, EV-11, EV-12, EV-20, EV-21	DAF (CD55)
	Enterovirus 70	DAF (CD55)
<i>Rhinovirus</i>	HRV-major receptor group (90 species)	ICAM-1
	HRV-minor receptor group (9 species)	LDLR and α_2 MR/LRP
	HRV-87	Sialylated membrane protein
<i>Cardiovirus</i>	Encephalomyocarditis virus	VCAM-1
	Encephalomyocarditis virus	Sialoglycoprotein(s) (70 kDa)
	Encephalomyocarditis virus	Glycophorin A ^d
	Theiler's murine encephalomyelitis virus	Not known (34 kDa and 90 kDa)
<i>Aphthovirus</i>	Foot-and-mouth disease virus (7 species)	$\alpha_v\beta_3$ (possibly others) ^e
	(Proposed member) Equine rhinovirus 1	2 polypeptides (>200 kDa)
<i>Hepatovirus</i>	Hepatitis A virus	α_2 -Macroglobulin
	Hepatitis A virus	HAVcr-1
<i>Parechovirus</i>	EV-22, EV-23	Integrin (possibly $\alpha_7\beta_1$ or $\alpha_7\beta_3$)
Unclassified	Equine rhinovirus 2	Not known (100–150 kDa)

^aCD44 (lymphocyte homing receptor) is a possible cofactor in poliovirus attachment.

^bCV-A9 has an extension to the C-terminal end of VP1 containing an integrin-binding RGD tripeptide; this extension can be removed by enzymatic cleavage. In some cells cleaved virus is able to use an alternative receptor which presumably attaches to another site (the canyon?) on the virus surface.

^cThe integrin $\alpha_v\beta_6$ is a possible cofactor.

^dOn human erythrocytes.

^eInvolvement of heparan sulfate in binding has been reported for FMDV-O.

PV, poliovirus; CV, coxsackievirus; EV, echovirus; HRV, human rhinovirus; PVR, poliovirus receptor (possibly NCAM-like); LDLR, low density lipoprotein receptor; α_2 MR/LRP, α_2 -macroglobulin receptor/LDLR-related protein; VLA-2, very late antigen 2; VNR, vitronectin receptor; DAF, decay-accelerating factor (CD55; a glycosylphosphatidylinositol-anchored complement regulatory protein); ICAM-1, intercellular adhesion molecule 1; NCAM, neural cell adhesion molecule; VCAM-1, vascular cell adhesion molecule 1; HAVcr-1, hepatitis A virus cellular receptor 1 (mucin-like class I integral membrane glycoprotein).

Immune responses of the host

Neutralizing antibodies play an important role in protection against picornaviruses since passively transferred and vaccine-induced antibodies can abrogate virus replication and disease progression. The early immunoglobulin M (IgM) response is less specific than later IgG and IgA responses. Infection by a number of different serotypes of the same group of viruses can induce antibodies which cross-react between a wide range of serotypes. Equally, epitopes conserved between viruses of the same or different genera induce cross-reactive responses. Virus neutralization by antibody occurs via Fc receptor-mediated endocytosis (opsonization) and/or interactions which prevent virus penetration and uncoating or cause lethal RNA unpackaging (disablement). In some situations opsonization may actually promote infection of Fc receptor-positive cells although the virus yield and cell types involved are unlikely to be

sufficient to sustain clinically important productive infection.

The paradigm of antibody-mediated protection against picornavirus infection has now been challenged by the demonstration that adoptive transfer of CD4 T cells can protect from lethal cardiovirus-induced disease in the absence of any detectable antibody response. Picornavirus infections induce a wide range of cell-mediated immune responses (e.g. T cell help, delayed-type hypersensitivity (DTH), cytotoxic T lymphocyte (CTL) and natural killer (NK)-like activity) with concomitant production of cytokines (e.g. interferon γ (IFN γ), interleukin 2 (IL-2), IL-4, IL-5, IL-10, IL-12, tumor necrosis factor α/β (TNF α/β)) thereby regulating virus load, antibody responses, and the expression of accessory molecules such as major histocompatibility complex (MHC), immunoglobulin supergene family, integrin and virus receptor molecules. Epitopes recognized by CD4 and CD8 T cells have been identified in mice (e.g.

Table 3 Location of antigenic epitopes on picornaviruses

<i>Location</i>	<i>PV-1</i>	<i>PV-2</i>	<i>PV-3</i>	<i>CV-B3</i>	<i>CV-B4</i>	<i>SVDV</i>
	Site 1	Site 1	Site 1	Site 1	Site 1	Site 1
VP1 N-terminus				1023–1027		
VP1 β B- β C	1097–1101 ^a	1094, 1095, 1097, 1099	1089–1100	1051–1065, 1069–1083 1099–1123	1083–1087	1087, 1088
VP1 β C- α A						
VP1 β D- β E	1144					
VP1 β E- β F		1174	1166			
VP1 β H- β I			1253			
	Site 2	Site 2	Site 2	Site 2	Site 2	Site 2
VP1 β G- β H	1221–1226	1217, 1221				
VP1 C-terminus						
VP2 N-terminus						
VP2 β E- α B ('puff')	2142, 2164–2170	2167	2164–2192			2163 (2A) 2154 (2B)
VP2 C-terminus	2270	2158				
	Site 3	Site 3	Site 3	Site 3	Site 3	Site 3
VP1 C-terminus			1286–1290			1272, 1275 (3A)
VP2 β B- β C	2072 (3B)	2072				2070 (3B)
VP2 β C- α VP2 β E- α B ('puff')						
VP2 β H- β I		2239, 2244				2233 (3B)
VP3 N-terminus		3066				3060 (3A)
VP3 β B ('knob')	3058–3060 (3A)	3056, 3061	3058, 3059			
VP3 β B	3071, 3073 (3A)		3070, 3071			
VP3 β B- β C	3076 (3B)		3077, 3079			3073, 3076 (3B)
VP3 β E- α B						
VP3 β G- β H						
VP3 β H- β I		3207				
<i>Location</i>	<i>BEV-1</i>	<i>HRV-14</i>	<i>EMCV (Mengo)</i>	<i>TMEV (BeAn)</i>	<i>HAV</i>	<i>FMDV-SAT2</i>
	Site 1	NIm-I				
VP1 N-terminus				1013–1027		
VP1 β B- β C		1091, 1095 (1A) 1083, 1085 (1B)			1102, 1104, 1105	
VP1 β C- α A				1101		
VP1 β D- β E		1138, 1139 (1B)				
VP1 β E- β F				1145–1167	1171, 1176	
VP1 β H- β I					1232	
	Site 2	NIm-II				Site 1
VP1 β G- β H	1202–1207	1210			1221	1147–1149, 1156, 1158
VP1 C-terminus				1268		
VP2 N-terminus				2002–2014		
VP2 β E- α B ('puff')	2152–2161?	2158–2162, 2136	2144–2148			
VP2 C-terminus				2165–2179		
	Site 3	NIm-III				
VP1 C-terminus		1287		1225–1276		
VP2 β B- β C			2075			

Table 3 Continued

<i>Location</i>	<i>BEV-1</i>	<i>HRV-14</i>	<i>EMCV (Mengo)</i>	<i>TMEV (BeAn)</i>	<i>HAV</i>	<i>FMDV-SAT2</i>
VP2 β C- α A						
VP2 β E- α B ('puff')						
VP2 β H- β I						
VP3 N-terminus				3024–2043		
VP3 β B ('knob')	3054–3063		3057, 3061		3065, 3070, 3071, 3074	
VP3 β B			3068			
VP3 β B- β C		3072–3078				
VP3 β E- α B						
VP3 β G- β H						
VP3 β H- β I		3203				
<i>Location</i>	<i>FMDV-O₁</i>	<i>FMDV-A₅</i>	<i>FMDV-A₁₀</i>	<i>FMDV-A₁₂</i>	<i>FMDV-A₂₂</i>	<i>FMDV-C₁</i>
	Site 3		Site 3	Site 3		
VP1 N-terminus						
VP1 β B- β C	1043–1045, 1048, 1059 (Site 3)					
VP1 β C- α A						
VP1 β D- β E						
VP1 β E- β F						
VP1 β H β I			1169	1173		
	Sites 1 & 5		Site 1	Site 1	Site 1	Site A
VP1 β G- β H	1138, 1144–1154 (Site 1) 1149 (Site 5)		1142, 1144, 1146, 1147, 1149–1153	1151, 1152	1138, 1140, 1142, 1144, 1149, 1150, 1153, 1154	1138–1150 (Site A)
VP1 C-terminus	1208 (Site 1)	1198	1204	1201, 1209		
VP2 N-terminus						
VP2 β E- α B ('puff')						
VP2 C-terminus						
	Sites 2 & 4		Sites 1 & 2		Site 1 (?)	Site D
VP1 C-terminus						1193 (Site D)
VP2 β B- β C	2070–2073, 2075, 2072, 2079 2077 (Site 2)					2072, 2074, 2079 (Site D)
VP2 β C- α A			2080 (Site 1?)		2082	
VP2 β E- α B ('puff')	2131, 2134 (Site 2)		2196 (Site 2)			
VP2 β H- β I						
VP3 N-terminus						
VP3 β B ('knob')	3056, 3058 (Site 4)					3058–3061 (Site D)
VP3 β B						
VP3 β B- β C						
VP3 β E- α B						
VP3 β G- β H			1175, 1178 (Site 1?)			
VP3 β H- β I						

*The four-digit numbers refer to residue numbers within each of the structural polypeptides plus either 1000, 2000 or 3000 for VP1, VP2 and VP3, respectively.

PV, poliovirus; CV, coxsackievirus; SVDV, swine vesicular disease virus; HAV, hepatitis A virus; EMCV, encephalomyocarditis virus; TMEV, Theiler's murine encephalomyelitis virus; FMDV, foot-and-mouth disease virus.

Adapted from Usherwood and Nash (1995).

CV-B3, HRV-2, PV-1, PV-3, EMCV, TMEV), humans (PV-1) and cattle (FMDV) (Table 3). In some picornavirus infections (e.g. CV-B, HAV, TMEV), T cell responses contribute to the immuno-

pathological sequelae such that depletion of T cell subsets can ameliorate clinical disease. The T cell receptor (TCR) repertoire of T cells infiltrating pathogenic lesions can show restricted V $_{\alpha}$ and V $_{\beta}$,

gene usage, suggesting that the target is a specific antigen.

Some picornaviruses can establish persistent infections in the throat (FMDV, equine rhinoviruses), CNS (TMEV), or muscles (enteroviruses). The role of the immune system in maintaining or controlling persistence is not yet understood. However, CD4 T cell-depleted mice infected with TMEV fail to produce antibody and to clear virus from the CNS. TMEV antigens have been used to induce split tolerance affecting the T_H1 subset function, suggesting that inappropriately regulated immune responses, which would include infection of cells in privileged sites, such as the eye and salivary glands, may contribute to the establishment of persistence.

Evasive strategies by the organism

Evasion of the immune system by picornaviruses is primarily by antigenic variation of neutralizing antibody epitopes but may also involve variation at T cell sites/MHC-binding motifs leading to compromised help for humoral immune responses and cell-mediated killing of infected cells. However, persistent infections may be another mechanism by which these viruses can survive in the presence of circulating antibody. Hepatitis A virus appears to be antigenically very stable and little is known about how this virus may evade the immune response. Antigenic epitopes lie on the surface of the virus and are generally located on the loops which join β sheets. These loops are less constrained in both amino acid type and length. Many of these epitopes have been mapped by sequence analysis of monoclonal antibody neutralization-resistant mutant viruses.

Vaccines

Vaccines against picornaviruses rely upon the induction of neutralizing antibodies in the host by the inoculation of either attenuated live virus or chemically inactivated intact virus. Successful inactivated (Salk) and attenuated (Sabin) cell culture vaccines have been used since the early 1950s against all three serotypes of poliovirus. Similar success has been achieved for avian diseases (duck virus hepatitis and avian encephalomyelitis). Inactivated FMDV vaccines are widely used but can sometimes be of limited value because of rapid antigenic variation within serotypes; attenuated FMDV vaccines have been of little use due to rapid reversion to virulence. A more extreme problem exists for HRV which numbers at least 100 serotypes and for which no vaccines are available.

Recent advances in the molecular biology of picor-

naviruses has led to the identification of many of the antigenic sites recognized by neutralizing antibodies. Short linear peptides representing some of these sites have been evaluated as subunit vaccine antigens for several picornaviruses. Protection using peptide antigens has proved elusive despite the capacity of the antigens to elicit neutralizing antibodies in model and natural hosts. It is now clear that in addition to antibody such antigens must also elicit CD4 and CD8 T cell effector responses and that the target antigens for these cells could be derived from non-structural or structural proteins. Furthermore, T cell epitopes will need to have promiscuous MHC binding to confer maximum efficacy in outbred species. Effective delivery of these sequences will also need to be solved. The use of poliovirus as a vector to carry small antigenic peptides of unrelated viruses (e.g. human immunodeficiency virus and human papillomavirus) either within their own natural antigenic sites or as extragenomic information has also been described. An effective vaccine has recently been developed for hepatitis A virus. Poliovirus vaccines have been improved by the specific introduction of additional attenuating mutations, although they are not currently in use.

Restricted TCR gene usage by infiltrating lymphocytes from some picornavirus disease lesions also introduces the possibility to prevent or treat T cell-mediated tissue damage (e.g. myocardium, pancreas) through specific immunotherapy.

Resolution of the three-dimensional structures of a number of human rhinoviruses has enabled the design of specific antiviral drugs which bind in a pocket beneath the canyon and inhibit viral uncoating; however, their use may be limited by the ease with which drug-resistant mutants are generated.

See also: Adhesion molecules; Attenuated organisms as vaccines; Cardiac disease, autoimmune; Decay-accelerating factor (CD55); Eye infections; Fibrinectin; Hepatitis A virus, infection and immunity; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Neurological infections; Respiratory and cardiac infections; Antibody-antigen complexes, three-dimensional structures; Vaccines; Viruses, immunity to.

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PLAQUE-FORMING CELL (PFC) ASSAYS

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Hemolytic plaque assay procedures

Developed in the early 1960s, hemolytic plaque assays are among the most sensitive biological measurement procedures. In principle, these assays resemble plaque techniques for the detection of bacterial viruses. Typically, agar-embedded (see below for variations) erythrocyte suspensions are mixed with lymphocyte populations which contain relevant B lineage cells. If these cells secrete antibody to antigenic determinants present on the surface of target red blood cells, the antibody will bind first to the nearest cells and then to erythrocytes further away. In the presence of a source of complement (usually rabbit or guinea pig serum), antibody-coated red cells lyse. Thus, after ~6–12 h incubation (1–3 h in the thin-/monolayer techniques, see below), the result is formation of clear zones where erythrocytes have been lysed, and in the center of the zone is the single antibody-producing cell.

To facilitate the lytic function of antibodies that are not able to or inefficient in complement fixation (e.g. immunoglobulin G, IgG), addition of a secondary (e.g. goat anti-IgG) antibody will 'develop' such 'indirect' plaque-forming cell (PFC) assays (Table 1). If only secondary (e.g. IgG/IgA) responses are to be evaluated, it is possible to prevent the formation of 'direct' (IgM-mediated) plaques by the inclusion of 2-mercaptoethanol in the assay mixture. An excellent and extensive review of PFC assay techniques used in rodent research has been written by its inventor, Niels Jerne, and updates have been published by colleagues in his institution (see Further reading).

Numerous antigens and haptens have been conjugated to various red cells to serve as specific antigen, and the assay lends itself to functional estimates of affinity through, for example, titration of the amount of free hapten required for blockade of 50% of PFCs. However, since such blocking procedures also titrate the amount of antibody, i.e. secretory rates, and

Table 1 Key words and terms

<i>Term</i>	<i>Definition</i>
PFC	Hemolytic plaque-forming cell: a B lineage cell which secretes antibody that binds to epitopes present on surrounding erythrocytes; if the antibody can fix complement added to the assay, a zone of hemolysis is formed around the secretory B cell.
Direct PFC	If the antibody produced by the PFC is of the IgM class, the added complement directly mediates lysis of surrounding red blood cells. Theoretically, a single, bound IgM molecule is sufficient for the lysis of a red cell.
Indirect PFC	If the antibody produced by the PFC is not of the IgM class (e.g. IgG, IgA) then a 'secondary' (or developing) antibody (e.g. goat anti-IgG, rabbit anti-IgA) is added late during the assay to achieve more efficient complement activation on target erythrocytes.
Fluid-phase PFC assays	Reduction of plaque assay volumes by omission of the agarose gel. The first of these assays ('Cunningham assay') uses hemocytometer chambers and coverslips to suspend appropriate mixtures of lymphocytes (i.e. PFCs) and target red blood cells. To avoid physical disturbance of these mixtures, another procedure uses red blood cell monolayers fixed to the bottoms of 96-well microtiter plates with poly-L-lysine. Relevant lymphocyte/complement mixtures are added to monolayers and close red cell/white cell contact can be achieved by gentle centrifugation.
Reverse PFC assays (rPFC)	Nonantigen-specific version of indirect plaque assays where the target red blood cells are conjugated with anti-Ig antibody (e.g. anti-IgG, anti-IgA or polyvalent antibody). One variation uses protein A instead of anti-Ig. A nearby B cell secreting an antibody of the relevant class becomes a plaque-forming cell as its Ig product is bound to the red blood cell surface. rPFC assays permit studies of polyclonal B cell activation. This assay can be modified to detect the secretion of molecules other than Ig.
ELISPOT assays	Reminiscent of rPFC. Replacement of conjugated erythrocytes with an enzymatic detection system typical of ELISA procedures allows enumeration of cells secreting molecules of interest such as specific cytokines. Instead of red cells, the primary (capture) antibody is coated to plastic and cells are incubated on top. Following addition of enzyme-conjugated secondary antibody and enzyme substrate, secretory cells are enumerated as dark spots surrounding a single lymphocyte.

since secretory rates can vary by orders of magnitude within a given immune response induced, results of hapten inhibition studies must be interpreted with caution.

The size of hemolytic zones is related to antibody affinity and secretory rate, but accurate measurements are cumbersome and have not been rigorously examined with cloned cell sources. Given that a single IgM molecule can fix one C1q complement molecule to the red cell surface and that this is sufficient for the formation of a lytic site, there is little doubt that hemolytic plaque assays are of exquisite sensitivity. The actual sensitivity is somewhat lower, probably due to antibody loss in the three-dimensional assay space, but cells secreting in the order of a few thousand antibody molecules per hour are clearly detectable.

Methodological variations of PFC assays

A number of variations of the original hemolysis-in-gel (agarose) procedure have been described with the goals to improve cell survival in the assay system, reduce the cell number and materials requirements and increase practical assay throughput and, perhaps, sensitivity. Agarose is still used extensively as

the plaquing medium but smaller assay vessels than standard Petri dishes (e.g. slides) are now being used more often. The development of efficient microcultures generated a need for micro-PFC assays. The first such modification was Cunningham's fluid phase assay that used red blood cell/lymphocyte mixtures suspended in hemocytometer chambers. In this procedure PFCs are read microscopically. It requires small cell numbers and eliminates the need for a solid carrier medium. One potential drawback of the procedure is the danger of accidental resuspension which, of course, obscures the round lytic zones.

Modified plaque assays were further developed under Cunningham's influence in Toronto, including a solid-phase procedure, and its micromodification which permitted fast (1 h) PFC assays in 96-well formats using very small numbers of cells (e.g. limiting dilution microcultures). In this procedure, monolayers of red blood cells, modified with haptens or unmodified, are immobilized on plastic surfaces with poly-L-lysine. To ensure intimate contact, lymphocytes are sedimented on to that monolayer by centrifugation. Small volumes of medium and complement are added with the lymphocyte preparation. Plaques in this system develop quickly but often are small

and a specific role of the poly-L-lysine itself modifying antibody release was suggested. A majority of experiments dealing with antigen-specific *in vitro* responses of human lymphocytes have used this system. Although successful in several laboratories these modifications were overall 'tender' techniques which are rarely used today.

Another, rather important development was the introduction of 'reverse' hemolytic PFC assays. This procedure is in essence the reverse of an indirect PFC technique where the secondary (developing) antibody becomes the primary reagent covalently coupled on to the surface of target erythrocytes. Any secretory B cell releasing an Ig bound by these anti-Ig-coated target red blood cells will now become a PFC. This is a rapid and elegant procedure for studies of polyclonal B cell activation, in particular in the human system where B cell activation has been less easily achieved and manipulated.

Reverse PFC assays have been further modified for the detection of cells secreting proteins other than Ig, broadening applications for this sensitive procedure beyond B lymphocyte studies. The latest of these modifications is a departure from the use of indicator erythrocytes and their replacement with an ELISA-type enzymatic read-out system. These ELISPOT assays are now increasingly used for the enumeration of T cells secreting T_H1 - or T_H2 -type cytokines. Reminiscent of rPFC assays, plastic dishes or plates are coated with an antibody specific for the cytokine of choice, T cells (fresh or stimulated) are added in culture medium, and the secreted cytokine is captured by the plastic-bound antibody. After some hours of incubation, the assays are developed through addition of enzyme-coupled secondary antibody to the cytokine. In the presence of relevant enzyme substrate, round areas of enzymatic activity (usually dark spots) are counted microscopically, each corresponding to one T cell producing the particular cytokine of interest.

Application of PFC assays

PFC procedures have long been a mainstay of cellular immunology research. Developed prior to the advent of high-sensitivity immunoassay procedures for secreted antibodies, the vast majority of early fundamental research into cellular aspects of humoral immune responses employed hemolytic plaque assays. PFC assay procedures turned out to be overall very robust, reproducible and practical. Although a high throughput screening method has been described (hemolytic drop procedure), the principal drawback of PFC assays is the need for visual inspection of each plaque. This reduces the number of vari-

ables that can be practically monitored and stands in some contrast to the now quite efficient tissue culture methods for multiple microcultures.

A computer-directed on-line literature search yielded over 3000 PFC papers published from 1970 to 1980. This number declined in the following decade to ~1000, with a majority published in the earlier 1980s. Besides the current expansion of molecular approaches towards cellular immunology research, this primarily reflects the development of more and more versatile and sensitive immunoassays which can easily be automated and require less experimental effort, in particular in processing larger sample numbers. The use of ELISPOT assays, however, is on the upswing.

The exquisite sensitivity of hemolytic plaque assays can become a drawback. For example, antibody present in serum or culture fluids and binding to leukocyte Fc receptors can easily be transferred to PFC assays. These Igs may be released to form a plaque; as well, small conglomerates of antibody-coated red cells used as antigen in preassay culture systems can be transferred to the assay system and produce small 'pseudoplaques' without a central leukocyte. Inclusion of controls such as cycloheximide (to block *de novo* protein synthesis) can point to such artifacts, since pseudoplaques obviously do not involve newly synthesized Ig. Fc receptor binding/release of Igs was exploited in the development of plaque assays for the characterization of Fc receptors on lymphoid and nonlymphoid cells.

PFC assays measure several aspects of the total cellular immune response induced in a given experiment, i.e. the product of clonal recruitment, clonal expansion and activation of enhanced antibody secretory rates. However, and in contrast to immunoassays of secreted immunoglobulin accumulation in supernatants, PFC assays detect only those cells which secrete their antibody *during the assay period*.

When combined with limiting dilution-type protocols, PFC precursor cell recruitment and clonal expansion can be distinguished and employed for estimates of response diversity. However, neither the PFC nor the immunoassay approach permit a complete analysis and understanding of a given immune response. For example, the induction of few, very high-rate Ig secretors may represent a 'large' secretory immune response, while enumerating the number of secretory cells in PFC assays would suggest a 'small' response – and vice versa.

This is illustrated in **Figure 1** which summarizes typical results of limiting dilution experiments with hapten carrier-stimulated human lymphocytes. When PFC assays were used in such experiments, it was

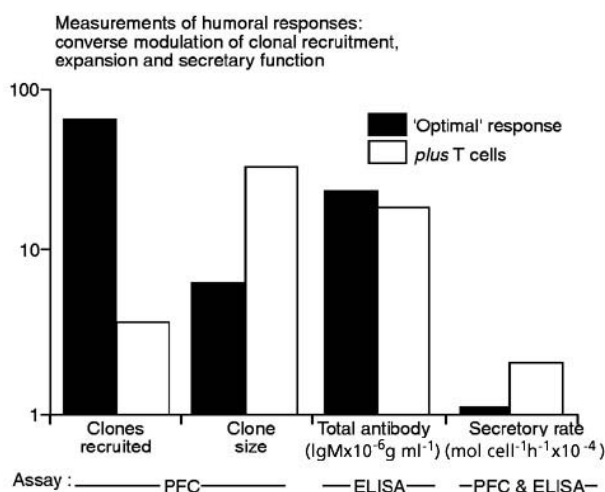


Figure 1 Measurements of B cell recruitment, clonal expression and activation of antibody secretion: cellular PFC and innoval (ELISA) assays generate different conclusions. Limiting dilution cultures of human blood or tonsil lymphocytes were stimulated with TNP-ovalbumin in the absence (■) or presence (□) of high-dose (100 µg ovalbumin per 24 h) primed, purified T cells. Cultures were assayed by hemolytic plaque assay for the frequency of TNP-specific PFC precursor cells and for the clone size present in positive cultures. A TNP-specific ELISA was used to measure specific antibody accumulated in supernatants over the 1-week culture period.

found that high-dose carrier (e.g. ovalbumin)-primed T cells appeared to actually reduce the optimal response of blood or tonsil cell cultures by 94–98% (Figure 1, columns 1 and 2, log scale). Closer analysis revealed that the few clones escaping this apparent suppression had much larger clone sizes (4–8-fold, columns 3 and 4). However, when immunoassays (e.g. ELISA) were used to measure specific antibodies secreted in these cultures, the suppressive effect of high-dose carrier-primed T cells was no longer apparent, since in addition to differential promotion of clonal expansion, these regulatory cells promoted higher secretory rates in these clones ($\sim 2\text{--}3 \times 10^4$ molecules cell⁻¹ h⁻¹). These latter two parameters are closely linked and vary concordantly during B cell maturation. Thus, depending on the assay system employed, such experiments may generate literally opposite conclusions.

It is therefore clear that the choice of assay system for immune responses can dramatically affect understanding and interpretations: in the given example, the apparent suppressor function following high-dose carrier priming identified in the PFC assay is not really reducing an immune response. Instead, these T cells recruit a clonally restricted portion of the hapten-specific B cell repertoire and promote its maturation more effectively. It is interesting to note that severe reduction of such apparent suppressor

cell function induced with high doses of protein antigen is typical for a number of autoimmune conditions.

See also: Antibodies, secretion; B lymphocytes; Erythrocytes; Immune response *in vitro*; ELISPOT assay.

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PLASMA

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Plasma is the noncellular fraction of blood which transports red cells, white cells and platelets. It is an aqueous solution of salts, proteins, lipids, sugars and various other substances serving a myriad of physiological functions. Plasma is obtained for clinical use either by centrifugation of anticoagulated whole blood and collection of the supernatant or as source plasma from a plasmapheresis program (see below). If plasma is allowed to clot, the residual liquid is called serum. Essentially, serum is plasma minus the clotting factors V, VIII and fibrinogen which are consumed during clot formation.

Plasma was first used as a treatment for shock in the 1930s after the development of sodium citrate anticoagulant made possible the separation of cellular blood components from plasma by centrifugation. After the development of lyophilization techniques, plasma from many donor units could be pooled and stored for an indefinite period at room temperature as a freeze-dried preparation to be reconstituted later with sterile water. Because pooled plasma was found to be associated with a high risk of viral transmission, in particular hepatitis B, this pooled lyophilized product is no longer used in current medical practice when plasma is needed. Rather, single donor units of fresh frozen plasma have become the standard therapeutic component. Given the small amount of some blood proteins in plasma, such as coagulation factors, obtaining commercial quantities of coagulation factor concentrates still

requires use of large pools of source plasma. These products, however, generally undergo one or more viral inactivation steps during processing.

In the early 1900s, serum was used medically as a source of immunoglobulins to treat infections including diphtheria, rabies, anthrax and tetanus. The first immune serums to be used were collected from naturally or artificially infected cattle or sheep; however, the frequency of anaphylactic reactions to these products, after the first injection, made them unsuitable for most human uses. Today, purified human immunoglobulins are available and serum is used mainly as a diagnostic laboratory reagent and in tissue culture media.

Plasma composition

Some of the normal constituents of human plasma are listed in Table 1. Proteins make up 6% of plasma weight; all other constituents make up 2%. Plasma serves as a vehicle for transportation and exchange of cells, nutrients and gases. Importantly, plasma is in dynamic equilibrium with the extravascular and intracellular spaces. Tissue oncotic pressure and capillary hydrostatic pressure, which tend to move fluid out of the intravascular space, are counterbalanced primarily by plasma protein oncotic pressure pulling fluid into the intravascular space. Albumin, a relatively small (60 kDa) globular protein manufactured by the liver, is the major plasma protein

Table 1 Some components of human plasma

Constituent	Normal concentration	
	mEq l ⁻¹	mg dl ⁻¹
Salts		
Cations		
Sodium	142	326
Potassium	4	16
Calcium	5	10
Magnesium	2	2.5
Anions		
Chloride	104	362
Bicarbonate	27	60
Phosphate	2	3.5
Sulfate	1	1.5
Organic acids	6	15
Nutrients and metabolites		
Proteins		7000
Albumin		4000
α ₁ -Globulin		250
Fibrinogen		300
α ₂ -Globulin		750
β-Globulin		900
γ-Globulin (immunoglobulins)		1100
Glucose		107
Total lipids		510
Cholesterol		190
Ammonia		0.2
Creatinine		0.85
Urea		18
Uric acid		5
Bilirubin		1

Total plasma volume: 3.2 litres (70 kg adult); 4.5% of body weight.

responsible for maintaining intravascular volume. Although it makes up only half of the protein mass, it provides 80% of the oncotic pressure. Patients with acquired hypoalbuminemia due to malnutrition, malabsorption, liver disease, protein-losing enteropathy or nephropathy, or burns develop peripheral edema when the albumin concentration falls to about half its normal value (<1.5 g dl⁻¹). Curiously, patients with congenital analbuminemia usually do not manifest this symptom, presumably because of a compensatory increase in other plasma proteins.

Immunoglobulins (Igs) as a class represent 15% of the total plasma proteins. They are produced by mature B lymphocytes, or plasma cells in a variety of molecular forms separable by electrophoresis and designated IgG, IgM, IgA, IgE and IgD. They are vital components of the immune system, being responsible for the recognition and opsonization of foreign antigens. Transport proteins, including albumin, transferrin and lipoproteins, are responsible for solubilizing hydrophobic moieties such as steroids and fats to permit their rapid distribution. They also serve to bind potentially toxic metabolites, such as

bilirubin, iron, exogenous drugs or poisons, thereby preventing peripheral tissue uptake and accelerating excretion.

That blood is able to remain liquid despite its ability to coagulate rapidly after damage to a blood vessel is due to a balance between opposing sets of plasma proteins: those initiating coagulation and those promoting fibrinolysis. The clotting factors (II, V, VII–XIII) form a cascade of activation that terminates in the precipitation of stable insoluble multimers of fibrin from its precursor fibrinogen. The fibrinolytic system degrades fibrin and interferes with several steps in the cascade. Together the two systems interact to produce normal hemostasis.

The major plasma electrolytes, listed in Table 1, serve to regulate total body water balance, maintain the transport and conduction properties of membranes, and mediate the resorption and reformation of bone. The pH of plasma is kept within a narrow range of about 7.35–7.45. This permits adequate binding with, and dissociation of oxygen from, hemoglobin, as well as optimizing many other pH-dependent physiologic reactions. Although overall acid–base balance is dependent on renal and pulmonary function, it is the buffering capacity of the plasma provided by bicarbonate and, to a lesser extent, by phosphate, sulfate and proteins, which stabilizes the system against transient fluctuations.

Plasma is responsible for the bulk transport of red cells and platelets, blood elements which function primarily within the vascular spaces. Plasma also carries white cells which are in transit between reticulo-endothelial and tissue sites. Plasma transports dissolved atmospheric gases such as oxygen, carbon dioxide and nitrogen, as well as anesthetic gases during surgery. Nutrients in the form of sugars, amino acids and fatty acids are transported from sites of absorption or storage to the tissues, and wastes such as urea and organic acids are carried to the excretory organs. Present in minute but critical amounts are the many blood-borne hormones, including those from the pituitary (gonadotropins, growth hormone, adrenocorticotrophic hormone, thyroid-stimulating hormone), the kidney (erythropoietin), the adrenals (aldosterone, epinephrine (adrenaline)), and the gonads (testosterone, estrogen, progesterone). In addition, there are literally hundreds of enzymes, interleukins, growth factors, biological response modifiers and a great number of as yet uncharacterized substances in the plasma. Although synthetic volume expanders exist (i.e. colloid solutions such as dextran and hydroxyethyl starch), the complexity of the nature and functions of plasma make it unlikely that a synthetic substitute which can truly mimic the

many functions of plasma will be developed in the foreseeable future.

Clinical uses

Fresh frozen plasma

A 'unit' of plasma is defined as that volume of plasma (180–270 ml) separated from a single unit (450 ml) of anticoagulated human whole blood. The therapeutic component fresh frozen plasma (FFP) is plasma separated from donated whole blood and frozen at -18°C within 8 hours of donation. When processed in this fashion, FFP maintains labile as well as stable coagulation factors in concentrations essentially equal to that of fresh whole blood for up to 1 year with proper storage (below -18°C). When plasma is stored in the liquid form at $1-6^{\circ}\text{C}$, the labile clotting factors V and VIII begin to deteriorate within hours; consequently liquid stored plasma is generally not used to treat clinical coagulopathies. Although individual factor concentrates are available, many clinical situations require the replacement of multiple clotting factors, thereby making FFP the therapeutic component of choice.

The primary indication for administration of FFP is to increase the level of coagulation factors in patients with a documented deficiency (i.e. prolonged prothrombin time (PT)/partial thromboplastin time (PTT)). FFP transfusion as replacement therapy may be indicated when there is a deficiency in a single factor for which a factor concentrate is not available, when a specific factor deficiency has not been determined, or when multiple factors have been depleted, as in hepatic failure or disseminated intravascular coagulation (DIC). Other indications for use of FFP include rapid reversal of warfarin sodium therapy, and as the replacement fluid during plasmapheresis for thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS). FFP should not be used as a volume expander, as a nutritional supplement, or prophylactically either for massive transfusion or following cardiopulmonary bypass. Aside from use in TTP or HUS, FFP is rarely indicated for use as a replacement fluid for plasmapheresis; 5% albumin in normal saline (0.9%) should be used instead. FFP dosage depends on which factors are depleted and the patient's plasma volume.

Cryoprecipitate

Cryoprecipitate is the cold-insoluble precipitate which is formed when a unit of FFP is thawed between 1 and 6°C . This precipitate is collected by centrifugation, refrozen and stored at -18°C for up

to 1 year. It contains predominantly factor VIII (80 units, with a unit of factor VIII being defined as that amount of factor contained in 1 ml normal plasma), fibrinogen (150 mg), factor XIII and von Willebrand factor. Consequently, cryoprecipitate is used as replacement therapy in patients deficient in these components (i.e. patients with classic hemophilia, or dysfibrinogenemia). As fibrinogen is depleted in DIC, cryoprecipitate may be used as a concentrated source of fibrinogen along with standard FFP therapy in the management of this coagulopathy. Use of cryoprecipitate alone in the management of DIC is inadequate, given the lack of factor V in this product.

Other uses of cryoprecipitate take advantage of the adhesive properties of fibrin. This adhesive action depends on the final stages of the coagulation cascade in which fibrinogen is converted to fibrin in the presence of thrombin, factor XIII, fibronectin and ionized calcium. In the USA, cryoprecipitate is often used in conjunction with topical thrombin (bovine or human) as a local hemostatic agent, referred to as 'fibrin glue' or 'fibrin sealant'. This 'glue' is felt by many to be the ideal operative sealant, with its primary use in cardiothoracic and general vascular surgery. It appears to have little tissue toxicity, promotes a fibrin seal in seconds to minutes, is reabsorbed in days and promotes local tissue growth and repair in general. Although commercially available in Europe, these commercial products are not yet licensed in the USA because the source of fibrinogen is pooled plasma, thereby introducing the theoretical risk of viral transmission. The American experience with fibrin sealant, therefore, is limited to that derived from individual units of cryoprecipitate based on methods which are cumbersome and variable in response.

In addition to infectious risks, other possible complications from the use of fibrin sealant include thrombosis, formation of antibodies to bovine thrombin, a bleeding diathesis due to an acquired factor V deficiency, adhesions and transient hypotension.

Coagulation factor concentrates and other products

Not all coagulation factors are available as individual concentrates. Concentrates are, however, available for the more common deficiency states i.e. factors VIII and IX. Lyophilized coagulant complexes containing factors II, VII, IX and X are also available. Factor concentrates are derived from either source plasma or recombinant technology. If prepared from pooled plasma, the concentrates are often highly purified. In addition, they undergo a variety of viral inactivation techniques (i.e. heat or

solvent/detergent treatment) aimed at destroying lipid-enveloped viruses. Although deemed to be safer, the risk of viral transmission continues to exist. In particular, outbreaks of nonlipid-enveloped viruses hepatitis A and parvovirus have been reported. For those patients who have developed inhibitors to factor VIII, other products are available which bypass factor VIII activity. These are presumed to act via factor VIIa, thus bypassing the need for factor VIII.

Albumin

Albumin, as used clinically today, is derived from plasma by a Cohn cold alcohol fractionation technique developed for large-scale plasma processing during World War II. The resulting product contains 96% albumin and 4% globulins and other proteins. During processing, the plasma is heated to 60°C for 10 hours, which serves to inactivate microorganisms, including hepatitis viruses and human immunodeficiency virus (HIV), thereby minimizing any infectious risk of this product. Albumin is used as a colloidal solution to provide an oncotic load and can serve to bind potentially toxic metabolites (i.e. bilirubin). Albumin is indicated as a replacement fluid during plasmapheresis and to support the blood pressure in shock. It is also used to reduce accumulated fluid, via an induced diuresis, in patients with ascites suffering from hypoproteinemic states (i.e. protein-losing nephropathy or enteropathy, liver disease or burns). Albumin is not a good nutritional source of protein because it lacks essential amino acids, is very slowly metabolized and is far more expensive than standard hyperalimentation products available with total parenteral nutrition (TPN) programs.

Immunoglobulins

A second major product of cold alcohol separation is the immunoglobulin fraction consisting of 95% IgG and 5% other proteins. When isolated by this technique, the IgG molecules tend to form multimers which, when injected intravenously, can activate complement, induce basophil degranulation and precipitate a severe anaphylactic reaction. Consequently, its use originally was limited to intramuscular preparations, which were painful and not ideal for patients requiring large doses or chronic therapy. Since the 1980s, however, treatment of immunoglobulin preparations with proteases or reducing agents, which serve to prevent these multimer formations without affecting the biological activity, have led to the availability of intravascular preparations as well. Intravascular administration of immunoglobulin has been used as prophylaxis against known viral and

bacterial infections in patients with either primary or secondary antibody deficiency syndromes. Intramuscular injection of immunoglobulin preparations containing antibodies to rhesus D (RhD) have been used since the middle 1960s to treat Rh-negative patients prophylactically to prevent Rh hemolytic disease of the newborn (erythroblastosis). A licensed intravenous Rh immune globulin is now available for that purpose, as well as for treatment of Rh-positive patients with idiopathic thrombocytopenic purpura (IPT). Intramuscular injections of hyperimmune serum globulins are still used for cases of acute antigen exposure in nonimmunized immunocompetent individuals. Hyperimmune serum globulins are pooled from donors with high specific antibody titers and are available with specificity for hepatitis B, herpes zoster, rabies, tetanus, vaccinia, mumps, pertussis and measles.

Serum

The therapeutic uses of whole human serum have been supplanted by plasma and its derivatives. As a reagent, however, serum is still important as a diagnostic tool in blood banking to detect specific red blood cell antigens. It is also used for the production of antihuman globulin, the basis of the direct antiglobulin (Coombs') test. Serum is also used in transplant medicine to determine the tissue (HLA) antigens of donors and recipients. Serum from humans or domestic animals continues to be an essential component of many tissue culture media because it contains many necessary but as yet undefined growth factors. Serum from animals immunized against human tissues provides the basis for immunohistochemical staining in both research and in surgical pathology.

Risks

The risks associated with the transfusion of plasma or its derivatives are similar to those encountered with any blood product. Allergic reactions to plasma-containing products are very common and occur in 1–3% of transfusions. Nonhemolytic febrile reactions may occur as well. Other risks include alloimmunization to plasma antigens, hypervolemia and viral disease transmission. Severe reactions include anaphylactic shock due to infusion of various allergens such as IgA transfused to IgA-deficient patients. Transfusion-related lung injury (TRALI), due to the transfusion of leukoagglutinins which activate complement, resulting in a pulmonary capillary leak syndrome causing acute noncardiogenic pulmonary edema, has also been reported. Some recent animal

studies suggest a deleterious effect of plasma transfusions in the setting of septic shock.

Many pathogenic microorganisms are known to be transmissible by plasma infusions including HIV-1/2, viral hepatitis types A, B and C, and, if not stored frozen, a variety of bacteria. The statistics on transfusion-transmitted disease risks from all transfused blood components, including plasma, are approximately as follows: hepatitis B 1:60 000 per unit transfused (50% of cases become symptomatic), hepatitis C 1:100 000 per unit transfused (50% of cases become chronic), human T-cell leukemia virus (HTLV) 1:600 000 per unit screened (4% lifetime risk of associated disease) and HIV 1:600 000 to 1:800 000 per unit transfused. Also of note is that, in the USA, all immunoglobulin preparations, both intramuscular and intravenous, must either undergo viral inactivation procedures or must be subjected to hepatitis C viral RNA testing.

A recent well-publicized theoretical risk of blood component use relates to the transmission of Creutzfeldt-Jakob disease (CJD). After the death of a frequent blood donor due to CJD in 1994, there was a voluntary market withdrawal of involved plasma-derived products pending further investigation of this issue. CJD is a rare neurologic disease believed by some to be caused by an abnormal proteinaceous infectious particle, the prion, which occurs in a sporadic fashion with an extremely low incidence. It has an inherited form (100 extended families identified) and an infectious form (80 cases identified). This disease has only been shown to be transmitted via direct inoculation of infected neural tissues, i.e. dura mater, cornea, human pituitary growth hormone and ingestion of infected brain. Although this disease has been transmitted to animals by direct intracerebral inoculation of the buffy coat of blood from a known infected human, there has never been a documented case of disease transmission via a blood or blood component transfusion, despite several retrospective studies of recipients of blood from donors who subsequently died of CJD. Nevertheless, because a theoretical risk of transmission via transfusion exists, new guidelines state that if a person has more than one family member who has succumbed to this disease, they will be permanently deferred from donating blood.

Attempts to eliminate the infectious risk of plasma products include various viral inactivation techniques. Solvent/detergent (S/D) treatment has been used in Europe since 1990. This technique involves the introduction/incubation of 1% tri-*n*-butyl phosphate and 1% octyl phenylpolyethylene glycol ether (Triton-X) with large pools of plasma (300–1250 litres from 1200–1500 donors). During incubation, the S/D strips the lipid envelope from viruses, thereby

inactivating them; it has no effect on nonlipid-enveloped viruses. The S/D reagents are then extracted/adsorbed from the plasma, which in turn is repackaged as a pooled product and frozen. Minimal residual S/D is present in treated products, but no detrimental effect on red cells or platelets has been shown. It has been documented that this process decreases procoagulant activity by 10–15%, decreases protein S and α_2 -antiplasmin and eliminates the presence of larger multimers of von Willebrand factor. Its main disadvantage lies in the need for large plasma pools, increasing the risk of transmission of known nonenveloped viruses as well as possible unknown infectious agents. Other technologies under investigation to reduce risk of disease transmission include use of pasteurized plasma and use of photochemically treated plasma, chaotropic agents, irradiation, chromatography, filtration or a combination of the above technologies. It is apparent, however, that absolute viral inactivation in plasma and plasma derivatives is difficult to obtain. The benefits of pooled processed plasma must be weighed against product yield, product function and cost.

After infection, the most common serious complication of plasma transfusion is circulatory overload. The risk depends on the volume infused, the rate of infusion and the cardiovascular status of the patient. Fluid overload is more likely to occur in the elderly and in infants. The overall incidence of transfusion-related pulmonary edema is very low, about 0.01%. This complication can often be avoided or controlled by a slow infusion or a simultaneous diuresis.

Plasmapheresis

Plasmapheresis is the selective removal of plasma from the blood, generally using automated machines, with the immediate return of the cellular components to the donor/patient. It is accomplished by removing anticoagulated whole blood via a closed system, sterile in-line centrifuge, and separating the collected plasma from the cellular components. The cellular components are resuspended with normal saline and/or albumin and reinfused back into the donor/patient. This technique is used to collect plasma (donated source plasma) for the manufacture of clotting factor concentrates as well as albumin or immunoglobulins. It is also used therapeutically for the removal of plasma containing toxins, antibodies and antigen-antibody complexes. Diseases treated by plasmapheresis include myasthenia gravis, Guillain-Barré syndrome, TTP/HUS and hyperglobulinemic conditions such as multiple myeloma and Waldenström's macroglobulinemia.

See also: ABO blood group system; Acute phase pro-

teins; Antilymphocyte serum; Antiserum; Clotting system; Cytokines; Fetal calf serum; Gammaglobulin; IgA; IgD; IgE; IgG; IgM; Kallikrein-Kinin system; Plasmapheresis; Tissue typing.

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PLASMAPHERESIS

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Historical background

The word 'plasmapheresis' (meaning the withdrawal of plasma) was coined by Abel in 1914 to describe a process by which whole blood is withdrawn from the body and separated into its constitutive parts, the cells being returned to the body while the plasma is retained. It should be noted that the word has no etymological relationship to 'electrophoresis'. Between the two World Wars Whipple used the technique to evaluate the dynamic equilibria between tissue and plasma proteins, but during World War II it was appreciated that plasmapheresis could provide a method of taking frequent plasma donations so as to satisfy military needs without depleting the donor's red cells. This spurred Cohn to modify a Swedish cream separator to produce the first semi-continuous flow cell separator. Developments of this machine are the mainstay of plasma donation today.

In response to the need for white cells to transfuse into patients with leukemia a continuous flow cell

separator was developed jointly by IBM and the National Cancer Institute, and subsequently a large number of different machines have been produced. Most separate the blood by centrifugation, but a new generation of machines which work by membrane filtration has been developed. These machines have turned what would otherwise be a tedious and time-consuming process into an easily applied automated technique and opened up the possibility of physically manipulating the immune response by the removal of large volumes of antibody.

Pathophysiological basis of plasmapheresis

Anything that appears in the plasma may be removed by plasmapheresis according to the equation $Y_t = Y_o \cdot e^{-x}$, where Y_t is the final concentration of a plasma component, Y_o is the initial concentration and x is the number of plasma volumes exchanged. Because of the exponential nature of this curve it is

usual to limit the volume exchanged to a single plasma volume on each occasion.

As fluid is lost from the intravascular space there is a flow of protein-free fluid from the interstitial tissues into the blood vessels to restore blood volume. Thus, within minutes there is a dilutional effect on plasma proteins which persists for about 6 h before both albumin and immunoglobulin G (IgG) begin to return to the circulation from the interstitium via the thoracic duct and capillary pores. Increased synthesis of albumin does not begin for 2 days, but the synthetic rate may increase threefold under severe hypo-oncotic stimulation. A loss of the albumin from 1 liter of plasma per week can be replenished by resynthesis alone by a well nourished 70 kg man. It is usual to use 5% human albumin solution as a replacement fluid when undertaking repeated plasmaphereses.

For IgM, 76% of which is intravascular and for which the fractional synthetic rate is only 8%, plasmapheresis is an efficient means of clearing it from the body. On the other hand only 45% of IgG is intravascular and repeated and frequent plasmaphereses are necessary to make an impact on plasma levels. Falls in IgG levels diminish the fractional catabolic rate, and for this reason some advocate adding IgG to the replacement fluid. Effects on the synthesis of specific IgG molecules vary, but during an immune response fueled by persisting antigen it is likely that plasmapheresis will be followed by a rebound and overshoot of antibody levels.

Other plasma proteins such as complement components and coagulation factors are also removed by plasmapheresis, and their removal may play a part in the modulation of some diseases by this form of treatment.

In many diseases characterized by the presence of circulating immune complexes, a defect in the clearance of such complexes by the mononuclear phagocytic system can be detected. Plasmapheresis may be shown to improve the function of the cells of this system in a way that is not totally clear.

Therapeutic effects

For many years the only clinical use of plasmapheresis was to alleviate the hyperviscosity syndrome of Waldenström's macroglobulinemia. In 1976 three articles in the *Lancet* detailed its use in Goodpasture's syndrome, systemic lupus erythematosus and myasthenia gravis. Since then reports of its use in over 180 different conditions have appeared. In most of these diseases plasmapheresis has been shown to be ineffective or to offer no clear benefit over conventional treatment. In 1981 a report that plasmapher-

esis was an effective treatment of rheumatoid arthritis was threatening to add \$28 billion to the American health care budget. Controlled clinical trials have delineated only very few clinical conditions in which plasmapheresis is an effective component of treatment. These include Goodpasture's syndrome, the Guillain-Barré syndrome, Waldenström's macroglobulinemia, myasthenic crisis, cryoglobulinemia, thrombotic thrombocytopenic purpura, polyarteritis nodosa associated with hepatitis B virus, homozygous hypercholesterolemia and certain poisonings. A case can also be made for its use in certain other autoimmune diseases when they are refractory to conventional treatment.

Plasmapheresis undoubtedly exerts an immunosuppressive effect, but is seldom safe to use alone because of the tendency to produce rebound with overshoot of antibody levels. It is usually used together with immunosuppressive drugs. Some have suggested that these are synergistic but this is unproven. The great advantage of plasmapheresis is speed so that vulnerable end organs such as the kidney or nerve may be spared from the onslaught of circulating antibody while immunosuppressive drugs are switching off its production. Thus plasmapheresis confers no additional benefit to conventional immunosuppression in cases of rapidly progressive glomerulonephritis with independent renal function, but is of significant benefit for those patients who are already oliguric. In a similar way, plasmapheresis is effective in polyarteritis nodosa associated with hepatitis B virus when combined with antivirals such as vidarabine or interferon α which, in eliminating the antigen, reduce the generation of immune complexes.

The considerable placebo effect of connecting a patient to a whirring machine with flashing lights tended by an attentive nurse should not be underestimated and some have recommended that controlled clinical trials should include a sham apheresis arm. The role of plasmapheresis is becoming clearer as more controlled trials report. Even where a clear role has been previously established new and cheaper methods of immune manipulation may usurp its position. A good example is the use of intravenous immunoglobulin in the Guillain-Barré syndrome.

See also: Cryoglobulin; Goodpasture's syndrome; IgG; IgM; Immune complexes; Immunosuppression; Lymphoma; Neuromuscular junction autoimmunity; Plasma.

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PLATELET-ACTIVATING FACTOR (PAF)

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Platelet-activating factor (PAF) is a potent autocoid mediator implicated in a diverse range of human pathologies including shock, ischemia, asthma, cardiac and systemic anaphylaxis, CNS and renal disorders, and a variety of inflammatory conditions. Originally isolated from antigen-stimulated rabbit basophils and characterized structurally as 1-O-alkyl-2(R)-acetyl-glycero-3-phosphocholine, the alkyl phospholipid is now known to be produced by, and act on, a variety of cell types including neutrophils, eosinophils, monocytes, macrophages, platelets and endothelial cells. Platelet-activating factor (PAF) acetylhydrolase is a physiological inhibitor of PAF which is widely distributed in plasma and tissues and inactivates PAF by removing the acetyl group at the sn-2 position. Studies on the pathophysiological role of PAF have been facilitated by a variety of compounds which can specifically inhibit the binding of PAF to its receptors in various cells and tissues. Such PAF antagonists include the triazolothienodiazepine WEB 2086, the 20-carbon terpene ginkgolide B (BN 52021), and ligands such as kadsurenone. Studies with these and other PAF antagonists have indicated that PAF also plays an important role in the regulation of the immune response.

Effects on lymphocytes: proliferation, cytotoxicity, interleukin 2 production and surface antigen expression

Although PAF has a highly hydrophobic structure, it binds to a cell surface-expressed receptor belonging to the seven transmembrane G protein-coupled receptor family (Figure 1). The PAF receptor is coupled to several second messenger pathways including activation of phospholipase A₂, phospholipase C, phospholipase D, MAP kinase, phosphatidylinositol-3 kinase and tyrosine kinases, and inhibition of adenylyl cyclase.

Generally, PAF appears to have an inhibitory effect on lymphocyte proliferation *in vitro*. The addition of PAF to human peripheral blood lymphocyte cultures stimulated with the mitogens phytohemagglutinin (PHA) or concanavalin A (Con A), induces a concentration-dependent inhibition of cell proliferation. The IC₅₀ ranges between 1 pM and 0.1 nM and inhibition is only observed when PAF is added early in the culture period. The effect of PAF can be reversed by the use of the PAF receptor antagonist BN 52051 or indomethacin, the effect of this latter drug suggesting that prostaglandins may be involved as secondary messengers in this response.

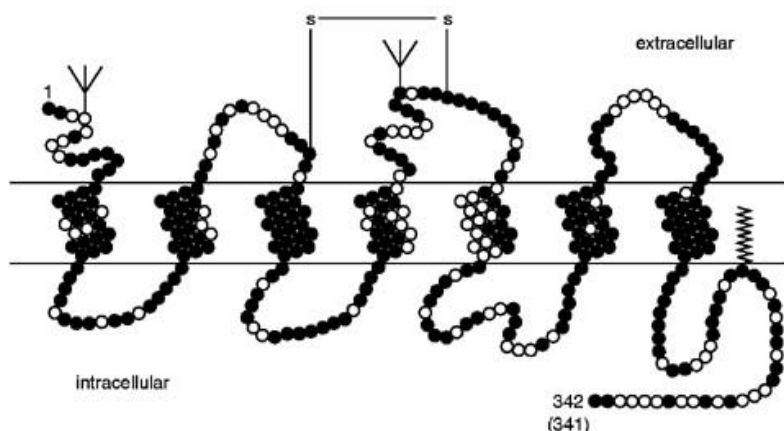


Figure 1 Structural model of the PAF receptor indicating the amino acids conserved among four mammalian species. It has a putative seven-transmembrane topology with the conserved amino acids shown as closed circles. Guinea pig and human PAF receptors are composed of 342 amino acids, while rat and mouse receptors are of 341 amino acids. The bracketed residue is deleted in the rat and mouse receptors. Potential glycosylation sites, a palmitoylation site, and a disulfide linkage between two cysteines are also shown. (Reproduced with permission from Ishii *et al* (1997) *Journal of Biological Chemistry* 272: 7846-7854.)

In contrast to these data, PAF and two nonhydrolyzable PAF analogs have been shown to increase the proliferation of interleukin 2 (IL-2)-stimulated human lymphoblasts, whereas some antagonists are inhibitory. Furthermore, it is possible that endogenously produced PAF may be involved in some step(s) of IL-2-induced proliferation of T lymphoblasts, as indicated by the inhibition of this process by the PAF synthesis inhibitor L-648,611. An important corroboration of some of these *in vitro* observations has been provided by the *in vivo* instillation of PAF into rats via subcutaneously implanted osmotic minipumps connected to the jugular vein. In this system, after 7 days of instillation, PAF-treated rat splenocytes showed enhanced IL-2 production in response to Con A.

Contradictory results have also been reported with regard to the effect of PAF on lymphocyte subsets, with some *in vitro* studies showing a suppressive effect of the mediator on CD4⁺ T cell proliferation or that PAF activates suppressor cells which inhibit lymphocyte proliferation and increases the CD8⁺/CD4⁺ T cell ratio. However, other studies indicate that PAF stimulates CD4⁺ T cell proliferation but has no effect on CD8⁺ cells. Little evidence is available concerning the effects of PAF on lymphocyte proliferation *in vivo*; however, in a randomized double-blind study on 18 healthy male volunteers receiving the ginkgolide mixture BN 52063 (80 or 240 mg day⁻¹) or placebo for 15 days, the results showed a dose-related reduction of up to 43% in the CD4⁺/CD8⁺ ratio in individuals receiving the PAF antagonist, but no change in the control group. The CD4⁺/CD8⁺ T cell ratio returned to normal values after the end of treatment.

Also, PAF has been implicated in the generation of cytotoxic lymphocytes, as BN 52021 potentiates alloantigen recognition in primary and secondary mixed lymphocyte cultures and enhances the generation of cytotoxic lymphocytes *in vitro*. PAF antagonists also exert a protective effect on *in vitro* rat splenic lymphocyte-mediated cytotoxicity of Langerhans islets. As cytotoxic and/or suppressor T lymphoblasts have been shown to accumulate around Langerhans islets in diabetes, PAF antagonists may be therapeutically valuable in the autoimmune form of this pathology.

Effects on monocytes/macrophages: production of cytokines and free radicals and CD23 receptor expression

Stimulated macrophages release both PAF and cytokines such as IL-1 and tumor necrosis factor (TNF),

which are essential regulators of immune cell interactions. *In vitro* experiments on human monocytes have demonstrated opposite effects of PAF on lipopolysaccharide (LPS)-induced IL-1 production depending on the concentration of the mediator. Low concentrations of PAF (1 pM to 0.1 nM) significantly enhance IL-1 production, whereas high concentrations (10 nM to 1 μ M) are markedly inhibitory. In *ex vivo* experiments using minipumps loaded with PAF placed under the back skin of rats and connected to the jugular vein, monocytes from animals receiving 1.0, 4.5 or 9.0 μ g PAF for 7 days exhibit an increased capacity to produce IL-1. In contrast, with 28 μ g PAF week⁻¹, IL-1 production by the monocytes is markedly decreased. IL-6 release by rat and human macrophages is also modulated by PAF.

Very low concentrations of PAF (1 pM to 10 nM) can activate peripheral blood-derived monocytes to produce TNF and significantly amplify the interferon γ (IFN γ)- or LPS-induced production of this cytokine. PAF also acts synergistically with IFN γ to increase monocyte cytotoxicity. Production of toxic oxygen species by monocytes is an important component of the bactericidal response of these cells. PAF can amplify superoxide generation in human monocytes preincubated with TNF, this effect being abolished by various structurally unrelated PAF antagonists. Interestingly, PAF antagonists can also partially inhibit superoxide generation elicited solely by TNF, suggesting the involvement of endogenous PAF in this response.

In addition to macrophages, natural killer (NK) cells also mediate part of their cytotoxic activity via the release of TNF. NK cell-mediated lysis of the erythroleukemic target cell line K562 is markedly enhanced by picomolar concentrations of PAF and significantly diminished by BN 52021. Furthermore, large granular lymphocytes, which comprise the effector NK cells responsible for lysis of K562 target cells, generate PAF under certain conditions and thus it appears that endogenous PAF may assist in the regulation of NK cell functions.

Finally, PAF has been implicated in the regulation of the expression of Fc ϵ R2/CD23 receptors, which are low-affinity binding sites for IgE. *In vitro* incubation of monocytes and B lymphocytes, but not T lymphocytes, with PAF induces a dose-dependent increase in Fc ϵ R2/CD23 expression. These results are supported by *in vivo* studies showing that aerosol administration of PAF to rats dramatically increases expression of Fc ϵ R2/CD23 receptors on alveolar macrophages collected from the animals by bronchoalveolar lavage.

Transplant rejection

Graft rejection is another area in which the immunomodulatory effects of PAF appear to be important. PAF is known to be released from renal allografts during hyperacute rejection and the PAF antagonist BN 52021 increases cardiac, liver, and skin allograft survival in rats, acting synergistically with azathioprine and cyclosporine. The PAF antagonist also prevents cyclosporine induced nephrotoxicity without altering the immunosuppressive effect of the drug. In addition, treatment with BN 52063 in combination with cyclosporine increases the immunosuppressive effect of the latter.

Conclusion

The experimental evidence described here indicates that PAF plays an important role in the regulation of the immune response. In addition to directly modulating immune processes and cytokine production as described above, PAF can also influence the activity of other mediators, which in turn can modulate lymphocyte function. These substances include cationic proteins and other suppressive factors released from activated eosinophils, and leukotrienes, prostaglandins and neuropeptides. Indeed, the ability of very low concentrations of PAF to both prime and amplify cell responses indicates that the mediator acts as an important amplifier of immunoinflammatory responses. Investigations with PAF antagonists will allow further elucidation of these processes and may lead to the development of these compounds as valuable immunomodulatory drugs.

See also: Eosinophil chemotactic factors; Fc receptors; Graft rejection; Macrophage activation; Monocytes; Natural killer (NK) cells; Neutrophils; Recruitment.

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PLATELETS

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Platelet structure and function

Hemostasis is initiated when endothelial cells are damaged, and platelets adhere to the collagen and von Willebrand factor (vWF) multimers in the sub-

endothelium. These events occur in flowing blood, with platelets exposed to different shear stresses in different areas of the circulation. The subendothelial vWF forms include unusually large vWF (ULvWF) multimers that have been released in a backward

direction from endothelial cells. The ULvWF multimers (which are also synthesized within megakaryocytes and present in the α granules of platelets) are larger than the vWF multimers in normal circulation, and have a greater capacity to bind to platelets.

Platelets adhere to subendothelial vWF multimers via glycoprotein Ib (GPIb) receptors, and to collagen via GPIa-IIa receptors. These are integral membrane proteins. GPIb is a disulfide-linked heterodimer that is complexed to other smaller platelet integral membrane proteins, GPIX and GPV. vWF multimers bind to the poorly glycosylated tail regions of GPIb molecules extending from the exterior surface of the platelet membrane. Both GPIb and GPIa span the platelet membrane and are linked to its cytoskeletal undersurface via actin-binding protein.

Following platelet-subendothelial adherence, platelets are activated to generate and transmit internal chemical signals. These transducing signals include G proteins, effector enzymes (phospholipase C, phospholipase A₂, adenylyl cyclase, protein kinase C, tyrosine kinases) and second messengers (inositol triphosphate, Ca²⁺, diacylglycerol, thromboxane A₂, cyclic AMP and cyclic GMP). One important result of platelet activation is the increased exposure of fibrinogen- and vWF-binding sites on platelet surface GPIIb-IIIa complexes. Under certain conditions, platelet GPIIb-IIIa complexes can also bind vWF multimers.

One example of platelet activation signaling is the following: membrane lipases hydrolyze arachidonic acid from ester bonds in platelet membrane phospholipids. Platelet fatty acid cyclooxygenase, the enzyme that is irreversibly inhibited when it is acetylated by aspirin, rapidly converts arachidonic acid to the cyclic endoperoxides PGG₂ and PGH₂. The platelet enzyme thromboxane synthase then produces thromboxane A₂ (TXA₂) from PGH₂. TXA₂ is a short-lived compound that potentiates the release of ADP, serotonin and Ca²⁺ from platelet-dense granules, as well as several proteins from α granules. These latter include platelet-derived growth factor that is mitogenic for smooth muscle cells and fibroblasts; β -thromboglobulin; platelet factor 4 that is capable of binding heparin; and fibrinogen and vWF multimers that attach, along with their plasma forms, to activated GPIIb-IIIa complexes in the platelet membrane. The result is platelet-platelet cohesion, or aggregation, of platelets onto those already adherent to subendothelial vWF and collagen.

Thrombin generated by activated coagulation pathways, as well as epinephrine, amplify platelet release and aggregation. Any TXA₂ that leaks from activated platelets may induce further platelet aggrega-

tion and stimulate vasoconstriction locally before it is hydrolyzed nonenzymatically into the inactive end-product, thromboxane B₂.

Platelet antigenicity

GPIb is a target on the platelet surface for the attachment of quinine/quinidine drug-dependent antibodies. The result of this drug-antibody binding to (or near) platelet GPIb molecules is usually severe thrombocytopenia. Some patients with 'idiopathic' autoimmune thrombocytopenic purpura (ITP) make autoantibodies that are directed against antigenic components of either GPIb or, more commonly, the GPIIb-IIIa complex. The platelet-specific alloantigens Lek^a and Pl^{Al} are located on GPIIb and IIIa, respectively. Following transplacental isoimmunization, anti-Pl^{Al} or anti-Lek^a can cause neonatal thrombocytopenia. Pl^{Al}-specific antibodies reactive with the Pl^{Al} antigenic locus on GPIIIa have also been implicated in the still enigmatic pathogenesis of post-transfusion purpura. It is perhaps important that GPIIIa molecules have been found on endothelial cell membranes and might also be a target of Pl^{Al}-specific antibodies (with resultant endothelial cell damage) in patients with this rare syndrome.

Platelets possess HLA antigens, some of which may be derived from other cells and become adsorbed to platelets from the plasma. Platelet HLA-A and HLA-B (but perhaps not HLA-C) antigens are capable of stimulating alloantibody formation. Repetitive transfusions of platelets concentrated from random normal donors will be HLA mismatched and will, with continuing exposure, elicit anti-HLA-A or anti-HLA-B alloantibodies that will bind to the transfused platelets and prevent their circulation and participation in hemostatic events. When this occurs, HLA-compatible or partially compatible platelets (often from siblings, parents, or grown children) will have to be substituted in order for transfusions to be effective.

Incompatible platelet transfusions do not produce the catastrophic effects associated with the intravascular hemolysis of transfused, incompatible red blood cells. Platelet transfusion reactions are characterized by chills and fever, lack of increment in the platelet count after transfusion, and no (or only transient) hemostatic effect.

Thrombocytopenia

Thrombocytopenia is most often caused either by defective platelet production or excessive platelet destruction. Defective platelet production is a common manifestation of many toxic (e.g.

chemotherapy), nutritional (e.g. folate or B₁₂ deficiencies), or neoplastic disturbances of bone marrow hematopoietic stem cell proliferation and differentiation. Thrombocytopenia that is caused by an increased peripheral destruction of platelets is characterized by a shortened platelet survival (normal survival is about 10 days), increased proliferation of marrow megakaryocytes in an effort to compensate for the decreased platelet lifespan, and an increase in the percentage of large (young) platelets in the circulation (normally there are about 10%). The process is frequently immune mediated.

Drug-induced immune thrombocytopenia

Some individuals produce antibodies against a drug or drug metabolite that can result in immune platelet destruction. The drugs implicated include quinidine and quinine (stereoisomers of each other), sulfonamides and structurally similar drugs, digitoxin, rifampicin, heroin, morphine, α -methyl dopa, gold salts, histamine-2 (H₂) receptor antagonists (cimetidine, ranitidine) and valproic acid. Either the drug or its metabolite, as a hapten, may bind non-covalently to platelet surface proteins and form complete antigenic structures. The susceptible patient produces antibodies that combine specifically with the platelet protein-bound haptenic drug or metabolite. Complement component C1 is fixed and activated if immunoglobulin M (IgM), IgG1, or IgG3 is involved in the immune complex. If the classical complement pathway on platelet surfaces is activated completely via C1, C4, C2, and C3 through to the terminal membrane attack complex (C5-C9), then platelets lyse in the circulation. Alternatively, the drug or metabolite may bind to (or near) a platelet surface structure and alter the antigenicity of the external platelet surface protein. This latter mechanism probably explains the presence of IgG molecules that bind, via their Fab portions, to platelet membrane GPIb or GPIIb-IIIa components in the presence of quinine or quinidine. In drug-induced immune thrombocytopenia, platelet counts are often $<10^4$ per μ l, and bleeding complications are frequently severe.

For immune reactions to occur on platelet surfaces, the drug or drug metabolite must be present continuously. The thrombocytopenia reverses within days to weeks following discontinuation and catabolism of the drug, and its complete removal (along with any haptenic metabolites) from the body.

A considerable percentage of patients anticoagulated with heparin in therapeutic, prophylactic or 'keep open' dosages will develop thrombocytopenia. Porcine heparin may be somewhat less likely to pro-

duce thrombocytopenia than bovine heparin. Heparin attaches to positively charged platelet factor 4 molecules that are secreted from the α granules of activated platelets on to their outer membrane surfaces. In susceptible patients, antibodies are produced against the heparin/platelet factor 4 complexes. It has been demonstrated that IgG antibodies bind, via their Fab regions, to the heparin/platelet factor 4 complexes. The Fc regions of these IgG antibodies interact with adjacent Fc receptors on the platelet surfaces and stimulate release of arachidonic acid from platelet membrane phospholipids. This is followed by the production of cyclic endoperoxides and TXA₂, the release of ADP and other platelet granule contents, ADP-induced binding of fibrinogen to activated platelet GPIIb-IIIa complexes, and intravascular platelet aggregation. The consequences are thrombocytopenia that becomes progressively more severe over the course of several days, often accompanied by arterial or recurrent venous thrombosis. Discontinuation of heparin leads to a reversal of intravascular aggregation within hours (as the heparin is catabolized and excreted), and the platelet count returns to normal within days.

Idiopathic (autoimmune) thrombocytopenic purpura (ITP)

A relatively common disorder in adults, ITP often complicates diseases associated with defective immune regulation (e.g. systemic lupus erythematosus, HIV infection, lymphoproliferative disorders, Hodgkin's disease). The onset of thrombocytopenia and bleeding is usually less abrupt than in drug-induced immune thrombocytopenia caused by drugs other than heparin. In adults, ITP is usually chronic and seldom remits spontaneously. IgG and/or IgM autoantibodies react with GPIb, GPIIb-IIIa, or other antigens present on the ITP patient's platelets, on platelets from normal donors and, sometimes, on the patient's megakaryocytes. The IgG or IgM antibodies may fix and activate complement components on platelet surfaces; however, complement fixation is often incomplete, and intravascular platelet lysis is much less common than in drug-induced immune thrombocytopenia (caused by drugs other than heparin). Macrophages have specific surface receptors for the Fc portion of IgG1 and IgG3 molecules, as well as for portions of the C3 molecule, and phagocytose IgG- or C3-coated platelets.

In chronic ITP, platelet counts may sometimes drop below 10^4 per μ l. Usually, however, platelet counts are in the range of 20 000-80 000 per μ l. Anemia, when present, is often caused by blood loss. An autoimmune hemolytic anemia caused by an IgG

autoantibody directed against a component of the Rh system occasionally may occur concurrently with ITP (Evans' syndrome).

Chronic ITP is treated initially with a glucocorticoid (prednisone or its equivalent in a dosage of 1–2 mg kg⁻¹ day⁻¹). Glucocorticoids are lipophilic cholesterol derivatives that enter the membranes of spleen and liver macrophages, and decrease the capacity of macrophage surface receptors to identify and phagocytose IgG- or C3-coated platelets. Platelet survival and platelet counts increase within days to weeks as a result of the inhibitory effects of glucocorticoids on splenic and hepatic macrophage IgG Fc and C3 receptors. It has also been claimed that glucocorticoids increase platelet production by megakaryocytes.

The dosage of glucocorticoids usually required to control chronic ITP compromises host defense against infection, as well as glucose and protein metabolism. Splenectomy is usually necessary to interrupt adequately phagocytosis by splenic macrophages of platelets coated with IgG (with or without C3). Platelet survival and platelet counts return to normal or near normal after splenectomy in about 75% of patients, although platelet autoantibodies often remain in the circulation and on patient platelets. These circulating platelet autoantibodies can cause neonatal thrombocytopenia in the offspring of mothers who have previously had their ITP brought under clinical control by splenectomy.

Because of blood flow patterns through the macrophage-packed splenic red pulp, the spleen is usually the major site of platelet destruction. Phagocytosis also occurs in the macrophage-lined sinusoids of the liver. In the latter circumstance, splenectomy may not be successful.

Some patients benefit transiently from the infusion of about 1.0 mg of vincristine. Vincristine depolymerizes platelet microtubules, and this cytoskeletal alteration may produce secondary changes in the exposure of platelet membrane antigens. Intravenous polyvalent IgG concentrate (0.4–0.5 mg kg⁻¹ day⁻¹ for 4–5 days) is transiently even more likely to be effective in treating ITP, either because splenic and hepatic macrophage IgG Fc receptors become blocked or because the concentrate contains anti-idiotypic antibodies capable of neutralizing some patient platelet autoantibodies. IgG concentrate ($\pm$ vincristine) is especially useful in producing short-term elevations in platelet counts in order to amelior-

ate a severe bleeding episode or to prepare a patient for splenectomy.

Transfusions of normal donor platelets are ineffective or only transiently effective in ITP because they also are attacked by the platelet autoantibodies in patient plasma. Platelet transfusions should be restricted to episodes of life-threatening (especially CNS) bleeding.

Danazol (400–800 mg day⁻¹), an attenuated androgen that may also interfere with macrophage Fc- and C3-receptor function, and immunosuppressive agents (6-mercaptopurine, azathioprine, cyclophosphamide) are useful in some patients with ITP who do not respond to the standard glucocorticoid/splenectomy regimen ($\pm$ IgG concentrate and vincristine).

See also: Blood transfusion reactions; Idiopathic thrombocytopenic purpura; Integrins; Platelet-activating factor (PAF).

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PNEUMOCYSTIS CARINII, INFECTION AND IMMUNITY

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Pneumocystis carinii is an extracellular pathogen that causes pneumonia in the immunocompromised host. Pneumocystosis is one of the leading opportunistic infections and causes of death in patients with the acquired immune deficiency syndrome (AIDS). No reliable *in vitro* culture system exists for *P. carinii*, which effectively hampers research on this pathogen. Until recently, the taxonomic status of this organism, based on morphological and ultrastructural characterization, was not clearly defined. However, molecular studies have shown that *P. carinii* is more closely related to fungi than protozoa. This classification was initially based on DNA homology studies using a single RNA ribosomal gene, but was subsequently confirmed by analysis of mitochondrial genes and protein coding sequences.

Two predominant forms of the organism have been identified: the trophozoite and the cyst. The trophic form is the smaller form (1–4 µm), pleomorphic, and exists in clusters. The cyst stage is larger (5–8 µm) and contains up to eight intracystic bodies. Two prominent groups of *P. carinii* antigens have been identified. A large surface complex, termed major surface glycoprotein (MSG), gpA or gp120 has been found in organisms derived from humans and other animals with a reported molecular weight of 95–140 kDa under reducing conditions. MSG is encoded by a family of genes, suggesting the possibility of antigenic variation. N-Linked carbohydrates comprise about 5–10% of the molecular mass of this glycoprotein. A major function of MSG is to mediate the interaction of *P. carinii* with the host. The other major antigen complex is a glycoprotein that migrates as a broad band of 45–55 kDa in rat *P. carinii* and 35–45 kDa in human *P. carinii*. The gene encoding the rat *P. carinii* 45–55 kDa antigen has recently been cloned but awaits further characterization.

Immune response in the host

P. carinii infection stimulates both humoral and cellular immune responses in the host. Seroepidemiologic studies have shown that 60–95% of all individuals contain detectable levels of antibodies to the

organism, suggesting widespread natural environmental exposure. Most patients infected with human immunodeficiency virus (HIV) have pre-existing serum antibodies to *P. carinii*, but these antibodies are not capable of providing protection against the development of *P. carinii* pneumonia. The 35–45 kDa band is the antigen recognized most frequently by the serologic responses.

Evasive strategies of the organism

P. carinii maintains an extracellular existence within the lung alveoli and adheres tightly to a specific lining cell, the type I pneumocyte. With immunosuppression, the organism proliferates slowly and initiates a series of changes in the alveolar microenvironment which culminate in damage to the type I cell. The mechanisms which permit *P. carinii* to evade the host defenses are poorly understood. Impaired cellular immunity is generally considered to be the main host factor that predisposes to infection, but impaired humoral immunity also plays a role. Even with successful treatment of *P. carinii*, the host remains susceptible to recurrent episodes of pneumocystosis as long as the immunosuppressive conditions persist.

Vaccines

Recent work has begun on the development of a *P. carinii* vaccine. Immunization of rats with whole organisms has not prevented the development of pneumocystosis but passive administration of a monoclonal antibody to MSG offers partial protection. Immunization with MSG also confers partial protection against the development of *P. carinii* pneumonia induced by immunosuppression. Additionally, an even more dramatic reduction in *P. carinii* infection has been achieved by the adoptive transfer of MSG-specific lymphocytes.

See also: Acquired immune deficiency syndrome (AIDS); Immunosuppression; Opportunistic infections; Respiratory and cardiac infections.

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POKEWEED MITOGEN (PWM)

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In 1964, Farnes reported that extracts of pokeweed (*Phytolacca americana*) induced transformation of human lymphocytes *in vitro*. Subsequently, Borjeson and colleagues isolated an active mitogen from the plant and demonstrated that pokeweed extracts contained materials that caused hemagglutination and leukagglutination and stimulated cell growth of both T and B cells. It was then demonstrated that pokeweed mitogen (PWM) induced immunoglobulin (Ig) production in peripheral blood lymphocytes and that this effect was dependent on both T cells and macrophages. Furthermore, interleukin 6 (IL-6) was shown to play an essential role in PWM-induced Ig production.

Preparation of PWM

The first isolation of an active mitogen from *Phytolacca americana* was published by Borjeson and colleagues, who purified PWM using the following procedure. Pokeweed roots were ground in a blender and active mitogenic molecules were extracted in PBS, then fractionated by heat coagulation, ethanol or trichloroacetic acid (TCA) preparation, and calcium phosphate column chromatography. The crude saline extract of PWM possessed the ability to agglutinate human red blood cells, white blood cells and to transform human peripheral blood lymphocytes. The TCA-precipitated fraction was further separated by the calcium phosphate column. The eluate from the column with 0.05 M phosphate buffer

at pH 7.5 was found to contain hemagglutinating, leukagglutinating and mitogenic activity.

Waxdal, using sequential hydroxyapatite chromatography and gel filtration, isolated five distinct fractions containing active mitogens, termed Pa1–Pa5. Yokoyama and colleagues also described a fractionation scheme consisting of DEAE-cellulose column chromatography, affinity chromatography on a column of desialylated human erythrocyte glycopeptide-Sepharose 4B, and gel filtration.

Characterization

The five fractions Pa1–Pa5 each contain a single molecule identified by SDS-PAGE and the mitogenic activity present in each fraction is stable to periodate and mercaptoethanol. Mitogens in fractions Pa2–Pa5 are probably in the form of a single polypeptide chain, whereas the molecule in Pa1 appears to be a polymer of 22 kDa subunits.

In comparison with phytohemagglutinin (PHA), the polypeptide part of the PWM is remarkable for its large number of internal disulfide bridges. The compositions of the molecules in fractions Pa2–Pa5 were found to have high half-cystine values, much higher than that of the molecule in the Pa1 fraction.

Yokohama and colleagues have reported that the mitogens in the Pa1 and Pa2 fractions recognize a core NN'-diacetylchitobiose moiety in asparagine-linked sugar chains of glycoproteins, and they demonstrated the presence of receptors for these

mitogens on the surface of human erythrocytes and murine lymphocytes.

Biological activity

Pokeweed mitogens have a mitogenic activity on murine T and B lymphocytes. All the fractions (Pa1–Pa5) stimulate murine T cells over a wide range of concentrations. The mitogen in the Pa2 fractions has the strongest activity and the Pa4 and Pa5 fractions have the least effective mitogens. However, only the molecule in the Pa1 fraction stimulates murine B cells in the absence of T cells.

The effects of PWM on human lymphocytes have been investigated mainly using impure mixtures of PWM. This has led to confusing and variable results, depending on the amounts of each active molecule present in the preparation. Although the Pa2 fraction showed the strongest activity for murine T cells, in studies using human T cells, both Pa1 and Pa2 had approximately the same potency. Peripheral blood B lymphocytes cultured alone with PWM did not respond with proliferation or cells was found to be dependent on T cells and macrophages. T Cell-depleted tonsillar mononuclear cells as well as peripheral blood lymphocytes had markedly diminished Ig production following PWM stimulation. The Ig production by T cell-depleted mononuclear cells could be reconstituted by cell-free supernatant obtained from PWM-stimulated peripheral blood lymphocytes or T cell-rich fraction. This probably accounts for the lack of HLA restriction for B cell stimulation. Furthermore, the PWM-induced

immunoglobulin production was found to be inhibited by anti-IL-6 antibody and thus is dependent on IL-6.

See also: Antibodies, secretion; Interleukin 6; Phytohemagglutinin (PHA); Polyclonal activators.

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POLYCLONAL ACTIVATORS

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Polyclonal activators are classically defined as agents that stimulate a large fraction of lymphocytes, regardless of their antigen specificity. The responding cells proliferate and/or produce end-products such as immunoglobulins or cytokines. Another attribute is the frequent lack of requirement for cell–cell collaboration, so that cell populations other than those activated do not limit responses to polyclonal acti-

vators. This property of autonomous activation has proven useful for studying the integrity of specific lymphocyte populations. Although polyclonal activators are widely considered to be experimental models for the activation of lymphocytes by antigen, they do not utilize the same activation pathways although the pathways overlap and interact with each other.

While reasonable, these classical definitions obscure the functional links between polyclonal activators, synergism by adjuvants, and costimulation. The combination of antigen-specific signaling via antigen receptors and polyclonal activation can lead to heightened immune responses compared to that caused by antigen receptor signaling alone. Polyclonal activators may influence signals by cell surface molecules other than antigen receptors. Furthermore, since the expansion of lymphocyte populations must be followed by contraction, polyclonal activation is not without an element of inhibition or inactivation. Both polyclonal activation and inactivation may be equally important in the maintenance of an intact immune system. The effect of nominal polyclonal activators depends on the cell activity measured and the conditions under which this activity is studied. As with positively acting functional links (activators/synergism/costimulation), important links in the opposite direction, that is, inhibition/antagonism/coinhibition, also influence immune responses.

Classes of polyclonal activators

There are a variety of polyclonal activators that operate by different mechanisms on cells of the immune system (Table 1). Many have varying abilities to stimulate a sizable fraction of lymphocytes, regardless of the lymphocyte antigen specificity. Polyclonal activators can both costimulate and induce costimulatory mechanisms, thus indicating the functional connection between polyclonal activators and costimulators. The physiology of costimulatory molecules to a large degree limits the function of these molecules to lymphocytes undergoing antigen-specific stimulation. This is also the case for a number of cytokines which may induce polyclonal activation under experimental conditions, but primarily function *in vivo* to augment antigen-specific responses. One exception to this generality is septic and toxic shock in which massive and damaging production of immune products causes severe illnesses. Lastly, activation is often followed by inactivation and lymphocyte death. The precise mechanism for inactivation and death, which allows lymphocytes to be removed when they are no longer needed, depends on the nature and mechanism of the initial activating stimulus.

Polyclonal inhibitors

While polyclonal activators possess polyclonal inhibitory potentialities, the polyclonal inhibitors listed in Table 2 are, for the most part, without strong activating properties. Intact antibodies to acti-

vating receptors that signal via an immunoreceptor tyrosine-based activating motif (ITAM) also bind to inhibitory Fc receptors, induce coaggregation of the two receptors and ultimately inactivate the cell. If the intact antibodies recognize invariant determinants, then the inhibition is polyclonal. If the antibodies react to variable determinants, such as those within the antigen-binding site, then inactivation is more restricted. Intact antibodies to antigenic determinants also inhibit by Fc-dependent mechanisms; this inhibition is not determinant specific but can involve any other determinants on the antigenic molecule or particle. All these forms of inhibition involve modulation of the positive signal sent through the ITAM. In some cases, polyclonal activation of one cell type inhibits other cell type(s). For example, concanavalin A polyclonally activates T cells, including T cells that suppress B cell responses in a polyclonal fashion.

Lymphocytes undergo explosive expansion in response to antigenic challenge. This provides a basis for active immunity, but presents two problems. How are responses to self antigens controlled? How are a majority of lymphocytes that respond to an antigenic challenge dealt with after the antigenic challenge has been eliminated? Lymphocytes have a range of cell surface molecules which transmit inhibitory signals. Beside the inhibitory Fc receptor, other receptors listed in Table 2 help to keep lymphocyte activity under control. While many of these receptors can, under experimental conditions, inhibit in a polyclonal fashion, under physiologic situations most act in an antigen-specific fashion due to regulation by the antigen receptor.

Polyclonal activators may also cause profound inhibition, an example being superantigens. Such superantigens, for example those derived from *Staphylococcus aureus*, activate T cells in a polyclonal fashion, but this activation can be followed by deletion of superantigen-binding T cells. This deletion creates holes in the T cell repertoire, potentially reducing the effective number of T cell antigen receptors available in immune responses.

Interaction between polyclonal and clonal activators

Synergism

Low doses of polyclonal activators increase responses to stimulatory cytokines or induce production of these stimulatory cytokines. This partly accounts for the effect of adjuvants. Adjuvants augment immune responses by a process of synergism between signals via the antigen receptor and those lymphoid system cell surface components that interact with the many elements that make up adjuvants.

Table 1 Examples of polyclonal activators

Name	Source	Cells activated	Cells required
<i>Mitogenic lectins – bind polysaccharide of surface structures</i>			
Phytohemagglutinin (PHA)	<i>Phaseolus vulgaris</i>	T cells (human)	Monocytes
Concanavalin A (Con A)	<i>Canavalia ensiformis</i>	T cells (mouse)	
Pokeweed mitogen (PWM)	<i>Phytolacca americana</i>	T and B cells	T and B cells
Streptolysin O (SLO)	<i>Streptococcus</i>	B cells	T cells
Nocardia water-soluble mitogen (NWSM)	<i>Nocardia opaca</i>	B cells	± T cells
Some oligonucleotides	Bacterial or synthetic	B cells	
<i>Bacteria cell wall products and analogs – bind a number of receptors on lymphocytes</i>			
Lipopolysaccharides (LPS)	Gram-negative bacteria	B cells	
Peptidoglycan	Bacteria	B cells	
Lipoproteins	Bacteria	B cells	
Porin proteins	<i>Neisseria</i>	B cells	
Dextran sulfate	Synthetic	B cells	
<i>Polyene antibiotics</i>			
Amphotericin B	<i>Streptomyces</i>	B cells (T cells)	
<i>Calcium ionophores – alter calcium signals</i>			
A23187	<i>Streptomyces</i>	T cells	
Ionomycin	<i>Streptomyces</i>	T and B cells	
<i>Phosphorylation modifiers – increase stimulatory phosphorylation events</i>			
Phorbol myristate acetate	<i>Croton tiglium</i>	T and B cells	
Pervanadate	Inorganic salt + H ₂ O ₂	T and B cells	
<i>Antigen–receptor ligands – bind to non-variable portions of the receptor mechanism</i>			
(Fab') ₂ anti-immunoglobulin	Mammalian	B cells	
anti-Ig-dextran	Mammalian + synthetic	B cells	NK/macrophages
(Fab') ₂ anti-T cell receptor	Mammalian	T cells	
Staphylococcal enterotoxin	<i>Staphylococcus</i>	T cells	APC
TSST – toxic shock toxin	<i>Streptococcus</i>	T cells	APC
Superantigens (MMTV)	Retroviruses	T cells	APC
Protein A	<i>Staphylococcus aureus</i>	B cells	
<i>Ligands for costimulator molecules – bind to lymphocytes and favor stimulation</i>			
Anti-CD28/CTLA4 and B7	Mammalian	T cells	
Anti-CD40 and CD40L	Mammalian	B cells	
Anti-CD21/complement	Mammalian	B cells	
Anti-CD2	Mammalian	T cells	
<i>Transforming agents infect cells and cause continued growth and/or activation</i>			
Epstein–Barr virus	Virus	B cells	
HumanT lymphotropic virus	Virus	T and B cells	
Human immunodeficiency virus	Virus	B cells	

APC, antigen-presenting cells; NK, natural killer cells.

Lymphocytes do not respond very well to purified antigens, but must be stimulated to do so with various adjuvants, such as Freund's complete adjuvant, an oil-in-water emulsion containing heat-killed bacteria, *Mycobacterium tuberculosis*. Bacteria contain elements that are unlike vertebrate structures. These elements interact with lymphocytes via components other than the antigen-specific variable region of the antigen receptor. An example of the adjuvant activity of a B cell polyclonal activator is the increased anti-

body production by purified B cells to antigen in the presence of bacterial oligo-DNA containing hypomethylated cytosine–guanine sequences.

Lipopolysaccharide (LPS, endotoxin), a classical polyclonal activator, favors the generation of an inflammatory T cell population that may provide immunity to the forms of bacteria that display LPS. Thus, under natural conditions, polyclonal activators may act to augment protective forms of immunity. Injection of LPS induces rapid polyclonal production

Table 2 Polyclonal inhibitors

Name	Source	Cells inhibited
<i>Intact antibody to antigen receptors – cross-links antigen and Fc receptors (FcγRIIB)</i>		
Anti-B cell antigen receptor	Mammalian	B cells
Anti-T cell antigen receptor	Mammalian	T cells
Anti-IgE on FcεRI	Mammalian	Mast cells
<i>Ligands or antibodies that activate inhibitory receptors on lymphocytes</i>		
Anti-CTLA4 without CD28	Mammalian	T cells
Anti-CD22	Mammalian	B cells
Anti-Ly49, anti-p58/p70, and others	Mammalian	NK and T cells
TGFβ	Mammalian	Many cells
FasL and anti-Fas (CD95)	Mammalian	T and B cells
TNFα (receptor = CD120)	Mammalian	T cells
Polymyxin B	Bacterial	B cells

TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α.

of immunoglobulin M (IgM) antibodies from short-lived naive B cells, while memory B cells are resistant to stimulation by LPS (possibly due to prior antigen receptor signaling). These observations suggest that lymphocytes may use nonclonal receptors to stimulate rapid, low-affinity, polyclonal responses to reduce microbial growth during the lag period before specific high-affinity immune responses are generated. Also, the products of innate immunity may induce suitable forms of antigen-specific immune responses which reduce the impact of invading microorganisms. However, polyclonal activators are two-edged swords in that too much activation of cells of the immune system can lead to septic or toxic shock.

Antagonism

LPS is considered to be a classical B cell activator in that it activates via a putative receptor that is not the antigen receptor, and shows very little requirement for cell types other than B cells. As such, it would seem to be totally independent of specific responses. However, activation by LPS in terms of immunoglobulin production is inhibited by prior ligation of the antigen receptor, and LPS exposure inhibits activation of B cells to antibody production via the antigen receptor. This has been termed mutual antagonism. Normally, LPS cannot polyclonally activate lymphocytes with receptors that bind self antigens to produce antiself antibodies. However, in some types of autoimmunity, antagonism between LPS- and antigen-signaling is defective. This results in increased polyclonal activation of lymphocytes and eventually leads to production of damaging antiself responses. Polyclonal activators commonly induce autoimmune disease in susceptible hosts. Normally

occurring antagonism is not likely to prevent induction of high-affinity secondary responses when triggered by bacterial antigens attached to LPS, since immune responses to antigens coupled to LPS are not inhibited by prior B cell receptor (BCR) signaling. Polyclonal activation by other agents may or may not be inhibited by ligation of the antigen receptor, suggesting that many mechanisms result in polyclonal activation.

Polyclonal activation by endogenous agents

Do functioning polyclonal activators or inactivators exist endogenously in unmanipulated animals? An indication of endogenous polyclonal activation is the considerable amount of both B and T cell activity that occurs in germ-free mice not exposed to exogenous antigens. However, the degree to which these activities are due to endogenous polyclonal activators versus multiple antigen-specific stimuli is not known. Another example is that of superantigens produced by endogenous retroviruses in certain mouse strains which result in activation followed by deletion of T cells with antigen receptors that react with the superantigens. The binding of superantigens by T cells does not involve the antigen-binding site of the T cell antigen receptors but conserved parts of the variable region. Thus, superantigens affect T cells with different antigen specificities. Superantigens link T cells to major histocompatibility antigen on antigen-presenting cells and induce the activation-in-activation cycle.

Costimulation and coinhibition are physiologically related to polyclonal activation and inhibition

The immune system did not develop in isolation, but in response to various infecting agents with which it had to deal. As discussed above, infecting agents possess polyclonally active agents that stimulate or inhibit immune responses. However, many infecting agents stimulate inappropriate responses, and others do not induce the appropriate responses. The immune system adjusts partly to this situation by making use of internal activators and inhibitors. Classical polyclonal activation may synergize with or antagonize antigen-specific immune responses. The endogenous functional counterparts of such activation and inactivation in the immune system are costimulation and coinhibition, both of which are mediated by polyclonally distributed receptors and ligands.

Costimulation

Synergistic interactions between a clonally restricted antigen receptor and a polyclonal receptor form the basis of costimulation. This is a physiologic process in which receptor–ligand interactions occur, allowing different types of cells to communicate with each other. T cells are activated by signals transmitted via the antigen receptor and other signals transmitted via a second receptor or class of receptors. The classical costimulator of T cells is the receptor CD28 whose ligand is B7 (CD80/86), but there are many others. B cells are activated by signals transmitted via the antigen receptor and other signals transmitted via a second receptor or class of receptors. The classical costimulatory receptor for B cells is CD40 whose ligand is CD40L which is found on T cells. CD40L also acts as a receptor for T cell signaling, so that a given cell surface element can serve as both receptor and ligand. Other costimulatory receptor–ligand systems have been defined, and evidence suggests others (as yet not characterized). It has been suggested that spontaneous autoimmune disease is due to an overabundance of costimulatory signals.

Coinhibition

Interactions between a clonally restricted antigen receptor and a polyclonal receptor can result in coinhibition. Because lymphocytes must be removed when they are not needed or, indeed, are dangerously autoimmune, they possess receptor–ligand pairs that result in lymphocyte death. The expression of these pairs is increased upon lymphocyte activation. In T cells, ligation of the antigen receptor and CTLA-4 leads to inactivation of the T cell. If, however, CD28

is also ligated, the result is pronounced T cell activation. Therefore, the outcome of contact with antigen via the antigen receptor is influenced greatly by what other connections the T cell makes. Mice that lack CTLA-4 develop a lethal autoimmune disease by one month of age.

B cells also have a defined coinhibitor system, the low avidity Fc receptor called FcγRIIB which binds the Fc portion of secreted antibody that is attached to antigen receptors. Antibody is able to suppress immunoglobulin production and to inactivate B cells. This inhibition requires the Fc portion of antibody and involves a coligation of the antigen receptor with the Fc receptor. This coligation results in phosphorylation of the Fc receptor, attachment of a phosphatase (SHP-1), and inactivation of the B cell. A strain of autoimmune mice lacks SHP-1, while mice lacking the Fc receptor have increased immune responses but are not noticeably autoimmune. CD22, another B cell coinhibitory receptor that signals via the recruitment of SHP-1, may take over the function of the Fc receptor when it has been deleted.

Defects in other coinhibitory mechanisms are associated with autoimmunity. Apoptosis defects result from the lack of the receptor Fas (lymphoproliferative, or *lpr* trait) or the lack of the Fas ligand (generalized lymphadenopathy, or *gld* trait). Excess of elements (e.g. Bcl-2) that prevent lymphocyte death or apoptosis also result in autoimmunity. All of these defects are associated with polyclonal activation followed by the emergence of specific antiself responses and clinical autoimmunity. Spontaneous clinical autoimmunity has been associated more with the lack of coinhibition than with augmented costimulation.

Conclusions

Polyclonal activators come from a variety of sources. These activators may stimulate one lymphocyte population and can be used to assess the functional status of that population. Some polyclonal activators require more than one cell type, and, despite their name, may induce profound inhibition and inactivation of lymphocytes. Many interactions occur between antigen-specific and polyclonal forms of lymphocyte activation and inactivation. Functional analogs and physiologic counterparts of classical polyclonal activators and inhibitors (antagonists) are costimulation and coinhibition which serve to regulate specific immune responses. Defects in interactions between these various signaling events, particularly that of antagonism and coinhibition, predispose towards autoimmune disease.

See also: Adjuvants; Autoimmune disease, pathogen-

esis; Autoimmunity; Bacterial cell walls; Bacterial immunoglobulin-binding proteins; B lymphocyte activation; CD22; CD28; CD40 and its ligand; B7 (CD80 & CD86); Complement receptors; Endotoxin (lipopolysaccharide (LPS)); Epstein-Barr virus, infection and immunity; Fas (CD95) and fas ligand; Fc receptors; Immunopotential; Immunosuppression; Lectins; Phorbol esters; Phytohemagglutinin (PHA); Pokeweed mitogen (PWM); Proliferation, lymphocyte; Septic shock; Superantigens; T lymphocyte activation; Viruses, infection of immune cells by.

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POLYENDOCRINE AUTOIMMUNITY

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There are two major autoimmune polyglandular syndromes (APS), type I and type II, comprising a constellation of endocrine diseases caused by autoimmunity. The key features of each syndrome are described below. Although additional features have been described in individual patients, it is difficult to know whether these are true associations or ascertainment artifacts. Rarely, patients have features of both types of syndrome. Other polyendocrine autoimmune disorders are considered after the two main syndromes.

APS type I

This is an autosomal recessive disorder, also known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy. The prevalence is around 1 in 25 000 in Finland (based on a national survey),

where the disease may be due to a single major mutation derived from a limited founder population. Linkage analysis has indicated that the autosomal locus is on chromosome 21. Iranian Jews may also have a high disease frequency (exact prevalence unknown), but type I APS is very rare elsewhere and may be sporadic.

The clinical components are shown in Table 1. The diagnosis rests on the presence of two of the three major components but the various features differ within and between families. Chronic mucocutaneous candidiasis and hypoparathyroidism usually begin in childhood and children with either of these apparently isolated disorders warrant careful screening. Addison's disease usually occurs 5–10 years later. Patients may die of untreated endocrine disease, severe infections or, in later life, hepatic failure. HLA alleles influence disease expression; HLA-A28

Table 1 Features of the autoimmune polyglandular syndromes types I and II

	<i>APS type I</i>	<i>APS type II</i>
Major components	Chronic mucocutaneous candidiasis Addison's disease Hypoparathyroidism	Addison's disease Type I diabetes mellitus Autoimmune thyroid disease Gonadal failure
Other endocrinopathies	Insulin-dependent diabetes mellitus Gonadal failure Hypothyroidism	Lymphocytic hypophysitis Isolated adrenocorticotrophic hormone or gonadotropin deficiency Lymphocytic infundibulo-neurohypophysitis
Other features	Vitiligo Alopecia Pernicious anemia Hepatitis Malabsorption Ectodermal dysplasia (enamel and nail dystrophy, keratopathy)	Vitiligo Alopecia Pernicious anemia Celiac disease Myasthenia gravis Chronic active hepatitis

is more frequent in those patients with hypoparathyroidism and diabetes mellitus.

Treatment consists of glucocorticoid and mineralocorticoid replacement for adrenal failure, 1α -hydroxycholecalciferol for hypoparathyroidism and ketoconazole for candidiasis.

There are some parallels in autoimmune models in mice and rats; neonatal thymectomy with or without irradiation can induce various endocrinopathies with a relative T cell deficiency, and it is possible that the defective gene in APS type I causes a similar impairment of T cell maturation. T cell responses have not been studied in detail, although nonspecific abnormal responses following mitogen stimulation and generalized cutaneous anergy to recall antigens have been reported. Patients with Addison's disease as part of APS type I have adrenal autoantibodies, predominantly against the cytochrome P450 side-chain cleavage enzyme. This enzyme is also found in the gonads, presumably accounting for the high frequency of premature ovarian failure in the disorder. Most patients also have antibodies against the pancreatic beta cell autoantigens glutamic acid decarboxylase and aromatic-L-amino-acid decarboxylase, irrespective of the presence of clinical diabetes. Parathyroid autoantibodies remain poorly characterized.

APS type II

This is usually defined as the occurrence of two or more of the major components listed in Table 1; some would widen this to encompass various other features such as pernicious anemia, whereas others would limit the syndrome to those patients who have Addison's disease with thyroid autoimmune disease (Graves' disease or autoimmune hypothyroidism) and/or type I diabetes mellitus. The clinical features

of the individual components do not differ from the isolated disorders in their severity or age of appearance. APS type II seems to be inherited as an autosomal dominant condition with incomplete penetrance. There is considerable variation, both within and between families, in the expression of the various components. Affected siblings usually share one or both HLA haplotypes. There is a strong association with HLA-A1, -B8, -DR3, which varies according to the disease components, but is generally greater than with the individual isolated diseases. In addition, HLA-DR4 is increased in frequency in these patients, due to its association with type I diabetes mellitus. However, not all patients have these HLA alleles (suggesting that other loci are involved in the predisposition to develop polyendocrinopathy) and some diseases known to be associated with HLA-DR3, such as systemic lupus erythematosus, do not appear to be unusually frequent in APS type II patients.

It is important to be aware that these autoimmune endocrinopathies have a long prodromal phase and may remain subclinical throughout a patient's life; the presence of appropriate antibodies in such patients may be a marker for future endocrine dysfunction and for the presence of a more extensive autoimmune process than can be appreciated clinically. In particular, occult pernicious anemia/atrophic gastritis may be more frequent than generally appreciated, as revealed by biopsy or achlorhydria after pentagastrin stimulation. Patients with pernicious anemia are especially prone to develop vitiligo.

When considering whether a patient has APS type II, those presenting with Addison's disease are the most likely to have another autoimmune endocrine disorder, 10–25% having gonadal failure, 10–25% type I diabetes mellitus, and 15–25% auto-

immune thyroid disease. In contrast, patients presenting with autoimmune thyroid disease (which is about 500 times commoner than Addison's disease) are much less likely to have the type II syndrome. This has implications for screening, since it is clearly worth testing patients with Addison's disease, but not thyroid autoimmunity, for the presence of other endocrinopathies. Since the separate components may occur 20 years apart in some individuals with the syndrome, there should be long-term follow-up. It is also essential to advise and, where appropriate, test family members for disease once a patient with type II APS has been identified. Apart from screening, the management of this syndrome is the same as that of patients presenting with the individual endocrine abnormalities.

There are no unique features of the T cell or antibody response against endocrine tissues in patients with APS type II compared to those with the isolated disorder. The major adrenal autoantigen in both APS type II and isolated Addison's disease is the 21-hydroxylase enzyme, although other P450 enzymes are also targets in a small proportion of patients. The closest animal models of APS type II are the non-obese diabetic (NOD) mouse and BB strain rat: both animals develop autoimmune diabetes mellitus and have a low but significant prevalence of autoimmune thyroiditis, but adrenalitis is absent. The obese strain (OS) chicken is a close model of Hashimoto's thyroiditis and adrenal and gastric autoantibodies have been reported although tissue destruction does not occur.

Other polyendocrine autoimmunity syndromes

Rarely, diabetes mellitus results from a decreased biological response to a normal amount of insulin. In type A insulin resistance, the problem lies in the insulin receptor, which is quantitatively or qualitatively abnormal, but in the type B syndrome autoantibodies to the insulin receptor produce diabetes mellitus. *In vitro*, using short-term culture experiments, these antibodies mimic the action of insulin, which may account for the occurrence of hypoglycemia in some patients. More typically, however, there is massive insulin resistance, so that even 15 000 units of insulin per day may not lower blood glucose, and prolonged *in vitro* experiments reveal the antagonistic properties of the antibodies. About one-third of these patients have other autoimmune diseases, including systemic lupus erythematosus, Sjögren's syndrome, scleroderma, primary biliary cirrhosis,

Hashimoto's thyroiditis and autoimmune thrombocytopenia. Serological markers include a raised erythrocyte sedimentation rate (ESR), hypergammaglobulinemia and hypocomplementemia.

The POEMS syndrome is a rare form of plasma cell dyscrasia with polyneuropathy, organomegaly (liver and/or spleen), endocrinopathy (hypogonadism, hypothyroidism or diabetes mellitus), monoclonal gammopathy and skin changes, these features giving rise to the acronym. It is likely that the POEMS syndrome represents a variant of osteosclerotic myeloma. The endocrine dysfunction may be caused by specific autoantibodies but this remains unproven.

See also: Adrenal autoimmunity; Autoimmune diseases; *Candida*, infection and immunity; Insulin-dependent diabetes mellitus, human; Systemic lupus erythematosus (SLE), human; Thyroid autoimmunity, human; Vitiligo.

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POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

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The polymerase chain reaction has revolutionized the study of molecular immunology. In principle it is very simple; DNA is extracted from a cell and a specific gene can then be amplified within 2 h by the addition of a synthetic oligonucleotide complementary to one strand of the DNA and a second oligonucleotide complementary to the opposite strand of the DNA. If the genomic DNA is denatured and made single stranded, cooled, and DNA polymerase added together with deoxynucleotide triphosphates, then the synthetic primers will be extended. As this synthesis is template specific, then the result is a doubling of the DNA sequences bounded by primers. If the DNA is denatured a second time, cooled (reannealed) and extended again, then the amount of DNA will again be doubled. As the procedure is repeated then this results in an exponential increase in the amount of the DNA sequence that is bounded by the primers. After 20 cycles of heating, cooling and extension, approximately a million copies of a target molecule will be produced. This is sufficient to see by agarose gel electrophoresis. Uses of the technique extends from HLA typing to the production of 'humanized' monoclonal antibodies.

Basic technique

The original PCR process was described by K. Mullis in 1983 working for the Cetus Corporation. The first publication describing the procedure appeared in 1985, and in 1993 Mullis was awarded the Nobel Prize for Chemistry. The significant factor which

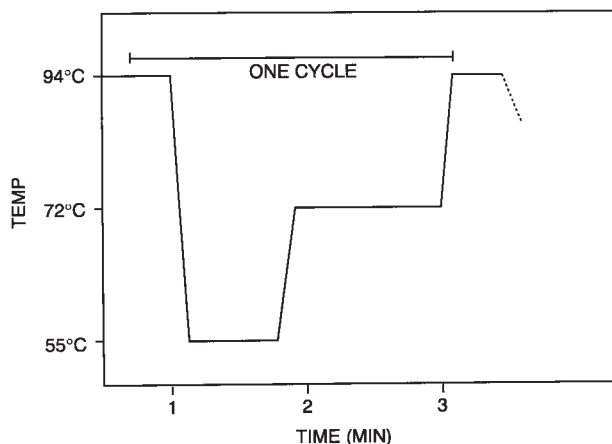


Figure 1 Typical cycle profile for a PCR reaction.

made PCR possible was the use of a previously described thermal stable DNA polymerase (Taq) isolated from the bacterium *Thermus aquaticus* YT1. This grows in the hot springs of Yellowstone National Park. The enzyme works optimally at 72°C and can withstand the denaturation steps at 94°C. The significance of this is that the enzyme does not have to be replenished between steps in the PCR procedure. The cycling profile is shown in Figure 1. This is performed in a controlled heating block so that each cycle is completed within approximately 3 min. The synthetic nucleotides used in the PCR process are either purchased commercially or are synthesized by the user. Again it must be stressed that in general only genes for which the sequence is already known can be amplified by the PCR process. The standard procedure is outlined in Figure 2.

Applications

The most important determinant for the suitability for tissue transplantation has been the HLA type of the individuals. Traditionally, HLA types have been

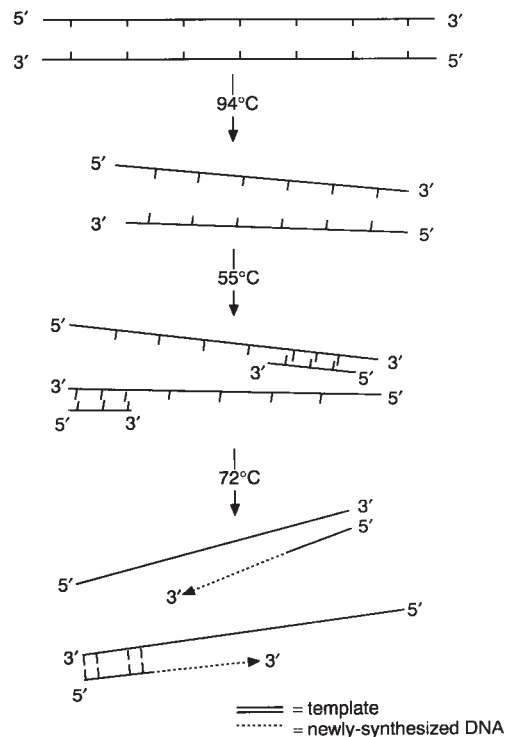


Figure 2 The principles of PCR amplification.

identified by the use of antibodies. Now it is common to use PCR; this procedure is faster and more accurate. Specific primers are used to amplify a number of HLA genes in a single tube. After the amplification procedure the products are analyzed by gel electrophoresis. The degree of similarity (and therefore compatibility) is indicated by the banding pattern of the donor and the recipient.

Within the human genome are a number of loci known as microsatellites or short tandem repeats (STRs). As these repeated sequences vary from individual to individual they can be used for genetic mapping. Most often these microsatellites vary in the number of CA nucleotide repeats; these repeats can be detected by using synthetic primers that amplify unique sequences either side of a CA repeat. Different allele sizes are seen by the migration of amplified DNA on a polyacrylamide or agarose gel. In a standard reaction seven primer pairs are used; these not only have a discriminatory power of greater than 1×10^{-9} but can also be used to confirm the sex of an individual using primer sequences based on the human gene amelogenin, a gene that encodes a protein that makes up part of the tooth enamel.

As PCR procedures become widespread, it may be that many of the antibody-based techniques used in immunology ranging from tissue typing to microbial identification will be replaced by DNA-based methods.

See also: Chromosome translocations of immune genes; Genetic analysis at the molecular level; Northern blotting; Southern blotting; Tissue typing.

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POLYOMAVIRUS, INFECTION AND IMMUNITY

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The polyomaviruses are widely distributed in nature. Infections are species specific and largely harmless in normal hosts. The viruses remain latent after primary infection and are reactivated when T cell functions are impaired. Most of the pathogenic consequences of polyomavirus infections are associated with virus reactivation in immunocompromised hosts. Two

polyomaviruses, JC virus (JCV) and BK virus (BKV), infect humans.

Characteristics of the organism and its antigens

The virion of polyomaviruses is small (diameter

45 nm), with a noneveloped icosahedral capsid and a circular, superhelical, double-stranded DNA genome of about 5000 base pairs. The viral genome is functionally divided into an early region (2.3 kb) which codes for the regulatory T proteins, a late region (2.3 kb) which codes for capsid proteins, and a noncoding regulatory region (0.4 kb). The early and late regions are transcribed from different strands of the DNA molecule. Although polyomaviruses and papillomaviruses are classified as two genera of the family Papovaviridae, the two are not related immunologically or genetically. The capsid is composed of three proteins. The major capsid protein, VP1, which constitutes more than 70% of the virion mass, mediates viral attachment to receptors on susceptible cells and contains epitopes for neutralization and hemagglutination inhibition. The large T antigen of polyomaviruses is a multifunctional protein. It binds to specific sites in the regulatory region of the viral genome to initiate viral DNA replication, stimulates host DNA synthesis by activation of cellular genes, and modulates early and late transcription. The T antigen of primate polyomaviruses is an oncogene which is required for the establishment and maintenance of virus-induced transformation.

Polyomaviruses multiply in the nucleus and produce nuclear injury and cell death in permissive cells. Nearly all of the pathogenic effects of polyomaviruses can be ascribed to lysis of infected cells. Nuclei of infected cells are enlarged and display inclusions. Infection probably occurs by the respiratory route, after which the viruses reach their target organs by viremia. The target organs for polyomaviruses may be urinary tract epithelium (BKV, JCV and simian virus 40 of Asian macaques), oligodendrocytes in the brain (JCV), and pulmonary endothelium (K virus of mice).

Progressive multifocal leukoencephalopathy (PML) is a subacute demyelinating fatal disorder of the central nervous system caused by JCV. It was once considered rare and occurred mostly in the fifth and later decades of life. With the advent of acquired immune deficiency syndrome (AIDS) and the large numbers of organ transplantations, the population at risk for PML has increased and the disease is seen frequently and in younger individuals. PML was identified in as many as 3.8% of AIDS patients who reported neurologic abnormalities and, in the USA, AIDS-associated PML cases outnumber all other cases of PML. PML occurs as an infrequent complication in many other conditions which include lymphoproliferative disorders, chronic diseases, primary immunodeficiency diseases and autoimmune diseases. The onset of PML is insidious and may occur at any time during the course of the underlying condition. Early signs and symptoms point to multifocal, asymmetric lesions in the brain in the absence of increased intracranial pressure. Impaired speech and vision and mental deterioration occur early in the disease. Paralysis of limbs, cortical blindness, and sensory abnormalities are common. Death occurs within 3–6 months of onset. Throughout the disease, the patient has normal cerebrospinal fluid and remains afebrile. Noninvasive techniques such as computed tomography and magnetic resonance imaging are effective in the diagnosis of PML.

The key pathogenetic event in PML is the cytotoxic infection of oligodendrocytes with JCV (Figure 1). Oligodendrocytes are responsible for the formation and maintenance of myelin and their destruction leads to demyelination. The PML brain shows widespread foci of demyelination of varying size. In advanced cases, the centers of the large areas may be necrotic and form cavities. The lesions are most frequent in the subcortical white matter and the

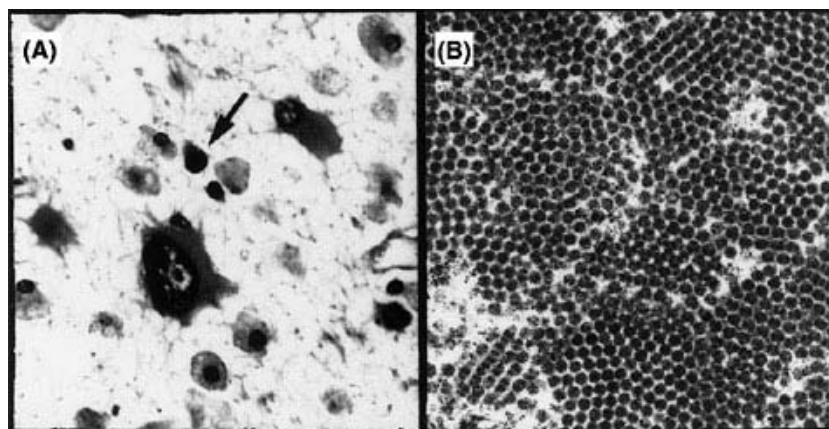


Figure 1 (A) PML lesion showing oligodendrocytes with enlarged deeply staining nuclei (arrow) and giant astrocytes. (B) Crystalloid array of JCV particles in an infected oligodendrocyte nucleus. (Courtesy of Dr G ZuRhein.)

cerebrum is almost always affected. Microscopically, the diagnostic feature of PML is the presence of enlarged oligodendrocytes located at the edges of the foci of demyelination with nuclei 2–3 times their normal size. The affected nuclei are deeply basophilic and contain abundant numbers of viral particles. In a majority of the cases, the centers of the demyelinated areas contain greatly enlarged, bizarre, giant astrocytes which have pleomorphic hyperchromatic nuclei. These astrocytes seldom contain viral particles or viral antigen and may represent cells 'transformed' by JCV. Inflammatory response is minimal or absent. Analyses of JCV genomes recovered from PML brains indicate that specific rearrangements in the regulatory region of the virus are associated with its ability to grow in the central nervous system.

PML patients have a generally depressed cellular immunity and a marked decrease in the specific ability of lymphocytes to respond to JCV antigen. The virus probably reaches the brain by the haematogenous route, thus accounting for the widespread and multifocal distribution of discrete foci of demyelination. In PML cases, JCV has been detected in mononuclear cells in the bone marrow, the spleen, and the Virchow–Robin spaces in the brain.

BKV excretion in urine is associated temporally with hemorrhagic cystitis which is a frequent complication in bone marrow transplant recipients, especially in those receiving allogeneic marrow. Ureteral stenosis, an uncommon late complication of renal transplantation, is associated with BKV infection. BKV was incriminated as the likely cause of a fatal tubulointerstitial nephritis in a child with a primary immunodeficiency disease. Nonfatal cases of cystitis in healthy children have been occasionally linked to BKV infection. BKV and JCV are reactivated in pregnancy but do no apparent harm to the women and are not transmitted to the fetus.

BKV genomic sequences have been recovered from brain tumors and pancreatic islet cell tumors but the significance of these observations is at present unknown.

Immune responses of the host

Primary infections with BKV and JCV occur in childhood. In the USA, 50% of the children acquire antibodies to BKV by the age of 3 years and to JCV by the age of 10–12 years. Antibody response is measured by neutralization, hemagglutination inhibition

and ELISA. Virus reactivation is often accompanied by an increase in antibody titers. Virus-specific immunoglobulin M (IgM) antibodies have been described in the sera of renal transplant recipients.

BKV infections generate cell-mediated immunity (CMI) as measured by lymphoproliferative assays. In bone marrow transplant recipients, both the humoral and CMI responses to BKV infections are severely depressed.

Evasive strategies by the organism

Following primary infections in childhood, BKV and JCV are not cleared from the body but remain latent in the kidney and in B lymphocytes for an indefinite period of time. Virus reactivation may occur in as many as 50% of the previously infected seropositive adults in the face of severe immunosuppression. It is likely that CMI prevents or limits virus reactivation in healthy individuals. Impaired CMI, as may occur in physiological states such as pregnancy and aging, or in more severe conditions such as organ transplantation, immunosuppressive therapy and AIDS, results in virus reactivation and viraemia.

Vaccines

The pathogenic effects of polyomaviruses are rare, even in the populations at risk, and they result from reactivation of infections latent since childhood. Therefore, no attempts have been made to develop vaccines against polyomavirus infections.

See also: Acquired immune deficiency syndrome (AIDS); Hematopoietic stem cell transplantation; Neurological infections; Opportunistic infections; Urinary tract infections; Viruses, immunity to.

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PORCINE IMMUNE SYSTEM

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Pigs belong to the order Artiodactyla (with camels, deer, cattle), which appeared abruptly with primates and odd-toed ungulates in the early Eocene. The pig is of considerable interest to immunologists because of its potential use in xenotransplantation; the absence of maternal immunoglobulins in newborns; its physiological similarity to humans; its phylogenetic proximity to cattle; and the major importance of both species in human diet.

The porcine immune system differs from that of mice and humans in the following features.

1. Pigs have inverted lymph nodes (dense medulla lacks sinuses and cords).
2. Porcine lymphocytes leave lymph nodes (in the paracortex) in the blood and not in efferent lymph. The $\gamma\delta$ T lymphocytes enter the lymph nodes directly from blood vessels and not via afferent lymph.
3. Pigs have four types of Peyer's patches (PPs): dispersed jejunal PPs, a continuous ileal PP (B organ in young piglets), spiral colon PPs, and numerous single small PPs distally in the colon.
4. Pigs have small tonsillar papillae in the down of the tongue along with palatine and nasopharyngeal tonsils.
5. Porcine epitheliochorial placenta prevents transfer of immunoglobulins. This means that maternal colostrum represents a vital source of antibodies for neonates. Colostrum-deprived newborn piglets contain only traces of immunoglobulins; cells forming IgM are present in the spleen and liver. The ability to reabsorb maternal immunoglobulins decreases during the first hours of feeding and between 2 h (in the duodenum) and 2 days (in the ileum) the epithelium of the gut changes and prevents any further absorption of intact colostral immunoglobulins.

Fetal and perinatal ontogeny

Numerous natural killer (NK) cells appear in the endometrium of pregnant sow (day 9 of gestation), i.e. before attachment of the blastocyst (day 14). Pig conceptus is capable of antiviral activity (interferon α (IFN α) and IFN γ) (day 14).

Hematopoiesis starts in the yolk sac (day 16) as in other mammals and lasts only for a short period

(day 24). The majority of cells are erythroid cells, stem cells and macrophage precursors. Erythropoiesis continues (day 20) in the liver (rudiment day 17) where platelets are also observed.

Lymphoid cells appear in the liver after macrophages (day 25), natural IFN α -producing cells (day 26), and myelocytes (day 28). The CD2 molecule and Thy-1 mRNA are detected in embryonic liver (day 30). Lymphatic vessels appear (day 32). Erythropoiesis is observed also (day 31) in the spleen (rudiment day 22); lymphocytes, however, occur later.

Thymic and cord blood cells are inducible to tumor necrosis factor α (TNF α) synthesis (day 34). The thymus (rudiment day 21) contains CD3⁺ T cells (day 40).

Other sites of hematopoiesis (e.g. para-aortic area) cannot be excluded. Lymphocytes occur very early in the omentum (day 40). Few MAC320⁺ lymphocytes appear early in the liver (day 40).

The sIgM⁺ B cells occur in the liver (day 44). The first serum immunoglobulins, specific antitflagellin antibody response, and CD4⁺ and CD8⁺ thymocytes were found in the same developmental stage (day 44).

By 50 days of gestation, lymphocytes occur in bone marrow and gut, liver B cells secrete IgM, T cells are in the spleen and skin, thymus cortex and medulla are formed and lymphocyte mitogenic response is detectable.

By 55 days antiparvovirus antibodies can be detected, purified protein derivative (PPD) of *Mycobacterium tuberculosis* elicits a T cell response *in vivo* and sIgM⁺ B cells are found in the spleen.

Hassall bodies appear (day 64), thymic B cells secreting IgM, IgG and IgA (only IgM in the spleen) arise (day 67), Peyer's patches are formed (day 77) and the first plasma cells appear in immunized fetuses (day 80).

Perinatally, lung alveolar and perivascular macrophages occur. Maternal immunoglobulins are transferred from the colostrum in the first 2 days after birth.

Macrophages gain functional maturity and germinal centers appear in the spleen at day 7. By day 10, a normal white blood cell picture with predominant lymphocytes is formed. By 2 weeks an influx of T cells into the lamina propria of the gut occurs, IgA⁺ and IgG⁺ B cells (mainly in the ileum) and new

germinal centers appear (in lymph nodes and Peyer's patches).

MHC

The genome of the pigs, unlike other species, contains a relatively small class I gene family. The porcine major histocompatibility complex (MHC) class I products encoded by seven genes designated PD are composed of a 44 kDa heavy chain (chromosome 7) and a 12 kDa β_2 -microglobulin, both with sequence homology to the human analogs. Six PD genes are highly homologous, whereas the seventh (PD6 gene) is distantly related.

The MHC class II products consist of a 32 kDa heavy chain and a 25 kDa light chain. SLA-DR and SLA-DQ subsets of class II antigens have been demonstrated. Human class II gene probes cross-hybridize with pig DNA.

Complement

Hemolytic complement C and components in adults in CH_{50} units ml^{-1} of serum: total C 50, C2 2000, C3 3200, C5 800, C6 6400, C7 40 000, C8 32 000, C9 14 000. Complement in newborn piglets $3.5 \pm 1.3 \text{ CH}_{50} \text{ ml}^{-1}$, by 10 weeks $35.7 \pm 25.5 \text{ CH}_{50} \text{ ml}^{-1}$.

Peripheral blood leukocytes

Throughout life the average leukocyte count is 16 million ml^{-1} (in the range 11–22), at birth 6 million (in the range 4.2–9). The differential distribution is as follows (mean and range in %):

Mature neutrophils	37%	(28–47)
Immature neutrophils	1%	(0–4)
Lymphocytes	53%	(39–62)
Monocytes	5%	(2–10)
Eosinophils	3.5%	(0.5–11)
Basophils	0.5%	(0–2)

Unique features of porcine blood leukocytes are the high proportion of $\gamma\delta$ T cells, the fact that the ratio of $\text{CD8}^+:\text{CD4}^+$ T cells is high in adults, and the high frequency of $\text{CD4}^+\text{CD8}^+$ double-positive T cells.

Granulocytes

The differential distribution of peripheral blood leukocytes in newborn pigs is in the order of 60% neutrophils and 38% lymphocytes. This ratio is reversed by the 10th day of life. The absolute number of leukocytes is only 3 million ml^{-1} in germ-free piglets

with a persisting deficit in lymphocytes. Functional maturity of neutrophils is accelerated in the presence of endogenous gut microflora. Some heterogeneity (in MIL4 expression) has been observed in peripheral blood neutrophils. Eosinophils can also be distinguished by membrane antigens expression ($\text{SWC1}^-\text{SWC3}^+$).

Monocytes/macrophages

Four clusters of differentiation (SWC4, SWC9, CD14 and CD16) have been defined so far. New monoclonal antibodies have confirmed the antigenic heterogeneity of tissue macrophages.

Phagocytic activity and metabolic burst are very low in fetal and perinatal macrophages. Maturation of macrophages seems to be heavily influenced by the endogenous gut microflora. The metabolic burst of monocytes measured by INT (iodine nitro-tetrazolium) increased in germ-free piglets one day after per oral *Enterococcus faecalis* challenge (the INT index is the ratio of stimulated:control absorbances).

B cells

Minute amounts of immunoglobulins appear in the serum of healthy nonstimulated pig fetuses by 44 days of gestation ($\text{IgM } 24 \text{ ng ml}^{-1}$, $\text{IgG } 26 \text{ ng ml}^{-1}$ and $\text{IgA } 15 \text{ ng ml}^{-1}$). These levels increase during fetal ontogeny independently of external stimuli. Naturally occurring fetal IgM antibodies react with phylogenetically conserved molecules (trypsin, ssDNA) autoantigens (FSH, thymocytes) and bacterial components (tetanic anatoxin, *E. coli* O86). The concentrations of immunoglobulins in piglets and adults are shown in Table 1.

IgM, IgA, IgG and IgE are coded for by constant region genes. There is a high degree of sequence similarity between all pig C_{H} genes and those in humans.

Table 1 Porcine immunoglobulins in piglets and adults (mg/ml)

	IgG	IgM	IgA
<i>Serum</i>			
Adult pigs	18.3–19.5	3.15–3.19	1.44–2.68
Sow, 1 week of lactation	28.2	4.8	2.0
Sow, 1 week of gestation	36.3	6.3	1.5
Piglets, 1 day	20.4–26.3	1.02–2.9	2.62–9.1
Piglets, 1 month	5.1–6.7	0.86–1.5	0.20–1.05
<i>Secretions</i>			
Colostrum	61.8	3.19	9.66
Milk, 8–35 days	1.35	0.89	3.04

A single gene for IgA has allelic forms IgA^a and IgA^b. Pigs have the largest number of IgG subclasses of all the animals so far studied. Gene conversion seems to create antibody diversity in pigs.

T cells

αβ T cells

Porcine TcRαβ is a dimer with a molecular mass of 46–48 kDa under reducing conditions. αβ T cells express the CD2 and CD3 molecules and can be further divided into several subsets according to the expression of the CD4 and/or CD8 molecules. Classical CD4⁺CD8⁻ and CD4⁻CD8⁺ cells are present and also unusual CD4⁺CD8⁺ memory helper cells can be found.

γδ T cells

The phenotype of γδ T cells is CD2⁻ or CD2⁺, CD3⁺, CD4⁺, CD8⁻, CD5⁻, γδ TCR⁺. TcR δ chain shows a molecular mass of 40 kDa. In association with this δ chain, three γ chains differing in their molecular masses could be identified by immunoprecipitation from sorted CD4⁻CD8⁻ T lymphocyte subsets.

One γ chain with a molecular mass of 38 kDa is expressed on CD2⁺CD4⁺CD8⁻ T cells characterized by an ungulate common epitope recognized by murine anti-sheep monoclonal antibody (mAb) 86D. These γδ T cells are found in peripheral blood. Another γ chain (37 kDa) could be allocated to the 86D⁻CD2⁺CD4⁺CD8⁻ T cells present in the circulation and lymphatic tissues. A third γ chain (46 kDa) is expressed on CD2⁺ TcRγδ T lymphocytes distributed in lymphatic tissues.

Porcine γδ T cells are frequent in peripheral blood (20–30% of lymphocytes in young pigs), in gut-associated lymphatic tissues and in the spleen, but rare in lymph nodes and thymus. They migrate into inflammatory sites in mucosae and skin.

NK cells

CD2⁺, CD4⁺, CD8^{low} cells are able to lyse K562 cells, porcine kidney cells and cells infected with pseudorabies virus. Cells with this phenotype form about 30% of blood lymphocytes, 10% of spleen lymphocytes and less than 5% in tonsils and lymph nodes in young pigs.

NK cytotoxicity is low in newborn pigs, whereas ADCC (antibody-dependent cellular cytotoxicity) is comparable with that of older piglets.

Differentiation antigens

A number of monoclonal antibodies reactive with porcine leukocyte membrane molecules has been characterized. These CD molecules, wCD (Workshop) molecules or SWC (swine-specific) molecules can be detected with standard monoclonal antibodies: CD2, 3, 4, 5, 14, 16, 18, 45, wCD1, 6, 8, 21, 25, 29, 44, and SWC1, SWC2 on T cells, SWC3 and SWC9 on macrophages, SWC4, SWC5, and SWC6 on null cells, SWC7 on B cells, and SWC8 on lymphoid cells. The 74-22-15 antibody (SWC3) recognizes macrophages and some granulocytes, MIL-1 recognizes alveolar macrophages, MIL-3 and MIL-4 neutrophils and eosinophils, and MAC 320 null/γδ T cells.

Humans and Old World monkeys have natural antibodies recognizing α-galactosyl epitopes on porcine endothelium. These antibodies mediate hyperacute rejection of porcine xenografts by primates.

Adhesion molecules

E-Selectin and some integrins were studied in the course of inflammatory reactions. There is a rapidly growing panel of monoclonal antibodies directed against these molecules. The 2F4/11 monoclonal antibody recognizes CD11b or α chain of the porcine complement type 3 receptor (CR3).

Cytokines

Interferons (IFNα, IFNγ and trophoblastic IFN) and inflammatory cytokines and chemokines (TNFα, IL-1, IL-6, IL-8, TGFβ) are being intensively studied in pigs. Other cytokines have also been cloned, including IL-2, IL-4, IL-5, IL-10, IL-12, GM-CSF, G-CSF, SCF, TNFβ.

Gut

Intestinal villi

1. Pig intestinal epithelium does not express MHC class II molecules.
2. Activated T cells and the endothelium (MIL-11⁺) express MHC class II.
3. CD4⁺ and CD8⁺ T cells are lacking in newborn and young germ-free piglets. Enterocytes in newborn piglets have large cytoplasmic vacuoles disappearing after gut closure (in germ-free piglets persisting for some 6 weeks).
4. Intraepithelial lymphocytes are MHC II⁺CD8⁻ (also adjacent to the epithelium).
5. Core cells are CD4⁺ T cells coexpressing the SLA-DR molecules when activated.

Deep lamina propria

1. Eosinophils among crypts are MIL-3⁺, MHC II⁺ cells.
2. Neutrophils (e.g. *Salmonella*-infected pigs) in the base of villi express MIL-4.
3. Macrophages (74-22-15⁺, MIL-3⁻, MIL-4⁻) are distributed mostly in the interfollicular area.

Peyer's patches (PPs)

	Jejunal PP	Ileal PP
Number	25-35	1
Size	small	large
Persistence	whole life	1 year
IgM ⁻ CD2 ⁻ cells	?	35%
B/T cell ratio	1	10

PP cells produce IL-2 and IFN γ , whereas lamina propria lymphocytes secrete IL-4, IL-5 and IL-6 cytokines. Peyer's patches play an important role in the differentiation of immune cells, antigen recognition, specific immune responses, and as the site of invasion for some pathogens.

Lungs

Clearance of bacteria from the circulation occurs in adult pigs predominantly in pulmonary intravascular macrophages (PIMs), and not in Kupffer cells and splenic macrophages as in humans or newborn piglets. A 2-month-old pig clears as much as 75% of *S. aureus* bacteria by PIMs.

Airborne particles are cleared by alveolar macrophages (AMs), cells with the phenotype CD45⁺, 74-22-15⁺, SLA-DR⁺, MIL-1⁺, MIL-2⁻, MIL-3⁻, MIL-4⁻. The AMs and PIMs both originate perinatally from circulating monocytes and produce inflammatory cytokines when stimulated. The full phagocytic activity of the AMs appear in 7-day-old piglets.

The INT indexes of AMs increased one day after per oral challenge of germ-free piglets with *Enterococcus faecalis*.

Differences between alveolar and intravascular macrophages:

	AM	PIM
Function	more phagocytic	more cytolytic
Quantity	10 ⁷	10 ⁹

The bronchus-associated lymphatic tissue (BALT) is absent in germ-free piglets. Lymphocytes occur in lungs in BAL (bronchial alveolar lavages), in BALT, intraepithelially, interstitially or in vessels.

The upper respiratory tract produces predominantly IgA, whereas lungs produce mostly IgG. Oral immunization elicits an increase of plasma cells in BAL (IgG > IgA > IgM) and leads to the protection against subsequent infection. The frequency of SLA-DR⁺ cells in alveolar lavages significantly increased 24 h after *E. faecalis* per oral challenge in one-day-old germ-free piglets (from 21% to 66%). An even greater increase was observed in young germ-free piglets colonized with *E. coli* microorganisms via airways (all alveolar cells in 3-day-old piglets were SLA-DR⁺ 24 h postchallenge).

See also: Ungulate immune systems; Xenotransplantation; T cell receptor, $\gamma\delta$; MHC, evolution of; Mucosa-associated lymphoid tissue (MALT); Phylogeny of the immune response.

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POXVIRUS, INFECTION AND IMMUNITY

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The poxviruses of vertebrates are classified into eight genera, determined by their antigenic characteristics (Table 1). Morphologically, only the parapoxviruses are distinct from the other groups, being oval rather than brick shaped, and being covered in a continuous tubular element rather than a large number of short projecting tubular elements. A precipitating 'nucleo-

protein' common to the parapoxvirus, orthopoxvirus, leporipoxvirus and avipoxvirus genera has been identified, prepared by alkaline extraction of the virion. Subsequent investigations have shown four cross-reacting polypeptides between orthopoxviruses and leporipoxviruses, and one between capripoxviruses and parapoxviruses. However, none of these are significant in cross-immunity between the genera.

Within the orthopoxviruses, there is good cross-immunity between all the members, exemplified by the use of vaccinia to protect against smallpox (variola). The antigens which stimulate protective antibodies are present in the surface tubular elements and in the envelope derived from modified cellular membrane that surrounds the naturally released virion. Virions released from disrupted cells lack this external membrane and are reported to be less infectious. The envelope also contains the glycoprotein hemagglutinin, which is common to all orthopoxviruses but absent from the other seven genera. Approximately 80 polypeptides can be identified by one-dimensional polyacrylamide electrophoresis of purified vaccinia virus. Sera prepared against the disrupted virion can be adsorbed with purified heterologous orthopoxviruses to distinguish between the different members of the group using agar gel immunoprecipitation. Monoclonal antibodies are available to characterize the major antigens.

All members of the capripoxvirus genus also cross-protect, and although differences have been identified between the viral polypeptides of different isolates, these have not yet been used to distinguish between them. As with the orthopoxviruses, polyclonal sera are equally reactive with homologous and heterologous strains of capripoxvirus. Distinction can also be made between enveloped, naturally released virion and unenveloped virion derived from a disrupted cell.

The poxviruses of birds were originally placed together in what was thought to be an antigenically closely related group. More recent investigations suggest that at least three immunologically distinct types of avian poxvirus exist, represented by type species: fowl poxvirus, quail poxvirus, and psittacine poxvirus.

The parapoxviruses form a morphologically distinct group, with shared antigens which cross-react

Table 1 The poxviruses of vertebrates

Orthopoxvirus	Vaccinia
	Buffalo pox
	Camel pox
	Ectromelia
	Monkey pox
	Rabbit pox
	Raccoon pox
	Taterapox
	Variola
	Cowpox
	Vole pox
	(Skunk pox)
	(Uasin Gishu disease)
Capripoxvirus	Sheep pox
	Goat pox
	Lumpy skin disease (Neethling)
Parapoxvirus	Contagious pustular dermatitis
	Bovine papular stomatitis
	Orf
	Pseudocowpox
	Parapox of red deer
	(Contagious ecthyma of camels)
	(Chamois contagious ecthyma)
Avipoxvirus	(Seal pox)
	Fowlpox
	Canary pox
	Junco pox
	Pigeon pox
	Quail pox
	Sparrow pox
	Starling pox
	Turkey pox
	Mynah pox
	Psittacine pox
	(Peacock pox)
	(Penguin pox)
Leporipoxvirus	Myxoma virus
	Hare fibroma
	Rabbit (shope) fibroma
	Squirrel fibroma
Suipoxvirus	Swinepox
Molluscipoxvirus	Molluscum contagiosum
Yatapoxvirus	Tanapox
	Yaba monkey tumor

in all standard serological tests. However, previous infection gives only limited protection against even homologous challenge, so that the cross-protection studies used to place new strains of poxvirus into any of the other poxvirus genera cannot be applied to novel strains of parapoxvirus.

The poxvirus genome appears to be very stable in the field situation. Isolates of capripoxvirus collected in Kenya over a 30 year period cannot be distinguished by comparing the genome fragments generated by *Hind*III restriction endonuclease digestion. The orthopoxvirus, vaccinia, has also persisted, little changed, in buffaloes as buffalo pox. Major genomic changes may occur when recombination takes place between poxviruses during a dual infection. This has been shown to occur not only in the laboratory between strains of orthopoxvirus and between strains of leporipoxvirus, but also in the field between strains of capripoxvirus. Whether recombination could occur between strains of different poxvirus genera is probably unlikely, but within genera there is potential for the evolution of new combinations with an enlarged host range or increased virulence.

Immune response

The humoral response to poxvirus infection results in the production of numerous antibodies which can be detected *in vitro* using complement fixation, immunofluorescence, virus neutralization, hemagglutination inhibition, immunoprecipitation, radioimmunoassay and ELISA tests. However, although many antibody specificities are produced, it is likely that only certain functional groups play a role in recovery and protection from poxvirus infection. These groups comprise antibodies which directly neutralize either enveloped or nonenveloped virion infectivity, those which complex with circulating antigens to aid immune clearance by phagocytes, and those which in conjunction with effector cells and/or complement mediate lysis of infected cells.

Neutralizing antibody may reduce or prevent viremia in primary and secondary infections by directly acting on extracellular virus. It is possible to protect susceptible mice from ectromelia infection by the passive transfer of serum from immune mice, and passive transfer of serum from sheep recovered from capripoxvirus infection protects sheep challenged with a virulent strain of capripoxvirus. This passive protection is short lived and limited in its effectiveness compared with active immunization with live virus, which provides far greater levels of long-lasting protection.

Highly cross-reactive neutralizing antibodies are

detectable in orthopoxvirus infections from days 6–8 postinfection and can remain elevated for over 20 years. No direct correlation appears to exist between the levels of neutralizing antibody and the immune status of the individual. Although antibody does exert some influence on poxvirus multiplication and spread, the cell-mediated response of the host has consistently proved to be the major factor contributing to protection and recovery from poxvirus disease.

In ectromelia infection of mice, there is an absolute requirement for immune T lymphocytes and cells of the monocyte/macrophage series for regression of infectious foci in the liver. Ectromelia-specific cytotoxic T lymphocytes are detectable from 2 to 4 days postinfection and reach peak levels by day 6. A delayed-type hypersensitivity reaction is detectable from day 2, whereas significant levels of neutralizing antibodies do not appear until day 8. *In vitro*, 3 hours after infection with ectromelia virus, target cells are specifically lysed by ectromelia-primed T cells, indicating that a population of cytotoxic T lymphocytes specific for determinants which appear early after infection are present.

The delayed-type hypersensitivity reaction comprising a T lymphocyte-mediated response to antigens injected under the skin was commonly used as an assessment of immunity to smallpox. In sheep, the presence of such a reaction is taken as evidence of immunity to capripox. However, the absence of a discernible delayed-type hypersensitivity response is not conclusive evidence of complete susceptibility to capripoxvirus infection.

Recovery from infection with vaccinia virus in rhesus monkeys and hamsters is mediated by a population of non-major histocompatibility complex (MHC)-restricted NK (natural killer) cells, whereas, in sheep, recovery from vaccinia virus infection is due to virus-specific MHC-restricted cytotoxic T lymphocytes.

Vaccination of individuals with vaccinia virus after they have been exposed to variola results in an accelerated immune response, with a similar reaction observed in sheep following reinfection with contagious pustular dermatitis. Vaccinia virus is sensitive to interferon (IFN), although its role in protection and recovery from poxvirus disease is unknown.

Immune interaction and evasion

During infection, the majority of newly synthesized poxvirus remains intracellular and is transmitted from cell to cell. This prevents circulating neutralizing antibody from coming into contact with rep-

licating virus. Only neutralizing antibody directed against external virus budding from infected cells has been shown to play a part in recovery. In most cases, a productive infection generally leads to host-cell death, although it has been reported under certain conditions that vaccinia virus can persist for unusually long periods, possibly in a benign form. Virus may also survive because of its ability to spread throughout the host, particularly in the skin. It is possible that the ability of the epidermal Langerhans cells to process and present poxvirus antigens to T lymphocytes may prove to be an important factor in initiating a protective immune response.

Poxviruses encode a variety of functional homologs of host cell factors which regulate the immune system. Some operate directly within infected cells, downregulating synthesis and processing of specific proteins such as interleukin 1 β (IL-1 β) and the expression of MHC class I antigens. Others, such as complement control proteins, soluble cytokine receptors and serine protease inhibitors, may be secreted into the extracellular environment and function some distance from the site of infection (Table 2).

Poxviruses replicate in the cytoplasm of infected cells and as a result encode many factors which manipulate and regulate the intracellular environ-

ment. It is now clear that by encoding a variety of virokines and other proteins which can interact with and modulate the host immune system, poxviruses can also exert a significant effect upon their extracellular environment.

Vaccines

In 1798, Edward Jenner published results of his experiments which showed that previous infection with cowpox – variolae vaccinae – protected humans against challenge with smallpox. Although the original observation can be traced back to country folklore, Jenner was the first to test the hypothesis scientifically. He had also to contend with the difficulty that cattle could be infected with either cowpox, an orthopoxvirus which did protect, or the clinically similar pseudocowpox, a parapoxvirus which did not confer protection. In 1881, Pasteur defined the term 'vaccination' to include any form of prophylactic inoculation.

At what stage cowpox virus was replaced by vaccinia is not clear. Even the origin of vaccinia is not known: suggestions include horse pox, which is now extinct, or a recombinant derived from cowpox and smallpox. Regardless of origin, cowpox vaccinia was

Table 2 Poxvirus encoded factors which interact with the host immune system

Host factor/mechanism	Virus-encoded homolog	Function
Complement control proteins	Vaccinia complement control protein (VCP) VV VV (B5R)	Secreted from infected cell binds C4b and C3b and blocks complement activation Sequence homology only, membrane bound
Cytokine receptors	IL-1 receptor VV, CPV IFN γ receptor M, SFV, VV, CPV TNF receptor SFV, M, VV IL-8 receptor SPV	Binds IL-1 β and abrogates function Binds IFN γ and abrogates function Binds TNF α and abrogates function Sequence homology only
Serine protease inhibitors (serpins)	SPI-1 (B24R) VV, RPV SPI-2 (crmA) CPV SPI-2 (B13R) VV SPI-3 (K2L) VV, RPV MYX SPI-4 (SRP1) M	Prevents apoptosis in RPV Inhibits pro-IL-1 β converting enzyme/alters arachidonic acid metabolism Inhibits cell-cell fusion; reduces inflammation
Interferon	VV (E3L) dsRNA binding protein VV (K3L) PKR binding protein	Blocks PKR activation by competitively binding dsRNA Functions as a substrate for PKR preventing phosphorylation of eIF2 α
MHC class I antigen presentation	VV Late protein M Late protein	Reduction in surface MHC class I expression and a block in the presentation of specific peptides to cytotoxic T lymphocytes
Growth factors	Epidermal growth factor Transforming growth factor α VV, SFV, M Vascular endothelial growth factor OV	Promote cell growth and virus replication
Steroid hormones	3 β -HSD VV	Synthesizes steroid hormones and reduces inflammation

CPV, cowpox virus; M, myxoma virus; OV, orf virus; RPV, rabbit pox virus; SFV, Shope fibroma virus; SPV, swinepox virus; VV, vaccinia virus; PKR, double-stranded RNA-dependent protein kinase; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; TNF, tumor necrosis factor. Gene designations are given in parentheses.

extremely valuable in helping to reduce the mortality rate due to smallpox and ultimately to the declaration, on 9 December 1979, that the disease had been eradicated from the world.

Complications associated with vaccination against smallpox were initially ignored as being insignificant compared with the consequences of infection. But as the industrial countries eradicated smallpox, it was seen that more patients were suffering the effects of vaccination than were being infected with smallpox. Complications included eczema vaccinatum in patients suffering from eczema, progressive vaccinia in patients with cell-mediated immune deficiency or agammaglobulinemia, generalized vaccinia, accidental infection, postvaccinal tonsillitis, postvaccinal encephalopathy in infants under 2 years old, and postvaccinal encephalitis in patients over 2 years old. In a very large survey carried out by the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia in 1968, 75 people per million given primary vaccination had associated complications, and, of these, 1 per million died, either of postvaccinal encephalitis or progressive vaccinia. Following secondary vaccination only 5 people per million had associated complications.

Vaccinia has also been used successfully in veterinary medicine to control camelpox, although it is less efficient in controlling ectromelia. However, less successful has been its apparent persistence in buffaloes as buffalo pox, and in rabbits as rabbit pox. How vaccinia was introduced to these species is not certain, but there are numerous examples of the transmission of the virus from recently vaccinated dairy farmers to the udders of their cows.

Sheep, goats and cattle can be effectively vaccinated against capripoxvirus infection, and single vaccines are available to protect all three species. Similarly, pigeon pox virus is effective in protecting chickens and turkeys against fowlpox and turkey pox. Separate vaccines are required against quail pox and against psittacine pox. Shope fibroma virus provides good protection in rabbits against myxomatosis, although it is less effective than homologous vaccine.

Vaccination against parapoxvirus infection is considerably less effective than that against ortho-, capri-, avi- or leporipoxvirus infection. Even against challenge with the homologous virus strain, contagious pustular dermatitis vaccine gives only limited protection.

Dead vaccines against smallpox and capripox have been developed, but at best these have provided only

temporary protection, compared with the prolonged protection provided by live vaccines. Part of the initial failure was due to the use of unenveloped virus derived from disrupted cells. Poxvirus in tissue culture is highly cell-associated, and yields of extracellular, enveloped virus are small. However, even when this problem had been recognized, further attempts to develop effective dead vaccines have been unsuccessful.

Poxvirus recombinants are valuable research tools; by facilitating the expression of individual gene products or particular epitopes, specific immune responses can be studied in isolation from the whole pathogen. This approach has helped to identify and decipher individual components of the immune system, including the relative functions of particular T cell subsets. More recently, there has been a revival in interest in vaccinia as a vector for the genes of pathogens such as hepatitis B, rabies, rinderpest, plasmodium, respiratory syncytial virus, vesicular stomatitis and human immunodeficiency virus (HIV). The advantages of having a replicating vector expressing the antigens of these agents are self-evident. Vaccinia virus is genetically stable, easy to produce and apply, and nononcogenic. The disadvantages of its use as a vector are its possible virulence in nontargeted hosts including humans, the possibility that it could recombine with other poxviruses in the field, and the effect it could have on immunodeficient individuals. Veterinary vaccines using capripoxvirus or avipoxvirus vectors may prove to be more acceptable than those incorporating vaccinia virus.

See also: Vaccines; Viruses, immunity to.

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PRECIPITATION REACTION

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Immunoprecipitation is a process that takes place when a solution of antigen is mixed with corresponding precipitating antibodies and, as a result, a visible precipitate or aggregating floccules are formed. The original observation of this serological phenomenon was made by R. Kraus in 1897, when he tested filtrates of cholera, plague and typhoid bacterial cultures with corresponding immune sera. A serological specificity of the reactions was observed. Since then, a multitude of precipitation techniques for qualitative and quantitative analyses has been developed. Their application has led to observations of fundamental value for revealing and understanding the immunospecific phenomenon of antigen-antibody binding, its physiochemical background and biological implications. Among pioneers in this field of research may be mentioned K. Landsteiner (serological specificity), G. Ramon (flocculation), M. Heidelberger, E.F. Kendall, E.A. Kabat (quantitative immunochemistry), J.R. Marrack, L. Pauling (molecular structure and intermolecular forces) and J. Oudin (diffusion-in-gel).

Precipitation in fluids

As mentioned, the phenomenon of immunoprecipitation appears when a solution of antigenic material is mixed with immune serum containing corresponding antibodies. The reaction is registered by a gradually increasing turbidity, changing to the appearance of precipitating or flocculating aggregates. A common procedure for immunoprecipitation analyses is to use a series of test tubes containing a constant amount of one of the reactants, and adding the corresponding reactant in serial, stepwise dilutions. A rough estimation of the grade of precipitation can be made by the naked eye or, more precisely, by turbidimetric or nephelometric measurements. The most adequate measurement of the intensity of the precipitation reaction is made by weighing the precipitate or by chemical analyses, e.g. N-determination, or by physical measurements when, for instance, dye- or isotope-labeled materials are involved in the reaction. Moreover, such measurements make it possible to determine the antigen:antibody ratios in the precipitated material. By extensive experimental work along these lines, basic information on immunoprecipitation and its characteristics has been obtained. In this connection, monospecific anti-

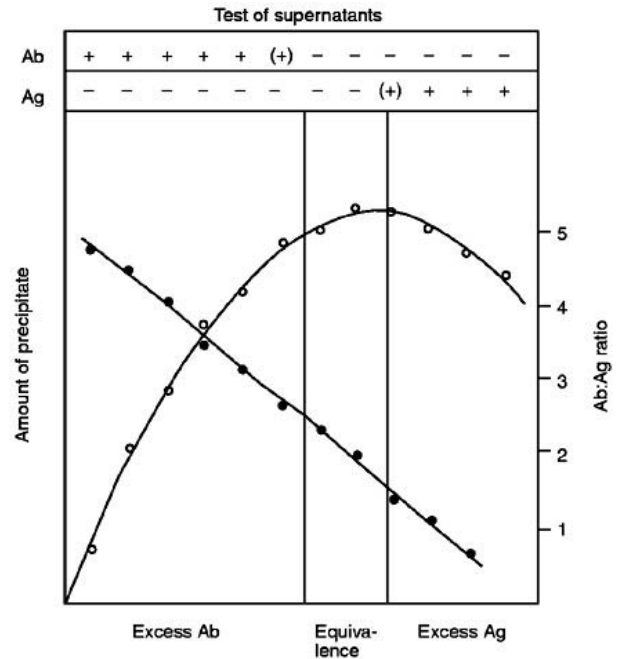


Figure 1 Schematic representation of a precipitation curve when a constant amount of antibody was tested against increasing amounts of corresponding antigen (ovalbumin-rabbit antiovalbumin). The amounts of precipitated material (○—○), the antibody:antigen (Ab:Ag) ratio in the precipitates (●—●) and the presence of unprecipitated antigen or antibody in the supernatants are indicated.

bodies, usually of the immunoglobulin G (IgG) class, and corresponding multivalent antigen have commonly served as a suitable model system.

The most important characteristics of the precipitation reaction may be summarized as follows. There are two stages to the reaction, the first being the very rapid binding of antibody to antigen, which occurs within seconds. The binding is governed by the degree of structural complementarity which provides serological specificity. Physically, van der Waal forces, hydrogen bonds, hydrophobic bonds and electrostatic forces are involved. The union is firm but reversible and at dissociation each reactant can be recovered unaltered. The strength of binding is measured by the grade of avidity of the antiserum, high avidity implying greater firmness. The second stage of the precipitation reaction develops more slowly, lasting minutes to hours, the primary antigen-antibody complexes forming larger secondary complexes in a lattice formation which end up as

visible precipitating aggregates. This aggregation is electrolyte dependent. A prerequisite for the formation of registrable aggregates is that the molecules of the participating reactants have a valency of at least 2 (e.g. bivalent IgG) and a multivalent antigen. An important observation is that in the immunoprecipitation reaction, antigen and antibody molecules combine in varying proportions depending on the ratio of reactants when they are initially mixed. This phenomenon of multiple proportions is in contrast to what happens in a common, pure chemical precipitation reaction where the reactants mostly combine in a nonvariable proportion.

The variation in the amount and composition of antigen-antibody aggregates in an immunoprecipitation system assayed by serial dilution of one of the reactants is represented by the precipitation curve in **Figure 1**. The amount and the antigen:antibody ratio of the precipitates are indicated, as well as the presence or absence of free antigen or antibody in the supernatants. The general characteristics of the reaction are, in short, as follows. At a certain antigen:

antibody ratio, maximal precipitation takes place, with neither free antigen nor antibody in the supernatant (zone of equivalence, a quantitatively balanced system). Where the antigen:antibody ratio differs from that of equivalence – zones of either excess antigen or antibody – the conditions are less favorable for the creation of an extended lattice formation. Consequently, at either side of the zone of equivalence, smaller amounts of precipitate are formed, and where the reactants are greatly out of balance a zone of complete inhibition may be observed. It should be noted that this phenomenon occurs even though primary antigen-antibody binding has taken place. Whether there is total or partial solubility of antigen-antibody complexes in the regions around the zone of equivalence depends on the species origin of the immune serum and the nature of the antigen. So, for instance, horse immune sera reacting with the corresponding protein antigens usually give a more sharply defined point of maximum precipitation, and complete inhibition occurs at excess of antigen as well as of antibody,

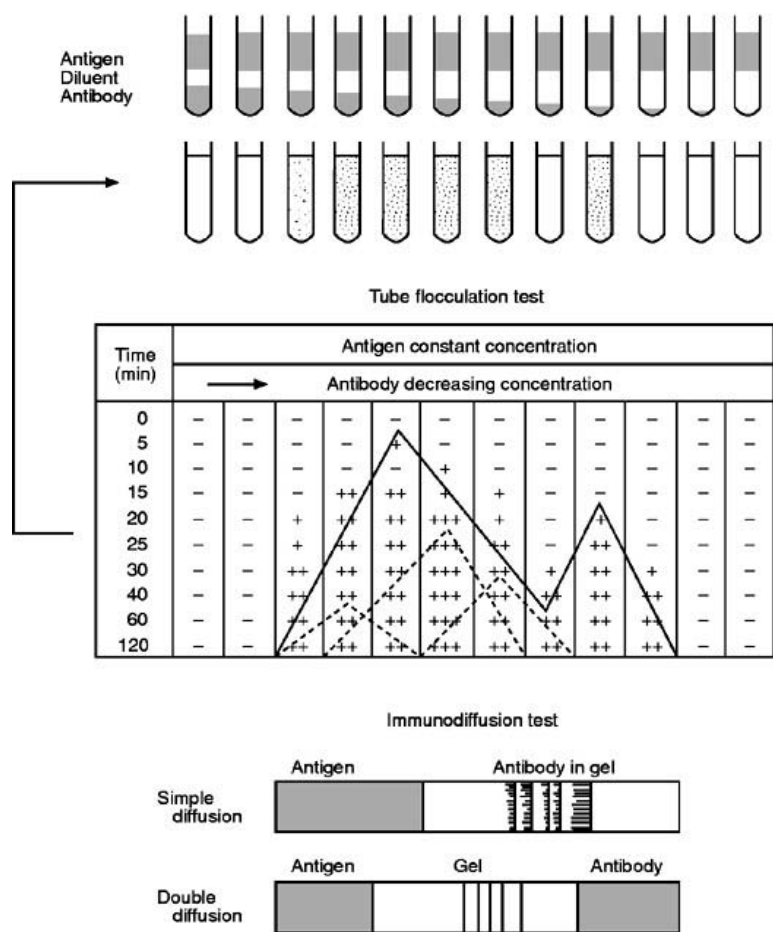


Figure 2 Complex serological system containing a set of five antigen-antibody systems of different specificities analyzed by tube flocculation (serial dilution) and immunodiffusion precipitation (concentration gradient) techniques.

whereas for rabbit immune sera reacting with polysaccharide antigens, the point of maximum precipitation is less distinct and complete inhibition is generally seen at excess of antigen.

In the flocculation reaction, which is very similar to the precipitation reaction except that the aggregates are formed as flocculent masses, the time at which visible aggregates appear is used for the evaluation of the serological system to be assayed. Flocculation is most rapid when the reactants are in approximately equal proportions (R_{opt}) for the formation of aggregates. This information, denoted as flocculation time (L_f), can be used for quantitative determinations. Either side of R_{opt} , flocculation proceeds more slowly and inhibition occurs when antigen is in excess and, for certain systems, also when antibody is in excess, as illustrated in Figure 2. The analytical procedure used to determine reaction velocity during flocculation is also applicable to precipitation reactions.

Precipitation in gels

If a gel is chosen as the medium in which the precipitation takes place, a continuous concentration gradient of reactants can be obtained by means of gel-stabilized diffusion, thereby eliminating the need for

stepwise dilution. The reactants are applied to the gel in a way such that one of the reactants, either antigen or antibody, diffuses into the gel which already contains the corresponding reactant (simple diffusion) or both reactants diffuse into a serologically neutral gel (double diffusion). By such an arrangement the precipitating material at the highest concentration down to zero will be tested by means of the time-dependent concentration gradients established. In a monospecific serological system, precipitation will appear as a band or a line at the site in the gel where the reactants satisfy R_{opt} . When materials containing two or more serologically different entities are analyzed, each system will form its individual precipitation band and most often their sites do not coincide.

In simple diffusion techniques such as the Oudin tube test, the external reactant, usually a solution of antigen, is applied at a relative concentration greater than that of the internal reactant, i.e. corresponding immune serum in the gel. Initially a precipitate is formed in the gel close to the interphase; however, it is gradually dissolved by the excess of the diffusing reactant. Reprecipitation occurs in the direction of the diffusion, resulting in a migrating precipitation band. When the external reactant is exhausted at its original source the 'migration' ceases and the

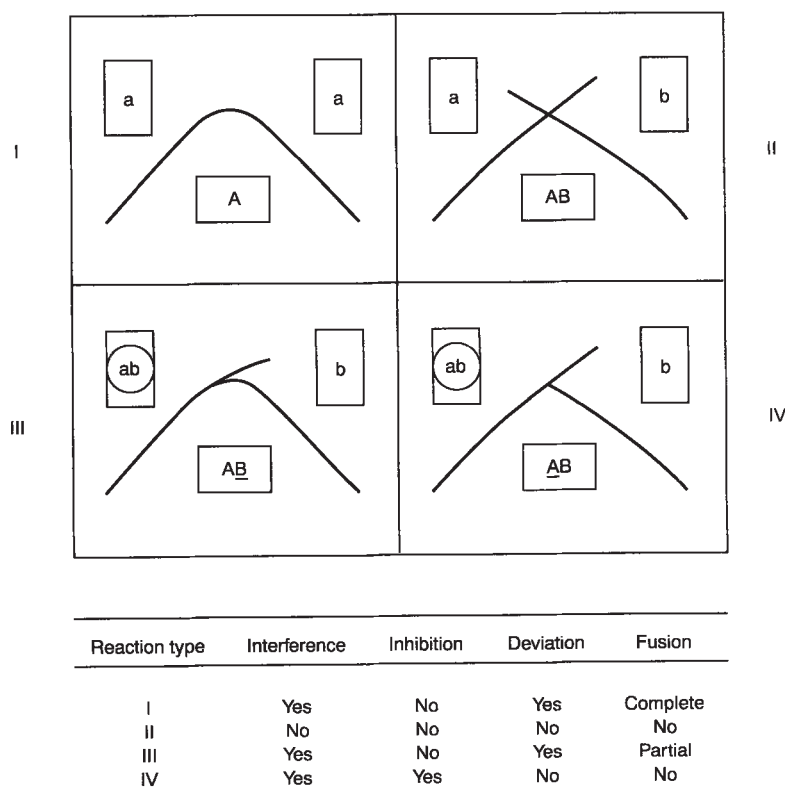


Figure 3 Basic precipitation patterns in combined systems (Ouchterlony comparative plate technique). For explanation see text.

precipitation band rests at its final site. This phenomenon is the base for quantitation by the simple radial diffusion method of Manicini and colleagues where a monospecific immune serum is used as the internal reactant.

In double diffusion techniques the precipitation bands are formed in a gel which initially is serologically neutral. With the reactants tested at equivalent concentrations (i.e. at R_{opt}), a stationary precipitate is formed at a site in the gel governed by the diffusion coefficients of the reactants. In a simple serological system only one band is formed. A multiple system will – provided that a superimposition of bands does not occur – create a series of bands corresponding to the number of serological systems present, i.e. a precipitation spectrum. The band sites are, apart from the degree of diffusibility, greatly dependent on the concentration of the reactants at their initial sources. Worth noting is that in the double diffusion technique a precipitation band, once stabilized as to site, acts as a specific barrier for further diffusion for the integrated antigen and its antibody but not for reactants with a different serological specificity. This phenomenon is taken advantage of in the Ouchterlony comparative plate technique in which the sources of diffusion are arranged in a way that permits a registration of possible interactions between two or more serological systems, as represented by their individual precipitation patterns. For the case where antigen preparations are compared by means of an immune serum there are, in principle, four alternatives for pattern formation: complete (I) or partial (III) fusion, interference without fusion (IV) or absence of interaction (II). These four types of reaction, illustrated in Figure 3, constitute the basis for the interpretation of comparative precipitation patterns. It should be noted that, when the analytical

system is reversed, i.e. antisera are compared by means of a multideterminant antigen, the interpretation of a complete fusion of lines as identity of antibody specificities is not justified.

For the analysis of very complex serological systems by means of precipitation-in-gel a greatly increased resolution can be obtained by using gel electrophoresis in combination with the double diffusion technique. This method, originally devised by Grabar and Williams and named immunoelectrophoresis (IE), has the advantage that it combines an electrophoretic characterization with the immunological identification of the material to be analyzed. It should be mentioned that the somewhat time consuming diffusion procedure in the original IE technique can advantageously be replaced by gel electrophoresis of one or both of the corresponding serological reactants, i.e. electroimmunoprecipitation.

See also: Antibody-antigen intermolecular forces; Autoantibodies, tests for; Immune complexes; Immunodiffusion, single radial; Immunoelectrophoresis; Valency of antigens and antibodies.

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PRIMARY BILIARY CIRRHOSIS

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Primary biliary cirrhosis (PBC) is an enigmatic chronic liver disease characterized by progressive inflammatory obliteration of the intrahepatic bile ducts. Clinical features and laboratory studies of the disease include data suggesting an underlying immunologic basis for pathology. These features

include a marked predominance of female patients, the presence of a variety of autoantibodies, circulating immune complexes, complement activation, defects in immunoglobulin class switching, T cell regulatory dysfunction, predominance of T cells in the hepatic periductular infiltrates, and an increased

association with scleroderma and the sicca complex. The autoantibodies found in PBC include antibodies to thyroid antigens, nuclear antigens, thymocyto-toxic autoantibodies, and mitochondrial-specific antibodies.

There are striking B lymphocyte abnormalities in PBC, including high levels of serum immunoglobulins, with an immunoglobulin G3 (IgG3) subclass restriction and a range of autoantibodies including, as noted above, those to mitochondrial antigens and occasionally antibodies to centromeric antigen and other undefined nuclear antigens. There is also increased serum complement consumption. The association with disease is closer for mitochondrial antibody and PBC than for any other autoimmune process; while PBC may occur in the absence of mitochondria-specific antibodies, such cases are most exceptional. Also, there are T lymphocyte immunoregulatory abnormalities that involve both CD4⁺ and CD8⁺ cells. In brief, the number of CD4⁺ and CD8⁺ cells in blood are decreased and various functional assays show deficiencies affecting both subsets of T cells. Histologically, biliary ductules in the liver are surrounded and penetrated by infiltrating T lymphocytes, mostly of the CD8 phenotype. Granulomata are prominent in the periductular infiltrates which is unusual for autoimmune pathology. The bile duct epithelium normally expresses only major histocompatibility complex (MHC) class I molecules but, in PBC, MHC class II molecules are also expressed in biliary epithelial cells, possibly under the influence of cytokines released by activated T cells. Such expression may be relevant to the autoimmune pathogenesis of PBC, since biliary epithelial cells which express MHC class II molecules could act in an inducer capacity as antigen-presenting cells for CD4⁺ helper T cells. The inducer processes and the peptide molecule(s) that stimulate T cells have yet to be identified and functional data are needed to substantiate the immunohistochemical evidence for the cytodestructive effects of CD8⁺ T cells.

The clinical signs of disease include features that are consistent with chronic intrahepatic biliary

obstruction including itching, jaundice, steatorrhea and osteomalacia. This classic presentation is now being replaced by early or asymptomatic disease recognized by the occurrences of liver functional abnormalities on autoanalyzer screening of serum. The treatment of patients is primarily directed at reducing itching as well as dietary replacement for malabsorption and osteomalacia. Treatment with corticosteroids and/or immunosuppressive agents has generally been unimpressive, perhaps because these were usually given only in advanced disease. Urso-deoxycholic acid (UDCA) is of established benefit according to the results of recent trials and there have been optimistic reports on the use of methotrexate. The results of liver transplantation for advanced disease are excellent.

The hallmark of PBC is the presence of high-titer mitochondrial autoantibodies in patients' sera. Such reactivity has been demonstrated using standard immunofluorescent assays, ELISA, complement fixation and inhibition of enzymic activity. Mitochondria-specific antibodies recognize major mitochondrial inner membrane proteins of approximately 74, 56, 52, 48 kDa and 36 kDa (Table 1). These four mitochondrial autoantigens have been identified as being protein X and the acyltransferases (E2) of three related 2-oxo acid dehydrogenase complexes: the pyruvate dehydrogenase complex (PDC), the branched-chain oxo acid dehydrogenase complex (BCOAH), and the oxoglutarate dehydrogenase complex (OGDC). The identification was established using either expressed polypeptides from DNA clones or purified enzyme preparations. Moreover, the mitochondrial autoantibodies of patients specifically inhibit the function *in vitro* of these enzymes.

These observations relating autoantigens to functional sites of proteins are of interest in that they are consistent with an emerging trend seen in a number of other autoimmune diseases. Autoantibodies to RNA synthetase in polymyositis sera are able to inhibit RNA aminoacylation and it has also been reported that synthesis of 28S and 18S RNA is inhibited by RNA polymerase I-specific antibodies in

Table 1 Molecular weights and functions of the mitochondrial autoantigens in PBC

Enzymes	Molecular weight (kDa)	Function
Pyruvate dehydrogenase (PDH)	74	Transfers acetyl group from E1 to coenzyme A (CoA)
E2 acetyltransferase		
Protein X	56	?
Branched-chain 2-oxo acid dehydrogenase (BCOAH) E1 α and β E2 acyltransferase	52	Transfers acyl group from E1 to CoA
Oxoglutarate dehydrogenase E2 succinyltransferase	48	Transfers succinyl group from E1 to CoA

scleroderma sera. In systemic lupus erythematosus (SLE) sera, autoantibodies specific for Sm and U1-RNP, which are both components of small nuclear ribonucleoproteins (snRNP), inhibit splicing of early RNA sequences, and autoantibodies to proliferating cell nuclear antigen (PCNA) inhibit DNA replication. Additionally, autoantibodies to the La (SS-B) nucleoprotein antigen, present in primary Sjögren syndrome, interfere with the transcription of small RNA molecules. The significance of these findings in terms of disease expression has yet to be determined, but two schemes are prominent. First, as for the mitochondrial antigens of PBC, many of the described autoantigens are intracellular enzymes and exist not as single proteins but as components of large complexes, and, second, the autoantibodies directed against these subcellular molecules appear to be directed against functional sites or domains, since they interfere with cellular function in assay systems *in vitro*. The capacity of PBC serum to abrogate the catalytic activity of the PDC enzyme is most impressive and is the basis of a diagnostic assay. Other assays, including the use of a hybrid recombinant protein that contains all the major epitopes of PDC-E2, BCOADH-E2 and OGDC-E2, are now also available.

Although there have been significant advances based on cloning, antigen definition and epitope mapping, there has been relatively little insight into the relevance of T cell reactivity in patients. The identification of T cell reactivity will be critical. The characterization of T cell reactivity in other autoimmune diseases has likewise been difficult because of the low frequency of antigen-specific T cell activity in peripheral blood. Moreover, even when liver tissue is studied, the frequency of antigen-specific T cells may be low because of the irrelevant inflammatory response. All of the identified mitochondrial autoantigens contain lipolate-binding sites and therefore

perhaps shared epitopes. Hence, a single common epitope could generate CD4⁺ cells to provide help for the oxo-cross-reactive B cell responses for each of the E2 subunits of the 2-OADH enzymes.

There are other theoretical considerations that should be applied to the understanding of the etiology and immunological responses in primary biliary cirrhosis. First, there is scanty data on specific etiology, i.e. environmental insult, toxin or infection. It remains plausible that the mitochondrial autoantigens are cross-reactive with a microbial antigen, but this is yet to be identified with a highly conserved component of an infectious agent. Second, PBC is often viewed as a ductal disease as lesions have been reported in other epithelial glandular tissues, i.e. salivary glands and pancreatic ducts. Third, mitochondrial autoantigens are synthesized in the cytoplasm and then transported into the mitochondria; therefore, mutations in leader sequence, or defects in breakdown, could lead to altered metabolism and expression on cell membranes. Finally, there are intriguing data that suggest that there is expression of either PDC-E2 or a cross-reacting molecule on the surface of biliary epithelial cells in patients with PBC but not controls. These data, if they reflect a primary not secondary feature of disease, would explain the specific targeting of biliary cells. The molecular basis of this molecule is still unresolved.

See also: Autoantigens; Autoimmune diseases.

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PRIMATE (NONHUMAN) IMMUNE SYSTEM

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Nonhuman primates have been widely used as animal models for human diseases. Simian immunodeficiency virus (SIV) infection of rhesus macaques provides an excellent model for human immunodeficiency virus (HIV) infections of humans. Similarly, experimental autoimmune encephalomyelitis

(EAE) induction in macaques and common marmosets has been utilized as a model for multiple sclerosis, and collagen-induced arthritis (CIA) in macaques is a good model for rheumatoid arthritis. Nonhuman primates have also been important in a variety of other studies attempting to delineate the

immune response to pathogens that infect humans. The use of nonhuman primates in such studies is largely due to the similarity between their immune systems and that of their human counterparts. In particular, the lymphocyte markers, major histocompatibility complex (MHC) molecules, and T cell receptors (TCRs) of Old World primates and great apes are very similar to those of humans. In this entry we will describe several animal models of human diseases and the similarities between the immune systems, MHC genes and TCR genes of nonhuman primates and humans.

Primates of the Anthropeidea suborder can be divided into two main infraorders. The first is the Catarrhini, which includes humans, great apes (e.g. chimpanzees, gorillas and orangutans) and Old World primates (e.g. baboons, colobus monkeys and the macaques). It is thought that chimpanzees and humans last had a common ancestor about 5 million years ago, whereas rhesus macaques and humans last had a common ancestor 35–40 million years ago. The second infraorder is the Platyrrhini, or New World primates. These include, among others, owl monkeys, squirrel monkeys and the marmosets and tamarins.

Nonhuman primates as animal models for human disease

Primates are widely used as models for studying HIV infection of humans. Although HIV does not infect all of the different species of nonhuman primates, it does infect chimpanzees. Thus far, however, there are few documented cases of chimpanzees succumbing to HIV-induced pathogenesis despite several chimpanzees having been infected for over 10 years with the virus. Therefore, combined with their endangered status, the use of chimpanzees is hardly practical for the study of HIV infection in humans. SIV infection of the rhesus macaque (*Macaca mulatta*), however, has proved to be a very effective animal model for studying HIV pathogenesis in humans. Due to the nucleotide sequence similarity between HIV and SIV and the similar course of disease progression between humans and rhesus macaques, SIV infection in rhesus macaques is widely used as an animal model of HIV infection in humans. More importantly, the immune systems of humans and rhesus monkeys have been found to bind and present peptides derived from very similar regions of HIV and SIV, respectively. Therefore, this animal model will be especially useful for conducting vaccine trials which cannot be carried out in humans.

Nonhuman primates have also been used to study the pathogenesis and immune response to other

viruses. Many of the New World primate species were extensively used to study the pathogenesis of viral infections in the early 1970s and 1980s due to their extreme susceptibility to these diseases. In particular, the cotton-top tamarin has been used to test vaccines for Epstein–Barr virus (EBV). Interestingly, the tamarin is susceptible to EBV-induced lymphoproliferative tumors upon inoculation with EBV whereas many of the other New World primate species are not susceptible to EBV. Owl monkeys, for example, develop a typical lymphocytosis similar to that seen in humans after infection with EBV. Rhesus macaques have also been used to study a variety of autoimmune diseases. These include experimental EAE and CIA. EAE can be induced in rhesus monkeys and the common marmoset by injection of whole brain homogenates or purified myelin basic protein. Also, rhesus macaques injected with collagen readily develop a disease that resembles rheumatoid arthritis. Thus, these animals can be used to understand the pathogenesis of this disease and can be used to test therapeutic modalities.

The similarities between human MHC molecules and those of nonhuman primates has facilitated a variety of transplantation-related studies. In particular, the rhesus macaque has been widely used to determine whether tolerance can be induced prior to organ transplantation. By manipulating the immune system, tolerance to kidney transplants was found to be dramatically increased in rhesus monkeys. Therefore, nonhuman primates provide preclinical large-animal models to determine whether studies conducted in mice can be extended into clinical applications in humans.

Similarities between lymphocyte markers of humans and nonhuman primates

The similarity between the various lymphocyte subsets of nonhuman primates and those of their human counterparts is one of the primary reasons that nonhuman primates are useful as animal models to study human diseases. Monoclonal antibody (mAb) reagents generated against human CD antigens can be used in many nonhuman primate species, and cross-reaction of these mAbs with the lymphocytes of nonhuman primates indicates that the structures of these cell surface molecules are likely well conserved. Table 1 gives a list of antibodies that are useful in functional studies of the immune system of the rhesus macaque. These cross-reactivities illustrate the conservation of functionally important cell surface molecules between humans and Old World primates.

A similar array of these human leukocyte-specific

Table 1 Monoclonal antibodies directed against human cell surface molecules which cross-react with Old World or New World primate cell lines (with thanks to Dr Keith Reinmann)

	<i>mAb</i>	<i>Source</i>	<i>Mamu</i>	<i>Caja</i>	<i>Saoe</i>
CD2	CD2 (IOT11a)	Immunotech	+	+	
	Leu-5b	Becton Dickinson	+	+	
	T11	Coulter	+	+	
	CD2	Exalpha	+		
	MT910	Dako			+
CD3	SP34,1	PharMingen	+		
	SP34	Dr C. Terhorst			+
	FN18 ^a	Biosource	+		
CD4	CD4	Exalpha	+		+
	Leu-3a	Becton Dickinson	+	+	
	OKT4	Ortho	+		
	Anti-CD4	Alpha Laboratories			+
	MT301	Dako			+
CD5	T1	Coulter		+	
CD6	T12	Coulter	+	+	
CD7	MT701	PharMingen	+		
	3A1	Coulter		+	
CD8	CD8 (IOT8a)	Immunotech	+		
	Leu-2a	Becton Dickinson	+		
	T-cell, T8	Dako	+		
	CD8	Exalpha	+		
	OKT8	Ortho	+		
	MT1014	Dr P. Rieber	+		+
	MT122	Dr P. Rieber	+		+
	T8	Coulter		+	
	Leu-2b	Becton Dickinson		+	
CD11a	CD11a (IOT16)	Immunotech	+	+	
	CD11a	Exalpha	+		
	MHM24	Dako			+
	LFA1a	Becton Dickinson		+	
CD11b	Mo1	Coulter	+	+	
	Leu-15	Becton Dickinson		+	
CD11c	Leu-M5	Becton Dickinson		+	
CD14	CD14	Immunotech	+		
	Leu-M3	Becton Dickinson	+	+	
	MY4	Coulter	+		
	TUK4	Dako			+
	Mo2	Coulter		+	
CD16	CD16	Immunotech	+		
	Leu-11a	Becton Dickinson	+	+	
	Leu-11b	Becton Dickinson			+
	ION16	Immunotech		+	
CD18	LFA-1 beta	Dako	+	+	
	CD18	Exalpha	+		
	MHM23	Dako			+
CD19	CD19	Immunotech	+		
	B4	Coulter		+	
CD20	CD20 (IOB20a)	Immunotech	+	+	
	Leu-16	Becton Dickinson	+		
	B1	Coulter	+	+	
	B-Lyl	Dako			+
	IOB20	Immunotech		+	
CD21	BU35	Binding Site			+
	BU36	Dr L. Young			+
	B2	Coulter		+	
	IOB1a	Immunotech		+	

Table 1 Continued

	<i>mAb</i>	<i>Source</i>	<i>Mamu</i>	<i>Caja</i>	<i>Saoe</i>
CD22	B3	Coulter		+	
CD23	EBV/CD4	Dr D. Crawford			+
	Leu-20	Becton Dickinson		+	
	B6	Coulter		+	
CD25	IOT14	Immunotech	+	+	
	IL2-R1	Coulter	+	+	
	CD25	Exalpha	+		
	ACT-1	Dako			+
	IL-2R	Becton Dickinson		+	
CD26	Tal	Coulter		+	
	IOA26	Immunotech		+	
CD28	CD28	Immunotech	+		
	Leu-28	Becton Dickinson	+	+	
CD29	4B4	Coulter	+	+	
	MAR4	PharMingen	+		
CD31	IOM31	Immunotech		+	
CD32	IOM32	Immunotech		+	
CD33	MY9	Coulter		+	
CD34	Q-bend10	Immunotech	+		
	IOM34	Immunotech		+	
CD36	IOP36	Immunotech		+	
CD38	OKT10	Ortho	+		
	IOB6	Immunotech		+	
CD44	Leu-44	Becton Dickinson		+	
	IOL44	Immunotech		+	
CD45	KC56	Coulter		+	
CD45RA	CD45Ra	Immunotech	+		
	2H4	Coulter	+		
	Leu-18	Becton Dickinson		+	
CD45RO	IOL-3	Immunotech		+	
CD49b	IOP49b	Immunotech		+	
CD49c	IOP49c	Immunotech		+	
CD49d	IOP49d	Immunotech		+	
CD49e	IOP49e	Immunotech		+	
CD49f	IOP49f	Immunotech		+	
CD51	IOP51	Immunotech		+	
CD56	MY31	Becton Dickinson	+	+	
	NKH-1	Coulter		+	
CD58	IOT58	Immunotech		+	
CD61	IOP61	Immunotech		+	
CD62E	IOP62	Immunotech		+	
CD62L	Leu-8	Becton Dickinson	+	+	
	TQ1	Coulter		+	
	LAM1-3	Coulter		+	
CD69	Leu-23	Becton Dickinson		+	
CD71	Transf.R	Becton Dickinson		+	
CD72	IOB72	Immunotech		+	
CD86	B70	Immunotech		+	
CD94	Kp43	Immunotech		+	
CD95	FAS	Immunotech		+	
CD105	Endoglin	Immunotech		+	
CD106	VCAM	Immunotech		+	
CDw124	IL-4rec.	Immunotech		+	

Table 1 Continued

	<i>mAb</i>	<i>Source</i>	<i>Mamu</i>	<i>Caja</i>	<i>Saoe</i>
MHC-I	HLA-ABC	Immunotech		+	
	W6/32	ATCC	+		
	BB7.7	ATCC	+	+	-
MHC-II	HLA-DR	Becton Dickinson	+	+	
	I2	Coulter		+	
	HLA-DR	Immunotech		+	
	CR3/43	Dako			-
	949	Todd <i>et al.</i> 1984	+		+
	DA6.231	EACC			+
	IOT2a	AMAC	+		
	I3	Coulter	+		
	HLA-DR	Exalpha	+		
	Anti-HLA-DR	Olympus	+		

Those antibodies which cross-react with the listed primate species are indicated by a +. The absence of a + does not necessarily indicate that the antibody does not cross-react, since not all antibodies have been tested for cross-reactivity in these primates. *Macaca mulatta* (Mamu) = rhesus monkey; *Callithrix jacchus* (Caja) = common marmoset, and *Saguinus oedipus* (Saoe) = cotton-top tamarin.

^aNote that the CD3-specific mAb FN18 is a rhesus-specific anti-CD3 mAb.

mAbs have been tested in two species of New World primates, the cotton-top tamarin (*Saguinus oedipus*) and the common marmoset (*Callithrix jacchus*). Not all monoclonal antibodies that cross-react with surface expressed molecules from the rhesus macaque work well in New World primates. Table 1 provides a list of those mAbs which also cross-react with lymphocytes from the common marmoset and cotton-top tamarin. However, these results were taken from a study in which only 62% of mAbs specific for human epitopes were found to cross-react with New World primate lymphocytes. Therefore, although a significant number of mAbs are able to bind to both human and New World primate cell lines, indicating the preservation of functionally important cell surface molecules between these species, the percentage of mAbs which cross-react decreases the further one travels across the phylogenetic tree.

The MHC and TCR genes of nonhuman primates

The products of the MHC and TCR genes represent some of the most important molecules involved in initiating and controlling an immune response against a pathogen. Although various mAbs specific for the human MHC molecules have been shown to cross-react with MHC molecules from humans, Old World primates, and New World primates (see Table 1), there has also been an extensive analysis of MHC molecules from these species at the nucleotide sequence level. Such analyses allow for more detailed comparisons of the conservation of the functions of these molecules than mAbs. Similarly, cloning and sequencing of the TCR genes in nonhuman primates

has revealed a striking similarity among the various TCR genes of primates.

While MHC class II genes are well conserved between humans, Old World primates, and even in some cases New World primates, the MHC class I genes appear to have undergone many more changes. The MHC class I loci can be separated into classical or nonclassical loci. Classical MHC class I molecules, such as the human leukocyte antigen (HLA)-A, -B and -C molecules, are highly polymorphic and their role in binding and presenting peptides is well established. The non-classical molecules from the *HLA-E*, *-F*, and *-G* loci, for which specific functions have not, as yet been described, are more limited in their polymorphism. The *HLA-J* and *-H* loci, however, represent inactivated MHC class I loci or pseudogenes.

Nonhuman primates belonging to the Pongidae family are the most closely related species to humans. This family includes the common and pygmy chimpanzees (*Pan troglodytes* and *Pan paniscus*), gorilla (*Gorilla gorilla*) and orangutan (*Pongo pygmaeus*). Due to their close phylogenetic relationship to humans, chimpanzees express homologs of the *HLA-A*, *-B*, *-C*, *-E*, *-F*, *-G*, *-H*, and *-J* loci (Table 2 and Figure 1). An *HLA-C* homolog has also been identified in the gorilla, while no such homolog has been found in the orangutan, which suggests that the *HLA-C* locus likely arose through gene duplication after gorillas and orangutans last shared a common ancestor.

Analysis of the MHC class I genes from the rhesus monkey reveals that homologs of both the *HLA-A* and *-B* loci are present. Since rhesus monkeys last shared a common ancestor with humans approximately 35 million years ago, this indicates that the

Table 2 MHC class I and class II loci in human, great ape, Old World primate, and New World primate species

Locus	CATARRHINI						PLATYRRHINI			
	Hominidae			Pongidae		Hylobatidae	Cercopithecidae		Cebidae	
	Human	Chimp	Gorilla	Gorilla	Orangutan		Rhesus monkey	Owl monkey	Squirrel monkey	Golden lion tamarin
A	+	+	+	+	+	+	+			
B	+	+	+	+	+	+	+			
C	+	+	+	+						
E	+	+	+		+		+	+		+
F	+	+	+				+			+
G	+	+		+			+			
AG							+			
G'										+
G''										
G'''										
G''''								+	+	
H	+	+	+							
J	+	+	+	+						
DRB1										
DRB1'					+		+			
DRB3	+	+	+							+
DRB3'							+			
DRB4	+	+								
DQB	+	+	+					+		
DPB	+				+		+		+	

Only positively identified loci are indicated. The absence of a + sign for a particular locus in a species does not necessarily indicate that the particular locus is absent, but simply indicates that the locus has not yet been identified in that species. Note that *HLA-B* homologs have been identified in two New World primates, the long-haired spider monkey and white-faced saki monkey (data not shown). Approximate divergence times of these various primate species is listed in Figure 1.

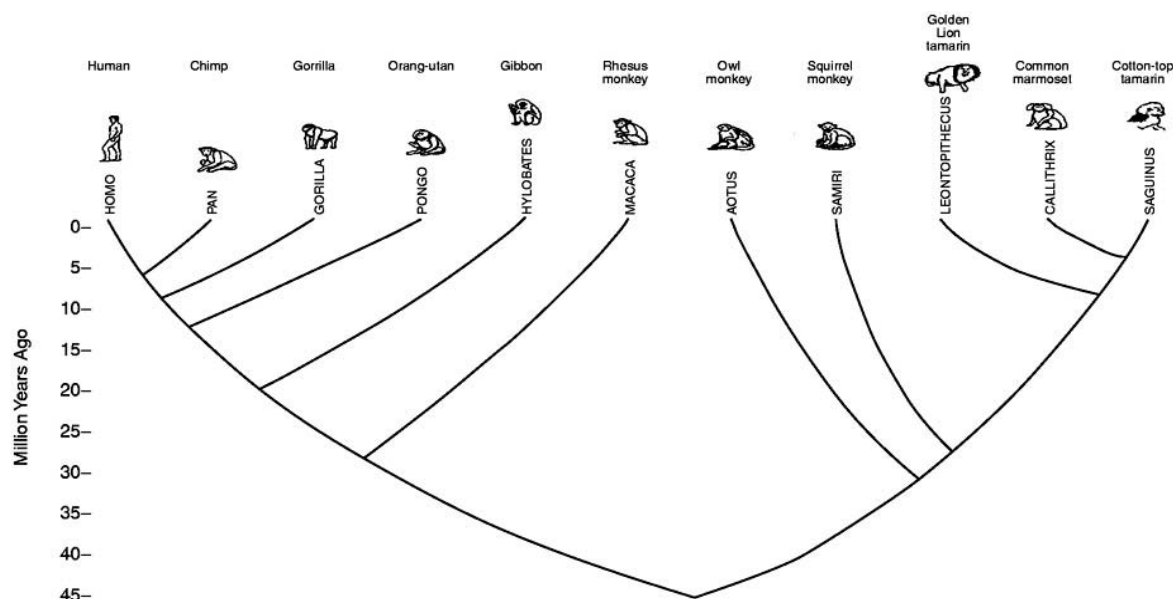


Figure 1 Evolutionary tree demonstrating the relationship and approximate divergence time of various primate species from the Catarrhini and Platyrrhini infraorders.

homologs of the *HLA-A* and *-B* loci are extremely stable. Interestingly, although the rhesus monkey does not appear to possess an *MHC-C* locus, both the *MHC-A* and *-B* loci can be duplicated depending on the haplotype. Because of the utility of the rhesus monkey as an animal model for several human diseases, the *MHC* class I genes and molecules of this species have been extensively characterized. As such, many nonclassical *MHC* class I genes and pseudogenes have also been identified in the rhesus monkey. Both *HLA-E* and *-F* homologs have been identified in the rhesus monkey along with an *HLA-G* homolog, which unlike *HLA-G*, has become inactivated in the rhesus monkey. Interestingly, another functional *HLA-A*-like gene in the rhesus monkey (*Mamu-AG*) possesses many of the unique features of *HLA-G* including expression in the placenta. Therefore, some of the *MHC* class I loci appear to be evolving rapidly through a pattern involving gene duplication events followed by gene deletion or inactivation events. In the case of *Mamu-AG*, this may represent a unique case in which an *HLA-A*-like gene was duplicated in the rhesus monkey with its protein products possibly serving the function of the original *Mamu-G* molecule. The presence of two genes, *Mamu-G* and *Mamu-AG*, each producing proteins capable of performing the same function may have allowed for inactivation of one of these genes in the rhesus monkey, in this case the original *Mamu-G* gene, which became a pseudogene.

The expressed classical *MHC* class I genes of New World primates are unique compared to those of the

great apes and Old World primates in that they are more closely related to the nonclassical *HLA-G* locus than they are to either the *HLA-A* or *-B* loci. Interestingly, one family of New World primates possess *HLA-G*-like loci that are distinctly different one from another. This expression of genus-specific classical *MHC* class I loci by the Callitrichidae may be due to the rapid turnover of *MHC* class I genes in New World primates. Recent examination of the *MHC* class I genes of two other species of New World primates from the Cebidae family, the long-haired spider monkey (*Alouatta seniculus*) and white-faced saki monkey (*Pithecia pithecia*), demonstrated the presence of a single *HLA-B* locus homolog in each of these species. This finding suggests that the *MHC-B* locus did indeed exist prior to the divergence of Old World and New World primates, and suggests that the lack of *HLA-A* and *-B* locus homologs in some species of New World primates is due to the deletion of these loci in these species. New World primates also possess *HLA-E* and *-F* homologs, demonstrating the stability of these loci in New World primates.

MHC class II genes

The human *MHC* class II molecules are encoded for by genes from the *HLA-DR*, *HLA-DQ* and *HLA-DP* loci. As illustrated in Table 2 and Figure 1, genes from these multiple *MHC* class II loci are present in humans, great apes, Old World primates and even some New World primates. The New World primates' *MHC* class II genes, however, do not likely rep-

resent homologs to those found in Old World primates and apes. Analysis of MHC class II genes at the nucleotide sequence level indicates that these genes are extraordinarily well conserved between most species of primates. Therefore, for the study of human MHC class II-associated diseases, various species of primates may serve as excellent animal models. For example, rhesus monkey B lymphoblastoid cell lines expressing a particular *MHC-DRB1* allele can present *Mycobacterium tuberculosis* peptides to human T cell clones. Additionally, in the rhesus monkey a clear association was demonstrated for the presence of the MHC class II allele *Mamu-DPB1 *01* with susceptibility to EAE.

TCR genes

TCR V_β (variable region of the β-chain) cDNAs have now been cloned from chimpanzees, rhesus macaques, cotton-top tamarins and common marmosets. Homologs of the various V_β families are easily recognizable in each of these primate species. Thus, even in New World primates homologs of the human TCR V_β genes are well preserved.

Summary

Due to the recent technical advances in molecular biology the number of human disease-associated genes identified will increase immensely. As such, it is now possible to investigate many of these diseases in animal models. This will facilitate the development of vaccine strategies or therapeutic modalities to control these diseases. Nonhuman primates represent the best animal models for many of these diseases due to the overall similarities of their immune systems to those of humans. This is best illustrated by the significant cross-reactivity of leukocyte-specific mAbs with both human and nonhuman primate lymphocyte subsets, and the similarities among the MHC and TCR genes of primates.

See also: Antibodies, specificity; CD antigens; Experimental autoimmune encephalomyelitis (EAE); HLA class I; HLA class II; Immunodeficiency, animal mod-

els; MHC, evolution of; MHC, functions of; T cell receptor, αβ.

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PRIVILEGED SITES

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Certain anatomic sites and/or tissues of the body exhibit the property of immune privilege: grafts of histoincompatible tissues placed within these sites are either not rejected at all, or only very slowly. Sites which have been considered to be privileged include brain, eye (anterior chamber, vitreous cavity, cornea, subretinal space), testis and ovary, adrenal cortex, hamster cheek pouch, and the pregnant uterine decidua and the fetal-placental unit. Privilege is usually extended permanently to grafts that are weakly antigenic, i.e. that display the products of only minor histocompatibility loci, whereas privilege may be transient if the immunogenetic disparity between graft and recipient includes major histocompatibility complex (MHC)-encoded antigens. The difference in survival times of MHC and non-MHC disparate grafts in privileged sites reveals that the phenomenon has quantitative features, i.e. privilege is not 'all or none'.

Until recently, immune privilege was not thought to be extended to histoincompatible grafts placed in privileged sites of recipients previously sensitized to antigens expressed on the graft. However, it now appears that there are factors operative within immune privileged sites that can suppress at least certain components of the alloimmune rejection response, even after they have been generated. Thus, immune privilege is a process that, on the one hand, alters the induction of immunity to antigenic materials first encountered via privileged sites, and, on the other hand, inhibits the expression of certain forms of alloimmunity in these same sites.

Common features of immune privileged sites

Distinctive anatomic, cellular and molecular features are commonly found at privileged sites: 1) a blood-tissue barrier (continuous capillaries with endothelial cells united by tight junctions) across which passage of blood cells and plasma only occurs as a facilitated process; 2) absence of a lymphatic drainage pathway, necessitating that tissue fluids drain directly into the blood vascular compartment; 3) reduced or even absent expression of molecules encoded by class I and class II genes of the major histocompatibility complex; 4) constitutive local expression of Fas ligand (FasL) which can induce apoptosis among activated Fas⁺ (CD95) lymphocytes; 5) paucity of

bone marrow-derived MHC class II positive cells that function conventionally as professional antigen-presenting cells; 6) deficit in the local microenvironment of molecular mediators of inflammation, such as complement factors and clotting factors, along with specific inhibitors of these tissue-threatening enzyme cascades; 7) constitutive presence of a distinctive array of molecules within the local immunosuppressive microenvironment (growth factors, cytokines and mediators) that inhibit conventional antigen processing and presentation, suppress antigen-driven lymphocyte activation, and/or down-regulate cellular and molecular mediators of inflammation. Not all of these features are found at any single immune privileged site, indicating that privilege is multifactorial in origin.

Recently it has been discovered that antigens can escape from grafts placed in privileged sites, and, as a consequence, evoke an unusual systemic immune response. Typically, delayed hypersensitivity and complement fixing antibodies are not elicited by such privileged allografts, although other immune effector modalities, such as precursor cytotoxic T cells, non-IL-4-secreting T helper cells, and non complement fixing antibodies (especially IgG1 in mice) are regularly elicited. Animals bearing foreign tissue grafts in privileged sites often (but not always) display concomitant immunity: the graft in the privileged site remains healthy, even though genetically identical grafts are rejected when placed at conventional body sites.

Immune responses to antigenic material in immune privileged sites

The manner in which these features create immune privilege and the associated 'deviant' systemic immune response (to privileged antigens) is the subject of intense investigation at the present. Current evidence indicates that privilege may result from an actively acquired, dynamically regulated, antigen-specific immune response that is able to express itself within the privileged site while avoiding nonspecific, tissue-destructive inflammation. Since this 'deviant' immune response is systemic in nature, the selective deficiencies (delayed hypersensitivity and production of complement fixing antibodies) are evident when the same antigens are encountered at other, nonprivileged sites of the body. The unique features of

immune responses to antigens expressed on grafts placed in privileged sites are also elicited when other types of nongraft antigens are inoculated into the privileged site, including antigens associated with tumors, haptens, virally encoded antigens, soluble heterologous protein antigens, and tissue-restricted autoantigens.

In the past, immune privilege has been regarded largely as a laboratory curiosity. However, it now appears that privilege is actively acquired and maintained, leading investigators to wonder whether privileged sites serve physiologic purposes. For example, it has been proposed that privilege in the anterior chamber of the eye, including the deviant systemic immune response to antigens placed within this site, serves to limit/prevent immunogenic inflammation at that site. As a consequence, the microanatomic integrity of the eye, which is essential for vision, is spared from blinding distortion by inflammation. Privileged sites may have in common a unique vulnerability to the nonspecific, destructive potential of immunogenic inflammation, and therefore immune privilege may be an evolutionary adaptation designed to mitigate this possibility. In addition, privileged tissues, such as the eye, contain

strong tissue-restricted autoantigens that can readily cause autoimmune disease. Immune privilege is considered to be one mechanism that is used by tissues with strong 'sequestered' autoantigens to avoid destructive autoimmunity.

See also: Autoantigens; Autoimmune diseases; Autoimmunity; Transplantation.

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PROKARYOTES, IMMUNITY IN

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In this article, we review a type of immunity which confers a highly specific insensitivity upon bacteria to the lethal action of a substance. The immunity is a genetic character carried by a bacterial chromosome, a bacteriophage genome or a transmissible plasmid.

Many antimicrobial agents are produced and released extracellularly by bacteria. These compounds have a specific action on the targeted bacteria. They can be classified as 1) enzymatically synthesized antibiotics, 2) post-translationally modified peptide antibiotics, 3) protein antibiotics as bacteriocins, protein exotoxins and bacteriolytic enzymes, and 4) temperate or temperate bacterial viruses. These agents penetrate into the bacterial cell and, once inside, they have to remain unmodified to exert their action.

Many levels of insensitivity to these lethal sub-

stances are reported for bacteria. For example, resistance to the antibiotic streptomycin can result either from a mutation in the bacterial genome which alters the 30S ribosomal subunit or from a plasmid encoding enzyme that chemically modifies and inactivates streptomycin. In such a case, the resistance can be overcome by adding a high external concentration of the antibiotic in the culture medium. Similarly, insensitivity to bacteriocins or phages may result from mutations of bacterial components involved in the uptake of the toxic macromolecules. Since many bacteriophages and bacteriocins share similar uptake pathways, a single mutation may confer insensitivity to various bacteriocins or phages. Bacteria carrying such mutations are termed resistant or tolerant (but not immune) to the particular toxic agent.

The system of restriction–modification directed against foreign genetic material is another example

of a bacterial defense mechanism. When the DNA of a phage is transferred from one bacterial strain to another bacterial strain with a different restriction–modification system, the phage DNA is immediately degraded after its injection by the host restriction endonuclease. The host DNA itself is protected from the action of its own restriction endonuclease by a strain-specific modification system. Bacterial strains able to restrict DNA are protected against the invasion of foreign DNA, but the specificity of the protection is not strictly restricted to a particular DNA. These protective mechanisms are not highly specific to the action of a particular killing agent and thus will not be considered as immunity. This entry will mainly focus on immunity against bacteriocins and bacteriophages.

Immunity to bacteriocins

Some diffusible substances released by bacteria into the medium inhibit the growth of other sensitive, generally closely related, bacteria. These substances, which are proteinaceous in nature, act at low concentrations. They have been called bacteriocins. A common characteristic of bacteriocin-producing strains is their ability to resist their own bacteriocin through specific-immunity peptides or proteins. The best-documented bacteriocins are the colicins produced by various members of the *Enterobacteriaceae*.

Colicinogeny appears to be the result of selection pressure among the *Enterobacteria*. Colicins are encoded by Col plasmids and their production is induced by agents which damage DNA. In most Col plasmids three genes have been identified: the colicin structural gene, the immunity gene, which protects the producing cells against the action of their own colicin, and the lysis gene, whose expression is required for efficient colicin release. Once secreted into the medium, colicins require at least three steps to kill sensitive cells of *Escherichia coli*: 1) binding to a specific receptor located in the outer membrane; 2) translocation across the membrane; and 3) interaction with their specific target in the cell. Three different domains linearly organized along the polypeptide chain are involved in each of these steps.

Pore-forming colicins kill sensitive bacteria by forming voltage-dependent ion channels in their inner membranes. This membrane depolarization causes a series of metabolic effects such as inhibition of active transport and of protein and nucleic acid synthesis, decrease of internal ATP concentrations, and leakage of potassium. Cell death results from all these events.

Pore-forming colicins form well-defined channels in the planar lipid bilayer. These channels are characterized by their sensitivity to the difference of the

electrical potential across the membrane. The channel domain is composed of 10 α helices and has to rearrange in order to insert into the membrane. The voltage-independent insertion of a hydrophobic helical hairpin, buried in the core of the structure, and the voltage-dependent insertion of a large voltage-responsive segment leads to channel formation.

Immunity genes are constitutively expressed and are transcribed in the opposite direction to the colicin genes. Immunity proteins are produced in very low amounts and protect the cell against external colicin only. The amount of colicin reaching its target, the inner membrane, depends on the number of uptake sites (400–1000); a very low amount of immunity protein is thus sufficient to protect the cell. Immunity proteins to channel-forming colicins are membrane proteins of 11–18 kDa that span the cytoplasmic membrane three to four times. They protect the cells against the colicin they produce by specifically interacting with the C-terminal domain of the colicins. Immunity proteins act in the cytoplasmic membrane by specifically preventing pore formation. The high specificity of the immunity protein suggests that a molecular complex forms between the colicin and its cognate immunity protein. The specific determinant for immunity protein recognition has been found to be the hydrophobic hairpin of the colicin channel domain. The immunity protein diffuses freely in the membrane and then recognizes within the lipid bilayer the colicin channel domain by interacting with its hydrophobic hairpin (Figure 1).

Colicins which do not form channels have enzymatic activity: some hydrolyze the bacterial chromosome while others cleave the ribosomal RNA or inhibit murein synthesis. For instance, colicin E3 is a

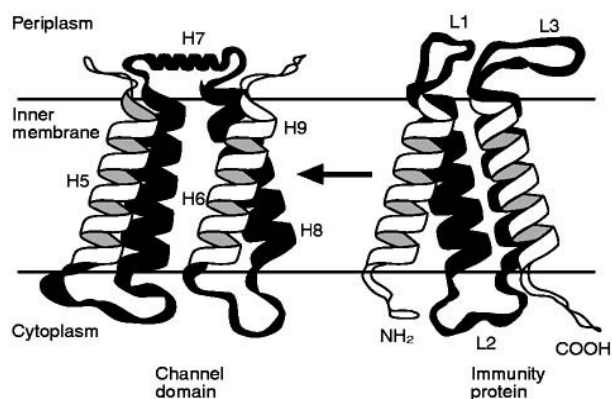


Figure 1 Colicin channels are made up of four helices: the voltage responsive segment (helices 5 and 6) and the helical hydrophobic hairpin (helices 8 and 9). The colicin A immunity protein inhibits channel formation by interacting within the lipid bilayer with the colicin channel. The immunity mechanism is based on intramembrane helix–helix interactions.

highly specific RNAase which cleaves 16S ribosomal RNA at a position that is conserved in all prokaryotes and many eukaryotes. Its action requires the presence of the 50S subunit in addition to the 30S subunit and the ribosomal proteins. Other colicins of the E class are nonspecific DNA endonucleases. They cause single- and double-strand breaks in the DNA of sensitive cells. Colicin M is the only colicin known to inhibit lipopolysaccharide O antigen synthesis and murein biosynthesis, leading to cell lysis. Bacteria producing RNAase and DNAase colicins are protected from their action by the presence of immunity proteins. They inhibit the RNAase and DNAase functions of their corresponding colicins. Immunity proteins (10 kDa) form a stoichiometric complex with their cognate colicin. This complex is extremely stable and can only be dissociated under strong denaturing conditions. For instance, the dissociation constant of the colicin E9 DNAase domain immunity protein is around 10^{-17} . Immunity proteins to colicins of RNAase and DNAase types are removed from the colicin C-terminal domain during the colicin entry into sensitive cells. NMR studies on the immunity proteins of colicin E9 DNAase and colicin E3 RNAase indicate that the colicin E9 immunity protein folds into a novel structure made of a distorted antiparallel four-helix bundle whilst colicin E3 immunity protein folds into a four-stranded, antiparallel β sheet connected by loops and a single short helix. The structure of the DNAase and RNAase domains remain unknown.

Gram-positive bacteria also produce bacteriocins. These are a heterogeneous group of antibacterial substances comprising a large number of small peptides. Generally, the level of immunity of the producing strain to its own bacteriocin is stronger for colicins than it is for bacteriocins of gram-positive bacteria. Lantibiotics (lanthionine-containing peptides) are the better characterized bacteriocins produced by gram-positive bacteria. The biosynthesis of lantibiotics is achieved by successive enzyme-catalyzed modifications of ribosomally synthesized precursor peptides. Lantibiotics act on the membranes of sensitive cells and form defined channels rather than a generalized membrane disruption. A minimum membrane potential is required for lantibiotic activity.

Lantibiotic-producing strains protect themselves from their own products by producing immunity proteins. Few immunity proteins have been described and their mode of action is not yet clear. The immunity proteins against the lantibiotics Pep5 and nisin, produced by *Staphylococcus epidermidis* and *Lactococcus lactis* respectively, have been identified. Both contain a hydrophobic N-terminus which might

serve as a membrane anchor. Immunity proteins interact with their cognate lantibiotic outside the cell. They act by preventing membrane depolarization caused by the peptide toxin. The mechanism which prevents pore formation is not identified. It is interesting to note that various mechanisms are used to transport immunity proteins to the outside of the producing bacteria.

Immunity to bacteriophages

Bacteriophages are viruses comprising one type of nucleic acid, either DNA or RNA, and a capsid which protects the encapsided nucleic acid. After a random collision between the virion and the bacterial cell, the phage becomes specifically and irreversibly attached to a receptor located on the outer surface of the host cell. The phage DNA is then injected into the cytoplasm of the host. Two consequences of phage infection are possible: 1) The bacteriophage replicates its DNA, produces the structural components of new phage particles, and packages the newly replicated DNA into the preheads. The progeny phages are released concomitant with lysis of the cell. This genetically well-regulated process is defined as the lytic cycle. 2) The phage structural proteins are not synthesized and the phage genetic material integrates into the bacterial chromosome and becomes dependent on the bacterial multiplication process. A cell that has integrated a phage DNA into its chromosome is referred to as a lysogen and the integrated phage DNA is termed a prophage. These two outcomes are possible for temperate phages, in contrast to the virulent phages which only go through the lytic cycle. Immunity to phage is the refractory state of an infected bacteria to be reinfected by a phage of the same type. Immunity to superinfection proceeds differently with temperate phages and with virulent phages.

Virulent phages

After infection of cells with the virulent T4 bacteriophage, genetic markers from a superinfecting phage of the same or closely related type are not recovered. The superinfecting phage adsorbs to the cell surface but superinfection immunity prevents entry of the bacteriophage DNA into the cytoplasm of the bacteria. The DNA is ejected from the phage capsid into the intermembrane space of the bacteria where it is degraded by an endonuclease. Ghosts of phage T4 which lack DNA are also capable of killing noninfected bacteria. They provoke a depolarization of the cytoplasmic membrane but, in contrast to the action of intact T4 phages, the membrane is irreversibly depolarized. It is thought that transmembrane

channels allow the penetration of phage DNA into the cytoplasm of the bacteria. On superinfection, binding of T4 to cells, tail-sheath contraction and penetration of the tail tube into the periplasm occur normally, but phage DNA is not liberated into the cytosol. An immunity mechanism causes the degradation of the ejected DNA.

The T4 bacteriophage immunity gene has been identified in the T4 genome. It encodes an immunity protein that acts 1–2 min after infection and which is responsible for most of the immunity to superinfection. The deduced amino acid sequence from the DNA sequence suggests that this protein has the structural features of an integral plasma membrane protein. The protein contains two hydrophobic segments which serve as membrane anchors. The action of the immunity protein specifically prevents superinfection by T-even phages. It is located at specific sites on the plasma membrane, bound to another protein. It may act indirectly by changing the conformation of the bound protein to prevent DNA transfer (Figure 2). The immunity protein acts stoichiometrically, therefore it must be located near the injection site or be able to diffuse very rapidly to this site since immunity develops in less than 2 min.

Temperate phages

Lysogenic bacteria are immune to infection by a phage of the carried type. Superinfecting phages of the same type as the integrated phage are unable to express their vegetative functions. This process is due to the expression of a prophage gene product called repressor which represses the expression of vegetat-

ive viral genes. The immunity thus depends on the synthesis of a repressor and on the sensitivity to this repressor, which is conferred by the repressor DNA binding sites. For the phage λ , these flank the gene encoding the repressor. The region delimited by the repressor binding sites is referred to as the immunity region because it determines the type of superinfection immunity conferred by the prophage. Immunity of temperate bacteriophages is thus based on a protein–DNA interaction which prevents the replication of the integrated phage and of superinfecting phages of the same type.

Immunity to bacteriophages λ , Mu and P1 is caused by a repressor which prevents transcription initiation at key promoters for lytic gene expression. Expression of the genes involved in the lytic cycle may also be prevented by the premature termination of transcription, as seen for phage P4. In this case, the trans-acting immunity factor is not a protein but a short RNA which causes transcription termination by pairing with complementary target sequences. For temperate bacteriophage P1, lysogenic bacteria do not carry the prophage integrated into the host chromosome, but the bacteriophage DNA remains as an autonomous plasmid in the cytoplasm of the host cell. Superinfection immunity is conferred by two repressors which prevent DNA replication associated to the lytic cycle: a protein repressor called C1 negatively regulates the expression of the lytic genes and an antisense RNA (C4) inhibits the translation of an antirepressor whose product interferes with the activity of the C1 repressor. Synthesis of only one repressor does not render a P1 lysogen immune to superinfection by P1 unless the other repressor is also expressed. In addition to this double control of immunity, C1 repression is strengthened by an auxiliary repressor protein which enhances the binding of C1 repressor to its operator sequence. These three components form a tripartite immunity system.

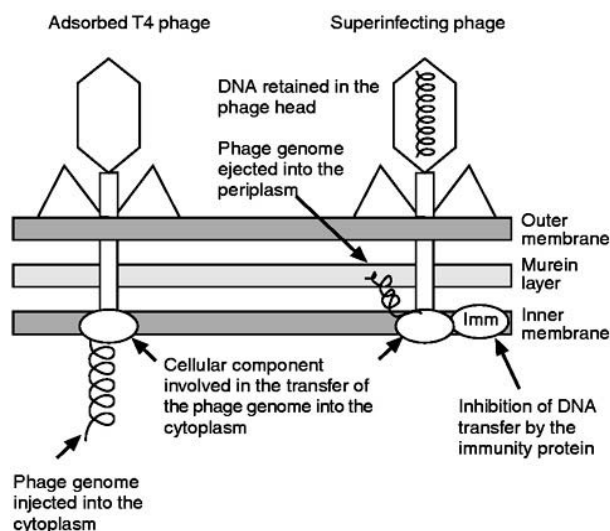


Figure 2 The immunity protein, probably bound to a plasma membrane protein, inhibits superinfecting phage DNA transfer. Its action may be indirect and remains poorly understood.

Conclusion

Colicins and bacteriophages are some of many agents that contribute to the relative competitiveness of bacterial strains. The immunity mechanisms are highly specific and based on protein–protein, protein–DNA or RNA–DNA interactions. Many different mechanisms have evolved to confer immunity to prokaryotic organisms against specific bactericidal agents. The mechanisms for some of them have been elucidated. However, other systems probably remain to be discovered. It is worth noting that the term immunity is also employed for the replicons carrying a transposon: a second copy of a transposon cannot

insert into a replicon that already carries one copy of the same element.

See also: *Escherichia coli*, infection and immunity.

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PROLIFERATION, LYMPHOCYTE

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Bone marrow-derived cells contain three classes of cells: first, those that continuously proliferate throughout the life of the animal (e.g. stem cells); second, those that divide during differentiation and then cease further division after terminal differentiation (e.g. red cells and neutrophils); finally, lymphocytes, which have a more complex regulation and have an extensive proliferative capacity involving a poorly characterized means for reversible cell cycle entrance and exit. Lymphocyte homeostasis requires the control of both lymphocyte proliferation and lymphocyte death rates. This process is controlled at the clonal level as well as at the organism level. The former results in shaping of the repertoire and is the cellular basis of immune memory while the latter process determines total lymphocyte mass.

Lymphocyte proliferation and homeostasis

Cytokine feedback systems control the compartmental size of some bone marrow-derived cell lineages. For example, erythropoietin produced in the kidney regulates red cell mass while thrombopoietin produced in the liver controls platelet mass. In contrast, total lymphocyte mass is precisely controlled by mechanisms that remain obscure. Thus, delineation of the processes that regulate the size of the lymphoid compartment remains a central problem in immunology. It takes years after transplantation or chemotherapy for an adult to repopulate lymphoid

mass, suggesting that the feedback system is either slow or else the regenerative capacity of the human adult system is limited. In contrast, rodents rapidly repopulate lymphoid compartments within several weeks after ablation of the immune system, implying a more robust regenerative power. In the mature, steady-state animal, lymphopoiesis is controlled by the rates of cell division and cell death (Figure 1). The processes of cell growth and cell cycle are independently controlled but are interrelated. For example, lymphocytes may respond to environmental signals by enlarging and by secreting cytokines, and then return to arresting state without entering the cell cycle. However, once a cell is recruited to

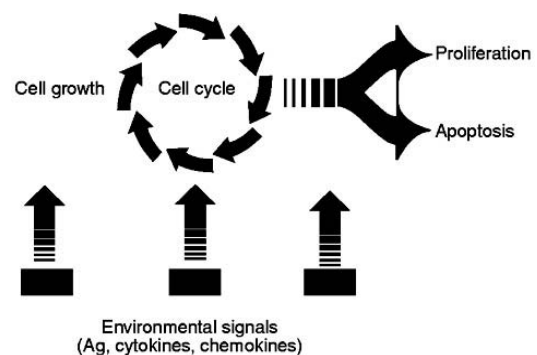


Figure 1 Mechanisms of lymphocyte proliferation and homeostasis. In response to environmental signals, lymphocytes grow in size and enter the cell cycle. Most cells leave the cycle and die by apoptosis within a short time, however some cells survive for extended time as memory cells.

enter the cell cycle, the lymphocyte must grow to double the size of the resting cell before completing cell division. Thus, the cell activation and growth process is reversible while the cell cycle process is irreversible once a cell has reached the S phase of the cell cycle. Here the lymphocyte is committed to cell division and cannot return. In fact, the lymphocyte will die by apoptosis if cell cycle arrest is induced.

The period between sequential mitotic events is known as interphase. In adult humans, the average interphase survival of memory T cells has been estimated as 22 weeks while the survival of naive T cells has been estimated as 3.5 years. This is counterintuitive as the expectation had been that memory cells would be longer lived, thus supporting the notion that maintenance of immune memory requires frequent lymphocyte proliferation. The survival of any given lymphocyte clone, however, can be considerably longer, based on the product of the intermitotic lifespan and the number of population doublings. Estimates based on measurements of the *in vitro* population doubling capacity are that the lifespan of a given lymphocyte clone is in a range similar to the lifespan of a rodent or human. Lymphocyte interphase survival in mice is considerably shorter than that in humans.

Antigen-induced lymphocyte proliferation of mature cells *in vivo* does not normally occur in the blood and lymphatic ducts but, rather, in lymphoid tissues such as tonsil, lymph nodes, and spleen. It begins about 1–2 days following inoculation of antigen, and by 7 days lymph nodes may increase as much as 15 times normal size, reflecting a striking increase in lymphocyte size and number, as well as in lymphocyte ingress (homing). Several key aspects of lymphocyte proliferation and homeostasis are summarized in Table 1.

Lymphocyte proliferation: the cell cycle

Conceptually, the cell cycle process occurs in a series of phases (Figure 2). Fundamentally, these exist to ensure that DNA replication and chromosome segregation are completed with high fidelity. Cells must ensure that chromosome duplication and segregation occur in the correct order, and that segregation only occurs after all sister chromatids are aligned on the mitotic spindle. Another concept is that cells have evolved surveillance mechanisms or ‘checkpoints’ to ensure that critical processes are completed correctly. Examples include the DNA damage checkpoint, the DNA replication checkpoint and the spindle assembly checkpoint (Table 2).

The eukaryotic cell cycle is controlled by a complex series of regulatory cascades involving proteins

Table 1 Key concepts in lymphocyte proliferation

1. The cell cycle time for lymphocytes in exponential division varies from 6 h to 24 h, and is somewhat shorter for mouse lymphocytes than human lymphocytes, and shorter for memory than naive lymphocytes.
2. The time from activation of quiescent human lymphocytes to the onset of DNA synthesis (G_0 to S phase) is about 30 h, considerably longer than that for fibroblasts. This implies that lymphocytes enter a ‘deeper’ resting (G_0) state or that the control process in lymphocytes is more complex than in fibroblasts.
3. Proliferation of mature lymphocytes occurs in many anatomic compartments. Lymphocytes in circulation do not usually proliferate. Lymphocyte proliferation with concomitant antigen receptor rearrangements occurs in a more tissue-restricted fashion, usually in the thymus in the case of T cells.
4. The total lymphoid mass of B and T lymphocytes in an adult human is about 10^{12} cells. About 2% circulate while the majority reside in secondary lymphoid organs.
5. Lymphoid mass is maintained by differentiation of precursor cells as well as proliferation of mature lymphocytes. In steady state this is controlled by balanced rates of production and apoptosis.
6. After cell division, most lymphocytes die by apoptosis, and only a small proportion (1–10%) survive to return to a resting state as memory cells.
7. Lymphocytes have a finite replicative capacity *in vitro*, estimated at 30–50 population doublings in the case of adult mature lymphocytes. Naive lymphocytes have longer telomeres than memory lymphocytes, suggesting a more extensive replicative history for memory cells *in vivo*.
8. The intermitotic lifespan of lymphocytes varies from days to years. The lifespan of an individual lymphocyte clone is the product of the number of cell divisions times the intermitotic survival time.
9. Lymphocyte proliferation can be antigen specific, superantigen mediated and polyclonal. An example of polyclonal proliferation is ‘bystander’ division induced by inflammatory cytokines.

termed cyclins and their associated cyclin-dependent kinases (CDKs). Cyclins are divided into a number of classes based on sequence similarity and physiological function: mitotic cyclins (cyclin B), S-phase cyclins (cyclin A) and G_1 cyclins (cyclins C, D, E and F in vertebrates, and called Cln in yeast). The stability of cyclin proteins varies, whereas the CDKs are stable throughout the cell cycle. The CDKs were first identified as the product of two cell division cycle genes: *cdc2* of the fission yeast, *Schizosaccharomyces pombe*, and *CDC28* of the budding yeast, *Saccharomyces cerevisiae*. CDKs are serine/threonine kinases and their activity is regulated by a series of complex post-translational reactions involving kinases, phosphatases and ubiquitin-dependent proteolysis. Most of these events appear to be highly conserved throughout evolution, however some forms of cell

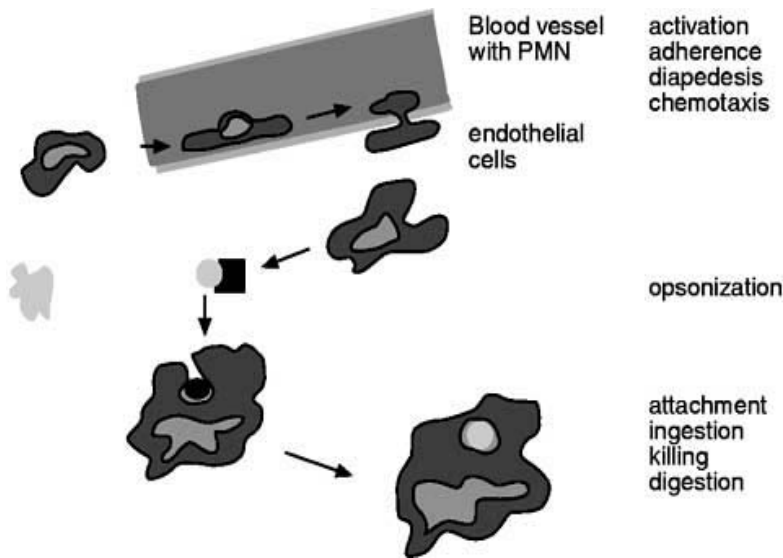


Figure 2 Lymphocyte cell cycle. Quiescent G_0 lymphocytes respond to stimulation by enlarging and then entering interphase. Interphase is divided into G_1 , S and G_2 . Mitosis (M phase) occurs and the daughter cells return to G_1 and either re-enter the cell cycle or exit to G_0 . The G_0/G_1 transition is regulated by antigen and costimulatory signals while the G_1/S -phase transition requires cytokine signal transduction. The CDKs CDC2, CDK2, 4 and 6, and the CDK1 regulators p27 and p21 are indicated.

cycle regulation appear to be tissue specific. For example, the D-type cyclins are expressed differentially in various tissues and thus are likely to confer cell-type specificity to proliferation.

Lymphocytes normally exist in the quiescent (G_0) stage of the cell cycle, but remain responsive to mitogenic signals and, therefore, can enter and exit the cell cycle in a reversible manner. The critical events that drive lymphocytes from G_0 to G_1 and reversal to G_0 are not known. Regulation of the cell cycle in lymphocytes occurs primarily in the G_1 phase, where cells can revert to a quiescent state (G_0) or proceed to the S phase. The transition of lymphocytes from G_0 to G_1 is a complex event. For example, human T lymphocytes express at least 70 new genes within 4 h (early G_1) after stimulation with the mitogenic lectin phytohemagglutinin.

Two major control points lie on the pathway that leads lymphocytes from the resting state into cell division. Stimulation of cells through the antigen receptor accompanied by appropriate costimulation permits passage through the first control point which takes the cells from the resting G_0 state into G_1 . However, in order to undergo mitosis, lymphocytes must also traverse a control point which restricts passage from G_1 phase into S phase, termed the 'restriction point' in animal cells and the 'start point' in yeast. In mammalian cells the restriction point is the principal point in the cell cycle where proliferation is regulated by growth factors. This signal is provided by the binding of cytokines including interleukin 2 (IL-2) and IL-4 to functional lymphocyte surface receptors. The first signal induces the expression of cytokine and cytokine receptor genes and other genes which render cells competent to respond to the second signal. The second signal, in

contrast, activates the cell cycle machinery required for cells to initiate DNA synthesis and to undergo mitosis.

Recent advances have identified the proteins that drive progression through the phases of the cell cycle. Cyclins of the D family and CDK6 are likely to play a major role in the entry of lymphocytes into G_1 , the beginning of the cell cycle. In G_1 , CDK1 (p34cdc2) lacks any associated cyclins and has no protein kinase activity. The activity of CDK1 is dependent on its association with certain regulatory molecules, the mitotic cyclins A and B, whose levels vary with the cell cycle. In mammalian cells the restriction point involves CDK2 and cyclin E. In cells in S phase, CDK2 is found complexed to cyclin A. Mitosis (M phase) is initiated by the activation of CDK1 bound to cyclin B. The precise removal of a tyrosine phosphate from CDK1 during late G_2 is one of the events that leads to the induction of mitosis. The discovery that mitotic cyclins must be degraded for cells to exit mitosis has shown that proteolysis is critical for control of CDK activity and regulating progression through the cell cycle.

B cell proliferation

Two major functions of B cells are to secrete immunoglobulins and to bind antigens, process them, and present them to T cells. Each B cell produces immunoglobulins with a single specific antigen-binding site. By virtue of immunoglobulin on its surface (slg), B cells bind specific antigens which then deliver a signal to these cells to proliferate and clonally expand into memory cells or to differentiate into Ig-secreting cells. Since only one in 10^7 – 10^8 normal B cells has specificity for a particular antigen, the

Table 2 Key concepts and terms in cell cycle

<i>Concepts</i>	
Timing	The eukaryotic cell cycle is a set of ordered events characterized by DNA replication and cell division. Prokaryotic organisms have overlapping periods of replication and DNA segregation, i.e. simultaneous S and M phases of replication
Checkpoints	Cell cycle arrest is induced if a particular condition at a checkpoint is not met. Mammals and other metazoan cells may also activate apoptotic pathways if a checkpoint is not met, while apoptosis is lacking in yeast and other lower eukaryotic cells
Conservation	Basic aspects of the cell cycle are highly conserved in eukaryotic cells. The major differences are increasing complexity in higher eukaryotic cells. For example, there is one CDK gene in yeast while there are seven in mammalian cells
<i>Terms</i>	
Restriction point	The point in G ₁ phase when commitment to DNA replication occurs. After a lag time, S phase begins. In yeast, the restriction point is called 'Start'
Cyclin	Regulatory subunits for CDKs. They encode cyclin destruction boxes that target cyclins for periodic or cyclic ubiquitin-mediated proteolysis, hence their name. Cyclins are classified into mitotic cyclins (cyclin B), S-phase cyclins (cyclin A) and G ₁ cyclins (cyclins C, D, E and F in vertebrates)
CDK	Cyclin-Dependent Kinases are heterodimeric serine/threonine protein kinases that require cyclin subunits for activity as well as positive and negative regulatory phosphorylations
CDKI	Cyclin-Dependent Kinase Inhibitors. A family of proteins that bind CDKs and block kinase activity of cyclin:CDK complexes. There are two families of CDKI: Cip/Kip (p21, p27, p57) and Ink4 (p16, p15, p18, p19)

number of potential antigen-binding B cells are too few in number to provide an appropriate model to study the mechanisms of cell activation.

In order to stimulate larger numbers of cells via sIg cross-linking without resorting to purification of specific antigen-binding cells, investigators have used antibodies which react with sIg on the B cell (anti-Ig). By themselves, anti-Ig antibodies stimulate a number of measurable events reflecting B cell activation. Within 3 h, B cells enlarge in size, show enhanced expression of a number of surface determinants including major histocompatibility complex

(MHC) class II molecules, and show a substantial increase in RNA synthesis. The enhanced expression of MHC class II by antigen is an immunologically relevant event since these molecules interact with receptors on T cells, and their enhanced expression leads to enhanced cognate interaction between B and T helper cells. This interaction enhances the ability of T cells to activate B cells. To drive B cells beyond G₁ into S phase more efficiently, anti-Ig must either be used at high concentrations or must be added in the presence of T cells. As noted below, physiologic B cell activation *in vivo* probably involves antigen binding to sIg with coligation of CD19 and CD21 by the action of complement breakdown products. Activation of B cells to proliferate does not lead to Ig secretion *per se*. For this differentiative event to occur, additional lymphokines including IL-4, IL-2 and IL-5 need to be added together with anti-Ig. These lymphokines support the stimulation of a polyclonal antibody response but not, in the case of T-independent antigens, an antigen-specific response, for which antigen-specific T cells are required.

Recently, the importance of CD40-mediated signaling in B cell proliferation and differentiation has been described. CD40 is constitutively expressed on B cells and engagement by CD40 ligand (gp39), which is primarily expressed on activated CD4⁺ T cells, induces proliferation in B cells. The level of proliferation can be enhanced by the addition of IL-4, IL-10, and in some systems, IL-2. CD40 stimulation can also rescue B cells from anti-Ig-induced apoptosis.

Discovery of the mechanisms of signal transduction by these cell surface molecules has led to the use of 'postmembrane' chemical stimuli to study B cell proliferation and activation. When surface Ig is cross-linked by anti-Ig, or by antigen, phospholipase C is activated to hydrolyze phosphatidylinositol biphosphate (PIP₂) into inositol triphosphate (IP₃) and diacylglycerol (DAG). In support of the hypothesis that protein kinase C (PKC) activation and [Ca²⁺]_i elevation may lead to B cell proliferation, is the finding that the combination of ionophores and phorbol esters leads to B cell proliferation (Table 3).

However, LPS (lipopolysaccharide) does not induce the hydrolysis of PIP₂ or increase [Ca²⁺]_i, but is nevertheless one of the most potent inducers of murine B cell proliferation. Moreover, unlike anti-Ig plus IL-4, LPS can further drive B cells to differentiate and secrete Ig in the absence of added lymphokines. It has been shown that when the intensity of cross-linking surface immunoglobulin is increased by coupling anti-Ig to a large molecular weight carrier molecule such as dextran, B cells can be activated at picomolar concentrations compared to micromolar

Table 3 Reagents and experimental systems used for lymphocyte proliferation *in vitro*

Antigen-specific T cell proliferation	
e.g. mixed lymphocyte culture, soluble recall antigens (tetanus toxoid, mumps, purified protein derivative, etc.), superantigens (staphylococcal enterotoxins, etc.)	
Antigen-nonspecific T cell proliferation	
Plant lectins, e.g. phytohemagglutinin, concanavalin A, pokeweed mitogen	
Antibody-induced proliferation, e.g. anti-T cell receptor antibodies, antibodies to CD2, CD28, Thy-1, and Ly-6, etc.	
Viral transformation, e.g. herpesvirus saimiri, HTLV-I	
Oxidative mitogenesis, e.g. sodium metaperiodate, neuraminidase and galactose oxidase	
B cell proliferation	
Plant lectins, e.g. pokeweed mitogen	
Receptor-induced proliferation, e.g. anti-IgM, anti-IgD, anti-CD40, SAC (<i>Staphylococcus aureus</i> Cowan I), LPS (lipopolysaccharide, endotoxin)	
Viral transformation, e.g. EBV (Epstein-Barr virus)	
B and T cell proliferation	
Pharmacologic agents, e.g. calcium ionophores (A23187, ionomycin), pervanadate to inhibit protein tyrosine phosphatases	
Agents to activate protein kinase C, e.g. phorbol esters, bryostatins, teleocidins	
Cytokines and chemokines	

concentrations that are required for unconjugated anti-Ig. Recently, an explanation for enhanced B cell activation at low antigen receptor occupancy levels was provided by studies of mice immunized with a recombinant model antigen, hen egg lysozyme (HEL), fused to the murine complement component, C3d. HEL bearing two and three copies of C3d was 1000- and 10 000-fold more immunogenic, respectively, than HEL alone. Thus, C3d is a molecular adjuvant of innate immunity that profoundly influences B cell proliferation. Understanding the mechanisms which lead to the various stages of B cell activation may ultimately provide the means to manipulate the immune response.

T cell proliferation

A fundamental property of the immune system is that cellular proliferation is required for most effector functions of T cells. Several requirements dictate that continued T cell proliferation must occur throughout life in order to maintain an intact immune response. First, at least half the peripheral T cells in the mouse have a short lifespan of only a few days. Steady-state levels of T cells are maintained by export of cells from the thymus ($\sim 1 \times 10^6$ per day) and, more importantly, by cell division of peripheral T cells. Second, since only a small proportion

of the potential T cell repertoire is expressed at any time, the organism must maintain continuous T cell proliferation and senescence in order to select for the optimal repertoire in the face of changing environmental conditions (Table 1).

T cell proliferation occurs as a consequence of interactions between a large number of molecules on the surfaces of both the antigen specific T cell and the antigen-presenting cell. These interactions result in the generation of intracellular signals in the form of biochemical changes which initiate a programmed set of cellular responses. After stimulation of T cells with antigen, or the binding of antibody to the T cell receptor or CD3 complex, protein tyrosine kinases activate at least three distinct signal transduction pathways: the phosphatidylinositol second messenger bifurcating pathway leading to calcium/calineurin and protein kinase C activation, the Ras pathway, and the phosphatidylinositol 3-kinase pathway. These biochemical pathways are also activated after B cell stimulation. These biochemical signals result in blast transformation (cell enlargement and G_0 to G_1 transition) and the expression of high-affinity receptors for IL-2. In the presence of IL-2, nearly all T cells will progress through at least one round of the cell cycle after appropriate stimulation by antigen.

Lymphocyte proliferation as a consequence of molecular mimicry by infectious agents

An increasing number of viruses are known to infect lymphocytes, and directly or indirectly induce lymphocyte proliferation. Both retroviruses and DNA viruses infect lymphocytes. Human $CD4^+$ T cells are infected by the nontransforming retroviruses human immunodeficiency virus type 1 (HIV-1) and HIV-2 and by the transforming retrovirus human T lymphotropic virus type I (HTLV-I) while CD8 cells are transformed by HTLV-I. Human B cells are infected with two herpesviruses: Epstein-Barr virus (EBV) and human herpesvirus 8 (HHV8). Likewise, human T cells are infected with at least three herpesviruses: HHV6 and HHV7 and herpesvirus saimiri. Mouse B cells are infected with murine mammary tumor virus (MMTV), resulting in augmented T and B cell proliferation. Other organisms have subverted lymphocyte signal transduction pathways for enhanced replicative fitness, to impair the host immune response or both. A list of some of these agents and their targets are found in Table 4.

Table 4 Examples of pathogens that can alter lymphocyte proliferation

Organism	Comment
Viruses	
HIV	Infects CD4 cells via CD4 and chemokine coreceptors <i>env</i> gp120 primes uninfected CD4 T cells for antigen-induced apoptosis <i>nef</i> p27 perturbs TCR signal transduction, and may predispose thymocytes to negative selection HIV gag proteins bind to cyclophilins A and B via a cyclosporine-inhibitable interaction, and gag:CyP binding may inhibit T cell activation Vpr gene product arrests infected T cells in the G ₂ +M phase of the cell cycle
HTLV-I, II	Transforms T cells. An immunosuppressive peptide homologous to the p15E product of FeLV, MuLV, and HTLV-I inhibits protein kinase C-dependent responses
Feline leukemia virus (FeLV)	FeLV-infected lymphocytes have impaired calcium mobilization to a variety of stimuli
Bovine leukemia virus (BLV)	BLV can transform bovine lymphocytes. The <i>env</i> gene for BLV encodes a TAM motif, and this may induce aberrant or constitutive signal transduction through the Src family of protein tyrosine kinases
Poxviruses	Poxviruses produce soluble IL-1 and tumor necrosis factor α (TNF α) binding proteins that inhibit cytokine signals by ligand:receptor competition
EBV	LMP2A, a transmembrane protein of Epstein-Barr virus, encodes a TAM motif, and signal transduction via the motif may contribute to immortalization of B cells The binding of EBV to the B cell receptor CR2 elevates calcium, and may be involved in the initial infection of B cells. EBV-transformed B cells have impaired antigen receptor-induced calcium mobilization
HHV-6	Infects T cells and increases CD4 expression. Transactivates HIV expression.
HHV-7	Infects human CD4 cells. Uses CD4 as a receptor
H. saimiri	Some strains transform human T cells
MMTV	Infects mouse B cells, and causes superantigen specific T cell proliferation
Bacteria	
<i>Staphylococcus</i>	T cell activation induced by microbial superantigens, and this may induce proliferation or apoptosis of T cells
Protozoa	
<i>Theileria parvum</i>	<i>T. parvum</i> is an intracellular parasite that can transform bovine lymphocytes and causes constitutive IL-2 mRNA expression

From Siegel JN and June CH (1995) Signal transduction and T lymphocyte activation. In: Rich RR (ed) *Principles of Clinical Immunology*. St Louis, MO: Mosby Year Book.

Experimental approaches to lymphocyte proliferation

Several experimental *in vitro* models exist to study antigen-specific and nonspecific (polyclonal) T cell proliferation (Table 3). The use of antigen-specific proliferation is limited by the fact that cell proliferation is limited to the frequency of cells having a clonally rearranged antigen receptor that is specific for the test antigen. For example, the frequency of circulating human T cells responding to alloantigen is on the order of 10^{-2} , the frequency of cells responding to soluble 'memory' antigens such as tetanus toxin is 10^{-5} , and the frequency of cells responding to a primary antigen is $<10^{-6}$. For this reason, T cell clones of antigen-specific cells are used to study antigen-specific responses, and have proved quite useful for analysis of the molecular basis of cellular interactions. A limitation of this approach, however, is that the triggering properties of T cell clones are much less stringent than primary T cells. Antibodies to the T cell receptor (TCR) are a con-

venient and effective way to cause polyclonal T cell proliferation. Antibodies to either the heterodimeric T cell receptor or the associated CD3 complex are effective mitogens. Antibodies may be added in solution or immobilized onto the surface of culture vessels or beads. The manner of addition of the antibody to the culture system is a critical experimental variable. For example, soluble antibodies are much more dependent upon the presence of accessory cells for their mitogenic effect than are immobilized antibodies. Furthermore, when using soluble antibody as a stimulus for human T cells, the isotype of the anti-TCR antibody is important due to the varying affinity of Fc receptors on accessory cells for murine monoclonal antibodies. Antibodies to the CD2 and CD28 coreceptors also cause polyclonal T cell proliferation.

Chemical reagents can also be used to mimic the biochemical signals provided by TCR-induced T cell activation, as was previously discussed with respect to B cell activation. This concept has been termed 'membrane bypass activation'. As in B cells, calcium

ionophores together with phorbol esters cause T cell proliferation, however the proliferation is accompanied by high rates of apoptosis unless appropriate costimulatory signals are provided. A general principle of lymphocyte proliferation is that multiple stimuli are required in order to elicit a full proliferative response and to avoid induction of apoptosis. For example, lectin-stimulated proliferation requires the presence of accessory cells such as monocytes. Pure populations of T cells will not divide unless the primary stimulus as well as the biochemical signals provided by the accessory cells are supplied. In practice, small and variable quantities of monocytes are required for *in vitro* proliferation (0.1% monocytes for phytohemagglutinin (PHA)-induced proliferation; 1% monocytes for anti-CD3 and calcium ionophore-induced proliferation).

Both B and T cell proliferation can be assayed in a variety of ways. [³H]Thymidine added to stimulated cells becomes incorporated into newly synthesized DNA, and thus is a measure of stimulation of the initial cell population. Bromodeoxyuridine measures proliferation using the same principle, but is detected by flow cytometry with a fluorescent antibody. Proliferation measured using these agents must take into account ongoing cell death in the population as a whole. Hoechst 33342, a DNA-binding dye and Pyronin Y, a RNA-binding dye can also be detected by flow cytometry and measure cell cycle phase. PKH-26 and other vital membrane dyes can discern divided and nondivided cells, but become less accurate the more a population of cells has divided. Tetrazolium salts are converted by metabolically active cells to formazan, which can be quantified by measuring absorbance at 490 nm.

See also: Apoptosis; B lymphocyte activation; B lymphocyte differentiation; CD3; CD28; CD40 and its ligand; Endotoxin (lipopolysaccharide (LPS)); Lifespan of immune cells and molecules; Memory, immunological; MHC restriction; Polyclonal activators; Superantigens; T lymphocyte activation; T lymphocyte clonal expansion; T lymphocyte differentiation.

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PROSTAGLANDINS

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Communication between cells involved in the immune response requires cell-to-cell interactions and the production of soluble messengers which may act in a paracrine or autocrine manner. The presentation of antigen by macrophages to T lymphocytes requires a direct cell-to-cell interaction, but cytokines are also produced during this process which control the induction of immunity. Lipid mediators derived from cell membranes, such as the prostaglandins, are also produced during T cell-macrophage interactions and may modulate the behavior of immunocompetent cells.

Pathways of prostaglandin formation

Prostaglandins are acidic lipids which can be enzymatically produced by most mammalian cell types in response to mechanical, chemical or immunological stimuli. The unsaturated fatty acid arachidonic acid is the precursor for the synthesis of the major classes of prostaglandins and leukotrienes, collectively known as eicosanoids. The level of free arachidonic acid in cells is very low, but it is stored in high concentrations in an esterified form in membrane phospholipids. The arachidonoyl moiety is located almost exclusively at the 2-acyl position and consequently, the action of phospholipase A_2 causes the liberation of arachidonic acid. In some cell types, arachidonic acid may also be liberated by the sequential action of phospholipase C and diacylglycerol lipase. As shown in Figure 1, the liberated arachidonic acid can then be converted to the cyclic endoperoxides prostaglandin G_2 (PGG_2) and PGH_2 by the cyclo-oxygenase enzymes present in the 'microsomal' fraction of most mammalian cells. There is a constitutive form of cyclo-oxygenase (COX 1) which is present in many cell types and prostaglandins produced by this enzyme play a role in physiological processes such as hemostasis and the regulation of blood flow in the gastrointestinal tract and the kidney. In recent years it has been discovered that a second form of cyclo-oxygenase (COX 2) can be induced in certain cells by infectious and immunological stimuli and this enzyme is believed to be largely responsible for the production of prostaglandins at sites of chronic inflammation. Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce prostaglandin production by inhibiting the cyclo-oxygenase enzymes and this action underlies both their therapeutic efficacy and side-effects, as discussed later. The unstable endo-

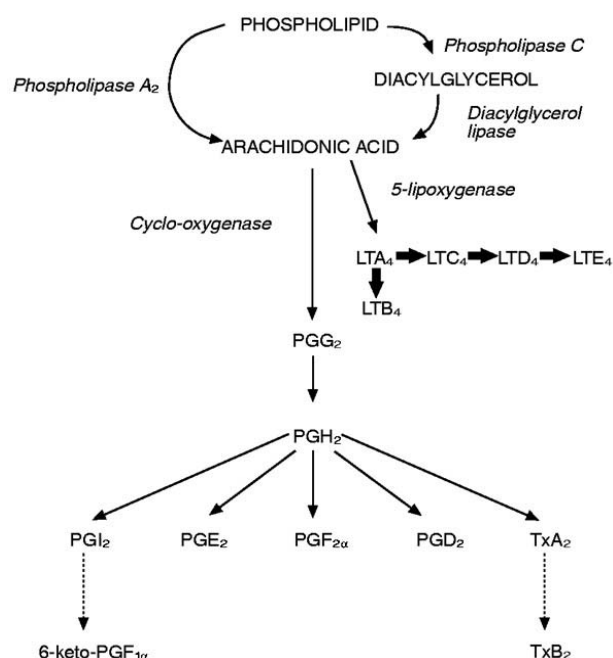


Figure 1 Pathway of eicosanoid production from membrane phospholipids. The solid lines represent enzymatic conversion and the dashed lines nonenzymatic breakdown. PG, prostaglandin; Tx, thromboxane; LT, leukotriene.

peroxides can degrade nonenzymatically to the primary prostaglandins PGD_2 , PGE_2 and $PGF_{1\alpha}$. However, there are distinct isomerases which catalyze the conversion of endoperoxides to PGD_2 and PGE_2 and the formation of $PGF_{2\alpha}$ may be catalyzed by aldoketoreductases. Some cell types, such as the mast cell, produce PGD_2 , but not PGE_2 , which suggests that the relative distribution of these isomerases is important in determining which prostaglandin is formed.

In endothelial cells, the major cyclo-oxygenase product is prostacyclin (PGI_2), which is enzymatically produced from endoperoxides by prostacyclin synthase. Prostacyclin is unstable under physiological conditions and is rapidly hydrolyzed to the considerably less potent 6-keto- $PGF_{1\alpha}$. Thromboxane A_2 (TxA_2) is produced from endoperoxides in platelets, macrophages, and some other cell types, by an enzyme termed thromboxane synthase. Thromboxane A_2 is highly unstable and decomposes rapidly to the biologically inactive TxB_2 . Macrophages are rich sources of prostaglandins, particularly PGE_2 , which they produce in response to a range of

immunological stimuli or during phagocytosis. In contrast, lymphocytes produce little if any prostaglandins, the low levels present in lymphocyte cultures being probably due to macrophage contamination.

Lymphocytes can take up arachidonic acid and incorporate it in their phospholipids but cannot convert that arachidonate into prostaglandins, presumably due to lack of cyclo-oxygenase enzyme activity. However, *in vitro* experiments have demonstrated that arachidonic acid can be donated by lymphocytes to other cell types and metabolized to biologically active prostanoids. It is not certain if this process can occur *in vivo* where protein binding is likely to dramatically reduce the concentration of free arachidonic acid.

Role of prostaglandins in inflammation

The primary prostaglandins such as PGE₂, as well as the breakdown products of prostacyclin and TxA₂, have all been detected in elevated levels at sites of acute and chronic inflammation. The most abundant cyclo-oxygenase product is usually PGE₂, but this depends on the nature and location of the inflammatory response. For example, in conditions where mast cell degranulation is central to the pathology, such as in systemic mastocytosis, PGD₂ and its metabolites can be detected in high amounts in plasma and urine. The enzymatic source of prostaglandins depends on the chronicity of the lesion. The early production (within minutes) of prostaglandins which occurs in acute pain models (zymosan-induced writhing in mice, for example) is likely to be solely due to the activity of the constitutive COX 1 while the delayed, cytokine-dependent production of prostaglandins is dependent upon the induction of COX 2.

Prostaglandins produced at a site of injury contribute to the signs and symptoms of inflammation. Prostaglandin E₂ and prostacyclin can sensitize nerve endings to painful chemical and mechanical stimuli, a process termed hyperalgesia. Consequently, inhibition of prostaglandin biosynthesis in inflamed tissues by NSAIDs accounts for the analgesic activity of this class of compound. Prostaglandin E₂ and prostacyclin are also potent vasodilators and are responsible in many circumstances for the increased flow of blood to sites of inflammation.

Prostaglandins are not chemotactic for neutrophils nor do they directly induce vascular permeability, but the increased blood flow may potentiate leukocyte accumulation and edema formation. The ability of NSAIDs to suppress edema formation has been attributed to inhibition of prostaglandin synthesis,

but the doses required to inhibit edema formation are much higher than required to inhibit the production of prostaglandins in many circumstances. Furthermore, it is well recognized that the antiedema or 'anti-inflammatory' doses of NSAIDs are much higher than the analgesic doses. It is puzzling why the doses of NSAIDs required to inhibit these end-points should differ if inhibition of prostaglandin synthesis is responsible for both effects. Differences in site of action, degree of inhibition required to inhibit each end-point or COX1/2 selectivity may explain the divergent potencies of NSAIDs as analgesic compared to anti-inflammatory agents.

Reduction in the tissue concentrations of vasodilator and cytoprotective prostaglandins also explain why NSAIDs cause gastrointestinal ulceration and kidney damage. These side-effects can be serious, particularly in the elderly population, and limit the dose of NSAID which can be used. Since inhibition of the constitutive COX 1 is likely to be responsible for the side-effects there has been a large effort in recent years to identify novel agents which selectively inhibit COX 2 in the hope that gastrointestinal and renal toxicity will be reduced while anti-inflammatory efficacy will be retained. Several companies have been successful in identifying such compounds and COX 2 inhibitors, a potentially new class of NSAID, are in the late stages of development. Preclinically, COX 2 inhibitors have shown efficacy in reducing edema and suppressing adjuvant arthritis but, unlike traditional NSAIDs, COX 2 inhibitors do not cause gastrointestinal ulceration. The results of clinical trials with COX 2 inhibitors are awaited with interest. It is possible that reduced side-effects may enable the doses to be increased to achieve more profound anti-inflammatory activity. On the other hand, COX 1 may contribute to prostaglandin production in inflamed tissues and/or spinal cord in some circumstances.

Role of prostaglandins in fever and infection

The febrile response which often accompanies infection is the result of the production of endogenous pyrogens from phagocytic leukocytes in response to microbial products. Endogenous pyrogens, which include interleukin 1 (IL-1) IL-6 and tumor necrosis factor α (TNF α), are released into the circulation during severe infections and stimulate specialized endothelial cells in the brain capillaries to produce PGE₂. This leads to increased levels of PGE₂ in hypothalamic tissue and in the third ventricle, which can elevate core temperature. The role of prostaglandins in mediating fever is well established; prostaglandins

of the E series are potent pyretic agents when given centrally, while the reduction of fever by antipyretic drugs, such as aspirin and paracetamol, correlates with their ability to inhibit prostaglandin synthesis in brain tissue.

Fever is a phylogenetically ancient response which may have a protective role in infection, since elevation of core temperature leads to activation of host-defense systems. At febrile temperatures, T lymphocyte proliferation is enhanced, as is the induction of primary humoral immunity. Consequently, one would predict that infective organisms can be eliminated more effectively at febrile temperatures and that treatment with antipyretic drugs may consequently prolong infection. Indeed, in mice, treatment with drugs that inhibit prostanoid synthesis and reduce fever, has been shown to exacerbate *Listeria* and *Coxsackie* virus infections. However, mechanisms other than reduction of fever may explain such effects and there is little clinical evidence that antipyretic drugs prevent or delay recovery from infection.

The local release of vasodilator prostaglandins may also contribute to increased blood flow to sites of infection. This may serve a physiological role in increasing the recruitment of phagocytic leukocytes. Thus, postcapillary venules become more permeable at inflamed sites and concurrent hyperemia will therefore lead to enhanced plasma extravasation. These processes lead to increased opsonization and engulfment of invading microorganisms. While inhibition of prostaglandin synthesis may account for the mild anti-inflammatory activity of NSAIDs, reduction in prostaglandin-mediated blood flow to sites of infection may be deleterious in some situations.

Prostaglandins and chronic autoimmune diseases

In addition to participating in some aspects of acute inflammation, prostaglandins of the E series and prostacyclin can suppress a number of chronic immunological responses. Systemic administration of PGE₂ or its analogs can suppress disease activity in animal models of chronic inflammation such as adjuvant arthritis, immune colitis or glomerulonephritis. These actions have been attributed to effects on lymphocyte function, since prostaglandin E₂ is a potent inhibitor of lymphocyte proliferation and can suppress the production of lymphokines from T cells.

Macrophage activation is also decreased *in vitro* in the presence of concentrations of PGE₂ that can be detected at sites of inflammation. In particular, there are many reports that PGE₂ can inhibit the pro-

duction of IL-1 and TNF α . These cytokines are found at sites of chronic inflammation and play a central role in the recruitment of neutrophils, macrophages and lymphocytes. Interleukin 1 and TNF α can also induce procoagulant activity on endothelial cells which leads to deposition of fibrin, a characteristic feature of chronic autoimmune reactions. Furthermore, these cytokines cause tissue damage by the induction and release of proteolytic enzymes from mesenchymal cells, which also respond by synthesizing prostaglandins. The prostaglandins so produced may act through a feedback mechanism to inhibit cytokine generation from macrophages. Prostaglandin E₂ has been convincingly demonstrated to inhibit the production of mature TNF α protein activity and downregulate the level of mRNA for TNF α in macrophages. Whether PGE₂ inhibits IL-1 secretion is less certain, but since PGE₂ inhibits T cell function, this could indirectly suppress IL-1 generation. These interactions between eicosanoids and cytokines are shown in Figure 2.

Since NSAIDs inhibit prostaglandin synthesis in inflamed tissues, treatment with these drugs may

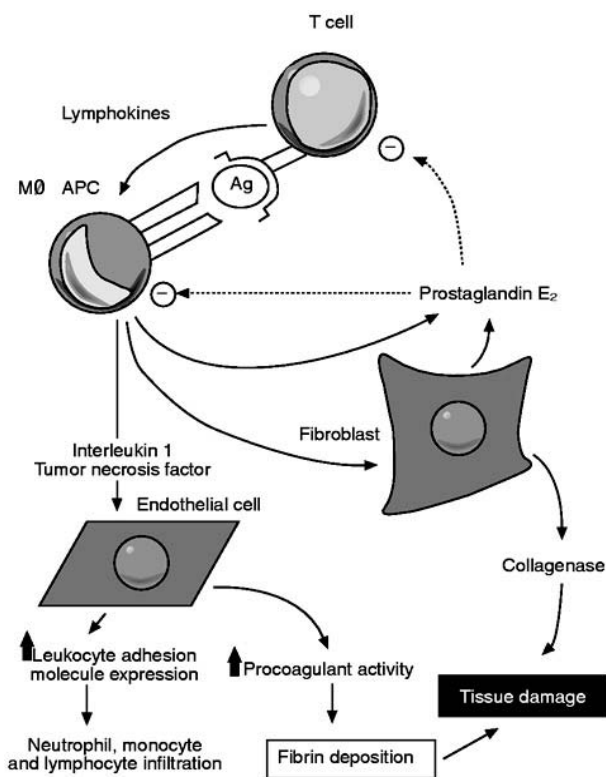


Figure 2 A proposed scheme to demonstrate the interaction between T lymphocytes and macrophages that can lead to cytokine production which, in turn, initiate the processes of leukocyte infiltration, fibrin deposition and tissue damage. Prostaglandin E₂ is also produced during this interaction which can downregulate T cell and macrophage activation.

enhance cytokine production and exacerbate some chronic aspects of autoimmune disease. Indeed, both indomethacin and ibuprofen have been shown to enhance TNF α production *in vivo* which is likely to explain why these agents increase mortality in murine endotoxic shock and enhance cartilage degradation in rabbits with antigen-induced arthritis. These findings have obvious clinical implications for patients receiving NSAID therapy for the treatment of chronic inflammatory diseases. It is tempting to speculate that in diseases such as rheumatoid arthritis NSAIDs, while ameliorating the disease symptoms may exacerbate the underlying disease process leading to enhanced bone and cartilage destruction. There have been several suggestions in the literature that NSAIDs may accelerate the radiological changes that occur in osteoarthritis but definitive proof is lacking because long-term trials with large numbers of patients comparing an NSAID with placebo have never been conducted since all patients require NSAID therapy to provide analgesia. However, there is clear clinical evidence that NSAIDs can exacerbate inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis. Such actions may reflect a reduction in the local biosynthesis of cytoprotective prostanoids in the intestinal mucosa. However, since increased cytokine production leading to fibrin deposition and multifocal infarction has been implicated in the pathogenesis of IBD, removal of immunosuppressive prostanoids may underly the exacerbation of colitis by NSAIDs.

Prostaglandin E₂ can also inhibit the expression of major histocompatibility complex (MHC) class II molecules, and consequently can interfere with the presentation of antigen to T cells by macrophages. Although PGE₂ and prostacyclin have potent effects on immunocompetent cells, there is little evidence that other cyclo-oxygenase products, such as thromboxane, can effect lymphocyte or macrophage function.

In addition to suppressing T cell and macrophage function, prostaglandins in some circumstances can stimulate immune responsiveness. The production of immunoglobulin M (IgM) rheumatoid factors by human rheumatoid mononuclear cells is inhibited by

indomethacin *in vitro*, an effect reversed by exogenous PGE₂. These observations can be explained by inhibition of T suppressor cell activity by PGE₂. In contrast to these findings, indomethacin has been shown to enhance humoral immunity in some animal models. Clearly, the site of production and the concentration of mediator produced is critical in determining whether prostaglandins have immunosuppressive or immunostimulatory effects.

Conclusions

Increased prostaglandin synthesis occurs in response to a range of immunological stimuli. The prostaglandins so produced mediate fever and increase blood flow to sites of infection and inflammation. Prostaglandins, particularly PGE₂, also modulate the function of immunocompetent cells. At concentrations detected at sites of inflammation, PGE₂ can downregulate the function of macrophages and T lymphocytes. Consequently, inhibition of PG synthesis by NSAIDs may exacerbate some aspects of autoimmune disease such as those occurring in chronic arthritis and inflammatory bowel disease.

See also: Annexins (lipocortins); Anti-inflammatory (nonsteroidal) drugs; Arachidonic acid and the leukotrienes; Autoimmune diseases; Cytokines; Fever; Macrophage activation; Protein kinases; T lymphocyte activation.

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PROTEIN KINASES

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Protein kinase is a general term to describe an enzyme activity that can catalyze the transfer of phosphate from a donor source (usually nucleotide triphosphate, either ATP or GTP) to an acceptor amino acid residue in a polypeptide substrate. The resulting covalent modification of peptide structure leads to a new activity status for the target protein. That protein kinase-mediated protein phosphorylation is of central importance in biology is clearly evidenced by the broad distribution of such enzymes in both the plant and animal kingdoms.

The following discussion will address the central role for protein phosphorylation in stimulus-response coupling, the broad spectrum and characteristics of the protein kinases which participate in such activity, and the involvement of various protein kinases in the function of myeloid cell types. The reader is referred to related entries on cell activation for a discussion of the role of protein kinases in non-myeloid immune cell types.

Role for protein kinases in cellular physiology: stimulus-response coupling

While at rest, most cells no doubt express a multitude of protein kinases whose activities are not subject to modulation and which phosphorylate proteins whose routine function requires such post-translational modification. Tremendous interest, however, has been focused on the role of protein kinases as major contributors in stimulus-response coupling or intracellular signal transduction. This process, whereby extracellular stimuli are transduced into intracellular behavioral change is a multistep sequence of events which begins with the interaction between the stimulus and the cell surface and ends with the acquisition of new functional competence. The intermediate events are now known to be multifactorial and frequently involve a sequential cascade of change in protein function which results from reversible or irreversible covalent modification of the target protein. Protein kinases are one of the most common enzymatic activities participating in intracellular signal transduction.

Sensitivity to extracellular stimuli

One major requirement for protein kinases as central mediators of signal transduction is sensitivity to

extracellular stimulation. Essentially all extracellular information is transduced through interaction of the stimulus with some form of receptor. In general, receptors are protein in nature, are localized in the plasma membrane and display a stimulus recognition structure on the exterior surface of the cell. Some receptor systems (e.g. steroids, retinoids, etc.) detect the presence of the stimulus after it has entered the cell. Receptors may couple with protein kinases either directly, or indirectly through the action of nonprotein second messengers. At least three categories of second messengers have been identified. These include the cyclic nucleotides (cyclic AMP and cyclic GMP), various phospholipid hydrolysis products, and alterations in localized concentrations of Ca^{2+} ions. Some protein kinases (particularly the protein tyrosine kinases, see below) are integral parts of the receptor structure and enzyme activity is subject to modulation by receptor-ligand interaction. Other tyrosine-directed protein kinases are structurally distinct but associate with and/or become activated by interaction with ligand-binding receptors in stimulus-dependent fashion.

An additional complexity is the well-documented existence of protein kinase cascades. In these, the response to direct or indirect stimulation of a kinase activity may be dramatically amplified through multiple cycles of protein kinase activation in which the activity of each sequential enzyme in the chain is modulated by phosphorylation. Thus there are many protein kinase activities in cells that participate in signaling pathways but which are not directly sensitive to the primary action of the extracellular stimulus.

Categories of protein kinase activity

Protein kinases can be divided into two broad classes on the basis of whether the amino acids serine/threonine or tyrosine are the primary phosphate acceptor sites. The following sections will consider first the serine/threonine-directed enzymes, subdivided on the basis of stimulus sensitivity, and second, the tyrosine-directed enzymes, either as receptor-linked or nonreceptor-linked.

Serine/threonine-directed protein kinases

Cyclic nucleotide-dependent protein kinases The first stimulus-sensitive protein kinases to be studied

were those regulated by cAMP. This second messenger is generated by adenylyl cyclase which couples to multiple cell surface receptors through heterotrimeric GTP-binding proteins. Cyclic nucleotide levels are also subject to control through the action of phosphodiesterases. cAMP-dependent protein kinases (PKAs) consist of two subunits: a catalytic subunit and a regulatory subunit. The catalytic subunit is normally inactive when complexed with the regulatory subunit. Interaction of the complex with cAMP results in subunit dissociation and subsequent activation of the enzyme. Multiple forms of each subunit class have been characterized. Substrate specificity of PKAs is quite broad and the response to elevated cAMP exhibits a diverse array of downstream consequences.

Ca²⁺/calmodulin-dependent protein kinases There are multiple protein kinase activities that are controlled by changes in intracellular Ca²⁺ levels through interaction with calmodulin, a Ca²⁺-binding regulatory protein. When Ca²⁺ levels are elevated, Ca²⁺-calmodulin complexes associate with several different protein kinases and such association results in enhanced catalytic activity of the enzymes. At least six different forms of calmodulin-dependent protein kinases have been defined. Three of these isoforms (phosphorylase kinase, myosin light chain kinase, and Ca²⁺/calmodulin kinase III) exhibit a high degree of substrate specificity. Isoforms I, II and IV exhibit much broader specificity and are referred to as multifunctional forms. The different forms exhibit substantial structural complexity, frequently being composed of multiple distinct subunits.

Phospholipid-dependent protein kinases Phospholipid hydrolysis is now a well-documented early event in intracellular signaling. The activation of different phospholipase activities with highly distinct substrate specificities is linked to multiple receptor systems in using a variety of distinct molecular coupling mechanisms.

There are two broad categories of protein kinase activities that are sensitive to specific phospholipid hydrolysis products which act as second messengers. The first of these families is a group of enzymes known collectively as protein kinase C (PKC). These enzyme activities were discovered as a Ca²⁺- and phospholipid-dependent activity and were subsequently found to be the target of the tumor promoter phorbol diesters. At least 11 structurally distinct forms of PKC-like enzymes are known and these can be divided into three subgroups on the basis of cofactor requirements. These include α , β and γ isoforms which require Ca²⁺, phospholipid,

and a diacylglycerol (DAG) activator, the δ , ϵ , η and σ isoforms which are Ca²⁺-independent but still require DAG, and the ζ and λ isoforms which require only phospholipid for activity. In addition, two structurally related enzymes known as protein kinase D and PKC μ are also DAG-sensitive and Ca²⁺-independent.

Activity of the Ca²⁺-dependent isoforms of PKC may require the concomitant elevation of intracellular Ca²⁺. These cofactor and activator requirements provide some opportunity for stimulus-dependent specificity in the function of the different isoforms of PKC. However, there appears to be little substrate specificity inherent in PKC structure and, at least *in vitro*, many proteins can serve as targets for the different forms of enzyme. One potentially important determinant of specificity may be subcellular localization. Some forms of PKC show preference for association with different membrane sites and may so associate in an activation-dependent fashion. Furthermore, PKC isoforms exhibit interaction with other nonprotein kinase proteins which may also be important determinants of selective function or activation. Following activation, some forms of PKC undergo Ca²⁺-dependent proteolysis. Cleavage may selectively remove the regulatory domain of the protein, rendering it constitutively active at least transiently.

A second form of phospholipid-dependent protein kinase has been identified whose activity is modulated by ceramide, a hydrolysis product of sphingomyelin. Several different extracellular stimuli (tumor necrosis factor α (TNF α), interleukin 1 (IL-1) and vitamin D₃) have been convincingly linked with the sequential activation of a neutral sphingomyelinase which generates ceramide, and at least some downstream signaling events are mediated by activation of the ceramide-dependent protein kinase. The enzyme is a member of a large family of serine/threonine-directed protein kinases which recognize substrate peptides in which the phosphoacceptor site is N-terminal to a proline residue. While specific substrates are not definitively known, activation of the ceramide-dependent signaling pathway(s) has been linked with the activation of the MAPK cascades, with activation of the NF κ B transcription factor, and with the stimulation of apoptosis.

The MAPK signaling pathway A major functional class of protein kinase activities which has been studied intensively in recent years is now known collectively as the mitogen-activated protein kinase (MAPK) cascade. While there are multiple structural forms of protein kinase activity which participate in the MAPK cascade, they are linked together in a

functional unit which can mediate information transfer from the exterior of the cell to the nucleus, resulting in stimulus-dependent alterations in gene expression. Indeed these signaling pathways can be initiated by a highly diverse array of important extracellular stimuli which induce both cell proliferation and functional differentiation.

Two general features of this signaling process deserve special comment. First, there are multiple forms of the MAPK cascade that may coexist in the same cell and which exhibit differential sensitivity to different forms of extracellular stimulation. Second, the different MAPK cascades exhibit at least five sequential layers of protein kinases. At each layer, multiple isoforms of kinase activity have been described. The nomenclature for these enzymes is quite complex and the simplest system relates all kinase activities to the originally identified mitogen-activated protein kinase termed MAPK. Thus the MAPK kinase is termed MAPKK while the next upstream enzyme is termed MAPKKK or MAP3K and so on. Enzymes which are downstream of MAPK are termed MAPK-activated protein kinases or MAPK-APK.

The MAPKs are members of the proline-directed protein kinase family (like ceramide-activated protein kinase, see above). MAPKKs are relatively unique enzymes which phosphorylate MAPK on both tyrosine and serine or threonine residues, both events being required for full activation of MAPK activity. The MAPK cascades can be initiated through multiple entry points and include action of receptor-linked protein tyrosine kinase activity (see below), phospholipid-dependent kinases, and intracellular receptor-initiated events (steroids). Furthermore, there is substantial cross-talk between the distinct cascades. Through such combinatorial mechanisms this system is capable of generating remarkable diversity in information transfer.

Other serine/threonine protein kinases There are no doubt many additional serine/threonine-directed protein kinases which participate in stimulus-response coupling. Prominent examples include the double-stranded RNA-dependent protein kinase known as PKR which is associated with response to interferon (IFN) and with acquisition of antiviral function. Recent studies using mice in which this enzyme has been deleted by gene targeting through homologous recombination indicate that this enzyme may play an important role in cellular response to extracellular stimulus.

Another class of enzymes known to exhibit change in activity in response to stimulation are collectively known as casein kinases based upon their preference

for casein as a substrate. Two classes of casein kinases have been studied. Both show relatively broad substrate specificity. Although casein kinase activities vary in response to extracellular stimulation, there is little mechanistic understanding of how enzyme activity changes during cell treatments nor how important these enzyme forms may be in processing such extracellular information.

Tyrosine-directed protein kinases (PTKs)

Protein kinases that utilize tyrosine residues as the principal phosphate acceptor site have been classified into two categories on the basis of whether the enzyme activity is an integral part of a cellular receptor or has no demonstrable receptor function.

Receptor-linked PTKs Although the first tyrosine-directed protein kinase discovered was not receptor associated, the linkage of tyrosine-directed phosphorylation with response to extracellular stimulation focused early attention on those enzyme activities which were integral parts of the receptor protein structure. This paradigm is now well-established and is associated most closely with receptors for growth factors such as insulin and insulin-like growth factors, epidermal growth factor, fibroblast growth factors and the macrophage colony-stimulating factor CSF-1. The linkage to cell proliferation is not exclusive as these stimuli are also capable of producing developmental changes in cells related to the acquisition of selective functions. Receptor-linked PTKs are activated upon ligand binding by receptor subunit aggregation. Activated enzymes phosphorylate the receptor itself as well as other substrates, at least some of which associate with the receptor via protein domains which recognize and bind to phosphotyrosine residues. Thus the phosphorylation induced by ligand-receptor interaction initiates a signaling cascade which may involve additional protein kinases as well as other enzymes. The receptor-linked PTKs are highly pleiotropic and can couple to many downstream signaling pathways, including those involving the serine/threonine protein kinases discussed above.

Nonreceptor-linked PTKs There are at least eight categories of PTK that are not part of any stimulus-binding protein and exhibit no extracellular domain. These eight subclasses are distinguished by specific structural features. All possess at least one kinase domain which contains the active site. Many possess regions that allow interaction with phosphotyrosine residues or with other specific amino acid sequence motifs which are expressed on the cytoplasmic domains of specific receptors. In addition, enzyme activity is frequently subject to positive or negative

regulation by phosphorylation on critical tyrosine residues. These latter two characteristics are critical to the function of at least three classes of nonreceptor PTKs. Cell surface receptors aggregate upon ligand binding, which results in the activation of PTKs associated with the cytoplasmic domains of the receptors. These enzymes phosphorylate themselves as well as the receptor subunits, leading to interaction with a multitude of downstream signaling intermediates in a fashion remarkably similar to that described above for the receptor-linked PTKs.

Nonreceptor PTK classes that function in this manner include the Janus family (JAK1, JAK2, JAK3, Tyk2), the Syk family (Syk, Zap), and the Src family (Src, Yes, Fyn, Yyn, Lck, Blk, Hck, Fgr, and Yrk). Many of these mediate signaling pathways restricted to hematopoietic cell types. Although the other classes of nonreceptor-linked PTKs have not been so closely associated with receptor-mediated events, they are also likely to be involved with intracellular communication and regulation.

Protein kinases in myeloid cells

While much of our current knowledge of protein kinase function in cellular control and stimulus-response coupling has been developed in cell types not considered to be components of the immune system, both lymphoid and myeloid cell types have frequently served as exceptionally useful models. Indeed, much of the current detailed understanding of the nonreceptor-linked PTKs as primary mediators of ligand-induced cellular response has emerged from studies utilizing antigen- and/or cytokine-mediated activation of T lymphocytes. The reader is referred to the entry covering T lymphocyte activation for more detailed coverage. Thus the focus of the remaining discussion here will be on the roles for different forms of protein kinase activity in mediating responses of myeloid lineage cell types to extracellular stimulation.

Because there are numerous myeloid cell derivatives and these may exhibit a highly diverse set of characteristics in different tissue settings, the discussion will be limited to mononuclear phagocytes (monocytes and tissue macrophages) and neutrophilic polymorphonuclear leukocytes (PMNs). These two categories of cells share many functional capabilities (though these may involve either markedly or subtly different mechanisms).

A major difference between the two cell types is the degree to which their functional competence is subject to modulation by extracellular stimuli. For example, PMNs are constitutively competent to exhibit certain acute secretory responses although such

function may be increased or decreased in quantitative terms following exposure to exogenous stimuli. In contrast, monocytes and macrophages are relatively quiescent cells in their resting or unstimulated state and they often require exposure to appropriate activating agents in order to acquire enhanced functional competence. Such adaptive behavior may require hours or days to develop following stimulation. Nevertheless, both acute and adaptive responses frequently require the participation of protein kinase activities.

Categories of extracellular stimulation

Three classes of extracellular stimuli will be considered. The first two of these are generally associated with the triggering of acute responses and have been frequently studied in detail using PMNs. These are the structurally diverse collection of agents which induce chemotaxis, and the opsonins for which specific receptors have been defined (e.g. Fc and complement receptors). The third class of stimuli are largely associated with induction of proliferation or adaptive functional activation of more plastic cell types such as mononuclear phagocytes. Stimuli responsible for such change include cytokines, growth factors and bacterial cell wall products such as lipopolysaccharide (LPS). The adaptive change following such stimulation is usually mediated in large part by alterations in gene expression. Studies of acute responses have been done more frequently in PMNs while analyses of adaptive behavior responses involving new gene expression have utilized macrophages (or other cell types) as models. Nevertheless both cell classes exhibit both forms of response.

Chemotactic factors Much of the current knowledge regarding the actions of chemotactic factors has been developed in PMNs using formyl-Met-Leu-Phe, the complement fragment C5a, leukotriene B₄, and members of the chemokine gene family, in particular interleukin 8. The principal common denominator for all of these agents is their interaction with structurally related receptors which are characterized by seven transmembrane-spanning domains. These receptors all couple to downstream signaling pathways using the heterotrimeric GTP-binding proteins. The responses to most chemoattractants in PMNs are quite similar and involve the activation of substrate-specific phospholipase C activity which selectively hydrolyzes phosphatidylinositol biphosphate (PIP₂), resulting in the generation of DAG and inositol triphosphate (IP₃), which activate PKC and release Ca²⁺ from intracellular storage sites, respectively. The consequences of this series of events include the initiation of one or more MAPK cascades

as well as the triggering of several acute response pathways which culminate in cell polarization and mobilization, in degranulation, and in the assembly of the NADPH oxidase complex.

Opsonins Responses very similar to those in PMNs stimulated with chemotactic agents are seen following exposure to opsonized materials. Opsonins are bivalent proteins which recognize foreign material and interact with specific receptor structures on leukocyte cell surfaces (e.g. complement fragments C3b and C3bi or immunoglobulin). The cell surface receptors which interact with these materials are quite diverse as well and in addition, initiate a broad spectrum of responses. These include the phagocytic uptake of opsonized particles, which is frequently accompanied by degranulation and activation of the NADPH oxidase system. Despite the strong similarity between response to chemotactic agents and opsonins, these processes appear to involve quite distinct initiating mechanisms. The best understood example of signaling in response to opsonins is that mediated by members of the receptor families that recognize the Fc portion of immunoglobulin chains. In contrast to the chemoattractant receptors, which utilize the seven transmembrane-spanning receptors, the Fc receptors generally contain one or more cytoplasmic domains that exhibit immunoreceptor tyrosine-based activation motifs (ITAMs). These sequence motifs are able to interact with several of the nonreceptor-linked PTKs, in particular members of the Src and the Syk families and ligand binding results in PTK activation. These kinase activities are then able to couple with various intracellular signaling pathways as outlined above.

Cytokines, growth factors and LPS Knowledge of the mechanisms responsible for stimulus-response coupling in immune system cell types following exposure to cytokines and growth factors has undergone an explosive expansion over the last 5 years. Cells of myeloid lineage have served as useful models for much of this work. Major progress has come from the structural characterization of the receptor systems with which many cytokines and growth factors interact and the immediate as well as the ultimate mediators of intracellular signaling following receptor occupancy. The following discussion will consider three distinct response categories: those mediated by cytokines and growth factors which bind to the collection of diverse but structurally related 'cytokine family' receptors, those mediated by the broad acting proinflammatory agents TNF and IL-1, and those mediated by bacterial cell wall products, prototypically lipopolysaccharide (LPS).

Members of the cytokine receptor family bind ligands through single or multichain structures. Interestingly, some individual subunits are shared by several different ligands. The ligand-induced aggregation leads to activation of tyrosine kinases of the Janus family, which are frequently associated with the cytoplasmic portion of the receptor. Some receptors utilize only one kinase isoform while others may involve the function of as many as three. Activated Janus kinases phosphorylate specific tyrosine residues on selected receptor subunits and these are able to serve as docking sites for other downstream signaling molecules which recognize and bind phosphotyrosine. Most prominent amongst these are members of a family of proteins termed the signal transducers and activators of transcription (STATs). These proteins dissociate from the receptor following phosphorylation and form homo- and hetero-oligomeric complexes which translocate to the nucleus where they are able to interact directly with specific sequence motifs responsible for regulated inducible transcription of nearby genes. Phosphorylation of STATs on tyrosine may also be mediated (though perhaps less efficiently) by other nonreceptor-linked PTKs, in particular those of the Src family. STAT protein function is also dependent upon serine-directed phosphorylation. The enzymes responsible have not been unambiguously identified but may be part of or depend upon activation of one or more MAPK cascades. The cytokine receptor family kinases have also been shown to initiate MAPK cascade activity.

TNFs and IL-1s are structurally dissimilar and interact with structurally unrelated receptor proteins. Nevertheless, both cytokines induce a diverse but overlapping set of responses in virtually all cell types. The signaling pathways utilized by these two agents are still only poorly understood but appear to share at least one important common feature. Both stimuli result in the rapid hydrolysis of sphingomyelin to release ceramide, thereby activating the ceramide-dependent protein kinase pathway. As discussed above, this may lead into a number of distinct downstream signaling events including activation of other phospholipid hydrolysis events (phospholipase A₂), the MAPK cascades and the activation of several nuclear signaling molecules, in particular members of the Rel homology group which participate in the formation of NFκBs.

LPS is the prototypic microbial cell wall-derived stimulus and may be among the most potent of stimulatory agents for myeloid cells. Indeed, while a variety of cells exhibit sensitivity to LPS, the mononuclear phagocyte family is clearly the professional cell type for this response. LPS stimulation of macro-

phage activation has been studied intensively for many years and yet understanding of signaling pathways remains limited. In the last 5 years, evidence from many laboratories have implicated serine/threonine- and tyrosine-directed protein phosphorylation events in the response to LPS. Several members of the Src family of nonreceptor-linked PTKs (Lyn, Hck, Fgr) and one or more of the MAPK cascades have been shown to be activated in LPS-stimulated monocyte/macrophages. The contribution of each of these events to particular functional endpoints remains to be fully explored. Although the responses to cytokines such as IFN γ and LPS overlap in many circumstances, there has been no evidence implicating participation of the Janus family of PTKs in response to LPS.

Perspectives – control of protein kinase function

Protein kinases play a major role in cellular activation processes. An important aspect of activation is the need to provide stringent controls which will allow for appropriate enhancement and diminution of function. Protein kinase activities are regulated by interaction with other proteins. Some of these may be protein kinases themselves as occurs during multi-layer protein kinase cascades. Other protein–protein interactions contribute to controlling kinase activity in a second messenger or receptor–ligand interaction dependent manner (e.g. Ca²⁺/calmodulin kinases). A third category involves enzyme activities that directly control the level of target protein phosphorylation. These enzymes are collectively termed protein phosphatases and are the functional antagonists of protein kinases. Frequently, protein phosphatase activities are regulated by the same stimuli which participate in controlling protein kinases activities. Thus protein phosphatases are sensitive to second messenger systems and receptor–ligand interactions.

Finally, subcellular localization is known to be an important determinant of both protein kinase (and phosphatase) activity and specific physiologic function. The impact of kinase or phosphatase activities may be dependent upon their being directed to subcellular regions where appropriate substrates are

localized. Such subcellular distribution may be determined by amino acid sequence motifs present in the kinase protein itself or by other proteins with which kinase activities associate. As the number of individual protein kinase activities expands, understanding the mechanisms through which they are regulated will become an increasingly important area of research interest.

See also: B lymphocyte activation; Cytokines; Endotoxin (lipopolysaccharide (LPS)); Phorbol esters; Polyclonal activators; Prostaglandins; T lymphocyte activation; TNF receptors.

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PROTEIN SEPARATION TECHNIQUES

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Our understanding of the biochemical processes involved in living systems has progressed at an increasingly rapid pace over recent decades. Underlying these developments has been the ability to understand and describe the structural basis of the biological function of biomolecules. The isolation, purification and characterization of these biomolecules, typically proteins, has been crucial to this process.

Separation of a protein is nowadays rarely a major project, but has become a routine requirement for many research programs. The separation must, therefore, be performed smoothly and efficiently to enable the mainstream work to continue. Similarly, we see the need for large-scale protein production from a wide variety of biological sources for subsequent clinical application (e.g. vaccines, antibodies, recombinant proteins). Here, too, the purification must be efficient, cost-effective and safe.

This constant demand on technologies to produce more efficient, easy-to-use and inexpensive techniques for protein separation has produced a fascinating array of media and instrumentation. However, to many workers coming into the area of protein separation for the first time, this selection must seem somewhat daunting rather than fascinating. It is hoped that this brief review will put into perspective the major techniques available and how they interrelate during a protein separation process. It is not intended to include precise technical details, but to outline the basic principles involved and their interrelationships.

Protein structure

Before progressing further, we must remind ourselves of the basic characteristics of all proteins, for it is the variation in these which provides the 'handles' by which we can eventually extract a single protein from a mixture of thousands. All proteins are comprised of amino acids, and it is the precise number, composition and distribution of these amino acids, together with any postsynthetic modifications the protein may undergo (such as glycosylation), which gives each protein its individual features. We can summarize these briefly:

1. *Size*: the molecular weight, which may range from $<10^3$ to $>10^8$ kDa.
2. *Charge*: the net charge at a particular pH is a

function of the individual pK_a values of the amino acids (note that a protein has zero net charge at its isoelectric point, pI).

3. *Hydrophobicity*: most hydrophobic amino acids will be positioned within the structure of the protein, well away from hydrophilic environments. However, some may appear on the protein surface, resulting in hydrophobic 'patches'.
4. *Biospecificity*: specific sites on a protein may interact reversibly with a complementary binding substance or ligand, e.g. the Fc fragment of an antibody reacts reversibly with protein A.

Preliminary considerations

To achieve successful results, the *total* separation problem must be considered from the beginning and, as more information accrues, the procedures in use must be frequently reassessed. The first step is to obtain as much information as possible from the current literature on the protein of interest and, secondly, to define the purpose of the purification. For example: Are large amounts required? What degree of purity is needed? It is then possible to set minimum objectives for the end-product in terms of purity, yield and economy. Decisions can now be made regarding more practical details:

1. *Selection of source*: primary considerations when selecting source material include ease of extraction together with quantity and stability of the protein within the material itself.
2. *Assay development*: throughout the separation, it is essential to determine total protein in the sample using routine assays such as Lowry or Bradford together with assaying for activity of the specific protein. These assays provide constant monitoring of recovery and reveal immediately any sudden loss of activity. This information can be used to modify and improve procedures.
3. *Protein stability*: both investigation of source material and development of an activity assay will yield information on the stability of the target protein. It is vital to be aware at an early stage of any protein sensitivity to temperature, pH, organic solvents, ionic strength, metal ions, inhibitors or contact surfaces; any one of these may result in significant loss of activity and should therefore be taken into account during

development work. Avoidance of protein disruption during initial extraction procedures will be discussed below.

Protein characterization

Size and charge

To determine both the size and charge characteristics of the protein and, indeed, of the contaminating proteins, we must use *electrophoretic separation methods*. Although preparative electrophoresis techniques have been developed, these can be costly, time-consuming and difficult to use on any large-scale purification. The value of electrophoresis lies in its ability to provide highly resolved, analytical separations of complex protein mixtures.

Many electrophoretic media are available; however, polyacrylamide gels are by far the most commonly used for high-resolution protein separations. These gels are easily adjusted, controlled and reproduced, easily stored, and can be scanned if required.

Native polyacrylamide gel electrophoresis (PAGE) is most suitable for studying the composition and structure of native proteins, as both their conformation and biological activity will remain intact during the analysis. However, it is often difficult to find standard proteins which resemble the shape, partial specific volume and degree of hydration of the native protein under investigation. Consequently, SDS-PAGE (sodium dodecyl sulfate) is easier and in most cases more reliable than native PAGE for molecular weight determinations.

In SDS-PAGE, proteins are first treated with SDS under reducing conditions at an elevated temperature. This treatment denatures the proteins, causing them to unfold and assume a rod-like structure coated with SDS molecules. SDS binds to protein in a constant weight ratio and provides a net negative charge. Thus when applied to the polyacrylamide gel in an electric field, all the SDS complexes will migrate at the same rate until the sieving properties of the gel take over. The outcome is a separation based on size. The molecular weight of the proteins can be calculated by comparing their electrophoretic mobility with that of protein standards of known molecular weight.

Isoelectric focusing (IEF) is a high-resolution electrophoretic technique for separating proteins on the basis of their isoelectric points (pI) and, as a consequence, can be used to determine the range of isoelectric points of proteins in a mixture. Homogeneous gels are prepared containing carrier ampholytes, such as Pharmalyte®. Under an electric field, the ampholytes create a stable, linear pH gradient across the gel (the pH range being dependent

upon the ampholytes selected) and proteins will then migrate, essentially unhindered by the gel, to a point in the pH gradient that corresponds to their pI. Markers indicate the pH positions across the gels.

Electrophoretic titration curve analysis is a two-dimensional technique for analyzing protein charge characteristics. In the first dimension, the pH gradient is generated, as in IEF. The gel is then rotated through 90° and the sample applied perpendicular to the pH gradient across the middle of the gel. The proteins become positively or negatively charged, depending on their pI, and will migrate toward the anode or cathode. The rate of their migration will depend on the magnitude of their charge.

From the titration curves, we can predict optimal conditions for further separation by chromatography, in particular ion exchange chromatography as shown in Figure 1. All of these electrophoretic techniques can be run on any horizontal electrophoresis system and it is advisable to use these powerful analytical tools not only for initial sample characterization but also for continual evaluation of all stages of purification. Considerable time and effort can be saved by using precast gels on dedicated equipment, such as PhastSystem™, which enables analyses to be performed in under 30 minutes.

Biospecificity and hydrophobicity

The biospecificity of the protein is likely to be known only if precise details of its activity and structure are known beforehand. The hydrophobicity of the protein is less easily defined. All proteins are, to some extent, hydrophobic, and it is only the difference in their *relative* hydrophobicities which makes selection

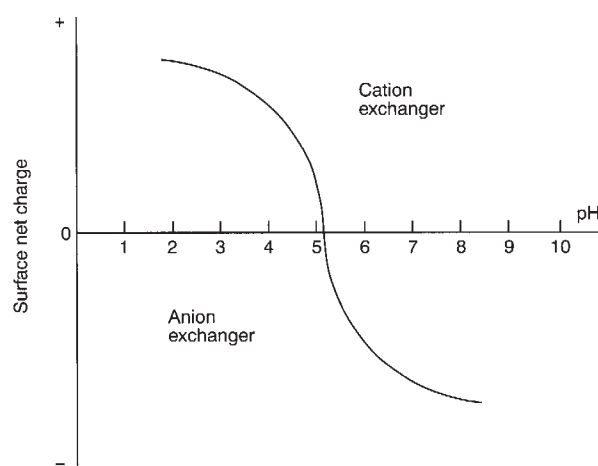


Figure 1 Electrophoretic titration curve: typical titration curve analysis showing pH-dependent charge characteristics of a protein. From this analysis, appropriate ion exchange media and buffer pH can be selected for subsequent chromatography.

possible. Addition of increasing salt concentration to a protein mixture to observe at what stage the protein of interest precipitates is the simplest investigative measure, i.e. high salt enhances hydrophobic interaction, in this case precipitation, hence the less salt required to cause precipitation the more hydrophobic is the protein.

Preliminary separation steps

At this stage, the *method of extraction* must be selected. Unless dealing with a secreted protein in cell culture, any extraction from a biological source will require mechanical disruption of cells. This leads to dispersion and dilution of cell contents resulting in release of proteolytic enzymes and general acidification. Appropriate buffer solutions can be used to guard against rapid acidification and addition of sucrose or maltose may help to stabilize lysosomal membranes and hence reduce the release of proteases.

It is advisable to maintain the ionic strength of the medium to aid protein stability. Many other additives can be included at this stage, e.g. protease inhibitors, or EDTA to chelate metal ions which can catalyse oxidation. However, with prior knowledge of the protein stability these can be kept to a minimum as they will inevitably have to be removed at a later stage and may cause unnecessary complications and delay. As a general rule, the preliminary extraction should be performed quickly, at sub-ambient temperatures.

Protein separations

Having acquired the crude extract, we can begin to separate out the protein of interest. To isolate a single component from this complex mixture usually requires a combination of fractionation techniques that can be divided into two categories: differential solubility and chromatography. Most schemes will start with techniques offering high capacity and low resolution, i.e. differential solubility methods. Later on, more highly resolving chromatography methods will dominate.

Differential solubility techniques are used to remove gross impurities, particularly if the protein of interest is less than 1% of the total sample protein. Several of these methods include:

1. *Salt fractionation*: typically using ammonium sulfate precipitation.
2. *Polyethylene glycol*: precipitation complementary to ammonium sulfate.
3. *Protamine sulfate*: very useful for removal of nucleic acids.

4. *Heat treatment*: occasionally beneficial for the isolation of heat-stable proteins.
5. *Isoelectric precipitation*: used as an alternative in some applications.

Chromatographic techniques are the vital part of virtually all preparative purification schemes. When combining different techniques using the 'handles' we have established (size, charge, hydrophobicity and biospecificity), four main parameters need to be considered: resolution, speed, recovery and capacity. They are closely interrelated – an improvement in one may lead to impairment in another. The aim is to achieve a desired balance – determined by the objectives set for the purification.

Resolution becomes increasingly important throughout the separation. Methods need to be more selective as the quantity of sample material is reduced. *Speed* and resolution are inversely related. An increase in speed above recommended specifications will decrease resolution. *Recovery* can only be maximized by using a minimal number of steps, and recovery of biological activity can only be achieved by maintaining a biocompatible environment throughout the separation. *Capacity* of a technique refers to the sample size (both in terms of mass and volume) that can be handled. This can have considerable significance in considering both the speed and cost of a separation procedure.

To achieve good resolution, recovery and speed (regardless of which chromatographic principle is being applied), it is essential that all matrices should be hydrophilic, inert, physically and chemically stable and provide reproducible results. Particularly for adsorptive techniques, the matrix must be porous and highly substituted with the chosen group, to give high capacity over a large molecular weight range. As a general rule, smaller bead size and narrower bead size distributions produce faster and more highly resolved results. However, these advantages may be compromised by decreasing capacity of the matrix, increasing cost due to more complex production, and, if bead sizes are sufficiently small, more sophisticated equipment is required to work against the back pressures created when the beads are packed in a column. In protein chromatography, all low-pressure *standard* chromatography media can be run using peristaltic pumps. The very fast, high-resolution matrices such as Superose or MonoBeads must be run on an FPLC[®] system specifically designed to pump against the medium pressure they create. All standard media can also be run on an FPLC system, frequently with advantage, as this system produces highly accurate and controllable gradient elution conditions.

With these considerations in mind, we can now review the chromatographic techniques available, their basic principle of action, and media selection.

Gel filtration separates proteins according to molecular weight (size). The basic principle is simple: as solute molecules pass through the column of gel filtration media, the smaller molecules are retarded to a greater degree than the larger ones. Sample components are thus eluted in order of decreasing molecular weight. Fractionation range is important when considering gel filtration gels. A suitable range must cover the molecular sizes of interest in the sample to be fractionated.

The gel medium most widely used is Sephadex[®], a cross-linked dextran polymer developed some 30 years ago. Sephadex G-25 is still the medium of choice when desalting is required, i.e. group separation by gel filtration. Here samples up to 30% of the total column volume can be rapidly desalted and/or buffer exchanged. However, since the first release of Sephadex, experimentation and production has continued resulting in improved media such as Sephacryl[®] High Resolution (HR), Superdex[®] and Superose[®]. These provide high resolution and high flow rates coupled with chemical stability, making them far easier to use than the original Sephadex matrix.

The main advantages of gel filtration are that it is a mild technique, predictable and simple. However, it is relatively slow and poorly resolving compared to alternative methods; more importantly, it has a very low capacity (maximum sample volume = 5% of column volume for optimum resolution) and the sample is always diluted after separation. Consequently, it is best used as a 'polishing' step at the end of a strategy when handling small sample volumes.

Ion exchange chromatography (IEX) separates proteins according to differences in their charge. It is based on the reversible interaction between a charged protein and an oppositely charged matrix. Consequently, a mixture of proteins can be bound to a gel and subsequently eluted differentially by a change of conditions, typically by altering salt concentrations or pH.

These changes may be made in a stepwise fashion, for example, if using older Sephadex (DEAE, CM, SP, QAE) or Sephacel DEAE media. However, far better resolution can be obtained from the modern media, particularly if gradient elutions are performed. Again, there has been continual improvement of IEX media culminating in the use of modified polymers (such as Sepharose Fast Flow, Sepharose High Performance and Monobeads[®]), providing more porous matrices than dextran and consequently giving higher capacity for binding techniques, higher flow rates and good chemical stability. The selection

of the charge group on these matrices is important. QAE and SP will maintain their full charge over a wide pH range, whereas DEAE and CM must be used within specified ranges. The latter can, however, offer high throughput and slightly changed selectivity which may be advantageous.

IEX is a powerful technique. It effectively concentrates the sample (as do all adsorptive techniques) and provides excellent resolution, capacity and yield. With knowledge of the protein characteristics, it is also highly predictable. It is ideal for use at an early stage in a strategy.

Chromatofocusing is a technique which separates proteins according to differences in their pI. This is achieved by passing the protein sample through a linear pH gradient formed automatically by simultaneous passage of a specific elution buffer (Polybuffer[®]) through a weak ion exchange matrix (Polybuffer exchanger or high-resolution Mono P[®]).

Chromatofocusing can be extremely highly resolving, and is therefore ideal, for example, in separating isoenzymes with differences of less than 0.05 pH units in pI. It is also very simple to perform as the gradient is self-generating, and hence no special equipment is required. As in ion exchange, prior knowledge of protein characteristics ensures predictable results and samples will be concentrated during the separation. It should be noted, however, that this is not a high-capacity technique and, as with gel filtration, is far better used at the final purification stages once all major contaminants have been removed.

Hydrophobic interaction chromatography (HIC) separates proteins on the basis of their differing strengths of interaction with a matrix bearing hydrophobic groups. To enhance these hydrophobic interactions, protein samples are loaded in high concentrations of salt, such as ammonium sulfate or sodium chloride. The unbound proteins are washed away and the adsorbed proteins differentially desorbed by elution with a decreasing salt gradient, thus separating individual protein species. As with ion exchange, stepwise elutions can be performed if comparatively low resolution is sufficient. Sepharose CL-4B, Sepharose High Performance or Superose are used as matrices to which a hydrophobic group is attached. A phenyl substituted matrix is usually the medium of choice for samples where the hydrophobic characteristics have not been established, as, in this uncertain situation, selection of a strongly hydrophobic gel could result in irreversible binding of the protein of interest. If, however, the protein fails to bind or elutes too early, Octyl Sepharose CL-4B may be used. This gel is particularly useful for membrane

proteins as it can be used in the presence of the solubilizing detergents often required.

The use of HIC is particularly suitable after a salt precipitation or after an IEX step as, in both instances, samples are already in higher salt concentrations. It is also complementary to gel filtration as it will have concentrated the sample and the filtration step will remove excess salt.

Reverse phase chromatography (RPC) also separates proteins on the basis of their differing strengths of interaction with a hydrophobic matrix. However, the nature of these matrices, usually silica or polymer-based substituted with carbon chains (most often C-8 or C-18), produces an interaction with proteins which is so strong that subsequent elution requires the use of organic solvents, such as methanol or acetonitrile. In most instances, this will destroy the biological activity of the protein. It should also be noted that all reverse phase media must be run on medium- or high-pressure equipment (FPLC or HPLC, respectively).

If retention of biological activity is not a requirement of the purification, then RPC, although a very low-capacity technique, is ideal for high-resolution analysis, or as a final polishing step in a strategy. It is ideal for separation and analysis of peptides and amino acids, as these are unlikely to be damaged by exposure to the harsh elution conditions used.

Affinity chromatography is a highly resolving technique in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilized on an insoluble matrix. Protein mixtures are loaded under conditions favoring the binding reaction, unbound proteins are washed away, and conditions altered to elute the protein of interest. Again this is a simple technique requiring no gradient formation and with a high capacity. Its high selectivity results in excellent separations and concentration of the sample. Porous agarose media such as Sepharose 4B, Sepharose CL-6B, Sepharose Fast Flow or Superose are used as matrices and are provided linked to a specific ligand (e.g. protein A or protein G for antibody separation) or as coupling gels where the required ligand can be easily attached, e.g. CNBr Activated Sepharose or NHS Activated Superose. When used with detailed prior knowledge of the interaction, the technique is predictable and fast. However, in some cases, harsh conditions may be required to elute the protein and cause damage to the protein itself (e.g. low pH); this must be carefully monitored. Difficulties in using affinity chromatography may arise when isolating a specific ligand as this in itself may be costly and time consuming. This effort may be worthwhile to establish such a powerful step in the overall strategy.

To summarize, if when we consider that the main contaminant in any protein solution is in fact water, then we need to bear in mind whether our chosen technique has a concentrating or diluting effect on the sample, as dilution in particular could cause later handling problems. Therefore, the order of chromatographic steps will, in most instances, be: 1) the use of adsorptive techniques (i.e. IEX, HIC, affinity) in the initial stage where the aim is concentration and group separation; 2) further adsorptive techniques such as IEX or HIC at an intermediate stage with the aim of high-resolution separation; and 3) the use of gel filtration, if required, as a final polishing step. For general examples, see Figure 2.

Conclusions

The purification of most proteins should be a relatively straightforward procedure, using both electrophoresis and chromatography to enable protein characteristics to be defined and a logical strategy

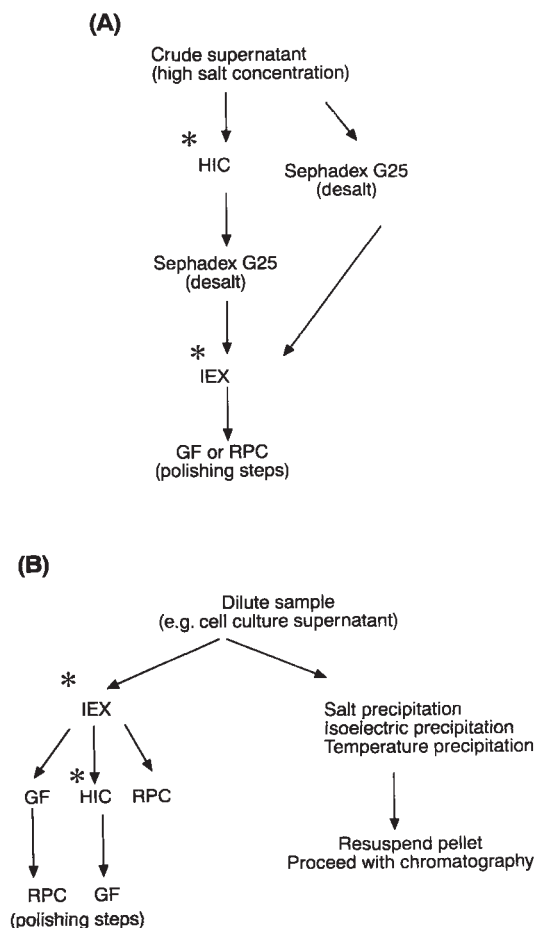


Figure 2 Examples of purification strategies. * Major concentration steps; GF, gel filtration; IEX, ion exchange; HIC, hydrophobic interaction; RPC, reverse phase chromatography.

developed. It must be stressed that both media and instrumentation are continually being improved. Consequently, considerable time and effort can be saved by an understanding of the basic principles involved, and by analyzing any established protocol and looking for possible improvements. For example, a lengthy dialysis may be avoided by using the faster desalting method of gel filtration, and using an improved matrix may reduce a separation fivefold or more.

When problems do arise, it should always be remembered that no matter what the media or the protein extract, the basic principles governing the behavior and interaction of proteins between themselves and with other media remain the same.

See also: Affinity chromatography; Isoelectric focusing; SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

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PROTEUS, INFECTION AND IMMUNITY

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Proteus mirabilis and *P. vulgaris* are motile, urease-positive, lactose-negative, indole-negative, gram-negative rod-shaped bacterial species of the family Enterobacteriaceae. They deaminate phenylalanine and produce abundant hydrogen sulfide. These species, which can differentiate from short vegetative cells to elongated, highly flagellated forms, are found in soil, water and the human intestinal tract. *P. mirabilis* is probably most familiar to clinical microbiologists who frequently observe agar plates on which this organism overtakes the entire surface as a result of swarming differentiation (Figure 1). *Proteus* was aptly named by Hauser in 1885 for the character in Homer's *Odyssey* who 'has the power of assuming different shapes to escape being questioned'.

Infections

Among the urease-positive genera of the Enterobacteriaceae, *P. mirabilis* is the most frequent agent of human infection. It is primarily associated with urinary tract infections (UTIs) and nosocomial infections (Figure 2). *P. mirabilis* causes approximately 5% of uncomplicated UTIs; *Escherichia coli* is the most prevalent pathogen identified in these cases. UTIs in patients with functional or anatomical defects in the

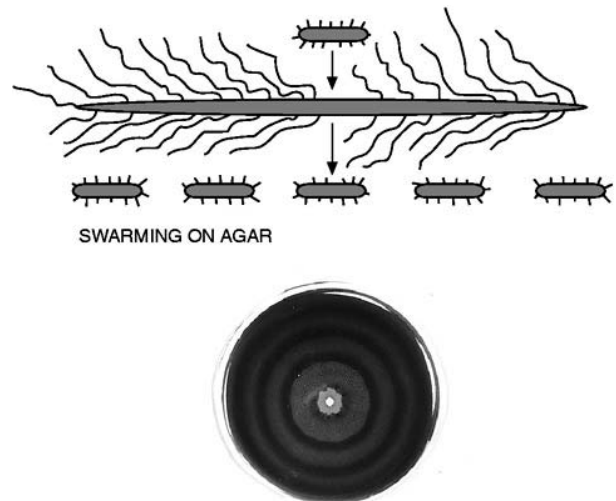


Figure 1 *P. mirabilis* morphology and swarming on Agar. Short swimmer cells, when plated on an agar surface differentiate into elongated, multinuclear heavily flagellated swarmer cells (up to 80 µm long). Swarmer cells associate with one another and spread away from the point of inoculation. After several hours, swarmer cells consolidate into numerous swimmer cells thus completing the cycle. Alternating cycles of differentiation and consolidation result in a bull's-eye pattern of swarming on an agar plate.

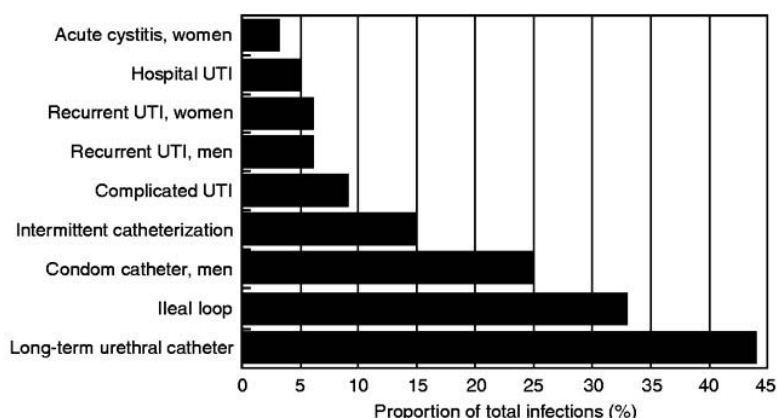


Figure 2 Percent of UTI caused by *P. mirabilis*. In specific studies with selected patient populations, the number of specimens for which *P. mirabilis* was cultured per total number of specimens is shown. Actual data are: acute cystitis, 4/136, 3%; hospital UTI-men and women, X/25371 (numerator not provided), 5%; recurrent UTI-women, 29/518, 6%; recurrent UTI-men, 16/262, 6%; complicated UTI, 12/137, 9%; intermittent catheterization 7/48, 15%; condom catheter, 5/20, 3%; ileal loop 11/34, 33%; and long term urethral catheter 512/1158, 44%.

urinary tract as well as immunosuppressed individuals are considered to be complicated. *P. mirabilis* infections account for nearly 10% of these UTIs. In one study, this pathogen was found at $>10^5$ colony-forming units (CFU) ml⁻¹ in approximately 44% of patients with urinary tracts complicated by long-term instrumentation such as catheterization. Clinical syndromes correlated with *P. mirabilis* infections of the urinary tract include urolithiasis, cystitis, acute pyelonephritis and asymptomatic bacteriuria. Characteristics of cystitis are frequent and urgent urination, dysuria and suprapubic pain. Acute pyelonephritis is often accompanied by fever, flank pain, sweats, nausea, vomiting and malaise. Asymptomatic bacteriuria is usually identified by two consecutive urine cultures with $\geq 10^5$ organisms ml⁻¹.

Immunity

Most of our understanding of host immunity to *P. mirabilis* has been extrapolated from rodent models of ascending UTIs and from rheumatoid arthritis (RA) patients. Sera collected from mice transurethra-ly challenged with *P. mirabilis* have been shown to react *in vitro* with purified lipopolysaccharide, flagella, outer membrane protein (OMP) and man-nose-resistant *Proteus*-like (MR/P) fimbriae. Partial immunity has also been observed following vac-cination in mice. In one study, animals were preim-munized with *P. mirabilis* and cleared using ampicil-lin. After one week of a secondary challenge *P. mirabilis* colonization in the bladder and kidney was significantly decreased compared with an unvac-cinated group. Intramuscular injections of purified OMP has also been demonstrated to protect mice from *P. mirabilis* renal infection.

Serum from patients with active RA also react with *P. mirabilis*. The immunogenic response may be due to a hexamer peptide within hemolysin which has a high degree of similarity to the susceptibility

sequences of HLA-DR1 and DR4. Future research is aimed at determining whether the production of antibodies against *P. mirabilis* hemolysin stimulates the autoimmune response observed in RA patients.

Virulence factors

A number of specific proteins encoded by chromo-somal genes have been implicated as virulence factors that allow *P. mirabilis* to colonize its host, evade host defenses, and cause damage to the host (Figure 3). The production of one of these proteins, urease, is a hallmark of *Proteus* infection. This enzyme, which catalyzes the hydrolysis of urea to carbon dioxide and ammonia, elevates urine pH to a level where normally soluble ions precipitate to form struvite or apatite stones. These stones, which form around the bacterium itself, can grow to a size that obstructs normal urine flow or prevents normal kidney func-tion. The gene cluster encoding urease is comprised of eight genes, ureRDABCEFG. UreR encodes a tran-scriptional activator that induces transcription of urease genes in the presence of urea; ureABC encode the structural subunits of the enzyme itself; ureDEFG encode accessory proteins that are responsible for inserting nickel ions into the active site of the urease metalloenzyme. Mutations in the urease genes of *P. mirabilis* attenuate the organism; the ability of the urease-deficient *P. mirabilis* to colonize the urinary tract in an experimental mouse model of ascending UTI is much diminished. In addition to reduced his-tological damage, the formation of kidney and blad-der stones is never observed for this mutant.

P. mirabilis also produces an Hpm hemolysin. This high molecular weight protein is secreted by the bac-terium, resulting in lysis of erythrocytes or mem-brane damage to other cultured epithelial cells. The *in vitro* effect of the hemolysin secretion is quite dra-matic. Cytotoxicity is observed within an hour of exposure of the cultured epithelial cells to hemolysin.

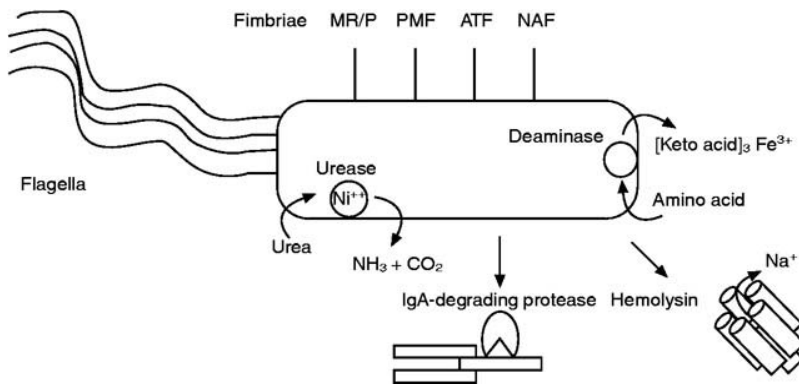


Figure 3 Virulence factors of *P. mirabilis*. Proteins produced by this species that have either been demonstrated or proposed to contribute to virulence are depicted. Mutants in urease, flagella, hemolysin, MR/P fimbriae, and PMF fimbriae have been constructed and tested in a mouse model of UTI. Of these factors, urease, flagella, and MR/P fimbriae appear to contribute most significantly to virulence. Abbreviations: MR/P, mannose resistant/*Proteus*-like; PMF, *Proteus mirabilis* fimbriae; ATF, ambient temperature fimbriae; NAF, non-agglutinating fimbriae (also called uroepithelial cell adhesin).

The *in vivo* effects of hemolysin are less well understood. Mutations in the hemolysin structural gene (*hpmA*) do not appear to significantly attenuate the organism in the experimental mouse model of UTI. Its cytotoxic effect may be more subtle *in vivo* and cannot yet be defined as an important virulence determinant.

P. mirabilis also produces at least four kinds of fimbriae, proteinaceous surface appendages that may play a role in adherence. Fimbrial types thus far identified for *P. mirabilis* include the MR/P fimbria, *P. mirabilis* fimbria (PMF), ambient temperature fimbria (ATF), and uroepithelial cell adhesin (also called nonagglutinating fimbria, NAF). MR/P fimbria, which has received the most attention, causes agglutination of mammalian and avian erythrocytes. Mutation of the MR/P genes result in loss of fimbrial production and inability to agglutinate erythrocytes. In addition, there is a slight, but significant diminishment of virulence of the organism. Numbers of bacteria recovered from infected mice drop about ten-fold in MR/P mutants. As a result, less histological damage is observed in the infected animals.

The organism also secretes an immunoglobulin A (IgA)-degrading protease and an amino acid deaminase which converts amino acids to keto acids. These keto acids are able to complex iron, and thus, may act as an iron-acquisition system for the bacterium. The contribution to virulence of the deaminase has not been determined due to the lethality of introducing this mutation into *P. mirabilis*.

Finally, *P. mirabilis* produces flagella for motility. In broth cultures, the organism produces polar flagella; on solid agar cultures, the organism converts into an elongated 'swarming cell' which produces thousands of flagella (Figure 1). Mutation of flagella genes that inactivate flagella synthesis significantly diminishes the virulence of the organism in a fashion similar to the mutation in urease. Thus, flagellum-mediated motility appears to be a critical virulence determinant for this organism.

Model for pathogenesis

From data accumulated from virulence studies, we are able to establish a model of pathogenesis for *P. mirabilis*, a virulent uropathogenic organism. *P. mirabilis*, which establishes itself around the periurethral area, can colonize the bladder epithelium via PMF fimbriae, which may bind to receptors on the bladder epithelium. We postulate that once established, highly flagellated differentiated swarmer cells ascend the ureters to the kidney. MR/P fimbria can then bind to the renal epithelium. Bacteria that are established in the kidney can hydrolyze urea, raising the local pH and cause precipitation of saturated polyvalent cations and anions to form kidney and bladder stones (struvite and apatite stones). Epithelial cell necrosis has been observed during this stage of infection. Hemolysin, a potent cytotoxin *in vitro*, may be involved once the organism has reached the kidney parenchyma. Bacteria may also invade or be internalized by renal tubule epithelial cells causing further damage, which may, in turn, allow the organism to breach this thin barrier and occasionally enter the bloodstream.

See also: ABO blood group system; Bacteria, immunity to; Bacterial cell walls; Molecular mimicry; Urinary tract infections.

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PSEUDOMONAS AERUGINOSA: INFECTION AND IMMUNITY

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Pseudomonas aeruginosa is a major cause of infections in Western society, being the causative agent of about 200 000 nosocomial infections per year in North America. Such infections tend to be rapidly progressing and pose serious risks to patients. However, *P. aeruginosa* can also cause long-term (up to 10 years or more) chronic infections in the lungs of patients with cystic fibrosis, and the onset of *P. aeruginosa* infection signals the eventual deterioration of lung function in such patients, leading to death from respiratory failure. *P. aeruginosa* shows high intrinsic antibiotic resistance. While there are antibiotics that show initial success against *Pseudomonas* infections, even modest mutations lead to clinically significant resistance, making development of antibiotic resistance common and successful treatment difficult. For this reason considerable effort has been expended in seeking vaccine or immunotherapeutic solutions to such infections. At the same time, diagnosis and epidemiology, each of which use specific antigens, has become important in monitoring the

onset and course of infection and can influence the eventual success of therapy.

Process of infection

P. aeruginosa causes rapidly progressing infections in animals and humans. Generally speaking, these infections only occur in those animals and people which have a reduced capacity to defend against infection (or at body sites that have intrinsically weaker defenses). Thus, in animal models, mild alcohol burns or neutropenia induced by cyclophosphamide lead to a reduction in the LD₅₀ (the concentration of bacteria that kill 50% of animals) by up to 100 000-fold. Similarly, the types of human conditions which tend to predispose towards *P. aeruginosa* infections include neutropenia induced by anticancer drugs, burns, major surgery, steroid therapy, etc. In such patients the likelihood of *P. aeruginosa* infection increases with length of hospital stay.

P. aeruginosa produces a multitude of virulence

Table 1 Immunogenicity and antibody response to major antigens of *Pseudomonas aeruginosa*

Antigen	Characteristics/function	Specific antibodies/use	Immunogenicity
<i>Cell associated:</i>			
Outer membrane protein OprF	Major pore-forming protein; structural role	Opsonic and protective; mAbs used in structural studies, diagnostics; antigenically conserved	Strong Ab response
OprL (= H2)	Peptidoglycan-associated lipoprotein (PAL); structural role	mAb reveals conserved antigen in type I Pseudomonadaceae	Strong Ab response
OprI	Braun lipoprotein	Protective; mAbs reveal antigenic conservation	Strong Ab response
Iron-regulated outer membrane proteins	Transport of iron-siderophore complexes		Ab response accompanies chronic infection
Variant lipopolysaccharide (LPS) A-band (D-rhamnan)	Common antigen, induces influx of PMN	Not neutralizing or opsonic	Ab response appears late in patients with chronic infections
LPS B-band (O antigen of LPS)	O-polysaccharide of conventional LPS; forms the basis for serotype-specificity	Serotype-specifically opsonic and protective; mAb/polyclonals against each serotype produced; useful in serotyping and epidemiology	Strong Ab response
LPS core polysaccharide	Conserved region which links O antigen polymers to lipid A	Cross-reactive; inner core epitope exposed on cell surface	Strong Ab response
LPS lipid A	Anchors LPS in the outer membrane; endotoxic moiety – causes fever and septic shock	Ab broadly cross-reactive even with other species; reacts poorly with intact cells	Weak immunogen, includes <i>P. aeruginosa</i> -specific response
Mucoid exopolysaccharide/alginate	Exopolysaccharide in the form of loose slime; associated with biofilm/microcolony formation	Both conserved and selective Abs available	Weak unless conjugated to protein
Flagella	Motility; chemotaxis and invasion	Two major serogroups (type a with 4 subgroups and type b); opsonic; inhibit motility; protective; mAbs available	Strong Ab response
Pili	Adherence and initial colonization	About 14 types; antigenically variable; Neutralizing and passively protective; many mAbs available	Weak to intermediate Ab response
Ribosomes	Protein synthesis	Protective property not confirmed; may be due to contamination	
<i>Extracellular proteins:</i>			
Elastase	Degrades connective tissues. Tissue invasion		Observed in chronic infections
Exotoxin A	Inhibition of host cell protein synthesis	Cross-reactive neutralizing activity	Strong Ab response
Exoenzyme S	Inhibition of host cell protein synthesis	Neutralizing activity	

factors including the extracellular toxins exotoxin A and exoenzyme S, a periplasmic cytotoxin, two secreted proteases elastase and alkaline protease, secreted lipase, phospholipase and heat-stable hemo-

lysin (also called rhamnolipid), a neurominidase, slime glycolipoprotein, mucoid exopolysaccharide, and endotoxin. Each of these has been shown in animal models to have a role in pathogenesis, making

Pseudomonas pathogenesis truly multideterminant, and in many cases in those same animal models, immune intervention directed against the virulence factor in question will reduce the impact of, or even prevent infection. In rapidly progressing infections, which can be common in nosocomial situations, perhaps the most important characteristic of *P. aeruginosa* is its ability to grow rapidly *in vivo*, with a doubling time equivalent to that observed on a nutrient medium *in vitro*. Thus in a relatively short time (24–36 h) such infections can become overwhelming.

In the more complex situation posed by the chronic infections observed with cystic fibrosis patients, it is still not entirely clear why this disease predisposes a patient towards a long-term, localized lung infection by a bacterium which normally causes rapidly developing invasive infections. The suggestion is that localized defects lead to establishment of infection. One newer concept is that the high salt concentration induced by the mutation of the cystic fibrosis transmembrane regulator (CFTR) protein, renders ineffective the natural antimicrobial defense peptides in the lung.

Antigenicity

As indicated in Table 1, there are a wide range of antigens that have been studied in detail in *P. aeruginosa*. Specific antisera and in many cases monoclonal

antibodies (mAbs) have been raised against these (and other) antigens and have been useful in demonstrating the varied levels of serological conservation in these antigens. Thus, there are 20 lipopolysaccharide (LPS) O-serotypes, about 14 pilin antigenic variants and about five flagellin variants (representative of two major serotypes) in *P. aeruginosa*. This makes these antigens ideal as targets for epidemiological studies. On the other hand, the outer membrane proteins OprF, OprL and OprI, LPS rough core and lipid A, exoantigens and mucosal exopolysaccharide have only one or two variant antigenic types. Such antigens therefore have substantial potential as targets for diagnosing *P. aeruginosa* infections and most of these are or have been considered as vaccines. The mAbs raised against *P. aeruginosa* antigens have also proven to be excellent tools for studying these antigens and have assisted in the study of the structure, function, molecular genetics and immunology of these proteins.

Immunogenicity and immunotherapy

All of the antigens listed in Table 1 have been shown to elicit antibodies in animal models, cystic fibrosis patients with *P. aeruginosa*-infected lungs, and/or humans with burn wound infections. This has provided the rationale for pursuit of vaccine and passive immunotherapy studies (Table 2). In many cases,

Table 2 Recent immunotherapeutic or immunoprophylactic preparations against *Pseudomonas aeruginosa*

Vaccine	Efficacy assessment and other remarks
Octavalent LPS	Engendered IgG response that was protective. However human intravenous immunoglobulins versus this vaccine did not show statistically significant protection in human trials
O-polysaccharide–toxin	
A conjugate vaccine	Serum antibodies from rabbits were opsonic and promoted killing of <i>Pseudomonas aeruginosa</i> , and had neutralizing activities towards toxin A
Alginate–toxin A conjugate vaccine	Induced LPS-specific response in mice
Anti-idiotypic antibodies	Significant antibody titers in human volunteers after immunization; protective in immunosuppressed mouse model study
OprF–OprI hybrid vaccine	
Alginate vaccine	Different sizes of alginate in preparation had variable effect in eliciting opsonic antibody response; large molecular size antigen preparation was tolerated well in volunteers; human trials apparently abandoned
Synthetic peptides of OprF epitopes	Linear B cell epitopes of OprF-conjugated with carrier protein as vaccine. Elicited protective response
ScFv of OprF-specific antibody	Modern technology; single-chain antibody specific against an OprF domain. Applicable for epitope mapping and further antibody engineering
Killed whole bacteria delivered mucosally	Demonstrated heterologous protection in mice
Pilin peptides	Limited cross-reactivity observed but attempts are being made to improve this by peptide engineering; protective in mice
Human mAbs	Five serotype-specific anti-LPS O-antigen human mAbs were opsonophagocytic and protective in mice; three flagella specific human mAbs were protective in mice
Human–Murine heterohybridomas	Human antibody specific against <i>Pseudomonas aeruginosa</i> , scale-up production successful, but efficacy is not defined at present

injection of the antigen into animals leads to a sufficient production of antibodies that promote opsonophagocytosis and protection against subsequent infections. Since *P. aeruginosa* is generally considered to be an extracellular infection, there is a general consensus that an antibody response is required for protection. However, this is not necessarily sufficient for protection since the lungs of patients with cystic fibrosis continue to remain chronically colonized despite exuberant serum antibody responses to several antigens. Furthermore, there are few circumstances in which *P. aeruginosa* infections are truly predictable, making active vaccination a less attractive prospect. For this reason passive protections with hyperimmune intravenous immunoglobulins from volunteers immunized with a *Pseudomonas* antigen has been pursued. Unfortunately, the most recent of these studies using an intravenous immunoglobulin with strong titers to the most predominant LPS serotypes and to exotoxin A, was abandoned due to its inability to show statistically significant protection in humans at the dosage used combined with a less favorable adverse reaction profile. While it is too early to discard the possibility for success with one of the other vaccine strategies being pursued, it may require a novel approach to yield success.

See also: **Bacteria, immunity to; Bacterial cell walls.**

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RABBIT IMMUNE SYSTEM

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The laboratory rabbit (*Oryctolagus cuniculus*) belongs to the order Lagomorpha and the family Leporidae. A recent analysis has shown that the order Lagomorpha is phylogenetically more closely related to Primates and Scandentia than to Rodentia. The family Leporidae includes the genera *Sylvilagus* and *Lepus* which contain cottontails and jackrabbits, some of which may be confused with the true rabbit. *O. cuniculus* existed in antiquity only at the borders of the Mediterranean and was probably first domesticated by the Phoenicians. Today feral rabbits exist in widespread locations including Australia and South America.

The rabbit was prominent in early studies of immunology and basic questions about immunoglobulin genetics, including the phenomenon of allelic exclusion and the inheritance of idiotypes, were first answered with this species. Although it is obvious that the rabbit cannot replace the mouse in investigations requiring numerous genetically identical animals there is a niche for an animal of this size in biomedical research, and there are good reasons to consider the rabbit for certain studies.

The rabbit was used for a number of studies in transplantation and histocompatibility. Disease models for rheumatoid arthritis, myasthenia gravis, atherosclerosis, syphilis, herpesvirus infection, adult T cell leukemia-lymphoma (ATLL), and human immunodeficiency virus (HIV) infection have been reported in this species.

In rabbits, arthritis can be induced by injecting a soluble antigen into the synovium of immune animals. This antigen-induced arthritis is similar to rheumatoid arthritis histologically and in its immune pathogenesis. Another autoimmune disease, myasthenia gravis, can be induced in the rabbit by immunization with nicotinic acetylcholine receptor. The immunized rabbits develop clinical signs of muscle weakness as do humans with the disease.

Experimental models for atherosclerosis involve the use of rabbits fed a high cholesterol diet or the Watanabe heritable hyperlipidemic (WHHL) rabbit.

The WHHL rabbit has a heritable defect in the LDL receptor gene resulting in the production of a non-functional protein.

Syphilis can be experimentally induced by infection with *Treponema pallidum*. In many small animal models of syphilis the disease follows the course of the natural disease in humans. In rabbits the infection persists and viable *T. pallidum* can be readily isolated for the life of the animal.

The rabbit is widely used as a model for human T lymphotropic virus type I (HTLV-I) infection. In the rabbit, infection has been transmitted via blood transfusion and orally through mother's milk with transmission rates similar to those found for humans. While most infected rabbits remain free of overt signs of disease, inoculation with an HTLV-I-transformed cell line (RH/K34) resulted in ATLL-like disease manifested in lymphomas, lymphocyte infiltration of the viscera and thymic atrophy. In one rabbit similarly infected, a skin disease with symptoms associated with human cutaneous lymphoma was reported. The current availability of infectious molecular clones of HTLV-I derived from rabbit cell lines will further the development of the rabbit as a model for HTLV-I infection.

The use of the rabbit as a model for HIV-1 infection has been hampered by lack of consistent signs of disease in infected animals. HIV-1 can be detected in certain tissues from infected rabbits and CD4 lymphocytopenia has been reported in rabbits transfused with HIV-1-infected blood. In addition, the function of tat, rev and the long terminal repeats of the virus has been demonstrated in rabbit cells. Rabbits transgenic for the human CD4 gene have been described and their efficacy as a model for HIV infection is being evaluated.

Rabbits have been used in studies of gene therapy for diseases such as atherosclerosis and hemophilia. Genes can be delivered to the rabbit via adenovirus or retroviral vectors.

Basic biology of the rabbit immune system

The rabbit immune system is comparatively similar to the human system with only minor differences in tissue and cellular organization. The rabbit gut-associated lymphoid tissue (GALT) is organized into a prominent appendix, obvious Peyer's patches and some diffuse lymphatic nodules. The human appendix is vestigial and has more diffuse lymphatic nodules. The GALT, as well as other peripheral tissues of the rabbit and human, are innervated with a well-developed lymphatic system of nodes and ducts. Also, similar to human, the rabbit has a prominent spleen and a thymus which atrophies in adulthood. Lymphopoiesis begins in the rabbit's bone marrow, and as the cells mature they populate the appropriate tissues and organs. Rabbit leukocytes are categorically similar to those in other mammalian reticulo-endothelial systems. The composition of lymphoid cell populations and circulation pathways in the normal dynamic nature of the immune system are also similar, an observation which accents the relevance of studying immunology in the rabbit.

Cytokines described in rabbits include migration inhibition factor (MIF) (which was first identified in the rabbit), chemotactic factor, migration stimulation factor (MSF), interleukin 1 (IL-1), IL-2 and tumor necrosis factor α (TNF α). It is interesting that both native and recombinant human IL-2 are effective at stimulating rabbit T cell proliferation, whereas rodent IL-2 is not.

Rabbit immunoglobulins

The complement of immunoglobulin (Ig) genes in the rabbit includes IgM, and IgE and is unusual in having only one IgG gene and 13 IgA genes; no IgD gene has been reported. A major distinguishing feature of the rabbit Ig family is the magnitude of structural differences between allelically determined immunoglobulin molecules. Several groups of allotypes have been described for the rabbit: the most important are the group *a* on the V_H region (*a*1, *a*2 and *a*3), groups *d* and *e* in the hinge region (*d*11, *d*12) and the C_{H2} portion (*e*14, *e*15) of the γ chain, and the group *b* on the constant part of the κ light (L) chains (*b*4, *b*5, *b*6 and *b*9). It should be pointed out that by extending the study of allotypes to include feral rabbits the number of alleles increases dramatically. This demonstrates that the domesticated pool represents only a fraction of the genetic diversity of the rabbit population.

The group *a* and *b* allotypes are distinguished by large differences in primary structure that charac-

terize the allelic forms. There is as much as 20% difference in amino acid sequence between two alleles of the *b* types and these differences reflect the strong serologic reactivity of the alloantisera used to detect them.

The κ/λ ratio is about 95/5, but this may vary (based on the allotype of the κ chain) with observations of λ chain as high as 15% in rabbits with certain *b* allotypes.

Molecular studies of the κ locus have indicated that there are two C_κ genes in the rabbit and that six of the seven J region genes of the *b*4 and the *b*5 types are not functional. Most of the κ light chains in the rabbit result from rearrangement of the C_κ 1 gene. Light chains derived from the C_κ 1 gene are of the K1 isotype, the five allelic forms of the K1 isotype determine the group *b* allotypes described above. The K2 isotype is encoded by the C_κ 2 gene and is expressed infrequently. There are six to seven λ chain constant region genes but expression seems to be restricted to only the C_λ 5 and C_λ 6 genes.

Antibody diversity in the rabbit is generated by somatic gene conversion. Rabbits have many germ-line V_H genes but only one is productively rearranged in most B lymphocytes. These V_H genes are members of one large V_H gene family and can function as donors in gene conversion. It has also been shown that all B lymphocytes in the rabbit are CD5⁺ and thus may be self-renewing with little or no B lymphopoiesis occurring in mature rabbits.

The major histocompatibility complex (MHC) genes

Studies at the molecular level have identified a number of genes of the class I and II type for the rabbit. The class I gene family consists of 8–14 members depending on the haplotype. Early analysis of protein products from spleen and transformed cell lines indicated that expression of the class I genes is limited to one or two molecular species. However, when class I RNA was studied, at least four transcripts were found indicating that more genes are expressed at the nucleic acid level. A comparative study of the primary structure of the class I genes reveals strong homology to human, porcine and murine genes. The similarity found with human genes is striking, with the exon 4 sequence of rabbit being over 90% identical to that of human class I; in contrast, it is only 75% similar to that of mouse.

The class II genes are likewise very similar to those of humans with homologs of the class II HLA-DR, -DQ, -DP α and β genes as well as DO β and -DZ (or DN) α described for the rabbit. Expression patterns of class II antigens follow the expected pattern

with high levels in lymphoid tissue and liver and low levels in brain and skeletal muscle.

Molecular genetic typing has defined 13 distinct haplotypes which resulted from combinations of 11 RLA-A (class I) and 10 RLA-D (class II) subregions. Among class II genes, DQ α showed the highest level of polymorphism; 9 of 10 D regions could be distinguished by DQ hybridization. Several haplotypes were found to be identical with respect to either class I or class II genes, suggesting that class I genes are clustered in the A subregion and class II genes in the D subregion, and that these haplotypes have resulted from recombinations between the A and D subregions. Unlike the murine MHC where class I gene clusters are localized on both sides of the H-21 subregion, the rabbit, like humans, appears to have discrete class I and class II regions. Results of random typing of colonies at the National Institutes of Health (NIH) and the Institut Pasteur suggest that there are only a limited number of molecularly defined haplotypes in addition to the 13 found in the recent survey.

T cell receptor (TCR)

Although there are no reported studies at the level of protein structure, molecular cloning of rabbit TCR α genes has been carried out using libraries prepared from thymus and from a transformed T cell line. Eleven rabbit V β genes have been cloned and have been assigned to nine V β families (family members have $\geq 75\%$ nucleotide sequence identity).

Approximately 25% of circulating peripheral blood mononuclear cells (PBMCs) in the adult rabbit express TCRs of the $\gamma\delta$ type. This is relatively high compared with TCR $\gamma\delta$ expression in humans and mice and is similar to levels seen in ruminants.

Genes for rabbit C γ , C δ , V γ and V δ have been cloned and sequenced. The deduced amino acid sequence of the rabbit TCR C γ gene shows 60% identity to the human C γ 1 gene. It was also shown that all rabbit TCR γ transcripts utilize this constant region gene and no rabbit homolog of the human C γ 2 gene has been detected. The deduced amino acid sequence of the rabbit TCR δ gene also showed 60% similarity to the human TCR δ gene.

Cell lines

Until recent years very few rabbit cell lines existed. Two of the most well known are RK-13, a rabbit kidney line, and SIRC, a rabbit corneal line; both are used in viral infectivity studies. Herpesvirus, SV40 and HTLV-I have been used to derive lines of lymphoid origin (Table 1). The derivation of rabbit T cell lines using HTLV-I as a transforming agent has become routine. There are numerous cell lines in

Table 1 Rabbit lymphoid cell lines

Line	Transforming agent	Cell type
RL-5	Herpesvirus ateles	T cell
MF/120	Sheep-associated malignant catarrhal fever virus	NK cell
Ra1-Ra5	HTLV-I	T cells
RHT16	HTLV-I	T cell
RH/K30	HTLV-I	T cell
RH/K34	HTLV-I	T cell
KSH-5a	HTLV-I	T cell
BJ-610	Alcelaphine herpesvirus-1	T cell
240E1-1-2	c-myc / v-abl	Plasmacytoma

existence that have been derived by transformation with HTLV-I. In addition, there are rabbit B cell lines derived from transgenic rabbits that carry the proto-oncogenes c-myc or v-abl conjugated to either the rabbit immunoglobulin heavy chain or κ chain enhancers. A rabbit fusion partner for the production of rabbit $\times$ rabbit monoclonal antibodies has been developed using plasmacytomas derived from the transgenic rabbits described above.

Lymphoid cell markers

Rabbit lymphoid cells can be divided into the general categories of T and B cells. Other cell types, such as macrophages and polymorphonuclear leukocytes (PMNs), can also be identified by differential staining and functional assays. A survey of recent literature revealed that there are over 60 different monoclonal antibodies reported to react with rabbit lymphoid cells. Unfortunately, most of these are not adequately characterized and claims of cellular specificity are supported precariously by available data. Table 2 lists monoclonal antibodies and nucleotide sequences for rabbit lymphoid markers. The use of these reagents will allow general characterization of leukocytes. There are, in addition, certain antibodies directed against markers of other species that cross-react with rabbit cells. The identification of T cell subsets is now possible with the development of both monoclonal antibodies (mAbs) and probes for CD4 and CD8.

Conclusions

The 'state of the art' in rabbit immunology reflects the combined effort of many investigators over many years. Use of the rabbit as a disease model is increasing as scientists realize the advantages of size, docility and biologic similarity to humans. The paucity of inbred rabbit strains may be overcome by the estab-

Table 2 Monoclonal antibodies and probes to molecules expressed by rabbit cells

Molecules	mAb	DNA probes ^a
CD1	NA	M26249
CD4	Yes	M92480
CD5	Yes	L03204
CD8b	Yes	L22293
CD11a	Yes	NA
CD11b	Yes	NA
CD11c	Yes	NA
CD14	NA	M85233
CD18	Yes	NA
CD25	Yes	L22292
CD28	NA	D49841
CD43	Yes	NA
CD44	Yes	NA
CD45	Yes	NA
CD58	Yes	NA
CD80	NA	D49843
CD86	NA	D49842
CTLA-4	NA	D49844
IL-1	NA	X02852
IL-8	Yes	M57439
IL-8R	NA	M82873
MHC class I	Yes	KO2819
MHC class II		
DP α /DP β	NA	M22640 M21465
DQ α	Yes	M15557
DR α	Yes	M28161
DO β	NA	M96942
TCR α	NA	M12885
TCR β	NA	M14577 M14576
TCR γ	NA	L22290 D38135
TCR δ	NA	L22291 D26555
TNF α	NA	M12846
Lymphotoxin (TNF β)	NA	M60340
Ig heavy chain		
C α	Yes	X00353
C γ	Yes	K00752
C μ	Yes	J00666
V H	Yes	J00666
Ig- κ	Yes	K00751
Ig- λ	Yes	M12762
RAG-1	NA	M77666
RAG-2	NA	M77667

NA, not available.

^aGenBank accession numbers for the sequences.

Adapted from Knight and Crane (1994).

lishment of rabbit MHC typing and MHC matched breeding colonies. Molecular genetics of Ig genes are reasonably well-defined, as are the MHC genes. Studies of T cell receptor genes and proteins are progressing in step with the development of probes and reagents for their characterization. The establishment of long-term cell lines should lead to more rapid progress of rabbit immunology including a better understanding of cellular interactions and functional definition of lymphoid cells and their surface markers.

See also: Allotypes, immunoglobulin; Antigens, cell surface; CD antigens; Cytokines; Lymphocytes.

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RADIATION, EFFECTS ON IMMUNE SYSTEM

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Ionizing radiation (e.g. X- and gamma rays, neutrons and charged particles) damages DNA and other cellular components. Organized lymphoid tissues and recirculating lymphocytes are the most radiosensitive cells in the body. Following exposure to radiation, most cells of other lineages maintain their viability for one or two mitotic cycles. Small lymphocytes are unique in that even small doses of radiation (as low as 0.25 Gy (25 rad)) result in programmed cell death (apoptosis). Myeloid progenitor cells, also vital to host defenses, are similarly radiosensitive, but the mode of their death remains to be established.

At the cellular and tissue level, sensitivity to radiation has been evaluated by determining cell viability and functional capacity. The sensitivity of the immune system to radiation is reflected by 1) the magnitude of the suppression of the primary or secondary immune response, 2) the reduction in size of the lymphoid organs, and 3) the decrease in the number of surviving lymphoid cells. Despite the exquisite radiosensitivity of most lymphoid cells, a number of radioresistant immune functions (and cells) are maintained, even after doses of radiation that may lead to the death of the animal. Thus, although radiation-induced death of mice, within a dose range below 16 Gy (1600 rad) is often caused by opportunistic infections, the reduced resistance of the host is caused primarily by the ablation of the hematopoietic tissues, rather than total ablation of the immune system. This condition is referred to as the hematopoietic syndrome.

Consequently, to understand the effects of radiation on the immune system and host resistance, we must consider the deleterious effects of radiation not only on lymphocytes but additionally on the leukocytes which mediate nonspecific host responses.

Radiosensitivity of cellular components of the immune system

The type and magnitude of specific immune responses is determined by the interaction of antigen-presenting cells such as dendritic cells and macrophages with T and B lymphocytes. However, the effector arms of the immune system depend on interactions of numerous leukocyte subsets. In addition to lymphocytes and macrophages, natural killer

(NK) cells, eosinophils and granulocytes contribute significantly to host resistance to infections.

Macrophages are relatively radioresistant, as are other nondividing cells in the body. Although phagocytic function and migratory activity of the macrophage are resistant to doses of up to 100 Gy (10 000 rad) given to cells *in vitro*, it is uncertain whether at these doses macrophages maintain their ability to catabolize, degrade, produce antigenic peptides or kill microbial organisms. In general, macrophages are activated following irradiation, as evidenced by enhanced RNA synthesis, enhanced production of cytokines such as IL-1, and increased production and release of lysosomal enzymes.

As in the case of macrophages, the phagocytic ability of polymorphonuclear cells (PMNs) is not decreased by radiation. Moreover, radiation given during or following phagocytosis increases the intracellular killing capacity of these cells. However, radiation results in profound depression of PMN production, due to the destruction of extremely radiosensitive progenitors (D_0 , the dose of radiation that reduces survival of cells to 37% on the exponential portion of the survival curve, is only 0.9 Gy (90 rad) in mice). Given the short lifespan of mature PMNs, a dose of a few gray (a few hundred rads) leads to greatly reduced numbers of PMNs in the circulation within days after irradiation and results in enhanced susceptibility to infections.

Lymphocytes are unique in that the majority of mature resting lymphocytes die rapidly, within hours, after low doses (0.8–5 Gy (80–500 rad)) of radiation. Lymphocytes stimulated by antigen or mitogen, however, become much more radioresistant and maintain their function and viability even after relatively high doses (e.g. 10 Gy (1000 rad)) of radiation (Table 1). The exact mechanism for such enhanced radioresistance remains unknown, but may be based on altered pathways leading to apoptosis, i.e. reduced expression of p53 and/or increased expression of apoptosis-preventing molecules, such as Bcl-2. Alternatively, because mitogen-stimulated lymphocytes contain elevated levels of DNA polymerase and ligase it has been suggested that these cells have an enhanced ability to repair single-strand scissions in DNA.

Table 1 Radiosensitivity of cellular components of the immune system

Type of cell	Radiosensitivity (D_0)	Radioresistance (D_0)
Macrophages	—	Resistant to <i>in vitro</i> doses of 100 Gy (10 000 rad)
B cells	0.8–1 Gy (80–100 rad)	—
Plasma cells	—	60–90 Gy (6000–9000 rad)
T helper cells	Unprimed 1–2 Gy (100–200 rad)	Primed >10 Gy (>1000 rad)
T suppressor cells	Unprimed 1–5 Gy (100–500 rad)	Primed >10 Gy (>1000 rad)
T cytotoxic cells	Minority 2–5 Gy (200–500 rad)	Majority >10 Gy (>1000 rad)
Granulocytes	—	Resistant to ~50 Gy (several thousand rad)
Natural killer cells	—	Resistant to 20 Gy (2000 rad)
Hematopoietic precursors	<1 Gy (<100 rad)	—

Radiosensitivity of lymphoid tissues

The thymus is the most radiosensitive lymphoid tissue, as changes can be detected even after exposure to a dose as low as 0.05 Gy (5 rad). The sensitivity of immature CD4⁺CD8⁺ thymocytes is based on upregulation of p53 expression leading to apoptosis, with thymocytes from p53^{-/-} mice being radioresistant. Although the exact pathways of p53-induced apoptosis remain to be determined, it is clear that p53 is a guardian of genetic integrity, and prevents potential malignant transformation.

Localized irradiation of human thymus, as shown by studies of individuals irradiated prophylactically in infancy for an enlarged thymus to prevent 'status thymolympathicus', resulted not only in an increased frequency of tumors, but also increased frequency of asthma and other autoimmune disorders (including sarcoidosis, enteritis, thyroiditis and others). However, no impairment of delayed hypersensitivity reactions was noted, suggesting that T cells with regulatory functions are more radiosensitive than those with effector roles.

The loss of spleen mass is less pronounced than that of the thymus, as only about 70% of splenic volume in the mouse is composed of lymphocytes. The apoptotic death after radiation of mitogen- or antigen-activated mature T and B cells and NK cells, depends on an antioncogenic transcription factor, interferon regulatory factor (IRF1) and is independent of p53. Necrosis and cell loss are more pronounced in B-cell-enriched areas than in T-cell-enriched areas. Germinal centers are the most sensitive, with necrosis occurring in the follicular outer cortex. Despite apparent rapid recovery of germinal centers following sublethal radiation, T cells in the spleen appear to recover more quickly than B cells. As with the thymus, the stromal elements of the spleen are considerably more radioresistant, thus providing a microenvironmental stimulus for the recovery of either surviving or exogenously supplied stem cells.

Irradiated lymphocytes lose their normal homing

ability. Although they retain their capacity to migrate to spleen and lymph nodes immediately after transfer to a syngeneic host, the subsequent cell traffic into the tissues is impaired, apparently due to membrane damage manifested within hours after irradiation. Thus, radiosensitivity of a given lymphoid cell subset can best be evaluated by *in vitro* assays of their function, because changes in the migration ability preclude accurate *in vivo* measurements in an adoptive host.

Radiosensitivity of the immune response

Humoral immunity

In general, radiation is more deleterious to the antibody response than to delayed hypersensitivity responses. In the primary antibody response, radiation suppresses the inductive more than the productive phase, because plasma cells, unlike immature B cells, are extremely radioresistant (D_0 values of 60–90 Gy (6000–9000 rad)), and radiation does not affect the biological activity of antibodies. However, most plasma cells are short-lived and survive only for several days, resulting in the eventual reduction of antibody titers. Despite reduced antibody titers, the antibody affinity is considerably higher in irradiated animals than in nonirradiated animals, possibly due to the relatively greater radioresistance of more differentiated antibody-producing cells.

Radiation has been used as a tool to elucidate regulation of antibody production. Depending on the time of irradiation in relation to antigenic challenge, depression or augmentation in primary antibody response may result. For example, irradiation of mice with 4.4 Gy (440 rad), a few hours or days *before* antigen exposure, results in immune suppression, whereas the same dose of irradiation 4 days *after* antigenic challenge leads to enhanced titers of antibody. This may be based on selective radiosensitivity of certain suppressor cell populations. In addition to timing, the type of antigen, its mode of presentation, the animal species or strain, and the class of antibody

are important in determining whether exposure to radiation will result in increased or decreased antibody titers.

Although the *in vivo* secondary response has repeatedly been found to be more radioresistant than the primary, the D_0 values for immunoglobulin M (IgM) and IgG plaque-forming cells (PFCs) were found to be similar in the primary and secondary responses, and ranged from 0.8 to 1 Gy (80–100 rad). These findings indicate that factors other than B cell survival, operating *in vivo*, account for the relatively greater radioresistance of the secondary response. The mechanism of this enhanced radioresistance may have a quantitative rather than qualitative basis.

Studies of mammalian T and B cells established that as far as death of dividing cells is concerned, the D_0 for T and B cells is similar. Apoptotic death, however, is more rapid for B cells than T cells. The T helper cell is critical in determining the magnitude of the T-dependent antibody response to most common antigens. Although different experimental procedures have shown heterogeneity in the radiosensitivity of T helper cells, several conclusions can be drawn. Unprimed helper T cells are quite radiosensitive. Priming renders helper T cells resistant to several thousand rad. This resistance, however, can be demonstrated primarily in *in vitro* assays. In contrast, D_0 values in *in vivo* adoptive transfer were found to be as low as 2.3 Gy (230 rad). These low D_0 values were explained by the loss of homing capacity of irradiated lymphoid cells. Radioresistant primed T helper cells can survive and function until stimulated to divide, at which time mitotic death occurs.

Regulatory suppressor cells may also be divided into radiation-sensitive and radiation-resistant populations, with antigen or mitogen-activated suppressor cells being more radioresistant than resting cells.

Cellular immunity

Delayed-type hypersensitivity (DTH) is relatively radioresistant when compared to antibody production. However, as measurements of skin test reactions cannot be evaluated with the same degree of sensitivity as antibody titers, the D_0 values for the DTH reactions are difficult to determine. The homing properties of the DTH subclass of T cells are impaired by radiation, similar to the T helper cells. Furthermore, irradiation of a recipient prior to cell transfer also results in reduced DTH.

The majority of cytotoxic T lymphocytes (CTLs) are radioresistant to doses of several thousands rad. However, a fraction of CTLs are sensitive to doses ranging from 2 to 5 Gy (200–500 rad). The radio-

resistant fraction of CTLs is particularly beneficial in resistance to those infections that depend on cell-mediated immunity, e.g. most viral infections.

Susceptibility to infection

Whole-body exposure to doses of radiation that are injurious to the hematopoietic system results in greatly increased susceptibility to infections, often leading to death. Susceptibility is increased not only to pathogenic microorganisms, but also to nonpathogenic normal flora of the gut and mucous membranes. Leukopenia, due to the stem cell killing, and the loss of physical integrity of mucous membranes, are the primary reasons for such opportunistic infections. Susceptibility to acute infections is most pronounced 3–4 days after radiation exposure, which correlates with the nadir in numbers of bone marrow and lymphoid cells. Increased permeability of biological barriers, such as skin, gastrointestinal tract and blood–tissue barrier, contribute greatly to disseminated infection. Radiation, however, may also alleviate symptoms of some viral infections (encephalitis in humans or choriomeningitis in mice) by destroying lymphocytes that contribute to the pathogenesis of the disease.

Immunotherapeutic applications of radiation

Whole-body irradiation has been used with increasing success for nearly two decades to ablate neoplastic cells in the bone marrow. It is followed by allogeneic bone marrow transplant or a purged autologous graft to restore normal hematopoiesis. Total lymphoid irradiation (TLI), with sparing of the bone marrow, a procedure developed a decade ago, has been used as a treatment of Hodgkin's disease and autoimmune rheumatoid arthritis, and is now being tested in multiple sclerosis patients. With this treatment, patients receive 20 Gy (2000 rad) delivered in doses of 1.5–2 Gy (150–200 rad) per fraction for 2–3 weeks, to their cervical, axillary, mediastinal and hilar lymph nodes and the thymus. The patients are immunosuppressed and show long-lasting remissions in disease activity. Several months after TLI patients show a marked decrease in the percentage of total T cells and $CD4^+$ cells, decreased mixed lymphocyte reaction (MLR) and decreased lymphocyte proliferation to T cell mitogens which is correlated with an increased ratio of $CD8^+$ to $CD4^+$ lymphocytes. Despite such marked immunosuppression, these patients do not experience any increase in the incidence of bacterial infections. In addition, allogeneic transplant survival was recently shown to be

enhanced following TLI due to the T cell-mediated, acquired tolerance. Thus, radiation may be considered as a means of promoting tolerance to grafts prior to transplantation.

Immunodeficiency and radiation sensitivity

Many immunodeficient states have been associated with increased radiation sensitivity. Ataxia telangiectasia (AT) is associated with an increased sensitivity of T cells to the cytotoxic effect of ionizing radiation, with defective cell-mediated immunity, and with nervous system disorders. The gene responsible for this disorder (the *Atm* gene) has recently been identified as being important in the regulation of cell division. Lymphocytes from patients with AT do not exhibit normal cell cycle arrest and do not rapidly accumulate p53 following irradiation. Patients with AT have a high incidence of T cell leukemias with aberrant chromosomal rearrangements involving T cell receptor (TCR) genes.

Certain types of severe combined immune deficiency (SCID) are characterized by an inability of lymphocytes to rearrange TCR and Ig genes due to a functional loss of a recombinase. There is an increased sensitivity of these lymphocytes to the killing effects of radiation. Two protein subunits, called Ku proteins (originally identified as a nuclear antigen in certain autoimmune diseases) mediate the rearrangement of Ig and TCR genes by binding to a 350 kDa protein (catalytic subunit) which functions as a DNA-protein kinase. This DNA-protein kinase activity is absent in SCID mice and in some forms of human SCID. The radiation sensitivity of SCID cells is corrected by transfecting the DNA-protein kinase

catalytic subunit, thus implicating this pathway in both Ig/TCR rearrangement and radiation sensitivity. Additional immunodeficient states such as Wegener's syndrome in humans, and the syndrome of 'wasted' mice are associated with radiation sensitivity. Studies of these abnormalities are likely to uncover more interactions between normal immune function and radioresistance.

See also: Bone marrow and hematopoiesis; Hematopoietic stem cell transplantation; Immunosuppression; Lymphoma; Rheumatoid arthritis, human.

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RADIOLABELING

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The incorporation of radioisotopes into macromolecules has increased by 30 000-fold the sensitivity of immunoassays, so that picogram amounts of an antigen or receptor can be detected and quantified. Radiolabeled antibodies can be used to detect the primary binding of antibody to antigen, providing speed, sensitivity and measurements of affinity.

In addition, radiolabeled antibodies may be used to identify the site of a tumor or the presence and topography of molecules in the membrane of an intact cell. Similarly, radiolabeled antigens were used to demonstrate that B lymphocytes bind antigen specifically via cell surface immunoglobulin receptors.

Radiolabeling of soluble molecules

Most proteins are iodinated externally with ^{125}I or ^{131}I (iodide), although other radioisotopes have been used. Radioiodine is used to label proteins usually by oxidation of iodide to iodine (I_2) or I^- which covalently halogenates the phenolic ring of accessible tyrosine or histidine residues. Oxidation is accomplished chemically with chloramine T or enzymatically with lactoperoxidase. Proteins can also be radioiodinated chemically with the Bolton-Hunter reagent, ^{125}I -N-succinimidyl 3-hydroxyphenyl propionate, iodogen (3,3',4,4'-tetrachloro-3a,6a-diphenyl glycoluril) or iodine monochloride. Generally, γ -ray emitting radioisotopes are used to radiolabel proteins, but β -emitting ^3H , ^{14}C or ^{35}S are sometimes used. In particular, these β -emitting isotopes are used to label proteins or antigens containing carbohydrate groups by biosynthetic incorporation of radiolabeled amino acid or carbohydrate precursors of macromolecules. The radiolabeled proteins are analyzed by various procedures in which radioactivity is detected with appropriate instrumentation. Microanalytical polyacrylamide gel procedures will resolve radiolabeled proteins which may be detected by autoradiographic procedures. Radiolabeled ligands or antibodies binding to cells are visualized by autoradiography with light or electron microscopes. The specificity of binding of radiolabeled ligand to cells or antibodies is determined by competition with unlabeled ligand.

Radiolabeling of living cells

In addition to radiolabeling proteins in solution, radioisotopes have been incorporated into cytosolic and/or cell membrane proteins of living cells. Lactoperoxidase or insoluble iodogen have been used to radioiodinate accessible cell membrane proteins, based on the principle that the labeling agent cannot penetrate the cell membrane. Ten million lymphocytes provide approximately 5–10 μg membrane protein, of which 0.5–5 ng might be a particular species. Radiolabeling, in combination with immunochemical procedures, facilitates the rapid molecular identification of individual membrane proteins.

For example, radioiodinated cells are lysed with nonionic or bile salt detergents and radiolabeled proteins immunoprecipitated with antibody. These procedures permit the isolation and identification of as little as 0.1% of the radiolabeled proteins. Once isolated, the radiolabeled proteins are analyzed by standard microchemical procedures such as polyacrylamide gels and autoradiography. Cell surface

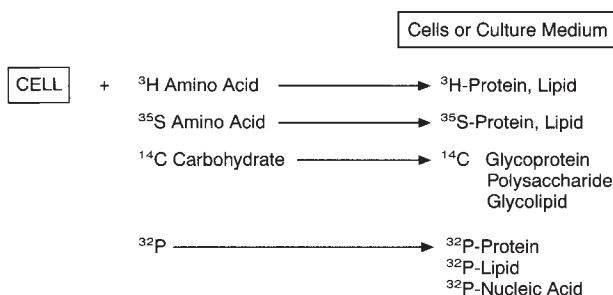


Figure 1 Biosynthetic labeling of cellular macromolecules. Cells are cultured 2–24 hr in complete cell culture medium or cell culture medium lacking the radiolabeled constituent (e.g., methionine for ^{35}S -methionine). The cells incorporate the radiolabeled precursor into macromolecules and after culture, the cells and culture medium are separated by centrifugation. Radiolabeled macromolecules are extracted from the cell or, if secreted, obtained from the cell culture medium. The radiolabeled macromolecules are then specifically isolated by immunoprecipitation.

radioiodination has identified the molecular properties of B and T cell receptors of antigen, major histocompatibility complex glycoproteins and differentiation antigens. Cell membrane glycoproteins can also be labeled vectorially with ^3H - NaBH_4 after oxidation of carbohydrate moieties. In addition, cellular proteins can be labeled by biosynthetic incorporation of ^3H , ^{14}C or ^{35}S -labeled amino acid precursors of protein or ^3H , ^{14}C -sugars as precursors of carbohydrates. Some proteins can also be labeled by incorporation of ^{32}P catalyzed by various kinases.

After these cells are biosynthetically labeled (see Figure 1), radiolabeled proteins are isolated immunochemically, and their structure determined by microanalytical procedures. In addition, the loss of vectorially-labeled membrane proteins by cultured cells and the increase in biosynthetically radiolabeled proteins can be taken as a measure of the turnover of cytosolic or membrane proteins.

See also: Antigen, cell surface; Cell surface molecules, immunoprecipitation of; Enzyme labeling of antibodies and antigens; Immunoassays.

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RAT, INBRED STRAINS OF

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Immunological investigations can be greatly enhanced by the availability of rat models. A number of inbred strains are available for such studies, and there is a growing body of genetic information about these strains. This entry summarizes what is known about the derivation of the most commonly used inbred strains, and provides references for genetic data that have been accumulated so far.

The common strains of laboratory rats belong to the species *Rattus norvegicus*, so named because some of their ancestors were observed in the Norwegian peninsula as they migrated from Asia into western Europe. The laboratory mouse (*Mus musculus*) and the laboratory rat are both members of the family Muridae in the order Rodentia, so the adjective 'murine' could be applied to either mice or rats (although in practice it usually refers to mice). In fact, the rather close relationship between mice and rats is very important to investigators who work with rats, because the wealth of information on the genetics of the mouse frequently provides useful guidance in rat studies.

Many of the rat strains in use today were originally inbred at the Wistar Institute in Philadelphia or the Crocker Institute of Cancer Research at Columbia University. The early investigators who were active at the Wistar Institute included Drs Henry Donaldson, Helen Dean King and Margaret Reed Lewis. One of the noninbred lines which they maintained was known as 'the Wistar rat'. Rats from this colony were sent all over the world, beginning as early as 1911. Inbred strains directly descended from these Wistar rats include AS, BUF, KYN, LEW, LOU, MNR, OKA, SHR, W, WAG, WF and WKY. Several other inbred strains also originated at the Wistar Institute, including PA, WKA and BN. The BN strain is especially interesting in that it was derived from wild rats, and is therefore useful whenever a strain is needed that differs as much as possible from other laboratory strains. Drs Frederick D

Bullock, Maynie Rose Curtis and Wilhelmina F Dunning at the Crocker Institute for Cancer Research worked for many years on the susceptibility of rats to the induction of sarcomas. In 1919, Dr Curtis initiated the inbreeding of the Fischer (F344), Zimmerman, Marshall (M520) and August (AUG) rat strains, which were named after commercial breeders who supplied the first mating pairs. The Copenhagen (COP), ACI, ACP, ACH and A990 strains were also initiated by these investigators. Many additional inbred strains were derived subsequently in numerous laboratories around the world. Genetic relationships among various inbred strains have been investigated by comparing genotypes at polymorphic markers. A tentative genealogic tree of 13 inbred rat strains was constructed on the basis of genotypes at 264 arbitrarily primed polymerase chain reaction products by Canzian and colleagues. In general, these results confirmed that the Wistar-derived strains form a cluster that is different from the Crocker Institute-derived strains, and that BN is quite different from both clusters.

The inbred strains developed by these and other pioneers have been used throughout the world for a variety of genetic and immunological studies. As new genetic loci were identified, sometimes independently in different laboratories, conflicting nomenclature systems were reported in the literature. These problems have not been fully resolved, but there is more uniformity in recent publications than occurred in some of the older literature. During the late 1970s, investigators working on the immunogenetics of the rat agreed to adopt a system of nomenclature in which genetic loci encoding surface antigens would be designated by 'RT' followed by an appropriate number. It was agreed that the first locus in the series, RT1, would be the major histocompatibility complex. The status of RT1 genetics has been reviewed by Butcher and Howard (1986) and the RT1 types of many strains are shown in Table 1.

Table 1 Genetic characteristics of common inbred rat strains

Strain	Synonym	RT1	RT2	RT3	RT6	RT7	Ig-k1	Igh-1	Igh-2	Igh-3
ACI	AXC9935 Irish	a	b		b	a	b	b	a	
ACP	ACP 9935 Irish piebald	a	a	a	b	a	a	b	a	
AGA		1								
AGUS		1					a	b	a	
ALB	Albany	b	b	b	b	b	b	a	a	
AO		u						a		
AS		1					a	a	a	
AS2		f	b				a	a	a	
AUG	August	c	a	a	b	a	a	b	a	a
AVN		a					a	b	a	
A990	August 990	1v4								
BDE		u					a			
BDI		d								
BDII		u		a	a					
BDIII		1								
BDIV		d								
BDV		d	b					b	a	
BDVI		d								
BDVII		e	b							
BDIX		dv1			b	a	a	b	a	
BDX		d					a	b	a	
BH	Black Hooded	1v3			b	b	a			
BI		i	b	a						
BN	Brown Norway	n	a	b	b	b	a	a	a	a
BP	Black Praha	b					a			
BS		1v2						a	a	a
BUF	Buffalo	b	a	b	a	b	a	a	a	a
CAP		c					a	a	a	
CAR	Hunt's Caries-resistant	1a					a			
CAS	Hunt's Caries-susceptible	1	b				a			
COP	Copenhagen 2331	a	b		b	a	b	b	a	a
F344	Fischer	1v1	a	b	b	a	b	a	a	a
HW		h	a							
KGH		g	b	a	a	a	a	a	a	
LE	Long Evans	uv2	b							
LEJ		j					a	a	a	
LEP	Long Evans Praha	uv1					a	b		
LEW	Lewis	1	a	a	a	a	a	a	a	a
LOU/C	Louvain	u	b				a	a	a	b
LOU/M	Louvain	u			b	b	a	a	a	b
MAXX		n	a		b	a	a			
MNR	Maudsley nonreactive	m	a	a			b			
MR	Maudsley reactive	o	a	a	a	b	a			
M520	Marshall 520	b			b	a	a			
NBR	NIH Black	1	b	a	a	b	a	b		
OKA		k	b	a	a	a	b	b	b	
OM	Osborne Mendel	uv3	a				b	b		
PETH		u	b							
PVG	HO	c	b	a	a	a	a	a	a	
R		u					a			
RP		p								

Table 1 Continued

Strain	Synonym	RT1	RT2	RT3	RT6	RT7	Ig-k1	Igh-1	Igh-2	Igh-3
SD	Sprague-Dawley		b							
SHR	Spontaneously hypertensive	k	b	a	a	a	b	b	b	b
WAG	Wistar albino Glaxo	u	b				a	a	a	
WF	Wistar Furth	u	b	a	b	b	a	a	a	b
WKA	Wistar King A	k	a	a	a	a	b			
WKY					a	a				
YO		u	b	a			b	a	a	
YOS					a	a				
Z61			a							

The second locus in the series, RT2, was originally designated AgC, and by linkage similarities involving serum esterase loci appears to be homologous to the mouse Ea-1 locus. RT3 is a blood group locus that was formerly designated AgD. The histocompatibility loci RT4 and RT5 were previously designated H-2 and H-5, respectively and unfortunately most of the common inbred strains have not been characterized for these loci. The RT6 antigens (formerly designated ART-2, AgF, Pta and RT LY-2) are expressed on T lymphocytes in peripheral lymphoid tissues but are absent from thymus and bone marrow. The RT7 antigens (previously designated ART-1 and RT LY-1) are expressed on all thymocytes and peripheral T lymphocytes, while RT9 is a blood group locus linked to RT2, and all strains tested so far except WKA have the a allele. A monoclonal antibody which defines RT10 has been reported.

The study of immunoglobulin allotypes in rats was greatly facilitated by the development of the LOU/C strains, which have a unique susceptibility to the development of immunoglobulin-secreting tumors. Nomenclature conflicts have occurred in this field as well, but a uniform system in agreement with World Health Organization (WHO) guidelines is now in use. Allotypes of many common strains are shown in Table 1.

In addition to the above antigens, a variety of other loci of immunogenetic importance have also been characterized. These include tubular basement membrane antigens, the rat homolog of the CD4 lymphocyte antigen, W3/25 and interleukin 3 (IL-3).

Immunologically unique features of some rat strains are summarized in Table 2. The BB rat is not included in Table 1, as it has not been rigorously inbred. This is a spontaneously diabetic population of rats that was first obtained from a noninbred Wistar-derived stock maintained at the Bio Breeding Laboratory of Canada, Ltd, in Ottawa, Ontario. As this model has become increasingly popular, a good deal of inbreeding has undoubtedly occurred, but its genotype has not been reported at many polymorphic markers.

Rat strains have also been characterized for a large number of polymorphic biochemical markers. These include aconitase, aldehyde dehydrogenase, two alkaline phosphatases, two α -amylases, twelve esterases, fumarate hydratase, α -glycerophosphate dehydrogenase, glyoxalase, hemoglobin, leucine aryl-aminopeptidase, peptidase, pepsinogen, 6-phosphogluconate dehydrogenase, and seminal vesicle proteins. Many of these genes have been mapped by the use of rat/mouse somatic cell hybrids. Although the genetic map of the rat is not nearly as complete as

Table 2 Rat strains with well-characterized immunological susceptibilities

Strain	Susceptibility	References
BB	Diabetes	Nakhooda <i>et al</i> (1977) <i>Diabetes</i> 26 : 100-112
BN	Nephritis	Mathieson <i>et al</i> (1993) <i>Journal of Experimental Medicine</i> 177 : 1309-1316; Hua <i>et al</i> (1993) <i>Toxicology</i> 79 : 119-129
BUF	Thyroiditis	Cohen <i>et al</i> (1988) <i>Cellular Immunology</i> 114 : 126-136
DA	Arthritis	Holmdahl and Kvick (1992) <i>Clinical and Experimental Immunology</i> 88 : 96-100
LEC	T cell maturation defect	Agui <i>et al</i> (1990) <i>Journal of Experimental Medicine</i> 172 : 1615-1624; Yamada <i>et al</i> (1991) <i>Immunogenetics</i> 33 : 216-219
LEW	Arthritis	Ulmansky and Naparstek (1995) <i>European Journal of Immunology</i> 25 : 952-957
LEW	Encephalomyelitis	Mor and Cohen (1995) <i>Journal of Immunology</i> 155 : 3693-3699

that of the mouse, a great deal of progress has been made. Investigators who wish to map additional genes will benefit from the large numbers of microsatellite polymorphisms that have already been mapped. A growing collection of the oligonucleotides needed for these tests is available from Research Genetics, Inc. (Huntsville, AL, USA).

In some experiments, it is important to know whether sublines of the same strain have diverged from one another. This question has been examined by Matsumoto and colleagues by testing numerous sublines of the SHR and WKY strains for polymorphic variants. The SHR sublines varied at six of the 30 loci that were tested, and the WKY sublines differed at 15 of the 30 loci. Many strains and substrains were also surveyed for their genotypes at 39 loci by Bender and colleagues. These investigators observed variation at only WAG sublines, but there was variation at two of the loci among 13 sublines of BN, at three of the loci among four WF sublines, and at four of the 39 loci among five sublines of BUF. Twelve LEW sublines showed no variation at any of the 39 loci, but LEW/Mol differed from the other LEW strains at eight of the loci. Investigators should therefore be aware that there is a varying degree of subline diversity among inbred strains, and that this may or may not affect any given experiment.

See also: Adjuvant arthritis; Alloantigens; Experimental autoimmune encephalomyelitis (EAE); Inbred strains; Insulin-dependent diabetes mellitus, animal models; MHC, evolution of; Mouse inbred strains; Rheumatoid arthritis, animal models; Thyroid autoimmunity, experimental models.

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RECRUITMENT

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Historical background

In the late nineteenth century, Metchnikoff first suggested that phagocytic leukocytes were important in host defense against infectious pathogens. After noting that leukocytes move toward bacteria and ingest them, he induced local recruitment of macrophages and demonstrated protection of the host against otherwise lethal doses of bacteria. However, Metchnikoff's concept of recruitment and activation of leukocytes, the cellular theory of immunity, sparked intense opposition from the humoralists who supported the theories advanced by Koch and others for an exclusive role of circulating antibodies in protective immunity. Indeed, proponents of the humoral theory of immunity considered the inflammatory reaction and the accompanying macrophages to be harmful rather than beneficial to the host, setting back the study of cellular mechanisms of immunity for decades.

Not until the 1950s and 1960s did a revival of interest in the mechanisms of cellular recruitment and activation occur as their importance in transplantation, delayed hypersensitivity and immunodeficiency was recognized. Moreover, investigators soon realized that inflammatory cell infiltrates were not merely exudative, but rather dependent on an active migration of leukocytes from the circulation to the site of inflammation. Intense investigation of the mechanisms involved in leukocyte adhesion and recruitment, particularly within the past decade, has revealed that these events are not only very complex, but importantly also provide potential targets for therapeutic intervention.

Leukocyte recruitment in inflammation

Recruitment of circulating leukocytes to a site of tissue injury, infection or antigen deposition is essential to the evolution of an inflammatory response. The inflammatory response, characterized by altered vascular permeability, the release of inflammatory mediators, and the mobilization and infiltration of leukocytes, serves to sequester and eliminate pathogens. As the leukocytes phagocytose and degrade the inflammatory agent, they, in turn, release additional inflammatory mediators that orchestrate additional recruitment and activation of leukocytes. If these recruited leukocytes succeed in eliminating the

immunologic challenge, the inflammatory response subsides and the tissue repair begins. Failure to eliminate the antigen, however, results in continued recruitment and activation of leukocytes, responsible for the development of chronic inflammatory lesions. These lesions may account for destruction of the host's own tissues.

In order for leukocytes to accumulate in an inflammatory site, circulating leukocytes must: 1) adhere to nearby vascular endothelial cells, and 2) migrate between cells and through the basement membrane toward chemotactic signals in the tissue site where they 3) become immobilized (migration inhibition) and activated to phagocytose, secrete cytokines and/or proliferate as they carry out their inflammatory functions. Thus, recruitment of leukocytes to a specific location is not random, but involves an ordered series of events.

Mechanisms of cell adhesion

The first cells to appear at an inflammatory focus are neutrophils which arrive rapidly after injury, followed by mononuclear cell egress shortly thereafter (**Figure 1**). Stimulation of leukocytes by substances immediately released at the injury site such as complement fragments (C5a), bacterial products (*N* formyl peptides (fMLP) and lipopolysaccharide (LPS)), cell-derived chemotactic cytokines (chemokines) and other inflammatory mediators (leukotrienes (LTB₄)) results in increased leukocyte margination and adherence to the vascular wall. Originally thought to be passive participants in these events, endothelial cells are now known to actively contribute to leukocyte adhesion. Adherence of phagocytes to endothelium, basement membranes and matrix constituents is a complex, reversible event which is augmented during inflammation. When circulating phagocytes encounter an inflamed postcapillary venule lined with activated endothelial cells, they become tethered and commence a transient adhesive rolling motion (**Figure 1**). Rolling is initially mediated by adhesion molecules of the selectin family, which are glycoproteins expressed on the surfaces of both the endothelial cells (E-selectin (CD62E) and P-selectin (CD62P)) and leukocytes (L-selectin (CD62L)), and their corresponding carbohydrate ligands on the target cells (L-selectin targets on endothelial cells: GlyCam-1, CD34 and MadCAM-1).

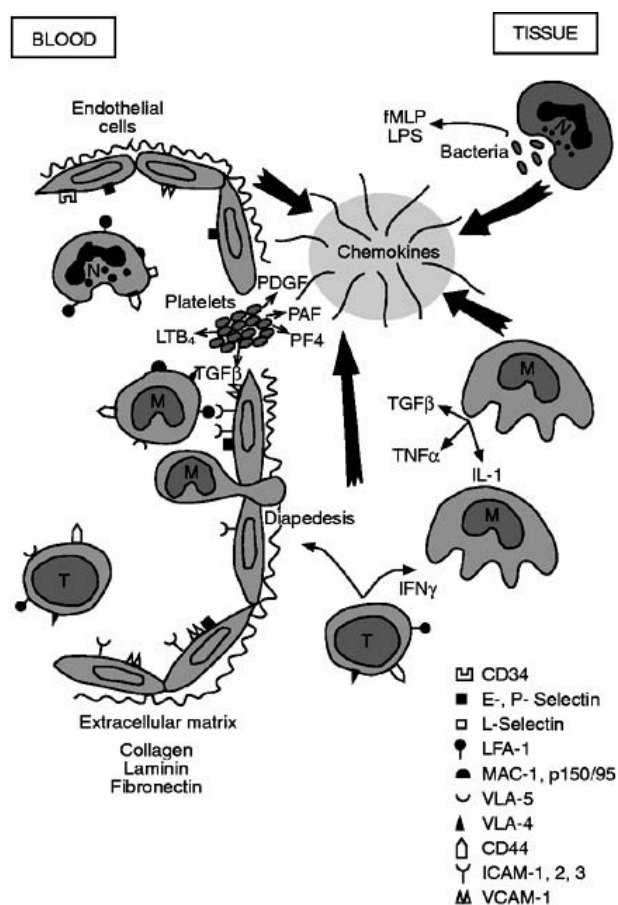


Figure 1 Leukocyte adhesion and recruitment. Leukocytes (N, neutrophils; M, monocytes; T, T lymphocytes) adhere to endothelial cells through cell surface adhesion molecules, leave the circulation by directed migration towards chemotactic stimuli, and enter the tissues where they secrete cytokines which recruit additional cells.

Rolling of leukocytes along the endothelium slows their movement through the circulation, allowing them intimate contact with the vessel wall where the leukocytes may be exposed to additional pro-inflammatory stimuli.

If sufficient stimuli are encountered, the weak selectin-mediated adhesion is replaced (due to shedding of L-selectin) by a more secure binding which precedes transendothelial migration (diapedesis) (Figure 1). This process is partially mediated by the integrins, a multi-membered family of cell surface glycoproteins each consisting of a noncovalently associated α and β subunit (Figure 2). The leukocyte β_2 (CD18) integrins interact with ligands displayed on other leukocytes and/or vascular endothelium, including members of the immunoglobulin superfamily, intercellular adhesion molecules 1 (ICAM-1, CD54) ICAM-2 (CD102), ICAM-3 (CD50) and vascular cell adhesion molecules (VCAM). Integrins,

particularly of the β_1 (CD29) family, also interact with extracellular matrix molecules (laminin, collagen, fibronectin) encountered in basement membranes and in tissues (Figure 2). Leukocyte-endothelial adhesion is augmented by the inflammatory cell-derived cytokines, interleukin 1 (IL-1), tumor necrosis factor α (TNF α), and interferon γ (IFN γ), as well as bacterial lipopolysaccharides (LPS), which can induce and/or activate integrin expression to facilitate leukocyte migration into local areas of immune reactivity. IL-1 and TNF α also stimulate endothelial cells to produce chemotactic cytokines of the chemokine superfamily of proteins, further promoting inflammatory cell recruitment (Figure 3). Chemokines, in turn, facilitate the upregulation of integrins on leukocytes. Thus, endothelial cells not only are targets of regulatory cytokines, but reciprocally produce these polypeptide mediators.

The importance of the adhesion molecules in leukocyte recruitment and host defense is underscored in transgenic mice in which the genes for selectins, integrins, or ICAM-1 and VCAM have been inactivated via homologous recombination with a mutant gene (knockouts) (Table 1). Genetically engineered deletions of integrin α chains or the β_1 chain of the integrins are embryonically lethal, demonstrating their additional critical roles in embryogenesis (Table 1). Mice lacking selectins develop normally, but have leukocytes with impaired adhesive and migratory capabilities. Likewise, null mutations of the β_2 chain result in an adhesion deficiency, and this deficiency causes manifestations similar to a genetic disease recognized in humans, leukocyte adhesion deficiency (LAD). Leukocytes from these patients have a defect in synthesis of CD18, with diminished leukocyte aggregation, adherence and chemotaxis. Consequently, these individuals display markedly diminished inflammatory responses with inadequate phagocytosis of microorganisms, resulting in recurrent, life-threatening infections.

In addition to integrins, adhesion molecules termed homing receptors (CD44) are important in directing lymphocyte-high endothelial venule (HEV) binding (addressins) central to lymphocyte recirculation and homing. Within an inflammatory site, areas of activated endothelium facilitate lymphocyte recruitment analogous to high endothelium in lymphoid tissues. In this regard, HEV-like structures which express VCAM-1 have been demonstrated in areas of chronic, but not acute, inflammation, consistent with the presence of IFN γ which promotes this HEV phenotype. Moreover, memory T cells express increased levels of adhesion molecules including CD2, CD44, LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$) which favor their adherence and enrichment

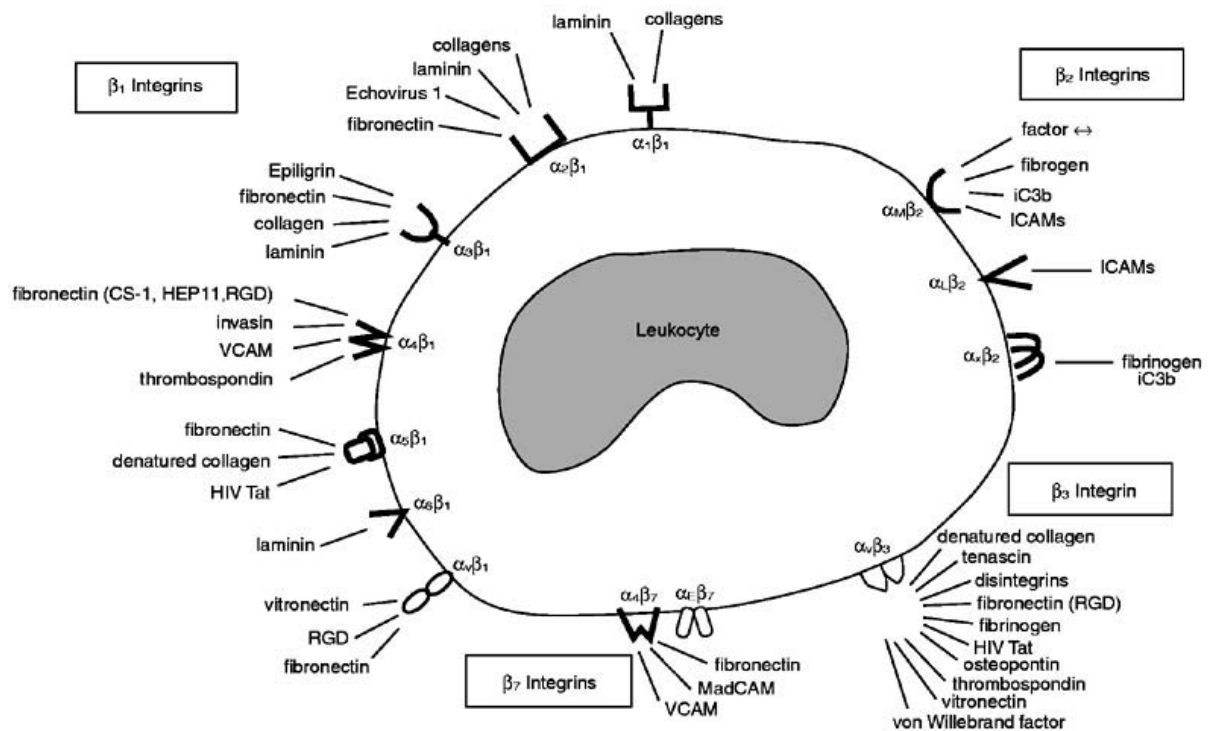


Figure 2 Leukocyte integrins and their ligands. In addition to selectins and members of the Ig superfamily, leukocytes express a plethora of integrin molecules which promote adhesive interactions with endothelium, extracellular matrices, other leukocytes and also infectious microorganisms. Shown are representative leukocyte integrins and their ligands.

at sites of secondary immune responses. Increased expression of CD44 is also found on neutrophils and monocytes, and antibodies to this receptor inhibit accumulation of these cells in inflammatory sites. Thus, homing receptors appear to cooperate with leukocyte integrins in mediating the large increase in transendothelial migration seen during inflammation.

Chemotaxis

To enter the inflamed tissues, adherent leukocytes have to cross the vascular endothelial lining and migrate along increasing concentrations of biochemical signals emanating from the site of injury. This locomotion (chemotaxis) may be initiated, augmented or directed by a variety of chemotactic stimuli such as humoral components (C5a) and coagulation products (thrombin), and those which are released by microorganisms (fMLP), platelets and other sources. Early events in inflammation trigger platelet aggregation and the release of platelet-activating factor (PAF), platelet-derived growth factor (PDGF), platelet factor 4 (PF4) and transforming growth factor β (TGF β) (Figure 1), all of which are potent inducers of leukocyte mobilization. Neutrophils, the first leukocytes to arrive at the site, contrib-

ute to complement activation (C5a), and also release chemoattractants (chemokines, PAF, TGF β), thereby amplifying inflammation and promoting monocyte accumulation. Monocytes, in turn, influence subsequent events by generating numerous pleiotropic products (IL-1, TNF α , chemokines, PAF, TGF β) which promote leukocyte-endothelial cell adhesion and recruit additional cells (Figure 1). These leukocyte-derived products have an enormous cascading impact on the evolution of an inflammatory or immune response.

Among these amplification products is the superfamily of nearly 30 chemotactic proteins, the chemokines (Figures 1 and 3). These small (8–10 kDa) proteins contain four cysteine residues in highly conserved positions, and have been subdivided structurally into two families based on the arrangement of the first two cysteines which are adjacent in β chemokines (C-C), but interrupted by a single amino acid in the α chemokines (C-X-C). Most α chemokines preferentially elicit and activate neutrophils to destroy foreign agents, while β chemokines mobilize mononuclear cells, enabling a progression of cell recruitment into an inflammatory site. Secreted by a wide variety of myeloid and non-myeloid cells including neutrophils, monocytes, endothelial cells, fibroblasts, and some tumor cells in response to

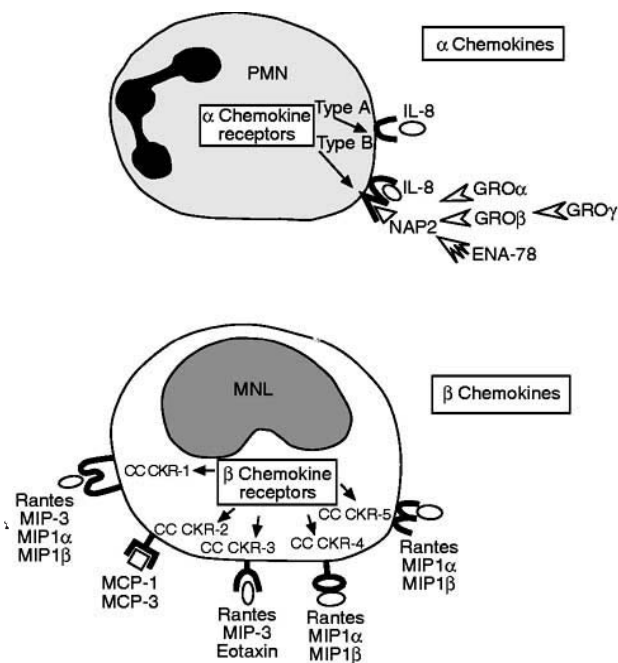


Figure 3 Chemokines and their receptors. Chemokines are represented by two subfamilies based on the arrangement of their first two cysteines, which are adjacent (C-C) in β chemokines and separated by a single amino acid (C-X-C) in α chemokines. Polymorphonuclear neutrophils (PMNs) express type A and B receptors for α chemokines, and mononuclear leukocytes (MNLs) express different combinations of the five β chemokine receptors which have been identified to date.

Table 1 Consequences of gene targeting (knockout) of adhesion molecules

Null mutation	Phenotype of homozygotes
<i>Selectin family</i>	
E-selectin (CD62E)	Normal phenotype
L-Selectin (CD62L)	Normal phenotype ↓ recruitment ↓ lymphocyte homing
P-Selectin (CD62P)	Normal phenotype ↓ recruitment
<i>Integrin family</i>	
α_3 (CD49c)	Day 1 postnatal lethal
α_4 (CD49d)	Embryonic lethal
α_5 (CD49e)	Embryonic lethal
β_1 (CD29)	Embryonic lethal
β_2 (CD18)	Similar to LAD
<i>Ig superfamily</i>	
ICAM (CD54)	Normal phenotype ↓ recruitment
VCAM-1	Embryonic lethal

proinflammatory factors, chemokines may also be produced in response to other tissue insults. Disruption of the cytoskeletal–nuclear architecture triggered by cellular stresses such as cell deformation, hypoxia,

ischemia–reperfusion and particulate deposition activates the nuclear transcription factor NF κ B to signal chemokine expression in the initiation, amplification and maintenance of inflammatory sequelae.

Chemokines and other chemotactic factors are recognized by specific cell surface receptors. Receptor numbers vary from a new hundred for TGF β , which is chemotactically active at femtomolar concentrations, to nearly 100 000 for fMLP and C5a (C5aR, CD88), active in the nanomolar range. Two α chemokine receptors, which belong to a superfamily of seven transmembrane G protein-coupled receptors, have been characterized on the surface of neutrophils: the type A receptor selectively binds IL-8, while the type B receptor binds all α chemokines which contain the sequence motif Glu-Leu-Arg (ELR) near the N-termini (IL-8, NAP2, GRO α , β , γ , and ENA-78) (Figure 3). The family of receptors for β chemokines is much more complex with at least five β chemokine receptors (CC CKR1–5) identified on mononuclear cells (monocytes, lymphocytes) and on basophils and eosinophils, each with varying specificities for different subsets of β chemokines. Interestingly, a chemokine receptor expressed on the surface of erythrocytes has broad specificity for both α and β chemokines, and may act as a scavenger receptor to remove chemokines from the circulation that might otherwise interfere with local chemotactic gradients. Occupancy of chemokine receptors, other than the erythrocyte chemokine receptor, transduces signals coupled to guanine nucleotide-binding (G) proteins which initiate a sequence of complex biochemical and cellular events. These events culminate in polarization of the cell with a redistribution of receptors and cytoskeletal elements (actin, myosin) towards the leading edge, extension of a lamellopod in the direction of the attractant (motile phenotype), and the initiation of directed movement toward the signal.

Migration inhibition, activation and resolution

Chemotactic signals are naturally self-limiting, since they have a narrow range of optimal concentrations for chemotactic activity. When chemotactic factors are present at high concentrations, they tend to activate target cells, resulting in dramatic changes in cellular physiology and in the repertoires of adhesion molecules displayed on the cell surface. Since the release of chemoattractants from a proinflammatory site results in a concentration gradient away from the site, leukocytes first encounter low, chemotactically active concentrations and as they migrate up the gradient, the cells reach concentrations which

activate other functions and inhibit motility. This migration inhibition, assuring leukocyte persistence at the site of inflammation, is also due to a downregulation of receptors for certain chemotactic ligands. When leukocytes, newly recruited to the source of a chemotactic signal, are not subsequently activated, they may undergo apoptosis, a process here serving to minimize unneeded leukocyte accumulation. However, upon exposure to relevant activation and differentiation signals, such as IFN γ or LPS, the cells are rescued from apoptosis, undergo maturation, and lose their ability to migrate. Under the influence of the activation signals, the leukocytes are directed to carry out their specific functions of proliferation, phagocytosis, cytokine secretion, and/or cytotoxicity necessary for host defense.

Clearance of the inciting stimulus leads to resolution of the inflammatory response, whereas persistence of the stimulus favors excessive recruitment of inflammatory cells with the potential for leukocyte-mediated tissue damage. In chronic inflammatory sites, lymphocytes constitute a substantial proportion of the infiltrating leukocytes. Moreover, once activated, the lymphocytes exhibit remarkable selective migratory behavior due to their specific adhesion molecules and chemotaxis receptors. Release of chemotactic ligands by these cells, including β chemokines selective for monocytes, appears to direct responses associated with delayed hypersensitivity and chronic inflammation. Consequently, excessive, self-perpetuating recruitment is the basis of many chronic autoimmune, allergic and inflammatory diseases. In contrast, deficiencies in leukocyte recruitment lead to recurrent life-threatening infections. Thus, regulated leukocyte recruitment from the bloodstream to sites of inflammation is an essential component of host defense. Consequently, the adhesion molecules, their ligands and the chemokine family have become key targets in identifying novel therapeutic approaches to control recruitment in aberrant inflammatory responses.

See also: Acute inflammatory reaction; Adhesion molecules; Chemokines; Chemotaxis; Cytokines; Diapedesis; Integrins; Intercellular adhesion molecules: ICAM-1, ICAM-2 and ICAM-3; Selectins (CD62-E/L/P).

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RELATIVE RISK

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A genetic marker association with a disease is demonstrated if a statistically significant difference is found in marker allele (or genotype) frequencies between ethnically matched unrelated patients and a group of unrelated control individuals. ABO blood group associations with certain diseases are well established, however the associations so far are consistently weak. In contrast, many strong associations of the HLA system with disease have been found. Association studies are increasingly relevant for mapping purposes in all disease studies. Association data have been used as a follow-up to linkage studies to further localize the disease-predisposing gene in a number of monogenic traits, and also to detect the role of candidate genes in complex diseases. To perform an association study between a disease and, say, a particular HLA antigen, or other genetic marker, a 2×2 table of antigen presence or absence versus disease presence or absence is set up as follows. Traditionally, the class antigen presence includes individuals who are homozygote and heterozygote for the marker allele, or HLA antigen, but also see below.

Marker allele			
Disease	+	-	
+	<i>a</i>	<i>b</i>	<i>N</i> ₁
-	<i>c</i>	<i>d</i>	<i>N</i> ₂
	<i>M</i> ₁	<i>M</i> ₂	<i>T</i>

The number of patients studied is *N*₁, the number of control individuals is *N*₂.

The most commonly used measure of the strength of an association between a disease and a marker allele is the relative risk (RR), or relative incidence ratio of Woolf (1955). The relative risk is simply estimated by the cross-product ratio of the entries in the 2×2 table of association, i.e.

$$RR = x = \frac{a \cdot d}{b \cdot c} \quad (1)$$

(Strictly, this measure in a retrospective study as described here is the odds ratio (OR), but when the incidence of the disease in the population is low, this is equivalent to the relative risk as defined in epi-

demiology for prospective studies.) A relative risk higher than 1 (a positive association) is seen when an antigen is more frequent in the patients than in the controls, whereas a risk below unity (a negative association) reflects a decreased frequency in the patients.

Under the null hypothesis of no association of the genetic marker with disease, the expected RR is 1, and observed values of the RR follow a chi-squared distribution with 1 degree of freedom, as follows

$$\chi^2 = (ad - bc)^2 T / (M_1 M_2 N_1 N_2) \quad (2)$$

Deviations of an observed RR value from the chi-squared distribution indicate a statistically significant association. For more details on statistical issues, including combining samples, see Svejgaard *et al* (1974).

In cases where an antigen, or marker, is absent or present in all patients or when sample sizes are small, the formula given by Haldane in 1955 should be used:

$$RR = x = \frac{(a + 1/2)(d + 1/2)}{(b + 1/2)(c + 1/2)} \quad (3)$$

For a number of HLA-associated diseases large relative risks are observed, for example in the case of ankylosing spondylitis the relative risk for B27 in Caucasians is 69.1, while for insulin-dependent diabetes mellitus (IDDM) the relative risks for DR3 and DR4 are 3.8 and 9.0 respectively (see Thomson, 1988).

Two possibilities exist to explain a marker-disease association. The first is that disease susceptibility is directly influenced by the presence of the marker allele, e.g. class II HLA-DRB1, DQA1 and DQB1 associations with IDDM. The second is linkage disequilibrium (nonrandom association) between the marker allele and a disease predisposing locus, e.g. the association of HLA class I A3 with hemochromatosis in Caucasians. In either case, the 'disease' locus may have alleles necessary for disease, or alternatively all genotypes at the locus may be disease susceptible, with different degrees of penetrance. Significant linkage disequilibrium values are usually not expected for loci with recombination distances greater than 2%, and sometimes less than this distance.

All subjects must be of the same homogeneous ethnic origin. Otherwise, spurious associations may result from a stratification effect if one of the subgroups had a higher frequency of the disease and the marker allele than did the rest of the population. Using family-based association studies, where the parental marker alleles not transmitted to an affected child in simplex families form the 'control' population, ensures that only associations of marker genes linked to a disease gene will be detected (for details, including statistical tests, see Thomson, 1995).

Multiple alleles at a genetic marker locus may show associations with a disease. To test this, it is preferable to test all k alleles simultaneously in a $k \times 2$ contingency table of number of alleles observed in patients and controls, than to perform multiple tests on each allele separately. Genotype specific effects can similarly be determined.

See also: Genetic analysis at the phenotypic level;

Insulin-dependent diabetes mellitus, human; Linkage disequilibrium; MHC disease associations; Multiple sclerosis.

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RENAL INFECTIONS

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The principal infections of the kidney and urinary tract occur in either the renal parenchyma (pyelonephritis) or in the bladder (cystitis). Other, or connecting, parts of the urinary tract may be specifically involved as in urethritis, prostatitis, epididymitis, or as part of a more widespread process, e.g. tuberculous ureteritis.

Patients with renal disease may be prone to urinary infection because of anatomic or pathological abnormalities which lead to stasis of urine (e.g. retention of urine in the bladder), or are more prone to infections in general because of 1) uremic immunosuppression, 2) concomitant immunosuppressive treatment (e.g. transplant recipients), 3) malnutrition, 4) iron overload (in patients after multiple blood transfusions), or 5) concomitant systemic disease, such as diabetes mellitus.

Definition

Urinary tract infection (UTI) is the presence of microorganisms in the urine (bacteriuria). Significant bacteriuria is considered as the presence of at least 10^5 colony-forming units (CFU) ml^{-1} of freshly voided urine. Symptomatic infection is associated with

inflammation and a white blood cell count higher than 10 cells mm^{-3} in the urine (pyuria, leukocyturia).

Epidemiology

Significant bacteriuria is found in about 1–2% of girls and 3–5% of adult women but seldom occurs in males. The incidence rises dramatically in old age, occurring in 25–50% of patients in geriatric institutions.

Etiology and pathogenesis

Urine is an excellent culture medium and contains sufficient nutrients to support bacterial growth to a density of 10^7 CFU ml^{-1} . Ideal conditions, of urine pH 4–7 and concentration of 300–800 mosmol kg^{-1} , are common. Bacteria most commonly enter via the urethra (ascending infection) or may enter via the bloodstream. Ascending infections, which account for the great majority of cystitis and pyelonephritis, are mainly due to organisms of the normal bowel flora – principally *Escherichia coli* (75% or more).

Predisposing factors are shown in Table 1. Bladder

Table 1 Predisposing factors for urinary tract infection

Cystitis	Pyelonephritis
Urinary stasis	Normal kidney
Outflow obstruction	Pregnancy
Impaired bladder contraction	Stones
Reflux into ureter or diverticulum	Diabetes
	Previous episodes
Foreign body	Abnormal kidney
Bladder stone	Scars (from previous reflux/infection)
Bladder tumor	Obstruction
Instrumentation	Duplex kidney
Catheter	Malformed (dysplastic) kidney
	Vesico-ureteric reflux

outflow obstruction is a major factor and may be due to bladder neck stenosis, urethral stricture or prostatic enlargement. The particular susceptibility of women is due partly to the short urethra and its proximity to the bowel.

The virulence of an organism is related to its ability to adhere to epithelial cells. Less virulent organisms may be pathogenic when the kidney is not normal, e.g. with vesico-ureteric reflux. The epithelial cell receptors for the majority of uropathogenic *E. coli* strains are glycolipids of the globo-series, which interact with adhesins on the bacterial surface. The adhesins are filamentous appendages called fimbriae. The two main types are distinguished by whether their binding to red cells is inhibited by mannose or not. The receptor glycolipids are antigens in the P blood group system with the phenotype P1 as the major binding sites. *E. coli* express both O and K antigens and the pathogenic strains are associated with relatively few serotypes of these two families.

There are a number of host defense mechanisms against UTIs. The bladder luminal surface is covered with a glycoprotein mucin layer. Disruption of this layer allows bacteria to colonize the epithelium. Bacterial washout by the normal urine flow and bladder emptying is a major defense mechanism. Urinary antibodies (immunoglobulin G (IgG) and secretory IgA) are formed against the O and K serotypes, and with pyelonephritis a systemic antibody response to O antigens occurs.

Clinical infection

Acute pyelonephritis

This is associated with fever, malaise, loin pain (which may be bilateral) and bacteruria and leukocyturia. There may also be symptoms of cystitis. In nor-

mal kidneys the organisms are *E. coli*, coagulase-positive *Staphylococcus*, *Streptococcus faecalis* and *Klebsiella*. The presence of other gram-negative organisms (*Pseudomonas*, *Proteus*) would suggest pre-existing disease or a stone.

Other acute parenchymal infections

Rarely, septicemia may lead to microabscess formation in the kidney (usually *Staph. aureus*). Occasionally, with very severe infection, or particularly associated with diabetes, there may be necrosis of the renal papillae (papillary necrosis). Rarely subacute or chronic infections may develop, particularly associated with pre-existing abnormalities of the kidney, and the abscess may extend to form a mass (renal carbuncle) or point through the capsule of the kidney to form a perinephric abscess.

Chronic pyelonephritis

Vesico-ureteric reflux with or without infection may cause scarring of the kidney, and the scarring varies with the degree of reflux. Much of the scarring may have occurred in the fetal or neonatal period, although further scarring in childhood is associated with UTI. In later life progressive damage and renal failure may occur in the absence of both UTIs and reflux and the term reflux nephropathy is now preferred for such cases, rather than the former term chronic pyelonephritis. Ureteric reflux is now recognized as a common inherited disease (autosomal dominant).

Other chronic infections

Complete obstruction may lead to severe infection in the renal pelvis (pyonephrosis) and loss of the kidney. Tuberculosis may involve the renal parenchyma, and this is generally associated with a ureteritis and cystitis. Obstruction with stone disease may be associated with a chronic granulomatous pyelonephritis (xantho-granulomatous pyelonephritis). Malakoplakia is a rare chronic infection of the renal substance which may simulate a renal tumor. It is associated with an inability of macrophages to kill *E. coli* successfully.

Cystitis

Cystitis is associated with frequency and urgency of micturition and painful micturition (dysuria). Bacterial infection is usually due to *E. coli*. Chronic cystitis with pyuria but cultures that are reported as 'sterile' is highly suggestive of tuberculous infection. In some women recurrent symptoms of cystitis occur in the absence of significant bacteruria and pyuria (acute urethral syndrome). The cause is unknown but

may be due to fastidious organisms (such as *Gardnerella vaginalis*) or organisms that are considered as part of the normal urethral flora.

Urethritis

Urethritis is associated with dysuria and a urethral discharge. Infection from *Neisseria gonorrhoeae* should be considered; nongonococcal infections (non-specific urethritis) include *Chlamydia trachomatis* and *Ureaplasma urealyticum*.

See also: **Chlamydia, infection and immunity; Escherichia coli, infection and immunity; Klebsiella, infection and immunity; Neisseria, infection and immunity; Staphylococcus, infection and immunity; Streptococcus, infection and immunity; Urinary tract infections.**

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REOVIRUS, INFECTION AND IMMUNITY

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Mammalian reoviruses are the prototype members of the *Orthoreovirus* genus of the Reoviridae family of segmented, double-stranded (ds) RNA viruses. Reovirus virions are nonenveloped and consist of two concentric protein capsids. While originally isolated from the respiratory and gastrointestinal tracts of humans, reovirus (reo – respiratory and enteric orphan virus) infections do not appear to be a source of major symptomatic respiratory or enteric disease. However, they are ubiquitous in the environment and the majority of the human population acquires a reovirus-specific antibody response in infancy or early childhood. Reovirus infection of rodents provides an excellent model for the study of viral pathogenesis and host immune responses at both systemic and mucosal sites.

Characteristics of the organism and its antigens

The mammalian reoviruses are classified into three serotypes based on neutralization and hemagglutination-inhibition tests. The prototypical strain(s) of the three reovirus serotypes are shown in Table 1. The reovirus genome contains ten discrete dsRNA segments which are resolved into three class sizes by polyacrylamide gel electrophoresis (four small, three medium and three large). Each segment is translated into a unique mRNA which codes for a unique structural or nonstructural viral protein. With the exception of the S1 gene, each of these RNAs appear to be monocistronic and produce a single primary gene product. These viral proteins are also resolved into

Table 1 Prototypical strains of mammalian reoviruses

Serotype	Prototype strain(s)	Source
Reovirus type 1	Lang (type 1/L)	Human GI tract
Reovirus type 2	Jones (type 2/J)	Human GI tract
Reovirus type 3	Dearing (type 3/D) Abney (type 3/A)	Human GI tract Human respiratory tract

GI, gastrointestinal.

class sizes (σ , small; μ , medium; λ , large). The major components of the inner protein capsid of reovirus are $\lambda 1$ and $\sigma 2$, with $\lambda 3$ and $\mu 2$ being minor components. Sigma 3 and $\mu 1C$ (a 72 kDa cleavage product of the $\mu 1$ protein) are the major outer capsid proteins. Twelve spikes extend from the inner to outer protein shells and are composed of five molecules of the $\lambda 2$ protein. The vertices of the 12 spikes extend to the outer capsid and are each associated with 2–4 molecules of the $\sigma 1$ protein. Figure 1 illustrates the structure of reovirus virions and the relationship of the viral genome and structural proteins. While morphologically similar, the three serotypes of reoviruses have varying levels of homology at the genetic level. RNA hybridization studies show that reovirus 1/L and reovirus 3/D are approximately 70% related, while reovirus 2/J is only 10% related to the other serotypes.

The segmentation of the reovirus genome has facilitated the making of reassortant virions. Simultaneous infection of tissue culture cells *in vitro* with two reovirus serotypes results in the generation of

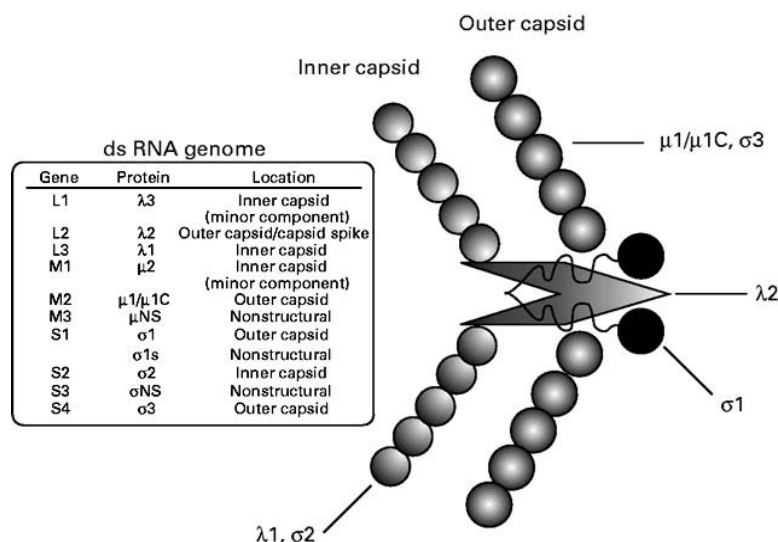


Figure 1 Reovirus virion structure and genome.

reoviruses which can have gene segments derived from either parent. The origin of individual gene fragments from reassortant clones can be determined by comparison of the pattern of dsRNA migration in polyacrylamide gel electrophoresis. The use of reassortant clones has allowed unique biological properties of particular serotypes to be mapped to individual reovirus genes and proteins. For example, by analyzing reassortant clones, the $\sigma 1$ protein has been found to be the viral hemagglutinin as well as the target of serotype-specific neutralization antibodies.

The primary pathway for reovirus penetration of host cells involves receptor-mediated endocytosis, fusion with lysosomes and degradation of two of the outer capsid proteins ($\sigma 3$ and $\mu 1/\mu 1C$), which results in the formation of intermediate subviral particles (ISVPs). In the gastrointestinal tract, ISVPs can be directly formed in the lumen of the small intestine and this form of the virion appears to be required for adherence to specialized absorptive cells (M cells) overlying the organized lymphoid follicles (Peyer's patches) of the gut. Full activation of viral transcriptase activity requires further digestion of outer capsid proteins in the cell to form cytoplasmic viral cores. Newly synthesized positive strands are capped and are used as messenger RNA for protein synthesis as well as templates to synthesize complementary negative strands in nascent progeny viral particles.

Viral spread and pathology

The mucosal routes of reovirus entry into the host are via the gastrointestinal and respiratory tracts. After entering Peyer's patches via M cells, reovirus 1/L spreads to the mesenteric lymph nodes (MLNs)

and the spleen. Reovirus 3/D infection of the gut is more localized, with infectious virions detectable only in the gut and MLNs. High inoculum doses of reovirus 1/L ($\sim 10^{10}$ PFU) by the oral or intravenous routes results in site-specific viral replication in the ileum with the resultant generation of ileitis. In contrast, intravenous infection with similar doses of reovirus 3/D induces duodenitis, jejunitis and ulcerative colitis, with the ileum being spared. Studies with reassortant clones suggest that the $\sigma 1$ protein is the major viral determinant of site-specific intestinal disease. Respiratory reovirus infections of rodent lungs may involve a similar mechanism of viral entry into organized lymphoid nodules via M cells overlying bronchus-associated lymphoid tissue. In addition, reovirus also replicates in alveolar type I epithelial cells of the lung which is associated with the generation of pulmonary pneumonia. Although the details of viral spread following respiratory infection have not been delineated, it is likely that the virus spreads from the lung to the lung-associated lymph nodes, then to the bloodstream, in a manner similar to the events following reovirus infection of the gastrointestinal tract.

In addition to mucosal routes of infection, experimental models of reovirus infection using alternate routes of infection have been used to study particular aspects of viral pathology and viral immunity. Reovirus-specific immune responses can be stimulated in adult, immunocompetent mice after inoculation through a number of routes including intraperitoneal, intradermal, subcutaneous, and by direct inoculation in the footpads. Studies utilizing typical viral inoculation doses of $\sim 10^7$ PFU are not associated with pathological responses in adult mice (via systemic or gut routes of immunization). In contrast

to reovirus infection of immunocompetent adult mice, intracerebral infection of neonatal mice results in reovirus serotype-specific central nervous system (CNS) pathology. In neonatal mice, intracerebral inoculation of reovirus 1/L results in a nonfatal ependymitis followed by hydrocephalus. In contrast, intracerebral infection of neonatal mice with reovirus 3/D results in the development of a lethal encephalitis. Studies utilizing reassortant viruses have demonstrated that the S1 gene product ($\sigma 1$) determines the specificity of infection for neuronal versus ependymal cells. In addition to the intracerebral route, reovirus 3/D also produces a fatal encephalitis after footpad inoculation via spread to the CNS following local replication in muscle tissue. While reovirus 3/D does not result in encephalitis after oral inoculation, a murine isolate (reovirus 3 clone 9) does produce encephalitis after oral inoculation. The use of reassortant viruses have demonstrated that the M2 gene product ($\mu 1$) is the determinant of viral replication and systemic spread from the gastrointestinal tract.

In addition to the infection patterns previously described, infection of neonatal mice with any of the three reovirus serotypes can result in hepatic and pancreatic involvement. Hepatic infection leads to zones of necrosis and hemorrhage, and reovirus 3/D infects the mucosal cells of the bile duct, leading to chronic obstructive jaundice. Reovirus infection of acinar cells of the exocrine pancreas results in cellular injury and an inflammatory infiltration which can progress to extensive necrosis. Infection of endocrine areas of the pancreas by reovirus 3/D (insulin-producing β islet cells) and reovirus 1/L (insulin-producing β islet cells as well as other islet cell types) results in cellular injury, and may also induce an autoantibody response through upregulation of major histocompatibility complex (MHC) expression. More recently, a reovirus type 2-induced diabetes-like syndrome in neonatal mice has been described which is partially mediated by immune mechanisms. In this model, immunosuppressive treatment reduced or prevented the development of diabetes-like syndrome.

Infection of neonatal mice with reovirus 1/L or 3/D can result in a 'runt syndrome' characterized by small size, reduced growth rates and an oily hair effect (coat hair stuck together in irregular tufts). It is thought that this syndrome is caused by severe malabsorption due to coexisting pancreatic and hepatobiliary disease (reovirus 3/D infection), and in the case of reovirus 1/L, additional infection of the growth hormone-producing cells of the anterior pituitary. Because of the impact of reovirus infection on many organ systems in rodents, and the presence of

reovirus-specific antibody in humans, it has been hypothesized that reovirus may be involved in similar human disease patterns. However, attempts to detect the presence of reovirus in human tissue have not been successful, and it appears at this time that reovirus does not play a significant role in human hepatic disease.

The availability of severe combined immunodeficient (SCID) mice has more recently demonstrated the innate pathogenicity of reovirus infections in the absence of functional B and T lymphocytes. While infectious virions are typically cleared within the first 2 weeks after inoculation in immunocompetent mice, young adult SCID mice infected with reovirus 3/D develop a chronic infection of multiple organ systems. SCID mice infected with reovirus 1/L develop a more severe infection with persistent high virus titers in multiple organs and the development of severe hepatitis, which is frequently lethal within the first 3 weeks of the infection.

Table 2 lists the uses of reovirus in probing the causes of viral spread and pathology.

Immune responses of the host

As is common with viral infections, reovirus has been shown to elicit vigorous humoral and cell-mediated immune responses. Serum antibodies specific for reovirus are common in human and animal populations and this demonstrates the ubiquitous distribution of reoviruses in the environment. The predominant specificity of reovirus-specific antibodies is against the outer capsid protein $\sigma 1$. However, serum antibodies to other viral proteins are also elicited. While reovirus-specific antibodies to the $\sigma 1$ hemagglutinin protein are predominantly serotype specific, reovirus-specific monoclonal antibodies (mAbs) against other external capsid proteins have demonstrated serotype nonspecific neutralizing and/or hemagglutinin inhibition abilities. In the case of reovirus 3/D, a panel of mAbs has identified distinct neutralization and hemagglutination inhibition domains. Similar domains have not been identified for reovirus

Table 2 Uses of reovirus to probe causes of viral spread and pathogenesis, delineate immune mechanisms and provide models of human disease

Pathogenesis of viral central nervous system disease
Pathogenesis and immunity of viral myocarditis
Gastrointestinal infection and immunity
Respiratory infection and immunity
Viral induction of autoimmunity
Delineation of the anti-idiotypic network
Induction of autoimmune diabetes

I/L. Reovirus-specific mAbs can inhibit virus attachment to host cells, or, after binding, inhibit internalization and intracellular proteolytic uncoating to the ISVP form. Systemic routes of reovirus infection also elicit viral-specific cytotoxic T lymphocytes (CTLs) as well as T cell-mediated delayed-type hypersensitivity (DTH) reactions. While reovirus-specific CTLs are predominantly serotype nonspecific, the reovirus-induced DTH response is serotype specific and is determined by the S1 gene product. Reoviruses can also induce both antigen-specific and antigen-nonspecific immunosuppression. The administration of nonreplicating virions by the intravenous or oral routes results in T cell-mediated suppression of the reovirus-specific DTH response *in vivo*. Reovirus 3/D inhibits mitogen-induced T cell proliferation, as well as T cell responses specific for unrelated viruses, via its interaction with a subset of reovirus 3/D receptor-bearing CD8⁺ T lymphocytes.

The ability of reovirus infection to elicit a number of pathological conditions in neonatal or immunocompromised mice provides a model for the study of the protective nature of viral-specific humoral and/or cell-mediated immunity. The administration of reovirus-specific polyclonal antisera protects neonatal mice from reovirus 3/D-induced encephalitis. Studies utilizing reovirus-specific mAbs of varying isotypes and specificities, and analysis of the effect of these antibodies on virus spread and replication in the CNS, have shown that their protective effect can be mediated through inhibition of primary replication, prevention of neural spread and inhibition of growth within the spinal cord. Further studies have shown that a single antibody may not work in all capacities, and also that isotype and avidity (above a certain threshold) does not determine which features any single antibody will possess. Protection against reovirus-induced encephalitis in neonatal mice can also be transferred from immune dams to neonates either transplacentally or by suckling, demonstrating the importance of maternal immunity in protection of neonates from infectious pathogens. In neonatal mice, adoptive transfer of reovirus-immune splenocytes as well as the passive transfer of reovirus-specific antibodies has demonstrated humoral and cell-mediated immune protection against reovirus-induced myocarditis.

While the generation of reovirus-specific T cell responses in systemic tissues is not unexpected, the ability of reovirus to elicit a gut mucosal infection via M cell entry into Peyer's patches has provided a valuable model to analyze viral-specific immunity in the context of the gastrointestinal tract. Gut mucosal application of reovirus generates a virus-specific B cell response in Peyer's patches that is dominated by

immunoglobulin A (IgA) memory cells. In addition, the gut mucosal application of reovirus results in the appearance of virus-specific CTLs in both Peyer's patches and the intestinal epithelium. Frequency analysis of CTLs occurring in Peyer's patches and peripheral lymph nodes 6 days and 6 months after gut mucosal stimulation has demonstrated that a persistent gradient of precursors is established, with higher frequencies present in Peyer's patches. These results suggest that, for enteric viruses, the generation of a CTL response in Peyer's patches may be important in preferentially repopulating mucosal tissues with effector CTLs, which could result in the local containment of infections in the gut. The ability of adoptively transferred reovirus-specific CD8⁺ CTLs from the intestinal epithelium to protect neonatal mice lethally challenged with reovirus 3/D demonstrates the *in vivo* relevance of viral-specific T cell immunity in the gastrointestinal tract. The adoptive transfer of reovirus-immune Peyer's patch lymphocytes before infection protects SCID mice from persistent viral replication in multiple tissues and also from hepatitis. Reovirus infection of the lower respiratory tract results in an infiltration of T and B lymphocytes, including reovirus-specific CTLs, and provides a model for the study of the inter-relationship of respiratory-mucosal immunity with gastrointestinal immunity and other components of the common mucosal immune system.

Vaccines

The ability of reovirus to enter Peyer's patches via M cells suggests that members of the Reoviridae family may prove useful as mucosal vaccines if they could be engineered to carry heterologous B and/or T cell epitopes. Such an approach may be feasible since both *Rotavirus* and *Orbivirus* (bluetongue virus) virus-like particles (produced by the expression of multiple viral capsid proteins in insect cells via a baculovirus expression system) are reported to be highly immunogenic in a number of animal species. Additionally, studies of the reovirus 3/D receptor, using an anti-idiotypic approach, have identified a method of inducing immunity to pathogens which is independent of *in vivo* immunization with pathogenic agents. In these studies, the immunization of mice with anti-idiotypic antibodies specific for an idiotypic marker of a major neutralization domain-binding mAb has been found to induce reovirus-specific B and T cell immunity *in vivo*. Thus, while reovirus infections are not associated with significant human disease, these approaches may be useful for the generation of vaccine strategies against other pathogens. In the Reoviridae family, such an

approach may be useful for the design of vaccine strategies for avian reovirus infections which are a significant source of morbidity in the commercial poultry industry.

See also: Mucosal immunity; Mucosa-associated lymphoid tissue (MALT); Viruses, immunity to.

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REPRODUCTIVE TRACT INFECTIONS

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The reproductive tracts of the male and female are anatomically distinct and present different sites for microbial colonization and pathogenesis. Primary infections of the reproductive tract are preceded by colonization of the tissue. A variety of microbial agents are frequently associated with the infections. These include *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Ureaplasma urea-*

lyticum, *Treponema pallidum*, mycoplasmas, human papillomavirus (HPV), herpes simplex virus (HSV), and the gram-negative enteric bacilli. Human immunodeficiency virus (HIV) certainly enters the host by way of the reproductive tract but will not be dealt with here because of its diverse and multiorgan clinical manifestations.

Neisseria gonorrhoeae

The gonococci are oxidase-producing gram-negative diplococci that cause sexually transmitted infections including urethritis and cervicitis. The local complications of the infection are salpingitis and pelvic inflammatory disease in the female and epididymitis and urethritis in the male. Disseminated gonococcal infection (DGI) occurs in 0.5–3% of infected patients. The gonococci have many virulence factors which contribute to their pathogenesis. Pili attach to receptors on the mucosal epithelial cells which line the reproductive tract of humans. Their expression is under genetic control and has a high degree of antigenic variability. In experimental infections of volunteers with nonpiliated gonococci approximately 80% did not develop active infections. Other surface-exposed molecules involved in the biological activity of the gonococcus are proteins I, II and III, lipooligosaccharides and peptidoglycan. 1) Protein I (PI) is a major outer membrane protein and functions as a porin. It has two structural subtypes which are designated IA and IB. Isolates express only one subclass of PI. However, antigenic variation allows for the further division of the IA and IB into other serotypes. 2) Protein II (PII) consists of several different proteins (PII a–h). Individual strains may possess as many as three different PIIs. These proteins are involved in attachment and the production of bactericidal antibodies and have a high degree of antigenic variability with rates approaching frequencies of 10^2 to 10^3 . 3) Protein III (PIII) is antigenically conserved and functions as a site for blocking antibody attachment. 4) Lipo-oligosaccharide (LOS) is present in the outer membrane and frequently is excreted in the form of blebs in actively dividing gonococci. This molecule is toxic for epithelial cells and has a particular affinity for ciliated epithelial cells. LOS disrupts tight junctions and the intact ciliated epithelial cells are sloughed intact from the epithelial surface following exposure to LOS. 5) Peptidoglycan is an integral component of the cell wall of these gram-negative organisms and is responsible for epithelial cellular cytotoxicity. These components contribute to the virulence of the organism and enhance its pathogenicity to mucosal tissue (Figure 1).

The ability of gonococci to evade immune surveillance resides in their extraordinary ability to undergo phase and antigenic variation. This variation leads to frequent changes in expression of the antigenic composition as no single serotype predominates. Therefore, the host is unable to mount an effective protective immune response. At this time there is no vaccine available against gonococcal infection and the potential for developing one is not close at hand.

Chlamydia trachomatis

Chlamydia are gram-negative bacteria which have a unique developmental cycle. *C. trachomatis* is the only reproductive tract pathogen. The *C. trachomatis* serovars D–K and L_{1–3} are involved in urogenital infections and cause in excess of 50 million cases of genital infection worldwide, including urethritis, mucopurulent cervicitis, and salpingitis (serovars D–K), as well as lymphogranulovenereum (LGV) (serovars L_{1–3}).

The chlamydia infect primarily columnar epithelial cells where the organisms undergo a unique replicative cycle. Multiplication occurs in intercytoplasmic vacuoles where they are energy parasites and use ATP produced by the host cell. The cell wall contains the major outer membrane protein (MOMP) which is responsible for the rigidity of the cell wall since chlamydia lack peptidoglycan. These molecules are responsible for the species-specificity of the organisms. The cell wall also contains a lipopolysaccharide (LPS) (Figure 2).

Although nonspecific host responses to *C. trachomatis* and its antigens exist, specific immune responses (both humoral and cellular) appear to be involved in modifying infection or conferring immunity to reinfections. The presence of serovar-specific antibody has been documented in women with upper genital tract infection but correlation between the humoral immune response and resistance to infection has been difficult to make. The role of the cell-mediated response is even less well understood in humans than in many animal models. In experimental infection both responses are involved in the resolution of infection. Most of the immune response involves local mucosal immunity as the infection is restricted to the epithelial layer of the cervix, endometrium and fallopian tubes. However, it is generally accepted that mucosal immunity to chlamydial genital infection does not exist and recurrent infections are common. T Cells are recruited to the area of infection and through the release of inflammatory cytokines there is host cell-mediated damage to the mucosa.

Although no vaccine is available for clinical use, a number of laboratories are evaluating vaccine components of *C. trachomatis* for their efficacy in inducing a protective immune response.

Treponema pallidum

T. pallidum is the etiological agent of syphilis. It is a long spiral-shaped rod encased in a protoplasmic cylinder. It has a peptidoglycan layer and is motile by means of an axial filament. The fact that this bacteria

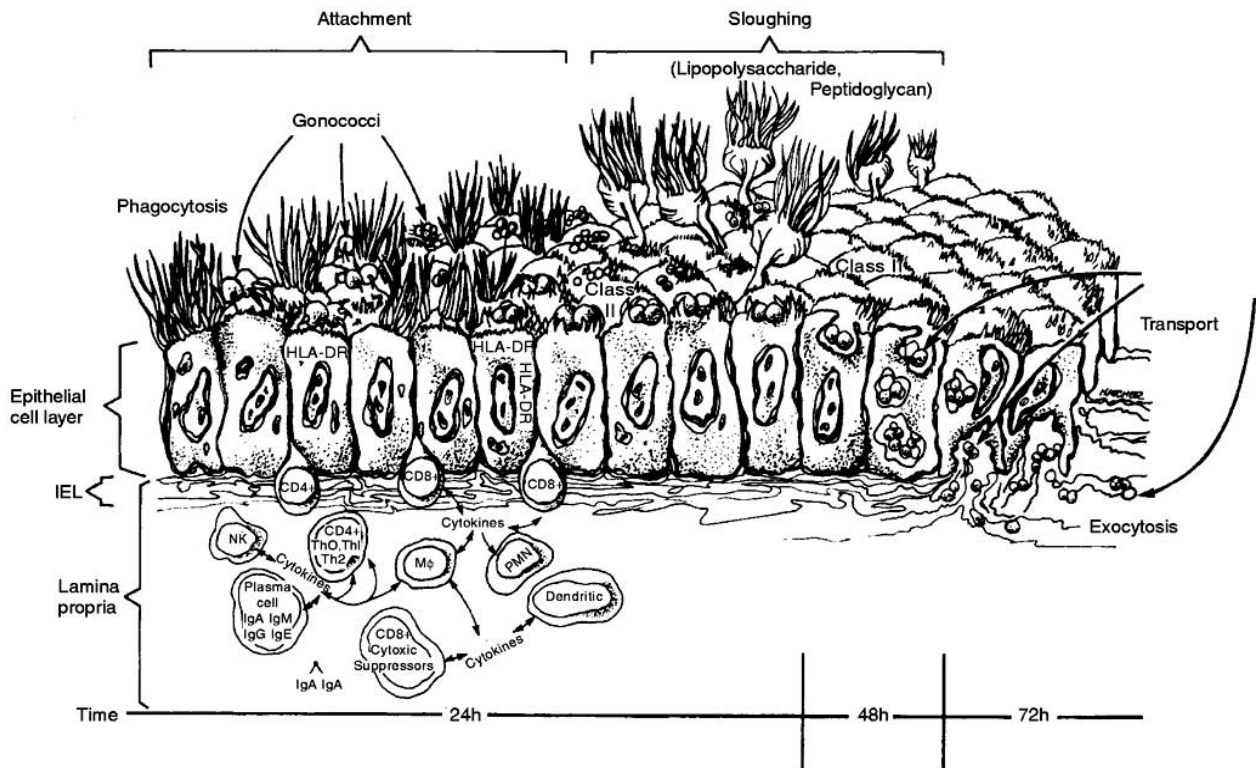


Figure 1 Immunobiology of gonococcal infection of the human fallopian tube mucosa. Gonococci attach to the microvilli on nonciliated epithelial cells and are internalized by a phagocytic mechanism that results in a membrane-bound vesicle. This process takes approximately 24 h. At the same time, the gonococci liberate from their surface two molecules (lipo-oligosaccharide and peptidoglycan) that are toxic to the ciliated epithelial cells and they slough as a result of this action. Gonococci continue to migrate through the epithelial cell and by 72 h after infection they have begun an orderly exocytosis from the epithelial cell. At this point in the infection, the gonococci are present in the lamina propria where they can encounter a variety of immunocompetent cells to initiate both B and T cell responses as well as the increased production of cytokines (e.g. TNF α). (Reproduced from Cooper and Moticka (1996) by copyright permission of Blackwell Science.)

cannot be cultured on artificial media has hampered studies to characterize the organism and study the immune response to the infection. No toxin production has been documented.

After infection with *T. pallidum*, polymorphonuclear leukocytes are attracted to the site where treponemas are killed. Activated macrophages also phagocytose and kill *T. pallidum*. Specific responses to treponemal infection involve both T and B cells. The initial humoral response is the production of antibodies which are nonspecific for *T. pallidum*. These are detected by serological tests such as the Venereal Disease Research Laboratory (VDRL) or the RPR (rapid plasma reagin) tests which are used to screen for syphilis.

By the time of the primary infection (chancre), specific humoral and cell-mediated immune mechanisms have been activated. Treponema-sensitized lymphocytes and activated macrophages are present in the lesions and antitreponemal antibody is detectable in serum. This leads to a decrease but not elimination of the treponemas. In the absence of treatment

incompletely understood events occur and a generalized infection called secondary syphilis occurs. The time and appearance of disseminated lesions suggest that there is a hematogenous spread throughout the body. The development of systemic immunity modulates the appearance and severity of the lesions. The infected individual has high nonspecific as well as specific antibody titers. However, delayed-type hypersensitivity (DTH) is usually absent.

Eventually the host suppresses the infectious process sufficiently and lesions are no longer apparent. This leads to a period of clinical latency. By this point, cell-mediated immunity is now present. Immobilizing treponemal antibody is present but it is difficult to demonstrate a protective humoral immune response. Even during latency, *T. pallidum* can be isolated from both human and experimentally infected animals. The spirochetes may be sequestered in an immunologically privileged site, e.g. the central nervous system. The infection may remain latent for the remaining natural course of the disease or manifest itself as tertiary syphilis.

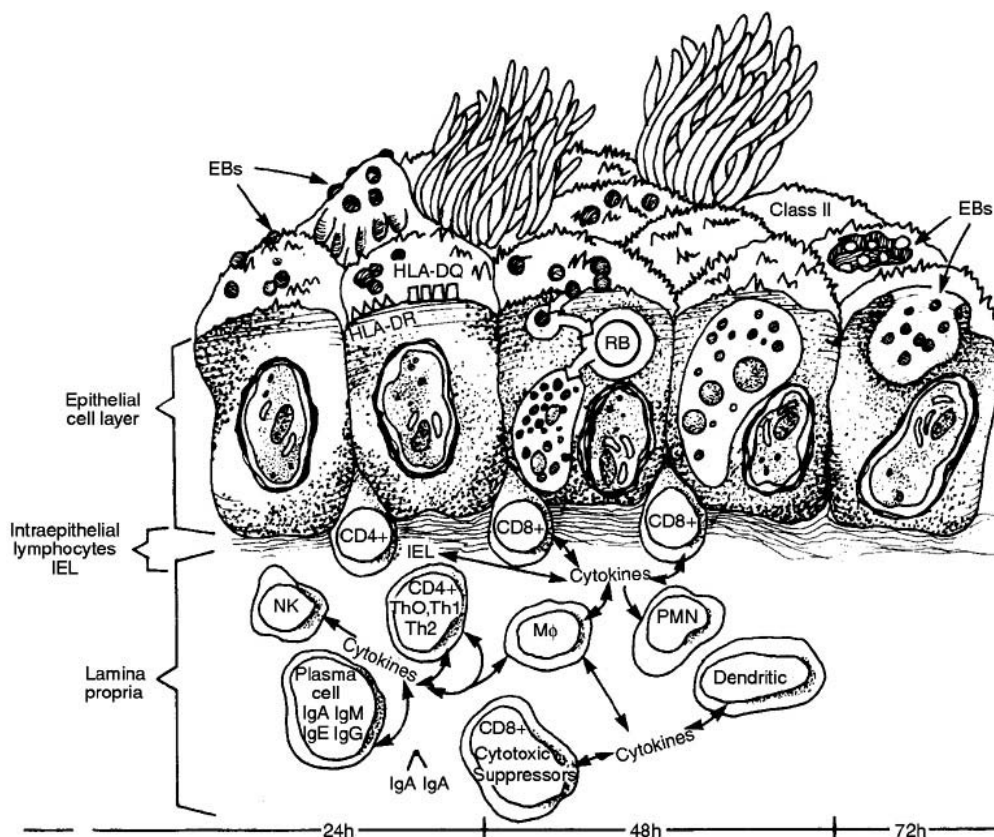


Figure 2 Immunobiology of chlamydial infection of the human fallopian tube mucosa. Elementary bodies (EBs) attach to the microvilli of epithelial cells prior to invasion of the epithelium. In some cases, where EB aggregates are large, a local cytotoxicity is seen. The chlamydia have a normal developmental cycle in the epithelial cells but do not usually penetrate the basolateral portion of the cell. The EBs cause a lesion in the luminal surface of the infected epithelial cells and are released where they are capable of infecting other cells. (Reproduced from Cooper and Moticka (1996) by copyright permission of Blackwell Science.)

Since the natural history of the disease is unique and much remains to be understood about the biology of the spirochete, discussions regarding the development of a vaccine are premature.

Human papillomavirus

The human papillomavirus (HPV) belongs to the family Papovaviridae and up to 100 types have been recognized. The HPVs are naked viruses with a double-stranded DNA genome. Clinically they cause genital warts or condyloma acuminata affecting the mucosal lining of the genital tract as well as cutaneous sites. Warts are generally benign, self-limiting tumors which may regress spontaneously. All layers of the epithelium are represented in the wart and the basement membrane is not breached. The virus enters one or a few cells in the epithelium (basal germinal layer) presumably through a traumatic process. The virus replicates exclusively in the nucleus and cell growth is stimulated.

Several human and animal papillomaviruses are

associated with naturally occurring cancers. There is a long latency period with the need for a number of cofactors for the progression of the papillomas to malignancy. This is consistent with a multistep mechanism of carcinogenesis. It is speculated that papillomavirus infection leads to 'immortalization' of the cells (the resulting warts show abnormal mitosis in the suprabasal layers of the epithelium). However, a second event (ultraviolet light, X-irradiation, cellular mutations, etc.) is required for the development of the invasive tumor. More than 90% of the cervical cancers contain HPV DNA, usually types 16, 18 or 31. These same HPV types are found in the precursor lesion or cervical intraepithelial neoplasia. The detection of HPV mRNA in cervical cancer tissues indicates that HPV genomes is expressed in these tissues. Many epidemiological investigations demonstrate a close relationship between HPV infection and cervical cancer.

Warts increase in number and size in conditions that suppress T cell functions (i.e. pregnancy and organ transplantation). Histological examination of

regressing warts indicates the presence of a cell-mediated immune response. Viral antigens which have been obtained from clinical lesions have been used to study humoral and cell-mediated immune responses to HPV infections with limited success. There is no vaccine currently available for HPV.

Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2)

There are several features which distinguish herpes viruses from other viruses. The genome is a linear double-stranded DNA ranging from 125 000 to 250 000 base pairs. The virion is composed of the DNA-containing core; an icosahedral capsid, which is about 100 nm in diameter and exhibits 162 morphological units (capsomeres) on its surface and an outer lipid-containing membrane or envelope. These viruses use both the nucleus and the cytoplasm of the host cell for their replication.

The pathology and epidemiology of herpes virus infections depend on the mode of viral replication, associated cytotoxicity as well as their capacity to cause latent infections. Clinically many of the infections caused by HSV-1 and HSV-2 are indistinguishable. Despite the fact that these strains are predominantly isolated from different anatomical sites, it is clear that strains of either type can initiate and establish infection at either genital or extragenital sites.

HSV rarely causes severe disseminated disease in adults, except when cell-mediated immunity is compromised. Among infected individuals who have no known immunologic defects, the responses to infection typically range from no clinical signs and symptoms to localized lesions. Mild to moderate systemic symptoms followed by periodic recurrence of localized lesions may occur. Asymptomatic genital infections are responsible for a significant number of transmissions. Genital HSV infections are a significant public health problem. It is estimated that 1 in 4 Americans are infected with HSV. Cell-mediated immune deficiency rather than antibody synthesis is associated with increased incidence of disseminated disease caused by HSV. It is likely that cell-mediated effector mechanisms are more important for preventing the spread of infections. Recurrent localized herpetic lesions can occur in the presence of high antiviral antibody titers. Despite this fact, antibodies are important. Many interrelated issues occur between cell-mediated immunity and the humoral immune response that regulate immediate antimicrobial effector responses.

It has been suggested by studies on protective immunity to HSV infections that the envelope glycoprotein antigens have the most likely potential to be

protective. However, it has been difficult to predict which antigens are most important in inducing protective cell-mediated responses. Some of the viral glycoproteins expressed by HSV may not only induce immune responses but also modulate effector mechanisms in ways that have not been precisely defined. Immune responses to viral antigens can, in some instances, cause as much or more damage in affected tissues as the cytopathology associated with viral replication.

Live attenuated as well as subunit HSV vaccines have been studied. The disadvantage of live vaccines is that even an attenuated HSV strains can establish a latent infection. The study of subunit vaccines revolves around the myriad of viral proteins, which is the best immunogen for protective immunity and methods to present them to the host. Therefore, the promise of an effective vaccine is not immediately apparent.

Other reproductive tract pathogens

Urethral colonization by gram-negative enteric bacilli, such as *Escherichia coli*, cause bacteriuria with no inflammation. The nonsexually transmitted diseases of the reproductive tract are usually caused by enteric organisms, e.g. *E. coli*, but can often be polymicrobial. The uropathogenic strains of *E. coli* possess pili that allow ready adherence to urogenital epithelial cells.

The mucosal surfaces which are exposed to the external environment are under a continual state of bacterial colonization. Local immunity is generated primarily by the secretory immune system and is characterized by the secretion of immunoglobulin (Ig) classes in levels different than in serum. The mucosal immune system of the reproductive tract has a different compartmentalization to that of other mucosal systems. In the genital mucosa there is a lack of organized tissue (i.e. Peyer's patches) and specialized cells (i.e. M cells). These differences in the construction of the local immune apparatus may account for the difficulty in generating local protective immunity against many reproductive tract pathogens. A better understanding of the local mucosal system in reproductive tract tissue will aid in the development of effective protective immune responses to the infections which predominate in the reproductive tract.

See also: Acquired immune deficiency syndrome (AIDS); *Candida*, infection and immunity; *Chlamydia*, infection and immunity; Cytomegalovirus, infection and immunity; Epstein-Barr virus, infection and immunity; *Escherichia coli*, infection and immunity;

Herpes simplex virus, infection and immunity; IgA; Immunodeficiency, primary; Mucosal immunity; Mucosa-associated lymphoid tissue (MALT); Mycoplasma, infection and immunity; Neisseria, infection and immunity; Opportunistic infections; Papillomavirus, infection and immunity; Treponema, infection and immunity; Urinary tract infections.

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REPTILIAN IMMUNE SYSTEM

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Reptiles evolved from primitive amphibians nearly 300 million years ago during the Carboniferous period. They sprang from a basic stock, the long-extinct order Cotylosauria or 'stem reptiles'. A side-branch of stem reptiles led to the turtles and tortoises: order Testudines = Chelonia. The closest surviving descendants of the cotylosaurs are the tuatara that live on a few islands off New Zealand. Descended from ancient forms related to the tuatara is the much more successful order of the Squamata comprising the lizards (suborder Lacertilia) and snakes (suborder Ophidia = Serpentes). Stem reptiles also gave rise to other lines. One of these lines led to the crocodilians and modern birds; another led to the mammals. Reptiles are not only the closest ancestors of the birds and mammals, but are also the first vertebrates to exhibit the change from free-living larvae, as in fish and amphibians, to an embryo completely protected by an amnion similar to that found in avian and mammalian embryos. Therefore, study of immune processes in modern-day reptiles holds the promise of offering significant clues to the origin of certain facets of the immune response.

Reptiles are moderately abundant in the tropics, but less common in temperate zones and absent from cold climates. They are often said to be 'cold-blooded' like fish and amphibians as opposed to the 'warm-blooded' birds and mammals. This means

that reptiles lack the ability to regulate their metabolic heat for the production of sustained body warmth and a constant temperature. So, their temperature changes with that of their surroundings, hence the name poikilotherms, and they depend on an external source, rather than their own metabolism, for body warmth, i.e. they are ectothermic. Consequently, the structure and functional activity of several reptilian organs, including those of the immune system, are greatly modulated by environmental conditions.

Lymphoid organs and cells

The yolk sac is the first and major hematopoietic organ in reptilian embryos. Later in development, blood cell formation occurs in fetal spleen and fetal liver. Hematopoiesis in bone marrow starts near the end of embryonic development and persists throughout life. The thymus, the first organ to become lymphoid during reptilian ontogeny, is a bilateral organ lying on either side of the neck in lizards and immediately anterior to the heart in snakes; it is usually multilobulated in turtles. The thymic primordium originates as dorsal outgrowths of pharyngeal pouches II and III in lizards, IV and V in snakes and III and IV in turtles that detach from the pharyngeal wall and appear as a condensation of epithelial cells

surrounded by mesenchyme, with no capsular device yet formed. Thymic lobes increase in size and fibroblasts condense around the organ to form a distinct capsule. The epithelial thymic rudiment is then invaded by bloodborne large, basophilic, undifferentiated cells, which divide and mature into large, medium and small lymphocytes. A complete demarcation into cortex and medulla is apparent just before hatching (or birth). Later on, the thymus lobes show a peripheral cortex loaded with lymphocytes, and central medulla with fewer thymocytes so that the epithelial groundwork is easily discernible. Numerous macrophages and large and small, round, oval or elongate myoid cells are seen in the medulla and corticomedullary junction; cross-striations similar to those of skeletal muscle are evident in some myoid cells. Dendritic-like cells, with numerous labyrinthine processes forming gap-like junctions with the neighboring lymphocytes, are frequent in the medulla of the turtle *Mauremys caspica*. These cells lack Birbeck granules and the extensive membrane-membrane contacts like those reported for mature interdigitating cells of mammalian lymphoid organs, but often contain abundant cell debris and dense bodies, suggesting a certain degree of phagocytic activity. It is important to note that the thymus appears so well developed only in reptiles collected from the field, or maintained in the laboratory under suitable conditions of temperature, photoperiod and feeding, during optimal, species-specific, seasonal conditions (Figure 1). Otherwise, the thymus shows various degrees of degeneration, whereby the number of lymphocytes in the cortex and medulla greatly decreases and numerous myoid cells and epithelial cysts are observed. Reptilian thymic cysts range from minute intracellular structures to formations over 0.1 mm in diameter. They probably represent foci of cellular degradation segregated from surrounding thymic parenchyma by a continuous layer of epithelial cells. Involution continues with persistence of the adverse environmental conditions. Cortical epithelial cells degenerate and dendritic cells are absent. Eventually, the thymus may transform into fibrous tissue and become scarcely detectable. This involution is totally reversible, as in suitable seasonal and other ambient conditions rebuilding of the thymus begins and progressively proceeds to a mammalian-like picture and organization. The issue of whether the degenerated epithelial cells found in involuted thymus are eliminated and substituted by a new cell population, or recover with the advent of optimal seasonal conditions, remains unresolved as yet; nor have the waves of thymocyte production in the rejuvenating thymus been investigated.

The spleen in reptiles is the most important peri-

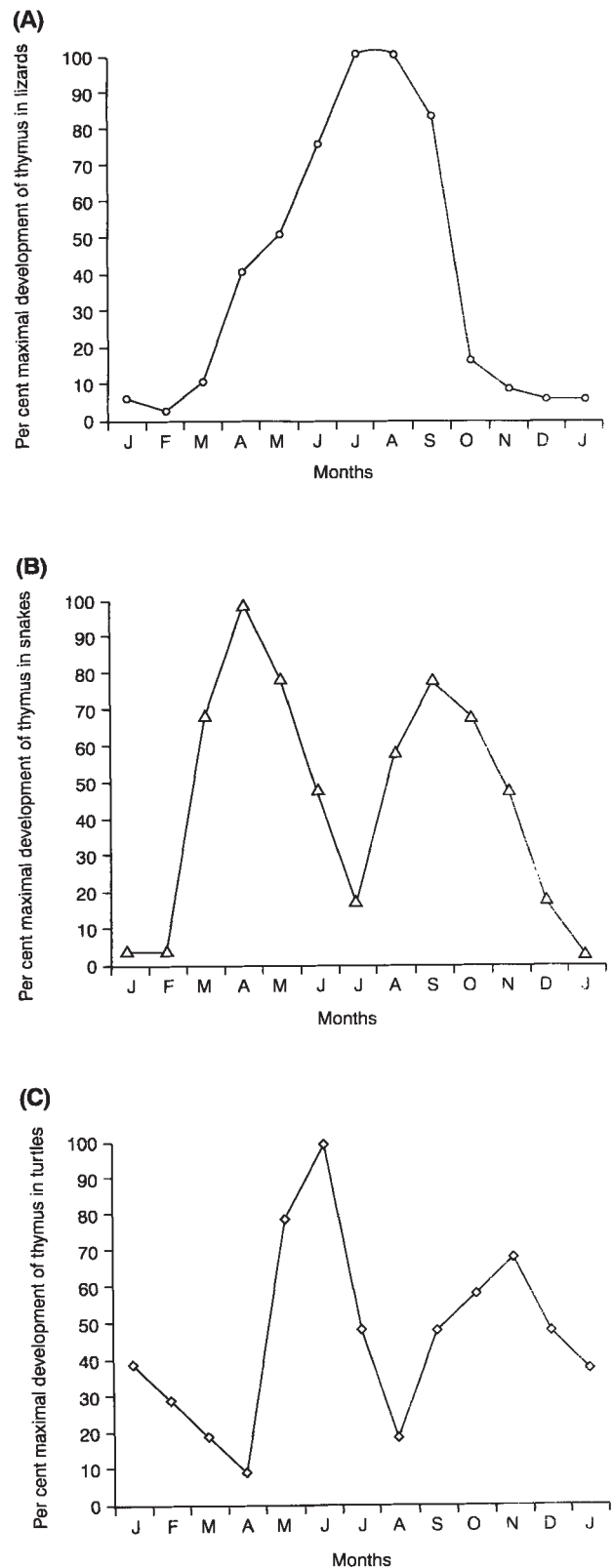


Figure 1 Development profile of thymus in: (A) lizards; (B) snakes and (C) turtles the year round.

pheral lymphoid organ. Histogenesis of the spleen commences with a thickening of the dorsal mesogastrium. The splenic rudiment then buds into the body cavity; later, blood sinuses develop as separate cavities in the mesenchyme, and spleen acquires its characteristic form by development of the capsule. The spleen in lizards, snakes and tortoises becomes lymphopoietic much later than the thymus, which is fully differentiated before any lymphocytes appear in the splenic rudiment. However, thereafter the spleen shows intense hematopoietic and lymphopoietic activity. After hatching and in adult life the spleen usually reveals well-developed and clearly defined white pulp, arranged around central arterioles, and red pulp, composed of blood sinusoids and cell cords. In the white pulp the framework of reticular cells contains macrophages, dendritic cells predominantly located in the periphery of the white pulp, and numerous densely packed lymphocytes. There is evidence to indicate that 50–70% of reptilian splenic lymphocytes are thymus derived (T-like cells) and are located in thymus-dependent juxta-arteriolar areas. The remaining lymphocytes are surface immunoglobulin (sIg) bearing, homologous to the B cells of aves and mammals. In lizards and tortoises, 25–50% of splenic lymphocytes are sIgM⁺, and 5–20% sIgY⁺. The sIgY⁺ lymphocytes coexpress sIgM. Germinal centers and nodules typical of mammalian secondary lymphoid organs are lacking in reptiles. As in the thymus, the “ideal” spleen architecture is only observed in reptiles living in optimal conditions. Otherwise, the splenic lymphoid aggregates regress in size, and the number of T-like cells sharply diminishes. A significant proportion of sIg⁺ lymphocytes survive even in animals exposed to rigorous environmental conditions (Figure 2). This suggests that reptilian T- and B-like cells differ in their sensitivity to environmental factors. The drastic depletion of splenic T-like cells is, however, reversible (Figure 2). It is indeed now established that the lymphoid elements of adult reptiles atrophy and regenerate, in a periodic manner, with the cycle of the species-specific adverse and optimal seasons.

Gut-associated lymphoid tissue (GALT) develops only after hatching in lizards, snakes and chelonians. Although reptiles do not possess tonsils, Peyer's patches or an appendix, numerous lymphoid aggregates occur in the mucosa and submucosa along the gut in most reptiles and consist of lymphocyte populations which exhibit differential sensitivity to seasonal conditions. In the lizard *Chalcides ocellatus*, esophageal lymphoid aggregates diminish considerably in size and number 1) during autumn and winter as compared with spring and summer, 2) after adult thymectomy, and 3) following hydrocortisone treat-

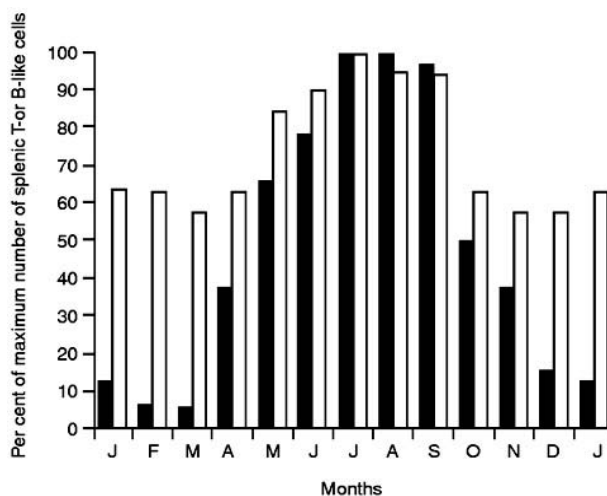


Figure 2 Number of splenic T-like (■) and B-like (□) cells of the lizard, *Chalcides ocellatus*, the year round. (Adapted from El Ridi R and Kandil O (1981) Membrane markers of reptilian lymphocytes. *Developmental and Comparative Immunology* 5(Suppl 1): 143).

ment, suggesting they consist essentially of T-like lymphocytes. Conversely the lymphoid accumulations of the small and large intestine are resistant to seasonal changes, adult thymectomy and hydrocortisone treatment, thus implying they are constituted essentially of B-like cells and that GALT in reptiles is a major reservoir of B cells. Additionally, adult splenectomy in lizards does not impair immune antibody responsiveness to foreign erythrocytes, and the nearly GALT-less lizards *Agama stellio* and *Chamaeleon chamaeleon* produce but a negligible amount of specific antibodies when confronted with various mammalian erythrocytes or human serum albumin, via the intraperitoneal, intramuscular or subcutaneous routes, and whether or not the antigen is incorporated in Freund's adjuvant. These findings indicate that the humoral immunological tasks in reptiles are shared between the spleen and GALT, and support the possibility that part of the GALT represents a bursa of Fabricius equivalent. T- and B-like cells constitute essentially 75–90% and 10–25% of peripheral blood lymphocytes (PBLs) of adult lizards, snakes and tortoises, with wide, season-dependent variations. In conclusion, despite the absence of true lymph nodes, the reptilian lymphoid system is homologous to that of mammals, from which it differs, however, by its exceptional sensitivity to ambient conditions.

T cell functions

Mitogen responsiveness in reptiles was first described in *Alligator mississippiensis* by Cuchens and Clem,

whereby PBLs were shown to respond by powerful proliferation to concanavalin A (Con A) and lipopolysaccharide. Significant Con A-mediated mitogenesis is consistently observed in thymocytes, spleen cells and PBLs of lizards and snakes. Acute graft-versus-host reaction is readily induced in newly hatched (or born) turtles and lizards following intraperitoneal injection with spleen cells from adult allogeneic donors. Reptilian lymphocytes are also able to respond powerfully to alloantigenic cells in the mixed leukocyte reaction (MLR). Significant responses are obtained in 50–90% of random MLR cultures performed, depending on the species examined, culture conditions and putative major histocompatibility complex (MHC) gene differences between responder and stimulator. Data gathered in several species suggest that the lymphocyte activating determinants in MLR are not extremely polymorphic in reptiles. Most importantly, seasonal and other environmental parameters, including captivity, malnutrition, etc., have an extremely strong impact on reptilian T cell functions. Deterioration in these ambient factors is paralleled by significant impairment, up to complete abrogation, of the different aspects of T cell responsiveness. So, in inadequate seasonal and laboratory conditions mitogen and MLR responsiveness is abolished (Tables 1 and 2). The severe impact of ambient factors on T cell functions, together with the putative low polymorphism of reptilian MHC class I/II loci, may explain why chronic graft rejection was thought to be the rule in reptiles. But in optimal seasonal conditions, a significant proportion of lizards and snakes reject skin allografts in the acute manner typical of mammals (Table 3). Anamnestic responses characterized by an earlier onset and a more rapid rejection time are invariably observed. These findings indicate that reptiles display the major functional markers of the

Table 1 Lizard lymphoproliferative responses to Con A and in MLR^a

Season	SI in response to			
	Con A		Allogeneic cells	
	Range	Mean	Range	Mean
Spring	1–4	2.2	1–10	5.1
Summer	2–7	3.1	2–21	6.8
Autumn	0.5–1.5	1.1	1–11	2.6
Winter	0.5–1.1	0.6	1–2	1.5

^aTypical lizard lymphoproliferative responses to Con A and to allogeneic cells in mixed leukocyte reaction (MLR) expressed as stimulation index (SI) (mean counts per minute of quadruplicate test cultures/mean counts per minute of mitogen- and alloantigenic cell-free control cultures.)

Adapted from Saad and El Ridi (1984, 1988).

Table 2 Snake lymphoproliferative responses to Con A and in MLR^a

Season	SI in response to			
	Con A		Allogeneic cells	
	Range	Mean	Range	Mean
Spring	2–20	9.4	1–15	5.5
Summer	1–2	1.7	1–4	2.0
Autumn	2–8	5.1	1–7	2.5
Winter	1–2	1.6	1–2	1.1

^aTypical snake lymphoproliferative responses to con A and to allogeneic cells in mixed leukocyte reaction (MLR) expressed as SI (mean counts per minute in quadruplicate test cultures/mean counts per minute in mitogen- and alloantigenic cell-free control cultures.)

Adapted from Farag and El Ridi (1985, 1986).

Table 3 Skin graft rejection in lizards and snakes^a

Species	Per cent	Rejection duration (days)	Mean $\pm$ SE (days)	Manner
Lizards	33	11–14	11.4 $\pm$ 0.7	Acute
	52	15–20	16.6 $\pm$ 0.1	Subacute
	15	21–24	21.7 $\pm$ 0.2	Chronic
Snakes	52	8–15	13.8 $\pm$ 0.3	Acute
	21	18–21	20.1 $\pm$ 0.4	Subacute
	27	25–40	31.6 $\pm$ 0.9	Chronic

^aTypical manner of primary skin allograft rejection in lizards *Chalcides ocellatus* ($n = 48$) and snakes *Psammophis sibilans* ($n = 48$) during spring and summer, respectively. Under nonoptimal seasonal and environmental conditions, skin allograft rejection is invariably chronic. Adapted from Farag and El Ridi (1990) and Afifi *et al* (1993)

MHC. These MHC markers were furthermore shown to be functionally linked in the snake *Psammophis sibilans*, as animals which reject skin allografts acutely manifest strong MLR and cell-mediated lympholysis (CML) to donor leukocytes, while animals which reject their skin allotransplant in a chronic manner do not react significantly to the donor leukocytes in MLR or CML. Genetic studies of the antigens inducing graft rejection, MLR and CML should be performed to examine whether these functionally linked markers are genetically linked as well. Some anti-chicken MHC sera (one against MHC class I α chain, two against heterodimer) immunoprecipitate putative class I molecules of 40, 45 and 90 kDa from iodinated *Psammophis sibilans* blood cells. The precipitation of the snake bands is immunogen inhibitable. Additionally, a 19 kDa snake band is recognized by snake alloantisera and by rabbit sera directed to chicken and human MHC class I complex or β_2 -microglobulin; the immunoprecipitation of this band is inhibited by chicken or human β_2 -microglobulin, but association with a class

I α chain has not been shown. However, the polymerase chain reaction was used by Grossberger and Parham to isolate clones with class I MHC sequences from lizards and snakes. All the sequences show the expected evolutionary relatedness. A small number of amino acid residues are conserved in the class I heavy chain sequences. These invariant residues are found symmetrically clustered in the first two domains contributing to intra- and interdomain contacts, but none are present at the peptide-, T cell receptor- or CD8-binding positions.

Antibody production

Reptiles synthesize at least two classes of immunoglobulins. An 850–900 kDa Ig related to mammalian IgM consisting of five disulfide-bonded 7S subunits, each of which is composed of two heavy (H) and two light (L) chains. The molecular mass of the H and L chain is approximately 70 and 21–23.5 kDa, respectively. The second Ig is of lower molecular mass (180 kDa) and shows a close antigenic relationship to the 7S Ig of birds and amphibians, but only a distant relationship to mammalian IgG. Thus, it has been termed IgY, and current evidence indicates that it is the evolutionary ancestor of mammalian IgG and IgE as well. IgY consists of two disulfide-bonded H chains and two L chains of, respectively, 50–63 and 21–26 kDa. The H (or ϵ) chain typically possess one variable and four constant (C) region domains. A truncated (120 kDa) IgY, lacking the two C-terminal domains, is found in some reptiles, particularly turtles.

All existing groups of reptiles are capable of powerful humoral immune response to a variety of antigens: bacteria, protozoa and heterologous erythrocytes and proteins. Primary antibody production is detectable after a lag period of 5–15 days and peaks at 20–60 days postimmunization, depending on the species, antigen type, route and dose, environmental factors (season, temperature, feeding) and hormonal status. Antibody titers then wane gradually. In several reptilian species a secondary response is demonstrable, characterized by a shorter latent period, a significantly higher peak level, and persistence for a long period of time. This indicates that immunologic memory is present in reptiles. It is important to note that immunologic memory is maintained through adverse seasonal conditions, as a very strong anamnestic antibody response can be induced in reptiles in adverse seasons, provided the animals have received a primary immunization in optimal environmental conditions.

Thymectomy of adult lizards has revealed that the thymus is a source of T helper cells that control the

kinetics and magnitude of the humoral immune response. This finding is supported by season-related circumstantial evidence. In fact, under adverse seasonal conditions, reptiles appear as adult thymectomized, do not possess T cells in the peripheral lymphoid organs and are unable to display primary antibody responses, despite the persistence of the majority of B cells in spleen and GALT. Thus, antigen recognition and binding (by B-like cells) occur normally, yet the subsequent proliferative and differentiating events are inhibited, and no serum antibody is detectable. This lack of antibody formation may well be attributed to an absence of T cell help and/or T cell-derived lymphokines, and appears not be due to an inherent inability of the lymphocytes to synthesize or release Ig as a result of low body temperature or malnutrition, as powerful secondary antibody responses can be elicited in reptiles, even during winter.

Mechanisms of changes in reptilian immune system

Body temperature is a salient factor regulating the performance of reptilian immunological functions. It must be noted in this regard that reptiles, in contrast to fish and amphibians, have become restricted in the temperature range over which physiological activities, including immune responses, can occur. Photoperiods, humidity and nutrition are also critical modulators of reptilian immune processes. Endogenous rhythmic factors that are not as yet characterized appear also to control immune reactivity independently of external conditions. Thus, it was not possible to induce thymus regeneration in winter reptiles even if they were maintained in optimal environmental conditions, including the species-specific preferred temperature.

Studies in lizards and turtles have documented that the impact of seasonal and other environmental factors on the immune system is mediated by fluctuations in levels of circulating corticosteroids and sex hormones. Therefore, reptiles represent unique natural models for studying the interrelations of the neuroendocrine immune systems in vertebrates.

See also: Bursa of Fabricius; IgE; IgG; IgM; MHC, evolution of; Mixed lymphocyte reaction (MLR); Phylogeny of the immune response.

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RESPIRATORY AND CARDIAC INFECTIONS

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Historically, infections of the cardiorespiratory system have resulted in substantial morbidity and mortality. This has been diminished but not eradicated by the introduction of antibiotics. Despite the continuing evolution of more sophisticated antimicrobial drugs, infections, particularly of the respiratory tract, are a major cause of hospital admission and death. Recent studies suggest that the annual incidence of community acquired pneumonia is 2–4 per 100 population in the UK while bacterial endocarditis has an estimated yearly incidence of 11 cases per 100 000 population in an urban area in the USA. There is a degree of overlap both in the type of organism that infects the respiratory tract and the heart and also in the host response to this challenge. However, the differing anatomy makes it more appropriate to consider each system in turn. Overall there is a greater prevalence of respiratory infections.

Respiratory infections

These are divided into two anatomically separate categories. First, there are those of the upper respiratory tract, which extends from the nose to the vocal cords. These include the common cold (coryza), sinusitis, pharyngitis, laryngitis and epiglottitis. Secondly, there are infections of the lower respiratory tract. These may affect the large airways (bronchitis), the alveoli and parenchyma (pneumonia) or the pleura space, leading to an empyema. The source of infection is variable. Droplet inhalation is the most frequent although pathogens may be introduced to the lung by alternative routes, such as the aspiration of pharyngeal contents as seen in neurological conditions leading to bulbar palsy and defective swallowing, haematogenous spread as in miliary tuberculosis or staphylococcal septicemia, and direct extension from surrounding tissues. In patients with comorbid illness, impaired host responses or damaged respiratory tract, organisms normally regarded as nonvirulent can become pathogenic and cause

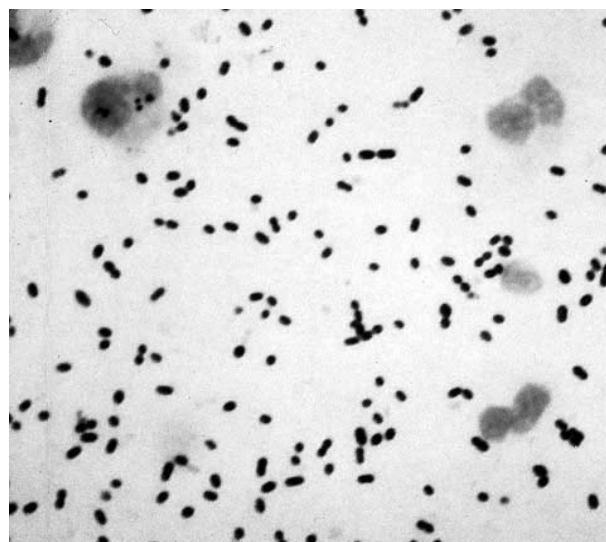
Table 1 Community-acquired pneumonia in British hospitals

Organism	Proportion of microbiological diagnoses (% total)
<i>Streptococcus pneumoniae</i>	34
<i>Mycoplasma pneumoniae</i>	18
Influenza A virus	7
<i>Haemophilus influenzae</i>	6
<i>Chlamydia psittaci</i>	3
Others	
<i>Legionella pneumophila</i>	
<i>Streptococcus</i> species (others)	
<i>Coxiella burnetii</i>	
<i>Staphylococcus aureus</i>	
Enterobacteriaceae	
Microbiologically negative	33

Adapted from the British Thoracic Society/Public Health Laboratory Service (1987).

infection. This is common in some immunocompromised conditions such as the acquired immune deficiency syndrome (AIDS).

The commonest cause of pneumonia encountered in the community is the bacterium *Streptococcus pneumoniae*, followed by *Mycoplasma pneumoniae* and influenza virus (see Table 1). The diagnosis is made clinically and with the aid of investigations such as chest radiographs (Figure 1), an elevated white cell count and the growth of an organism on culture of sputum, respiratory secretions (Figure 2) or blood. Indirect techniques such as complement fixation or indirect immunofluorescence tests are

**Figure 1** Chest radiograph of right lower lobe pneumonia. (Courtesy of Dr S Fowlie.)**Figure 2** Gram stain of *Streptococcus pneumoniae* in bronchoalveolar lavage. (Courtesy of Dr F Donald.)

used to detect infection by some organisms e.g. *Mycoplasma*, *Legionella*, *Chlamydia* and influenza virus, while pneumococcal capsular antigen can be detected in sputum, urine, pleural fluid and blood. Isolation of an organism is helpful in assessing the appropriateness of treatment, but in practice most cases are treated empirically on a 'best guess' policy as many laboratory results appear too late to aid initial clinical management.

Cardiac infections

The commonest infection of the heart affects the valves. Previously damaged valves when exposed to bloodborne infection may become colonized by microbes. The organisms involved are listed in Table 2. Untreated, the consequence of progressive destruction of the valve with associated thrombus formation and risk of embolization can be fatal. Even with prompt antibiotics and valve replacement surgery the

Table 2 Organisms that can cause infective endocarditis (in order of frequency)

<i>Streptococcus</i> species (e.g. <i>S. viridans</i> , <i>S. bovis</i> , <i>S. pneumoniae</i>)
<i>Enterococcus faecalis</i>
<i>Staphylococcus</i> species (e.g. <i>S. aureus</i> , <i>S. epidermidis</i>)
Gram-negative bacteria
Fungi
Other organisms
Culture-negative

Adapted from Kaye (1992).

outlook is still uncertain. The myocardium is vulnerable to infection, leading to myocarditis. Viral infection, particularly by coxsackievirus B and echovirus, can lead to myocardial damage, possibly secondary to the immune response elicited. Infectious pericarditis is usually of viral etiology but can be secondary to tuberculosis which, if untreated, leads to typical calcification seen on radiography.

Immune responses of the host

The host has a variety of mechanisms developed to prevent infection of the respiratory tract. These include a number of physical barriers, such as the filtration of large particles by nasal hairs and the branching structure of the bronchial tree causing impaction of inhaled particles which are subsequently removed by the epithelial ciliary action. The epithelium is coated with a layer of secretions, including surfactant which has antibacterial properties, immunoglobulins, complement and mucus which also acts as a physical barrier to pathogens. All components of the immune system have a role in protecting the respiratory system. The principal phagocytic response initially comes from the resident alveolar macrophages and also neutrophils that are recruited by the inflammatory response via the complement system to concentrate in areas of infection. The lymphocytes appear to coordinate the antigen-specific immunity, while the role of other components, such as eosinophils, mast cells and dendritic cells, are not yet fully understood. All antibody subtypes are found in the lung although immunoglobulin A (IgA) predominates in the bronchial secretions.

Clinically, the importance of the immune system is illustrated during its impaired function secondary to disease. Thus patients with generalized abnormalities of immunoglobulin production such as asplenic states, agammaglobulinemia, and myeloma are prone to recurrent bacterial sepsis. This particularly involves encapsulated microorganisms such as *Streptococcus pneumoniae* and some strains of *Haemophilus influenzae*, leading to repeated sinusitis, pneumonia and bronchiectasis. IgA deficiency may be asymptomatic although there can be an increase in upper and lower respiratory infections. Neutropenia increases the risk of many infections, especially those by gram-negative enteric bacilli (GNEB), such as *Escherichia coli* and *Pseudomonas aeruginosa*. An impaired T helper cell response, as associated with human immunodeficiency virus (HIV) infection, results in a greater frequency of opportunistic infections such as *Pneumocystis carinii*, a protozoan that

colonizes normal lung. These patients have a higher frequency of sinusitis, pneumonia and tuberculosis.

Evasive strategies by the organism

The ability of any pathogen to evade the host response continually evolves as the species adapts in response to its environment. To avoid opsonization and phagocytosis various strategies have developed. *S. pneumoniae* and *H. influenzae* type B both possess a polysaccharide capsule that provides greater resistance to phagocytosis. Other factors affecting the virulence of the different serotypes of *S. pneumoniae* include the production of the lytic enzymes pneumolysin and autolysin and also the presence of certain cell surface antigens. The roles of these molecules are currently poorly understood but they will have significance in the design of pneumococcal vaccines. The development of microbial resistance to chemotherapeutic agents is another response of the pathogen to environmental pressure, often due to the widespread use of antibiotics. This leads to changes in the structure of the microbe, preventing antimicrobial action. The mechanism varies. For instance, erythromycin resistance can arise when methylation of the ribosomal RNA, the site of antibiotic action, prevents antibiotic binding and hence activity, while in *Staphylococcus aureus* the production of a β -lactamase destroys penicillin and other antibiotics.

Vaccines

The development of vaccines has focused upon those organisms that cause respiratory rather than cardiac infections due to the greater prevalence of the former and thus the greater benefit of disease prevention. The two most often used are the pneumococcal and the influenza vaccine. The pneumococcal vaccine is a polyvalent compound covering the 23 most common of the 80 serotypes. In healthy adults under the age of 55 it is over 90% effective in preventing infection although in an immunocompromised population, particularly at risk of infection, the benefit is less clear. Current recommendations include those for whom the risk of contracting pneumococcal pneumonia is unusually high or dangerous. The influenza virus is constantly changing antigenically and any vaccine needs to be redesigned annually to provide immunity against this antigenic variation. Inactivated virus is used and leads to the development of serum antihemagglutinin and neuraminidase antibodies.

In the target population, the elderly and infirm, it is only 30–40% effective in preventing clinical influenza illness, although it also provides benefit by

reducing the severity of disease and its complications. Current recommendations for usage include nursing home residents and those with chronic underlying conditions such as diabetes, alcohol abuse and renal, cardiac or respiratory disease. A newer capsular conjugated polysaccharide vaccine against *H. influenzae* type B has proved highly effective in preventing meningitis in young children but also may have a role in conditions that predispose to infections with encapsulated bacteria (see above). The live attenuated BCG vaccine protects against tuberculosis infection although its usage is being re-evaluated. Other vaccines preventing respiratory disease are the attenuated live virus vaccines against measles and rubella that are standard for young children in the UK.

See also: Chlamydia, infection and immunity; Circulatory system infections; Coryneform bacteria, infection and immunity; Epstein-Barr virus, infection and immunity; Haemophilus, infection and immunity; Legionella, infection and immunity; Mycobacteria, infection and immunity; Mycoplasma, infection and immunity; Influenza virus (orthomyxovirus), infection and immunity; Paramyxoviruses, infection and immunity; Picornavirus, infection and immunity; Staphylococcus, infection and immunity; Streptococcus, infection and immunity.

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RETROVIRAL VECTORS

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Viral vectors have become one of the most widely used agents for gene transfer studies. These vectors have received so much attention primarily because they are more efficient at mediating gene transfer than other methods such as electroporation, transfection, lipofection, and particle acceleration. They act like Trojan horses to carry and deliver therapeutic transgenes into target cells. Viral vectors have been made based on retrovirus, adenovirus, adeno-associated virus (AAV), Epstein–Barr virus (EBV), herpes simplex virus (HSV), and alphaviruses including Semliki Forest virus and Sindbis virus.

Of these, retroviral vectors have received the greatest attention.

Retroviral vectors are retroviruses which have been genetically engineered to deliver therapeutic genes rather than retroviral genes to target cells. Retroviral vectors are particularly attractive because the transgenes they carry integrate into the target genome and can potentially provide permanent gene expression. Other viral vector systems have disadvantages in that they do not integrate (adenoviruses, EBV), are more pathogenic (adenoviruses, EBV and HSV), require helper viruses which are difficult to

eliminate (AAV), may be toxic to target cells (EBV, adenovirus and alphaviruses), or require transient production strategies which are subject to variability (AAV, adenovirus and alphaviruses). For these reasons, retroviruses have been the vector of choice in most of the clinical gene therapy trials to date.

Retroviruses

Retroviruses are characterized by RNA genomes which go through a DNA intermediate during replication. Retroviruses have been found throughout evolution including invertebrates and vertebrates. Retroviruses have been divided into three subfamilies based on the diseases they cause: lentivirinae, spumavirinae and oncovirinae. Of these, the oncovirinae have the simplest genomes, are the best understood, and are the most widely used as gene transfer vectors. Specifically, most retroviral vectors are based on murine type C oncoviruses such as the Moloney murine leukemia virus (Mo-MuLV).

The prototypical murine type C retroviral particle

The retroviral particle contains two copies of the RNA genome. Both RNA molecules are about 8 kb, are polyadenylated, and are positive (+) stranded, meaning they are templates for translation. These two RNA molecules are packaged in a core of retroviral proteins along with integrase, ribonuclease H, and reverse transcriptase (Figure 1). The retroviral core buds off from the producing cell, taking the

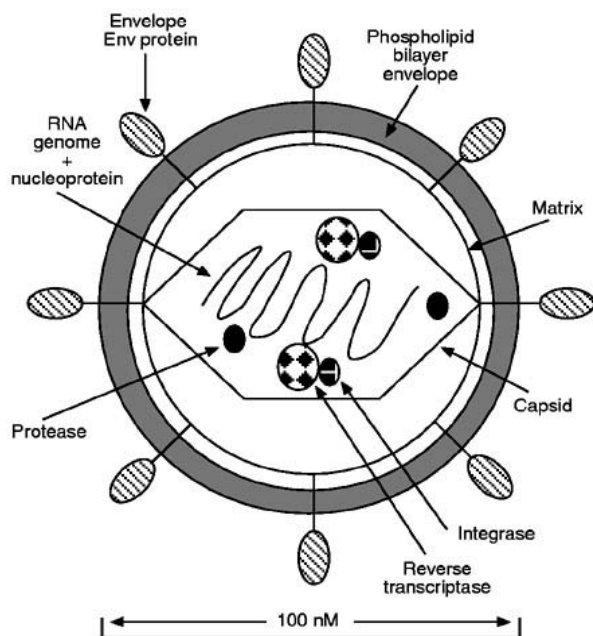


Figure 1 The prototypical mammalian type C retrovirus particle.

phospholipid bilayer with it to serve as the virus envelope. Most, but not all, cell surface proteins are excluded from the viral envelope during the budding process. A retrovirally encoded envelope protein (env) is incorporated into the phospholipid bilayer of the virus which determines the host range. This retroviral particle is highly unstable and has a half-life of around 4–8 h at 37°C *in vitro*. The half-life of murine retroviral particles *in vivo* in primates, including humans, is in the range of a few minutes due to complement and antibody-mediated inactivation. The virion is about 100 nm in diameter.

The retroviral life cycle

The retrovirus particle attaches to the target cell via a specific receptor which binds to the env glycoprotein on the viral surface. Upon binding to the receptor, the env protein undergoes a conformational change in which the fusion peptide of the env protein is thrust into the lipid bilayer of the target cell membrane. Membrane fusion results, leading to release of the viral core into the cytoplasm of the target cell. Lentiviral cores can traverse the nuclear membrane but the oncovirus cores can not. This is the reason that lentiviruses are capable of infecting nondividing cells whereas oncoviruses, including most retroviral vectors, can only infect replicating cells (they enter the nucleus as the nuclear membrane breaks down during cell division). The RNA genome is reverse transcribed into DNA as it is transported to the nucleus. The double-stranded retroviral DNA is called the provirus and it integrates randomly into the host genome after it has been replicated. Integration is mediated by host factors and the integrase enzyme from the retrovirus. The provirus then behaves like any other endogenous genetic element and is maintained through cellular division by passage to half of the progeny cells. Transcription from the integrated proviral promoter generates retroviral mRNA, allowing the life cycle to begin again.

It should be noted that reverse transcriptase lacks proofreading activity and results in a number of genetic rearrangements including point mutations, frameshifts, deletions, homologous recombinations, and nonhomologous recombinations. These mutations are responsible for generating wild-type viral diversity and may have undesirable consequences, albeit at a low frequency, in gene transfer studies.

The retroviral genome

The construction of retroviral vectors requires an understanding of which viral components are required *in cis* and which can be supplied *in trans*. The retroviral genome is approximately 8 kb in length, most of which can be deleted to make vec-

tors. The *trans*-acting elements can be deleted and are the gag, pol and env genes located in the middle of the genome. The gag gene encodes protease and structural proteins (including capsid, matrix and nucleoprotein), pol is reverse transcriptase (which includes the integrase function), and env is the envelope protein. All three of these genes are necessary for production of infective virions but can be supplied as 'helper' functions in packaging cells.

The only retroviral elements required in *cis* are the two long terminal repeats (LTRs), the packaging site (ψ), and the reverse transcription initiation sites found in the terminal portions of the provirus. The 5' LTR acts as the promoter and enhancer while the 3' LTR contains the poly-A signals, both are necessary for transcription of the retroviral mRNA. The ψ site located just downstream of the 5' LTR is the binding site for the gag polyprotein which packages the RNA into the viral core. These *cis*-acting elements need to be included in all retroviral vector designs.

Retroviral vectors

The first step in making retroviral vectors is to engineer a 'packaging' cell line. Most of the early packaging cell lines have been based on the Moloney murine leukemia virus. Investigators constructed retroviral packaging cell lines to constitutively express the Mo-MuLV helper gag, pol and env proteins by stable transfection of gag, pol and env expression plasmids into murine fibroblasts (NIH3T3 cells). Cells engineered to constitutively express gag, pol and env secrete 'empty' virion particles into the media and are called 'packaging' cells.

The next step is to get these 'empty' virions to package recombinant retroviral vector sequences. The recombinant retroviral vector is made by cloning a transgene between the LTRs in a plasmid-based retroviral vector construct. Transfection of the retroviral vector plasmid DNA into packaging cell lines results in production of retroviral vector mRNA, packaging into the retroviral core, and secretion of recombinant viral particles into the culture media. Packaging cells secreting recombinant retroviruses are called 'producer' cells and the media containing the secreted retrovirus is generally called 'supernatant'. The supernatant can then be harvested, titered, and used to infect target cells.

A number of retroviral vector designs have been developed including single gene, multigene (with additional promoters, ribosome binding sites, or IRES sequences), splicing, reverse orientation, self-inactivating (SIN), and double copy vectors (Figure 2). All of these vector constructs are plasmids

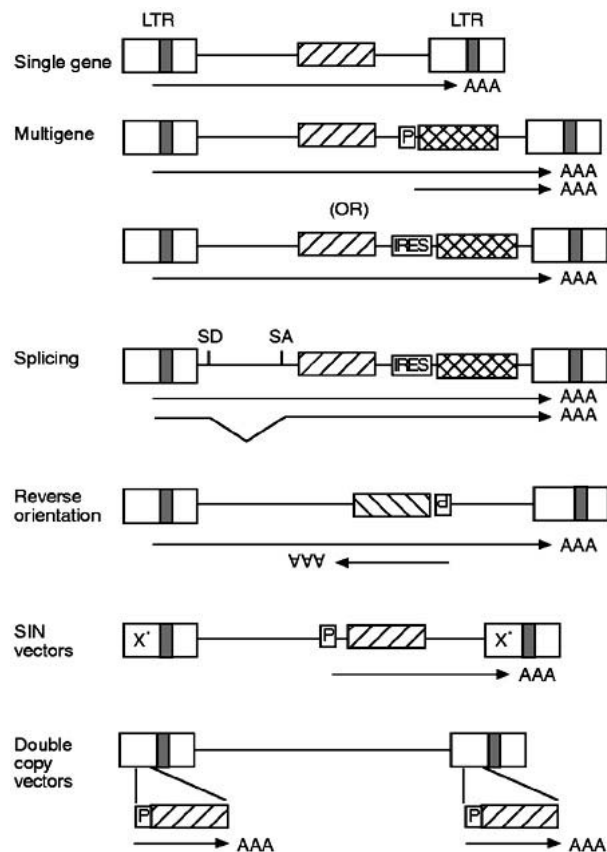


Figure 2 Types of retroviral vectors. LTR; therapeutic gene (forward orientation); therapeutic gene (reverse orientation); dominant selectable marker gene; promoter; Internal Ribosome Entry Site; AAA retroviral mRNA (forward orientation); retroviral mRNA (reverse orientation).

which contain a prokaryotic origin of replication and a prokaryotic selectable marker (AmpR).

Single and multigene retroviral vectors

Single-gene vectors contain one transgene, often transcriptionally regulated by the 5' LTR, in a retroviral construct. Multigene vectors can also be made since retroviral particles can accommodate up to 7 kb of insert DNA without affecting production of recombinant viral particles. Expression of the upstream gene is usually regulated from the 5' LTR while the downstream genes are expressed from either additional promoters or internal ribosome entry sites (IRES). The advantage of internal promoters is that one can vary expression of the downstream gene by choosing between strong viral promoters for high expression (Rous sarcoma virus (RSV) or cytomegalovirus (CMV)), tissue-restricted promoters for tissue-specific expression (albumin), or inducible promoters for inducible expression

(tetracycline promoters). IRES sequences are derived from picornaviruses (either encephalomyocarditis virus or polio virus), are about 500 bp in length, and allow for ribosomes to initiate translation at internal start codons on polycistronic mRNA. The advantage of IRES sequences is that they make expression of the downstream gene dependent on expression of the upstream gene. A selectable marker gene is often used in this downstream position so that clones isolated after selection for the marker gene are guaranteed to express the upstream therapeutic gene. As many as three genes have been expressed from a single retroviral vector utilizing combinations of IRES and internal promoters. These tricistronic constructs seem to give consistently lower expression than the bicistronic constructs, but whether this is a translation or RNA stability issue remains unclear.

Splicing retroviral vectors

Expressing genes from the 5' LTR promoter can be suboptimal since the transgene is cloned about 600 bp downstream of the transcriptional initiation site. Based on the scanning model for translational initiation, one would predict that this would lead to inefficient translation of the transgene. To minimize this problem, splicing vectors have been engineered in which a partially used splice acceptor sequence is cloned just upstream of the transgene. In the spliced messages, the translational start site of the transgene is brought closer to the 5' end of the message, which should result in higher translation efficiency and expression of the transgene. These splicing vectors give consistently higher expression of transgenes compared to vectors without the splice acceptor.

Reverse orientation vectors

Reverse orientation vectors are constructed by cloning a transcriptional unit (including promoter, cDNA, optimal introns, and poly-A sequences) in the opposite orientation to the retroviral LTR transcript. The primary reason for doing this is that introns can be incorporated in the reverse orientation but not in the sense orientation and intron-containing constructs tend to give higher expression than constructs lacking introns. Introns in the sense orientation would be spliced out in the nucleus of the producer cell before the vector was packaged. Problems with reverse orientation constructs include promoter interference between the LTR and the transgene promoter and antisense effects leading to lower expression and titers. A second problem that has been observed is frequent deletion of all or parts of transgenes from aberrant splicing due to splice sites in the antisense transcriptional unit.

Self-inactivating (SIN) vectors

Two types of vectors were generated predominantly to address early safety concerns: self-inactivating (SIN) vectors and suicide vectors. SIN vectors have a deletion in the 3' LTR enhancer which renders both LTRs transcriptionally inactive in the target cell. These vectors were initially designed for safety reasons to prevent the 3' LTR from promoting or enhancing spurious transcription of a gene adjacent to the site of integration. The specific concern was inappropriate activation of a cellular oncogene. Early concerns about insertional mutagenesis, however, have proven unfounded due to the enormous size of the target genome, so these SIN vectors have not been used extensively. SIN vectors are currently used in reverse orientation constructs to prevent the problem of promoter interference and in cases where an internal promoter is preferred to the 5' LTR.

Double copy retroviral vectors

Double copy vectors are designed to provide two copies of a therapeutic gene in one retroviral vector by inserting the transgene into the U3 region of the 3' LTR. The process of reverse transcription copies this sequence segment such that, when the provirus is formed, the transgene appears in both the 5' and 3' LTRs. Having two copies of the gene should result in higher expression than a single copy, but these vectors have not proven better in most cases.

Retroviral vectors with and without selectable markers

Most of the gene therapy studies and clinical trials have used retroviruses containing dominant selectable marker genes such as drug-resistant markers. The most common selectable marker gene has been the bacterial neomycin phosphotransferase (neo) gene. Selectable marker genes have been used for selecting and characterizing retroviral producer cell clones and for enriching target cell populations for transduced cells. However, investigators have recently suggested that bacterial drug-resistant markers should be eliminated or replaced with natural human proteins less likely to elicit an immune response that would eliminate targeted cells *in vivo*. The ideal dominant selectable marker would be a human cell surface protein that is not normally expressed on the target cell. Cell surface markers would allow more rapid selection of targeted cells by immunomagnetic selection or FACS compared to drug resistance. Human CD24 and NGFR (p75, nerve growth factor receptor) are examples of human cell surface proteins currently used as dominant selectable markers.

Eliminating selectable markers altogether may have advantages because including two genes in one vector may lead to lower titers or reduced therapeutic gene expression by promoter suppression. For these reasons, many investigators are turning to 'simplified' retroviral vectors which lack dominant selectable markers. Work with simplified vectors had been limited by the laborious methods required to isolate, characterize and titer useful producer clones but recent efforts to simplify this process may encourage greater use of these vectors.

Packaging cell lines

Retroviral packaging cells are designed to supply the *trans*-acting proteins required for replication and packaging of the engineered retroviral vector construct. Engineering these packaging cell lines has been accomplished by stable transfection of gag, pol and env into suitable cell lines. Constitutive high levels of helper expression are ensured by selection for drug resistance genes which are linked to the gag, pol and env expression cassettes. Interestingly, the gag and pol proteins from murine retroviruses can be used with a number of different env proteins

(including nonmurine env proteins) to create 'pseudotyped' vectors with different host ranges. A series of increasingly safer generations of retroviral packaging cell lines have been engineered to prevent the generation of replication competent retroviruses (RCR, see below) (Figure 3, Table 1).

Evolution of retroviral packaging cell lines

The first generation The earliest retroviral packaging cells were constructed by stable transfection of a complete provirus from which only the ψ (packaging) site had been removed. These cell lines were prone to replication competent retrovirus (RCR) generation since only one recombination event was needed to restore wild-type virus. Examples of these first-generation packaging cell lines include ψ -2, ψ -AM, and PA12.

The second generation The second generation of retroviral packaging cells replaced the 3' LTR with the SV40e poly-A signal to reduce the probability of generating RCR. In addition, the helper genome 5' LTR was mutated to destroy the integrative ability of this helper genome. Thus, even if a defective helper genome happened to get packaged, it would not be

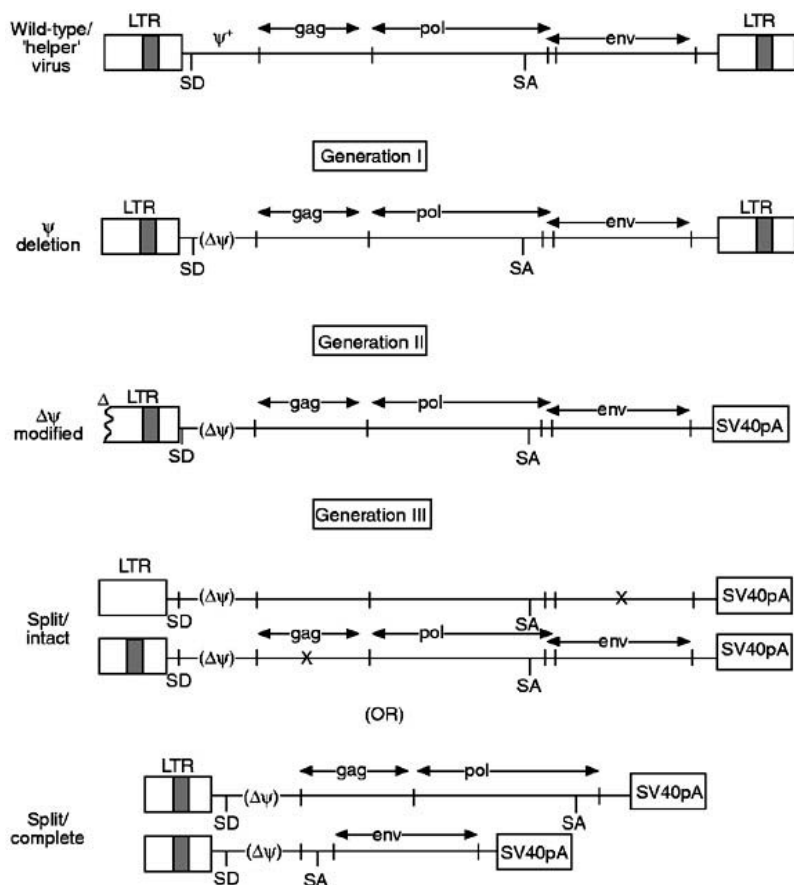


Figure 3 Evolution of retroviral packaging cell constructs.

Table 1 Retroviral packaging cell lines

<i>Envelope</i>	<i>Name</i>	<i>Generation</i>	<i>Parent cell</i>	<i>Resistance genes</i>	<i>Selectable media</i>
<i>Murine</i>					
Ecotropic	ψ-2	I	Murine NIH3T3	gpt	XMAT
	PE501	II	Murine NIH3T3	HSV-tk	HAT
	ψ-CRE	III	Murine NIH3T3	hph, gpt	Hygromycin + XMAT
	GP+E86	III	Murine NIH3T3	gpt	XMAT
Amphotropic	Y-AM	I	Murine NIH3T3	gpt	XMAT
	PA12	I	Murine NIH3T3	HSV-tk	HAT
	PA317	II	Murine NIH3T3	HSV-tk	HAT
	ψ-CRIP	III	Murine NIH3T3	hph, gpt	Hygromycin B + XMAT
	GP+AM12	III	Murine NIH3T3	hph,gpt	Hygromycin B + XMAT
	FLYA13	III	Human HT1080	bsr, phleo	Blasticidin S + phleomycin
	ProPak-A	III	Human 293	hph, puro	Hygromycin B + puromycin
	pkat AMPAC	(transient)	Human 293		
<i>Avian</i>					
Rev-A	C3A2	II	Canine D17	neo	G418
SNV	DSN	III	Canine D17	neo	G418
	Isolde	III	Quail QT6	hygro, phleo	Hygromycin + phleomycin
	DSH29B	III	Canine D17	neo, gpt	G418, XMAT
	DSH134G	III	Canine D17	neo, gpt	G418, XMAT
<i>Cat</i>					
	FLYRD18	III	Human HT1080	bsr, phleo	Blasticidin S + phleomycin
<i>Primate</i>					
	PG13	III	Murine NIH3T3	HSV-tk, dhfr	HAT + methotrexate
	PG53	III	Murine NIH3T3	HSV-tk, hph	HAT + hygromycin

gpt, xanthine-guanine phosphoribosyltransferase; hph, hygromycin phosphotransferase B; dhfr, mutant dihydrofolate reductase; HSV-tk, herpes simplex virus thymidine kinase; bsr, blasticidin S resistance; phleo, phleomycin resistance; puro, puromycin resistance; XMAT, XMAT media containing xanthine, hypoxanthine, aminopterin, mycophenolic acid and glutamine.

capable of integrating into a target cell genome and making new virus. These packaging cell lines require two recombination events to generate helper virus and, if used with care, have proven relatively safe. In fact, the amphotropic PA317 cell line has been used extensively to generate supernatant for many clinical trials. Examples of the second generation of retroviral vectors include PE501 and PA317.

The third generation A third generation of retroviral packaging cell lines has been engineered in which gag and pol are expressed from one transcriptional unit while env is expressed from a second transcriptional unit. These 'split' packaging cell lines require three recombination events to generate RCR and are thus 'safer' than the second generation cell lines. Examples of split packaging cell lines include ψ-CRE, ψ-CRIP, GP+E86, GP+AM12, PG13, PG53, FLYA13, FLYRD18 and ProPak-A.

Mammalian type C retroviral packaging cells

Retroviral packaging cells can be categorized in terms of their host range. The three types of murine retroviruses are ecotropic (murine host range),

amphotropic (broad host range) and xenotropic (nonmurine host range). Only the first two of these have been used extensively for gene transfer studies. The host range is specified by the env protein on the viral surface which binds to a specific receptor on target cells. Interestingly, many of the receptors for retroviruses are membrane transport proteins.

Understanding receptor function has proven valuable in gene transfer studies for two reasons. First, one can directly test for the presence and levels of different receptors on target cells of interest. Receptor levels should be predictive of which pseudotyped vector should be chosen to achieve the highest gene transfer efficiency. Second, receptor biology has provided insights into upregulating receptor levels on target cells to increase gene transfer efficiency. For example, phosphate depletion prior to infection with PG13-derived vectors has been shown to increase gene transfer efficiency presumably due to upregulation of the 1+5 receptor.

The ecotropic receptor (ecoR or rec-1) is found on most murine cells with the notable exception of mouse hepatocytes. Rec-1 is a basic amino acid transport protein 622 amino acids in length with 14

transmembrane domains. The envelope proteins of the murine ecotropic retroviruses bind to this receptor. The cell lines GP+E86, ψ -2, ψ -CRE and PE501 are examples of ecotropic packaging cell lines.

The amphotropic receptor (Ram-1) is a sodium-dependent phosphate symporter. The gene for this protein was cloned from amphotropic MuLV strain 4070A and used to construct packaging cell lines. Almost all human gene therapy trials to date involve amphotropic pseudotyped retroviral vectors. Packaging cell lines with the amphotropic env gene include ψ -AM, ψ -CRIP, PA317, GP+AM12 and *pkat* AMPAC.

Another mammalian type C retrovirus is the gibbon ape leukemia virus (GaLV). The GaLV receptor (Glv-1) is also a sodium-dependent phosphate symporter with ten transmembrane domains. GaLV pseudotyped vectors cannot infect murine cells but seem to infect some human cells better than amphotropic vectors. Packaging cell lines incorporating the GaLV envelope protein gene include PG13 and PG53.

The reticuloendothelial viruses (Rev) are avian type C retroviruses that have also been used to make retroviral vectors. Both the reticuloendotheliosis virus strain A (Rev-A) and the spleen necrosis virus (SNV) have been used. One drawback is that Rev vectors only infect avian and not human cells. Therefore, investigators have used site-directed mutagenesis of the Rev env sequence to extend the host range to include human cells. One advantage of these vectors is that the packaging site does not extend into the gag coding sequence, thereby minimizing generation of replication competent retrovirus.

Pseudotyped vectors using the env gene from the feline endogenous virus RD114 have also recently been reported. The RD114 receptor remains unidentified but it has been mapped to the long arm of human chromosome 19. The host range of RD114 pseudotyped vectors has not been fully explored, but they do infect human hematopoietic cells with high efficiency. In addition, retroviruses containing the RD114 env are much more resistant to complement inactivation in human serum compared to murine amphotropic vectors. FLYRD18 is an example of an RD114-pseudotyped packaging cell line.

Type C retroviral vectors in human packaging cells Generating novel retroviral packaging cell lines has been one of the most active areas of retroviral vector research over the past few years. One problem with the commonly used murine-based retroviral packaging systems is that they generate vectors that are rapidly inactivated by serum from Old World primates (including humans). The prim-

ary mechanism is activation of the classical complement cascade initiated by binding of C1q to the retroviral surface protein p15e, although a natural human antibody to the α -galactosyl carbohydrate epitope has also been implicated. This will severely limit the efficacy of *in vivo* delivery of the murine-based vectors. To overcome this problem, investigators have engineered complement-resistant packaging cell lines in human rather than murine cells. Retroviruses produced from human cells (which naturally lack the enzyme responsible for producing the α -galactosyl structure) are resistant to serum inactivation and may be better suited for *in vivo* applications. The FLY packaging cell lines (FLYA13 and FLYRD114) derived from human HT1080 cells (human fibrosarcoma) and ProPak-A derived from 293 cells (human kidney carcinoma) are examples of human packaging cell lines.

Lentiviral and foamy viral vectors A major limitation of the type C vectors currently in use is that they are unable to infect quiescent cells. Many quiescent cells (including neurons, airway epithelia, hepatocytes and bone marrow stem cells) are attractive targets for gene therapy. Lentiviral retroviruses can, however, infect nondividing cells and investigators have begun to explore their use as gene transfer vehicles. The lentiviral virion core can traverse intact nuclear membranes whereas the type C virion core cannot. This feature has been attributed to karyophilic determinants in the two lentiviral proteins matrix (MA) and Vpr. Pseudotyped lentiviral packaging cells have been made by transient cotransfection of three plasmids. One 'helper' plasmid contained the human immunodeficiency virus (HIV) genome with deletions or mutations in the 5' LTR, packaging signal, envelope gene, and 3' LTR. The second plasmid contained either the murine amphotropic env or the VSV-G protein to make pseudotyped virus. The third 'vector' plasmid contained the transgene of interest. Supernatant from this three-plasmid transient packaging system was shown to infect nondividing cells including terminally differentiated neurons. Eventual human applications may require development of nonhuman (non-HIV) lentiviral vectors such as bovine immunodeficiency virus (BIV), equine immunodeficiency virus (EIV) or simian immunodeficiency virus (SIV).

The use of foamy viral vectors has also been proposed but work in this area is still preliminary. Foamy viruses are found in several animal species and are generally nonpathogenic. Foamy viral vectors may have some advantages over Mo-MLV-based vectors including a broader host range, a

larger packaging size, and more efficient transduction of nondividing cells.

Manipulation of retroviral vector titers

Most of the standard murine type C retroviral vectors can routinely produce titers of 10^5 – 10^7 antibiotic-resistant colony-forming units per ml of supernatant (CFU ml⁻¹). These titers are suboptimal for many gene transfer experiments requiring high multiplicities of infection (MOIs) for success. For this reason, investigators have explored a number of techniques to increase absolute or effective viral titer.

VSV-G pseudotyped vectors

The relatively low titers of retroviral vectors is, in part, due to instability of the retroviral env protein. Retroviruses, as mentioned above, have the ability to incorporate heterologous proteins into their envelope membranes. This observation has been exploited by incorporation of the vesicular stomatitis virus envelope glycoprotein (VSV-G), into retroviral vectors. VSV-G pseudotyped vectors can be concentrated by ultracentrifugation by as much as 3 logs up to around 10^9 CFU ml⁻¹. In addition, whereas env-containing vectors require interaction with a specific receptor, VSV-G interacts directly with a phospholipid component of the target cell plasma membrane. The result is that VSV-G pseudotyped vectors have an extremely broad host range including most vertebrate and many insect cells. However, one problem with VSV-G is that it cannot be constitutively expressed in cell lines because it causes cell fusion and syncytia formation leading to cell death. Therefore, the VSV-G protein must be made transiently in the retroviral packaging cell line by either transient transfection or inducible expression.

Temperature, centrifugation, lyophilization

As noted above, retroviruses are relatively unstable entities. Investigators have attempted to increase the stability of retroviral vectors by growing producer cells at 32°C instead of 37°C. Titers obtained at 32°C were found to be 5–15 times higher than titers at 37°C. This correlated to release of six vector particles per cell at 32°C but only two vector particles per cell at 37°C and is most likely the result of increased viral stability at 32°C. Investigators have also attempted to increase effective viral titer by centrifugation. The theory is that centrifugation can overcome the Brownian motion which normally limits retroviral movement to bring more viruses near the target cells. Transduction with centrifugation at $1000 \times g$ has been shown to increase gene transfer

efficiency in both adherent (fibroblasts) and suspension cells (lymphocytes) 3–10-fold. Lyophilization has also been tried as a method to increase viral titer. This method has not proven effective in generating higher titers but has demonstrated that titers can be maintained by lyophilization which may have important implications for large-scale production and widespread delivery of clinical retroviral vectors.

Safety

Replication competent retrovirus

One important safety consideration in packaging cell design is to minimize the likelihood of generating replication competent retrovirus (RCR). RCR can form when the vector construct is introduced into the packaging cell and recombination occurs to regenerate a wild-type retroviral genome on a single transcript. Recombination leading to development of RCR is extremely rare with the newer packaging cell lines. However, the presence of RCR can have devastating consequences. One study reported development of viremia and T cell lymphomas in three out of ten monkeys which had been transplanted with bone marrow cells transduced with RCR-contaminated vector stocks. The level of RCR given to these immune suppressed animals was extraordinarily high, however, and other immunologically normal monkeys infected with 10^9 ffu MLV showed no pathology up to 7 years later.

The probability of generating RCR has been greatly reduced in second- and third-generation packaging cell lines by minimizing sequence homology between helper and vector genomes, by removing the 3' LTR from the helper transcripts, and by splitting the helper functions on to multiple transcripts. Some of these 'split' packaging cell lines require three homologous recombinations to generate PCR and have proven extremely safe *in vitro* and *in vivo*.

Assays have been developed for the detection of RCR in preparations of retroviral supernatant. The two RCR assays most frequently used are the 'S+L-' assay and the 'marker rescue' assay. The marker rescue assay is more sensitive and involves mobilization ('rescue') of an integrated vector, usually containing a selectable marker such as neo, from a target cell line which lacks gag, pol and env. Therefore, the indicator cell line constitutively makes vector message but it does not make any retrovirus. Supernatant to be tested for RCR is used to infect the indicator cell line. If, and only if, the test supernatant contains RCR (with functional gag, pol and env) will the indicator cell line start producing retroviruses with the indicator vector sequences. Detection of the 'rescued'

indicator vector is determined by selection of target cells for drug resistance.

See also: **Bone marrow and hematopoiesis; Gene conversion; Hematopoietic stem cell transplantation; Immunodeficiency, primary; Retrovirus, infection and immunity.**

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RETROVIRUS, INFECTION AND IMMUNITY

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Classification and ultrastructural appearance

Retroviruses are enveloped viruses that contain two copies of a single-stranded RNA genome. Their characteristic feature is an RNA-dependent DNA polymerase, termed reverse transcriptase, which allows the virus to reverse transcribe its RNA genome 'backwards' into a DNA copy, hence the name retrovirus. This DNA copy becomes integrated into the host genome during retroviral infection and serves as the template for the transcription of viral genes and the production of viral proteins (see below).

Retroviral genetic information can be transmitted by two distinct mechanisms. 'Horizontal' or infectious transmission occurs when the virion fuses with uninfected cells. Retroviruses which utilize this mechanism of infection are known as exogenous retroviruses. 'Vertical' transmission occurs when germline cells become infected and the integrated viral genome is then transmitted to subsequent generations as an endogenous genome. The term 'vertical' transmission is also used to describe an exogenous infection of the fetus occurring *in utero* or perinatally. Both exogenous and endogenous retroviruses are found in many species (see Table 1 for some examples). Retroviruses are conventionally classified as oncoviruses (subdivided morphologically into B-type, C-type, D-type viruses), lentiviruses, and foamy (spuma) viruses. B- and D-type viruses bud from the cell membrane with their cores already assembled in the cytoplasm whereas C-type viruses, lentiviruses

and foamy viruses assemble their cores during the budding process. Mature lentiviral particles differ from C-type viruses by the conical shape of their core (the C-type core has a more spherical shape).

A-type particles are intracellular, retroviral core-like particles which are occasionally seen in some tissues or cell lines. In cells infected with D- or B-type retroviruses they occur in the cytoplasm and represent intracellular B- or D-type particles. In contrast, intracisternal A-type particles are localized in the endoplasmic reticulum and have been described in a strain of diabetic mice, in some human cell lines, in preterm placenta of some primates, including humans, as well as in short-term cocultures of biopsies from patients with Sjögren's disease.

Genomic structure and function

Common features

All retroviruses share a basic pattern of genomic organization but differ in other features which are characteristic for individual groups of retroviruses. As shown in Figure 1, all retroviral genomes are flanked by two identical elements, called LTRs (long terminal repeats). An LTR is itself subdivided into three domains: U3, R and U5. The U3 region contains promoter and enhancer elements which control retroviral transcription. Transcription starts at the beginning of R in the 5' LTR and terminates at the end of R of the 3' LTR. Thus the R region is represented twice in the viral RNA genome, hence R for repeat, whereas the U regions are unique to either

Table 1 Examples of retroviruses

<i>Retrovirus genus</i>	<i>Name</i>	<i>Acronym</i>
<i>Simple retroviruses</i>		
Avian C-type viruses	Avian leukosis virus	ALV
MLV-related C-type viruses	Murine leukemia virus	MLV
Mammalian B-type viruses	Mouse mammary tumor virus	MMTV
PTLV/BLV group	Primate T lymphotropic virus I	PTLV-I: HTLV-I, STLV-I
('tax type' viruses)	Primate T lymphotropic virus II	PTLV-II: HTLV-II, STLV-II
	Primate T lymphotropic virus L	PTLV-L
	Bovine leukosis virus	BLV
D-type viruses	Simian retrovirus 1,2,4,5	SRV-1,2,4,5
	Mason-Pfizer monkey retrovirus	MPMV (SRV-3)
<i>Lentiviruses</i>		
Primate lentiviruses	Human immunodeficiency virus 1	HIV-1
	Human immunodeficiency virus 2	HIV-2
	Simian immunodeficiency viruses	
	from macaques	SIV _{MAC}
	from sootey mangabeys	SIV _{SM}
	from mandrills	SIV _{MN}
	from African green monkeys	SIV _{AGM}
Other lentiviruses	from chimpanzees	SIV _{CPZ}
	Feline immunodeficiency virus	FIV
	Visna virus	
	Equine infectious anemia virus	EIAV
	Caprine arthritis encephalitis virus	CAEV
<i>Foamy/spumaviruses</i>		
	'Human' foamy virus	HFV
	Simian foamy viruses from macaques, African green monkeys, mangabeys, baboons, squirrel monkeys, spider monkeys, orang-utangs, chimpanzees, etc.	SFV-1 – 11 (defined by serotype)
	Bovine syncytial virus	BSV
	Feline syncytium-forming virus	FSFV
	Hamster foamy virus	HFV

the 5' end (U5) or the 3' end (U3). Reverse transcription of the viral RNA is initiated by a tRNA primer which is hydrogen-bonded to a region of the genome known as the primer binding site (PBS), localized between the end of the 5' LTR and the beginning of gag. Reverse transcription then proceeds towards the 5' end of this LTR and then jumps to the 3' end of the same or another viral genome. The resulting linear DNA copy is flanked by two LTRs and is then integrated into the host genome.

The core structural proteins of replication-competent retroviruses are encoded by the gag gene, the viral enzymes reverse transcriptase, proteinase and integrase are encoded by the pol gene, and the two envelope proteins by the env gene (Figure 1). The gag and pol gene products are translated from a single full-length viral RNA as a precursor polyprotein which is then cleaved by the retroviral proteinase to yield the individual proteins. In the case of murine leukemia virus (MLV), this common precursor is produced by occasionally suppressing the stop codon at the end of gag. For the other retroviruses 'ribosomal frameshifting' is required to change the reading

frame between gag and pol. In the case of human T lymphotropic virus type I (HTLV-I), a similar frameshifting event occurs between the proteinase gene at the 5' end of pol and the reverse transcriptase gene.

Typically, gag encodes three proteins, although in some cases there are further processing products. The first of these, the matrix protein, is encoded at the N-terminus of the gag precursor protein and serves to anchor the core complex underneath the cell membrane, often by a myristylated N-terminal glycine residue. The second protein encoded by gag, the capsid protein, is the major structural core protein, whereas the third, C-terminal gag protein interacts with the genomic RNA and serves to dimerize the genomic RNA copies and to package them selectively in the viral particle. In the dimerized state the genomic RNA cannot serve as a template for translation.

For C-type viruses and lentiviruses, the cleavage of the gag-pol precursor, performed by the viral proteinase, takes place mostly after budding from the cell membrane. This cleavage is accompanied by the 'maturation' of viral particles, as visualized by electron microscopy. In contrast, B-type and D-type

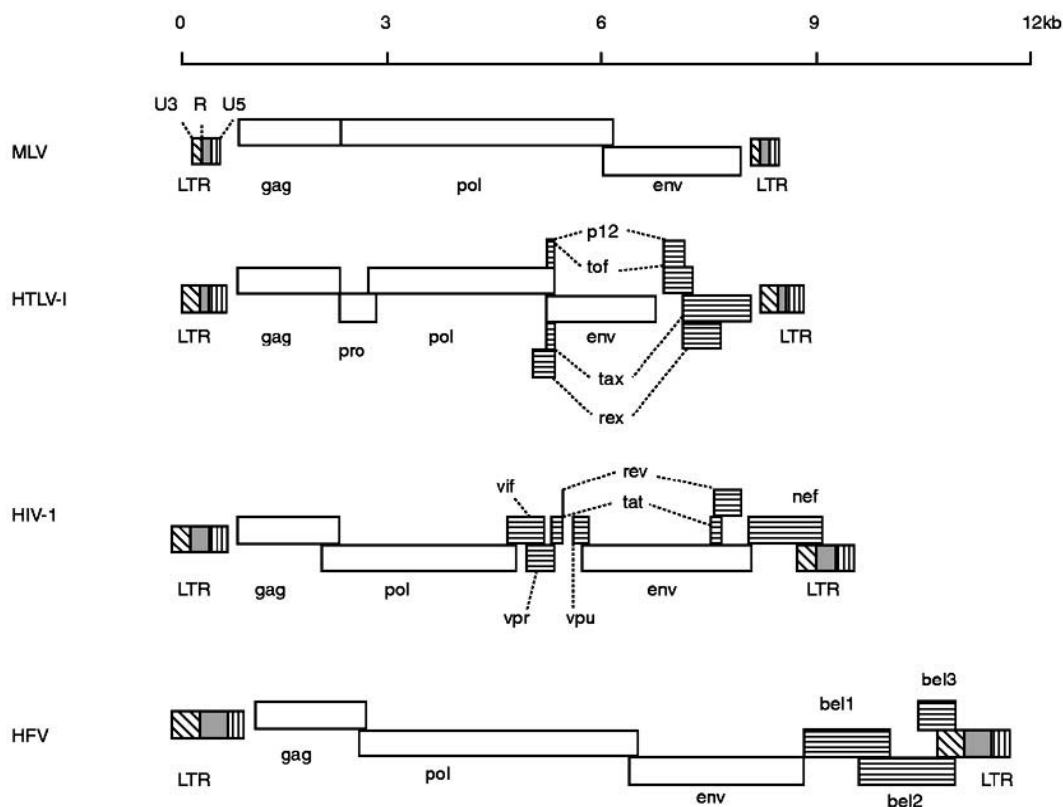


Figure 1 Genome structure of a C-type retrovirus (MLV), a 'tax-type' retrovirus (HTLV-I), a lentivirus (HIV-1) and a foamy virus (HFV). Structural genes are represented by open boxes, auxiliary (regulatory) genes by hatched boxes. The three compartments of the LTR (U3, R, U5) are indicated by different patterns.

retroviruses assemble viral particles with mature core structures inside the cell. Retroviral core particles can be released from the cell in the absence of envelope glycoproteins or coated in envelope proteins from some other viruses. This feature allows the packaging of envelope-deficient virus mutants into the envelope proteins of another retrovirus and has been exploited in the construction of retroviral vectors targeted at cells expressing particular receptors.

The env gene encodes two proteins which are derived from a common precursor protein. One of them extends through the lipid membrane of the envelope and is therefore referred to as TM (transmembrane) protein. It anchors the second envelope protein, termed SU (surface protein) to the virion membrane. This anchorage is mostly mediated by noncovalent bonds, although in the case of some C-type retroviruses a proportion of the SU protein is covalently linked to the TM protein by disulfide bonds. In the case of tissue culture-adapted strains of human immunodeficiency virus type 1 (HIV-1) the interaction between the SU and TM proteins is weak and these viruses therefore shed their SU proteins easily.

The SU proteins mediate the attachment of virions to their target cells by binding the virion to the cellular receptor. Several retroviral receptors have been identified so far. The CD4 glycoprotein serves as the primary receptor for primate lentiviruses, including HIV-1 and HIV-2 (Table 1). HIV-1 and HIV-2 require additional 'coreceptors' to gain entry into cells, and recently several chemokine receptors (mainly CCR5, CXCR4, CCR3, CCR2b) have been shown to play this role. Binding to CD4 induces conformational changes in the HIV envelope protein complex which can then interact efficiently with one of these chemokine receptors. Sequence variation in several regions of the HIV SU protein determines the tropism of individual isolates, presumably by influencing the choice of coreceptor. For 'non human' retroviruses, the cellular receptors have been identified for ecotropic and amphotropic MLV, for Gibbon ape leukemia virus (GaLV), for bovine leukosis virus (BLV), and for avian leukosis virus (ALV). All are membrane glycoproteins. As for HIV, the different tropism, due to the use of different receptors, of ecotropic and amphotropic MLV is determined by minor sequence variation in an N-terminal domain

of the SU protein. Although this is not completely resolved, it seems as if in most instances retroviruses enter the cell by fusing with the cell surface membrane. This is in contrast to some other envelope RNA viruses, e.g. influenza, which require the lower pH of endosomes to trigger the fusion event with internal (endosomal) membranes. The sequence responsible for the fusion ('fusion domain') is located at the N-terminus of the TM protein and is most likely hidden within the tri- or tetrameric envelope protein complex, until the fusion event is triggered by contact with the cellular receptor.

Characteristic features of individual groups of retroviruses

MMTV has a long U3 region in its LTR which, unusually, contains a protein-encoding region. This protein, mls (minor lymphocyte stimulating), has properties of a superantigen and stimulates B cells. Some retroviruses carry oncogenes in their genomes (see under *Neoplasia*). An example of this is Rous sarcoma virus (RSV) which carries the *src* oncogene between *env* and the 3' LTR.

The primate T lymphotropic viruses (HTLV-I, II, STLV) and bovine leukemia virus (BLV), as well as primate lentiviruses (HIV-1/2, SIV_{mac}, SIV_{AGM}, SIV_{MN}, SIV_{SM}) possess an extensive genomic region downstream of their envelope gene (Figure 1). In addition, the lentivirus group is characterized by a 'central region' between their *pol* and *env* genes (Figure 1). Both regions contain genes coding for control proteins which regulate viral transcription, processing of viral RNAs or maturation of viral particles. These control regions are translated from subgenomic, multiply spliced RNAs. Their mode of action can only be briefly summarized here.

Transactivating genes (*tat*, *tax*) The primate T lymphotropic viruses (PTLVs) and BLV as well as the primate lentiviruses (HIV-1, HIV-2, SIVs) produce nuclear activating proteins which act by enhancing transcription from the viral LTR. The Tat of primate lentiviruses acts by binding directly to the nascent viral RNA at a region termed *tar* (for *tat* responsive) which is localized in the R region of the viral LTR and promotes transcription. PTLV and BLV Tax achieve their effect through interaction with cellular transcription factors of the CREB, NF κ B, SRF families. By binding to these transcription factors Tax also affects the expression of cellular genes: it enhances the expression of the genes for interleukin 2 (IL-2), the IL-2 receptor α chain, granulocyte-macrophage colony-stimulating factor GM-CSF, *c-fos* and others and suppresses the gene for β -polymerase, a cellular DNA repair enzyme. The activation of some of these

cellular genes is thought to be important for the transforming properties of HTLV-I and the inhibition of β -polymerase may contribute to DNA damage and subsequent chromosomal aberrations commonly found in adult T cell leukemia/lymphoma, the malignancy associated with HTLV-I. So far, the effects of this kind have never been observed for the lentiviral *tat* protein.

Genes affecting RNA processing (*rev*, *rex*) Rev (primate lentiviruses) and Rex (HTLV-I/II, STLV, BLV) have a common mode of action and facilitate the export from the nucleus of unspliced viral RNA. They thus increase the generation of full-length genomic RNA (encoding Gag/Pol proteins), as well as singly spliced RNA (encoding Env), at the expense of multiply spliced RNAs which code for control proteins.

Other control proteins The Nef protein of primate lentiviruses, encoded between the second exons of *tat* and *rev* and the 3' LTR (in some cases partially overlapping with the latter) is important for efficient replication *in vivo*, but its function is poorly understood. Vif, found in all primate lentiviruses, is encoded upstream of the first Tat exon and required for the infectivity of cell-free virions. Vpu is only present in the HIV-1 genome and may play a role in the release of virus particles from infected cells. Vpx is found in HIV-2 as well as nonhuman primate lentiviruses and Vpr in HIV-1, HIV-2, SIV_{mac}, SIV_{sm}. Vpx plays a role during the nuclear import of the pre-integration complex. Vpr of HIV-1 and SIV_{AGM} is important for nuclear import and induces cell cycle arrest, whereas in HIV-2 these functions are split, and Vpr only causes cell cycle arrest.

Foamy viruses, the third major subfamily of retroviruses, also have a set of open reading frames downstream of the *env* gene, termed *bel* genes. The Bel-1 protein also serves as a transcriptional transactivator.

Retroviral antigens

All major structural proteins encoded by the *gag*, *pol* and *env* genes elicit antibody production and, in some cases, also a T cell response. Antibodies to *gag* proteins show some cross-reactivity among diverse retroviruses such as all strains of avian leukosis virus or gibbon leukemia virus and all murine C-type viruses – hence the designation *gag* for group-specific antigen. In contrast, antibodies to envelope antigens are more specific. Virus-neutralizing antibodies are directed against envelope antigens – both linear and

conformational epitopes have been shown to be 'neutralizing' epitopes in HIV, HTLV-I and BLV. The variable regions of the HIV envelope protein, in particular V1/V2 and V3, as well as its (conformational) CD-4-binding site and a region in the TM protein, are targets for neutralizing antibodies. In contrast to laboratory strains of HIV-1 which have been adapted to grow in established T cell lines, 'primary' HIV-1 strains are more resistant to antibody-mediated neutralization. Neutralizing antibodies to HTLV-I cross-neutralize all HTLV-I variants, but not (or much less efficiently) HTLV-II. The nonstructural regulatory proteins of HIV-1/2 and HTLV-I/II are also immunogenic although in patient sera antibodies to these antigens are found much less frequently than antibodies to structural proteins.

A cytotoxic T cell response to structural proteins has also been demonstrated for some retroviruses, e.g. HIV-1, HTLV-I, friend leukemia virus. In HIV-1 infection cytotoxic T cells are thought to limit viral replication during the early disease stages and to protect against primary infection. Immunization attempts against retroviral disease have been successful for feline leukemia virus and experiments with SIV_{mac} in macaques suggest that immunization with viral structural proteins may confer limited protection against infection. However, in the macaque model efficient protection against SIV infection has only been achieved using attenuated viruses from which some auxiliary genes (e.g. *nef*) had been deleted, but the mechanism of protection is still unclear.

Pathogenesis/involvement in disease

Whereas animal retroviruses have been known for a long time to cause a variety of diseases, including benign and malignant neoplasms, anemias, wasting syndromes, neurological disease and immunodeficiency states, the role of retroviruses as human pathogens has only been substantiated during the last 15 years. HTLV-I is etiologically associated with adult T cell leukemia (ATL) and tropical spastic paraparesis (see below). Although HTLV-II was originally isolated from patients with a T cell variant of hairy cell leukemia, its role in human disease has not been established. The two human lentiviruses HIV-1 and HIV-2 cause the acquired immune deficiency syndrome (AIDS). Foamy or spuma retroviruses are commonly found in several simian species, chimpanzees, cats, cattle and hamsters. There is no known association with disease in these animals. Infection of humans can occur through contact with animals

but is not passed on and proposed associations with human disease have not been substantiated.

The following examples of diseases caused by retroviruses are meant to illustrate some of the mechanisms by which retroviruses cause disease.

Immunodeficiency diseases/wasting/anemias

A number of retroviruses are known to cause immunodeficiency states. The closest analog to human AIDS is the disease caused in macaques by a number of simian lentiviruses. Simian lentiviruses have been found in several species (Table 1). SIV_{AGM}, SIV_{MN}, SIV_{SM} do not cause disease in their natural host but are pathogenic when inoculated into other monkey species. SIV_{mac} is pathogenic to macaques which, however, are not its natural host. SIV_{SM}, SIV_{mac} and HIV-2 are highly related and a simian virus from this group may be the ancestor of HIV-2 in humans and SIV_{mac} in captive macaques.

Many other species also contain lentiviruses. A wasting disease in sheep with some features of human AIDS is caused by visna virus. In goats and sheep caprine arthritis encephalitis virus (CAEV) causes arthritis, pneumonia and meningoencephalitis, and an infectious anemia in horses is due to equine infectious anemia virus (EIAV). A common feature of all lentiviral infections is the chronic, protracted course of the disease which affects many organ systems and the long latency of the virus in the infected host. During the early and late stages of HIV infection virus turnover is very high with 10^9 – 10^{10} virions produced and cleared, and a corresponding number of CD4⁺ T cells destroyed each day. This massive destruction of immune effector cells as a direct result of viral infection probably accounts for most of the damage to the immune system. In addition, immune mechanisms may contribute. Circulating immune complexes may play a role in the glomerulonephritis of equine infectious anemia and the thrombocytopenia purpura of AIDS patients. The phagocytosis and hemolysis of erythrocytes coated with viral antigen, antiviral antibody and complement is at the root of equine infectious anemia and an inflammatory response is responsible for lesions in the lungs and joints of animals infected with visna virus and CAEV. A T cell response to HIV-1-infected T cells may be involved in the destruction of this group of cells.

Some members of a subgroup of oncoviruses, D-type viruses, cause an immunodeficiency state in macaques which was referred to as SAIDS (simian acquired immunodeficiency syndrome). Five viral serotypes belonging to this group are known and have been termed SRV-1–5 (for simian retroviruses).

SRV-3 is also known as Mason–Pfizer monkey virus (MPMV).

In cats and mice, defective forms of feline leukemia virus (FeLV) and MLV have been shown to cause immunodeficiency disease with some features similar to human AIDS. These defective forms have small deletions and insertions in *env* (FeLV) or large deletions in *env* and *pol* (MLV) and are therefore replication defective, i.e. they require the presence of intact helper viruses in order to propagate their genome and package it into infectious particles. The diseases induced by these defective viruses differ in some respects from human or simian AIDS: the murine disease is accompanied by a rapidly progressive B cell hyperplasia and this and the immunosuppression may be T cell dependent. Exactly why these defective viruses are pathogenic is only incompletely understood. There is evidence that minor changes in the envelope gene may affect post-translational processing of the envelope protein core.

Neoplasia

Ever since P. Rous demonstrated in 1911 that cell-free tumor filtrates could transmit a chicken sarcoma, the list of animal tumors caused by retroviruses has continued to grow. Several pathogenic mechanisms have been elucidated over the last 20 years. Retroviruses can cause tumors by transducing oncogenes, i.e. cellular genes involved in the control of cell proliferation and differentiation. Cellular oncogenes, often in a mutated form, can be 'picked up' by retroviruses and become part of recombinant retroviral genomes. Oncogene-carrying retroviruses are usually replication defective since the foreign gene interferes with the normal retroviral life cycle and requires the presence of intact 'helper viruses' to package the oncogene-containing viral genome into viral particles. Other examples include Avian sarcoma virus (*fps*, *yes* oncogenes), feline sarcoma viruses (*fes*, *fms*, *sis*), avian erythroblastosis virus (*erbA*, *erbB*), murine sarcoma virus (*mos*, *Ha-ras*, *Ki-ras*), simian sarcoma virus (*sis*). In this situation the transduced oncogenes are under the control of viral promoters and enhancers and these viruses tend to cause rapidly appearing and progressing tumors.

However, retroviruses need not transduce host genes to cause neoplasia. In the case of avian leukosis virus viral integration occurs in the vicinity of the cellular oncogene *c-myc* and places this oncogene under the control of the retroviral 3' LTR. Mouse mammary tumor virus integrates preferentially in two genomic loci, termed *int-1* and *int-2*, and affects expression of these genes which resemble fibroblast growth factor. In the case of murine leukemia virus the situation is more complex: minimal differences

between the U3 regions of different virus strains (presence or absence of certain tandem direct repeats) seem to be important for leukemogenicity and tissue tropism, most likely by affecting the enhancer function of the viral LTR. An increased enhancer activity may affect the expression of cellular genes in whose vicinity the virus has integrated. Feline leukemia virus may use several of these mechanisms – in some cases integration near the *c-myc* locus and/or recombinant viruses carrying the cellular *c-myc* gene have been observed.

The only human neoplasia associated with a retrovirus is adult T cell leukemia (ATL) caused by HTLV-I. Although the majority of ATL cases are associated with HTLV-I, the existence of rare cases of HTLV-I-negative ATL, the long latency period between infection and the emergence of ATL, and the low proportion of seropositive individuals developing ATL indicate that other events may be involved. The oncogenic role of HTLV-I is related to the viral transactivating Tax protein, which, as outlined above, activates a number of cellular genes and may thus create a pool of proliferating cells. These cells are more prone to further genetic changes which may constitute the actual transforming event. The recently described inhibitory effect of Tax on the repair enzyme polymerase I may contribute to the likelihood of genetic 'accidents'.

Neurological disease

In humans, HTLV-I is etiologically associated with tropical spastic paraparesis (TSP/HAM), but TSP can occur in the absence of HTLV-I infection. The presence of large numbers of activated (HTLV-I infected and uninfected) T cells may play a role in disease development. However, the precise role of HTLV-I in the pathogenesis of this disease is not yet understood. Among lentiviruses both HIV and visna virus cause CNS lesions characterized by inflammatory and degenerative changes. For both viruses, macrophages and microglia seem to be important virus reservoirs in the CNS. A temperature-sensitive mutant of MLV also causes a neurological disease and this may be due to minor sequence changes in the envelope protein which interfere with the normal processing of the envelope in the endoplasmic reticulum and Golgi apparatus of neural cells.

Autoimmune disease

In addition to the 'autoimmune' mechanisms found in immunodeficiency and wasting disorders discussed earlier, murine leukemia viruses are thought to exacerbate the autoimmune disease of (NZB × NZW)F1 mice that resembles human systemic lupus erythematosus (SLE), as well as the necrotizing

arthritis and glomerulonephritis of SL/Ni mice. Retroviral particles have been observed in animal models of autoimmune diabetes, and a new endogenous avian leukosis virus gene locus has been found in obese chicken with spontaneous autoimmune thyroiditis. However, the pathogenic role of retroviruses in these diseases remains to be established.

See also: Acquired immune deficiency syndrome (AIDS); Antigenic variation; Human immunodeficiency viruses; Leukemia; Superantigens.

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RABDOVIRUS, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Characteristics of the family Rhabdoviridae

Rhabdoviridae are enveloped RNA viruses characterized by their shape (Gr. *rhabdos*, rod), and by the presence of helical nucleocapsids that are enclosed in a lipid envelope bearing surface projections. The genome is a single molecule of negative single-stranded RNA which is noninfectious and is transcribed into five mRNAs, each of which codes for a single protein. The gene order in the genus *Vesiculovirus* is 3'-N-NS-M-G-L-5', representing respectively the nucleocapsid protein N, the nonstructural protein NS, the matrix protein M, the envelope glycoprotein G, and a large protein L.

About 80 members of the family infect vertebrates, mostly mammals, but also fishes and reptiles, and several of the viruses which infect vertebrates also infect invertebrates. Others in the family infect plants and/or invertebrates. The family contains two genera: the genus *Vesiculovirus*, derived from the name of the virus causing vesicular stomatitis (VSV), a disease of domestic animals observed in the Americas, and the genus *Lyssavirus*, named after rabies (Gr. *lyssa*, madness).

The Indiana serotype of vesicular stomatitis virus is the type species of the vesiculoviruses, whereas rabies virus that of the lyssaviruses. In addition, a number of other viruses of vertebrates, invertebrates, and plants are placed within the family but have not yet been placed in a genus.

Certain rhabdoviruses have been isolated only from blood-sucking arthropods, but some of these

are believed to infect vertebrates on the basis of serological surveys.

Characteristics of lyssaviruses and their antigens

Among the Rhabdoviridae, lyssaviruses are of special interest. Rabies has been known for centuries as a disease of humans and of domestic and wild animals and for many years rabies virus was thought to be unique. It is now clear that antigenic variation exists within rabies virus isolates, and the existence of several distinct rabies-related viruses is now recognized.

The genome of rabies virus, which has been completely sequenced, contains 11 932 nucleotides. An infectious virus has been obtained from cDNA. The presence of a pseudogene between the G and L cistrons implies that rabies virus is evolutionarily intermediate in the Rhabdoviridae family; apart from that, the gene order is similar to that of the *Vesiculovirus* genus. The helical core of ribonucleoprotein (RNP) contains RNA complexed with about 1325 molecules of nucleoprotein N, 50–70 molecules of transcriptase L; and 700 molecules of phosphoprotein NS. The nucleocapsid structure is surrounded by a lipid envelope with about 1150 molecules of membrane protein M, through which project the surface spikes of the only transmembrane protein, the glycosylated G protein, present at 1350 molecules.

Purified N protein elicits group-reactive antibodies detectable by complement fixation, immunofluorescence and immunoprecipitation that react with rabies and the rabies-related viruses. The immune response against N protein plays a secondary protective role, notwithstanding the fact that it plays the role of a superantigen.

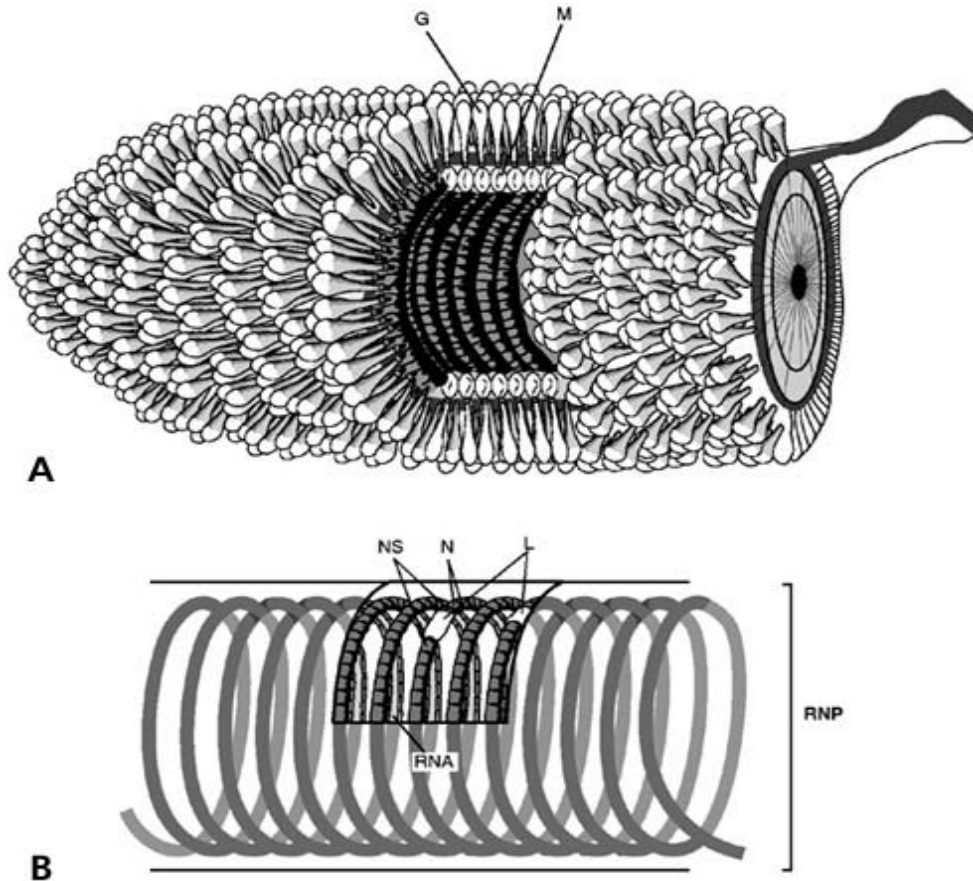


Figure 1 Drawing of the rabies virus (top) showing surface glycoprotein (G) projections (peplomers) extending from the lipid envelope that surrounds the internal nucleocapsid. Matrix (M) protein is shown lining the viral envelope and interacting with the cytoplasmic domain of the surface glycoprotein. The helical nucleocapsid core (bottom) comprises the ribonucleoprotein RNP (i.e., the single-stranded RNA genome plus nucleoprotein [N]), phosphoprotein (NS), and virion transcriptase (L) molecules. The membrane 'tail' of the virus represents the frequently observed irregular shape of particles budding from the plasma membrane of the infected cell. Reprinted after modification from *Rev Infect Dis* 10: (4): S771-S784, 1988, with kind permission of Professor William H. Wunner, Wistar Institute, Philadelphia, USA, and the publisher.

Purified G protein elicits neutralizing antibodies. Glycoprotein can, alone, elicit a protective immunity as has been shown by using a vaccinia virus recombinant containing the rabies virus glycoprotein gene. The antigenic determinants of the glycoprotein vary. Pathogenicity of rabies virus is partially related to the G glycoprotein since the introduction of a mutation at arginine 333 of this protein (selection of mutants resisting neutralization by appropriate monoclonal antibodies), renders the virus avirulent for mice and other species. The sequence of amino acids of the glycoprotein deduced from the nucleotide sequence contains residues that show analogies with sequences of curaromimetic neurotoxins from snake venom.

Rabies-related viruses have been described that can be distinguished from rabies virus using both conventional serums and monoclonal antibodies. In Africa, Lagos bat virus was isolated from fruit bats

(*Eidolon helvum*), Mokola viruses first from *Culicoides*, then from a child, and from shrews (*Crocidura* sp.) whereas Duvenhage virus was isolated from a man who died after having been bitten by an insectivorous bat. More recently, new viruses have been isolated from European insectivorous bats. Although closely related, they differ somewhat antigenically from Duvenhage virus.

Rabies virus infection and its epidemiology

The dog is the principal source of infection of humans where urban rabies exists. On a worldwide basis, the reservoir of rabies is often to be found in wildlife, for instance in Europe the red fox (*Vulpes vulpes*) is the main vector, whereas it is the striped skunk (*Mephitis mephitis*) or the raccoon (*Procyon lotor*) in the USA. Vampire bats (*Desmodus rotundus* for instance) are responsible for cattle rabies in Latin America. Rabies is usually acquired from the bite of

an infected animal, but aerosol transmission has also been described. The incubation period can range from a few days to several years but in most cases its duration is 2–3 months. The disease pattern may be of a classical ‘furious rabies’ or a ‘dumb’ or paralytic rabies.

Immune responses of the host

Genetic control of resistance to rabies

The first demonstrations that different strains of mice varied in their susceptibility to rabies virus were reported independently in 1940 by Johnson and Leach and Habel. This resistance is a dominant trait, controlled by one or two genes and not linked to the H-2 locus. The mechanisms involved are not elucidated. Several factors could play a role including differences between the multiplication rate of defective interfering particles. There is also a differential species susceptibility to rabies virus infection; the amount of infectious virus needed to reproduce the disease experimentally differs markedly according to the virus strain and the species.

Nonspecific immunity

Rhabdoviruses readily induce interferon production in the host and are fairly susceptible to interferon action as shown in various models of infection. Interferon production, which is temperature dependent, seems for instance to play a crucial role in the protection of the poikilothermic rainbow trout (*Salmo gairdneri*) against viral hemorrhagic septicemia. Interferon seems also to play a role in the protection against rabies virus infection. Interferon production could be involved in the protection conferred by the curative vaccination procedure discovered by Louis Pasteur.

A paradoxical phenomenon may be observed when using high doses of infectious virus for challenge infection. Animals infected with high doses resist challenge whereas animals infected with lower doses succumb. This paradoxical phenomenon may be linked to a better interferon response in the first case.

Specific immunity

Natural rabies infection is characterized by a variable incubation period, the absence of serological evidence of infection before onset of the disease, and the mostly fatal outcome of the disease when declared. Nevertheless, recovery from CNS infection can occur (albeit rarely) in both humans and other animals. Therefore information on the specific mechanisms involved in protective immunity has mainly been gained through vaccination studies.

Vaccination during the incubation period, if not repeated, leads to the so-called ‘early death phenomenon’, i.e. a reduction of the incubation period compared with infected control animals. The rabies virus glycoprotein alone can produce this phenomenon since it can be reproduced by using a recombinant vaccinia virus containing the rabies virus glycoprotein gene. Protection against rabies can be obtained by passive transfer of specific antibodies. Many questions remain to be answered, therefore, concerning the role of the different immunological mechanisms involved in the protection against rabies virus.

The importance of cell-mediated immunity in the protective mechanisms is not yet fully evaluated. There is an excellent correlation between antibody titers and protection; nevertheless vaccinated animals devoid of detectable neutralizing antibodies may resist a challenge infection which kills the control animals. Moreover, the protective mechanisms following postexposure treatment of humans with rabies vaccine involves T cells. Structural studies of B and T cell epitopes of the rabies glycoprotein will help to answer this question.

Evasive strategies by rabies virus

Rabies virus infection is characterized by a silent progression of the virus in the nervous system during the incubation period without any detectable humoral immune response. Moreover, infection can lead to asymptomatic carriers which excrete the virus, and the saliva from clinically normal animals may be infective for a few days before clinical disease. It is also noteworthy that viremia does not normally occur before the onset of clinical symptoms. Rabies virus has also been demonstrated in the brown fat of experimentally or naturally infected bats. Bats maintained in simulated hibernation retain a viable virus (multiplying at a very low rate or not at all) for weeks or months, and when returned to a warm environment, multiplication is initiated or increased.

Vaccines

Conventional vaccines

As far as antirabies vaccination is concerned, we must distinguish preventive vaccination and post-exposure treatment. The first human vaccination published by Louis Pasteur in 1885 was a post-exposure one. Postexposure treatment for humans nowadays requires several vaccine injections together with specific immunoglobulin injection when badly exposed. In veterinary medicine preventive vaccin-

ation is the rule. Most of the current vaccines confer partial or no protection against some rabies-related virus infections.

Conventional vaccines for human use Only inactivated vaccines have been licensed for humans. Although some countries still use vaccines produced in nervous tissues, these are not safe because of their myelin content; foreign myelin may be responsible for hypersensitivity reactions of the recipient leading to paralysis. Since rabies virus can be multiplied in cell culture, most of the vaccines used nowadays are derived in this way and are perfectly safe. They can be used both for preventive vaccination or for post-exposure treatment accompanied by serological control.

Conventional vaccines for domestic animals Attenuated virus vaccines have been widely used in the past for immunization of domestic animals; most of them were still pathogenic for some species and cases of vaccine-induced rabies were often reported. Humans exposed to attenuated vaccine strains for veterinary use are treated in the same way as after wild virus exposure.

New potent inactivated vaccines for veterinary use have now replaced attenuated vaccines. Rabies virus glycoprotein has been successfully produced in the baculovirus expression system and will serve as a source of inactivated subunit rabies vaccine. Anti-idiotypic immunization has also been tested with results showing that such a vaccination procedure would be ineffective. In contrast, DNA vaccination shows promising results.

Conventional vaccines for wildlife vaccination In Europe and North America emphasis is placed on the vaccination of the wildlife rabies reservoir to control rabies from the start. Wildlife such as red fox, striped skunk and raccoon must be vaccinated by the oral route using baits. Inactivated vaccines are therefore not useful and attenuated ones must be used. The most extensively used attenuated strain is the SADB19. Unfortunately, it is still pathogenic for several species such as micromammals and striped skunk, and ineffective for raccoons. A new attenuated SAD variant has been recently obtained by arginine 333 mutation. This is apathogenic for small rodents.

Recombinant vaccines

A vaccinia recombinant virus harboring the rabies virus glycoprotein gene has been constructed and shown to induce the synthesis of virus glycoprotein

in infected cells and to induce rabies virus-neutralizing antibodies and protection in susceptible animals. Active when orally administered, this recombinant is a good candidate for vaccination of wild animal rabies vectors. It is stable, perfectly safe for target and nontarget animal species and confers protection to most of the rabies vectors. This recombinant, after extensive experimental studies conducted under controlled conditions, has already been used in open field trials. The results obtained confirm its basic properties and potential interest for rabies eradication.

See also: Vaccines; Viruses, immunity to.

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Rh ANTIGENS

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In 1939, Levine and Stetson reported a case of erythroblastosis fetalis and recognized the causative role of the maternal antibody, to which they gave no name. In 1940, Landsteiner and Wiener described production of an antibody in rabbits and guinea pigs immunized with *Macacus rhesus* blood that reacted with the red cells of 85% of random humans. Between them, these observations represented discovery of the Rh blood group system that is now recognized as one of the most complex polymorphisms known in humans.

By 1996, 45 purportedly different Rh antigens had been named (see Table 1) and several potential additions are currently under investigation. As far as is known, the Rh antigens are present only on red cells. Because of early disagreement about the possible genetic control of Rh antigen production, two distinct terminologies, CDE and Rh-Hr, were used. In 1962, a third terminology that used numbers for the antigens was introduced to enable presentation of phenotypic data without genetic implications. Most recently, names unrelated to these terminologies have been used. Sometimes this occurred because the antigen was named before its place in the Rh system was recognized, at other times a numerical and an alphabetical term were introduced simultaneously. The hybrid terminology currently used is shown in Table 1.

The clinical importance of the Rh antigens was quickly recognized. First, the antigen D is by far the most immunogenic of all red cell antigens. If persons who lack D are transfused with one unit of D+ red cells, some 80% make anti-D. Before the introduction of Rh immunoglobulin (RhIg, see below), in the order of 20% of D– women who bore D+ children became immunized to D. Second, when hemolytic disease of the newborn (HDN) is considered, virtually 100% of *in utero* deaths are caused by anti-D (that is sometimes accompanied by other Rh antibodies), and of seriously affected live-born infants, some 97% are D+ children born to women whose antibodies are, or include, anti-D. Third, anti-

D and almost all other Rh antibodies detected by standard serological techniques are capable of causing (usually extravascular) *in vivo* destruction of antigen-positive red cells.

The terms Rh-positive (Rh+) and Rh-negative (Rh–) are used to describe persons with or without D on their red cells, respectively; the other 44 Rh antigens are ignored in the definitions of Rh+ and Rh–. Although considerably less immunogenic than D, other Rh antigens stimulate the production of immune antibodies via transfusion and much less frequently via pregnancy. Indeed, of all clinically significant antibodies to red cell antigens, that is those capable of bringing about accelerated *in vivo* clearance of antigen-positive red cells, some 50% define Rh antigens, even after anti-D is excluded from the calculation. In spite of this, immunization is sufficiently rare, i.e. approximately 0.5% of all transfusion recipients, that donor blood need not be matched to the recipient's phenotype, save for D, in nonimmunized patients.

The introduction of RhIg in the early 1960s dramatically altered the incidence of HDN. The product is a concentrated form of IgG anti-D that is injected into D– women either during or following a D+ pregnancy. Used in the correct amount and at an appropriate time, RhIg approaches 100% efficacy in the prevention of immunization to D in this setting. While there is no doubt that RhIg is effective, there is dispute as to its mode of action. Possible mechanisms include rapid clearance of D+ red cells from the maternal D– circulation; interference in the processing of D antigen in the presence of passively administered anti-D; and influence on the feedback mechanism between B and T cells, again in the presence of injected anti-D. It seems more likely that use of RhIg to prevent immunization to D in an Rh– patient accidentally transfused with Rh+ blood involves rapid clearance of the D+ red cells.

In addition to Rh system alloantibodies, some 40–50% of autoantibodies in patients with 'warm' antibody-induced hemolytic anemia have Rh specificity.

Table 1 Rh antigen names in different terminologies

Numerical	CDE	Rh-Hr	Other
Rh1	D	Rh _o	
Rh2	C	rh'	
Rh3	E	rh''	
Rh4	c	hr'	
Rh5	e	hr''	
Rh6	f or ce	hr	
Rh7	Ce	rh _i	
Rh8	C ^w	rh ^{w1}	
Rh9	C ^x	rh ^x	
Rh10	V	hr ^v	
Rh11	E ^w	rh ^{w2}	
Rh12	G	rh ^G	
Rh17		Hr _o	
Rh18		Hr or Hr ^s	
Rh19		hr ^s	
Rh20	VS		
Rh21	C ^G		
Rh22	CE		
Rh23	D ^w		
Rh26	c-like		
Rh27	cE		
Rh28		hr ^H	
Rh29		rh _m Total RH	
Rh30	D ^{Cor}		Go ^a
Rh31		hr ^B	
Rh32			
Rh33			
Rh34		Hr ^B	
Rh35			
Rh36			Be ^a
Rh37			Evans
Rh39	C-like		
Rh40			Tar
Rh41	Ce-like		
Rh42	Ce ^s		
Rh43			Craw
Rh44			Nou
Rh45			Riv
Rh46			Sec
Rh47			Dav
Rh48			JAL
Rh49			STEM
Rh50			FPTT
Rh51			MAR
Rh52			BARC

Blank spaces indicate no equivalent term.

LW^a (previously Rh25) and Duclos (previously Rh38) are no longer considered to be Rh system antigens since their encoding genes segregate independently of Rh.

Rh13, Rh14, Rh15, Rh16 and Rh24 are considered obsolete since the reagents to define them are no longer available.

In about 3% of patients the autoantibodies react with a well-characterized Rh antigen, such as e, c, C or even, on rare occasions, D. More frequently, the causative antibodies have a more complex specificity and react with 'core' Rh antigens that are present on all red cells with a common Rh phenotype, but are lacking from red cells with 'deleted' Rh phenotypes, when some or all Rh antigens are absent, e.g. Dc-, D- and Rh_{null}. There is as yet no immunological explanation of involvement of the Rh system in such a high proportion of persons autoimmunized to red cell antigens.

At the molecular level, much has been learned since the red cell membrane components that express the Rh antigens were first isolated in 1982. In persons with D+ red cells two Rh genes, RHD and RHCE, are present. The term RHCE includes (at least) the alleles RHcE, RHcE, RHce and RHCE. In most D- persons, at least those of Caucasian extraction, RHD is deleted. Both RHD and RHCE appear to be organized into 10 exons over 75 kb of DNA. Exons 1-7 each encode 50-60 amino acids, exons 8-10 encode the 58 residues of the C-terminal end of the proteins. The fourth intron of RHD contains a 60 bp deletion as compared to RHCE. Both RHD and RHCE encode production of nonglycosylated, fatty acid acylated polypeptides comprised of 417 amino acids (the initiating methionine is then cleaved from the mature protein). Hydrophathy analysis forecasts that the polypeptides cross the red cell membrane 12 times, thus suggesting an, as yet unidentified, transporter function. There is considerable homology between the RHD- and RHCE-encoded proteins; of the 416 amino acids only 36 differ. The N- and C-termini of the proteins are highly conserved. At the N-terminal end, the first 42 amino acids encoded by RHD and RHCE are identical. At the C-terminal end, only four of the last 84 amino acids differ. Some amino acid differences in the proteins encoded by the various alleles of RHCE have been correlated with red cell Rh antigen expression; such changes are summarized in Table 2. The partial D situation, in which persons with some but not all epitopes of D on their red cells are able to make allo-immune anti-D, is also understood at the molecular level. Table 3 summarizes the situations in which gene conversion (exon swapping), gene deletion and point mutation can result in an RHD gene that fails to encode all epitopes of D.

Isolation of the nonglycosylated Rh polypeptides results in loss of antigen integrity. It is believed that *in situ*, the Rh polypeptides are part of a complex that includes noncovalently bound Rh-associated glycoproteins (that are coprecipitated with the nonglycosylated polypeptides) CD47, LW glycoprotein,

Table 2 Amino acid changes in the CcEe polypeptide and antigen correlates

Polypeptide carries	Amino acid at position							
	16 ^a	36	41	60 ^a	68 ^a	103 ^b	226 ^c	245 ^c
C and e	Cys	Ala	Gln	Ile	Ser	Ser	Ala	Leu
C and E	Cys	Ala	Gln	Ile	Ser	Ser	Pro	
c and e	Trp	Ala	Gln	Leu	Asn	Pro	Ala	Leu
c and E	Trp	Ala	Gln	Leu	Asn	Pro	Pro	
C and C ^w		Ala	Arg			Ser		
c and C ^w		Ala	Arg			Pro		
C and C ^x		Thr	Gln			Ser		
c and C ^x		Thr	Gln			Pro		
VS							Ala	Val

^a The differences between C and c at positions 16, 60 and 68 are usual but not invariant.

^b Ser103 in C, Pro in c appears invariant.

^c Assumes that all VS⁺ red cells are e⁺.

Blank spaces indicate that residue present may vary dependent on other antigens expressed.

Table 3 Characteristics of partial D red cell categories

Category of partial D	Epitopes of D present ^a	Genetic change in RHD	Low incidence Rh antigen expressed
II	epD1,3,5,6/7,8 ^b	nk ^c	None ^d
IIIa	All	nk ^c	None ^d
IIIb	All	Exon 2 from RHc	None ^d
IIIc	All	Exon 3 from RHCE ^e	None ^d
IVa	epD4,5,6/7,8	Exon 3 and probably exon 7 from RHCE ^e	Go ^a (Rh30)
IVb	epD5,6/7,8	Exons 7 to 9 from RHCE ^e	Rh37 ^f
Va	epD2,3,4,6/7,8,9	Exon 5 from RHCE ^e	D ^w (Rh23)
VI.I ^g	epD3,4,9	Exons 4 to 6 of RHD deleted	None ^d
VI.II ^h	epD3,4,9	Exons 4 to 6 from RHCE ^e	BARC (Rh52)
VII	epD1,2,3,4,5,6/7,9	T to C substitution at nucleotide 329 in exon 2 of RHD causes Leu → Pro 110 change in protein	Tar (Rh40)
DFR	epD3,4,9 ⁱ	Exon 4 from RHCE ^e	FPTT (Rh50)
DBT	epD8 ^j	Exons 5 to 7 and perhaps exon 8 from RHCE ^e	Rh32
D of R ^{oHar}	See ^k	Exons 1 to 4 and 6 to 10 from Rhc ^l	Rh33 and FPTT (Rh50)

^a Based on a nine epitope, epD1 to epD9, model.

^b Red cells give variable reactions with monoclonal anti-D ostensibly directed against epD2.

^c nk = not known.

^d None yet detected.

^e Allele of RHCE donating exon to RHD, not known.

^f Probable but not yet proved.

^g Usually encoded by cD^{VI}E.

^h Usually encoded by CD^{VI}e.

ⁱ Red cells give variable reactions with monoclonal anti-D ostensibly directed against epD1, 2, 5, and 6/7.

^j Red cells give variable reactions with monoclonal anti-D ostensibly directed against epD6/7.

^k Red cells give variable reactions with monoclonal anti-D ostensibly directed against epD5 and 6/7.

^l Gene is essentially an RHce allele converted by the replacement of exon 5 of RHce with exon 5 of RHD.

glycophorin B and a component that carries the antigen Fy5. These various components are encoded by genes unlinked to Rh and to each other. That the nonglycosylated proteins make the major contribution to Rh antigen structure is clear from a number of lines of investigation. First, family and linkage

studies have shown that the Rh locus is on chromosome 1. Second, somatic cell hybrid analysis and *in situ* hybridization have localized the RHD and RHCE genes to 1p36.1–p34. Third, similar studies have localized the genes that encode the Rh glycoproteins to 6p21–qter. Fourth, transfection studies

using RHD and RHCE cDNA resulted in Rh antigen expression by K562 cells. In a number of instances it has been shown that the Rh genotype, as determined by studies on DNA, does not correlate with Rh phenotype as determined by typing tests on red cells. It is clear that in rare instances RHD and/or RHCE can be present but not expressed.

The Rh_{null} phenotype represents total absence of all 45 Rh antigens, the red cells are also LW(a-b-), Fy:-5 and have reduced expression of the antigen U. The Rh_{mod} phenotype represents marked depression but not total absence of the Rh antigens. The Rh_{null} phenotype can arise due either to the inheritance of recessive unlinked modifier genes (X^{or}), or to recessive amorphic genes ($\bar{r}$) that are at the Rh locus. The Rh_{mod} phenotype arises due to the inheritance of recessive unlinked modifier genes (X^Q). Recent evidence strongly suggests that X^{or} and X^Q are mutant alleles of the genes that encode production of the Rh-associated glycoproteins. Thus, presence of RHD and/or RHCE genes in 'modifier' Rh_{null} and Rh_{mod} individuals (those genes function normally in the parents and offspring of such individuals) but lack or reduction of Rh antigen expression, indicates that presence of normal Rh-associated glycoprotein is also required for Rh antigen expression.

The Rh_{null} and Rh_{mod} phenotypes are associated with a condition known as the Rh deficiency syndrome. The red cells are stomatocytic, have slightly increased osmotic fragility, and have a perturbation of membrane lipid organization. At the clinical level, these changes are associated with a reduced *in vivo* red cell lifespan and an often well-compensated form of red cell deficiency hemolytic anemia. Although several red cell membrane defects of Rh_{null} red cells have been identified it is not clear that any of them is responsible for the accelerated rate of *in vivo* red cell clearance. First, the clinical effects of the Rh deficiency syndrome are completely cured by splenectomy. Second, Rh_{null} and Rh_{mod} red cells survive normally when injected into persons who have been splenectomized following injury. In other words, perhaps the hemolytic anemia of this syndrome relates simply to the altered shape of the red cells and the filtering action of the spleen. Thus the clinical syndrome associated with the lack of Rh antigens throws no light on the function of the Rh antigen-bearing membrane components. Indeed, no definite conclusions about the function of these components can yet be drawn.

See also: Anemia, autoimmune hemolytic in human;

Blood transfusion reactions; Erythrocytes; Hemolytic disease of the newborn; Transfusion.

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RHEUMATOID ARTHRITIS, ANIMAL MODELS

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Rheumatoid arthritis (RA) is a relatively common and disabling form of inflammatory arthritis. The etiology of RA remains unclear, but it is postulated that in genetically susceptible individuals, an unknown antigenic agent is presented by a major histocompatibility complex (MHC) class II molecule to CD4⁺ T cells. These T cells then become activated and initiate an inflammatory cascade involving macrophages, synovial cells, cytokines and growth factors, which in turn cause joint inflammation and destruction. In recent years, considerable progress has been made at the molecular level in understanding the pathogenesis of RA. Although advances have been made in understanding the pathogenesis and genetic associations of RA, studies have so far failed to identify a putative infectious agent or antigenic peptide common to the development of RA.

Experimental animal models of RA have provided a valuable tool to dissect underlying mechanisms involved in the disease process. Numerous models of experimental arthritis are currently being employed to study RA. Disease can either be induced by immunization with a variety of antigens or arthritis has been shown to occur spontaneously in a wide variety of animals including mice, rats, guinea pigs, rabbits and monkeys. Although none of these animal models exactly mimic human RA, several of the disease models studied to date do possess similarities with RA (Table 1). Collagen-induced arthritis (CIA)

in the mouse has been intensely studied in the recent past and will be the focus of this article.

Collagen-induced arthritis: a versatile model

Immunization with type II collagen (CII), a major constituent of hyaline cartilage, was first demonstrated by Trentham and colleagues in 1977 to be arthritogenic in rats. In the years to follow, CIA has been induced in several animals including mice, goats and rhesus monkeys. One of the hallmark features of CIA is the production of CII-specific antibodies and CII-reactive T cells during the arthritogenic process, as seen in some patients with RA. Furthermore, the histopathological features of CIA resemble RA, with the formation of synovial infiltrates, that lead to pannus formation, marginal bone erosions and joint deformity.

Pathogenesis of murine CIA

Both humoral and cellular responses have been implicated in the pathogenesis of CIA. Antibodies against CII are detectable 9 days following immunization and an immunoglobulin M (IgM) response peaks within 2 weeks; peak IgG levels are attained at 4–5 weeks postimmunization. Anti-collagen IgG levels coincide with the onset of arthritis, suggesting a role for autoantibodies in the pathogenesis of CIA.

Table 1 Comparison between animal models and RA

	<i>Collagen arthritis</i>	<i>Adjuvant arthritis</i>	<i>Pristane arthritis</i>	<i>Cell wall arthritis</i>	<i>Antigen arthritis</i>	<i>Proteoglycan arthritis</i>	<i>RA</i>
Animal species	Mice, rats, monkeys, goats	Rats	Mice, rats	Mice, rats	Rabbits, rats, mice, guinea pigs	Mice	Humans
Genetic predisposition	+	+	+	+	+	+/-	-
Mediators							
T cells	+	+	+	+	+	+	+
Antibody	+	-	-	-	+	+	-/-
Joint involvement							
Peripheral	+	+	+	+	+	-	-
Axial	-	-	-	-	+	-	-
Relapsing/remitting	-	+	-	+	-	-	+
Antigen?	Type II collagen	Mycobacterium?	?	Group A Streptococcal cell wall	Ovalbumin BSA	Proteoglycan (aggrecan)	?

Further, passive transfer of transient arthritis is seen with the transfer of sera from arthritic mice or rats to naive animals. More profound is the ability of sera from a human with polyarthritis to induce arthritis similar to CIA in mice. As with the passive transfer of murine antibodies against CII, this arthritis is also transient in nature. This is in sharp contrast to CIA where chronic arthritis develops, culminating in joint destruction. Thus, autoantibodies do play a role in the pathogenesis of CIA, but their specific role in disease induction is yet to be clarified.

Cellular responses to CII are also involved in the pathogenesis of CIA. T cells and specific T cell receptors are clearly involved in the initiation of CIA. Mice with genomic deletion of specific T cell receptors have been shown to be resistant to the induction of CIA (see later). Furthermore, treatment of mice with anti-T cell agents prevents CIA and athymic nude mice are resistant to CIA.

Immunogenetic regulation of CIA

The immunogenetic control of CIA in mice has been extensively studied, providing considerable knowledge regarding the predisposition to CIA in mice and other models. Susceptibility to CIA in mice has been shown to be dependent on the presence of a trimolecular complex including: 1) a susceptible MHC haplotype, 2) the presence of self-reactive T cells utilizing selected T cell receptor (TCR) V_{β} chains, and 3) arthritogenic epitopes on the collagen molecule. MHC genes have been mapped to the H-2A locus, using inbred, congenic and recombinant mouse strains. Mouse strains expressing the MHC haplotypes H-2^a and H-2^r are susceptible, while other haplotypes of mice are resistant to the induction of CIA. In addition, studies utilizing CIA-resistant B10.P (H-2^p) and CIA-susceptible DBA/1, B10.G (H-2^a) strains of mice have localized a critical restriction site on the H-2A β chain that is important in CIA susceptibility.

Comparison between sequences in the A β first domain of H-2^a and H-2^p genes has revealed amino acid differences at positions 85–89 which are critical in CIA susceptibility. This region of the A β molecule is suggested to be localized at the end of an α helix, important for interaction between the TCR, the MHC molecule and an antigenic peptide. This 85–89 amino acid stretch has been further analyzed in CIA susceptibility. H-2^p mice transgenic for an A β^p gene mutated to resemble the A β^a develop CIA, supporting the role of the A β gene in predisposition to CIA. While considerable progress has been made in identifying a critical role of the H-2A molecules in CIA susceptibility, recent work from our laboratory has demonstrated a protective role for the H-2E mol-

ecules in CIA. Experiments were performed using a recombinant mouse strain (B10.RQB3), which possesses functional H-2A^a and E α^k molecules, but lacks an intact H-2E molecule due to a nonfunctional E β gene. However, B10.RQB3 mice transgenic for a E β^d gene express an intact E β^d /E α^k molecule. Upon immunization with bovine CII to induce arthritis, these transgenic mice showed a marked decrease in both incidence and severity of disease. This protective phenomenon was shown to be dependent on specific E β molecules, where only the E β^d and E β^r were associated with protection while other E β genes have no effect on CIA. Further analysis of the E β^d molecules has revealed that the HV3 region of the first domain in this molecule probably contains critical elements involved in the protection phenomenon. Lymph node cells from B10.RQB3 and B10.Q (H-2^a) respond to a peptide (65–79) encompassing the HV3 region of the E β^d and E β^r molecules, while peptides from E $\beta^{b,k}$ and E β^p failed to induce a T cell response. Thus, a correlation exists between the T cell response to the HV3 peptides and protection of the corresponding H-2E molecule against CIA, implicating that these peptides bind to the H-2A molecules and thereby mediate the protection effect. H-2A polymorphisms also have been suggested to play a role in the E β -mediated CIA protection. CIA-susceptible B10.RIII (H-2^r) mice transgenic for E β^d fail to protect mice from CIA and fail to mount a T cell response when lymph node cells are challenged with the E β^d 65–79 peptide, implying that the E β^d mediated protection is targeted to the H-2^a molecule and not the H-2^r.

In addition to a susceptible MHC haplotype, T cells expressing specific V_{β} chains are also important in CIA susceptibility. TCR involvement was first implicated by the observation that SWR mice, which possess a susceptible haplotype (H-2^a) but have a genomic deletion of 50% of their TCR- V_{β} genes, remain resistant to CIA. When SWR mice were crossed to B10 mice, which are resistant to CIA due to their nonsusceptible MHC haplotype (H-2^b) but possess a normal repertoire of V_{β} genes, the F1 offspring developed CIA, demonstrating gene complementation. The influence of TCR- V_{β} s on CIA has also been demonstrated using TCR- V_{β} congenic mice strains. Products of the minor lymphocyte-stimulating (Mls) loci have been shown in part to regulate the expression of specific V_{β} TCRs by T cells. Mice which have a clonal deletion of T cells because of certain Mls loci (e.g. mammary tumor virus), show a reduction in the incidence of CIA. For example, CIA-susceptible B10.Q (H-2^a, Mls-1^b) have a normal TCR repertoire, while CIA-resistant BALB.D2 (H-2^d, Mls-1^a) do not possess T cells bearing $V_{\beta}6$, $V_{\beta}7$,

V β 8.2, or V β 9 TCRs due to clonal deletion. An F1 cross between these mice (H-2^{d/q}) has a deletion of T cells utilizing the specific V β genes mentioned above and shows a lower incidence of CIA. In summary, the absence of T cells expressing certain V β TCRs, either by clonal deletion via Mls gene products or genomic deletion, clearly affects susceptibility to CIA.

Susceptibility to CIA is also dependent on the source of type II collagen. Immunization with either bovine, deer or porcine collagen can induce arthritis in H-2^d and H-2^r mice. However, B10.RIII (H-2^r) mice are resistant to disease induction with chick CII. B10.Q mice are resistant when immunized with porcine CII but are susceptible to chick CII. These findings suggest that at least two different collagen epitopes have the capacity to induce disease. Identification of such arthritogenic epitopes on CII has been pursued using cyanogen bromide fragments. A 245 amino acid CB fragment (CB11) from chick type II collagen has been shown to induce CIA in DBA/1 mice. Additionally, autoantibodies directed against murine CII are predominantly restricted to determinants on CB11, suggesting this fragment may contain arthritogenic epitopes. Although synthetic peptides corresponding to amino acids 122–147 and 245–270 of the CB11 molecule have been shown to induce tolerance and/or inhibit CIA, the arthritogenic potential of these peptides to date have yet to be demonstrated.

Immunomodulation of CIA

CIA in mice and rats has provided an excellent model for the development of several novel therapies of arthritis. These include the use of CII oral tolerance, antibodies and agents that affect T cells, MHC molecules, and the TCR. Modulation of cytokine levels in arthritic mice has also proved to be fruitful in the therapy of disease. Therapeutic protocols to date for the treatment of CIA are presented in Table 2. All of the above-mentioned interventions are able to

modulate disease to some extent and a few of the listed agents are currently in clinical trials for human RA.

HLA-transgenic mice: new models for human RA

Genetic studies have linked predisposition in RA in humans to the MHC class II HLA-DRB1 locus. Analysis of HLA-DR alleles associated with RA have revealed that DRB1*0401 (Dw4) and DRB1*0404 (Dw14) confer genetic predisposition to RA, while DRB1*0402 (Dw10) does not. In addition, these alleles bear a similar amino acid sequence within the HV3 region (amino acids 67–74) of the DRB1 molecule, which prompted the 'shared epitope' hypothesis of RA susceptibility. The hypothesis states that a critical element in conferring an increased risk for RA susceptibility is possession of this common epitope within the DRB1 molecule. Linkage disequilibrium between certain DQ and DR genes has revealed a much stronger association at the DQ and DR locus in some autoimmune disorders. In RA, the DQ7 allele has been shown to be associated with a majority of DR4 alleles (in Caucasoid populations), whereas in Asian populations, DQ8 is in linkage disequilibrium with DR4. The above data support a role of HLA-DQ alleles in genetic predisposition to RA.

Based on these findings and studies on the role of H-2E in CIA, we had proposed that an extended DQ/DR haplotype is responsible for RA predisposition and that polymorphism of the HLA-DRB1 alleles dictates disease protection. The molecular basis of this protection is through the presentation of a DRB1 HV3 peptide by RA-associated DQ molecules. It should be noted that HLA class II-derived peptides indeed constitute a major fraction of naturally processed peptides bound to class II molecules. According to our hypothesis, the negative association of DQ8/DRB1*0402 haplotype with RA should be reflected in the capacity of the DQ8 molecule to present the HV3 peptide from the DRB1*0402 chain during thymic education and induce a T cell response (Figure 1). Alternatively, the lack of DQ8 restriction of HV3 peptides derived from DRB1*0401, *0404, or *1402 allele should correlate with the RA association of the respective Dw4, Dw14, or Dw16 subtypes.

To test this hypothesis, we used HLA-DQ8 transgenic mice which express a functional DQB1*0302/A1*0301 dimeric molecule in the absence of endogenous mouse class II molecules. HLA-DQ8 molecule expression in the peripheral blood lymphocyte (PBL) population is approximately 25%. These

Table 2 Therapeutic protocols used for treatment of CIA

Anti-MHC class II, Anti-CD4
Anti- $\alpha\beta$ TCR, Anti-V β TCR
Anti-CD40, Anti-gp39
IL-1R antagonist protein, IL-1R:IgG fusion protein
Soluble IL-2R, IL-4, IL-10, IL-13
Anti-TNF α , TNFR, TNFR:IgG1 fusion protein
Recombinant human TNFR:Fc fusion protein
Oral tolerance (native type II collagen or peptide)
H-2E β transgene, DR2 (DRB1*1502) transgene

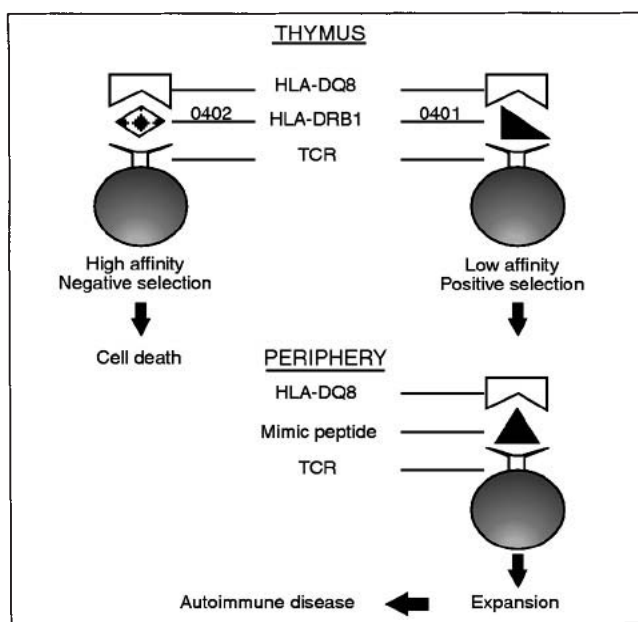


Figure 1 Presentation of HLA-DRB1 HV3 peptides by HLA-DQ molecules shape T cell repertoire. During thymic selection, potential autoreactive T cells are deleted following presentation of the DRB1*0402 HV3 peptide by HLA-DQ8 molecules. Conversely, positive selection of the same autoreactive T cells occurs due to the low affinity of DRB1*0401 peptide for the HLA-DQ8 molecule. These T cells are then activated and expand in the periphery by presentation of a foreign peptide and subsequently (via molecular mimicry) cause autoimmune inflammatory disease.

mice also induced a specific selection of CD4⁺ T cells with a normal V_β TCR repertoire. Upon immunization with bovine type II collagen, HLA-DQ8 transgenic mice mount a strong immunoglobulin G (IgG) response specific for type II collagen as well as proliferate vigorously against bovine CII. Bovine CII-immunized DQ8 mice also develop a severe polyarthritis with massive inflammation and swelling in the affected joints. Histological analysis of joints in these mice indicated synovitis, mononuclear cell infiltration with eventual bone and cartilage erosions leading to joint deformation. These findings demonstrated for the first time that expression of the RA linked HLA-DQ8 molecule in class II-deficient mice confer susceptibility to the induction of CIA.

Immunogenicity of peptides encompassing the amino acid sequence 65–79 of the HV3 region of known HLA-DRB1 alleles was analyzed in HLA-DQ8 transgenic mice. Peptides derived from alleles not associated with RA, namely DRB1*0402 and DRB1*1103, showed the strongest response whereas alleles associated with RA susceptibility, for example, DRB1*0101 and *0401, were not stimulatory. These results showed that a correlation exists between peptides derived from the HV3 regions of DRB1 chains and nonassociation of the corresponding alleles with RA predisposition. Polymorphisms in the DRB1 alleles were further analyzed in the DQ8 mice. RA-protective DRB1*1502 (DR2) gene was introduced in arthritis-susceptible DQ8 transgenic mice. The DR2.DQ8 double transgenic mice were then immunized with bovine CII and monitored for arthritis. Arthritis incidence was significantly lower

in these mice as compared to DR2-negative littermates. A similar reduction of disease incidence was found in CIA-susceptible B10.RQB3 (H-2^u) mice transgenic for the DRB1*1502 gene. HLA-DQ transgenic mice were further investigated to study polymorphisms within the DQ molecules in CIA susceptibility. RA nonassociated HLA-DQ6 transgenic mice generate a weak response to CII and do not develop CIA upon immunization with bovine CII. In contrast, bovine CII-immunized double transgenic mice expressing HLA-DQ6 β and HLA-DQ8 $\alpha\beta$ genes (DQ6/8) develop mild arthritis with delayed onset and lowered incidence. These studies using HLA-transgenic mice demonstrate that polymorphism in the HLA-DRB1 alleles probably modulate predisposition to RA by shaping the T cell repertoire, whereas susceptibility to human RA may be determined by polymorphism in the DQ loci.

Human type II collagen (HuCII) is a candidate autoantigen involved in human RA. Since arthritis-associated human MHC haplotypes (e.g. DQ8, DR4) may recognize unique sets of determinants on HuCII, HuCII peptide determinants recognized by DQ8 transgenic mice were analyzed. Using a series of 27 overlapping peptides (20 mer) encompassing the residues 124–403 of HuCII (CB11 fragment), T cell responses were analyzed both in DBA/1 and DQ8 transgenic mice. Unique peptide determinants were identified in DQ8 transgenic mice, namely peptide residues 174–193, 194–213, 284–303 and 314–343 when compared to responses in DBA/1 mice. Oral administration of HuCII peptides as well as native CII have been reported to suppress CIA in mice and rats, and to be beneficial in human RA patients. DQ8

transgenic mice orally tolerized with bovine CII show a marked reduction in incidence, severity, and delay in onset of disease.

Summary

Although significant progress has been made in recent years in understanding the pathogenesis of RA, a cure for this debilitating autoimmune disease still awaits the human population. It is quite clear that animal models only mimic certain aspects of human RA, yet they have proved invaluable in both the dissection of immunopathological mechanisms and in the development of novel therapies for RA. Certainly, the availability of the recently developed HLA transgenic mice opens exciting new avenues to pursue in understanding the role of HLA molecules in RA and their potential use in the search for therapeutic treatments of RA.

See also: Adjuvant arthritis; Adjuvants; Annexins (lipocortins); Antigen presentation via MHC class II molecules; Anti-inflammatory (nonsteroidal) drugs; Arachidonic acid and the leukotrienes; Autoantigens; Autoimmune disease, induced animal models; Autoimmune disease, spontaneous experimental models; Autoimmune diseases; Autoimmunity; Bacterial cell walls; Cytokines; Fever; Macrophage activation; Molecular mimicry; Muramyl dipeptide; *Mycobacteria*, infection and immunity; Protein kinases; Rheumatoid arthritis, human; *Streptococcus*, infection and immunity; Stress proteins; Systemic lupus erythematosus, experimental models; T cell vaccination; T lymphocyte activation.

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RHEUMATOID ARTHRITIS, HUMAN

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Rheumatoid arthritis (RA) is a chronic multisystemic autoimmune disease of unknown etiopathogenesis. Several different mechanisms probably overlap on a defined genetic background in triggering and perpetuating the disease (Table 1).

The disease

Incidence

Rheumatoid arthritis affects about 1% of worldwide population. The incidence of this disease increases with age in females, whereas is unusual in men under 45. First-degree relatives of rheumatoid arthritis patients have increased risk of developing rheumatoid arthritis, and siblings of severely affected patients are at highest risk. Monozygotic twins have a concordance rate of about 12–15% but dizygotic twins have only about 3% concordance rate.

Clinical picture and diagnosis

Progressive, symmetrical joint destruction is the main clinical feature of rheumatoid arthritis. It is the consequence of autoimmune inflammation, described

later in this article. Rheumatoid arthritis, however, is a systemic disease, as shown by the involvement of skin and internal organs, and from the presence of fever and nonspecific laboratory findings of systemic inflammation. Diagnosis is currently based on criteria established by the American College of Rheumatology (Table 2).

Table 2 The 1987 revised criteria* for the classification of rheumatoid arthritis

1. Morning stiffness in and around the joints, lasting one hour before maximal improvement
2. Arthritis of three or more joint areas
3. Arthritis of hand joints
4. Symmetric arthritis: simultaneous involvement of the same joint areas on both sides of the body
5. Rheumatoid subcutaneous nodules
6. Abnormal amounts of serum rheumatoid factor
7. Radiographic changes including erosions or unequivocal bony decalcification localized in, or more marked adjacent to, the involved joints

*Four of seven criteria are required to classify a patient as having rheumatoid arthritis. Criteria 1–4 must be present for at least 6 weeks. Criteria 2–5 must be observed by a physician.

Table 1 Immunological abnormalities which have been described in rheumatoid arthritis

- Hyperactivity of B lymphocytes
- Production of autoantibodies
- Rheumatoid factor
- Antinuclear antibodies: mainly associated with early onset of pauciarticular juvenile arthritis (JA) and with the onset of uveitis
- Antiperinuclear and anti-rheumatoid arthritis nuclear antigens (RANA) antibodies. Both associated with EBV
- Anti-single-stranded DNA: present. Crossreactive between human and other species. No documented diagnostic or pathogenic significance
- Anti-double-stranded DNA: absent
- Abnormalities in the cytokine network
- Increased secretion of IL-1, TNF α , IL-6, IL-15, IFN γ
- T_H1 pattern of cytokine secretion from T lymphocytes infiltrating the synovium
- Abnormal immune responses to infectious agents
- Increased T and B cell responses to EBV antigens
- Retrovirus-mediated immune dysregulation
- Abnormal immune responses to heat shock proteins such as hsp60 and dnaJ
- Antigen-independent mechanisms
- Synovitis, once initiated, may be self-perpetuating
- Mutations in the p53 gene
- Role of the shared epitope
- Binding site for 'arthritogenic' peptides
- Responsible for selection and activation of 'arthritogenic' T cells

Treatment

Pharmacological approach Nonsteroidal anti-inflammatory drugs (NSAIDs) and low-dose corticosteroids are currently used for the symptomatic treatment of inflammation in rheumatoid arthritis. Second-line antirheumatic drugs such as anti-malarials, sulfasalazine, gold, D-penicillamine, azathioprine and cyclosporine are now utilized early in the course of disease to modify its clinical activity. These drugs may stabilize and improve functional activity, including reduction in tender and swollen joint counts and levels of acute phase reactants. The most effective drug available for preventing joint destruction is currently methotrexate. It is often used in combination with other drugs.

Immunotherapy The increasing knowledge of immune mechanisms involved in the etiopathogenesis of rheumatoid arthritis has led to several attempts to correct immune anomalies by immunotherapy. Most approaches have been based on the usage of monoclonal antibodies specific for certain receptors on immune-competent cells, such as CD4 and CD44. Other targets include inflammatory mediators. Anti-tumor necrosis factor α (TNF α) therapy appears particularly promising. Two alternative approaches, both of extreme interest but as yet at a very preliminary stage, are: 1) gene therapy by intra-articular DNA injection; and 2) oral tolerization to putative targets of the disease process, such as collagen and heat shock protein-derived peptides.

Genetics

HLA and rheumatoid arthritis

Many studies have described the strong association between certain subtypes of HLA-DR4/DR1 and rheumatoid arthritis. By comparing the sequences of the various RA-related alleles, it has been observed that a five amino acid stretch, QK/RRAA, is common among all HLA alleles correlated to rheumatoid arthritis (Table 3). The QKRAA sequence was called

'shared epitope', with the idea that it may be involved in the etiopathogenesis of rheumatoid arthritis (Figure 1). Interestingly, the 'shared epitope' seems to be influencing susceptibility to rheumatoid arthritis and also the severity and the outcome of the disease. Together, these observations show that HLA genes play an important role in the etiopathogenesis of rheumatoid arthritis, probably by providing a sort of 'prearthritic' immunological background on which the triggering factors may thrive.

T cell receptors

Recognition of an antigen presented by the HLA molecule is mediated by the T cell receptor (TCR). The genes encoding the α/β receptor consist of variable (V), diversity (D), joining (J) and constant (C) regions. During T cell development, germline genes encoding one for each V, D, J, C region rearrange to form the functional gene. The preferential usage of some of these combinations in rheumatoid arthritis may be related to recognition of a specific autoanti-

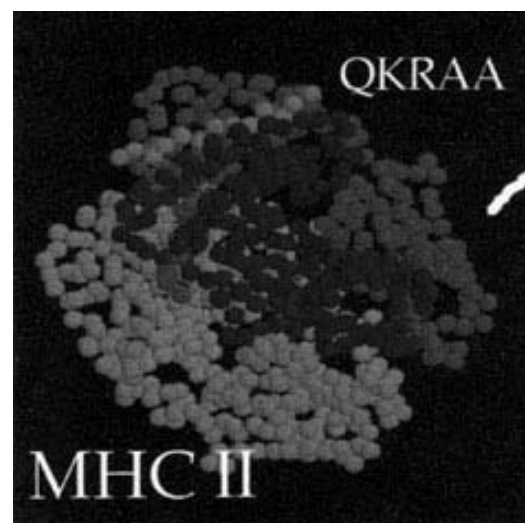


Figure 1 Three-dimensional representation of HLA class II molecule containing the antigenic processed peptide in the antigen-binding groove (light blue stretch, top of the figure). (See also color Plate 4.)

Table 3 Major sites of sequence polymorphism among DR β chain alleles associated with rheumatoid arthritis

Locus-allele	Serologic specificity	Determinant	Amino acid position						
			67	70	71	72	73	74	86
DRB1*0101	DR1	Dw1	L	Q	R	R	A	A	G
DRB1*0401	DR4	Dw4	L	Q	K	R	A	A	G
DRB1*0404	DR4	Dw14	L	Q	R	R	A	A	V
DRB1*0405	DR4	Dw15	L	Q	R	R	A	A	G
DRB1*0408	DR4	Dw14	L	Q	R	R	A	A	G
DRB1*1402	DR6	Dw16	L	Q	R	R	A	A	G

L, leucine; Q, glutamine; R, arginine; A, alanine; G, glycine; V, valine.

gen. To date, TCR repertoire studies in rheumatoid arthritis have shown that, in general, no specific TCR V gene is specifically associated with rheumatoid arthritis. Analysis of the antigen binding site of the TCRs from synovial fluid cells, as opposed to peripheral blood, has shown the presence of conserved amino acid motifs that may play a role in determining the affinity for the HLA-peptide complexes. TCRs bearing these motifs may have a higher affinity for the still undefined 'arthritogenic' peptide that triggers inflammation in the joint.

Proteasomes, transporters and other HLA-linked genes

Antigen processing and peptide transport may also contribute to the induction of abnormal cellular immune responses in rheumatoid arthritis. Allelic differences in the transporter genes, in linkage with specific HLA genes, could be responsible for the selection of a pathway of antigen processing and transport for the generation of peptides having auto-immune potential. For instance, TAP2*0101-1693.A, an allele of TAP-2, is significantly more represented in rheumatoid arthritis patients than in controls. Interestingly, the same allele has significant association with HLA-DR4 only in patients with rheumatoid arthritis. The prevalence of both TAP2*0101-1693.A and HLA DRB1*0401 alleles gives the highest relative risk for rheumatoid arthritis.

Immune abnormalities

Autoantibodies

Rheumatoid factors Rheumatoid factors are autoantibodies directed against the Fc fragment of the immunoglobulin G molecules and are found in approximately 70% of patients with rheumatoid arthritis. Rheumatoid factors produced in normal individuals, during lymphoproliferative or transitory inflammatory conditions, are structurally and functionally dissimilar from the ones produced by rheumatoid arthritis patients. They differ, in the first place, genetically. Rheumatoid factors produced during lymphoproliferative disorders are generally encoded by a set of variable region genes that are germ-line. Hence, these autoantibodies have not been developed by selective antigenic pressure. On the contrary, genes encoding rheumatoid factors derived from patients with rheumatoid arthritis show a wide range of variable region usage. These genetic discrepancies underline strong functional differences. Rheumatoid factors found in normal individuals are part of a family of natural antibodies that have low affinity for the self antigen. They are often polyspecific,

reacting with self and exogenous antigens. On the contrary, rheumatoid factors isolated from rheumatoid arthritis patients are often of high affinity for their target. B cells that express high-affinity rheumatoid factors are very effective in presenting immune complexed antigens to T cells, contributing therefore to the generation of a specific secondary immune response that may be part of the etiopathogenesis of the disease.

Other autoantibodies Autoantibodies other than rheumatoid factors are frequently found in rheumatoid arthritis patients. Their presence, in most cases, can be associated with polyclonal B cell activation, one of the immune abnormalities of the disease, rather than with a specific pathogenic pathway. Antiperinuclear factors and anti-rheumatoid arthritis-associated nuclear antigen (RANA) are specifically associated with rheumatoid arthritis and have been associated with Epstein-Barr virus (EBV) infection. Anticollagen antibodies constitute another family of autoantibodies that may play a role in rheumatoid arthritis by mediating joint damage through the activation of various inflammatory pathways.

Abnormalities of the cytokine network

Proinflammatory cytokine family Interleukin 1 (IL-1) is mainly produced by activated macrophages in response to inflammatory stimuli. IL-1 has both systemic and localized effects on the immune system. For its role in septic shock and as a pyrogen, it is considered as the endogenous pyrogen. At inflammatory sites, it induces T and B cell activation and proliferation, and release of proteolytic and chemotactic substances from macrophages, and has a stimulatory effect on osteoclasts, with consequent cartilage reabsorption.

TNF α is another potent proinflammatory cytokine. It is produced mainly by inflammatory cells, such as activated macrophages. T cells can also produce TNF α in response to antigenic stimuli. TNF α is a stimulator of T cell activation. It upregulates expression of interferon γ (IFN γ) and IL-2 receptor expression in T cells. It induces B cell activation and immunoglobulin (Ig) production. It has chemotactic properties. TNF α interacts with several other proinflammatory cytokines, including IL-15. This amplifying effect may be particularly relevant in the pathogenesis of rheumatoid arthritis.

Immunomodulatory cytokines IFN γ is a potent proinflammatory cytokine produced by T cells and natural killer (NK) cells. It has a stimulatory effect on many cells of the immune system. It plays a major role in controlling infection, in particular of viral

origin. It is also the cytokine primarily associated with T_H1 -type responses. The immunomodulatory cytokine which is the natural antagonist of $IFN\gamma$ is IL-4. IL-4 is produced by $CD4^+$ T cells and eosinophils and is a potent stimulator of B cell-mediated immune responses. IL-2 and IL-6 are both intermediate mediators. They modulate immune responses by acting on other immune cells and stimulating production of other cytokines. Their effects are mainly stimulatory and both cytokines are therefore implicated in the mechanisms of autoimmune inflammation.

Growth factors Transforming growth factor β (TGF β) is a cytokine produced mainly by macrophages and epithelial cells. It is a potent chemoattractant and a very effective inductor of wound healing and fibroblast proliferation. With the other cytokines in this group, its role in rheumatoid arthritis is probably restricted to the late pathogenic steps, when synovial hyperplasia occurs. TGF β also has an important role in mucosal immunity, by boosting specific IgA responses. Another cytokine of this group that has been significantly implicated in the pathogenesis of inflammation is granulocyte-macrophage colony-stimulating factor (GM-CSF). This growth factor is produced by T cells; it is a potent stimulator of inflammatory responses and is involved in processes related to hyperplasia and inflammation.

Balance between pro- and anti-inflammatory cytokines: the T_H1/T_H2 paradigm The control of the balance of immune response mediators secreted by differing T cell subsets is emerging as a major immunoregulatory mechanism. T Helper (T_H) cells have been subdivided into T_H1 , secreting mainly $IFN\gamma$ and IL-2, and T_H2 , secreting mainly IL-4. This subdivision, originally described in the mouse, has also been applied to humans. It has an important functional significance in the physiology of immune responses. T_H1 -type cells are generally involved in cytotoxic T cell-mediated responses, while T_H2 cells are elevated in allergic and parasitic diseases. An efficient immune response rests on a balance between the two cell types. Any alteration of this equilibrium would affect the quality of the immune response toward exogenous antigens, therefore favoring a possible loss of tolerance toward self antigens. In rheumatoid arthritis a T_H1 pattern is found. Proinflammatory cytokines are upregulated. IL-6, IL-1 β and TNF α stimulate the secretion of prostaglandins, collagenase and oxygen radicals, and sustain synovocyte proliferation. All these anomalies in the cytokine network are probably secondary to the trig-

gering events that elicit autoimmune inflammation, and they certainly represent an important part of the mechanism of amplification and perpetuation of the autoimmune damage.

Adhesion molecules

Any anomalous expression of adhesion molecules, or their ligands, could affect the normal pattern of migration of immune response cells to the synovium. ICAM-3, an adhesion molecule and a costimulatory signal for both resting and activated lymphocytes, is overexpressed in rheumatoid synovium. In parallel, adhesion ligands such as ELAM-1 and VCAM-1 are overexpressed on endothelial cells in rheumatoid arthritis patients. CD44, the ligand for hyaluronic acid, is also overexpressed in synovial fluid lymphocytes. These anomalies in leukocyte trafficking and homing are present in rheumatoid arthritis. They may be part of the disease pathogenesis.

Toward a comprehensive model for etiopathogenesis of rheumatoid arthritis

Infectious agents

To date, no convincing evidence has been provided for a single infectious agent fulfilling Koch's postulates as an etiologic agent for rheumatoid arthritis. Nevertheless, some interesting observations have been made that correlate some pathogens with rheumatoid arthritis. This is the case, for example, with EBV. Patients with rheumatoid arthritis have increased titers of unusual antibodies to EBV, such as antirheumatoid arthritis nuclear antigens (anti-RANA). EBV antigens have been identified in the synovium and as discussed later, EBV may also participate in mechanisms of abnormal reactivity to the 'shared epitope'.

Another family of viruses that some authors claim to play a role in etiopathogenesis of rheumatoid arthritis is represented by retroviruses, which might activate 'autogenes' that may trigger autoimmune disease. Bacterial antigens are believed to be triggers of various arthritides, such as Reiter's disease. In these diseases, chronic arthritis is associated with abnormal immune responses to bacteria such as *Shigella*, *Salmonella* or *Yersinia*. These responses can be detected at the synovial sites of inflammation, without demonstration of the intact microorganism in the synovial space. Bacterial antigens with autoimmune potential may be transported into the joint, and initiate, and/or sustain, abnormal immune responses. Among the possible candidates, special attention has been given to heat shock proteins. Bacterial heat shock proteins, including hsp65 and *Escherichia coli*

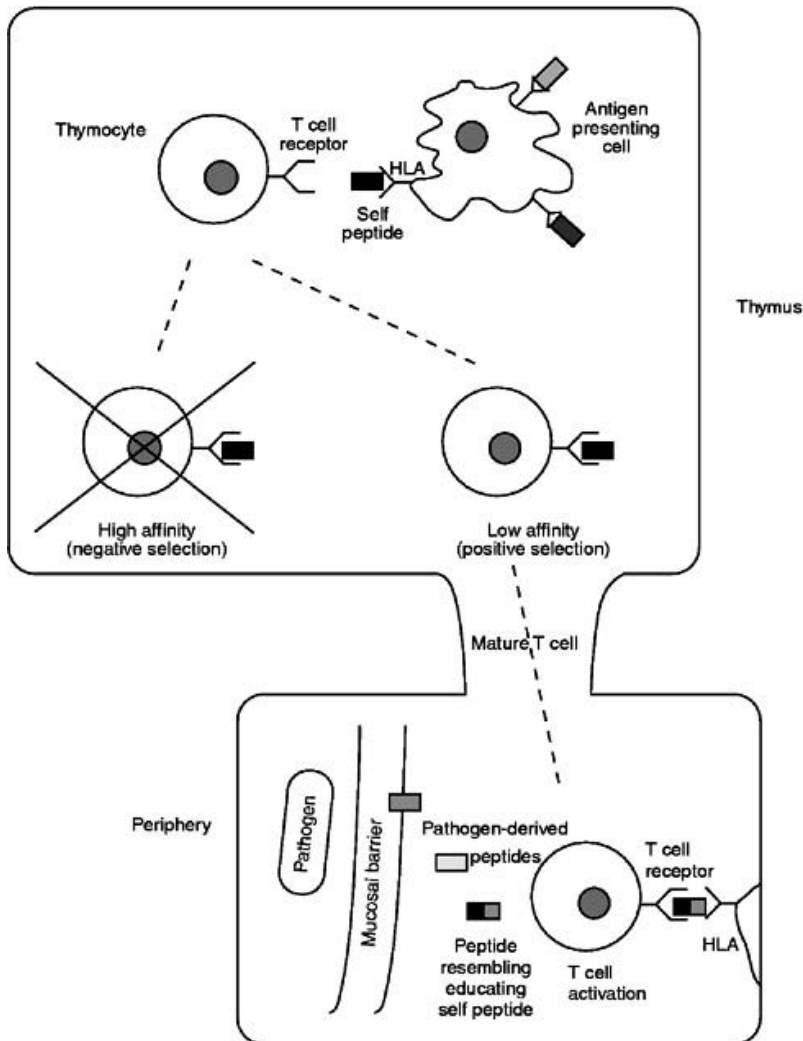


Figure 2 Multistep molecular mimicry mechanism.

dnaJ, are targets of specific humoral and cellular responses in patients with rheumatoid arthritis.

Role of the 'shared epitope': Binding and/or 'multistep molecular mimicry'?

Binding hypothesis One etiopathogenetic hypothesis takes into account the special binding properties conferred to the disease-related HLA alleles by the 'shared epitope' sequence. The QKRAA amino acid stretch is part of the third hypervariable region of the $\beta 1$ chain of the HLA molecule. This region is an interaction site both for the bound peptide and for the TCR. Recent data have shown that nonconservative amino acid substitutions in this region of the HLA molecule lead to strong differences in the HLA-peptide binding patterns. A first explanation for a role of the QKRAA sequence in rheumatoid arthritis may therefore be based on the idea that the presence of this sequence could affect the binding of still undefined 'arthritogenic' peptides in subjects

bearing the HLA alleles correlated to rheumatoid arthritis. Variations of the binding hypothesis support a role for the QKRAA sequence in binding to peptide transporters, or to a superantigen from viruses or bacteria.

Multistep molecular mimicry hypothesis HLA molecules not only bind the immunogenic peptide and present it to the T cell, but they can also be considered antigens themselves. Peptides derived from RA-related HLA molecules, and containing the 'shared epitope' sequence, may participate in mechanisms of T cell thymic selection and peripheral activation (Figure 2). Immature T cells are selected in the thymus on the basis of their ability to bind HLA-peptide complexes presented by the 'nurse' cells of the thymic epithelium. Peptides encompassing the 'shared epitope' sequence can be naturally processed and therefore are part of the peptide pool that positively selects T cells during fetal life. The

presence of HLA genes encoding for the QKRAA sequence provides the genetically predetermined background for the selection of potentially self reactive T cells. These T cells, previously quiescent, could be activated by the encounter with homologous peptides of exogenous origin. Interestingly, common human pathogens have been found that express the 'rheumatoid arthritis cassette' in the context of highly immunogenic proteins. This cassette is a target of specific immune responses in patients with rheumatoid arthritis. Upon generation of the QKRAA-driven abnormal T cell responses, the perpetuation and amplification of autoimmune inflammation is the last, and probably the most diverse, step of the process.

Antigen-independent mechanisms

In rheumatoid arthritis, the chronic granulomatous rheumatoid response is the late stage of inflammation that leads to destruction of the normal architecture of the joint. According to several investigators, this stage of rheumatoid arthritis is antigen independent. Several mechanisms related to chronic inflammation can indeed sustain the destructive process without necessarily requiring constant restimulation of T cells. Also, recent interest has come from the description in the rheumatoid synovium of mutations of p53, a tumor suppressor protein involved in the control of the cell cycle.

Pathogenesis of the destructive process in rheumatoid arthritis

Intermittent deposition into the joints of antigen-antibody complexes and sometimes infectious agents (often carried by the precursors of the synovial type A macrophages) leads over time to the release of cytokines and growth factors that cause fibroblast hyperplasia and angiogenesis. At the same time, T and B lymphocytes localize to the synovial space. The most damaging agents are the ones that induce secondary immune responses leading to intermittent immune complexes formation and/or stimulation of low-affinity T and B lymphocytes.

Rheumatoid factor B cells efficiently present antigens trapped in immune complexes. Once such cells undergo somatic mutation and localize to the joints, rheumatoid arthritis becomes severe and progressive. At this stage, synovitis is maintained by conventional immune and inflammatory responses to different environmental antigens. The progression to seropositive erosive arthritis may then occur more frequently in 'shared epitope' positive individuals, as this epi-

tope is present on different microorganisms that induce high-titer antibodies.

See also: Adjuvant arthritis; Autoimmune diseases; Autoimmunity; Cytokines; Glycosylation of immune system molecules; HLA class II; MHC disease associations; Rheumatoid arthritis, animal models; Rheumatological disorders.

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RHEUMATOLOGICAL DISORDERS

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The myriad of rheumatological diseases encompass those that are primarily degenerative, secondary to infectious agents, metabolic or endocrine-based diseases, neoplasms, neurovascular disorders, bone and cartilage disorders, and miscellaneous disorders in addition to those which have an immunological basis. Those diseases with an immunological basis constitute a large percentage of the rheumatological disorders, and in many cases, the most severe. Currently our understanding of these diseases is limited. However, it is believed that in the majority of instances, environmental factors are coupled with immunogenetic factors which determine first, susceptibility for a particular disorder and, second, severity. It is clear that many specific major histocompatibility complex (MHC) class I and II susceptibility genes have been identified for various rheumatic diseases, and certainly other susceptibility genes which are not part of the MHC gene complex also exist. In short, these diseases are polygenic in their basis. Thus, different degrees of immunogenetic susceptibility coupled with environmental factors capable of triggering an immunological disease can lead to loss of tolerance, followed by cellular and molecular auto-reactivity, which in turn can result in clinical autoimmune disease.

Of the immunologically mediated rheumatological disorders, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Sjögren's syndrome are discussed in detail in other sections of this encyclopedia. The discussion which follows will center on other, less common immunologically-based rheumatological disorders.

Systemic sclerosis (scleroderma) and related disorders

Systemic sclerosis (SSc) is a diffuse connective tissue disease of unknown etiology which is characterized by fibrosis of skin and internal organs with associated autoantibodies and microvascular pathology. The peak ages of incidence are in the fifth and sixth decades of life, females, particularly women of child-bearing years, are affected more commonly than males. Related disorders include CREST syndrome, mixed connective tissue disease (MCTD) and undifferentiated connective tissue disease (UCTD).

Fibrosis of the skin and other organs, including

blood vessels, is a pathologic hallmark of this disease. The underlying pathogenesis of scleroderma is a widespread disorder of microvasculature which results in extensive, proliferative fibrosis. Microvascular lesions in arterioles and capillaries generally precede fibrosis and proliferative changes in vital organs such as the lungs, heart, gastrointestinal tract and kidneys.

Although the exact immunopathogenesis is poorly understood, this is an immunologically mediated disease; for example, autoantibodies abound in scleroderma. Several of these autoantibodies are listed in Table 1.

T cell abnormalities have also been described in scleroderma, suggesting a role for both T_H1 and T_H2 cells. In particular, T_H1 cell responses are present and there is an increased expression in serum of both interleukin 2 (IL-2) and IL-2 receptors and endothelial cell growth-inhibition factor (a T_H1 granzyme). Data also exist to suggest activation of T cells by collagen. This suggests a scenario whereby cytotoxic T cells could directly attack endothelial cells based on an autoreaction to vascular antigens including type IV collagen. Moreover, a role for T_H2 cells is also suggested by increased expression in SSc serum of CD30, IL-4, IL-10 and IL-13.

CREST

CREST syndrome is considered a 'benign' variant of scleroderma (C = subcutaneous calcification, R = Raynaud's phenomenon, E = esophageal dysmotility,

Table 1 Autoantibodies in Scleroderma

Antinuclear antibodies (especially nucleolar, others)
Antinucleolar antibodies
RNA polymerase III
U2 RNA protein complexes (nucleolar 4-6S RNA)
U3 RNA protein complexes (fibrillarin)
Anti- T_H ribonucleoprotein
Anti-topoisomerase 1 (Scl-70)
Anti-collagen antibodies (type I and type IV)
Anti-ribonucleoprotein (RNP)
Anti-Ro, Anti-La
Ku
Anti-centromere antibodies
Anti-laminin

S = sclerodactyly, T = telangiectasia). It is most commonly associated with anticentromere antibody.

MCTD

MCTD shares clinical features of scleroderma, polymyositis and SLE, and represents an 'overlap' syndrome characterized by a very high titer of antibody to ribonucleoprotein and an absence of other antinuclear antibodies (ANAs). It has been considered to be a more benign CTD than scleroderma but many cases have evolved into typical scleroderma.

UCTD

Diffuse CTDs with shared features of more than one but with no specific ANA or other autoantibody profiles are termed UCTD.

Idiopathic inflammatory myopathy

The idiopathic inflammatory myopathies, which include polymyositis, dermatomyositis and inclusion body myositis, are inflammatory diseases of muscle which cause weakness, and less frequently pain, of the large proximal muscles of the trunk and limbs. The inflammatory lesion with primarily lymphocytes results in damage and subsequent attempts at repair of the muscles with release of the muscle enzymes creatine kinase, aldolase, aspartate aminotransferase and lactate dehydrogenase. The muscle damage also results in characteristic changes on electromyography.

Dermatomyositis is distinguished from polymyositis with its characteristic rashes. These include a heliotropic rash of upper eyelids, a violaceous rash over the finger joints (Gottron's sign) and erythematous rashes about the periarticular areas, neck and anterior chest (V sign) or back and shoulders (shawl sign). The skin manifestations may occur without evident myositis.

There are differences in the inflammatory process in the idiopathic inflammatory myopathies that may explain their differences in clinical presentation. In dermatomyositis, the lymphocyte infiltration is predominantly CD4⁺ cells localized to the perivascular area, with complement activation and deposition with a resultant vasculopathy. This may also be associated with a vasculitis of the gastrointestinal tract seen most often in childhood dermatomyositis.

Dermatomyositis may be associated with an antibody to Mi-2, a not yet fully characterized nuclear antigen. Patients possessing the anti-Mi-2 antibody usually present with acute onset of disease with good response to treatment with anti-inflammatory agents. Of the idiopathic inflammatory myopathies, derma-

tomyositis is associated with increased incidence of malignancies.

In polymyositis, the inflammation is characterized by CD8⁺ cells and macrophage invasion of the endomysium and there is cytotoxicity against muscle cells. A vasculopathy is not a prominent feature in polymyositis. A number of autoantibodies have been detected in polymyositis, primarily directed against aminoacyl-transfer RNA synthetases which function to bind amino acids to the transfer RNA. The Jo-1 antibody is directed against histidyl-tRNA synthetase; PL-7 against threonyl-tRNA synthetase; PL-12 to alanyl-tRNA synthetase; OJ to isoleucyl-tRNA synthetase; and EJ antibody to glycyl-tRNA synthetase. These antisynthetase antibodies identify a syndrome which includes polymyositis and increased incidence of pulmonary fibrosis, polyarthritis, Raynaud's phenomenon and mechanic's hands (with cutaneous thickening, roughening and fissuring). Patients possessing antibodies to signal recognition particle (SRP), an RNA-protein complex which functions in the recognition and regulation of the movement of newly synthesized proteins, have severe polymyositis that is unresponsive to usual treatment.

Inclusion body myositis (IBM) differs from the other idiopathic inflammatory myopathies in several ways. Clinically, there may be asymmetric involvement of both proximal and distal muscles, with a more frequent involvement of the muscles of swallowing. Like a patient possessing anti-SRP, IBM is very resistant to anti-inflammatory treatment. But it is the histopathology that defines inclusion body myositis. Though there may be endomysial inflammation, as seen in polymyositis, there may be at times only few inflammatory cells. However, characteristically, the biopsy specimens show 'rimmed vacuoles' with amyloid within or next to the vacuoles. Electron microscopy may demonstrate filamentous inclusions.

Though viruses have been suspected as the etiology for the idiopathic inflammatory myositis, at present the etiology is not known.

Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is a disorder of recurrent arterial and/or venous thrombosis and thrombocytopenia associated with the presence of antiphospholipid antibodies (aPL). Although scattered case reports existed, the term APS was first used in 1985 with tentative criteria being proposed in 1987. Approximately 50% of these patients are considered to have the primary APS (PAPS) and 50% secondary APS (SAPS). SAPS seems to be clinically

indistinguishable from PAPS except for an association with SLE.

The major clinical findings include venous occlusion with deep venous thrombosis, often recurrent, which is complicated in one-third of patients by pulmonary thromboembolism. Other venous thrombotic phenomena include pulmonary hypertension, hepatic vein occlusion producing a Budd–Chiari syndrome and renal venous occlusion. Arterial occlusions are also common. The most common manifestations are either transient ischemic attacks or strokes, often recurrent, and the frequency exceeds that of other arterial events such as myocardial infarction or peripheral large artery occlusion with gangrene. Mesenteric, retinal and renal infarctions also occur and migraine headaches with aPL may precede fully expressed APS. Recurrent fetal loss in non-SLE patients represents the most frequent clinical presentation of PAPS in young women.

Serologically these individuals are characterized by having the lupus anticoagulant (LA), anticardiolipin antibody (aCL) and/or antibodies to other negatively charged phospholipids such as phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA).

aPL are a heterogeneous group of antibodies which occur in certain autoimmune diseases, especially SLE, PAPS, syphilis and other infectious diseases, and in normal individuals. Autoimmune aPL include the LA and aCL and, as mentioned above, are associated with thrombosis, recurrent fetal loss and thrombocytopenia. Although aPL are heterogeneous in their specificities, those that react with anionic phospholipids and β_2 -glycoprotein 1 (β_2 GP1) (β_2 GP1-dependent aCL) are associated with the clinical manifestations of APS. In addition to LA and aCL, antibodies to the other anionic phospholipids include antibodies to PS (aPS), PI (aPI) and PA (aPA).

APS patients have been reported with aPL of the immunoglobulin G (IgG), IgM and IgA isotypes. In addition, patients may have one isotype alone or a mixture of one or two of the others. The majority of APS patients have aPL of the IgG isotype, usually at moderate to high levels. Some patients with APS are IgM positive only, and some data suggest that IgA aPL associate closely with thrombosis, but this latter observation requires further analysis. Some of the problems with IgM and IgA aPL isotype determinations may be the frequency of false-positive results.

The distribution of IgG aCL subclasses in APS patients is unclear. For example, in some patients, IgG1 and IgG3 aCL are the predominant subclasses, but in others, IgG aCL subclasses are approximately

the same as that found in normal human sera. Moreover, both κ and λ light chains are present in the same proportions as found in antibodies in normal human sera.

The specificity and affinity of aPL may distinguish one antibody from another and may be important in their pathogenic roles in the APS. APS aPL show binding cross-reactivity with various anionic phospholipids. For example, aPL binding to cardiolipin have equivalent binding to other anionic, phospholipids. Thus, aCL in patients with APS cross-react with a variety of anionic phospholipids and β_2 GP1. This is in contrast to aCL in syphilis sera that bind to CL alone (β_2 GP1-independent aCL) and are not associated with the APS.

The specificity and function of aCL and LA antibodies are closely related but slightly different. In particular, both LA and aCL act as anticoagulants by inhibiting PL-dependent coagulation reactions *in vitro*. LA recognize the complex of lipid-bound (human) prothrombin and aCL interact with β_2 GP1 absorbed on to a phospholipid surface. Moreover, antibodies to β_2 GP1 (and/or to aCL– β_2 GP1 complex) and LA associated with antiprothrombin activity are better predictors of thrombosis and likely have a pathogenic role in thromboses occurring in APS. In this regard, antibody binding to PL membrane-bound antigen could alter the kinetics of PL-dependent reactions involving that antigen. Thus, autoimmune aCL require β_2 GP1 to exert their anticoagulant effect, and they appear to amplify the anticoagulant effect of β_2 GP1. LA associated with antiprothrombin activity could produce similar functional effects.

Factors resulting in the induction of aCL and other aPL are not well understood. For example, data exist to suggest the importance of T cell dependence and antigen drive, including MHC allele and Ig, variable region (V) heavy chain (H) gene restriction and T cell subset abnormalities. However, experimental PAPS can be induced by passive transfer of human polyclonal IgG and IgM aCL and mouse monoclonal aCL in naive BALB/c mice. Furthermore, APS and SLE can also be induced both clinically and serologically in naive BALB/c mice with a human monoclonal anti-DNA antibody bearing the pathogenic 16/6 idiotype (id). PAPS is also inducible in BALB/c mice following immunization with a human monoclonal aCL (H-3). Cytokine analysis in these mice indicates low production of IL-2, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). This suggests that aCL and APS may be induced by anti-idiotypic antibodies generated in naive mice in response to immunization with anti-DNA or aCL antibodies bearing pathogenic idiotypes. However,

other data argue against this because aCL can be produced in mice by immunization with β_2 GP1. In this case, aCL always appears before anti- β_2 GP1, excluding the possibility that aCL are directed primarily to β_2 GP1 or are induced by the formation of anti-idiotypic to anti- β_2 GP1. In addition, IgG aCL from patients with APS can induce aCL in mice (this latter observation is consistent with an anti-idiotypic response). Thus, it is possible aCL may be induced by proteins with high avidity for phospholipid such as β_2 GP1.

Although the way in which aPL mediate thrombosis is not known, data exist to support several potential mechanisms for the procoagulant effect of aPL. These include reducing endothelial cell production of prostacyclin (PGI_2), increasing tissue factor activity, decreasing activated protein C, and/or activated protein S, and/or antithrombin III activity, increasing platelet activating factor and/or thromboxane A_2 production, increasing adhesion molecule expression, and decreasing placental trophoblast implantation. It is likely that more than one, and possibly several, of these mechanisms are important for aPL to mediate a clinical hypercoagulable state.

Wegener's granulomatosis and other diseases associated with antineutrophil cytoplasmic antibodies

Wegener's granulomatosis is a disease in which a granulomatous vasculitis results in inflammatory lesions of the upper and lower airways, particularly the paranasal sinuses and the lung. Other organs which may be involved include the kidney, with disease ranging from mild focal glomerulonephritis to a diffuse necrotizing glomerulonephritis, the joints with frank arthritis, small vessel vasculitis of the skin, and ocular disease ranging from conjunctivitis to scleromalacia perforans.

The severity of the disease can range from very mild and limited to severe multiorgan disease, which, prior to cytotoxic therapy, was uniformly fatal. While the biopsy demonstrating a granulomatous vasculitis remains a gold standard in the diagnosis of Wegener's granulomatosis, the ability to make the diagnosis has been facilitated with the recent identification of an antibody to cytoplasmic antigens detected in ethanol-fixed neutrophils.

These antibodies identified by indirect immunofluorescence are seen in two primary patterns. One is a coarse granular cytoplasmic pattern (c-ANCA) and the second a perinuclear pattern (p-ANCA). Further research has identified proteinase 3 as the antigen causing the c-ANCA. The p-ANCA is a result of an antibody to myeloperoxidase. Depending on the

extent and severity of the disease, c-ANCA is seen with 60–90% sensitivity and greater than 90% specificity in Wegener's granulomatosis. c-ANCA is seen in much lower frequency in other vasculitis, as well as infectious diseases.

p-ANCA is less specifically associated with the disease and is seen in Churg–Strauss vasculitis, as well as other vasculitides and autoimmune diseases.

With the sensitivity/specificity of c-ANCA/antiproteinase 3 antibody in Wegener's granulomatosis, aggressive investigation as to its etiopathologic role has been undertaken. However, its definitive role remains to be elucidated. It is hypothesized that the antiproteinase 3 antibodies interact with surface proteinase 3 on granulocytes and endothelial cells, subsequently triggering the inflammatory cascade that results in the granulomatous vasculitis and inflammation. However, contrary to what one would conclude, neither the level of c-ANCA nor the change in c-ANCA levels predict severity of disease or changes in disease activity. Further studies are necessary to more completely define the role of the antineutrophil cytoplasmic antibodies in their associated disease states.

Spondyloarthropathy

The spondyloarthropathies are a group of arthritides which share common features and include ankylosing spondylitis, reactive arthritis (including Reiter's syndrome), enteropathic arthritis (the arthritis associated with inflammatory bowel disease), psoriatic arthritis and unclassified spondyloarthropathy.

The shared features include arthritis of the axial skeleton and particularly the sacroiliac joints, peripheral arthritis and enthesitis (the inflammation of the transitional tissue between ligament and bone and, similarly, tendon and bone). The inflammatory lesions may evolve with development of bony structures which may ankylose a joint through a yet undefined process. Also shared are extraarticular features, including inflammatory diseases of the eye, heart, lung, skin and mucocutaneous tissues, gastrointestinal and genitourinary tracts. Both conjunctivitis and anterior uveitis are seen in the eye. The skin is involved with the obvious psoriasiform rash, as well as the closely related lesion of keratoderma blennorrhagicum, and with oral ulcerations. Cardiac lesions include an aortitis which may lead to valvular insufficiency, particularly with long-standing disease and conduction disturbances with various forms of heart block.

Whereas pulmonary fibrosis in other rheumatic diseases more frequently involves the lower segments of the lung, those seen in the spondyloarthropathies

involve the upper lobes and may further compromise the patient's respiratory status, which may be affected by involvement of the costovertebral and costochondral joints. Lesions of the gastrointestinal tract that are seen with inflammatory bowel disease and dysentery may also be seen in other spondyloarthropathies without clinical symptoms. In addition to the urethritis seen in Reiter's subset of the reactive arthritis, there is an increased occurrence of prostaticitis in the spondyloarthropathies.

Immunologically, there is a definite association of the spondyloarthropathies with the MHC class I molecule HLA-B27, particularly in ankylosing spondylitis, where more than 90% of white patients possess this antigen. Recently, there have been advances which have better defined the role of HLA-B27 in the pathogenesis of the spondyloarthropathies. For example, transgenic rat and mice models exist whose cells express human HLA-B27 antigen with animal/human β_2 -microglobulin. When these genes are significantly expressed, the animals develop articular and extraarticular lesions similar to the human diseases.

Environmental influences have also been defined, as animals raised in sterile conditions do not develop disease but, when allowed to reassociate with their normally-raised litter mates, subsequently develop disease. Studies are ongoing to identify which of the common enteric bacteria poses the biggest risk for development of HLA-B27-associated disease.

The structure and sequence of the HLA-B27 molecules have also been recently defined. The peptide-binding region of HLA-B27 interacts with nonapeptides which may derive from gram-negative enteric bacteria. Of importance is that the HLA-B27 has the typical MHC class I molecular structure with parallel helical chains that form a peptide-binding groove

and a β -pleated sheet forming the floor. The HLA-B27 molecule itself possesses a similar sequence of amino acids to the enteric bacterial nonapeptide. The differences in the amino acid sequences of the currently identified nine different HLA-B27 molecules are being investigated to determine how they influence the risk for development of disease, as identified by epidemiologic studies.

Although there have been significant advances in the understanding of the immunology of the spondyloarthropathies, as yet it is unclear how a chronic inflammatory process develops and is perpetuated.

See also: Antinuclear antibodies; Autoimmune disease, spontaneous experimental models; Rheumatoid arthritis, animal models; Rheumatoid arthritis, human; Sjögrens syndrome; Skin, autoimmune diseases; Systemic lupus erythematosus, experimental models; Systemic lupus erythematosus (SLE), human.

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RHINITIS, ALLERGIC

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When hayfever is seasonal or environmental, it will almost certainly be allergic, but the first distinction must be made between infectious and noninfectious rhinitis. In both of these types nasal polyps may be present and the patient is often anosmic. Lack of smell (and taste) may be so insidious in onset that only when leading questions are posed from the doctor taking the history may these symptoms be mentioned. In noninfectious rhinitis that is perennial, it may be very difficult to decide whether there is an

allergic basis to the symptoms. A rational classification of symptoms is not always easy in rhinitis, which is a heterogeneous group of diseases with multiple etiologies.

History, skin tests and possibly cytology of the nasal smear when symptoms are present should allow a basic classification of the appropriate subgroups (Tables 1 and 2). The term used in the past when rhinitis was not obviously allergic or infective was 'vasomotor rhinitis', however the International

Table 1 Rhinitis diagnosis: rhinorrhea, sneezing, obstruction, itching

<i>Allergic</i>	<i>Infective</i>	<i>Structural</i>	<i>Other</i>
Seasonal	Acute	Deviated septum	Multifactorial
Perennial	Chronic	Large turbinates	Stress
Occupational		Polyps	Hormonal
Environmental		Anatomical	Ingestants
			Smoke
			Smoke
			Hyperreactivity
			Systemic disease

Table 2 Classification of rhinitis

<i>Type</i>	<i>Skin test</i>	<i>Steroids help</i>	<i>Antihistamines help</i>	<i>Predominant cell</i>
<i>Noninfectious</i>				
Seasonal allergic	+	+	+	Eosinophil
Perennial allergic	+	+	+	Eosinophil
Perennial nonallergic	—	—	—	Few cells
Eosinophilic	—	+	+/-	Eosinophil
Noneosinophilic	—	—	—	Few cells
Polyps	+/-	+	—	Eosinophil
<i>Infectious</i>				
Common cold	—	—	—	Neutrophil
Rhinosinusitis	—	—	—	Neutrophil
Polyps	—	—	—	Neutrophil

Consensus Report on the Diagnosis and Management of Rhinitis decided this word should not be used. Clinical examination is not always helpful; the nasal mucosa, which may be described as looking 'typically allergic', should be more accurately described as looking pale and wet. Unfortunately allergic causes are not the only factors that may make the mucosa pale and swollen; indeed the nasal turbinates may be so swollen that they look like, but are not polyps – they are correctly described as polypoid. When a patient with allergic rhinitis has recently been sneezing, the mucosa looks red and congested and not unlike an infective rhinitis. A nasal airway that is obstructed may have had decongestive nasal sprays or drops used many times a day or for long periods, so that even if the condition causing the original nasal obstruction was allergic, the drop-treated mucosa is usually very red (the nasal cilia have been denuded), particularly on the nasal septum.

Causes of allergic rhinitis

Both the microenvironment indoors and also the environment out of doors have to be separately considered. All animals are strong sensitizers to the allergic-prone individual. Cats are stronger sensitiz-

ers than dogs, but all very small animals such as guinea pigs or hamsters can cause symptoms. Laboratory allergy is a significant problem since rats, mice, guinea pigs and rabbits in an animal laboratory setting, may quickly sensitize the allergic-prone individual.

There are many occupational allergic hazards which can give rise to allergic rhinitis and, more important, to the associated asthma. Workers dealing with castor beans (*Ricin* spp.), whether in the fields, during transport, at the dockside or in ships, are particularly at risk. They are such a strong sensitizers that they will cause allergic inhalent problems to the so-called 'normal' population when downwind from a castor bean factory or when actively working with the beans.

In a hospital setting, latex allergy occurs in 8–17% of health work personnel. Wherever latex gloves are in use in hospital there will be airborne latex allergen which may sensitize particularly the atopic individual.

It must be remembered that ingestants as well as inhalants may cause rhinitis. Traditionally, milk is supposed in some people to cause catarrh but this symptom is difficult to evaluate. Both the mediators of nasal allergy and also the resulting clinical symptoms including nasal obstruction can be measured

after provocation tests. However, the nose, which has its own neurogenic system and complicated blood supply, is exposed to so many stimuli, only some of these specifically allergic and only some from the external environment, that it is not surprising that rhinitis has many pathophysiological forms.

Hayfever on the increase

It is remarkable that in 1934 it was found that 3.5% of the Japanese population resident in southern California were found to have allergic rhinitis due to pollen, a disease that had never been recognized in Japan. However, in 1986 not only was the increase recognized, but an incidence as high as 33.3% for children aged between 6 and 17 years had been noted. The major allergen in Japan comes from trees – the Japanese cedar tree. The frequency of allergy to this particular pollen is more common in people living within 50–100 m of a motorway, compared with those living in the country who are surrounded by trees.

In the UK, it has been shown that the number of people consulting their doctors for seasonal hayfever almost doubled between 1970 and 1981. The increase is still continuing and is occurring in all age groups, but between 1961 and 1985 records on the content of the air over Central London have shown a decline in the cumulative grass pollen count. This is largely because fewer farmers make hay these days, and the grass is cut for silage before it flowers to produce pollen. The sown pastures are of ryegrass (*Lolium*) which is a poor pollen producer compared with the previously sown cocksfoot (*Dactylis glomerata*) and timothy grass (*Pleum pratense*). These grasses may be seen pollinating in the summer along uncropped roadside verges.

A decline in pollen concentration during the last two decades has been accompanied by an increase in air pollution. Hayfever is common, according to the Japanese, in people living near motorways. When pollen grains are collected from industrial areas it can be seen that they are coated with a variety of chemicals. It may be that for allergy to develop, additional factors are necessary rather than just the allergens. These same chemicals may act as irritants to the lining of the nose and lungs. Moreover, it has been shown that diesel exhaust particles have an adjuvant effect on the development of antibodies to pollen in mice.

The immune system and the allergic nose

Bone marrow-derived mast cell precursors prolifer-

ate locally due to stimulation by antigen-activated T cells. The precursors migrate to regional lymph nodes and acquire surface immunoglobulin E (IgE) under the regulation of helper T (T_H) cells. These mast cells reach the nasal secretions mainly by passive diffusion. Mucosal synthesis of IgE may also occur locally. The eosinophilia is probably due to ECF-A (eosinophil chemotactic factor of anaphylaxis) which is one of the degranulation products of mast cells, as is leukotriene B_4 (LTB_4). In effect, the manifestations of IgE-mediated hypersensitivity in the nasal mucosa are basically an inflammatory protection. It seems likely, however, that a vicious circle may be set up by mechanisms that depend on complement-activating IgG antibodies and perhaps both type I and type III hypersensitivity reactions participating in the development of nasal polyps. It is now clear that interleukin 4 (IL-4) plays a pivotal role both in the induction and the effector phase of the IgE-mediated allergic response.

Mediators and nasal allergy

Mediator release after nasal airway challenge with allergen has now been extensively investigated. Nasal lavage for the recovery of secretions on the surface of the nasal mucosa has made it possible to identify a postallergen challenge increase in a vast number of putative mediators of inflammation in humans. As well as experimental provocation tests, the inflammatory response of the nasal mucosa to natural exposure to pollen can also be measured. Much can be learned about the immediate (early) phase and the late (delayed) phase reaction so that a hypothesis can be generated of the involvement of different trigger cells during the different parts of the allergic reaction (Figure 1). It would seem that mast cells are the main source of histamine in the early phase, and basophils the main (but lesser source) in the late phase, during which there is no similar increase in prostaglandin D_2 . During the pollen season there is a large increase in the number of nasal mast cells, but perhaps there has been too much focus on these cells, and now membrane-derived mediators of inflammation are being studied following nasal allergen challenge. These include arachidonic acid products from the lipoxygenase pathway leukotrienes LTC_4 , LTD_4 and LTB_4 , and some of those from the cyclo-oxygenase pathway such as 5-hydroxyeicosatetraenoic acid (5-HETE) and thromboxanes. It is not yet certain whether platelet-activating factor (PAF) increases or decreases after allergen challenge. It is of interest that N - α -tosyl-L-arginine methyl ester (TAME)esterase, a nonspecific marker of inflammation, best reflects the symptomatology obtained at nasal challenge. This

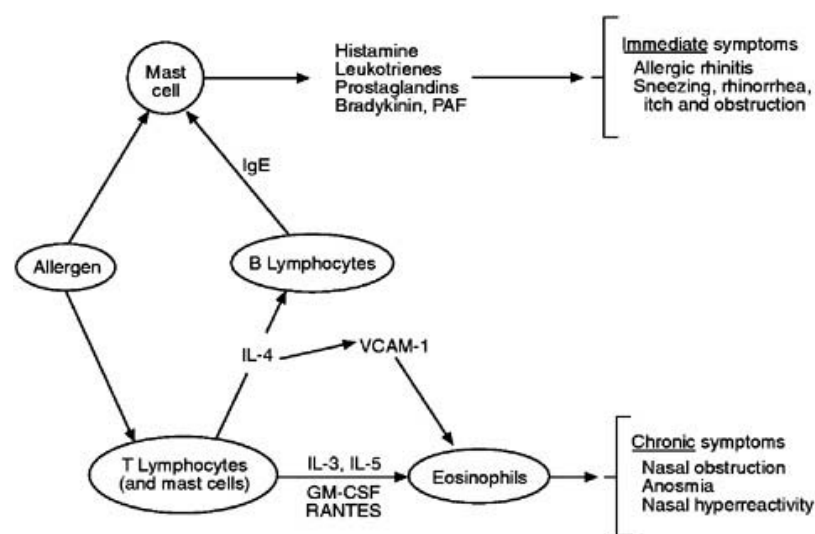


Figure 1 Mechanism of allergic rhinitis. (Reproduced with permission from Durham SR Hypothesis on mechanisms of allergic rhinitis.)

suggests that plasma leakage is a crucial marker of the pathogenesis of a nasal allergy. This plasma leakage may contain many potent peptide mediators and augment the inflammatory response. Certainly bradykinin can be demonstrated in the nasal secretions and would explain the ongoing chronic nature of nasal symptoms both in challenge models and during natural exposure.

To understand the role of different mediators that generate the signs and symptoms of allergic rhinitis, provocation tests with the mediator in question have been done, and the symptoms monitored. The other way to elucidate the problem is to use specific mediator antagonists which should produce further clues as to the effects of the mediator itself. So long as the effect is on the receptor mechanism it will be relevant. This is certainly the case as far as histamine with the antihistamines is concerned. There are variable amounts of histamine in nasal secretions in normal as well as in allergic nasal secretions. During a nasal infection some bacteria will increase the histamine level. Even so, histamine still remains the only mediator that behaves consistently in terms of release with local challenge, as well as producing most of the major symptoms. Also, specific H_1 antagonists influence the symptoms *in vitro* and *in vivo*. It remains to be seen whether antagonists against other putative mediators such as leukotrienes, PAF and bradykinin will be found.

There is some evidence that a nonsedative antihistamine compound (cetirizine), which may act against the eosinophil cell and PAF, confers greater benefit to the patient. Clinically, when nasal obstruction is a major symptom in allergic rhinitis, antihistamines are not very helpful in providing relief. This would seem to depend on the complicated mechanisms sub-

serving the blood supply to the nasal mucosa. It must also be remembered that there are functional differences between mucosal and connective tissue mast cells. This mast cell heterogeneity has clinical implications in rhinitis. The nasal mast cells contain tryptase and are designated MC_T . Prophylactic topical corticosteroids greatly reduce the seasonal increase of nasal mast cells, probably by an action on the $CD4^+$ (helper) cells that secrete mast cell colony-stimulating factors such as IL-3. Nasal, as opposed to human lung, mast cells have not been observed to degranulate under provocation. Only recently has it been shown that Langerhans cells are present in the nasal mucosa especially in allergic rhinitis. Also it has been observed that 20–40% showed IgE positivity. Whether Langerhans cells plus IgE that binds to aeroallergens will give a clue to the pathogenesis of atopic dermatitis remains to be answered, but their presence in the nasal mucosa may serve as a model to study atopic dermatitis and the function of these cells.

Nasal blood vessels

In order to understand the pathological changes that occur in allergic and vasomotor rhinitis, it is necessary to consider in some detail the vasculature of the nose. It consists of four groups of blood vessels: 1) precapillary resistance vessels, 2) capillaries, 3) veins and venous erectile tissue with venous sinusoids, and 4) arteriovenous anastomoses. Transudation of albumin occurs in nasal inflammation. This very stable protein may give a more reliable estimate of the inflammation than the relatively unstable mediators. The venous erectile tissue may be filled with blood. This filling determines the state of con-

gestion of the mucosa which will regulate the nasal resistance to air flow.

Blood flow through the blood vessels is controlled by the autonomic innervation of the nasal mucosa. The nasal blood vessels receive a dense sympathetic innervation so that stimulation causes reduction in nasal blood flow and pronounced decongestion. The sympathetic tone of the nasal venous erectile tissue exhibits a cyclic reciprocal activity. The nasal mucosa seems to undergo a rest period every 2–4 h in humans. It is not surprising, therefore, that patients may state that one and then the other side of the nose becomes obstructed. The tickling sensation, sneezing and hypersecretion caused by allergen challenge is due to stimulation of the sensory nerve endings in the nasal mucosa with histamine from the mast cells being the main mediator.

Prostaglandin E_2 is formed in an allergic response but as it is a potent constrictor of nasal venous erectile tissue, it presumably does not reach this tissue. There is an increase in nasal secretion and glandular vasodilation which is caused by reflex activation of the parasympathetic nervous system so that unilateral stimulation produces a bilateral effect yet the vascular response remains local and unilateral. Nerve fibers containing vasoactive intestinal polypeptide (VIP) are richly distributed in the nasal mucosa. The fibers are especially numerous around seromucous glands and beneath the surface epithelium. Probably VIP is mainly involved in vasodilation of blood vessels serving the glands.

The neuromediator substance P seems to be involved in many of the inflammatory responses elicited in the nose in animals, but not at all in humans. Much more information is required about the anatomy and physiology of nasal blood vessels. It may be that prostaglandin E_2 , which is known to inhibit norepinephrine release from sympathetic nerve endings, has a role as a modulator of sympathetic tone to nasal blood vessels.

Treatment of rhinitis

Find the cause of the rhinitis and get away from the cause is easy advice to give, but often impossible to follow. There should be obvious causes of symptoms if they are strictly seasonal or environmental, as has already been mentioned. When the symptoms persist throughout the year the most likely cause is the major allergen of the house dust mite. Dust mites of the genus *Dermatophagoides* are the most important indoor allergens found worldwide in dust samples. The two major mite species are *D. pteronyssinus* (Der. p) and *D. farinae* (Der. f). Although monoclonal antibodies are available for groups I, II and

III antigens, most information is available about Der. p I. The fecal pellets are relatively large, 10–30 μm , and cause allergic conjunctivitis as well as allergic rhinitis and asthma. Airborne levels of Der. p I give a more appropriate idea of allergen level than are obtained in dust, where the highest concentrations are in the mattress and bedding. The allergens are also present in soft furnishing and carpets and especially toys. Many methods used to control house dust mite rhinitis have been amazingly disappointing.

Symptomatic treatment is now much more effective since the availability of nonsedative antihistamines. The most commonly used one is terfenadine, but others are available and there are some undergoing clinical trials. Antihistamine tablets are not very effective if the worst symptom is that of nasal obstruction. Antihistamine nasal sprays (azelastin) may be useful in rhinitis. Decongestants can be used sparingly for a short time, but the topical use of nasal sprays containing sodium cromoglycate or nedocromil sodium or the use of corticosteroids (Beconase, Rhinocort, Syntaris, Betnasol and Flixonase) usually give adequate relief. Patients must be told of the anti-inflammatory nature of these drugs – they do not give quick relief of symptoms.

Specific injection treatment is indicated when symptomatic treatment is not giving enough help, especially when symptoms are increasing in severity with time. The UK was the first to use this method of treatment in 1911, but since 1986 because the Committee on the Safety of Medicines decided that the treatment was both dangerous and also ineffective, their recommendations mean that the UK has virtually ceased to use this form of treatment. The pros and cons of such treatment have been well-reviewed in debate (Kay, 1989). More recently it has been shown that immunotherapy in a double-blind controlled trial does give benefit in severe summer hayfever uncontrolled by antiallergic drugs. In such studies it could be shown that immunotherapy may act by altering the T_H2 – T_H1 balance in favor of T_H1 responses. There is also a marked increase in interferon γ (IFN γ) mRNA expressing T cells which correlates with the beneficial clinical response to immunotherapy. Also IFN γ has the potential to inhibit IL-4-induced IgE expression and favors B cell IgG production.

Noninjection treatment including oral and topically applied allergens, acupuncture and other forms of treatment, are being increasingly used, but double-blind trials either have not been employed or give equivocal results.

See also: Allergens; Arachidonic acid and the leuko-

trienes; Atopic allergy; Glucocorticoids; Histamine; Hypersensitivity reactions; IgE; Mast cells.

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RICKETTSIA, INFECTION AND IMMUNITY

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Characteristics of the organisms and their antigens

The term rickettsia is often used indiscriminately to describe very small gram-negative bacteria with an apparent obligate requirement for growth within eukaryotic host cells and regular association with arthropods. The usage of rickettsia has even been broadened occasionally to include small bacteria associated with plants and other invertebrates. Even in the more narrow sense employed here, the group is highly heterogeneous, and contains exceptions to the general definition in that some species can grow without host cells and the arthropod association of others has not been proven. Rickettsiae belong to three families, the Rickettsiaceae, and the hemotropic erythrocyte-associated bacteria in the Bartonellaceae and Anaplasmataceae. Although the latter families contain species causing disease in humans (e.g. *Bartonella bacilliformis*) and some are of major veterinary economic importance (e.g. *Anaplasma marginale*), they have been less extensively studied than the Rickettsiaceae and are not further discussed.

The Rickettsiaceae include numerous species pathogenic for vertebrates. Nine different diseases of humans are caused by members of the genus *Rickettsia*. The louse- and flea-transmitted typhus group rickettsiae cause epidemic and endemic (murine) typhus, respectively. The genetically closely related tick-transmitted spotted fever group rickettsiae cause Rocky Mountain, Mediterranean (boutonneuse),

North Asian (Siberian), Japanese and Israeli spotted fevers. Mites transmit the spotted fever rickettsia causing rickettsialpox and the scrub typhus rickettsia causing tsutsugamushi fever. Some other species of *Rickettsia* have varied pathogenicity for vertebrates and potentially for humans but are largely maintained in nature by transovarial and transtadial passage in their host ticks. The major serotype-specific antigens of typhus and spotted fever group rickettsiae are large (>120 kDa) proteins which form regular crystalline arrays on the rickettsial surface. The lipopolysaccharides are also major immunogens, comprise a cross-reactive antigen which distinguishes these groups, and contain epitopes similar to those of selected *Proteus* strains used in the Weil–Felix agglutination test for rickettsial disease. In contrast, antigenically heterogeneous scrub typhus rickettsiae appear to contain no lipopolysaccharide and differ primarily in the properties of their immunodominant 54–56 kDa protein antigens. The etiologic agent of trench fever, *Rochalimaea quintana*, exhibits a distant genetic relationship to the typhus rickettsiae and has been used as a model for rickettsiae since it can be grown free of host cells. However, its antigens differ from those of the genus *Rickettsia*. *Coxiella burnetii*, the etiologic agent of Q (or query) fever, differs markedly from the other rickettsiae in its extreme resistance to physical and chemical agents. This may be partially due to its developmental cycle which includes formation of an endospore-like cell. In addition, both its major antigen, the phase I lipo-

polysaccharide, and some cell wall proteins appear to contribute to the physical stability of *Coxiella*. Rickettsiaceae also include pathogens of invertebrates such as *Wolbachia* and *Rickettsiella* as well as the important veterinary pathogens in the genera *Ehrlichia*, *Cowdria*, and *Neorickettsia*. *Ehrlichia sennetsu* and a newly discovered agent *E. chaffeensis* related to *E. canis* are also pathogenic for humans. The Ehrlichiae have a distant genetic relationship to the genus *Rickettsia* but their dominant surface protein antigens are different.

Immune responses of the host

Rickettsiae are remarkable in their proficiency in invading a variety of hosts and diverse cell types. A single organism invading the dermis is able to cause disease. They are transmitted efficiently both by arthropod bite and by scarification of arthropod feces at the bite site. They can also infect the host by aerosol exposure of the lung or contamination of the conjunctiva. The immune response that is generated is generally vigorous and prolonged, involving both humoral and cell-mediated immunity. Antibody is efficient in opsonizing these bacteria for destruction by macrophages although complement plus antibody is not bactericidal. In addition, antibody-dependent cellular cytotoxicity has been demonstrated for *Coxiella burnetii*. Macrophages activated by interferon γ and other cytokines are also able to destroy rickettsiae. Interferon γ and, to a lesser extent, both tumor necrosis factor and interferons α/β can limit the replication of rickettsiae in nonprofessional phagocytes. Passive transfer studies in animal models have demonstrated that cell-mediated immunity but not antibody alone is important in protective immunity. In mice, T-independent and dependent antibody responses to infection have been demonstrated. Specific T helper cells also respond actively to rickettsial antigens in elaborating interferon γ . Natural killer (NK) cells are activated by rickettsial infection. Finally, both lymphokine (IL-2)-activated killer (LAK) and specific major histocompatibility complex (MHC)-restricted cytotoxic T cells able to recognize and kill rickettsia-infected cells have been demonstrated.

Evasive strategies by the organism

Despite the vigor and number of cellular immune reactions to rickettsial infection, persistent rickettsial infection and recrudescence has been demonstrated for *Rickettsia prowazekii*, *R. tsutsugamushi*, *Rochalimaea quintana*, and *Ehrlichia canis*. Of parti-

cular importance in human disease is the ability of strains of *Coxiella burnetii* with particular plasmid (or sometimes chromosomal) sequences to persist in the patient and later cause chronic hepatitis or endocarditis. The basic evasive strategy of the rickettsiae is their ability to invade host cells to escape the activated macrophages and antibody-dependent destruction mechanisms arrayed against them. Most rickettsiae appear able to induce their own phagocytosis in a variety of cells which are not professional phagocytes. Species of *Rickettsia* then escape from the phagosome directly into the cytoplasm and sometimes even invade the nucleus. In contrast *Ehrlichia* remain in the phagosome but prevent its fusion with lysosomes. Finally, *Coxiella* not only permits acidification of the phagosome by lysosome fusion but is actually activated metabolically by this step. How *Coxiella* persists in the liver and endocardium, despite antibiotic treatment, is not understood. Each of these genera appear to have developed specific transport mechanisms for regulating their metabolism extracellularly to maintain essential functions and only undergo replication once they are in their appropriate intracellular milieu. Indeed, in some cases these organisms barely damage their host cells while replicating. This equilibrium appears greatest in their arthropod host. The extent of imbalance between some rickettsiae and the vertebrate host (poor adaptation) contributes to the morbidity and lethality they induce. Although antigenic transformation as exhibited by *Borrelia* appears insignificant for rickettsiae, the binding of host cell components both intracellularly and extracellularly may be important in evading host recognition. Indeed, scrub typhus is particularly efficient in budding from the host cell in a plasma membrane blanket from which it can infect adjacent cells. *Rickettsia typhi* and spotted fever rickettsiae appear to rapidly spread from cell to cell with minimal damage on exit or entry. *Coxiella* appears to bind host cell proteins like non-immune antibody and ceruloplasmin that target it to specific host cells for phagocytosis. *Coxiella* also possesses a cell wall complex which can suppress host immune responses. Finally, strains of both interferon γ and α/β -resistant *R. prowazekii* have been discovered, suggesting that host cells may be unable to mount adequate attacks on intracellular rickettsiae. Indeed, cytotoxic T cells and LAK cells may merely act to disseminate the rickettsiae from heavily infected cells.

Vaccines

While protective immunity to rickettsiae may not always lead to complete clearance of the organism,

a large number of attenuated rickettsiae and killed subunit vaccines have been developed which can prevent lethal infection and reduce morbidity due to these agents. However, the commercial availability of these vaccines has been severely restricted by the expense and hazard of growing and purifying these agents and their antigens and the lack of understanding of the genetic basis of rickettsial attenuation. These vaccines also do not meet modern vaccine expectations in their reactogenicity, stability and standardization. Development of rickettsial vaccines is further complicated by the multiplicity of agents involved and the low degree of cross-protection between species and some serotypes. Recombinant DNA methods are rapidly permitting attacks on each of these problems. In a number of laboratories, both purified recombinant protein vaccines based on the immunodominant rickettsial antigens and live viral and bacterial vectors which express the recombinant rickettsial proteins are being investigated aggressively as candidate vaccines for the rickettsiae.

See also: **Bacteria, immunity to; Cytokines; Endotoxin (lipopolysaccharide (LPS)); Phagocytosis.**

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ROSETTING TECHNIQUES

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The interaction between an indicator particle and a surface molecule is among the most useful techniques in immunology. Indicator particles may exhibit natural receptors, or receptors may be attached by use of ligands. Erythrocytes of various species are used extensively by virtue of their availability, ease of detection and simplicity. Newer techniques have taken advantage of particles synthesized with hydrophilic materials with available hydroxyl and carboxyl groups to provide protein-binding sites for use in these assays. A rosette refers to the ability of sev-

eral cells, such as an erythrocyte, to react with and form a circle around another cell, much as an ornamental ring surrounds a centerpiece. Most workers require at least three particles adhering to the cell of interest as the definition of a rosette. The technique may be used to identify and/or to separate and purify a cell population. The relative simplicity of performance and equipment requirements make these techniques useful for field work. They only require a source of erythrocytes, minimal refrigeration capacity, a laboratory centrifuge and a microscope.

Erythrocyte (E) rosette

The most widely used rosetting technique in human immunology is the interaction between the sheep erythrocyte and human lymphocytes of the thymic or T cell lineage. This interaction, first appreciated by the laboratories of Brain, Coombs and Wybran, was a major step in the capacity to identify, separate and characterize the T and B cell lineages of lymphocytes in normal physiology and disease. The assay is performed by the incubation of a population of mononuclear cells obtained by a preliminary processing of blood, usually by differential density centrifugation of peripheral blood or of organ suspensions, with sufficient sheep erythrocytes to have a ratio of 50:1 or 100:1 erythrocytes to mononuclear cells. The suspension is incubated briefly at 37°C then centrifuged at 200 g and a further incubation for 1–2 hours or overnight at 4°C. The cells are gently suspended and counted in a hemocytometer.

The rosettes formed are more stable in a high protein solution such as 10% human AB serum or 25% bovine serum albumin. The prior treatment of the sheep erythrocytes with neuraminidase also increases the stability. Kaplan found that prior treatment of the sheep erythrocytes with neuraminidase also increases the stability. Kaplan found that prior treatment of the sheep erythrocytes with a sulfhydryl reagent such as 2-amino-ethyisothiuronium (AET) obviates the differences between batches of sheep erythrocytes and enhances the stability of the rosette. Recently Ocklind suggested the use of 1% w/v of polyethylene glycol 10 000 molecular weight in the medium allows for the detection of 97% of T cells. These modifications have proved very useful, particularly when the method is used to isolate T cells in preparative techniques.

The molecule on the surface of T cells responsible for this interaction is the 50 kDa glycoprotein CD2, present on more than 95% of thymocytes and all peripheral T cells. It binds to a protein present on sheep erythrocytes designated T11TS, which is the homolog of the human protein, lymphocyte function-associated antigen 3 (LFA-3). This latter protein is present on human erythrocytes and accounts for the observation that human erythrocytes can form rosettes with human thymocytes. The utility of the sheep erythrocyte over the human in this assay and its reaction with peripheral T cells is due to a three- to five-fold higher ligand density on its surface. The CD2 molecule is a T cell activation molecule involved in one alternate pathway. Thus T cells isolated by this technique may under certain circumstances be activated by the procedure. This phenomenon must be kept in mind when designing

experiments or making conclusions about the *in vivo* state of such populations.

In other species E rosette formation is less successful. Limited use of the technique occurs with sheep E and primate T cells. The cat T cell forms rosettes with guinea pig and gerbil erythrocytes.

Erythrocyte Antibody (EA) rosette

The identification of hematopoietic cells by their formation of rosettes with erythrocytes coated with antibody molecules is complex. Careful attention to the type of erythrocyte and of the antibody coating on that cell is required. Seven types of hematopoietic cells have receptors for immunoglobulin (Ig) on their cell membrane. An additional layer of complexity is that there are receptors for IgM, IgA, IgE and IgG and its subclasses. The difference between these receptors and their affinities is covered in the entry on Fc receptors. The most prominent Fc receptor-bearing cells are polymorphonuclear leukocytes and monocytes. In most immunologic studies of lymphocyte function the neutrophils are removed by density gradient centrifugation. They are readily identified by their characteristic morphologic appearance in smears or cytocentrifuge preparations. Monocytes are similarly identified as EA rosette-forming cells in such preparations. Monocytes are removed by glass or plastic adherence or passage through columns of Sephadex G-10. Platelets have Fc receptors and care must be taken to remove these cells as well from preparations. Eosinophils, basophils and mast cells are removed along with the neutrophils. B lymphocytes may be identified and isolated once contaminating cells are removed by their rosette formation with ox erythrocytes coated with anti-ox cell antibody. This method is inferior to their identification by anti-surface membrane immunoglobulin antibody techniques, but may be performed when fluorescence microscopy is not available. The results obtained should be correlated with the results of complement receptor assays (see below).

The T cell population can be subdivided into two populations by rosette formation with erythrocytes coated with IgM or erythrocytes coated with IgG. It is important in this assay to incubate freshly isolated T cells overnight in immunoglobulin free media to allow the *in vivo* passively absorbed IgM and IgG to be shed. Then rosette assays with appropriately coated erythrocytes can be performed. T cells with receptors for IgM were originally described as helper T cells, whereas cytotoxic T cells were said to have receptors for IgG. Subsequently it has been shown that these two classes of T cells cannot be distinguished on this basis. However, multiple cell types

Table 1 Rosette markers of human peripheral blood mononuclear cells

	<i>T cells</i>	<i>B Cells</i>	<i>Monocytes</i>
E Rosette	+	—	—
EA-Ox	—	+	+
EA-Human	—	(+)	+
EAC-CR1	—	+	+
EAC-CR2	—	+	—
EAC-CR3	—	—	+

can be identified by EA rosette formation but careful attention to details of preparation is essential.

Erythrocyte Antibody Complement (EAC) rosette

The use of erythrocytes coated with complement components, especially C3, to form rosettes is an extensively utilized technique. However, other particles such as zymosan or bacteria can also be used. There are a number of separate and distinct complement receptors on lymphocytes. Assays for CR1 receptor cells are performed using sheep erythrocytes sensitized with IgM antibody and purified complement components to build up a EAC1-4b or EAC1-3b. Alternative methods using heat inactivated serum to prepare appropriate reagents are available. Detection methods for CR2 use the EA cells and whole serum to prepare an EAC3d. Reagents for the detection of CR3 utilize the EAC3b cell subsequently treated with P1H and C3 inactivator. In such assays purified isolated mononuclear cells are incubated with the appropriate reagent, enumerated in a hemocytometer, and the percentage of positive rosettes determined. These rosette methods can also be used to isolate populations of cells for additional experiments. These methods are inexpensive and allow an excellent yield of cells. A summary of these tech-

niques and the mononuclear cell populations so identified is presented in Table 1.

See also: CD2; CD22: Cell separation techniques; Complement receptors; Fc receptors.

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SALMONELLA, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Salmonellae (family Enterobacteriaceae, genus *Salmonella*) are facultatively anaerobic, motile gram-negative rods. They are now classified into three main species (*S. typhi*, *S. cholerae-suis* and *S. enteritidis*) with over 2000 different serotypes considered different varieties of *S. enteritidis*. Pathogens of humans and animals, they can be found in the digestive tract, blood and internal organs, and can survive in the environment to cause water- or food-borne infections. Host-adapted salmonellae can cause severe invasive disease, e.g. *S. typhi* or the paratyphoid bacilli causing typhoid fever in humans, *S. gallinarum* or *S. pullorum* causing fowl typhoid. Others have a broader host range, causing invasive disease in one host species and more limited infections in others, e.g. *S. typhimurium* or *S. enteritidis*, which cause invasive disease in rodents and are common agents of food poisoning. These salmonellae of animal origin can also cause invasive disease in humans in the extremes of age or in situations of lowered host defences. Salmonella septicaemias are invasive infections with abscess formation, typically by *S. choleraesuis*. Asymptomatic carriers of *S. typhi* are the reservoir of human typhoid fever. Carriage of other serotypes in animals, including ovarian carriage in birds with infected eggs, causes contamination of foodstuffs and gastroenteritis.

Serological classification of salmonellae is based on the main determinants present on the somatic (O) and flagellar antigens. O antigens are present on the long side-chains of lipopolysaccharide (LPS or endotoxin). The lipid A of LPS is the main determinant of toxicity. 'Rough' mutants lacking the O side-chains are serum sensitive and avirulent. Some salmonellae (e.g. *S. typhi*, *S. dublin*, *S. paratyphi* C) express the Vi capsular antigen, a homopolymer of α -1,4-deoxy-2-N-acetylgalacturonic acid, which can mask the O antigen and is important in virulence and immunity. Enterobacterial common antigen (ECA)

may also be present. With few exceptions, salmonellae are motile with peritrichous flagella. Many serotypes show flagellar phase variation, reversibly expressing flagella with one or two different antigenic specificities. Important in classification, the relevance to pathogenicity of this highly conserved variability is unknown. Many salmonellae express fimbriae; other somatic components are also antigenic, and renewed attention is being given to the role of cell wall proteins in pathogenesis, immunity and diagnosis.

Evasive strategies by the organism

It is only in recent years that the molecular basis of virulence and the genetic determinants of pathogenicity which allow salmonellae to invade and multiply in the hostile environment of the host have begun to be elucidated. As *S. typhi* does not infect conventional laboratory rodents, much of the information is gained from animal models (mouse typhoid) and cell culture systems. Invasive salmonellae penetrate through the epithelial cells and M cells of the distal ileum and Peyer's patches; the brush border degenerates and the organisms pass through the cell enclosed in a membrane to exit through the basal pole to the submucosa. Flagella, motility, chemotaxis and smooth LPS contribute to invasion. Salmonella invasins and a cytotoxin have been described; invasion of and replication within epithelial cells are under separate genetic control. In gastroenteritis bacterial multiplication does not proceed beyond the intestine; some but not all strains will cause fluid accumulation in the rabbit ileal loop model. The precise mechanism of fluid loss remains unclear.

In invasive disease (e.g. enteric fevers) bacteria disseminate to the liver, spleen and bone marrow, grow within and kill hepatocytes. They also grow inside macrophages *in vitro* and can kill them, and the ability of salmonellae to grow inside cultured mouse macrophages correlates with mouse virulence. However, the ability of salmonellae to grow inside

macrophages in the tissues is debated, as macrophages in lesions have been seen to contain mainly dead salmonellae. Polymorphs are prominent in the early stages of infection and may contribute to the lysis of infected hepatocytes. In chickens purulent pericarditis caused by, for example, *S. enteritidis* is not uncommon. The precise mechanisms of survival within macrophages are still unclear; resistance to lysosomal enzymes and inhibition of phagolysosome fusion have been described, with the Vi antigen increasing the resistance of *S. typhi* to killing by leukocytes. Transposon-generated macrophage-sensitive mutants were found to be auxotrophic or sensitive to serum or oxidative stress. Mutations in genes which respond to environmental changes affect survival in macrophages; *ompR* regulates expression of outer membrane porins in response to osmolarity changes (*ompR* mutations are attenuating), while the *phoP phoQ* operon responds to phagolysosomal signals (low pH, cationic peptides), increasing resistance to phagocyte defensins. The large serotype specific ('cryptic') plasmid present in some but not all salmonellae (absent in *S. typhi*) affects serum resistance and persistence and growth in the tissues.

Immune responses of the host

Both innate and adaptive immune response mechanisms are crucial in survival following a primary infection with virulent organisms in the naive host. The ID₅₀ of *S. typhi* in humans is *ca.* 10⁵ organisms. Salmonellae initially grow exponentially in the reticuloendothelial system (RES). In lethal infections, growth continues unchecked until 'lethal numbers' of approximately 10⁸ organisms are reached; endotoxin poisoning is believed to contribute to death. Both strain virulence and host resistance determine the rate of increase of bacterial numbers in the RES; in mice, the *lty/lsh/bcg* gene on chromosome 1 regulates the growth rate of salmonellae in the tissues in the first days of the infection and determines the incubation period; the same gene modulates resistance to mycobacteria and *Leishmania*. In sublethal infections, exponential early growth is checked by a host response (the plateau phase) which is mediated by an influx of radiation-sensitive bone marrow-derived cells, tumor necrosis factor α (TNF α), interferon γ (IFN γ) and interleukin 12 (IL-12), but not T cells. LPS unresponsive mice, such as C3H/HeJ (*Lps*^d), are more susceptible.

After the plateau phase, bacteria are cleared from the tissues by an immune response phase (influenced by host genes within and outside the major histocompatibility complex, MHC) which can leave solid immunity to reinfection. CD4⁺ and CD8⁺ T cells,

TNF α , IFN γ and IL-12 are involved in bacterial clearance from the tissues, with TNF α involved in monocyte recruitment and granuloma formation, whereas T cells and IFN γ are needed for expression of increased antibacterial resistance. However, in contrast to immunity to mycobacteria and *Listeria*, immunity to salmonellae in mice also requires the participation of antibodies in addition to cell-mediated immunity (CMI), which is not common with intracellular bacteria. With the 'animal' salmonellae the antibody is probably directed against the LPS; in human typhoid antibodies to Vi are probably important. The targets of the cell-mediated response are, as yet, unclear. Antibodies to O and H antigens are diagnostic in typhoid fever, and carriers have elevated Vi antibodies.

Antibody alone or immunization with killed organisms, which do not elicit CMI, confer less protection than immunization with live organisms, which induce CMI as well as humoral immunity. Protection is best conferred by immunization with organisms which can persist and grow for a limited period in the tissues. Live organisms induce delayed-type hypersensitivity (DTH) to a crude salmonella elicitin, but the correlation of DTH with immunity is poor. The protective immunogen(s) may be the main LPS O antigen (or Vi in *S. typhi*), other protein antigens, or a combination of both. Mucosal immunoglobulin A (IgA)-mediated immunity confers a degree of protection. The precise mechanisms of immunity in human typhoid fever remain unclear, but the encouraging results obtained with live vaccines suggest that CMI is important, as well as Vi-specific antibodies. Cytotoxic T cells which lyse salmonella-infected target cells have been described in animals and humans.

Vaccines

Conventional parenteral salmonella vaccines consisting of killed organisms have been widely used; however, they confer incomplete protection and are excessively reactogenic. Human trials of oral vaccination with large doses of killed organisms did not show protection. A typhoid vaccine consisting of highly purified *S. typhi* Vi antigen is now available; it does not provoke the unacceptable side-reactions of killed whole-cell vaccines, and also confers significant but incomplete protection.

The superior efficacy of live vaccines in experimental models has led to the development of new candidate oral live attenuated salmonella vaccines for humans and animals. Mutations in the *galE* gene cause an LPS core defect with impaired synthesis of smooth LPS. The efficacy of *galE* mutants in mice

led to the development of *S. typhi* Ty21a, a nitroguanidine-induced Vi⁻ mutant which provided significant although incomplete protection. This is available as a human oral typhoid vaccine; it is very safe but also very attenuated; a Vi⁺ variant of strain Ty21a did not exhibit greater immunogenicity. More recently, an engineered *galE* Vi⁻ *S. typhi* vaccine candidate strain caused typhoid in volunteers, demonstrating that *galE* is not a fully attenuating mutation in *S. typhi*. It is now clear that chemical mutagenesis induced other unknown mutations responsible for the attenuation of Ty21a. Seroconversion rates in humans following oral immunization with Ty21a are not high. Vaccinees show positive proliferative responses in peripheral blood leukocytes stimulated with *S. typhi* antigens, suggesting induction of cellular immunity.

Other candidate live vaccines are also being developed. The use as vaccines of 'animal' salmonellae cured of the large virulence plasmid, which show reduced multiplication in the tissues, has been proposed. Mutants with lesions affecting adenylate cyclase and cAMP receptor protein (*cya crp* mutants) are attenuated and effective oral vaccines in mice. Aro salmonellae harboring lesions in genes of the aromatic pathway are attenuated, owing to their requirement for *p*-aminobenzoic acid which is unavailable in the mammalian host. Aro mutants grow slowly in the tissues, from which they are eventually cleared. Aro mutants of *S. typhimurium*, *S. dublin* and *S. enteritidis* are effective vaccines in mice, calves, sheep and chickens. Mutations in purine biosynthesis are also attenuating; *S. typhi* *aro pur* mutants are innocuous in humans, but the *pur* mutation appears to be excessively attenuating, making the vaccines poorly immunogenic. Mutations in *htrA*, a stress protein gene, are attenuating and provide good vaccines; human volunteer trials with an *S. typhi* *aroC aroD htrA* mutant are yielding promising results.

A further use of live attenuated salmonellae is their

potential as single-dose live oral multivaccine delivery systems for recombinant antigens from other pathogens. Salmonella vaccines can elicit humoral, secretory and cellular immunity to recombinant antigens. They have been used to express over 40 different antigens from viruses, bacteria and parasites, and with some antigens positive immune responses, toxin-neutralizing antibodies and protection from disease has been obtained, e.g. antibody to enterotoxins, fimbrial adhesins, and antigens from HbSAg, human immunodeficiency virus (HIV) and SIV, *Bordetella pertussis*, papilloma viruses and many others. Protection against *Plasmodium* spp., streptococci, tetanus and influenza infection in mice have been observed. Trivalent vaccines expressing recombinant antigens as genetic fusions to the immunogenic C fragment of tetanus toxin have conferred protection in mice against salmonellae, tetanus and schistosomiasis or herpes simplex virus. A recombinant Aro *S. typhi* expressing the *Plasmodium falciparum* circumsporozoite antigen elicited antibody and cytotoxic T cells in human volunteers.

See also: Bacteria, immunity to; Bacterial cell walls; Gastrointestinal tract infections.

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SARCOIDOSIS

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Sarcoidosis is a disease of unknown etiology characterized by epithelioid granulomas and T cell infiltrations in multiple organ systems. The disease most commonly affects the lungs and intrathoracic lymph nodes. Eye and skin involvement is seen in approxi-

mately 20% of patients, and symptomatic involvement of other organ systems occurs less frequently. Sarcoidosis can be asymptomatic or present in an acute or subacute form. Spontaneous remissions occur in about 50% of cases; in other patients,

chronic, progressive disease ensues with an overall >5% mortality rate. Corticosteroids are the mainstay of treatment for threatened organ failure or chronic progressive disease. Sarcoidosis occurs worldwide, primarily in 20–40-year-old adults, with a frequency that varies among different populations and ethnic groups. Epidemiologic and family studies suggest that both environmental and genetic factors play a role in the etiology of sarcoidosis. Recent immunologic studies provide evidence for oligoclonal expansion of $\alpha\beta$ T cell subsets and predominant expression of type 1 cytokines (interferon γ (IFN γ), interleukin 2 (IL-2), IL-12) at sites of inflammation, suggesting that sarcoidosis is an antigen-driven, T_H1-mediated immune disorder. The fundamental question regarding the etiologic stimulus driving this inflammatory process remains to be elucidated.

Historical perspective

In 1899, Caesar Böeck described a patient with lymphadenopathy and multiple skin nodules with characteristic epithelioid granulomas on histologic examination; he proposed the term 'multiple benign sarkoids' for this condition. The view that sarcoidosis is a multisystem disease was first clearly expressed by Jörgen Schaumann in 1914. Thirty years later, Sven Löfgren observed that sarcoidosis frequently presents with asymptomatic bilateral hilar adenopathy or with erythema nodosum. For many years, sarcoidosis was conceptualized as a disease of suppressed immunity with peripheral lymphopenia and cutaneous anergy. Landmark studies of pulmonary sarcoidosis in the late 1970s led to a fundamental reappraisal of sarcoidosis as a disease of enhanced, cell-mediated immune activity associated with granulomatous inflammation.

Epidemiology

The prevalence of sarcoidosis ranges from 10 to 40 cases per 100 000 population in North America, southern Europe and Japan. Higher prevalence rates have been noted among Swedes in Sweden (64/100 000) and African-Americans in southeastern US. Erythema nodosum has a particularly high prevalence among Irish women in London and Puerto Rican women in New York City. Lupus pernio, a disfiguring facial skin condition associated with chronic sarcoidosis, is more frequent in patients of African descent. Worldwide, there is a slight female predominance.

Genetics

Familial clustering of sarcoidosis occurs in approximately 5–16% of patients. Within the same family, sibling pairs are most common, followed by mother-daughter pairs and father-son pairs. Monozygotic twins appear more likely to develop sarcoidosis than dizygotic twins, strongly suggesting a genetic component to the disease. The lack of a clear genetic pattern suggests that susceptibility to sarcoidosis is polygenic and associated with environmental factors. Worldwide, HLA studies have shown inconsistent results with associations reported for HLA-DR3 and bilateral hilar adenopathy or erythema nodosum in Swedish patients, B27 and uveitis, B13 and chronic disease, DR5 in Italian patients and DRw52 in Japanese patients.

Etiology

Given the clinical and histologic similarities to tuberculosis, investigators have searched for an infectious etiology of sarcoidosis since its original description, with inconclusive results. Since the 1960s there have been unconfirmed reports of cultivable mycobacterial or cell wall-deficient organisms in sarcoid tissue. Other investigators reported finding acid-fast staining material in pathologic specimens, but these findings are contrary to general experience. High titers of antibodies against many viral and bacterial antigens are found in sarcoidosis but probably reflect generalized B cell activation and not a causal relationship. Recently, polymerase chain reaction methods have been used to search for traces of microbial DNA in sarcoid tissue. Different studies have found mycobacterial DNA in 0–50% of biopsy specimens or bronchoalveolar lavage fluids, with false positive rates ranging from 0 to 9%. These divergent results have done little to clarify a possible causal link between sarcoidosis and mycobacterial disease.

The possibility of a noninfectious, environmental cause of sarcoidosis is suggested by the association of beryllium dust exposure with a chronic, interstitial granulomatous lung disease that is indistinguishable from sarcoidosis. Other investigators suggest that sarcoidosis is the result of autoimmunity (perhaps due to molecular mimicry of microbial antigens or to altered self antigens) or immune dysregulation. The presence of antinuclear antibodies, rheumatoid factor, hypergammaglobulinemia and immune complexes in sarcoidosis is cited as supporting evidence for these latter possibilities.

Kveim–Siltzbach test

In 1941, Ansgar Kveim, a dermatologist from Oslo, demonstrated that intradermal inoculation of a suspension of sarcoid tissue resulted in firm red papules containing sarcoid-like granulomas in patients with suspected sarcoidosis, but not in control individuals including patients with tuberculosis. In the following decades, Louis Siltzbach pioneered worldwide efforts that demonstrated a single, validated reagent gave positive reactions in 70–80% of patients early in their disease with a <1% false-positive rate. Similar

reactions can be elicited by dermal injection of autologous bronchoalveolar lavage cell suspensions.

The Kveim reaction takes 2–4 weeks to develop well-defined granulomas, similar to the Mitsuda reaction to lepromins in patients with tuberculous leprosy. Local CD4⁺ T cell and monocyte infiltration precedes the development of epithelioid granulomas. In the search for clues to the etiology of sarcoidosis, many attempts have been made to identify the granuloma-inducing factor contained in the Kveim–Siltzbach reagent. The component appears to be a heat-stable, cell wall-associated microparticulate, but precise characterization remains an elusive goal.

Table 1 Major immunologic features of tissue inflammation in sarcoidosis

Epithelioid granulomata

CD4⁺ T cell infiltration

- Activated phenotypes (IL-2R⁺, VLA-1⁺, DR⁺)
- Biased, oligoclonal αβ T cell expansions
- Enhanced T_H1 cytokine production (IFNγ, IL-2)

Monocyte/macrophage infiltration

- Activated phenotypes (increased surface HLA-DR, -DQ, -DP, -ICAM-1, -LFA-1)
- Enhanced reactive oxygen species, angiotensin-converting enzyme, lysozyme production
- Spontaneous cytokine production (IL-12, TNFα, IL-6, ?IL-1)
- Enhanced chemokine production (MIG, MIP-1α, MCP-1)
- Enhanced production of fibronectin, IGF-1
- Enhanced antigen presentation (alveolar macrophages)

Pathology

The histologic hallmark of sarcoidosis is the presence of discrete, noncaseating, epithelioid cell granulomas typically at different stages of development (Table 1, Figure 1). The dominant cell in the central core is the epithelioid cell, thought to be a differentiated form of a mononuclear phagocyte. CD4⁺ lymphocytes and mature macrophages are typically interspersed throughout the epithelioid core, whereas both CD4⁺ and CD8⁺ lymphocytes are seen in the periphery of the granuloma. Approximately 5–10% of T cells at sites of inflammation are IL-2R⁺ and 20–50% are HLA-DR⁺, indicative of their recent activation.

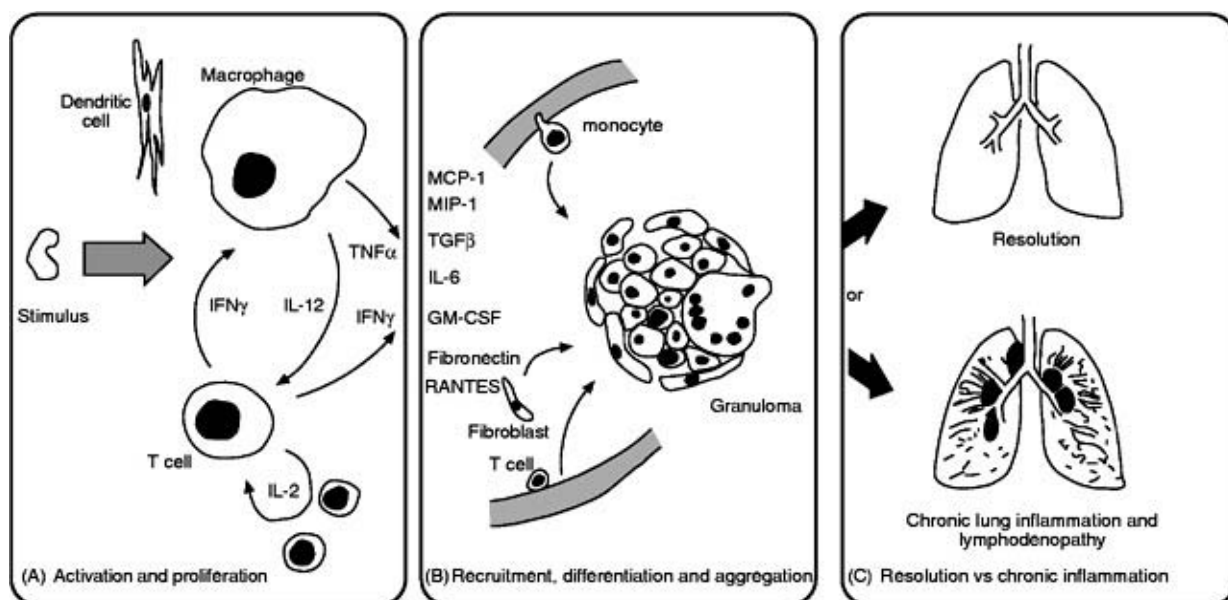


Figure 1 Principal events involved in granuloma formation in sarcoidosis. (A) In response to an unknown stimulus, activated macrophages and oligoclonal CD4⁺ T cells accumulate at sites of inflammation. (B) Epithelioid cells and giant cells, derived from differentiated macrophages, aggregate with fibroblasts, other CD4⁺ and CD8⁺ T cells and mononuclear phagocytes to form granulomas. These events are hypothesized to be driven by antigen-specific, T_H1-mediated immune responses that produce type 1 cytokines (IFNγ, IL-2, IL-12), TNFα and other proinflammatory chemokines and cytokines. (C) Granuloma formation may resolve spontaneously or persist, leading to chronic inflammation and fibrosis.

Giant cells, often containing cytoplasmic inclusions such as Schaumann bodies, are scattered throughout the inflammatory locus. In the lung, granulomas tend to form along bronchovascular regions, areas rich in lymphatic vessels. Granulomas and T cell infiltrations are also typically present in alveolar and bronchial walls.

Immunopathogenesis

The pathologic features of sarcoidosis are typical for 'immune-mediated' granulomatous diseases of known etiology such as mycobacterial or fungal infections and chronic beryllium disease. In these disorders, granulomatous inflammation is regulated by a complex interplay among T cells, mononuclear phagocytes, fibroblasts, dendritic cells and other accessory cells. In sarcoidosis, this inflammatory process results in pathologic changes in organ function by disrupting normal architecture, damaging local tissues and producing cytokines and chemical mediators with local and systemic effects. Current information on the immunopathogenesis of sarcoidosis has been derived in large part from studies of lung cells and fluid from the lower respiratory tract obtained by bronchoalveolar lavage.

T lymphocytes in sarcoidosis

Lung T cells recovered from the alveolar surface are predominantly of the CD4⁺ phenotype, typically with a CD4:CD8 ratio of 3–10:1 compared with a ratio of 2:1 in healthy individuals (Table 1). Sarcoid and normal lung T cells are almost all memory T cells expressing CD45RO⁺/CD29⁺ surface markers. Greater numbers of lung T cells express activation markers such as IL-2R, VLA-1 (CD49a) or HLA-DR molecules than circulating T cells. Sarcoid lung T cells demonstrate reduced surface density of the CD3/T cell receptor complex, a hallmark of antigen-stimulated T cells. Consistent with recent activation, greater numbers of lung T cells express the cell cycle-related nuclear antigen Ki67 and a small proportion of lung T cells are spontaneously proliferating. Lung T cells in sarcoidosis spontaneously produce the type 1 cytokines IFN γ and IL-2 *in vitro*.

In contrast to the lung, patients with sarcoidosis often demonstrate CD4⁺ T cell lymphopenia in peripheral blood. Increased numbers of circulating T cells are IL-2R⁺, suggesting that these cells are trafficking from inflamed tissues.

T cell receptor gene expression

Investigators have analyzed T cell receptor gene expression in sarcoidosis in order to define disease-

specific T cells and their reactivity. Results of these studies demonstrate the presence of expanded numbers of V β ⁻ or V α ⁺ T cell subsets (e.g. V β 8⁻, V β 19⁻ or V β 5⁺ T cells) in the lung, blood or skin (sites of Kveim-Siltzbach reactions) of subgroups of patients. Sequence analyses demonstrate that expanded $\alpha\beta$ T cell subsets are oligoclonal and sometimes contain shared amino acid motifs in the CDR3 region of their V β genes, consistent with local antigen-driven T cell responses. Expanded numbers of V α 2.3⁺ lung T cells are found in Swedish patients with DR3/DQ2 haplotypes, perhaps reflecting T cell responses to a specific peptide-MHC complex. Despite intense efforts, the reactivities of these expanded T cell clones have yet to be identified.

Studies of $\gamma\delta$ ⁺ T cells in sarcoidosis have led to divergent results. In some reports, as many as 30% of patients have greater proportions of circulating $\gamma\delta$ T cells, predominantly of the V γ 9⁺V δ 2⁻ subset. Other studies have found that the increase in $\gamma\delta$ T cells is secondary to a reduction in circulating $\alpha\beta$ T cells or have not found any difference from control populations. Since $\gamma\delta$ T cells are rarely found around granulomas in lymph nodes or skin, a role for $\gamma\delta$ T cells in the pathogenesis of sarcoidosis remains uncertain.

Mononuclear phagocytes in sarcoidosis

Sarcoid alveolar macrophages appear activated, producing increased amounts of lysozyme, angiotensin-converting enzyme and reactive oxygen species (Table 1). Reports of enhanced lung macrophage expression of the IFN γ -inducible chemokine MIG (monokine-induced by IFN γ), provide evidence of cellular activation by IFN γ . Sarcoid alveolar macrophages contain a twofold increased density of surface class II MHC (DR,DQ,DP) molecules and the adhesion molecules LFA-1 and ICAM-1. These characteristics probably contribute to the enhanced ability of sarcoid alveolar macrophages to present antigen to autologous T cells compared with normal alveolar macrophages.

Sarcoid alveolar macrophages spontaneously produce IL-12, tumor necrosis factor α (TNF α), IL-6, and possibly IL-1, cytokines known to regulate granuloma formation. These cells also release increased amounts of fibronectin and insulin growth factor 1 (IGF-1), molecules important in fibroblast recruitment and replication. In contrast, sarcoid alveolar macrophages produce lesser amounts of the counterinflammatory prostaglandin E₂, than normal alveolar macrophages. In a subgroup of patients, enhanced *in vitro* production of transforming growth factor β (TGF β) by lung macrophages has been as-

sociated with remission of active disease, suggesting this cytokine is critical to downregulating the inflammatory process.

Multiple chemokines including MCP-1, RANTES, MIP-1 α and IL-8 are found in bronchoalveolar lavage or tissue specimens in sarcoidosis. These chemokines probably assist in the recruitment of monocytes, macrophages, memory T cells and granulocytes to inflammatory loci in sarcoidosis. Natural killer (NK) cells, B cells, eosinophils and mast cells are also occasionally seen around granulomas; their contributions to the inflammatory response are uncertain.

Cytokine interactions in sarcoidosis

Substantial evidence indicates that granulomatous inflammation in sarcoidosis is characterized by a predominant type 1 cytokine response. Bronchoalveolar lavage studies demonstrate increased mRNA and protein levels of IFN γ , IL-2 and IL-12, low or undetectable levels of IL-4 and IL-5, and normal or low levels of IL-10 in pulmonary sarcoidosis. The known dependence of many granulomatous disorders on type 1 cytokines suggest they function in a similar manner in sarcoidosis. One hypothesis suggests that successful removal of the inciting agent by this T_H1-dependent response allows for TGF β inhibition of IL-12 and IFN γ production and resolution of the disease. If the immune response is ineffective in removing the inciting stimulus or T cell autoimmune responses develop, dysregulated cytokine production driven by a positive feedback loop between IFN γ and IL-12 results in maintenance of the granulomatous inflammation. A fibrotic outcome from accumulated tissue injury may theoretically be prevented by suppressing the inflammatory response early in the course of the disease. Consistent with this hypoth-

esis, drugs capable of suppressing IFN γ and IL-12 production (e.g. corticosteroids, pentoxifylline) are often beneficial in the treatment of sarcoidosis.

See also: Chemokines; Cytokines; Effector lymphocytes; Granuloma; Immunopathology; Macrophage activation; MHC disease associations; Mononuclear phagocyte system; *Mycobacteria*, infection and immunity; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T lymphocytes.

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SCHISTOSOMIASIS

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Schistosomiasis is the generic term given to diseases caused by digenetic trematodes, or blood flukes, of the genus *Schistosoma*. The three main species of schistosome that are pathogenic for humans, *S. mansoni*, *S. haematobium* and *S. japonicum*, are estimated to affect some 200 million people throughout the tropics and cause extensive chronic morbidity and some mortality, especially among children and young adults. *Schistosoma haematobium* causes urinary schistosomiasis in Africa and the Middle

East, *S. mansoni* causes intestinal schistosomiasis in South America, the Caribbean, Africa and the Middle East, and *S. japonicum* causes intestinal schistosomiasis in the Far East, especially China and the Philippines. Two other species of schistosome, *S. intercalatum* and *S. mekongi*, are also significant human pathogens, but have relatively restricted geographical distribution, and the important parasites of African cattle and sheep, *S. bovis* and *S. matthei*, occur in this genus.

Characteristics of the organism and its antigens

Figure 1 is a representation of the schistosome life cycle. A phase of sexual maturation and mating in the definitive mammalian host alternates with a phase of asexual replication in an intermediate molluscan host, i.e. freshwater snails of the genera *Biomphalaria*, *Bulinus* and *Oncomelania* for *S. mansoni*, *S. haematobium* and *S. japonicum*, respectively.

The free-living cercaria is released from infected snails and swims actively in fresh water for several hours. Upon contact with the skin of an appropriate mammalian host it penetrates directly through the skin, sheds its tail and outer glycocalyx coat and transforms into the young schistosome or schistosomulum. After a 2 day sojourn in the skin of experimentally infected animals the schistosomulum enters the bloodstream. The larvae can be found in large

numbers in the lungs of experimental hosts between 4 and 7 days after skin penetration, but it is believed that during this time they are circulating throughout the body, lodging only temporarily in highly vascularized tissues such as the lungs. From 7 to 10 days after infection the larvae are found in increasing numbers in the hepatic portal system. Maturation and pairing of the sexually differentiated worms occurs, and the paired worms take up their final position in the small mesenteric veins (*S. mansoni* and *S. japonicum*) or the veins of the vesical plexus (*S. haematobium*).

The mean lifespan of the adult worm is estimated to be 2–8 years, depending on species, but there is evidence of *S. mansoni* worms being able to survive 2–3 decades. Egg-laying starts about 6 weeks after infection, the females releasing between 300 eggs (*S. mansoni*) and 3000 eggs (*S. japonicum*) a day. A proportion of the eggs released by *S. mansoni* and *S.*

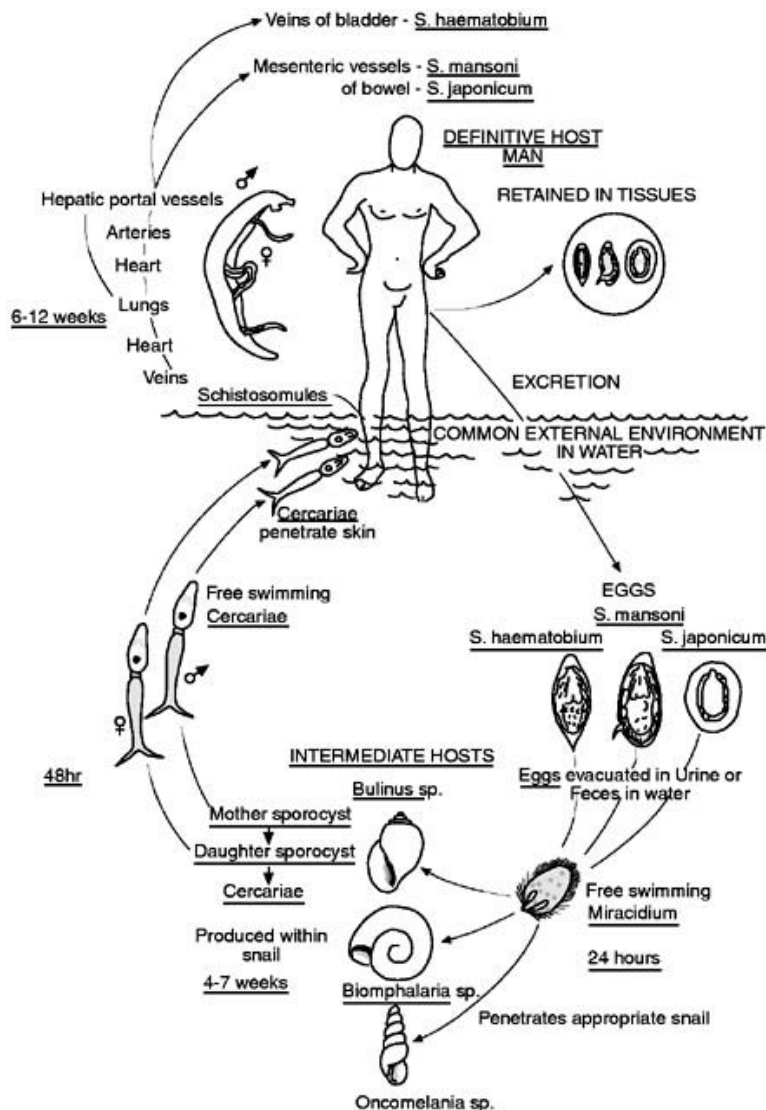


Figure 1 The schistosome life cycle.

japonicum worms are carried by hepatic portal blood flow to lodge in the liver, or they can be trapped locally in the intestinal walls, from which some pass into the gut lumen to be excreted. *S. haematobium* eggs are trapped in tissues of the urinogenital system, from which some leave the body in urine. The eggs hatch upon contact with fresh water, releasing the ciliated miracidium, a second free-living stage which is infective for the snail intermediate host. An asexual replicative phase in the snail leads to the production, after 4–6 weeks, of large numbers of cercariae.

A common feature of virtually all helminth parasites is that they do not replicate in their definitive hosts. In schistosomiasis, morbidity is largely attributable to the retention of eggs in the tissues, and the severity of disease is dependent on the number of eggs that are retained. This in turn depends on the number of worms acquired by the host (the intensity of infection) and their lifespan. Infection loads build up during childhood and intensities of naturally acquired infections are characteristically overdispersed, with a small proportion of individuals harboring most of the worms. These are the individuals who are most likely to develop severe disease. Although schistosome worms have a long lifespan the parasites acquired during childhood spontaneously die after several years. In addition, adult humans fail to become heavily reinfected, because of either lack of exposure or acquired immunity, or both. Intensities of infection in endemic areas therefore characteristically show a convex distribution with age, with a peak during the second decade of life.

It is accepted that eradication is an unachievable objective in the short to medium term, and the aim of control, whether by chemotherapy or eventually by vaccination, is now rather to reduce intensities of infection to levels sufficiently low that severe morbidity is not observed.

The several stages of the parasite that occur in the mammalian host are morphologically distinct and express both common and stage-specific antigens and epitopes. The young schistosomulum, about 100–150 μm long, has a syncytial tegument, containing spines bounded by an outer membrane composed of a double lipid bilayer. The tegument surrounds layers of circular and longitudinal muscle. Differentiated regions, in particular an oral and a ventral sucker, can be distinguished, but there is no functional gut, and nutrient and metabolite exchange takes place across the tegumental membrane. Development of a functional intestine and of specialized reproductive organs occurs during maturation into the adult worm: the sexes are morphologically distinct and permanently paired, with the flattened male (about

1 cm long) enclosing the longer and thinner round female in a gynaecophoric canal or schist. The egg comprises the larval miracidium enclosed by a rigid shell that bears, according to the species, a lateral spine (*S. mansoni*), a terminal spine (*S. haematobium*), or virtually no spine (*S. japonicum*) (see Figure 1). Micropores in the eggshell permit the passage of macromolecular antigens from the inside of the egg into the surrounding tissues.

Immune responses of the host

Repeated exposure to schistosome larval antigens, including those of nonhuman species, can result in hypersensitivity and cercarial dermatitis or 'swimmer's itch'. Initial exposure to a heavy primary infection of *S. mansoni* or *S. japonicum* can result some weeks later in an acute febrile syndrome, Katayama fever, which some have considered analogous to serum sickness or immune complex disease.

Immune reactions to the later stages of infection have different consequences for the host and are associated primarily with morbidity or in mediating resistance against infection.

Egg-induced immunopathology

Most of the pathological consequences of schistosome infection are attributable to prolonged deposition of eggs in the tissues, with the ensuing granulomatous reactions that the eggs induce leading to fibrosis. This in turn may lead to portal hypertension or urinogenital dysfunction, depending on the parasite species. The disease symptoms of schistosomiasis are therefore attributable to immunopathology.

The egg granuloma (Figure 2) is elicited in response to stage-specific egg antigens, though pre-

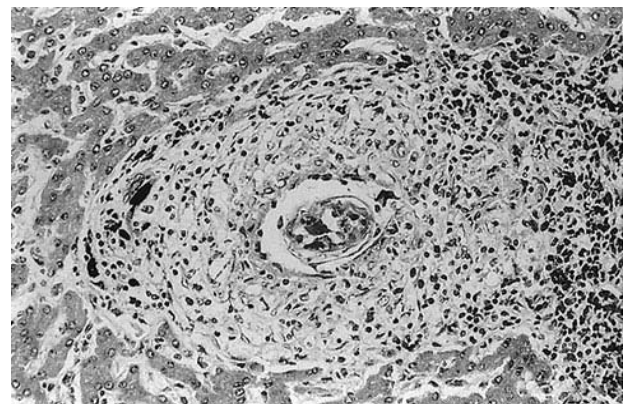


Figure 2 The schistosome egg granuloma. Section through an inflammatory lesion surrounding a *Schistosoma mansoni* egg in the liver of an infected mouse. Schistosome eggs are approximately 150 μm long, and in early infection the volume of a granuloma can be 100 times that of the egg.

sensitization can be achieved with unisexual male worm alone infections. The granuloma is a T cell-dependent, delayed-type hypersensitivity reaction comparable to that seen in other chronic granulomatous conditions, except that eosinophils as well as lymphocytes and macrophages are abundant in the lesion. The etiology of the subsequent fibrosis is also assumed to be an egg antigen-induced, T cell-dependent event, attributable to stimulation of fibroblasts by T cell-derived mediators. In mice, the granulomata that are induced around freshly deposited eggs during chronic infection are smaller and lead to less fibrosis than those that develop during acute infection. Such reactions are said to be 'modulated' and the capacity to mount a modulated reaction can be reproduced *in vitro* and adoptively transferred to acutely infected animals with CD8⁺ lymphocytes. There is evidence that modulation also occurs in chronically infected humans, in whom a variety of potential immunoregulatory mechanisms have been demonstrated, including serum factors, adherent and nonadherent suppressor cells, and T cells with idiotypic specificity for anti-egg antibodies.

Host reactions to egg antigens may play a protective as well as a deleterious role. Mice depleted of T cells show an enhanced mortality during *S. mansoni* infections, associated with hepatic parenchymal damage. This has been attributed to the action of an egg-derived toxin, the pathogenic effects of which can be prevented by passive transfer of immune serum.

In mice, excretion of *S. mansoni* eggs via the feces depends on an intact immune system, with both antibodies specific for egg antigens and immune lymphocytes having been implicated in this process. It has been postulated that the principal biological role of the egg granuloma is to facilitate egg excretion, and there is recent evidence that *S. mansoni*-infected humans with concurrent HIV infection have reduced schistosome egg excretion rates. Tumor necrosis factor α (TNF α) has also been identified as an important mediator in both the egg excretion process and female worm fecundity, as well as in host morbidity.

Anti-infection immunity

In contrast to the pathological effects of the egg granuloma, reactions to the schistosomulum are largely host-protective. Early studies by Smithers and colleagues showed that in rhesus monkeys adult worms of a primary infection continued to survive while the host was demonstrably resistant to reinfection by a secondary challenge of fresh cercariae, a phenomenon referred to as 'concomitant immunity'. Subsequent work in the mouse has shown that the

resistance induced by chronic patent infections may, however, be in part attributable to a nonspecific trapping or elimination of schistosomula following the formation of egg granulomas, i.e. as a result of disruption of the vascular migration routes normally used by incoming larvae.

Attempts to induce protective immunity with crude antigenic extracts of schistosomula, adult worms or eggs, or antigens purified therefrom have given variable results depending on the antigen(s), host animal species, type of adjuvant, etc., that were used. Partial, but specific protection against cercarial challenge can, however, be induced routinely in mice and other experimental hosts by immunization with irradiated cercariae which fail to mature into egg-laying adults. Relatively nonpermissive hosts such as the rat, as well as developing a specific resistance to cercarial challenge following immunization with irradiated larvae, tend also to become more immune after primary infection with unattenuated larvae or immunization with isolated antigens.

Although immune responses are elicited by both the adult and egg stages, the principal target of destructive immune attack is the migrating schistosomulum, and mechanisms of expression of antischistosomula immunity differ in host species. In most murine models, elimination of schistosomula occurs during or after the lung stage of migration, and is largely attributable to a T cell response to released schistosomulum antigens leading to macrophage activation and nonspecific killing of the parasite as a result of entrapment in inflammatory cellular foci or nitric oxide protection. In some circumstances, however, mice can also be protected against challenge by the passive transfer of monoclonal antibodies or of serum from donors that have been multiply vaccinated with irradiation-attenuated larvae. In the rat, death of schistosomula may occur either in the skin or after migration through the lungs, and may be attributable to antibody-dependent cellular cytotoxicity (ADCC) reactions against schistosomulum surface antigens. These reactions can involve eosinophils as well as macrophages or platelets, and immunoglobulin E (IgE) as well as IgG2a antibodies, suggesting a functional role for the long-recognized association between helminth infections, eosinophilia and IgE-mediated immediate hypersensitivity reactions.

In humans, the convex distribution of age-specific intensities of infection suggests the slow development of a concomitant immunity, and recent studies have demonstrated an age-dependent acquired resistance to reinfection after chemotherapy that is distinguishable from age-dependent changes in exposure to infection. In *S. haematobium* infections the expres-

sion of this resistance is correlated with peripheral blood eosinophilia, and human eosinophils are particularly potent mediators of ADCC reactions against schistosomula *in vitro* (Figure 3). This effect is enhanced by activation of the eosinophils *in vivo* or *in vitro*. Recent studies on both *S. mansoni* and *S. haematobium* infections in humans have demonstrated an association between the expression of infection-protective immunity and the development in older individuals of IgE antibodies against adult worm antigens. However, resistance in humans develops only in late childhood, and this may be attributable to the presence in younger children of 'blocking' antibodies of an inappropriate isotype and with specificity directed against carbohydrate epitopes on larvae.

Antigen carbohydrate epitopes are present in abundance on schistosome polysaccharides, glycoproteins and glycolipids, such residues being generally shared between the different parasite stages, and also between different schistosome species. Carbohydrates account for over 90% of the antigens expressed at the surface of the young schistosomulum, and antibodies, particularly IgM, IgG2 and IgG4, elicited early in infection in response to egg polysaccharide antigens may cross-react with the schistosomulum: this cross-reactivity may modulate or block the expression of potentially protective anti-schistosomular (ADCC) reactions. In contrast, peptide antigens tend to be both species- and stage-specific, although there is some sharing of these antigens by the schistosomulum and adult worm. Of the multiplicity of different proteins and glycoproteins that have been identified in the schistosomulum and adult worm, some have been implicated in the expression of protective immunity (see under Vaccination).

Cytokines and the T_H1/T_H2 cell axis

The identities of the populations and subpopulations of cells involved at various stages of schistosome infection, and of the cytokines they produce and respond to, are currently the subject of intensive investigation. Adult worms activate mainly T_H1-type responses, while eggs induce T_H2 responses. Protective responses in mice vaccinated with irradiated larvae appear to be driven principally by T_H1 cells, and can be enhanced by administration of interleukin 12 (IL-12).

TNF α mediates in egg granuloma formation in early patent infections and granulomatous activity is inhibited by treatment of normal mice with IL-4-specific antibody or exogenous administration of IL-12. In contrast to many other delayed-type hypersensitivity reactions the early egg granuloma is thus T_H2-dependent, which would account for the presence of large numbers of eosinophils in granulomas. Anti-IL-5 treatment results in the absence of eosinophils in egg-induced lesions which are otherwise of normal size. Hepatic fibrosis (collagen deposition) also appears to be T_H2 cell-mediated in mice.

The downmodulation of granulomatous activity that occurs as infections become chronic is mediated by T_H1 cells, as well as by CD8⁺ T cells.

Evasion of the host response

While in some combinations of host and parasite species there is evidence for an immunologically mediated suppression of egg output by adult females, the adult worm is generally not susceptible to immune attack, and can continue to survive for many years even in hosts that are immune to reinfection.

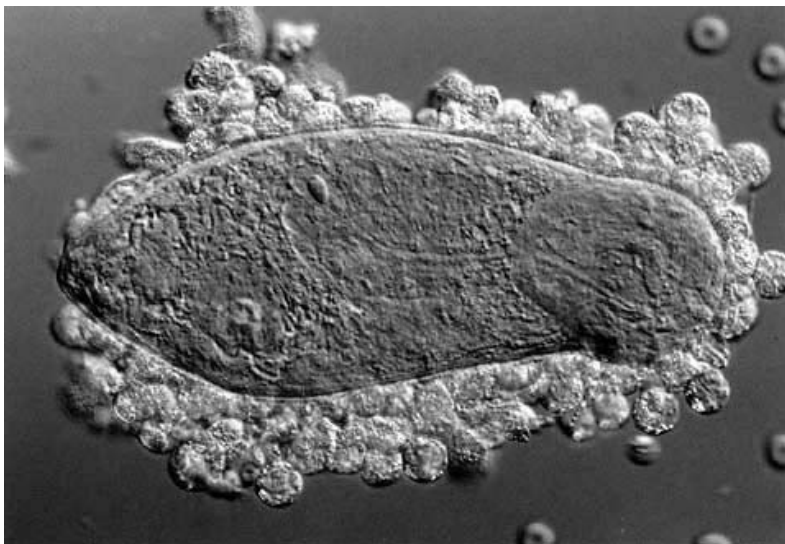


Figure 3 Antibody-dependent cellular cytotoxicity (ADCC) immune reactivity against a schistosomulum. The larval schistosome was incubated in culture medium together with a purified population of human eosinophils and human infection serum. After adhering to the parasite surface the eosinophils release the toxic constituents of their granules, resulting in damage and death to the former.

tion. Early work suggested that the loss of susceptibility to immune attack is attributable to the passive uptake onto the worm's surface of host-derived macromolecules that mask the expression of parasite surface antigens. Host constituents which have been shown to be present on or in adult worms include erythrocyte antigens, products of the MHC gene complex, immunoglobulins and plasma protease inhibitors. In addition the developing worm may simply lose antigenic macromolecules from the outer of the two lipid bilayers of the tegument, with adults thus having immunologically inert outer surfaces. Results showing that the schistosomicidal activities of drugs such as praziquantel are partially immune-dependent are consistent with this, in so far as the drugs erode the outer surface of the adult worms, thus exposing antigens which in normal worms are concealed from immune attack. Another explanation for immune evasion, currently less in favor, is that the parasite expresses epitopes which are shared with its host (the 'antigen mimicry' hypothesis). The developing schistosomulum may also partly evade immune damage, although less successfully than the adult, by releasing mediators that interfere with mast cell and lymphocyte function, proteolytic enzymes that cleave bound immunoglobulins, or lysophospholipids that kill effector cells of ADCC reactions.

Immunodiagnosis

Diagnosis of schistosomiasis is still done mostly by detection of the characteristically shaped eggs in human excreta. Indirect diagnosis using antibody detection assays, particularly ELISA are, however, becoming increasingly routine in the clinic, with antibodies against schistosome egg antigens showing somewhat more specificity and sensitivity than those against adult worm or larval antigens. The presence of at least two schistosome-derived carbohydrate-rich antigens in the blood of infected patients is enabling more direct immunodiagnostic tests to be developed using 'sandwich' ELISA.

Vaccination

No vaccine is yet available for protection against schistosomiasis in humans, though cattle have been protected against field challenge with *S. bovis* by immunization with irradiated larvae. The demonstration that experimental hosts can be partially protected against the human schistosomes by immunization with irradiated larvae, killed organisms or individual antigens suggests that vaccination in humans should be feasible.

Monoclonal antibodies with specificity for either carbohydrate or polypeptide epitopes confer protection after passive transfer in mice and rats. Carbohydrate antigens are not amenable to molecular cloning, but vaccination of rats with an anti-idiotypic monoclonal antibody that mediates ADCC reactions against the schistosomulum surface has been reported. This monoclonal antibody recognizes a carbohydrate epitope that is shared between schistosomula, eggs, and various molluscan macromolecules including keyhole limpet hemocyanin.

Various candidate polypeptide vaccine antigens have been prepared, following either affinity purification of the native molecule with monoclonal antibodies or monospecific sera, or molecular cloning and expression of relevant genes in *E. coli* or other hosts. Indeed, the application of recombinant DNA techniques now allows the production of parasite polypeptide antigens in sufficient amounts for large-scale use, and the World Health Organization has recently conducted preliminary independent laboratory trials on the immunoprophylactic potential of six recombinant *S. mansoni* proteins: 97 kDa paramyosin, a 62 kDa irradiation-associated vaccine antigen IrV-5, 28 kDa glutathione S-transferase, 28 kDa triose-phosphate isomerase, a 23 kDa membrane-bound antigen and 14 kDa fatty acid-binding protein.

Complete protection against challenge has not so far been demonstrated in any experimental host, either with attenuated organisms or with crude, purified or cloned antigens. However, in schistosomiasis a vaccine need not necessarily be completely effective in order to be of value. Since the adult worms do not proliferate within the human host, even a partial limitation on the establishment of adult worms will reduce the *intensity* of infection and may therefore prevent the development of severe morbidity.

See also: Antibody-dependent cellular cytotoxicity; Cytotoxicity, mechanisms of; Delayed-type hypersensitivity; Granuloma; Immunopathology; Parasites, immunity to; Vaccines.

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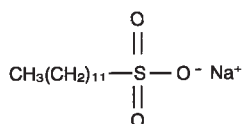
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SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

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The ability to separate a polypeptide of interest and to have an indication of its molecular size is a crucial element in any study involving mixtures of proteins. A variety of biochemical techniques are available to fractionate and analyze protein mixtures. These include electrophoretic, chromatographic and ultracentrifugation methods. The most relatively simple and powerful technique involves sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). In this method, separation of proteins occurs on the basis of their molecular size where the SDS-protein complexes are sieved through a polyacrylamide gel matrix. The combination of SDS and sieving properties in the molecular sized pores of the gel matrix, leads to an exceedingly high resolution of separation which is unattainable with any other separation method based upon protein size.

In SDS-PAGE, the protein(s) react with the anionic detergent SDS:



The binding of SDS destroys the tertiary structure of the protein, leading to partial unfolding of the polypeptide chain. This gives rise to a denatured protein whose biological function, with a few notable exceptions, is usually lost. SDS binds to both the hydrophobic and hydrophilic areas of the polypeptide chain to give a negatively charged, 'cigar'-shaped molecule, that is studded along its entire surface with negatively charged sulfonate ions. The protein, with bound SDS, thus has a net negative charge which overrides the intrinsic charge in the protein molecule contributed by the individual amino acids of the primary sequence. Generally, with a few exceptions, the amount of SDS bound per gram protein occurs at a constant SDS:protein ratio of approximately 1.4 g SDS g⁻¹ protein. This ratio is achieved under reducing conditions (when the inter- and intramolecular disulfide bonds are cleaved and the free thiol groups

blocked) and under these circumstances, the amount of SDS bound is proportional to the size of the protein giving a constant charge to mass ratio. Carbohydrate-containing proteins usually bind less SDS than other comparatively sized proteins.

Once SDS-protein complexes have been obtained in a protein mixture, they have to be separated into different polypeptide components. This is achieved by using a supporting medium called polyacrylamide and applying an electric field to the negatively charged complexes.

Polyacrylamide is a synthetic polymer formed by polymerization of acrylamide monomer with bifunctional cross-linking agents – the polymerization is usually aided by a catalyst. The final polymerized polyacrylamide matrix comprises a three-dimensional network of pores whose size is regulated by the percentage degree of acrylamide monomer and cross-linker concentration in the polymerizing mixture. The molecular size of the pores in the polyacrylamide gel is comparable to the size of protein molecules. Thus during electrophoresis, the proteins are sieved through the pores of the gel with large proteins having a slower migration than the smaller proteins. The choice of acrylamide concentration is important for a good separation and generally for proteins between the molecular weights of 10 and 200 kDa, an acrylamide gel concentration of 8–12% is sufficient. A single concentration of acrylamide in the gel for separating proteins will usually suffice, although the resolution of the separation can be increased even further by incorporating a gradient of acrylamide in the gel. The presence of an acrylamide gradient in the gel (usually 5–15% gradient) leads to a gradual reduction in the pore size accompanied by a greater sieving effect during electrophoresis and hence sharper protein bands.

Various forms of SDS-PAGE protocols have been described with electrophoresis in tube or slab gels with a variety of different buffer systems. The most common with the highest resolution is the buffer sys-

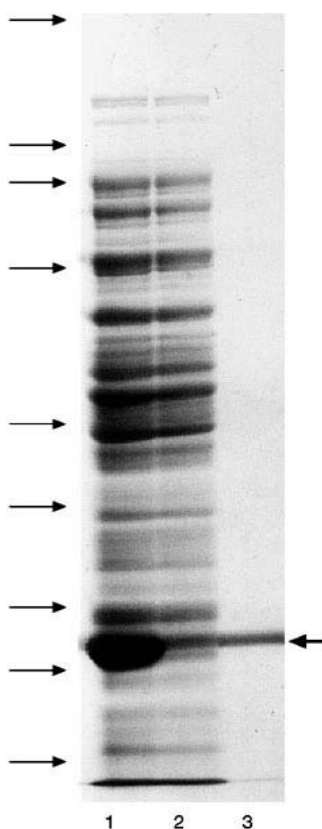


Figure 1 SDS-PAGE to show affinity purification of a recombinant protein from bacterial extracts. The gel is a 10% polyacrylamide gel and stained for protein with Coomassie brilliant blue. The arrows indicate molecular weight derived from electrophoresing a known mixture of proteins – from top to bottom, 205, 116, 97, 66, 45, 36, 29, 24 and 20.1 kDa. Lane 1 shows the presence of over 50 protein staining bands in the bacterial extract, with a strong band of glutathione-S-transferase at approximately 26 kDa (arrowhead). Lane 2 shows the same bacterial extract after passing through a column of glutathione agarose, where the 26 kDa protein is considerably reduced following the affinity chromatography step. Lane 3 shows the purified glutathione-S-transferase which was bound to the affinity column and specifically eluted with reduced glutathione. It is clear from this electrophoretic analysis that the purity of the recovered enzyme is >95%.

tem described by Laemmli and colleagues and commonly referred to as the Laemmli SDS-polyacrylamide gel system. The Laemmli gels use a discontinuous pH buffer system which allows the proteins (which are in a large volume when the sample is loaded on the

gel) to concentrate into a narrow, sharp band in the stacking gel during electrophoresis. Briefly, the principle of this system is based upon the use of two different buffers containing chloride ions (in the gel) and glycine ions (in the buffer reservoir). At the pH of the stacking gel (pH 6.8), the SDS-protein complexes have an intermediate mobility between the leading chloride and trailing glycine ions such that during electrophoresis the proteins are swept into sharp, narrow zones at the trailing glycine ions accelerate to keep up with the leading chloride ions. There is no separation of protein as they stack and concentrate into narrow zones in the stacking gel. Separation occurs when these stacked proteins enter the separating gel, which is at a different pH (pH 8.8) to give the chloride and glycine ions a comparable mobility.

SDS-PAGE has played a central role in biochemical fractionation procedures. Its role in assessing the purity of antigen or immunoglobulin preparations together with defining the molecular weights of target antigens by immunoprecipitation or immunoblotting (western blotting) techniques has given it an important role in immunochemistry. This is illustrated in Figure 1 which shows various fractions during the affinity purification of recombinant glutathione-S-transferase to yield a virtually pure preparation of the enzyme (Figure 1, lane 3). Additionally, combined with other fractionational procedures such as isoelectric focusing (two-dimensional electrophoresis), SDS-PAGE combines high resolution and simplicity not achieved with other electrophoretic methods.

See also: Cell surface molecules, immunoprecipitation of; Isoelectric focusing; Protein separation techniques; Western blotting.

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SECOND SIGNALS FOR LYMPHOCYTE ACTIVATION

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Lymphocyte activation requires the recognition of antigen by a lymphocyte receptor of unique specificity. Antigen is recognized in soluble form by the B cell surface immunoglobulin receptor (BCR), or complexed to a major histocompatibility complex (MHC) molecule on an antigen-presenting cell (APC) by the α/β heterodimer of the T cell receptor (TCR) complex. In each case the antigen receptor chains are coupled to a transmembrane signaling complex (Ig- α and Ig- β in B cells, CD3 in T cells) responsible for triggering a cascade of intracellular biochemical events that initiate the process of lymphocyte activation. However, under most circumstances, lymphocytes that receive only this signal do not become fully activated, but rather are turned off and become unresponsive to subsequent receptor stimulations, or undergo apoptotic cell death. In order to generate a productive immune response, lymphocytes must normally receive a second signal that increases lymphocyte proliferation, promotes cell survival and/or prevents lymphocyte receptor unresponsiveness. The hypothesis of a required second signal for lymphocyte activation was first proposed for B lymphocytes by Bretcher and Cohn in 1970. Second signals for lymphocyte activation can now be divided into two

categories: first, those that potentiate the signal transduced by TCR ligation and trigger enhanced proliferation; second, those that not only augment antigen-driven lymphocyte proliferation but also prevent the induction of lymphocyte unresponsiveness, and/or of programmed cell death (Figure 1). The latter are termed costimulatory signals, and activate intracellular pathways distinct from those triggered by the antigen-receptor complex. Some ligands can serve both functions. Second signals can be provided by different surface molecules depending on the type of lymphocyte considered as well as its stage of differentiation.

Second signals for T lymphocyte activation

Adhesion molecules

Certain adhesion molecules have been shown to enhance TCR-mediated T cell proliferation. It has been difficult to determine whether the increased proliferative response results from greater strength of the signal mediated by the TCR because of increased

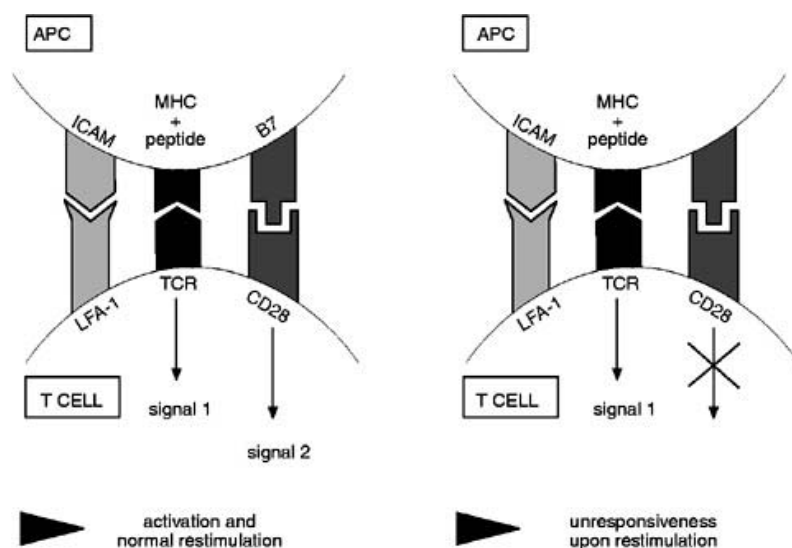


Figure 1 Two signals are required to induce full activation of T cells. T Lymphocytes receive signal 1 from specific interaction of their TCR with the peptide-MHC complex on an APC. This signal is increased by the simultaneous ligation of adhesion molecules such as LFA-1. Signal 2 results from the binding of a costimulator receptor such as CD28 with its ligand. Signaling through the TCR in the absence of a costimulatory signal results in hyporesponsiveness of the T cell upon restimulation.

cell-cell adhesion, or from direct transduction of a similar signal by the intracellular portion of the adhesion molecule. It has been proposed that adhesion molecules from the integrin family may prevent lymphocyte apoptosis through their binding to extracellular matrix components.

LFA-1 LFA-1 is a member of the integrin family of adhesion molecules, and is expressed constitutively on naive T cells, as well as on activated T cells and most other leukocytes. It is a heterodimeric structure composed of an $\alpha 1$ chain (CD11a) and a $\beta 2$ chain (CD18). Its described ligands, intercellular adhesion molecule 1 (ICAM-1) (CD54), ICAM-2 and ICAM-3 are expressed on a variety of cell types, including B cells, macrophages and endothelial cells. LFA-1 binding is thought both to increase adhesion between T cells and APCs and to potentiate the TCR signal in naive T lymphocytes. Binding of LFA-1 to ICAM-1 has been shown to augment TCR-induced interleukin 2 (IL-2) production and proliferation of naive T cells. In addition, simultaneous ligation of the TCR and LFA-1 augments activation of phospholipase $C\gamma 1$ (PLC $\gamma 1$) and Ca^{2+} flux over that induced by TCR ligation alone. However, disruption of LFA-1 interaction with its ligand by antibodies does not result in T cell unresponsiveness upon restimulation. Mice deficient in the expression of ICAM-1 are resistant to lethal doses of *Staphylococcus aureus* endotoxin B (SEB) as a result of reduced cytokine production in response to the toxin. T cells from these mice display diminished responses to alloantigen and to substances mediating contact hypersensitivity reactions. Mice deficient in CD18 (which is the common β chain shared by LFA-1, Mac-1 and p150/95) have delayed cardiac allograft rejections and impaired delayed-type hypersensitivity.

VLA-4 VLA-4 is also a member of the integrin family ($\alpha 4\beta 1$). VLA-4 can bind to vascular cellular adhesion molecule 1 (VCAM-1). VLA-4 ligation has been shown to augment TCR-mediated T cell proliferation, presumably in a similar manner to ligation of LFA-1, by increasing the strength of the signal delivered by the TCR. Gene disruptions of the α chain of VLA-4 or of VCAM-1 have lethal consequences, because of the involvement of these molecules in placental and umbilical cord generation.

CD2 The CD2 receptor on T cells binds to LFA-3 (CD58) or CD48 expressed on a wide variety of cells. Antibodies to CD2 have been shown to induce T cell proliferation, but their function is dependent on TCR expression. CD2 ligation results in augmented tyro-

sine phosphorylation of PLC $\gamma 1$ and Ca^{2+} mobilization, suggesting that interaction of CD2 with LFA-3 also acts by increasing specific signaling through the TCR. However, mice made deficient in CD2 by gene targeting lack obvious T cell defects, indicating that the function of CD2 may be redundant *in vivo*, or its function may be replaced by a different molecule when development takes place in the absence of CD2.

CD43 Recent data have attributed activating properties to human and murine CD43 (leukosialin), a molecule expressed on T cells, B cells, thymocytes, monocytes and fibroblasts, and capable of binding ICAM-1. Antibodies to CD43 increase TCR-mediated proliferation. CD43 is constitutively phosphorylated in T lymphocytes and monocytes, and becomes hyperphosphorylated upon activation. A possible association of CD43 with protein kinase C (PKC) has been suggested. However, the exact role of CD43 in T cell activation remains to be established.

B7 receptors

CD28 The best characterized costimulatory receptor is probably CD28, a homodimeric glycoprotein composed of two 45 kDa subunits and a member of the immunoglobulin gene superfamily. CD28 is constitutively expressed on virtually all murine T cells, 95% of human CD4⁺ cells, 50% of human CD8⁺ lymphocytes, as well as on developing thymocytes and plasma cells. Its ligands are members of the B7 family. B7-1 is expressed on activated B cells and monocytes, as well as on dendritic cells and Langerhans cells. B7-2 is detected on resting monocytes and dendritic cells, and on activated natural killer (NK) cells, T cells and B cells. Signaling T cells through CD28 with CD28-specific monoclonal antibodies (mAbs) in the presence of suboptimal TCR stimulation has been shown to enhance dramatically T lymphocyte proliferation, as well as to increase production of IL-2, tumor necrosis factor α (TNF α), lymphotoxin, interferon γ (IFN γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF). This augmentation of cytokine production may occur predominantly via stabilization of cytokine mRNA. Furthermore, prevention of the CD28/B7 interaction during TCR stimulation using blocking antibodies of CTLA-4Ig, a soluble fusion protein that binds with higher affinity to both B7-1 and B7-2 than does CD28, results in the induction of T cell unresponsiveness in T_H1 lymphocytes and IL-2-producing CD8⁺ T cells.

CD28-deficient mice have impaired T cell proliferation and IL-2 production in response to TCR stimu-

lation, as well as absent germinal center formation and reduced immunoglobulin class switching. CD28-deficient mice are resistant also to lethal injections of *Staphylococcus aureus*, due to reduced production of TNF α and IFN γ in response to the toxin injection. In contrast, these mice display normal cytotoxic responses.

CD28 signaling has been suggested to be necessary for differentiation of naive T cell towards a T_H2-like phenotype. In fact, repeated stimulation with B7-2 but not B7-1 has been reported to preferentially generate cells that produce IL-4. In addition, costimulation through CD28 has been shown to prolong T cell survival. Part of this effect is mediated by the ability of CD28 costimulation to upregulate Bcl-x_L, a member of the Bcl-2 family of antiapoptosis genes. As expected, T lymphocytes from CD28-deficient mice express lower levels of Bcl-x_L protein upon activation than do T cells from wild-type animals.

Cross-linking of CD28 with CD28-specific mAbs has been shown to augment the elevation in [Ca²⁺]_i induced by TCR ligation. However, in contrast to signals mediated by the TCR, the increased IL-2 production induced by CD28 cross-linking is resistant to the inhibitory effects of cyclosporine, indicating partially non-overlapping signaling pathways for these two surface molecules. The cytoplasmic tail of CD28 contains a phosphatidylinositol 3'-kinase (PI3K)-binding site, and stimulation via CD28 has been reported to activate PI3K. Transfection studies have demonstrated that p56^{lck} and p59^{fyn} can phosphorylate CD28 at Tyr191, which correlates with the recruitment of PI3K and the adapter protein GRB-2.

However, several studies have demonstrated that PI3K activity is not necessary for the effects of CD28 on IL-2 production. It has been suggested that signaling CD28 with CD28-specific mAbs can be different from that observed with normal B7 ligands. Ligation of CD28 with anti-CD28 in Jurkat cells results in tyrosine phosphorylation of p95^{vav} and a 36 kDa protein that associates with a complex of GRB-2 and the guanine nucleotide exchange factor SOS, and results in activation of p21^{ras}. In contrast, B7-1^{-/-} stimulator cells induce tyrosine phosphorylation of Vav but not p36, and do not trigger activation of p21^{ras}. CD28 signaling also activates the protein tyrosine kinase ITK. However, mice made deficient in ITK by gene targeting do not have impaired CD28 signaling. CD28 has been shown also to cooperate with CD3 to activate the mitogen-activated protein (MAP) kinases JNK1 and JNK2, enzymes involved in enhancing the transcriptional activity of AP-1, which is a factor directly involved in the transcription of the IL-2 gene. In addition, CD28 engagement has been shown to activate an acidic sphingomyelinase, resulting in the proteolytic degradation of I κ B. This event is necessary for nuclear translocation of NF κ B and has been detected both in Jurkat cells and in primary human T cells. Several mAbs against human CD28 have been reported to induce the nuclear translocation of the different components of NF κ B, namely c-Rel, NF κ B1 and RelA, that participate in IL-2 gene transcription (Figure 2).

CTLA-4 CTLA-4 is not constitutively expressed on T lymphocytes, but is induced following T cell

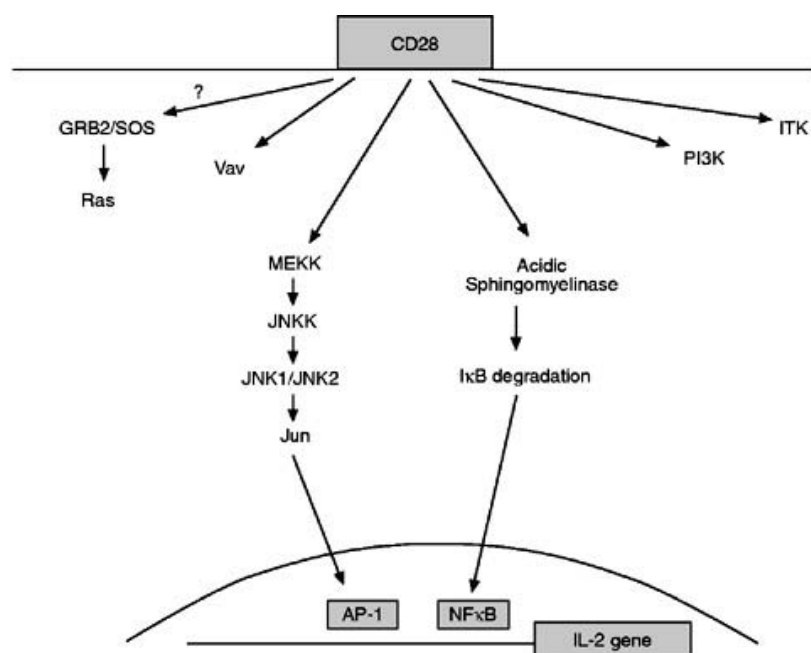


Figure 2 CD28 signaling. Schematic representation of some of the enzymatic and transcription factor activities induced by CD28 ligation.

activation, with peak levels being reached after 48–72 h. CTLA-4 is a second receptor for B7 family members, that shares 30% homology with CD28, but that binds both B7-1 and B7-2 with higher affinity. A third ligand for CTLA-4 and presumably CD28 has been implicated recently in mouse, the binding of which also promotes costimulation of T lymphocytes. Several reports suggest that CTLA-4 acts as a negative regulator of T cell activation rather than as a positive costimulatory molecule. Indeed, cross-linking of CTLA-4 molecules on the cell surface results in decreased T cell proliferation and IL-2 production. Conversely, blockade of CTLA-4 interactions using anti-CTLA-4 mAbs increases T cell responses *in vitro*, and leads to the rejection of implanted tumors in murine models *in vivo*. Furthermore, in previously activated human T cells, cross-linking of CTLA-4 has been reported to induce apoptosis, perhaps upon binding to a ligand different from B7-1 and B7-2. Consistent with these data, CTLA-4-deficient mice generated by homologous recombination develop a fatal lymphoproliferative syndrome and autoimmune disease, manifested by myocarditis, pancreatitis and high levels of circulating immunoglobulins. Both CD28 and IL-2 signaling can independently upregulate CTLA-4 expression. Therefore, the most potent signals for T cell activation appear to be the most powerful inducers of CTLA-4 expression. In addition, upregulation of CTLA-4 requires entry into the cell cycle, as agents capable of arresting T cells in G₁/S phase also inhibit CTLA-4 expression. These data suggest a negative feedback role for CTLA-4 that could control cell division after proliferation has occurred. Surprisingly, much more CTLA-4 is found intracellularly than on the cell surface at all time points. It appears that CTLA-4 is efficiently transported to the cell surface, but rapidly endocytosed, suggesting that CTLA-4 molecules cycle to and from the cell surface, with only a minimal fraction of the pool expressed transiently on the cell surface at any one time.

Little is known about the signals transduced upon CTLA-4 ligation. Like CD28, the cytoplasmic tail of CTLA-4 contains a PI3K-like binding motif, and it has been shown that CTLA-4 can associate with PI3K. Recently, T cells from CTLA-4-deficient mice have been reported to have an increased pattern of tyrosine phosphorylation, and the cytoplasmic portion of CTLA-4 has been found to interact with the protein tyrosine phosphatase SHP-2 (PTP1D).

Tumor necrosis factor (TNF) and TNFR family members

All known ligands of this family (CD40L, CD30L, TNF α , lymphotoxin β (TNF β), CD70, FasL and 4-

1BBL) as well as their receptors (CD40, CD30, TNFR1, TNFR2, CD27, Fas, 4-1BB and O $\times$ 40) can be expressed by T cells. CD40, CD30, CD27, TNF α , lymphotoxin and 4-1BB are also found on B cells, and TNF α , CD30L, and 4-1BB are also detected on activated macrophages. Expression of CD40L and O $\times$ 40 appear to be restricted to CD4⁺ T cells. Human peripheral blood T cells as well as CD4⁺ T cell clones show enhanced proliferation when stimulated simultaneously through the TCR and any of the TNF-like and TNFR-like proteins. These molecules can also trigger T cell death, and their ability to induce apoptosis or proliferation depends on the cell type, the stage of differentiation, the transformation status, and the presence of other stimuli. Because T cells express both TNF-like and TNFR-like molecules, T lymphocytes could potentially costimulate themselves or commit themselves to programmed cell death. The intracellular domains of CD40, TNFR2, and CD30 have been shown to interact with members of the TNFR-associated factor (TRAF) family. The TRAF proteins are now known to induce survival signals, as well as to activate the transcription factor NF κ B. In contrast, Fas and TNFR1 have been shown to associate with proteins containing a 65 amino acid domain termed a death domain. These receptors appear able to assemble a signal transduction complex that initiates apoptosis through the activation of proteases in the IL-1-converting enzyme (ICE) family.

CD40L CD40L (gp39) is a 33 kDa protein almost exclusively expressed by activated CD4⁺ T cells, although some expression has been described on CD8⁺ T cells, basophils and mast cells. Therefore, CD40–CD40L interaction can induce proliferation of CD4⁺ T cells upon TCR activation. CD28 stimulation induces upregulation of CD40L. Recent data indicate that CD40L/CD40 may also be implicated in negative selection of thymocytes. Mice made deficient in the CD40L gene by homologous recombination appear to have normal T cell responses to mitogens, but reduced antigen recall responses in terms of T cell proliferation.

CD30 CD30 was first described as a marker for Hodgkin lymphoma cells, but is also expressed by a subset of normal T lymphocytes. CD30 has been implicated as a costimulatory molecule on activated but not resting T cells. It has also been suggested to promote the differentiation of T cells into T_H2 effector cells, and is thought to play a role in the transcriptional reactivation of latent human immunodeficiency virus (HIV). Mice made deficient in CD30 by gene targeting show a defect in negative selection.

Cytokine receptors

IL-2R IL-2R is a trimeric receptor composed of an α chain, a β chain shared by IL-2R and IL-15R, and a γ chain, that is also shared by IL-4R, IL-7R, IL-9R and IL-15R. IL-2 is a growth factor and a survival factor for most T cells. Furthermore, the induction of TCR unresponsiveness by TCR stimulation in the absence of CD28 ligation can be prevented, in most cases, by the addition of exogenous IL-2. Proliferation of T cells from CD28-deficient mice in response to CD3-specific mAbs can be restored by the addition of exogenous IL-2. Similarly, high doses of IL-2 bypass the requirement for CD28 costimulation, as exogenous IL-2 can overcome blockade by CTLA-4Ig. On the other hand, IL-2 can sensitize T cells to activation-induced cell death. Consistent with this result, mice made deficient for IL-2 or IL-2R α by gene disruption develop a lymphoproliferative disorder and autoimmune disease.

IL-2 signaling requires the heterodimerization of the β and γ cytoplasmic domains. Neither the β chain or the γ chain have known intrinsic catalytic activity. Therefore, signaling operates via the recruitment of proteins containing SH2 domains. JAK3 has been shown to interact with the γ chain, whereas the β chain can associate with Syk, PI3K, SHC and JAK1. Recruitment of JAK1 and JAK3 is followed by activation of STAT3 and STAT5. IL-4R and IL-7R, which share a common γ chain with the IL-2R, have also been reported to be important in regulating lymphokine survival during the immune response.

IL-1R IL-1R functions as a costimulatory receptor for most murine T_H2 but not T_H1 clones, and allows T_H2 clones to respond to the IL-4 that they produce. Blocking IL-1 during TCR stimulation of T_H2 clones may result in TCR hyporesponsiveness upon restimulation, as determined by a decreased ability to provide B cell help. Proliferation of those cells remains intact, indicating that not all functions are desensitized under those conditions.

Heat stable antigen (HSA) receptor

HSA (CD24) is expressed on dendritic cells and B cells and binds to an unknown ligand on T lymphocytes. Some antibodies to HSA can induce T cell unresponsiveness upon restimulation. In addition, the combination of antibodies to HSA and CTLA-4Ig has been reported to completely block T cell proliferation induced by activated B lymphocytes, suggesting additive effects provided by B7 and HSA receptors. The effect of HSA receptor stimulation on T cell survival has not been investigated thus far. The level of expression of HSA does not correlate with

its costimulatory function, as expression is higher on resting B cells that are poor providers of costimulatory activity, than on activated B lymphocytes. This could result from a conformational change of HSA upon B cell activation, and differentially glycosylated forms have been described. Alternatively, an inhibitory molecule might be produced by naive but not activated B cells.

Second signals for B lymphocyte activation

Soluble antigens are recognized by surface immunoglobulins on B lymphocytes, processed into peptides, internalized and presented to CD4⁺ T cells in the context of MHC class II. Engagement of the BCR transmits a signal (signal 1) that induces a number of changes on the surface of B cells, including upregulation of B7-2, thus promoting T cell activation if antigen-specific T cells are encountered. This signal also appears to direct B lymphocytes to the outer region of the periarteriolar lymphoid sheath in the white pulp of the spleen, a region rich in T cells. During T/B cell contact, activated T lymphocytes provide second signals to B cells in the form of soluble cytokines, as well as membrane-bound ligands and adhesion molecules. This serves to enhance B cell activation, proliferation, differentiation, and immunoglobulin isotype switching. In return, engagement of MHC class II induces upregulation of B7-1 and other molecules capable of delivering second signals to T cells. Several surface molecules have been implicated in augmenting B cell signaling, the most important one being CD40, that plays in B cells a similar role to CD28 in T cells. In addition, monoclonal antibodies to LFA-1, ICAM-2, CD2, CD4, CD19, CD38, CD58 and MHC class II have all been reported to partially inhibit T-dependent B cell activation to different degrees.

In some situations, mouse and human B cells can be protected from apoptosis by CD38-specific mAbs. Following successful T/B lymphocyte interaction, oligoclonal proliferative foci can be detected that reach maximum size after 3–4 days, and then dissipate. Many B cells in these foci secrete IgM antibodies and undergo isotype switching, others migrate to the primary follicles and initiate the formation of germinal centers. CD40, B7-2 and lymphotoxin have all been shown to be required for germinal center formation, as mice made deficient for any of these molecules or their ligands by gene targeting are devoid of germinal centers.

TNF and TNFR family members

CD40 is a 45–50 kDa molecule and is a member of the TNFR family. It is expressed on the surface of B cells, macrophages, follicular dendritic cells and some T cells. The importance of CD40 in B cells has been exemplified by a naturally occurring disease in humans. The hyper-IgM syndrome is an immunodeficiency due to a mutation in the gene encoding CD40L, that either prevents expression of CD40L, or renders it incapable of binding to CD40. This results in the absence of serum IgG, IgA and IgE, the absence of germinal centers, and the inability to mount secondary antibody responses to thymic-dependent antigens. Similar phenotypes are found in mice made deficient for CD40 or CD40L by homologous recombination. CD40 has different functions in B cells, depending on the cell type considered. Resting B cells enter the cell cycle upon CD40 ligation. In contrast, CD40 signaling has been reported to trigger proliferation in some chronic leukemic B cells, programmed cell death in some B lymphoma cell lines, and prevention of apoptosis in germinal center B cells. In fact, CD40 ligation has been shown to induce upregulation of Bcl- x_L in B cells, similarly to engagement of CD28 in T cells. In addition, several studies have documented a role for CD40 in the induction of B cell memory. Finally, CD40L can induce upregulation of B7-1 and B7-2 on resting B cells and thus indirectly regulates T cell activation.

The cytoplasmic chain of CD40 does not possess intrinsic enzymatic activity. Rather, CD40 triggering is thought to induce an association with TRAF proteins such as TRAF-3 as well as lead to recruitment and activation of tyrosine kinases. Stimulation of transformed B cell lines with antibodies to CD40 induces tyrosine phosphorylation of several substrates, including Lyn and Syk. It also triggers turnover of inositol phosphates and activation of different serine/threonine kinases. Possible substrates of these protein tyrosine kinases are PLC γ 2 and PI3K, as these molecules have been shown also to undergo activation upon CD40 ligation with antibodies in the Daudi B cell line. In murine resting B cells, cross-linking of CD40 induces activation of ERK and JNK in a PKC-independent manner. This contrasts with the PKC-dependent activation of these enzymes observed after BCR stimulation. The two pathways seem to converge at the level of MEK in mediating ERK activation (Figure 3). However, in human tonsillar B cells, CD40 ligation has been found to activate JNK but not ERK. The discrepancy in these results may result from species differences, or antibody epitope variations.

CD40 binding to CD40L, in combination with

surface Ig cross-linking, has been reported to result in activation of NF κ B through its association with TRAF proteins. In addition, CD40 signal transduction can induce *c-jun* and *c-fos* although the mechanism for this activation has not yet been defined. In addition, CD40 ligation has been shown to result in decreased tyrosine phosphorylation of several cellular proteins, suggesting that CD40 also activates phosphatases. Increased cAMP and ornithine carboxylase activity have also been described following CD40 ligation. Signaling through CD40 may be different depending on the stage of differentiation or activation of B cells. It seems that in germinal center B cells, the CD40 pathway is coupled to protein tyrosine kinases but not to PI turnover. Tyrosine phosphorylation appears to be necessary for the antiapoptotic effect of CD40 in these cells. In addition to CD40L, other ligands in the TNF family, such as TNF α , have been shown also to enhance B cell proliferation after T cell contact.

Cytokine receptors

The response to CD40 ligation is enhanced by lymphokines such as IL-4. Exposure of B cells to IL-4 and IL-5 in the mouse or IL-4/IL-13, IL-2 and IL-10 in the human induces differentiation, antibody secretion and immunoglobulin isotype switching. Although surface immunoglobulin engagement by soluble antigen (signal 1) is necessary to attract B lymphocytes to T cell-rich zones, studies *in vitro* have shown that B cell proliferation and antibody production can be achieved in the presence of activated T lymphocytes and of the relevant cytokines. Through the cytokines that they produce, T cells help

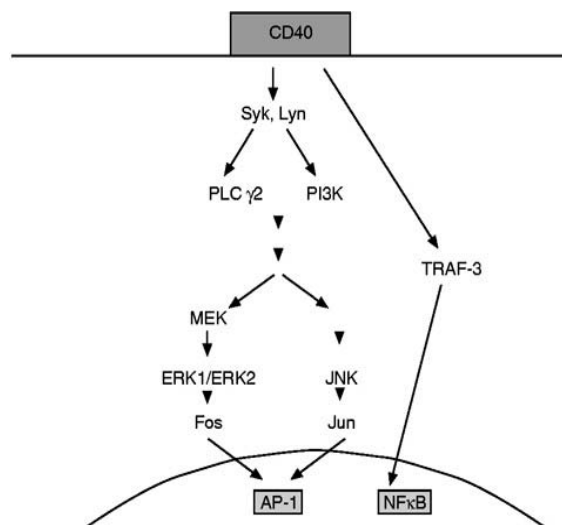


Figure 3 CD40 signaling. Schematic representation of some of the intracellular events triggered by CD40 ligation.

to determine the isotype switch that occurs following B cell activation. Thus, T_H1 clones have been shown to promote immunoglobulin switching to the IgG2a isotype. T_H2 clones induce IgG1 and IgE secretion. IFN γ inhibits the secretion of IgG1 and promotes that of IgG2a. IL-4 is required for IgE production and has also been found to induce expression of B7-1 and B7-2 on resting murine B cells.

The cytoplasmic domain of the IFN γ R has been shown to associate with JAK1 and JAK2 and to trigger activation of STAT1. Mice made deficient in STAT1 lack IFN γ signaling. The IL-4R interacts with JAK1 and JAK3, inducing activation of STAT6. STAT6-deficient mice display a similar phenotype to IL-4-deficient mice, showing diminished production of IgE and IgG1 antibodies.

Conclusion

Major advances have been made in the understanding of lymphocyte activation. Participation of costimulator molecules is an essential component of the initiation of productive lymphocyte activation. When lymphocytes are stimulated in the absence of second signals, an unresponsive state results. This knowledge has enabled immunologists to design therapeutic strategies aimed at blocking or triggering second signals, thus allowing for antigen-specific suppression or augmentation of immune responses, respectively. Indeed, treatment with CLTA-4Ig and/or CD40Ig in several animal models (including nonhuman primates) of transplantation has proven very efficacious at immunosuppression, and, in some cases, is capable of inducing donor-specific tolerance. Administration of CLTA-4Ig or CD40Ig can also prevent or treat some autoimmune diseases. Conversely, activation of the CD28 pathway of signaling has been shown to promote tumor rejection in other

experimental models. Application of these new therapeutic strategies to the treatment of human diseases holds considerable promise for the future.

See also: Adhesion molecules; B lymphocyte activation; CD2; CD28; CD40 and its ligand; B7 (CD80 & CD86); Cytokine receptors; Integrins; Interleukin 2 receptor; Lymphocyte function-associated antigen 1 (LFA-1); T lymphocyte activation; Tumor necrosis factor α ; TNF receptors.

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SECRETORY COMPONENT (THE POLYMERIC Ig RECEPTOR)

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The existence of an adaptive mucosal immune system was proposed in 1919 by Besredka when he demonstrated that rabbits, after oral immunization with killed *Shigella*, were protected against fatal dysentery irrespective of the serum antibody titer. In 1922 Davies was able to detect antibodies against the dysentery bacillus in stools from infected patients several days before such activity was present in serum. The interest in local immunity was significantly revived after 1965 when it was reported by Tomasi's group and also other laboratories that the predominating immunoglobulin (Ig) in external body fluids was IgA; this secretory IgA (S-IgA) was shown to have unique immunochemical and physicochemical properties because of its polymeric nature and association with an epithelial glycoprotein called 'secretory piece' or 'secretory component' (SC). At the same time Crabbé and coworkers demonstrated a class distribution of intestinal Ig-producing plasma cells remarkably different from that in peripheral lymphoid tissues; mucosal IgA immunocytes were strikingly predominant – being more than 20 times as numerous as IgG-producing cells. This finding has been repeatedly verified for most categories of normal exocrine tissues. Moreover, in the early 1970s Brandtzaeg and coworkers obtained evidence suggesting that the mucosal IgA immunocytes produce dimers or larger polymers (collectively called pIgA) rather than monomers.

The latter workers also pointed out that local production alone does not explain the preferential appearance of pIgA in exocrine fluids. They showed that serum IgG is abundantly represented in interstitial tissue fluid but is not internalized by secretory epithelia, whereas pentameric IgM (pIgM) is trans-epithelially translocated to the lumen in a preferential manner like pIgA. A common receptor-mediated external transport mechanism for S-IgA and secretory IgM (S-IgM) was therefore proposed by Brandtzaeg in 1973–74. The SC was suggested to be the transmembrane receptor responsible for the epithelial binding and uptake of the pIg ligands. The J chain was taken to be a key factor in the initial noncovalent binding process. This polypeptide was in 1971 shown by Mestecky and coworkers to be a common disulfide-linked component of pIgA and

pIgM; it is known to be selectively incorporated into these polymers within the Ig-producing plasma cells. Secretory immunity thus depends on a fascinating cooperation between the mucosal B cell system and the secretory epithelia.

Molecular biology and function of secretory component

The human SC gene locus (PIGR) has been assigned to the long arm of chromosome 1 (region q31–q42). This region contains other genes of immunological interest and is involved in a significant number of recombination events. We have detected restriction fragment length polymorphism (RFLP) for the SC gene; Pvu II revealed a two-allele RFLP with an autosomal codominant inheritance pattern.

Human SC occurs in three molecular forms: as a basolaterally expressed transmembrane protein (~100 kDa) functioning as pIg receptor (pIgR); as a stabilizing integrated component (~80 kDa) in S-IgA and S-IgM (bound SC); and as an excess of free SC (~80 kDa) in most exocrine fluids (Figure 1). Cell biology studies first substantiated in the rabbit that SC is produced as a transmembrane protein 25–30 kDa larger than the secreted form.

Transmembrane SC (pIgR) is composed of five homologous extracellular domains, a transmembrane anchor, and a cytoplasmic tail (Figure 2). Proteolytic cleavage near the apical cell membrane releases SC (bound or free) into exocrine secretions. Free SC appears to exhibit eosinophil-activating properties and may block adhesion of *Escherichia coli*. Such additional functions suggest that SC has originated phylogenetically from the innate defense system.

The five repeating domains of pIgR are stabilized by intrachain disulfide bridges and show considerable amino acid homology with several members of the Ig supergene family, particularly with Ig V domains. The full-length receptor characterization obtained by cloning of cDNA from different species has allowed identification of conserved regions and experimental definition of the functionally most important sequences. The cDNA for human pIgR first isolated in our laboratory, encodes a transmem-

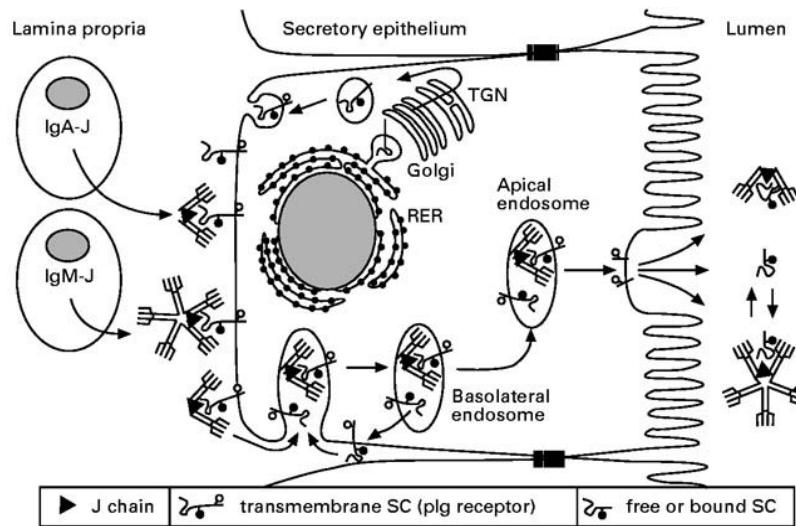


Figure 1 Schematic representation of various steps involved in the generation of human secretory IgA (top right) and secretory IgM (bottom right) via plgR-mediated epithelial transport of J chain-containing polymeric IgA (IgA-J) and pentameric IgM (IgM-J) secreted by local plasma cells (left). Transmembrane SC is synthesized in the rough endoplasmic reticulum (RER) of secretory epithelial cells and matures by terminal glycosylation (—●) in the Golgi complex. After being sorted through the trans-Golgi network (TGN), it is phosphorylated (○) and expressed as plgR at the basolateral plasma membrane. Endocytosis of ligand-complexed and unoccupied plgR is followed by transcytosis to apical endosomes and finally by cleavage and release of secretory Ig molecules with bound SC as well as excess of free SC at the luminal cell face. However, some basolateral recycling may first take place for unoccupied plgR. During the external translocation, covalent stabilization of the IgA–SC complexes regularly occurs (two disulfide bridges indicated in secretory IgA), whereas free SC in the secretion apparently serves to stabilize the noncovalent IgM–SC complexes (dynamic equilibrium indicated for secretory IgM).

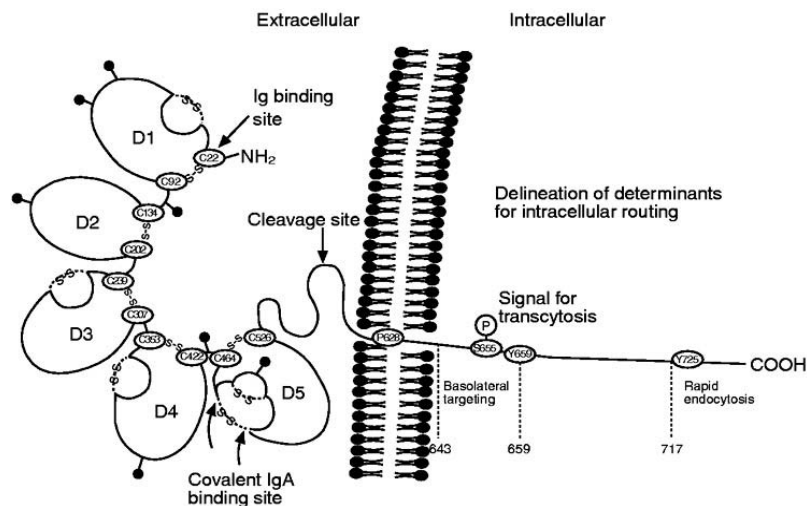


Figure 2 Schematic depiction of the mature human transmembrane SC or plgR. The position of cysteines participating in disulfide bonds stabilizing the extracellular Ig-like domains 1–5 (D1–D5) are indicated. Arrows mark the initial Ig-binding site in D1, the site for secondary covalent plgA bonding in D5, and the cleavage site near the epithelial cell membrane. Glycosylation sites (—●) and disulfide bonds (—S—S—) are also depicted. Putative determinants in the cytoplasmic tail critical for proper cellular routing are delineated on the basis of findings reported for the rabbit plgR, including the phosphorylated serine residue (P) S655 (counterpart to rabbit S664) and the two tyrosine-based endocytosis motifs. The kink-inducing proline in the membrane-spanning segment suggests signal-transducing capacity of the receptor.

brane protein of 746 amino acids (Figure 2) and a signal peptide of 18 residues. The average overall amino acid homology between human pIgR and the rabbit, rat, mouse and bovine counterparts is 73%. However, functionally important sequences such as the putative initial Ig contact site in domain 1 (see below), the transmembrane segment, and the basolateral targeting sequence in the cytoplasmic tail (see below), show average homologies of 93%, 87% and 92%, respectively.

Northern blot analysis has revealed that pIgR mRNA is 3.5–3.9 kb in all investigated species. Curiously, rabbit pIgR mRNA contains a second smaller form (~3.1 kb) generated by an alternative splice that gives rise to a functional receptor protein lacking domain 2 and domain 3. In humans, pIgR mRNA is expressed most strongly in the gut but can also be detected in other exocrine tissues. The weak expression revealed in the human liver probably reflects the activity of biliary duct epithelium. By contrast, rodents express almost as high levels of pIgR mRNA in the liver as in the intestinal mucosa because their hepatocytes produce SC (see below).

Intracellular sorting of the pIgR

After synthesis and core glycosylation in the rough endoplasmic reticulum of the epithelial cell, the pIgR further matures in the Golgi apparatus; it is then sorted in the trans-Golgi network for delivery to the basolateral plasma membrane (Figure 1). Both ligand-complexed and unoccupied pIgR is internalized by endocytosis in coated pits and sorted for transcytosis in the basolateral endosomal compartment; binding of pIg to the receptor is in fact not mandatory for its transport to the luminal cell surface. Here bound and free SC molecules are released by cleavage from the transmembrane anchor by a leupeptine-sensitive protease, while the remaining segment is degraded. Transmembrane SC has therefore been called a 'sacrificial receptor'.

The complex intracellular routing of the pIgR through the epithelial cell has made it a model protein for the characterization of intracellular sorting signals. Several studies by Mostov and coworkers based on mutants of rabbit pIgR have characterized the function of its cytoplasmic tail (103 residues in human) as an effector segment for routing through the epithelial cell; it apparently carries all the information needed to provide proper delivery at the different cellular locations, including an autonomous basolateral targeting signal, two tyrosine-based endocytosis motifs, and one major phosphorylation site (Figure 2). Phosphorylation of this serine (Ser664) in rabbit pIgR (equivalent to Ser655 in

human) stimulates transcytosis, which for pIgA-receptor complexes in addition is upregulated by ligand binding, probably through second messenger systems. The high conservation of the α helical membrane spanning domain, including a kink-inducing proline, further suggests that the pIgR is capable of mediating signal transduction.

Interactions between pIg and SC/pIgR

Various research groups have determined the affinity (K_a) of pIg for free SC in solution to be about 10^8 M^{-1} . This value is similar to that for a variety of antigen-antibody reactions. Also pIgR-expressing epithelial cells bind pIg with high affinity. Such non-covalent interactions of pIgA and pIgM with SC/pIgR depend on their incorporation of J chain. Evidence supporting this notion has recently been obtained both in transgenic plants and in J chain knockout mice.

There are much stronger noncovalent interactions between pIgM and free SC than between pIgA and free SC, the K_a being several times higher for the former ligand. The two subclasses of human IgA (IgA1 and IgA2) are apparently similar in this respect. It remains to be clarified whether a 'bonus' effect of a higher molar J chain content in pIgM than in pIgA explains the different properties of their SC-binding sites. Nevertheless, the *in vivo* efficiency of external translocation appears to be much better for pIgA than for pIgM (see below).

The I region of SC ('I' stands for 'inaccessible' antigenic determinant in bound SC) appears to be primarily responsible for the noncovalent binding of pIg molecules; this region corresponds to the N-terminal domain 1. The highly conserved sequence in this region (amino acids 15–37) probably reflects its functional importance as an initial Ig contact area; binding studies with synthetic peptides have demonstrated that this sequence alone shows 'promiscuous' relatively low affinity not only for pIg molecules but also for monomeric IgA and IgG. This binding site forms a loop similar to the first complementarity determining region (CDR1) of Ig V $_{\kappa}$ domains. Complexing with pIgA further appears to depend on the CDR2 and CDR3 loops, followed by noncovalent interactions extending beyond domain 1. Although the C $_{\alpha}$ 2 and particularly the C $_{\alpha}$ 3 domain of pIgA are involved in these interactions, the exact part of the pIg molecules that initially contacts the pIgR remains undefined. The subsequent covalent stabilization, which appears to occur selectively for S-IgA, depends on disulfide exchange reactions between one C $_{\alpha}$ 2 domain and domain 5 of pIgR (Figures 1 and 2),

although other possibilities have not been completely excluded.

Purified J chain shows only marginal affinity for free SC. However, one computer-assisted model of this peptide in humans and mice has suggested that, despite a low degree of primary structure homology with Ig domains, the J chain may nevertheless show an Ig domain-like folding of its secondary structure. It seems likely that appropriate binding of pIgR to the Fc portion of the pIg molecules is determined by complementary interdomain interactions both with the J chain and the C region domains of the α or μ chains. The role of J chain in the binding process may thus be to stabilize the noncovalent pIgR-pIg complexes as a requirement for their endocytic internalization by secretory epithelial cells.

Efficiency of pIg transport through secretory epithelia

Immunohistochemical studies have shown that SC is expressed mainly by serous types of epithelial cells in exocrine tissues. In the human liver, SC is restricted to the bile duct epithelium whereas abundant SC expression is shown by hepatocytes in the rat and some other animals. This species difference has been supported by Northern blot analysis of pIgR mRNA (see above) and harmonizes with the fact that ~90% IgA in duodenal fluid is derived from bile in the rat but <15% in humans.

Exocrine tissues constitute quantitatively the most important effector sites of specific humoral immunity with the gut as the major contributor. It has been estimated that at least 80% of all Ig-producing cells in humans and mice are located in the intestinal mucosa. Nevertheless, there is normally very little spillover of locally produced IgA from the human gut lamina propria to lymph and venous blood. The capacity of the pIgR-mediated external transport through human intestinal crypt epithelium is hence remarkable; more pIgA is translocated to the gut lumen by this mechanism every day (40 mg kg^{-1} body weight) than the total daily production of IgG (which is about 30 mg kg^{-1}). An *in vitro* model system established in our laboratory to characterize this process has shown that the epithelial transport of pIgA and pIgM mediated by the human pIgR is equally efficient. However, *in vivo* external translocation of pIgA is on a molar basis favored 6–12 fold over that of pIgM because the latter ligand is severely impeded when diffusing through the lamina propria and basement membrane. Nevertheless, S-IgM is an important compensatory antibody class in external secretions of newborns and patients with selective IgA deficiency.

Regulation of secretory immunity

The biological significance of the striking J chain expression shown by Ig-producing cells in exocrine tissues is that pIgA and pIgM are generated locally and thus become readily available for pIgR-mediated external transport. This important goal, in terms of clonal B cell differentiation, may be sufficient justification for the J chain to be expressed also by a variable fraction of B cells terminating in exocrine tissues with IgD or IgG production; these immunocytes may be considered as 'spin-offs' from recently stimulated memory B cells that through clonal isotype switching are on their way to IgA expression. The J chain appears to be a marker of fairly recently differentiated B cell memory clones, and no other function has been identified for this peptide than incorporation into the quaternary structure of pIgA and pIgM, thereby contributing to their SC pIgR binding site (see above). This incorporation takes place intracellularly as shown by the preferential binding of free SC to the cytoplasm of mucosal IgA and IgM immunocytes in affinity tests performed on tissue sections (color plate).

The precursors for Ig-producing cells in exocrine tissues are apparently in the main derived via lymph and peripheral blood from organized lymphoepithelial structures where the primary mucosal B cell responses are induced. The most important inductive site seems to be the gut-associated lymphoid tissue (GALT), particularly the Peyer's patches; but bronchus-associated lymphoid tissue (BALT) and probably the tonsils also contribute, especially to plasma cell populations in the upper alimentary and respiratory tracts.

It has been known for more than two decades that Peyer's patches are enriched in IgA-producing cell precursors. Some immunoregulatory mechanism(s) must exist in GALT and similar mucosa-associated lymphoid tissue (MALT) that facilitates expansion of early memory clones with activated J chain gene while allowing very little terminal B cell differentiation *in situ*. The final maturation to pIg-producing plasma cells will therefore mainly take place after precursor distribution to exocrine effector sites. Although uptake of antigens by lymphoepithelial MALT structures, and the involvement of antigen-presenting cells, T cells and various cytokines have been subjected to extensive studies, it remains obscure how B cells of the mucosal immune system normally are tuned for activation of the J chain gene and preferential pIgA responses.

Studies in our laboratory as well as by other groups have shown that SC/pIgR expression by secretory epithelium may be modulated by cytokines

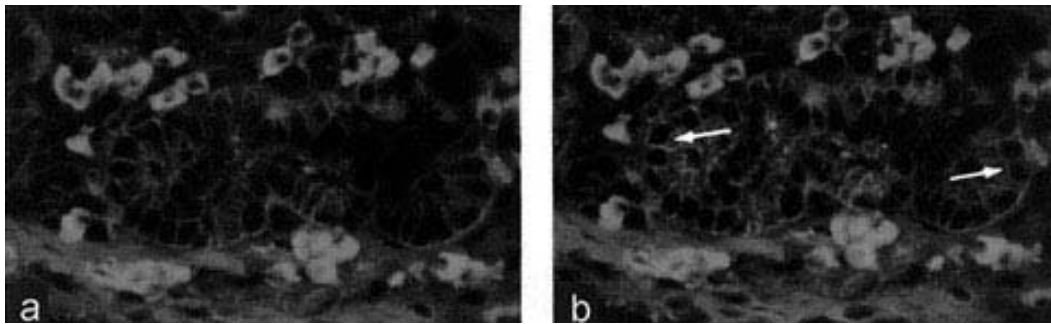


Figure 3 Paired immunofluorescence staining for IgA and SC in tissue section from human nasal mucosa; the same field is shown after incubation with 'red' anti-IgA + 'green' anti-SC, (a) (red filtration) and (b) (double exposure). IgA alone is present in immunocytes and throughout the stroma; SC alone is present in the Golgi zones (arrows) adjacent to nuclei of glandular acinar cells; both IgA and SC (mixed color) are present basolaterally on acinar cells and apically in their cytoplasm. Original magnification $\times 180$. (See also color Plate 5A.)

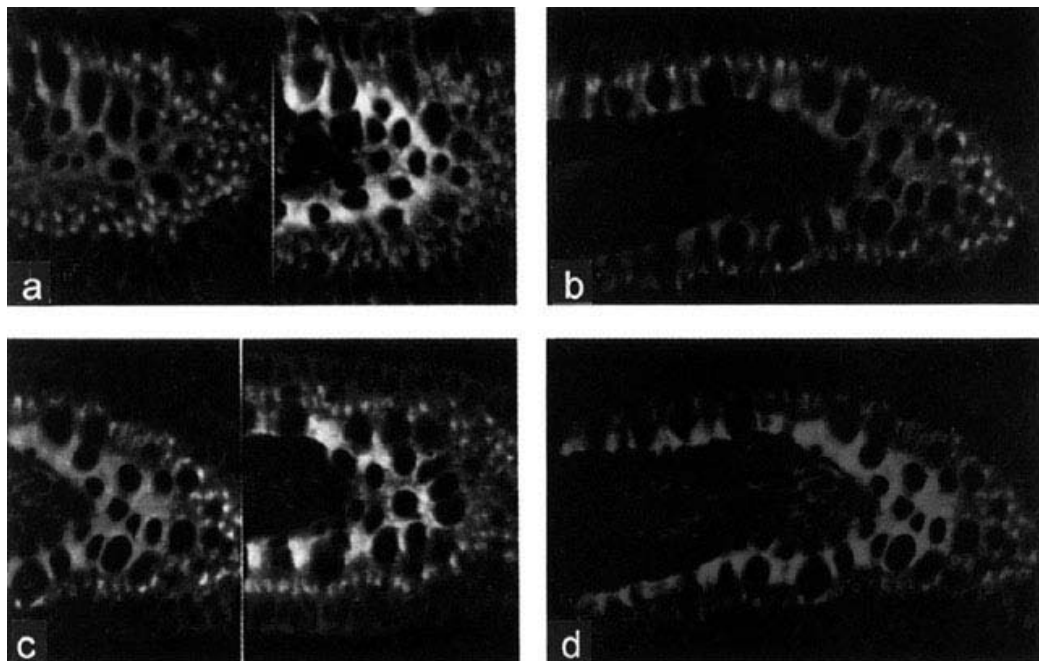


Figure 4 Paired immunofluorescence staining for IgA and total SC, or for unoccupied and bound SC, in tissue sections of human colonic mucosa. (a) left part (red filtration) and right part (double exposure), comparable fields in adjacent sections of gland incubated with 'red' anti-SC and with 'red' anti-IgA + 'green' anti-SC, respectively. Prominent SC-containing granules are present in the Golgi zones, whereas the rest of the cytoplasm and the basolateral aspects of columnar epithelial cells are positive for both SC and IgA (mixed color). Goblet cells are devoid of both markers. (b) (red filtration), (c) (left part, double exposure), and (d) (green filtration), same field in a section of gland incubated with 'red' anti-I determinant (specific for unoccupied SC) + 'green' anti-A determinant (accessible on free as well as bound SC). The two antigenic determinants show distinct differential distribution with a relative dominance of I in the Golgi zones, and of A in the remaining cytoplasm and weakly also basolaterally. Right part of (c) (double exposure) shows a comparable field after prolonged exposure time for red (anti-I) emission. Note that neither of the two anti-SC reagents produced fluorescence of elements in the lamina propria. Original magnification $\times 180$. (See also color Plate 5B.)

such as interferon γ (IFN γ), interleukin 4 (IL-4) and tumor necrosis factor α (TNF α). Experiments with the HT-29 adenocarcinoma cell line have suggested that IFN γ , IL-4 and TNF α all induce transcriptional upregulation of pIgR, and IL-4 may additionally stabilize the specific message. We and others have recently cloned and characterized the promoter

region of the SC/pIgR gene. DNA elements that bind transcription factors belonging to the basic helix-loop-helix leucine-zipper family or the interferon regulatory factor family appear important for constitutive or IFN γ -enhanced transcription, respectively.

There thus appears to exist a positive immunoregulatory link between the function of the pIgR and

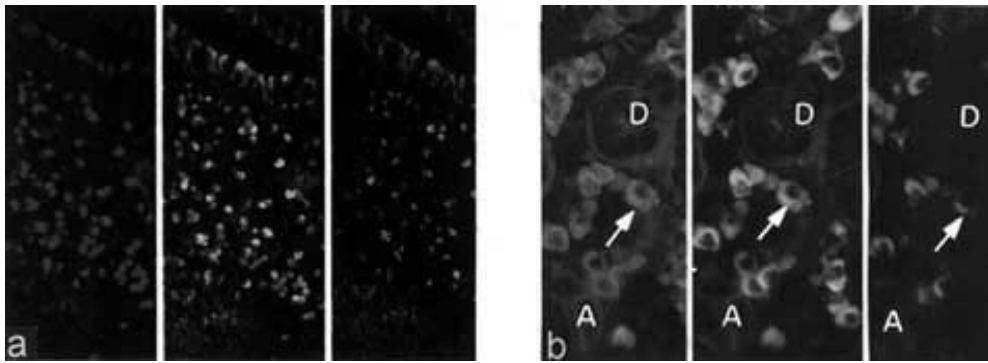


Figure 5 (a) Paired immunofluorescence staining to demonstrate *in vitro* SC affinity to IgA immunocytes in section of human colonic mucosa preincubated with free SC followed by 'red' anti-SC + 'green' anti-IgA. Note in double exposure (center) that most IgA cells show varying degree of mixed color as evidence for binding of SC to cytoplasmic pIgA, whereas a few cells (probably pure monomer producers) lack SC-binding capacity and are therefore only green. The purely red cells in the double exposure are SC-binding IgM immunocytes. The columnar epithelial cells of the glands at top and bottom contain innate SC and transport pIgA; they therefore show mixed cytoplasmic color with the exception of the Golgi zones which contain SC but no IgA. Magnification $\times 110$. (b) Similar SC-affinity test on section of human salivary gland. Double exposure (center) shows various tints of yellow in IgA immunocytes located between acini (A) and duct (D); in several immunocytes the yellow color is restricted to areas close to the nucleus (arrow), suggesting accumulation of pIgA. One immunocyte (at the bottom) is purely green, suggesting production of only monomeric IgA. Acini and duct are faintly double-stained for innate SC and translocated pIgA. Magnification $\times 430$. (See also color Plate 5C.)

the magnitude of the local immune response. Our immunohistochemical observations on Sjögren's syndrome and chronic gastritis harmonize with this notion; signs of increased SC expression and enhanced uptake of IgA are seen in glandular structures surrounded by dense infiltrates of mononuclear cells. Similar epithelial features are seen in celiac disease; and enhanced external transport probably explains that there is little increase of the serum IgA level compared with the remarkably expanded jejunal IgA cell population. Nevertheless, the pIgR-mediated epithelial transport capacity may be insufficient in certain patients with an unusual intestinal IgA cell expansion, resulting in excessive amounts of pIgA in serum.

See also: Antibodies, secretion; IgA; IgM; Joining J chain; Mucosal immunity; Mucosa-associated lymphoid tissue (MALT).

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SELECTINS (CD62-E/L/P)

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Selectins are a family of three Ca^{2+} -dependent transmembrane lectins i.e. L-selectin (CD62L), E-selectin (CD62E), P-selectin (CD62P) that initiate the tethering of leukocytes to activated endothelium under shear forces. They have a characteristic structural motif consisting of an N-terminal lectin domain, a domain with homology to epidermal growth factor (EGF), and a variable number of complement regulatory protein repeat sequences (Figure 1). L-Selectin is constitutively expressed on the majority of leukocytes, while E-selectin is often expressed by activated endothelium. P-Selectin is normally stored in α granules in platelets and the Weibel–Palade body in endothelial cells, and transported to the cell surface upon activation where it is expressed transiently. All selectins bind specific sialylated, fucosylated carbohydrates such as sialyl Lewis^x (sLe^x; Neu5Ac α 2–3Gal β 1–4(Fuc α 1–3)GlcNAc). Presentation of these carbohydrates in a clustered form on the protein backbone appears to confer high affinity to the sel-

ectin ligands. As shown in Figure 2, the glycoprotein ligands for L-selectin, GlyCAM-1 and CD34 are expressed on high endothelial venules (HEVs) in peripheral lymph nodes. Another L-selectin ligand, MAdCAM-1, is expressed specifically on HEVs in Peyer's patches and mesenteric lymph nodes. Both E-selectin glycoprotein ligand, ESL-1, and P-selectin glycoprotein ligand, PSGL-1, are expressed in neutrophils.

L-Selectin (CD62L)

L-Selectin (previously referred to as LECAM-1, LAM-1, Mel-14 antigen or Leu8 antigen) is expressed constitutively on leukocytes. It was

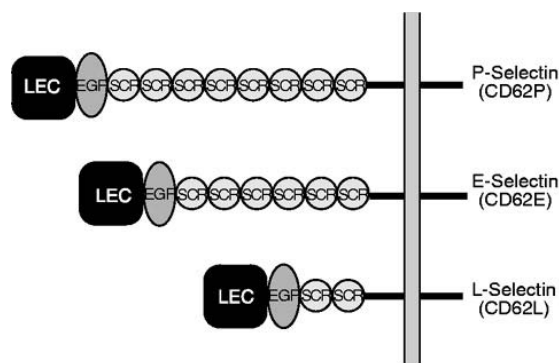


Figure 1 The selectins. The selectin family members have a unique domain structure consisting of the c-type lectin domain (LEC), epidermal growth factor-like domain (EGF) and complement regulatory protein domain with multiple short consensus repeats (SCR).

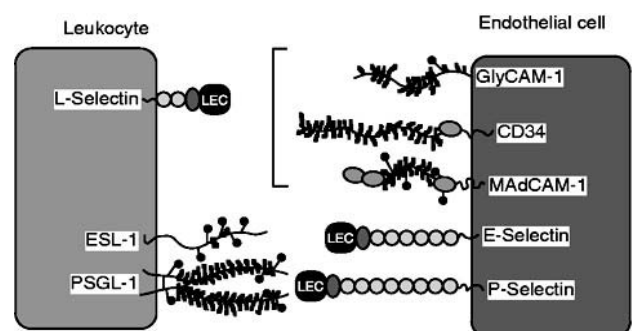


Figure 2 Selectins and their glycoprotein ligands. The lectin domains of the selectins (LEC) bind to clustered carbohydrate epitopes present on the protein backbone of sialomucins. L-Selectin recognizes at least three distinct sialomucins, GlyCAM-1, CD34 expressed on high endothelial venules of peripheral lymph nodes and also MAdCAM-1 expressed on high endothelial venules of mesenteric lymph nodes and Peyer's patches. Two endothelial selectins, E- and P-selectin recognize ESL-1 and PSGL-1, respectively, both of which are expressed on neutrophils. With GlyCAM-1, CD34, MAdCAM-1 and PSGL-1, O-linked sugars are recognized by selectins, whereas with ESL-1, N-linked sugars are recognized by E-selectin.

initially characterized by monoclonal antibody (mAb) Mel-14 as the murine homing receptor mediating lymphocyte binding to HEVs in peripheral lymph nodes. However, recent studies indicate that L-selectin is expressed on nonlymphoid leukocytes and is involved in the leukocyte traffic to inflamed tissues.

cDNA encoding L-selectin has been cloned in mice, humans, rats and cattle. L-Selectin has a characteristic lectin domain, an EGF domain, and two consensus repeats of the complement-regulatory protein domains. Human L-selectin has a core protein of 37 kDa with seven potential N-glycosylation sites, but no O-linked glycan appears to be present. Post-translational glycosylation yields L-selectin of different molecular sizes that are differentially expressed in various leukocyte subsets; 75 kDa in lymphocytes, 95–105 kDa in neutrophils and 110 kDa in monocytes. The L-selectin gene is located on human chromosome 1 and on murine chromosome 1 in close association with E- and P-selectin genes in both species. The proximity of these genes and similarity in the sequence of selectin genes (40–60% at the nucleotide level) suggest that they originated from a common primordial ancestor.

A unique feature of L-selectin is that it is rapidly shed from the cell surface upon activation. It is cleaved at the membrane-proximal site as a result of proteolysis, and increased levels of soluble L-selectin are present in the plasma of patients with sepsis, leukemia and the acquired immune deficiency syndrome (AIDS). Although the biochemical nature of this proteolytic enzyme, 'secretase' or 'shedase', remains to be fully elucidated, a novel type of zinc-dependent metalloprotease, similar to the one involved in the processing of membrane-bound tumor necrosis factor α (TNF α), appears to play a key role in proteolysis. Inhibition of the activity of this enzyme leads to reduced neutrophil rolling velocity under hydrodynamic flow conditions, resulting in increased accumulation of neutrophils. This suggests that the shedding process of L-selectin occurs very rapidly during rolling interactions and may be necessary for subsequent leukocyte adhesion and transmigration.

L-Selectin recognizes sialylated Lewis^x and sialylated Lewis^a (sLe^a; Neu5Ac α 2–3Gal β 1–3(Fuc α 1–4)GlcNAc), as do other selectins. Similar to P-selectin, L-selectin also binds to sulfatides and sulfated polysaccharides such as fucoidan and heparin. A group of O-glycosylated mucins decorated with sialic acid, termed sialomucins, represents the protein ligands for L-selectin. They are GlyCAM-1, CD34 and MAdCAM-1. GlyCAM-1, which bears sLe^x-type carbohydrates, is a secretory molecule expressed specifically on HEVs of peripheral lymph nodes and

probably acts as a regulator for L-selectin-mediated cell adhesion. CD34 is a transmembrane glycoprotein expressed extensively on blood vessels and hematopoietic stem cells, neither of which bind L-selectin under normal circumstances. Appropriate glycosylation appears to confer the ability to bind to L-selectin, and a specifically glycosylated form of CD34 expressed on HEVs serves as a ligand for L-selectin. However, mice deficient of CD34 by gene targeting possess almost normal lymph node architecture, and hence, CD34 is apparently not the sole ligand responsible for L-selectin-mediated lymphocyte trafficking into peripheral lymph nodes. MAdCAM-1 is expressed on HEVs of Peyer's patches and venules in intestinal lamina propria, and has been shown to bind L-selectin and $\alpha_4\beta_7$ -integrin. Recognition of vascular MAdCAM-1 by lymphocyte $\alpha_4\beta_7$ -integrin plays a crucial role in lymphocyte trafficking into intestinal lymphoid tissues. The physiological significance of L-selectin binding to MAdCAM-1 is unclear at present.

P-Selectin (CD62P)

P-Selectin (previously referred to as PADGEM or GMP-140) is normally stored in the Weibel–Palade bodies in endothelial cells and in α granules inside the platelets. It is rapidly mobilized to the cell surface upon appropriate activation, such as exposure to histamine, oxygen radicals, interleukin 1 (IL-1) and TNF α . P-Selectin has a domain organization common to all selectins, and nine consensus repeats in the complement regulatory protein domain in humans, six and eight repeats in cattle and mice, respectively. Alternative splicing of mRNA can yield two isoforms in humans; one variant lacks the seventh consensus repeat, whereas the other lacks the transmembrane region. Soluble P-selectin is detected in normal human plasma in concentrations of approximately 100 ng ml⁻¹, and structural analysis indicates that the plasma P-selectin is derived from alternative splicing of mRNA and limited proteolysis of the intact transmembrane form. In certain pathological conditions, such as the adult respiratory distress syndrome (ARDS), the plasma concentration of P-selectin may increase to 1.0 μ g ml⁻¹, a level that may be sufficient to modulate P-selectin-mediated cell adhesion *in vivo*. Data obtained from gene-targeted mice and mAb blocking studies indicate that endothelial P-selectin mediates the initial capture of leukocytes from the flowing blood, while the synergistic action of leukocyte L-selectin and endothelial P- or E-selectin is required for optimal leukocyte rolling (Figure 3).

Similar to other selectins, the lectin domain of P-

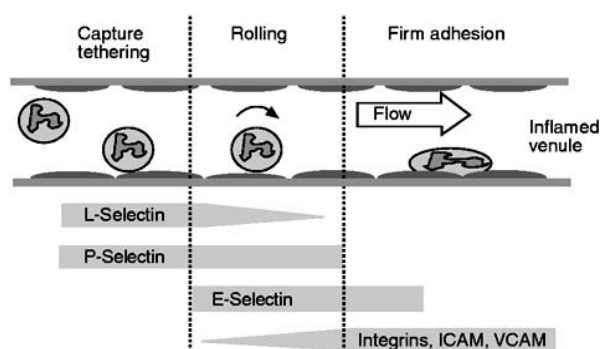


Figure 3 Synergy in selectin function in leukocyte rolling along the endothelium. Leukocyte L-selectin or endothelial P-selectin mediates the initial capture of leukocytes from the flowing blood as well as tethering of leukocytes to the endothelial cells. Subsequently, leukocyte rolling is mediated optimally by synergistic action of L-, E- and P-selectin. Leukocyte rolling is facilitated by leukocyte integrins and endothelial ICAM under low shear forces, leading to a firm adhesion of leukocytes to endothelial cells, a process also mediated by integrins, ICAM and VCAM (Modified from Ley and Tedder (1995)).

selectin plays a crucial role in the recognition of sLe^x- or sLe^a-type carbohydrate chains present on membrane glycoprotein counter-receptors. The appropriate presentation of the carbohydrate by glycoprotein scaffolds apparently allows high-affinity binding of the selectins. Several glycoproteins have been reported to be ligands for P-selectin. PSGL-1 is a transmembrane sialomucin of 110 kDa expressed on various types of leukocytes including T and B lymphocytes as homodimers. However, the majority of PSGL-1-positive peripheral blood lymphocytes do not display significant P-selectin binding. Only stimulated T cells show significant binding to P-selectin, and PSGL-1 is the predominant counter-receptor for P-selectin on this cell type. On the other hand, myeloid cells constitutively express an active form of PSGL-1. Thus, activation-dependent and/or cell-lineage specific post-translational events appear to contribute to the expression of functional PSGL-1. *In vitro* studies indicate that L-selectin may also act as a P-selectin ligand by presenting specific carbohydrate side-chains, although its significance *in vivo* remains unknown. Another P-selectin ligand on neutrophils was reported recently in the mouse. It is a 80 kDa glycoprotein (160 kDa; nonreduced), and its binding to P-selectin is sensitive to EDTA and O-sialoglycoprotease. P-Selectin also recognizes 3-sulfated galactosyl ceramides, sulfatides, which are expressed on the surface of granulocytes and certain tumor cells, and this may influence pathological interactions between tumor cells and activated endothelium or platelets.

E-Selectin (CD62E)

E-Selectin (CD62E or previously referred to as ELAM-1) is an inducible endothelial cell surface molecule of 115 kDa. Its expression on endothelial cells is transcriptionally upregulated by various pro-inflammatory substances such as IL-1, TNF α and lipopolysaccharide (LPS). Cytomegalovirus infection also induces E-selectin expression. The expression peaks 4–6 h after stimulation, and is rapidly down-regulated by internalization, returning to basal levels after 48 h, although sustained levels of expression may occur in the presence of interferon γ (IFN γ). E-Selectin has the C-type lectin domain, EGF-like domain and the complement regulatory protein homology domain consisting of six consensus repeats. E-Selectin mediates leukocyte rolling on the endothelium, and thus is involved in the recruitment of neutrophils, monocytes and T cells to inflammatory foci. However, blocking of E-selectin alone appears to have a limited effect on inflammatory responses *in vivo*. Results from gene-targeted mice deficient for expression of individual selectin also indicate that E-selectin alone is unable to recruit these inflammatory cells *in vivo*. Migration of neutrophils into the peritoneal cavity still occurs in E-selectin-deficient mice, but can be almost completely abrogated by pretreatment with mAb against P-selectin. Similarly, although E-selectin-specific mAb shows little effect in blocking leukocyte rolling in normal mice, it has a significant blocking effect in P-selectin-deficient mice. These results indicate that E- and P-selectin are partially redundant as mediators of leukocyte rolling *in vivo* (Figure 3).

Certain types of sialylated sugars, such as sLe^a and sLe^x have been identified as ligands that bind to the N-terminus lectin domain of E-selectin. CLA (cutaneous lymphocyte antigen) recognized by mAb HECA-452 is a fucosylated, sialylated structure closely related to sLe^x that can bind to E-selectin, and is expressed in a subset of skin-seeking memory-type lymphocytes. Expression of CLA in certain skin-infiltrating lymphocytes and the preferential expression of E-selectin in infiltrating foci suggest that CLA functions as a skin-homing receptor for lymphocytes. Several glycoproteins bearing sLe^a or related structures have been reported to bind E-selectin, although its significance *in vivo* remains unknown. These include L-selectin, CD66 and CD11/CD18. T Cells expressing $\gamma\delta$ -type T cell receptors (TCR) express a 250 kDa glycoprotein ligand in cattle. Furthermore, a 150 kDa glycoprotein ESL-1 (130 kDa nonreduced) was identified as a high-affinity ligand for E-selectin on murine neutrophils and human leukemia cell line HL60. This ligand appar-

ently lacks O-linked sugars, and utilizes N-linked sugars to bind E-selectin.

See also: Adhesion molecules; Endothelium; High endothelial venules; Integrins; Lymphocyte trafficking.

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SEPTIC SHOCK

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Sepsis is a systemic inflammatory response syndrome resulting from and complicating an infection. The clinical expression is extremely variable, but temperature dysregulation, tachypnea, tachycardia and leukocyte redistribution are common features. When the situation deteriorates, severe sepsis and septic shock may develop, with circulatory insufficiency, inadequate organ perfusion, capillary leak, an imbalance between oxygen demand and supply, and metabolic dysregulation. This may be further complicated by diffuse intravascular coagulation and multiple organ dysfunction syndrome.

The incidence of sepsis is increasing, presumably due to the improved possibilities of medical technology, which allows the survival of patients with important dysfunctions. Although this syndrome and related entities have attracted the attention of numerous (pre)clinical researchers, both from academies and industrial companies, the resulting gain in our understanding of sepsis has so far not been translated into an improved efficacy of treatment. The prognosis of patients with severe sepsis, septic shock and multiple organ dysfunction syndrome remains poor.

The question whether the data collected from (pre)clinical research during the last decades have brought more insight into the relevant pathways leading to lethality is appropriate. The complex pathophysiology of this entity, in which almost all organs and all physiological systems may become

involved in a redundant, concerted and interacting way, perhaps leads to a self-sustaining chaotic situation, in which our mechanistic approaches are inadequate. Furthermore, the highly variable clinical expression of these syndromes has led some authors to propose that these syndromes only exist in the mind of clinicians and researchers, and are in fact a variety of disparate pathological processes. Or, in other words, every patient with sepsis has his or her own subtype of the syndrome with a unique pathophysiology. The latter is not only determined by the original provoking challenge, but maybe more importantly by underlying, concomitant disorders and the reactivity of the individual patient. This not only makes it difficult to design an adequate treatment, but also hampers the development of relevant animal models and the design of clinical trials. Perhaps the most devastating concept which inhibited progress was the acceptance of the existence of 'black knights', central mediators of sepsis that would be responsible for the major deleterious, and only for deleterious, events. During the last years, the insight has grown that these presumed central mediators, such as tumor necrosis factor and nitric oxide, are in fact also indispensable to survive major challenges. It is only when the reaction of the body becomes malignant, i.e. uncontrolled, self-sustaining and chaotic, that the deleterious effects become predominant.

Table 1 Definitions

Infection = microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms

Bacteremia = the presence of viable bacteria in the blood

Systemic inflammatory response syndrome (SIRS) = the systemic inflammatory response to a variety of severe clinical insults. The response is manifested by two or more of the following conditions: 1) temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$; 2) heart rate >90 beats per minute; 3) respiratory rate >20 breaths per minute or $\text{Paco}_2 <32$ mmHg; and 4) white blood cell count $>12\,000$ per cu mm, <4000 cu mm, or $>10\%$ immature (band) forms

Sepsis = the systemic response to infection, manifested by two or more of the following conditions as a result of infection: 1) temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$; 2) heart rate >90 beats per minute; 3) respiratory rate >20 breaths per minute or $\text{Paco}_2 <32$ mmHg; and white blood cell count $>12\,000$ per cu mm, <4000 cu mm, or $>10\%$ immature (band) forms

Severe sepsis = sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to lactic acidosis, oliguria, or an acute alteration in mental status

Septic shock = sepsis-induced with hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status. Patients who are receiving inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured

Sepsis-induced hypotension = a systolic blood pressure <90 mmHg or a reduction of ≥ 40 mmHg from baseline in the absence of other causes for hypotension

Multiple organ dysfunction syndrome (MODS) = presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention

Reproduced with permission from Bone *et al* (1992).

It may be expected that this cluster of syndromes will remain a major challenge for the biomedical community. The results from such a major effort most likely will also find applications in less dramatic inflammatory responses.

Definitions

Being a syndrome ('a set of signs and symptoms') with an important interindividual variation, the decision as to who should be identified as a 'septic patient' relies on the presence of a sufficient number of preset symptoms, which may be attributed a weight factor. Especially since no single sign or symptom is explicitly required, it is not difficult to conceive that the great differences observed in epidemiology, outcome, efficacy of interventions and presence of mediators between the distinct clinical studies might be more a result from different definitions than from anything else.

Recently, an American College of Chest Physicians/Society of Care Medicine (ACCP/SCCM) consensus conference has proposed a new set of definitions of sepsis and related disorders that is becoming more and more accepted by the scientific community. 'Sepsis' is defined as a systemic inflammatory response to an infection. When sepsis is associated with organ dysfunction, hypoperfusion or hypotension, it is termed 'severe sepsis'. When the hypotension induced by sepsis persists despite adequate fluid resuscitation, in the presence of perfusion abnormalities, the term 'septic shock' is adequate. The recommendations of this conference

and the interrelationship of the distinct entities are shown in Table 1 and Figure 1, respectively.

Since sepsis and related disorders may present themselves in an important variation of severity, a severity-of-illness scoring system is an essential part of the description of patient populations. APACHE (Acute Physiology and Chronic Health Evaluation) scores are the most widely used severity scores, but a number of other scores exist.

Epidemiology and prognosis

Although there is, depending on the definitions used, quite some variation in the published numbers, there is a general agreement of increasing incidence. A reasonable estimate of sepsis complicating infections with gram-negative organisms is 500 000 cases/year in the USA. A recent trend is an increasing incidence of sepsis resulting from infections with gram-positive organisms. The majority of cases occur in patients who have already been admitted to hospital. Mortality rates range from 10 to 90%, septic shock having a higher mortality rate than uncomplicated sepsis. The presence of underlying organ dysfunction also contributes to a poor prognosis.

Pathophysiology

Table 2 is an incomplete listing of the various physiological systems involved in the pathophysiology of sepsis. A theory has evolved comprising successive waves of mediators and events which might result in lethality. In a first stage, bacterial products

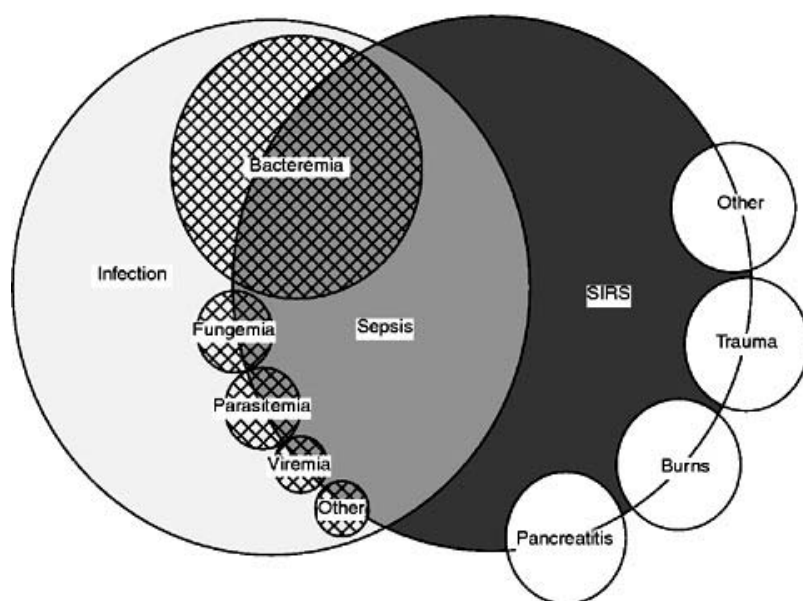


Figure 1 Interrelationship between systemic inflammatory response syndrome (SIRS), sepsis and infection. $\times\times\times\times$ = blood-borne infections (Reproduced with permission from Bone *et al* (1992).)

Table 2 Mediators of sepsis

Bacterial factors
Lipopolysaccharide
Peptidoglycans
Exotoxins
Cytokines
TNF
IL-1
IL-6
IL-12
IFN γ
Colony-stimulating factors
MIF
Complement
Fibrinolytic system
Blood clotting system
Platelet-activating factor
Neuropeptides
Eicosanoids
Thromboxanes
Leukotrienes
Prostaglandins
Kallikrein-kinin system
Adhesion molecules
Phospholipase A ₂
Endothelins
Oxygen radicals
Lysosomal enzymes
Nitric oxide

IFN, interferon; IL, interleukin; MIF, migration-inhibiting factor; TNF, tumor necrosis factor.

induce cytokine mediators. These in turn would activate endothelial cells and white blood cells, in this way inducing neutrophil/endothelial cell adhesion and the release of a second wave of mediators, including nitric oxide, elastase and products of the coagulation pathway. This second stage would then result in microcirculatory inflammation, capillary leak and circulatory insufficiency. The end-result is tissue hypoxia and multiple organ dysfunction. The latter events could result in a new trigger, either by inducing bacterial translocation in the gut or in other ways, and so be responsible for the vicious self-feeding character of the disease. On the other hand, negative feedback is induced: cytokine inhibitors, anti-inflammatory cytokines and acute-phase proteins. The balance between the proinflammatory events and the negative feedback would then determine whether sepsis will be transient or is going to develop into an uncontrolled disaster.

As a result of this theory, 'magic bullets' have been sought which might stop the deleterious activity of presumed 'central mediators' presented as 'black knights'. It was hoped that such an approach would allow the addition of an anti-inflammatory component to the supportive and source-controlling components of the therapy. Clinical trials could not confirm this approach. In the mean time, it has been recognized that at least two of the presumed 'black knights', nitric oxide and tumor necrosis factor, in fact are Janus-faced and also have indispensable protective properties. Perhaps this concept should be extrapolated to the septic reaction as a whole.

What prospects do we have? There is a general feeling that one should go back to basics, accept the complexity and the redundancy of the system, and become aware that the pure hierarchical mechanistic way of thinking is inadequate to approach this chaotic system. More insight should be gained into the factors that are responsible for the switch from a transient sepsis to an irreversible self-sustained syndrome. Alternatively, one could focus on the intracellular signal transduction pathways, rather than on intercellular mediators.

See also: Acute inflammatory reaction; Adhesion molecules; Arachidonic acid and the leukotrienes; Colony stimulating factors; Complement, alternative pathway; Complement, classical pathway; Complement, membrane attack pathway; Endotoxin (lipopolysaccharide (LPS)); Interferon γ ; Interleukin 1 and its receptors; Interleukin 6; Interleukin 12 and its receptor; Kallikrein-Kinin system; Macrophage migration inhibitory factor (MIF); Neuroendocrine regulation of immunity; Nitric oxide; Tumor necrosis factor α ; Platelet-activating factor (PAF); Prostaglandins; Lymphotoxin.

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SEROTONIN

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In addition to its role in neurological processes, the biogenic amine 5-hydroxytryptamine (5-HT) or serotonin (Figure 1) can be active as a vasoactive amine causing vascular permeability, smooth muscle contraction, and also playing an important role in the regulation of immunological responses. The following entry will summarize the most relevant information on storage and release of this catecholamine

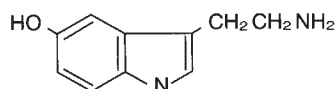


Figure 1 Structure of 5-hydroxytryptamine (serotonin).

by cells that belong to the immune system, or are associated with that system, and on the activity of serotonin in the context of the immune system.

Serotonin is released by rodent but not human mast cells, and also by basophils, platelets, and certain T cell populations with serotonin-positive granules. Upon activation, mast cells release a variety of mediators, including serotonin. Release occurs by a noncytolytic process that under most circumstances can be described as granule exocytosis. A large series of agents (secretagogues) are able to induce this release. The most well-studied example of mast cell release is induced by protein allergens in immunoglobulin E (IgE)-sensitized mast cells participating in

hypersensitivity reactions of the immediate, or anaphylactic type. Upon atopic sensitization, allergens induce specific antibodies of the IgE class (so-called anaphylactic, or reaginic, or cytotoxic IgE antibodies). Mast cells possess surface membrane receptors that specifically bind the Fc part of the IgE antibody with high affinity. Bridging of these Fc receptors by cross-linking of the bound IgE by the bi- or multivalent allergen, or anti-IgE, or otherwise, results in a transmembrane signal that causes activation of membrane-associated enzymes, leading ultimately to degranulation and release of mediators, among which is serotonin.

Apart from the classical IgE-mediated release of mast cell products, activation and mediator release can be initiated by a number of other potent secretagogues. Among those are products generated during inflammatory reactions such as the anaphylatoxins C3a and C5a which are produced during complement activation, and leukocyte-derived factors, i.e. products released from neutrophils, eosinophils, macrophages and lymphocytes, some of which are IgE-binding factors. Complement receptors have been demonstrated on mast cells. Other, non-immunological, secretagogues include several neuropeptides such as substance P, somatostatin, neurotensin and nerve growth factor, which is interesting in view of the fact that there is a close anatomical relationship between mast cells and nerve fibers. Further stimuli are opioids, smooth muscle relaxants and other drugs, highly charged basic polypeptides, the calcium ionophore A23187, compound 48/80, dextran and enzymes like chymotrypsin and phospholipase. Also physical factors like ultrasound, heat, cold, pressure and physiological conditions such as hypoxia, osmotic alterations and stress are considered stimuli for mast cell activation.

The ultrastructure of anaphylactic degranulation of mast cells in various species has been described in detail both *in vivo* and *in vitro*. Briefly, degranulation proceeds by swelling of the granules, converting the electron-dense granule contents into a more particulate appearance, and fusion of perigranular membranes with each other and with the plasma membrane. Thus, extensive channels, labyrinths or vacuoles opening to the exterior are formed, from which the granular contents are extruded into the environment. Degranulation requires calcium uptake and consumption of metabolic energy, i.e. it is a non-cytolytic process. After degranulation, mast cells still possess a full complement of cytoplasmic organelles, recover and regranulate.

In basophils and also in mast cells, a process of exocytosis that differs from the process described above has been described called 'piecemeal' degranu-

lation. Here, partial release of granular contents occurs by a vesicular transport mechanism involving budding of loaded vesicles from the granule membrane, transit through the cytoplasm, and fusion of these transport vesicles with the plasma membrane. This mechanism is thought to cause mild or gradual and time-spaced degranulation from basophils and differential release of serotonin from mast cells in mice. Differential release of serotonin can occur if mast cells are pretreated with certain drugs (such as the tricyclic antidepressant amitriptyline) and may occur *in vivo* in delayed-type hypersensitivity (DTH) responses.

Serotonin and delayed-type hypersensitivity

Thus far, substantial evidence has been obtained indicating that some type of antigen-specific T cell factor is capable of activating mast cells to release mediators, especially vasoactive amines in the early and late phases of DTH responses. The molecular nature of such factors remains to be fully defined. Activation of the mast cell may, conceivably, be achieved through specific receptors for the T cell factors. The events that take place during DTH responses can be summarized as follows. Early after immunization, antigen-specific T cell factors are produced and released by T cells in the lymphoid organs. These factors are transported in the circulation and leave the circulation to sensitize the peripheral extravascular tissues, i.e. bind to the mast cells that possess putative specialized receptors for these factors. Upon local antigen challenge the sensitized mast cells, and perhaps other serotonin-containing cells, release serotonin. This acts on serotonin receptors of postcapillary venules causing activation and the formation of gaps between endothelial cells, which results in increased vascular permeability. This allows circulating T cells, some of which are antigen specific, to leave the circulation and enter the site of the reaction where interaction with antigen takes place, probably in the context of major histocompatibility complex (MHC) class II antigens on local antigen-presenting cells such as Langerhans cells. Activation of these T cells by antigen-MHC complexes, and also by serotonin through 5-HT₂ receptors on their membranes, leads to local production of non antigen-specific lymphokines, which ultimately attract circulating bone marrow-derived leukocytes, such as monocytes, to also enter through the gaps between endothelial cells and constitute the classical perivascular infiltrate of DTH reactions.

There are several lines of evidence that favor a role of serotonin released from mast cells or platelets in

DTH-associated cellular immune responses in the mouse. First, these responses are elicited preferentially at those cutaneous sites that are enriched in mast cells, such as ears and footpads. In contact-sensitivity to trinitrochlorobenzene, as well as in hematorporphyrin derivative-induced phototoxicity, evidence of release of mediators by mast cells was obtained; in these studies histologic signs of hypogranulation of mast cells were noted. Secondly, pretreatment of cutaneous sites with serotonin causes tachyphylaxis, i.e. specific desensitization of vascular serotonin receptors on the endothelial cells, and this inhibits subsequent elicitation of DTH by local antigen challenge. Thirdly, treatment with the cromoglycate-like drug proxicromil, which prevents release of mediators including serotonin, or treatment with serotonin antagonists, like methysergide or ketanserin, suppresses contact sensitivity and also T cell-mediated inflammatory responses due to infection with *Trichinella spiralis*. Fourthly, studies employing the drug reserpine, which depletes serotonin from mast cells, platelets and neurons, showed suppression of DTH responses, contact photosensitivity, T cell-dependent immune responses to tumor cells, and the development of allergic encephalomyelitis. Finally, defective DTH responses have been observed in two strains of mast cell-deficient mice. This pertains to contact sensitivity to picryl chloride, delayed-type hypersensitivity to sheep red blood cells and to allogeneic tumor cells, and in mice sensitized to 3,3',4',5-tetrachlorosalicylanilide.

A potential role for mast cells and serotonin in contact sensitivity has been challenged. In contrast to defective DTH responses in mast cell-deficient mice, normal and even greater than normal responses were noted in other studies. Local reconstitution of mast cell-deficient ears with mast cells did not increase the responsiveness of such ears with respect to contact sensitivity. It can be concluded from these conflicting results that DTH can be elicited in mast cell-deficient mice, but that it is sometimes diminished. Crucial in this respect is how serotonin-dependent DTH responses are elicited in mast cell-deficient mice. The skin of these mice is not serotonin deficient. The source of serotonin is undoubtedly platelets, which could thus play a role in DTH. Therefore, recent findings concerning the role of platelets in IgE-dependent hypersensitivity responses could be extended to indicate an important role for platelets in DTH responses. It is noteworthy that active systemic anaphylactic responses were shown to occur in mast cell-deficient mice. Since such immediate-type IgE-dependent hypersensitivity reactions are shown to involve mast cells, these data can lead to two types of conclusions: 1) It may be that the relative low number

of mast cells (less than 1% of mast cells of normal litter mates) provides sufficient amounts of mediators, including serotonin, for systemic anaphylactic reactions and also can support DTH responses. Thus, mast cell-deficient mice may be a poor model to study the role of mast cells in such responses. 2) It is possible that other nonmast cell sources of serotonin are available for the elicitation of responses. In the skin this could be the rich supply of serotonin in platelets in the vessels and in systemic anaphylaxis this could be the extensive enterochromaffin tissue of the gut.

Serotonin and macrophages

At sites of inflammation, macrophages are exposed to mediators released by activated lymphocytes and aggregated platelets. The lymphokine interferon- γ (IFN γ) is a well-known activator of macrophages and induces, for example, increased expression of MHC class II on the plasma membrane of macrophages, rendering these cells capable of participating as antigen-presenting cells. Serotonin, which is likely to be present at sites of inflammation, is capable of suppressing the induction of the IFN γ -induced class II expression on macrophages. This inhibition is specific and is blocked by 5-HT $_2$ receptor antagonists.

Phagocytosis by macrophages is differentially modulated by serotonin. At low concentrations of IFN γ , serotonin augments phagocytosis, whereas at high concentrations of IFN γ , serotonin suppresses phagocytosis. These effects were blocked by the 5-HT antagonists spiperone, ketanserin, LY53857, mCCP and PAPP. Thus 5-HT could either counteract or augment the stimulatory effects of cytokines, depending on the overall balance of 5-HT and IFN γ , or other inflammatory mediators.

Serotonin and natural killer cells

Natural killer (NK) cell-mediated cytotoxicity against tumor target cells is enhanced by the addition of 5-HT to the incubation mixtures. The stimulation by 5-HT is dependent on the presence of monocytes and is presumably due to the abrogation of the monocyte-induced inhibition of NK activity. These effects are mediated through the 5-HT $_1$ receptor, although they were only evident at relatively high concentrations of 5-HT, suggesting that 5-HT may only have a role as an immunomodulator in local host defense against neoplastic cells and microorganisms.

Serotonin and lymphocytes

Serotonin has dual effects on lymphocyte function and proliferation. Addition of relatively high concentrations of 5-HT to phytohemagglutinin-stimulated lymphocytes suppresses *in vitro* proliferation. This effect could not be reversed by addition of interleukin 2 (IL-2) to the culture and was most likely due to inhibition of the activation of lymphocytes, since proliferation of other cells was not affected. In other studies stimulatory effects of serotonin on T cell activity were reported. Depletion of endogenous sources of serotonin reduced the capacity of splenic T cells to proliferate and to express IL-2 receptor in response to concanavalin A stimulation. Similarly, 5-HT₂ receptor antagonists had the same suppressive effect on the activation of normal spleen cells. Activated differentiated T cells express a 5-HT_{1a} receptor with an inhibitory regulatory effect on adenylate cyclase activity. Thus, 5-HT may have a T cell regulatory role at sites of inflammation by interfering with the suppressive effects of compounds that elevate cAMP (e.g. prostaglandins, β -receptor agonists, etc.). Serotonin may also act on other 5-HT receptors, such as 5-HT₁ and 5-HT₃, resulting in modulation of voltage-gated conductance in lymphocytes. Potassium channels are thought to play an important role in a variety of lymphocyte effector functions, including secretion of lymphokines and mitogenesis.

Serotonin and secretion of lymphocyte chemoattractant factors

At sites of inflammation, the accumulation of T lymphocytes is regulated by the sequential effects of multiple cytokine networks, resulting in the increased expression of adhesion molecules, production of chemoattractants and growth factors to induce cell migration and proliferation, respectively. Recently, a possible role for serotonin has become evident in these processes. Stimulation of aortic endothelial cells by serotonin results in the production of a novel 13–15 kDa T lymphocyte-specific chemotactic cytokine, endothelial cell-derived lymphocyte chemoattractant activity (ED-LCA), which is distinct from other endothelial derived cytokines (e.g. IL-1, IL-6 and IL-8). ED-LCA is not stored in an active form in endothelial cells; its secretion occurs within 60 min after stimulation with serotonin. No secretion of ED-LCA was found after stimulation with histamine or angiotensin II. ED-LCA acts as a chemoattractant for both CD4⁺ and CD8⁺ T cells. In addition, ED-LCA also primes T lymphocytes to respond to IL-2 by virtue of upregulation of IL-2 receptor expression. Another lymphocyte chemoattractant factor (LCF or

IL-16) is produced after stimulation of CD8⁺ T lymphocytes with serotonin or histamine. IL-16 is preformed and stored in biologically active form and release from CD8⁺ cells is essentially complete at 4 h after stimulation with serotonin. This release is mediated through 5-HT₂ receptors. IL-16, identified as a protein with a molecular weight of 56 kDa, has selective chemoattractive activity for CD4⁺ T lymphocytes and may play an important role in the recruitment of CD4⁺ T lymphocytes into sites of inflammation. Interestingly, it has been demonstrated that IL-16 is released in bronchoalveolar fluid following subsegmental allergen challenge of atopic asthmatics but not in normal individuals.

See also: Allergens; Anaphylatoxins; Basophils; Degranulation; Delayed-type hypersensitivity; Exocytosis; Mast cells; Lymphocytes; Macrophage activation; Natural killer (NK) cells.

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SERUM SICKNESS

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Serum sickness is a type III hypersensitivity reaction that develops after exposure to xenogeneic proteins. It was first described by Von Piquet and Schick in 1905, who noted the development of rash, fever, lymphadenopathy and arthralgia in some recipients of xenogeneic sera – horse serum for diphtheria. The pathogenesis of this serum sickness was attributed to the host's immunological reaction to injected antigens and has been described as a generalized Arthus reaction.

Serum sickness was proclaimed as an important experimental model of human immunological disease. In the 1950s several elegant animal studies using radiolabeled serum proteins conclusively demonstrated the association of the pathological lesions of serum sickness with the detection of circulating immune complexes. Circulating and tissue-bound immune complexes form at the time of immune elimination: for a number of factors determine whether circulating immune complexes are deposited in tissues rather than cleared by the reticuloendothelial system. These factors include vascular permeability, local hemodynamic flow, size of complexes and affinity of the antibody. Once deposition takes place, there is activation of the complement cascade and

release of chemotactic factors with resultant leukocyte infiltration which results in a characteristic inflammatory response in the target organ.

Experimental models of serum sickness

Acute serum sickness

This is the classical serum sickness model and is also known as acute immune complex disease. It is normally induced by a single intravenous injection of xenogeneic protein – most commonly bovine serum albumin (BSA) (250 mg kg⁻¹) into rabbits. Antibody is formed and binds to circulating antigen to form immune complexes. These complexes are observed in the sera and from day 10 there is no detectable antigen in the circulation. The events occurring in the 2 weeks following radiolabeled antigen administration are depicted in **Figure 1**.

Characteristic histopathological lesions are described in acute serum sickness; vasculitis with prominent neutrophil infiltration is seen in large and medium-sized vessels and endocarditis is observed in the cardiac valves. The type of lesions observed in the glomeruli of the kidney are dependent on the size of the immune complexes. In conditions of antigen

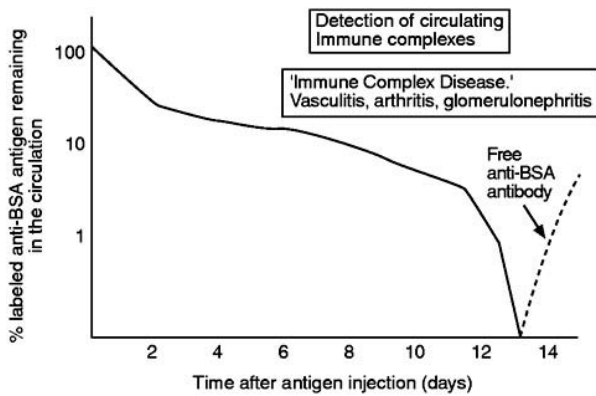


Figure 1 A graph showing events occurring in the 2 weeks following radiolabeled antigen administration.

excess, complexes are small and deposit in the subepithelial space; when complexes are large they deposit in a subendothelial location. There is an associated monocyte macrophage infiltration which is unique to the renal lesion. Proteinuria normally accompanies the glomerular lesion.

The mediation of injury in serum sickness appears to involve several mechanisms. Vasoactive amine-induced increase in vascular permeability appears to be of initial importance as is the infiltration of neutrophils in the vascular lesions. Macrophage depletion and interruption of the terminal complement components are capable of preventing nephritis. Cytokines, in particular tumor necrosis factor α (TNF α) and interleukin 1 (IL-1), have been shown to play a role in the activation of endothelial and mesangial cells and in leukocyte recruitment.

Chronic serum sickness

This model was developed as the prototype of human immune complex glomerulonephritis; it does not feature the vascular lesions of acute serum sickness. Repeated antigen injection (again normally in rabbits) creates a state of antigen excess and maintains immune complexes in the circulation. The main site of immune complex deposition is the kidney; joints, arteries and skin are rarely involved. The type of glomerulonephritis that develops is dependent on the antigen dose, antibody affinity and the resultant size of immune complexes. In states of antigen excess and low antibody affinity, complexes are small and the renal lesion is characteristic of membranous glomerulonephritis or severe endocapillary proliferative nephritis. With intermediate and high-affinity antibodies the complexes are large and insoluble and deposit in the mesangium with some juxtaglomerular subendothelial deposition. The glomerulonephritis associated with this is a mild mesangial proliferative nephritis.

If the antigen is chemically modified to impart a cationic charge, glomerular immune complex deposition and rapid onset of glomerulonephritis occurs without relative equivalence of antigen and antibody. *In situ* immune complex formation in the glomerulus is likely to be important here. Interestingly, heparin has been shown to enhance the removal of immune complexes, an action not mediated by its anticoagulant properties, but most likely related to its charge.

Serum sickness in humans

With the decline in use of heterologous serum products since the beginning of this century, there has been a marked reduction in the incidence of serum sickness in humans. Current heterologous proteins still in use are antithymocyte globulin and antivenom which carry with them a dose-dependent risk of serum sickness. Reactions resembling serum sickness have been associated with various nonprotein drugs, in particular cefaclor, β -lactam antibiotics, streptokinase and β blockers. This serum sickness-like illness has been postulated to be due to drug-related hapten antibodies, but this has never been proven. Other plausible alternatives are direct toxicity to the vessel wall, development of autoantibodies to endothelial cells and cell-mediated cytotoxicity. Most cases of drug-induced serum sickness are not associated with hypocomplementemia, which is a feature of classic serum sickness.

The typical clinical presentation of serum sickness in humans is seen 8–14 days after antigen exposure unless sensitization has occurred. The commonest clinical presentation is the development of fever, lymphadenopathy and morbilliform rash which normally starts in the extremities. Urticaria may occur, but is less common. Fifty per cent of cases have associated visceral involvement of which arthralgia and arthritis are the most common. Significant systemic vasculitis and glomerulonephritis are uncommon although transient abnormalities are often noted on urinalysis. Classically there is depletion of complement; investigations reveal low C3 and C4 levels.

Treatment involves withdrawal of the suspected antigen, symptomatic relief with antihistamines, analgesics and steroids if indicated. Effective treatments in animal studies include antioxidants, cyclosporine and plasmapheresis; these have not been subject to clinical trials in humans.

See also: Heterophile antibodies; Hypersensitivity reactions; Immune complexes.

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SEVERE COMBINED IMMUNODEFICIENCY

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Severe combined immunodeficiency (SCID) is the most devastating form of the human primary immunodeficiencies, characterized by the absence of both cellular and humoral immunity. Although the phenotype is similar, genetical heterogeneity has been observed in the group of patients and several genes causing SCID have recently been identified.

The mode of inheritance may be X-linked recessive (X-SCID) or autosomal recessive (AR-SCID). X-SCID is caused by mutations of the IL-2R γ chain (common γ chain, γ c). AR-SCID are reported to be caused by mutations of various genes, including JAK3, RAG-1/RAG-2, adenosine deaminase (ADA) and purine nucleotide phosphorylase (PNP). In addition, HLA class II deficiency caused by the defects of *trans*-acting regulatory molecules for the expression of class II, and T cell receptor–CD3 signaling deficiency caused by defective CD3 γ , CD3 ϵ , and ZAP-70, are also included in the category of AR-SCID. The approximate percentages of SCID categories classified on the basis of genetic etiology are shown in Figure 1.

In this article, we give an overview of the general symptoms and treatment of human SCID, and describe the various SCID entities on the basis of the responsible genes. In addition, we will briefly discuss mice with the SCID phenotype, including spontaneously derived SCID mice, RAG-1/RAG-2 targeted mice, JAK3 targeted mice, and common γ chain targeted mice.

Clinical phenotype of human SCID

As expected from the severe defects affecting both cellular and humoral immunity, SCID patients commonly have persistent and recurrent infections. Patients with SCID often present with oral candidiasis, diarrhea, otitis, sepsis, cutaneous infections and

pneumonia. Failure to thrive is observed secondary to chronic infection, malabsorption and diarrhea. Patients often suffer from infections by opportunistic microorganisms including *Pneumocystis carinii*, *Cryptosporidia* and *Candida albicans*. Herpes virus, adenovirus and cytomegalovirus often cause lethal systemic infections. Live vaccines such as BCG, oral polio vaccine, and varicella vaccine are contradicted in patients with SCID. Since SCID patients lack the ability to reject foreign tissue, chronic GVHD (graft-versus-host disease) caused by maternal T cells that cross the placenta may occur in affected infants. Similarly, nonirradiated blood transfusions can cause GVHD.

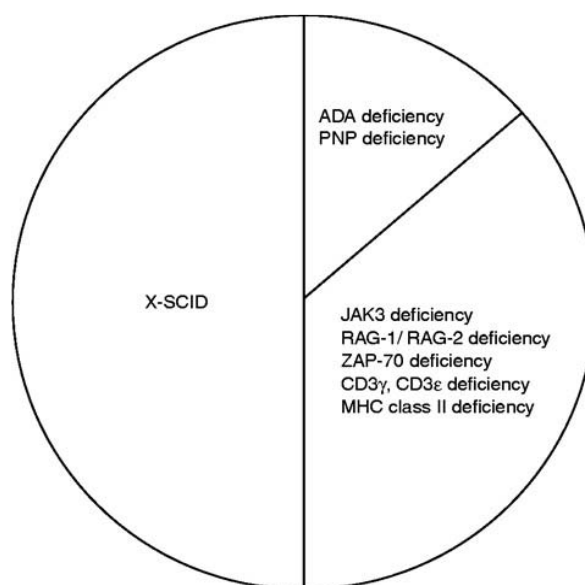


Figure 1 Classification of human SCID.

X-SCID

The gene that causes X-SCID has been mapped to Xq13 by linkage studies. The cloning of IL-2R γ chain (common γ chain, γ c) and the localization of this gene to Xq13 led to the hypothesis, subsequently confirmed, that mutation of γ c causes X-SCID. γ c is a subunit of the IL-2 receptor which consists of an α , β and γ chain, and is shared by IL-4R, IL-7R, IL-9R and IL-15R (Figure 2). Thus, γ c chain plays an important role in the signal transduction of multiple cytokine receptors. Subsequent studies of the γ c gene confirmed that all patients with X-SCID have mutations within the γ c gene. Approximately 100 unique mutations of γ c have been reported and registered in the worldwide database. They include missense and nonsense mutations, deletions, insertions and splice site mutations and affect all domains of γ c. The missense mutations of the extracellular domain of γ c result in impaired binding of IL-2. Mutations affecting the four cysteine residues that are conserved in the cytokine receptor family and the mutations affecting the WSXWS motif disrupt the configuration of the γ c chain. Missense mutations at the transmembrane domain cause unstable expression of the γ c chain either by the replacement of hydrophobic amino acids or the disruption of anchoring residues. Mutations within the intracellular domain affect the signal transduction pathway of IL-2R. Deletion of 62 amino acid residues at the C-terminal region of γ c caused a severe phenotype of X-SCID. A missense mutation at amino acid residue 271 (L271Q) interferes with the association of γ c and JAK3 (Figure 3).

Although T cells and natural killer (NK) cells are absent in X-SCID patients, B cell numbers in the peripheral blood are normal or elevated. The phenotypes of B cells are relatively immature and the diversity of Ig rearrangement is limited. This could be caused by the lack of appropriate T cell help or by the fact that γ c is shared by IL-2R, IL-4R, IL-7R, IL-9R and IL-15R, resulting in a lack of B cell activation by cytokines. B cell function after allogeneic bone marrow transplant (BMT) of patients not undergoing conditioning is known to be impaired when

major histocompatibility complex (MHC)-matched normal donor-derived T cells but not B cells were engrafted. Immunoglobulin (Ig) rearrangements of recipient-derived B cells are often limited.

γ c-deficient mice made by gene targeting show defective lymphocyte development. However, in contrast to human X-SCID, the number of mature B cells is decreased and CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes are present, although the absolute numbers are low. The discrepancy between the phenotype of γ c-deficient mice and that of human X-SCID indicates that the requirement of cytokines during lymphocyte development is different between human and mouse. The difference can be explained in part by the observation that IL-7 is indispensable for murine but not human B cell development. IL-7 receptor knockout mice show severe reduction of both T cells and B cells. The exact role of cytokines for human lymphocyte development has not been determined. *In vitro* culture systems that allow the maturation of functional B cells and T cells from human lymphoid stem cells will facilitate our understanding of defective lymphoid development in X-SCID.

JAK3 deficiency

JAK3 is a member of the Janus family kinases (JAK), which include JAK1, JAK2, JAK3 and Tyk2. These kinases are known to play important roles in the signal transduction via cytokine receptors, and transmit signals by binding to STAT (signal transducers and activators of transduction) family proteins. Of the JAK family kinases, JAK3 is the only kinase whose expression is limited to hematopoietic cells and is associated with γ c. In addition, mutations of γ c in the position that associates with JAK3 have been identified in patients with X-SCID. These observations suggested the possibility that AR-SCID might be caused by a defect of JAK3. This hypothesis was confirmed when female SCID patients were found to have mutations in both JAK3 alleles. The phenotypes of immunodeficiency observed in these patients are similar to those observed in X-SCID. B cells are present in normal or elevated numbers, while T cells

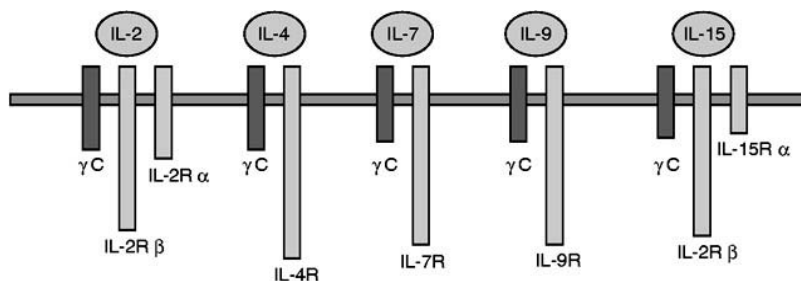


Figure 2 Structure of cytokine receptors sharing the common γ chain (γ c).

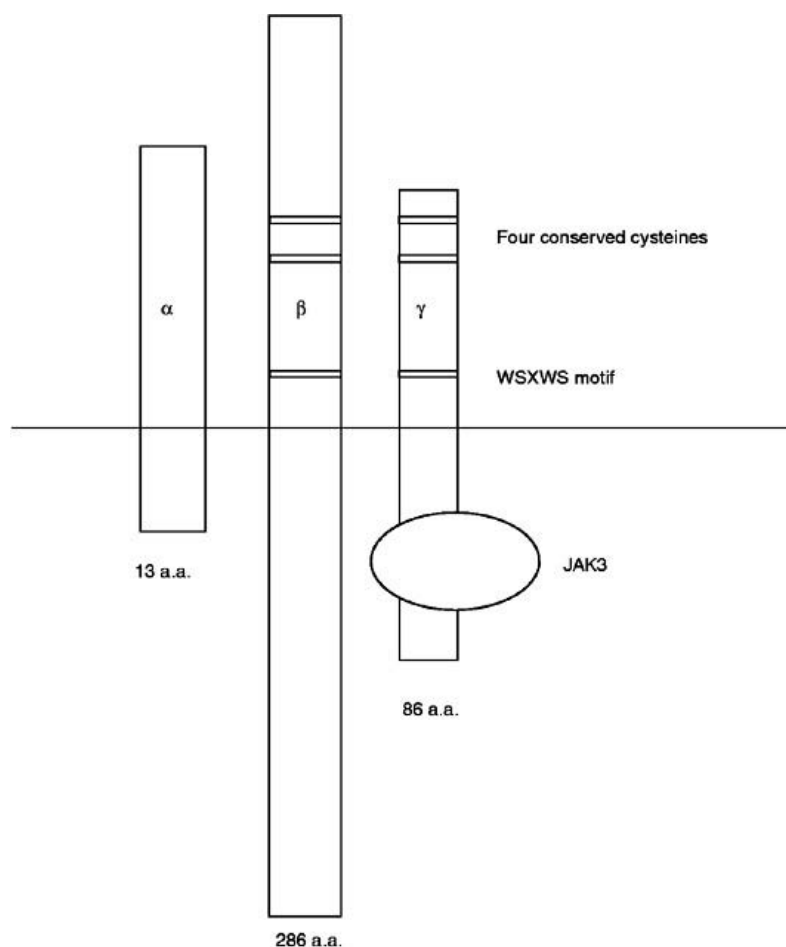


Figure 3 Structure of the IL-2 receptor and the binding site of JAK3.

and NK cells are absent. JAK3-deficient B cells are functionally defective. STAT6 could not be activated by IL-4 in JAK3-deficient B cells, suggesting the indispensable role of JAK3 in signal transduction from the IL-4 receptor to STAT6. These findings are consistent with the hypothesis that the signal transduction pathway from γ c to JAK3 is linear and that there is a direct association of these two molecules (Figure 3).

JAK3-deficient mice made by gene targeting have demonstrated abnormalities of lymphoid development identical to those described in γ c-deficient mice.

RAG-1/RAG-2 deficiency

RAG-1/RAG-2 is a recombinase activating protein that plays an important role in the initiation of V(D)J gene rearrangement of both the T cell receptor (TCR) and B cell receptor (BCR). Mice deficient in RAG-1 or RAG-2 made by gene targeting were found to show complete absence of both mature T cells and B cells. In human AR-SCID, RAG-1 and/or RAG-2 mutations were identified. The characteristic of this

form of SCID is absence of B cells, which is similar to RAG-1/RAG-2 knockout mice. The recombination frequency found in patients with RAG-1/RAG-2 mutations was decreased if determined in human 293 fibroblasts by the extrachromosomal V(D)J assay.

ADA deficiency and PNP deficiency

Adenosine deaminase (ADA) is an amino hydrolase that deaminates adenosine to inosine. Absence of ADA results in the accumulation of toxic metabolites including deoxy-ATP interfering with DNA synthesis, causing lymphopenia and SCID. ADA deficiency has been observed in approximately 15% of patients with AR-SCID. Although most patients with ADA deficiency demonstrate severe reduction of T cells, B cells and NK cells, T cells are more sensitive to the toxic metabolites than other lymphocyte subpopulations.

Purine nucleoside phosphorylase (PNP) is an enzyme that converts inosine to hypoxanthine. PNP deficiency results in the accumulation of toxic meta-

bolites including deoxy-GTP. Generally, the effect of PNP deficiency on lymphocyte number and function is less severe than that seen in ADA deficiency.

ZAP-70 deficiency, CD3 γ deficiency and CD3 ϵ deficiency

ZAP-70 (zeta-associated protein 70) is a non-src family protein kinase which associates with phosphorylated CD3 ζ chain, and plays an important role in TCR-CD3 complex signaling (Figure 4). In patients with ZAP-70 deficiency, CD8 $^{+}$ T cells are almost completely absent while CD4 $^{+}$ T cells and B cells are present. Thymocytes expressing both CD4 and CD8 (double positive) are present while CD4 $^{+}$ CD8 $^{-}$ thymocytes are absent, indicating that ZAP-70 is indispensable for the development of CD8 single-positive cells. Lack of ZAP-70 may be partly compensated by the presence of syk, allowing the development of CD4 $^{+}$ T cells but not CD8 $^{+}$ T cells. However, CD4 $^{+}$ T cells fail to respond to anti-CD3, mitogens or allogeneic cells *in vitro*, indicating defective signal transduction. NK cell activity is normal.

Mice with defective ZAP-70 lack both CD4 $^{+}$ and CD8 $^{+}$ T cells in peripheral lymphoid organs. This observation suggests that the requirement of ZAP-70 and syk for T cell development is different between mice and human.

A few patients with CD3 ϵ deficiency and CD3 γ

deficiency were reported, demonstrating the importance of these components of CD3 for TCR signal transduction (Figure 4). This is further supported by the observation that T cells from these patients proliferate poorly to anti-CD3 stimulation.

MHC class II deficiency

MHC class II deficiency is a rare primary immunodeficiency disorder characterized by defective expression of MHC class II antigens. Patients with MHC class II deficiency have a severe defect of cellular and humoral immune responses. CD4 $^{+}$ T cells are often decreased, while CD8 $^{+}$ T cells and B cells are present in normal numbers. In spite of the presence of B cells, serum immunoglobulin concentrations of one or more isotypes are decreased in the patients. The hypogammaglobulinemia observed in MHC class II deficiency seems to be caused by the lack of appropriate T cell help and/or by the defective activation of B cells, since class II molecules play important roles in antigen presentation to T cells and transduce activation signals into B cells. Specific antibody responses are severely impaired.

This deficiency is caused by abnormal *trans*-acting proteins that regulate the transcription of the MHC class II gene. At least four complementation groups (A, B, C and D) have been identified by cell fusion experiments. The genetic complementation approach

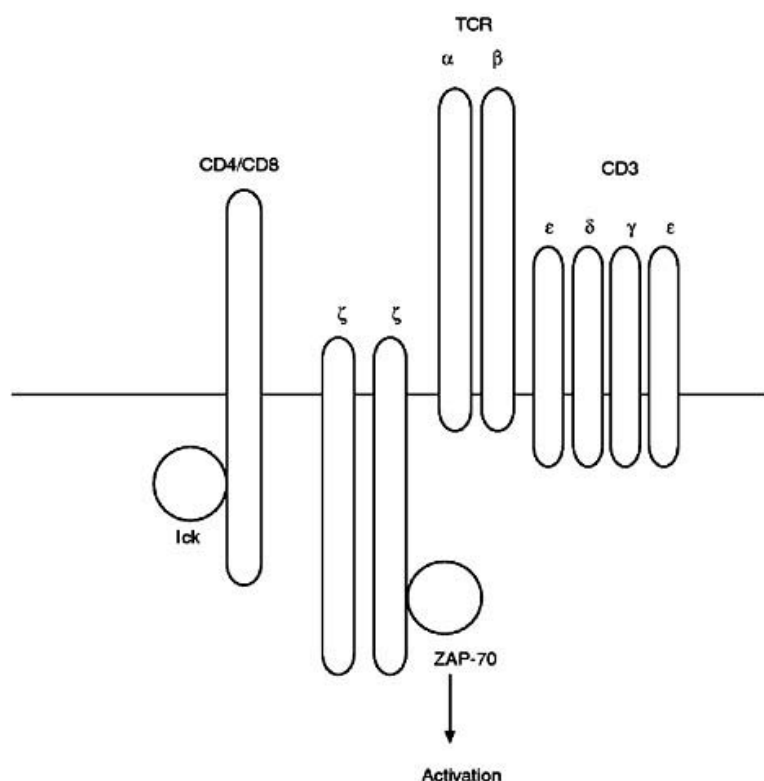


Figure 4 Association between the TCR-CD3 complex and ZAP-70.

has led to the cloning of MHC class II transactivator (CIITA), X box binding factor regulatory factor X (RFX5), and RFXAP genes. Group A is caused by mutations of the CIITA gene, group C is caused by mutations of RFX5 and group D is caused by mutations of RFXAP. The gene responsible for group B has not yet been identified. Some patients with MHC class II deficiency also have decreased expression of MHC class I for unknown reasons.

SCID mice

Mice with SCID mutation lack the appropriate rearrangements of TCR and Ig, which results in the absence of mature T and B cells. SCID mice also show abnormal sensitivity to ionizing radiation, which causes DNA double-strand breaks. A recent report suggests that murine SCID cells lack a DNA-dependent protein kinase (DNA-PK_{cs}), which is involved in the repair of DNA double-strand breaks during V(D)J rearrangement. Defective DNA-PK_{cs} has not been demonstrated in human SCID.

Treatment of patients with SCID

Patients with SCID have a poor prognosis and in most cases do not survive more than a few months without appropriate treatment. Supportive treatment with intravenous immunoglobulin infusions at regular intervals, and prophylaxis for *Pneumocystis carinii* is indicated. Bacterial or fungal infections are treated with intravenous infusions of antibiotics or antifungal agents. Epstein-Barr virus, herpes virus and cytomegalovirus infection may result in systemic and lethal disorders and should be treated with antiviral reagents. Prophylactic infusions of immunoglobulin with high titer for cytomegalovirus has been advocated. For ADA deficiency, enzyme replacement with polyethylene glycol-modified ADA (PEG-ADA) resulted in limited but significant improvement of the immunodeficiency.

To reconstitute the impaired immune system in SCID, bone marrow transplantation is the treatment of choice. If successfully engrafted, BMT is a curative treatment, although incomplete reconstitution of the immune system and post-transplant lymphoproliferative disorders caused by Epstein-Barr virus are major problems that need to be solved. To improve engraftment, various conditioning regimens including chemotherapy, total body irradiation, and anti-lymphocyte globulin (ALG) have been tried. HLA-haploidentical marrow from a parent can be used for BMT, although T cell depletion is necessary to prevent GVHD. Recently, a successful *in utero* transplantation of parental CD34⁺ hematopoietic progenitor cells into an X-SCID fetus has been reported.

Gene therapy has been attempted in patients with ADA deficiency. Two patients repeatedly received infusions of autologous T cells transduced with the ADA gene inserted in a retroviral vector. Circulating T cells producing ADA were detected, and a possible improvement of the immune responses were observed, including increased numbers of T cells, appearance of T cell cytotoxicity, positive delayed-type hypersensitivity skin tests, and proliferative responses to antigen by specific T cells. The fact that these patients also received PEG-ADA makes the interpretation of these findings difficult. Although many technological breakthroughs are needed, gene therapy has the potential to become a curative therapy for some primary immunodeficiencies whose genetic bases have been identified.

Concluding remark

Although the clinical symptoms of SCID patients are largely similar, they represent genetically heterogeneous disorders. SCID patients can be phenotypically classified into B⁻SCID and B⁺SCID. Approximately half of patients with B SCID are caused by RAG-1/RAG-2 deficiency, the genetic basis of the other half is unknown. Seventy per cent of all SCID cases are B⁻, and several genes that cause B⁺SCID have been identified. Selective lack of CD8⁺ T cells is associated with ZAP-70 deficiency. The presence or absence of NK cells also varies among the different types of SCID. Thus, a phenotype/genotype correlation exists in SCID. The functional analysis of proteins encoded by the affected genes will further clarify the pathogenesis of SCID.

See also: CD3; Cytokine receptors; Diversity, generation of; Gene therapy; Hematopoietic stem cell transplantation; HLA class II; Hu-SCID mice; Immunodeficiency, animal models; Immunodeficiency, primary; Interleukin 2 receptor; Interleukin 4; Interleukin 7 and its receptor; Interleukin 9 and its receptor; Interleukin 15 and its receptor; Opportunistic infections.

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SEX HORMONES AND IMMUNITY

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In addition to their profound effects on sexual differentiation and reproduction, sex steroids also influence the immune system's function. The effects of these hormones on the immune system initially attracted the attention of investigators working on autoimmunity, as a sexual dimorphism exists in human autoimmune diseases – that is, one gender is often more susceptible than the other. Although the precise mechanisms through which sex steroids interact with the immune system remain unknown, we do know that these hormones can act directly on certain of the system's cells, or indirectly either through cells controlling the immune system's growth and development or through organs eventually destroyed by autoimmune diseases. Understanding such mechanisms will allow new insight into autoimmune diseases and also into the normal development and function of the immune system and its responses to challenge.

Autoimmunity

In certain individuals, the immune system fails to recognize one's healthy tissue as self, and a humoral and/or cellular immune response develops, leading to the afflictions collectively known as autoimmune diseases. Historically, women have suffered from a greater (up to ninefold) prevalence of many autoimmune diseases. Such female-predominant diseases include systemic lupus erythematosus (SLE), rheuma-

toid arthritis, primary biliary cirrhosis and Graves' disease. This gender preference makes sex steroids an obvious starting point for investigations into the issue. Yet, despite clear-cut results in a number of laboratories, our knowledge of how sex steroids influence the development of autoimmune disease remains largely conjectural.

There are two well-studied, sexually dimorphic autoimmune diseases in laboratory mice modeling SLE and diabetes. In both, the manipulation of sex steroids profoundly influences the course of disease. The first example involves offspring (F1 hybrids) of NZB and NZW strain mice. Although almost all females from this NZB/NZW breeding die of a lupus-like disease within the first year of life, tenfold fewer males die of disease. However castration of NZB/NZW males causes them to suffer a similarly high death rate, and treatment with the male hormone androgen (testosterone or dihydrotestosterone) protects female mice from early fatality. In this experimental model of human lupus, the thymus is an important participant in androgen-induced protection. The second example is the NOD mouse strain, which suffers a high incidence of insulin-dependent diabetes mellitus. By 6 months of age, approximately 80% of NOD strain females are diabetic, whereas only 20% of the males have obvious disease. After castration, these male mice undergo a significant increase in the incidence of diabetes, an effect heightened by removal of the thymus. As in

the NZB/NZW hybrid mice, NOD females given androgen do not get the disease. However, when the androgen-treated females are injected with spleen cells from diabetic animals, diabetes develops. Therefore, the hormone is likely acting on immune cells, not the pancreas – the organ attacked by this disease.

Immune interactions

Studies such as these indicate that sex steroids can influence an abnormally functioning immune system or contribute to the cause of its abnormalities. Yet, the effect of these steroids on the normal immune system is less clear. Still, a few hints have come from studies of humans as well as experimental animals. For example, female mice can produce substantially more antibody in response to particular antigens than their male counterparts; however, humans inoculated with vaccines have virtually identical antibody responses, whether female or male. Also, in mice, cell-mediated responses to certain antigens were notably stronger in females than in males. When researchers mimicked the conditions of pregnancy by combined estrogen and progesterone treatment in mice, the result was a lowered production of B cells by the bone marrow. Additionally, antigen presentation in the uterus and spleen of female rats is highest at proestrus, when estrogen levels peak, and estrogen treatment of oophorectomized rats increases antigen presentation by uterine epithelial cells. Additional studies on the female reproductive tract in mice have shown that during the luteal phase, when there are high levels of progesterone, the number of dendritic cells increases in the vaginal epithelium.

Sex steroids can also profoundly affect the thymus. Studies of rodents and other animals indicate that administration of the hormones androgen or estrogen result in thymic involution, whereas castration leads to thymic enlargement in comparison to gonadally-intact animals. During pregnancy, marked thymic involution occurs, with recovery to normal size and shape after delivery, perhaps caused by markedly elevated levels of progesterone, although other factors could be responsible.

Compared to rodents, primates have fewer reported gender- and hormone-related differences. However, two differences are notable in the human immune system: women have higher levels of plasma immunoglobulin M (IgM) than men and also an elevated CD4:CD8 T cell ratio in the peripheral blood, due to an increase in the absolute number of CD4⁺ lymphocytes. Interestingly, in a nonhuman primate (rhesus macaque) model of acquired immune deficiency syndrome (AIDS), progesterone treatment

increased the number of animals infected by vaginally inoculated simian immunodeficiency virus (SIV) (the monkey homolog of human immunodeficiency virus, HIV), and the female animals so treated developed a more rapid disease course. Although the increased percentage of infected animals may be due to a progesterone-induced thinning of the vaginal epithelium and/or effects on dendritic cells, which play an important role in HIV infection, the nature of the accelerated disease course is unknown.

Targets of action

The potential targets of sex steroids are numerous. The studies cited above point to potential effects on sites where the precursors of immunity originate and differentiate (bone marrow, thymus), as well as on organized lymphoid tissue, circulating immune cells or organ-resident cells. To clarify where the sex steroid hormones have possible direct or indirect effects, lymphoid organs and cells of the immune system have been examined for the presence of specific receptors for sex steroids. However, pursuing this objective has been problematic, in general, because sex steroid receptors are relatively scarce on lymphoid cells compared to more traditional targets of sex steroid activity, such as the reproductive tract or breast. Still, the thymus clearly contains receptors for androgen, progesterone and estrogen. Although the cellular location of these estrogen receptors remains unclear, androgen receptors are present in immature thymocytes, and progesterone receptors are expressed in epithelial cells; it is possible that additional thymic cell types also express these receptors. In the major categories of mature immune cells, it appears that CD8⁺ T cells can express estrogen receptors and that macrophages express receptors for estrogen and also androgen. Two additional sites, endothelial cells and bone marrow stromal cells, also express estrogen receptors, and interactions with these cell types affect the immune system.

Cytokines

Given the prime importance of cytokines in the development, regulation and effector functions of immune reactions, numerous investigators have examined how sex steroids control cytokine genes. Additional impetus to perform such studies stems from experiments revealing gender differences in cytokine production by mice given experimental immune challenges. In one such study, 3 weeks after BCG sensitization, mice were challenged with purified protein derivative (PPD) – constituting a strong antigenic challenge. Four hours later, the resulting

immune response to this challenge was that serum from female mice contained 2.5–4-fold more interferon γ (IFN γ), an immunostimulatory cytokine produced mainly by T cells, than did sera from male mice. A second experimental protocol examined cytokine production four hours after lipopolysaccharide (LPS) administration, which induces a septic shock-like syndrome and proinflammatory cytokine expression. Plasma from female mice contained 4–5-fold more interleukin 1 (IL-1), and 2.5-fold more IL-6, than did plasma from male mice, although equivalent amounts of tumor necrosis factor α (TNF α) were produced. Since LPS is thought to first induce TNF α in macrophages, which then helps stimulate production of IL-1 and IL-6 production in macrophages, this equivalence of TNF α levels indicates that the effect may occur in a common pathway for IL-1 and IL-6 production, such as receptor signaling by TNF α , gene transcription, protein processing or protein release. Finally, androgen treatment of castrated mice leads to increased transforming growth factor β (TGF β) production in the thymus, potentially contributing to androgen-induced thymic involution.

In addition to establishing these gender-based differences in living animals, the role of sex steroids has

been examined directly *in vitro*, using cells from the immune system and elsewhere. When lymph node cells from mice were stimulated by anti-CD3 antibody then treated with androgen, the result was decreases in IFN γ , IL-4 and IL-5 production. However, in a separate study, androgens induced IL-5 expression in lymphoid cells. Elsewhere, estrogen enhanced IFN γ production by stimulated mouse spleen cells. Estrogen also enhanced IFN γ and IL-10 production by clones of CD4 $^{+}$ human T cells, and IL-1 production by macrophages. IL-6 production by bone marrow stromal cells was inhibited by estrogen, an effect with important implications for osteoporosis, considering the stimulatory effect of IL-6 on osteoclasts (macrophage-like cells that resorb bone) and the decreased estrogen content of postmenopausal women. A second sex steroid-regulated cytokine is also likely to be important in osteoporosis, as in osteoblasts (bone-forming cells), estrogen stimulates TGF β production, and TGF β can induce apoptosis (programmed cell death) of osteoclasts. Molecular studies of estrogen's ability to regulate cytokine gene expression have revealed this hormone's stimulatory influence on the IFN γ and TGF β promoter, and inhibitory effect on the IL-6 promoter.

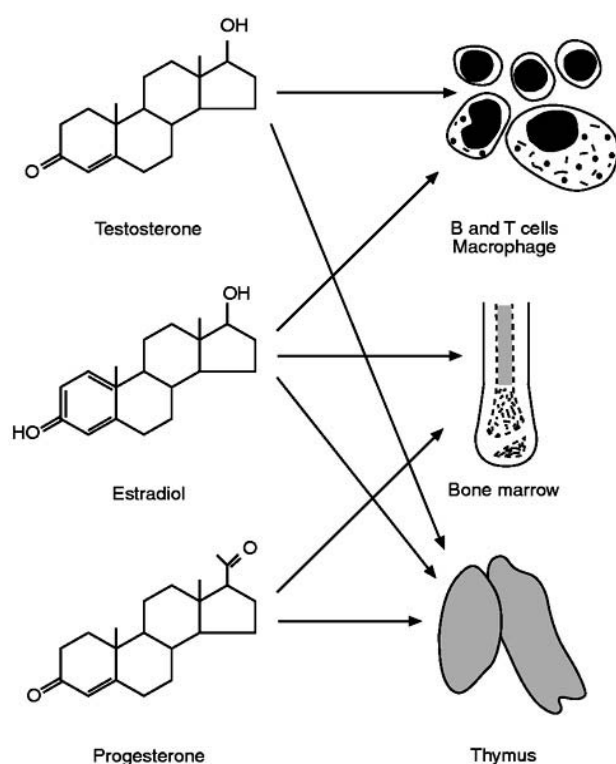


Figure 1 Immune organs/cells known to be affected by sex steroids. These hormones can act during the development of immune cells, as well as directly on mature effector cells.

Table 1 Some of the effects of sex and/or sex steroids on immune system properties or molecules

Parameter	Sex/sex steroid effect
CD4 $^{+}$ T cells	Higher in women
IgM levels	Higher in women
B cell production	Decreased by estrogen plus progesterone
Autoimmune diseases	Higher in women Ameliorated by androgen in mice
SIV infection	Enhanced by progesterone in monkeys
Thymic size	Decreased by estrogen or androgen
IL-1	Higher in female mice Increased by estrogen in macrophages
IL-6	Higher in female mice Decreased by estrogen in bone marrow stromal cells
IL-10	Increased by estrogen in T cell clones
IFN γ	Higher in female mice Increased by estrogen in T cell clones Increased by estrogen in spleen cells
TGF β	Decreased by androgens in T cells Increased by androgen in thymus Increased by estrogen in osteoblasts

Perspectives

Clearly, sex steroid hormones are capable of affecting the development and function of a variety of immune cells (Figure 1 and Table 1). With our increasing knowledge of immune system development and function, testing of hypotheses regarding the mechanisms of sex steroid actions should be increasingly fruitful. Understanding the etiology of these differences may help in the development of therapies for a variety of conditions where the immune system's actions are critical, such as in transplantation and autoimmune and infectious diseases.

See also: Autoimmune disease, pathogenesis; Autoimmune disease, spontaneous experimental models; Autoimmune diseases; Autoimmunity; Cytokine genes, regulation of; Cytokines; Insulin-dependent diabetes mellitus, animal models; Neuroendocrine regulation of immunity; Systemic lupus erythematosus, experimental models.

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SHIGELLA, INFECTION AND IMMUNITY

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Characteristics of the organism and its antigens

Shigella species are gram-negative bacteria of the family Enterobacteriaceae. They cause watery or bloody diarrhea, or the dysentery syndrome and occasionally lead to systemic complications, such as

the hemolytic-uremic syndrome, leukemoid reactions, and autoimmune phenomena such as reactive arthritis. *Shigella* are pathogens only for humans and primates with a low ID₅₀ of a few hundred to a few thousand organisms. Ubiquitous in distribution, they principally cause illness in children, especially where the infection is endemic, suggesting immunity is

acquired. Less commonly, shigellosis occurs in non-immune adults. Severe persistent bacteremic disease has been documented in immunocompromised patients with the acquired immune deficiency syndrome (AIDS).

Shigella invade the colonic mucosa, a step essential for virulence, and divide and spread from cell to cell within the intestinal epithelial cell layer. This process requires both chromosomal and plasmid genes, many of which have now been determined. Cytokines are produced, neutrophils recruited and there is an intense inflammatory response. The most characteristic lesions involve inflammation and ulceration of the colonic mucosa, resulting in the clinical presentation of dysentery, characterized by both blood and pus in the stools of infected patients.

There are four species of *Shigella*: *S. dysenteriae* (group A, 10 types), *S. flexneri* (group B, 6 serotypes and 14 subtypes), *S. boydii* (group C, 18 serotypes), and *S. sonnei* (group D, 1 serotype and 20 colicin types). *Shigella* are nonmotile and generally nonencapsulated. Like other Enterobacteriaceae, they display O (somatic) and K (capsular) antigens. Accurate serotyping of *Shigella* isolates using specific anti-O antigen antibody is best done after heating test organisms to denature K antigens which can obscure the seroreactivity of O antigens. Many immunologically defined *Shigella* O antigens are shared with serotypes of *Escherichia coli* or other gram-negative bacilli. Specific antibody responses occur to *S. dysenteriae* 1 (the only member of the genus to produce Shiga toxins), *S. flexneri* 6, and *S. sonnei*; group responses to *S. flexneri* 1–5 also develop.

Immune responses of the host

Despite the major advances in relation to the pathogenesis of *Shigella* infection in recent years there is still a relative paucity of knowledge of the host protective immune responses. Despite the fact that the bacteria appear to be taken up by M cells, the nature and the role of the ensuing immune responses in controlling the infection or in preventing subsequent infections by the same organism are poorly understood. It is reasonable to assume that the immunoglobulin A (IgA) secretory immune response should be protective in shigellosis, although it has been difficult to convincingly demonstrate this in humans. The only well-established principal is that exposure to one serotype generally induces type-specific immunity, such that reinfection occurs only with different *Shigella* species or serotypes. This is presumed to be associated with immune responses to the oligosaccharide structure of the lipopolysaccharide (LPS), and is the basis for a resurgence in interest in LPS

determinants for immunization, even by the parenteral route. The fact that *Shigella* are taken up by M cells may be a mixed blessing in terms of the immune response. While the M cells deliver antigens to underlying lymphoid tissues (Peyer's patches) in the gut, pathogenic *Shigella* also replicate in M cells and may destroy the cells involved in antigen presentation. Invasion of neighboring cells by *Shigella* clearly occurs before an immune response can develop, thereby allowing systemic exposure to *Shigella* antigens and toxins early in the course of the disease.

In the past, parenteral immunization with killed *Shigella* bacterial vaccines or extracts have demonstrated that serum IgG has no protective effect. In contrast, experimental oral vaccination of humans with attenuated *Shigella* produces high degrees of type-specific protection, suggesting that mucosal immunity is key. Indeed, specific intestinal IgA levels increase during *Shigella* infection. In *S. sonnei*-challenged volunteers, IgA-secreting lymphocytes are present in the circulation 8–10 days after infection, probably *en route* to other mucosal sites, such as the mammary glands. Prior infection with *Shigella* results in specific IgA secretion in breast milk, thereby offering some protection to breastfed infants.

Antibody responses to the somatic antigens of *Shigella* develop early in infection and follow the typical course for antibodies to LPS. The initial IgM response peaks within weeks and wanes after 1–2 years. Assays have been developed using specific antigens for the detection of antibodies against O antigen. While such assays are of use in epidemiological studies they are of little value as diagnostic tests. *S. dysenteriae* type 1 LPS has unique O-repeat units shared only with *E. coli* expressing O-1 or O-120 antigens. Since neither *E. coli* O-1 nor O-120 is common among bacterial pathogens, testing for antibody to *S. dysenteriae* 1 LPS is quite specific. *S. flexneri* types 1a–5b contain a tetrasaccharide repeat and extensive cross-reaction occurs amongst them. Despite this, antibodies to *S. flexneri* are so unusual in developed countries that even such a nonspecific immune response can be a useful tool to detect current infection. In contrast, the LPS from *S. flexneri* type 6 is distinctive. The *S. sonnei* antigen is an unusual disaccharide, also allowing specific serodiagnosis.

Shiga toxin, a multimeric protein composed of a single enzymatically active A subunit and five B subunits responsible for toxin binding, is produced only by *S. dysenteriae* type 1 strains and is considered to be an important virulence factor in the pathogenesis of the hemolytic-uremic syndrome, a devastating complication of infection. IgM antibody to Shiga

toxin has been documented both in field and experimental settings. The relative lack of an IgG response is consistent with the observation that toxin binds to CD77⁺ IgG- and IgA-producing B lymphocytes, and not T lymphocytes, and inhibits immunoglobulin synthesis in these cells. Another endogenous B lymphocyte surface protein, CD19, is reported to have sequence homology with Shiga toxin and the cocapping of CD19 and CD77 that occurs in the presence of CD19-specific antibodies is prevented by the prior binding of Shiga toxin B subunit to CD77. The effect of this would be to inhibit B cell activation. There is also some *in vitro* evidence to suggest that the Shiga toxin B subunit induces apoptotic changes in the high CD77-expressing Burkitt lymphoma cells, which could also account for the lack of an IgG and IgA immune response to the toxins. In animal models, however, it is possible to raise serum IgG to Shiga toxin, and such animals are protected against *in vivo* challenge with toxin. The humoral response to LPS or plasmid-encoded protein antigens have been principally implicated in the protection against *Shigella*. Recent work has, however, suggested that T cells become activated *in vivo* following infection with *Shigella*, and that there may indeed be a correlation between the degree of T cell activation and disease severity. This is supported by other recent studies suggesting that the T cell receptor V β repertoire may be skewed in *Shigella* infection toward certain V β types in CD4⁺ and CD8⁺ T cells. Such events would be compatible with an oligoclonal activation of certain T cell subsets, which would be related to the presence of either immunodominant peptides or superantigen-like molecules. Intestinal lymphocytes from Peyer's patches are able to kill *Shigella* when armed with IgA, but not with IgG. Peripheral blood lymphocytes are cytotoxic to *S. flexneri*-infected HeLa cells *in vitro*, especially when stimulated with interleukin 2 or interferon γ .

Local cytokine production may also be important in shigellosis, which is a reflection of the intense inflammatory response in the intestinal mucosa in this disease. Elevated cytokine levels have been detected in stool and plasma of infected patients, particularly interleukin 1 (IL-1), IL-6, tumor necrosis factor α (TNF α) and interferon γ (IFN γ) producing cells in those with severe inflammation. Thus both T_H1 and T_H2 response cytokines are modulated in shigellosis and there may be both a humoral and a cytotoxic defense mechanism involved which could be activated during and after infection. Systemic cytokines may also play a major role in the pathogenesis of the thrombotic microangiopathy which occurs during the hemolytic-uremic syndrome (HUS). Certain cytokines (IL-1 β and TNF α) are

known to increase expression of the Shiga toxin receptor on certain human endothelial cells. The importance of this is that endothelial cells are considered to be the major target of the toxin in HUS. Despite the role of Shiga toxin in HUS, high serum antitoxin titers do not protect against intestinal disease in monkeys challenged with live *S. dysenteriae*. However, antibodies to the toxins do protect animals following parenteral challenge with Shiga toxin.

Postinfectious reactive arthritis has been theorized to be caused by cross-reactivity between bacterial envelope proteins and class I HLA antigens. Reactive arthritis is particularly associated with infection due to *S. flexneri*, which express antigens similar to synovial membrane antigens. The fact that reactive arthritis also occurs following infection with several other enteric bacterial pathogens such as *Yersinia*, *Salmonella* and *Campylobacter* suggests that there may also be some nonspecific component of reactive arthritis pathogenesis associated with mucosal inflammation common to all.

Vaccines

In view of the clinical severity of shigellosis and the ever-changing status of antibiotic resistance there has been a significant effort to develop effective vaccines. The original *Shigella* vaccines were heat- or acetone-killed whole-cell preparations for parenteral administration, designed to induce circulating antibody. These failed to provide significant protection when administered either enterally or parenterally. Attention then focused on secretory immunoglobulin A and mucosal lymphocytes. A major role of secretory IgA bathing a mucosal surface is to prevent attachment of microorganisms to the mucosa, and secondarily to neutralize microbial products such as toxins. It is not certain that either mechanism is relevant to shigellosis, since secretory IgA collected from the gut of rabbits immunized with an invasive *E. coli*-*shigella* hybrid vaccine strain failed to inhibit the invasion of cultured mammalian cells by *S. flexneri* 2a. Current efforts have been focused on the construction of *S. flexneri* live attenuated strains. This has generally taken one of three routes: 1) *Shigella* strains attenuated by a chromosomal mutation, 2) *Shigella* strains attenuated by mutations in plasmid virulence genes, or 3) hybrid *Shigella* or *E. coli* strains expressing cloned *Shigella* virulence determinants. Despite these various approaches none have been very successful as human vaccines either because of instability, failure of protection or reactogenicity. One principal emerging from the various vaccine studies however, is that the ideal vaccine will probably have to be invasive. The noninvasive

candidates generally elicit an inadequate immune sponse.

Several groups have continued to explore the use of killed vaccine preparations. Such preparations include the conjugation of O-specific side-chains of *Shigella* LPS with carrier proteins. The idea is that this would generate such a high serum IgG titer that enough IgG antibody would leak into the lumen to kill organisms at, and within, the mucosa, and that circulating antibody will be bactericidal for organisms gaining entrance to the circulation.

See also: Bacteria, immunity to; Bacterial cell walls; Gastrointestinal tract infections; Molecular mimicry.

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SJÖGREN'S SYNDROME

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Clinical descriptions of patients with dry eyes and a dry mouth associated with swellings of lacrimal and salivary glands began to appear in the medical literature towards the end of the nineteenth century. In all probability a heterogeneous group of disorders such as sarcoidosis and lymphoma was included in these early descriptions, but in 1888 Mikulicz gave a histo-

pathological account of the glands from a patient with the characteristic histological picture of Sjögren's syndrome (SS). In 1933, Sjögren, a Swedish ophthalmologist, published a thesis on patients with dry eyes, dry mouth and a deforming arthritis, mostly without swollen lacrimal or salivary glands. Correlations between dry eyes, a dry mouth and

abundance of round cell infiltration in lacrimal and salivary tissue were noted, as was the association with rheumatoid arthritis in some patients.

Sjögren's syndrome has been defined by a workshop on diagnostic criteria held in Pisa in 1988 under theegis of the Commission of the European Community as a 'chronic inflammatory disorder of tear and salivary glands, reflecting general involvement of exocrine tissues, leading to dry eyes and a dry mouth and its sequelae. It may or may not be associated with systemic features and/or a defined autoimmune disease and connective tissue disease.' The same group subsequently developed diagnostic criteria (the Vitali criteria) which are currently considered to be the best evaluated. They are based on a short questionnaire of 1) ocular symptoms and 2) oral symptoms. Other essential criteria are 3) ocular signs (by Schirmer's test or Rose Bengal staining; 4) lymphocytic infiltrates on lip biopsy; 5) salivary gland involvement (scintigraphy, sialography or decreased salivary flow rate); and 6) demonstration of serum autoantibodies (rheumatoid factors, antinuclear antibodies and/or Ro or La antibodies).

Primary SS can exist with dry eyes and dry mouth as the sole disorder. However, many such patients exhibit extraglandular clinical features which do not in themselves constitute a distinct connective tissue disease and are included in the spectrum of primary SS. These include fatigue, fever, Raynaud's phenomenon, arthralgia, intermittent or chronic synovitis, myalgia, myositis, interstitial pneumonitis, purpuric vasculitis, renal tubular acidosis, chronic liver disease and peripheral neuropathy.

Bunim and Talal in 1963 first reported an increased incidence of lymphoma in SS, and subsequent follow-up at the National Institutes of Health, USA, of a cohort of patients over 8 years led to the conclusion that the risk of lymphoma in SS was about 6.4 cases per thousand patients per year, and represented a 40-fold higher incidence over a control population. Malignant lymphoma needs to be distinguished from pseudolymphoma, which also occurs in SS and is characterized by lymphoproliferation in exocrine tissues and peripheral lymphoid organs, giving rise to lymphadenopathy and splenomegaly but which clinically behaves as a benign disorder. Monoclonal immunoglobulins are detectable in many patients with SS, including those with pseudolymphoma and malignant lymphoma, emphasizing that hidden in the polyclonal B cell response are individual expanded clones which occasionally undergo oncogenic transformation.

The basis of classification into 'primary' and 'secondary' SS has received support from clinical, serological, and immunogenetic studies. For example, in

patients with primary SS, the occurrence of arthritis and immunoglobulin M (IgM) rheumatoid factor simulates diagnostic features of rheumatoid arthritis (RA), but such patients do not develop deforming arthritis with cartilage and bone erosions of the type seen in rheumatoid arthritis. Furthermore, in primary SS antibodies to Ro and La occur in about 50% of patients whereas these antibodies are rare in RA. In Caucasians with primary SS, the human leukocyte antigen (HLA) phenotype is usually HLA-B8, DR3, DRw52, whereas in Caucasian patients with secondary SS and RA, HLA phenotyping usually reveals either HLA-DR4 and/or DRw53.

Patients with primary SS and extraglandular features may, however, be more difficult to distinguish from secondary Sjögren's syndrome associated with systemic lupus erythematosus (SLE) since clinical, serological, and immunogenetic markers overlap considerably. Similarities include the presence of Raynaud's phenomenon, purpura, leukopenia, anti-La and anti-Ro antibodies, hyperglobulinemia, hypocomplementemia, and the presence of HLA-B8, DR3, DRw52 and C4 null genes. However, anti-double-stranded DNA in high titers and anti-Sm antibodies are markers of SLE and are not normally found in the serum of patients with primary Sjögren's syndrome. Features which distinguish SLE from primary SS include an immune complex-mediated diffuse glomerulonephritis, photosensitive rashes, alopecia, pleurisy, pericarditis, hemolytic anemia and thrombocytopenia.

Epidemiology and immunogenetics

Since only a handful of studies have been performed, only tentative conclusions can be drawn about the prevalence of the disorder. Disease prevalence had previously been estimated to be 0.2% of the population, with a female:male ratio of 9:1. However, in more recent studies in elderly populations, the prevalence of SS was estimated to be approximately 2% in the USA, 3.3% in Scotland and 2.7% in Sweden. Since it has been also estimated that 10–15% of the rheumatoid population suffer from SS, it may be predicted that an additional 0.1–0.4% of the population suffer from SS, assuming 1–3% prevalence of RA, giving an overall prevalence of approximately 3%. Thus Sjögren's syndrome may be one of the commonest autoimmune disorders in the general population.

Family studies of patients with SS carried out by Arnett and his associates have revealed a higher than expected prevalence of autoimmune diseases, including SS, RA, SLE and thyroid disease, in up to 30% of relatives, suggesting that genetic factors are

important in susceptibility to disease. Several studies have shown an association with HLA-DR3 and the linked genes B8, DQ2 and the C4 null gene. This haplotype is common in the healthy population (20–25%) and its components are in tight linkage disequilibrium so it has been difficult to identify the component which confers risk of SS. It is now known that the immune response genes are more strongly linked to autoantibody specificity than disease. In West European Caucasian patients with anti-Ro/La antibodies the frequency of DR3 is increased to 60–90% in both SS and SLE. In Black patients with anti-Ro/La antibodies the frequency of DR3 is increased to 80%. One of the first studies which attempted to identify the part of the haplotype associated with anti-Ro and La found that patients heterozygous for HLA-DQw1 and DQw2 had the highest anti-Ro antibody levels. These findings might explain studies of Greek and Israeli patients with primary SS in whom the only significant HLA association was with DR5 as opposed to DR3. This apparent discrepancy can be explained by the fact that the HLA-DQA1 allele is in linkage disequilibrium with both HLA-DR3 and HLA-DR5 haplotypes. Based on a structural analysis of the disease-related alleles Reveille proposed that glutamine at position 34 of the DQA1 chain and leucine at position 26 of the DQB1 chain are important in regulating anti-Ro and La responses. Scofield and Harley confirmed and extended these studies by sequence analysis and showed that the presence of both leucine and glutamine substitutions in a total of four alleles was significantly associated with anti-Ro antibodies.

The etiological role of viruses

In common with other autoimmune diseases, it is believed that polygenic genetic susceptibility factors interact with environmental factors to induce SS. Potential viral triggers include sialotropic and lymphotropic viruses such as the herpesviruses, in particular Epstein-Barr virus (EBV), cytomegalovirus (CMV) and human herpesvirus type 6 (HHV-6). Evidence for involvement of any of these viruses has been conflicting, with some studies finding elevated titers of antibodies to all three viruses in patients with SS but others finding them normal. EBV has been detected in parotid and labial salivary glands by DNA hybridization techniques. Salivary epithelium can contain up to 50 copies of EBV DNA per cell in healthy people without inducing an immune response, suggesting that the gland is an important site of persistence. The extent of EBV infection load in salivary glands in SS is still controversial with conflicting reports. While these inconsistent data may

reflect the different sensitivities and specificity of the methods or a bias introduced by studies based on small numbers of patients, a role for EBV and the other herpesviruses in the induction of Sjögren's syndrome remains unsubstantiated.

Retroviruses are known to infect cells of the immune system and can cause abnormalities of immune regulation including suppression, destruction or stimulation of T cells, excess antibody production and lymphomas. A subgroup of patients with human immunodeficiency virus (HIV) infection develop diffuse infiltrative lymphocytosis (DILS) of salivary glands and other organs with predominantly CD8⁺ T cells. They may present with a clinical picture almost indistinguishable from primary SS. In addition there is a high frequency of extraglandular disease including lymphocytic interstitial pneumonitis, lymphocytic hepatitis, lymphocytic interstitial nephritis and lymphocytic aseptic meningitis. In spontaneously occurring SS retroviral A-type particles were identified in lymphoblastoid cells cocultured with homogenates of salivary glands from SS patients but there has not been any further information such as sequence data to characterize the virus. A number of studies have detected antibodies to HIV- or human T lymphotropic virus type I (HTLV-I)-related *gag* sequences but these appeared to be broadly cross-reactive rather than indicating the presence of a specific retrovirus.

Animal models provide further support for the role of retroviruses in SS. Mice with the murine acquired immune deficiency syndrome (MAIDS) caused by the murine leukemia virus develop lymphocytic infiltrates of their salivary glands and other organs. The HTLV-I *tax* transgenic mouse develops a sialadenitis characterized by focal proliferation of ductal epithelial cells within the major and minor salivary glands followed by lymphocytic infiltration. Recently, a possible role for HTLV-I *tax* has been suggested in human disease. Using polymerase chain reaction (PCR) and *in situ* hybridization techniques, Mariette and coworkers detected HTLV-I *tax* but not *gag*, *pol* or *env* genes in labial salivary gland sections from two of nine patients with SS and none of nine control subjects. A Japanese study has found very similar results. Using PCR they found expression of HTLV-I *tax* gene without *pol*, *gag* or *env* in four of 14 patients with SS. In this case confirmation of identity was provided by sequence data which showed 100% homology between the nucleotide sequences from the four SS patients and that of the HTLV-I pXIV gene derived from the MT-2 cell line. These studies have not been confirmed in a series of 49 British patients from our unit. Using a highly sensitive PCR-based assay for proviral DNA

in salivary gland biopsies, *tax* was not detected in any of the samples, except one from a patient who was known to be seropositive for HTLV-I.

Humoral and cellular immune reactions

In addition to a polyclonal hyperglobulinemic response during active phases of disease, primary SS is characterized by the presence of many autoantibodies. Typically, IgM, IgG and IgA rheumatoid factors (RF) are observed in blood. Antinuclear antibodies are detectable in most patients with primary SS, and speckled, homogeneous, and nucleolar patterns by immunofluorescence have all been observed in individual patients. Anti-Ro antibodies are more frequently detectable than anti-La antibodies, but the latter are more diagnostically specific for primary SS. Anti-La almost invariably coexists with anti-Ro but anti-Ro may exist on its own in primary SS. The frequency of detection is dependent on the technique used, with solid-phase binding assays being more sensitive than precipitation reactions. Purification of Ro and La antigen by biochemical techniques and the availability of recombinant cDNA-derived antigens has led to tests of greater specificity and diagnostic accuracy.

In SLE, the finding of anti-Ro antibodies in renal eluates of patients with nephritis, correlation with skin rashes, and the development of congenital heart block in infants born to mothers with anti-Ro, some of whom have primary SS, has been cited as evidence for pathogenicity of those autoantibodies. Similarly, anti-La antibodies have been found to be associated with heart block and are enriched in the saliva of patients, suggesting that these too may be pathogenic.

The occurrence of anti-La and anti-Ro has led to extensive work on the molecular identity of La and Ro antigens and much speculation on the mechanisms of induction of autoantibodies. La is a 48 kDa polypeptide with several potential autoepitopes and Ro antigen exists as two distinct polypeptides with molecular masses of 60 kDa and 52 kDa. La and the two Ro polypeptides are complexed together with cytoplasmic RNAs of unknown function though La can also exist in free form where it is thought to be involved in termination of RNA polymerase III transcription. Antibodies to all of these polypeptides and to multiple epitopes exist in individual patients, suggesting that these antigens, possibly in the form of the Ro/La/RNA ribonucleoprotein complex, are involved in driving the antibody response. In resting eukaryotic cells La is predominantly intranuclear in location, but following cellular activation or viral infection, La redistributes to the

nucleoli, cytoplasm and the cell membrane. Recent work has suggested that the transport of La to the membrane is an event in apoptosis when La, along with other nuclear matrix proteins is transported to the cell membrane in the form of cytoplasmic blebs. In virally infected cells these blebs also contain viral antigens which may direct the immune response towards La in susceptible individuals. Currently, there is no direct evidence for this mechanism, though the observation of surface expression goes some way towards explaining how the immune response can be directed towards what was previously thought to be an intranuclear antigen.

Cellular studies have been performed on peripheral blood mononuclear cells and on infiltrating cells in salivary tissues. In most patients, CD4⁺ memory T cells predominate in the tissue infiltrates and many T cells are activated, as judged by the expression of HLA class II antigens. However, the T cells express little if any IL-2R α chain (Tac antigen) which is another T cell activation marker. HLA class II antigens are increased on ductal and acinar tissue and this has raised the possibility that T cell-derived interferon γ (IFN γ) may be inducing these molecules. Some support for this has been the recent demonstration that T cell clones from salivary gland produce high levels of IFN γ . By immunohistochemistry the cytokine is detected on ductal and acinar tissue suggesting it is bound to its receptors. These findings support the current view that interactions between the activated epithelial cell and T cells in the gland are central to the pathogenesis of SS. They are also consistent with the hypothesis that the T cells are responding to viral and/or autoantigens presented by the glandular epithelial cells.

Treatment

Treatment of dry eyes and a dry mouth is essentially symptomatic, with tear and saliva substitutes, and in the case of a dry mouth, an emphasis on dental and oral hygiene and early detection and treatment of infections. Some benefit has been claimed from the use of bromhexine and evening primrose oil, but this has not been validated by extensive clinical trials. More recently attention has turned to disease-modifying drugs and several studies have suggested benefit from treatment with hydroxychloroquine. Corticosteroids and immunosuppressive drugs are indicated in some patients with extraglandular disease.

See also: Antinuclear antibodies; CD5; Epstein-Barr virus, infection and immunity; Herpesvirus-6, infection and immunity; Rheumatoid arthritis, human;

Rheumatological disorders; Systemic lupus erythematosus (SLE), human.

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SKIN, AUTOIMMUNE DISEASES

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Autoimmune diseases of the skin may be divided into several groups according to different criteria. For example, one may make a distinction between autoimmune diseases that are systemic in nature but have prominent skin involvement and those that are primarily cutaneous in nature and are only rarely associated with symptoms in other organ systems. An alternative way of distinguishing subsets of dermatological autoimmune diseases is by dividing them into groups that are certainly autoimmune, probably autoimmune, and possibly autoimmune (Table 1). In this entry, emphasis will be on auto-

immune diseases of the skin that have clinical pathology mainly limited to the integument and which are almost certainly autoimmune in nature. Thus, cutaneous lupus erythematosus, autoimmune bullous diseases and scleroderma will be described in some detail.

Cutaneous lupus erythematosus

The existence of skin diseases related to sun exposure is an ancient observation as exemplified by the description of a condition inflicted by sun and called

Table 1 Autoimmune diseases of the skin^a

Autoimmune	Probably autoimmune	Possibly autoimmune
Pemphigus	Psoriasis	Pyoderma gangrenosum
Pemphigoid	Lichen ruber planus	Parapsoriasis
Epidermolysis bullosa acquisita	Alopecia areata	(Sarcoidosis)
Linear bullous IgA disease	Actinic reticuloid	
Herpes gestationis	Morphea	
(Cutaneous lupus erythematosus)	(Scleroderma)	

^aAll except those in brackets are mainly limited to the skin.

'herpes esthiomenos' by Hippocrates. The distinction between different photodermatoses came in the nineteenth century, when Casenave was the first to use the term lupus erythemateux for a skin disease characterized by scarring, violaceous lesions especially in sun-exposed parts of the face. Kaposi (1872) recognized that this was not only a skin disease but that systemic manifestations may also occur (lupus erythematosus disseminatus et aggregatus). The recognition of circulating antinuclear factors in 1958 by Friou and the subsequent detection of immunoglobulin deposition at the dermo-epidermal junction (DEJ), both resulted in the concept of lupus erythematosus (LE) as a disease resulting from autoimmune processes.

At present, we are aware of the existence of a heterogeneous group of diseases belonging to the LE spectrum. These may be categorized as cutaneous LE, subacute LE, systemic LE, drug-induced LE, ANA-negative systemic LE, lupus-like syndrome in homozygous C2 and C4 deficiency, late onset systemic LE, Japanese LE and neonatal LE.

Specific and nonspecific forms of cutaneous LE

In cutaneous LE, a primary distinction is made between specific and nonspecific dermatological abnormalities. 'Specific' refers to the histopathology of the lesion. Characteristic microscopic signs of specific cutaneous LE include epidermal atrophy; hydropic degeneration of cells of the epidermal basal layer (apoptosis); follicular plugging; thickening of the basal membrane zone (BMZ); a mononuclear cellular infiltrate localized subepidermally around the postcapillary venules and around the (capillary venules of) skin appendages; and finally, dilatation of vascular capillaries. All these histopathological signs may occur in variable intensity. A secondary distinction in the specific LE group is made by recognizing acute, subacute and chronic forms and variants (Table 2). Rare clinical manifestations of chronic cutaneous LE include LE profundus (Kaposi-Irgang), hypertrophic discoid LE, lupus tumidus and mucosal LE. These variants will not be discussed further.

Nonspecific cutaneous LE then is a collection of dermatological abnormalities that do not show the specific histopathological signs described above. Included in this group are vascular abnormalities such as Raynaud's phenomenon; (necrotizing) vasculitis; livedo reticularis; erythromelalgia; perniones; thrombophlebitis; diffuse (frontal) alopecia; sclerodactylia; hyper- or hypopigmentation; calcinosis cutis; urticaria; Rheumatoid nodules; and finally, oral ulcers. Nonspecific dermatoses are especially common in systemic lupus erythematosus (SLE). Overlap syndromes such as mixed connective tissue disease (MCTD), pemphigus erythematosus and scleroderatomyositis, in which some of these abnormalities may also be encountered exemplify the complexity and wide spectrum of pathogenic events that form part of the SLE syndrome.

Autoantibodies and cutaneous LE

A basic finding in lupus erythematosus is the occurrence of a wide variety of circulating autoantibodies. It is unclear whether these autoantibodies are primarily or secondarily formed. Primary autoantibody formation may be the result of intrinsic dysregulation of antibody formation or, alternatively, may be triggered by exogenous antigens, such as from retroviruses. Secondary autoantibody formation may occur as a result of tissue damage with the subsequent release of previously hidden autoantigens. In NZB/NZW mice, that develop antibodies to DNA and glomerulonephritis, anti-DNA titers precede the development of renal disease. In patients, anti-DNA titers rise before exacerbation of systemic disease occurs. These findings indicate that autoantibody formation is not a secondary event.

The phenomenon of neonatal LE, in which autoantibodies are passively acquired from the mother, and in which cutaneous disease disappears when these autoantibodies fade away from the child's circulation, also emphasizes the primary role of autoantibodies in LE pathogenesis.

Table 2 Clinical forms of histopathologically specific cutaneous lupus erythematosus (LE)

<i>Clinical types</i>	<i>Acute LE</i>	<i>Subacute LE</i>	<i>Chronic LE</i>
Subtypes	Butterfly rash Disseminated erythema Bullous LE	Annular Psoriasiform Exanthematous	Localized (face) Cutaneous Disseminated LE profunda and others
Photosensitivity	High	Medium to high	Medium
SLE association	>95%	50%	<5%
Natural course	Days to weeks	Months	Years

Immunopathogenesis of cutaneous LE

The spectrum of cutaneous LE, including neonatal LE lesions, specific LE and variants, and nonspecific LE is large and difficult to describe in general immunopathological terms. Vasculitis resulting from immune complex deposition (leukocytoclastic) may be found in some of these conditions. The best studied form of cutaneous LE is the histopathologically specific type of lesion, where two main hypotheses have been formulated and tested. In one, emphasis is on immunoglobulin deposition at the DEJ, with complement activation and formation of the membrane attack complex (MAC) C5b-C9. In the other, emphasis is on cytotoxic T cells which, through their Fc receptors for immunoglobulins, may recognize autoantibodies bound to epidermal cells (antibody-dependent cellular cytotoxicity – ADCC).

The presence of immunoglobulins and complement at the DEJ is highly suggestive for the presence of immune complex (IC) formation at that site. Thus, a pathogenetic model of cutaneous LE based on initial IC deposition, complement activation and acute inflammation, followed by a chronic inflammatory state has been proposed. Such a model could be supported by the identification of C5-C9 in LE skin lesions. In a recent direct immunofluorescence study of cutaneous LE, granular MAC deposition at the DEJ was found in 29 of 38 biopsies (76%).

Complement activation and deposition of MAC thus are common events in cutaneous lesions of LE. In view of the existence of complement regulatory proteins, both in soluble form as well as inserted in cell membranes, it is interesting to look at studies performed in this area. At least clusterin and S protein have been found to be colocalized with MAC, indicating inhibition of complement activation as part of MAC formation. A major problem with the complement activation model of cutaneous LE pathogenesis is that acute inflammation, starting with influx of polymorphonuclear granulocytes, is not a feature in any stage of skin lesions. Also, human intact keratinocytes have been found to be resistant to complement-mediated lysis, probably because they express the membrane-inserted complementary regulatory proteins (CRPs) decay-accelerating factor (DAF), membrane cofactor protein (MCP) and CD59.

The ADCC hypothesis has been worked out by Norris and coworkers. It is based on the lesional presence of immunoglobulins and effector cells, mainly of the T cell type. These lymphocytes may act in ADCC reactions. The damage to epidermal cells with keratinocyte necrosis might follow influx of CD8⁺ cytotoxic T cells which can adhere to increased

numbers of adhesion molecules (ICAM-1) expressed on keratinocytes as a result of stimulation by interferon γ secreted by cutaneous activated CD4⁺ T_H1 cells. The intraepidermal cytotoxic T cells would exert their effect through production of perforins or tumor necrosis factor α .

The ADCC hypothesis is especially interesting in view of the photosensitivity in LE. In general, the action spectrum of photosensitivity is in the ultraviolet B (UVB) (280–320 nm) range, but UVA (320–400 nm) may also lead to flares, and testing has shown sensitivity to UVA, UVB as well as to visible light in most patients. After UV exposure, certain autoantigens are expressed on keratinocyte cell membranes, including Ro/SSA and nRNP. These autoantigens then become available for binding by their specific antibodies in the extracellular fluid. Subsequently Fc receptors on effector lymphocytes recognize these keratinocyte-bound immune complexes, with subsequent lysis of these cells. It may be that both membrane attack formation and cytotoxicity may occur due to UV induction of nuclear antigen expression on basal keratinocyte membranes and subsequent binding of interstitial antibodies directed against (some of) these nuclear antigens (Figure 1).

Immunotherapy of cutaneous LE

Therapy in specific cutaneous LE starts with general measures such as avoidance of sun irradiation and the use of sunscreens that filter out UV. Immunosuppressive therapy consists of the administration of strong topical steroids, preferably in intermittent schemes. If unsuccessful, systemic agents such as chloroquine, hydroxychloroquine, prednisone, acitretin, diaminodiphenylsulfon (DDS, dapsone), gold

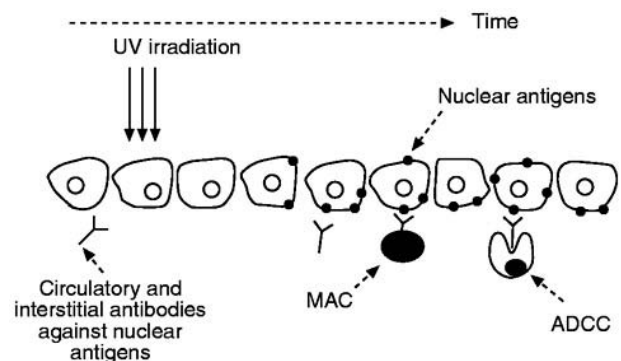


Figure 1 In specific cutaneous lupus erythematosus, ultraviolet irradiation leads to expression of previously hidden nuclear antigens on keratinocyte cell membranes. These antigens become available for binding with antibodies to nuclear antigens that these patients have circulating as well as in interstitial fluid. Antigen-antibody complex formation may then lead to membrane attack complex (MAC) formation and cellular cytotoxicity (ADCC) leading to keratinocyte degeneration.

preparations, thalidomide, clofazamine, azathioprine, cyclophosphamide and methotrexate are of use. Flowcharts or clinical protocols for the use of these systemic agents have not been developed as yet.

Autoimmune bullous dermatoses

Since the recognition of circulating antibodies against skin components as well as antibody deposition in the skin of pemphigus and pemphigoid, a large number of immunological studies have been performed to identify the precise subtypes of these presumed autoantibodies, their role in pathogenesis, and the antigens to which they bind in the skin. It has become apparent that the most important group of autoimmune skin diseases is characterized by blister formation as a result of interference by autoantibodies with the normal intercellular adhesion of epidermal keratinocytes (intraepidermal bulla formation) or with the structures responsible for adhesion of epidermis to dermis (subepidermal bulla formation).

Clinical forms of autoimmune bullous dermatoses

The pemphigus group of autoimmune bullous dermatoses is characterized by loss of adhesion of epidermal keratinocytes (acantholysis). As a result, there is intraepidermal blister formation. This follows the binding of autoantibodies to important adhesion proteins, particularly the desmoglein subfamily of the cadherin supergene family. Included in the pemphigus group are pemphigus vulgaris (PV), pemphigus vegetans (PVe), pemphigus foliaceus (PF), pemphigus erythematosus (PE) and the relatively new variants paraneoplastic pemphigus and IgA pemphigus. The pemphigoid group of autoimmune bullous dermatoses have autoantibodies against various components of the epidermal-dermal junction complex, mainly localized around the BMZ. Included in this group are bullous pemphigoid, cicatricial pemphigoid, herpes (or pemphigus) gestationes, linear IgA bullous disease (including chronic bullous disease of childhood), and acquired epidermolysis bullosa acquisita. Bullous pemphigoid is the most common of the immunobullous disorders, mainly affecting the elderly.

Detection of autoantibodies in autoimmune bullous dermatoses

Autoantibody detection generally follows advances in immunological methods. The standard approach is by direct immunofluorescence (DIF) techniques using lesional, perilesional, and/or normal uninvolved skin and detecting antibody subclasses and

complement cascade components using labeled anti-human antibodies. With this method, a fisherman's net type of fluorescence is observed in the pemphigus group, highlighting binding of autoantibodies to components of the intercellular adhesive structures of the epidermis. In the pemphigoid group, DIF reveals fluorescence at the BMZ, mostly in a linear fashion. Indirect immunofluorescence (IIF) is used to detect circulating or blister fluid-derived autoantibodies, using normal human skin, cavia lip, or monkey oesophagus as substrate. Titers of antibodies correlate with disease activity in the pemphigus group but not in the pemphigoid group. IIF can be useful in the pemphigoid group to detect the precise level of binding in the BMZ. Immunoelectron microscopic techniques have been used to further define the precise location of autoantibody binding, while immunoblotting and immunoprecipitating techniques are available in specialized centers to determine the pattern of antigens to which autoantibodies are directed in a given patient.

Pathogenesis of autoimmune bullous dermatoses

The pathogenesis of immunobullous diseases as far as their induction is concerned remains unknown. Immunogenetic associations (HLA), drug exposure and infections have been indicated to play various roles alone or in combination. Once the disease is induced, cellular and humoral mechanisms play different roles in different entities.

Eosinophilic granulocytes and mast cells are thought to be important in bullous pemphigoid. Proteolytic enzymes derived from eosinophils, such as metalloproteases (gelatinase), may be involved in destruction of components of the BMZ. T cells have been implicated in the chronic stages of bullous pemphigoid and have been linked to the scarring in cicatricial pemphigoid (especially of the conjunctivae). Neutrophilic granulocytes and T cells have been implicated in the pathogenesis of dermatitis herpetiformis and in linear bullous IgA disease. Acquired epidermolysis bullosa seems to have a spectrum in which neutrophils play some role in certain subtypes but none in others. Finally, in the pemphigus group, evidence for a role of cellular mechanisms is generally lacking.

Humoral factors, particularly autoantibodies, are of course essential in the pathogenesis of autoimmune bullous disorders. As described above, they can be found bound to the tissue and/or in the circulation in all entities. The precise role of autoantibodies in dysadhesion is best studied in the pemphigus group. Various experimental models have clearly shown that pemphigus sera can induce acantholysis, and complement activation is not necessary to obtain

that effect. Another indication for the primary role of autoantibodies in disease formation is the correlation between titers of autoantibodies and disease activity. In pemphigoid, it has been more difficult to precisely establish the dysadhesive effects of autoantibodies, due to differences in the species-specific epitopes involved. Recent studies have, however, indicated a role for pemphigoid autoantibodies in the immunopathogenesis of blister formation.

Immunotherapy of autoimmune bullous dermatoses

Pemphigus and pemphigoid are primarily treated with high doses of oral prednisone, up to 200 mg per day. In limited cases, topical steroids may be given a try. In pemphigoid, a combination of tetracyclines and niacinamide has been shown to have good results and this combination might be given a try before deciding to give systemic steroids. Azathioprine is given as a corticosteroid-sparing drug in prednisone-treated patients. Other systemic drugs used are cyclophosphamide and cyclosporine. The IgA-mediated diseases dermatitis herpetiformis and linear IgA disease, the rare bullous variant of SLE and IgA pemphigus respond to treatment with dapsone or the sulfonamides sulfapyridine and sulfamethoxypyridazine.

Scleroderma

Scleroderma is a chronic disease with variants limited to the skin only and systemic forms. Systemic scleroderma is discussed elsewhere in this volume. From a dermatological point of view, subvariants of systemic scleroderma include progressive systemic sclerosis, CREST-syndrome, and mixed connective tissue disease (MCTD). Cutaneous variants of this disease are also known as localized scleroderma, but generally called morphea.

Morphea

Essentially, in cutaneous manifestations of scleroderma, there is an increase in the connective (collagen) compartment of the dermis. These may be

secondary to vascular abnormalities that precede this fibrotic process. Recent findings have implicated decreased expression of complement regulatory proteins (CRPs) on endothelial cells in involved skin of systemic as well as localized scleroderma. As a result, naturally formed immune complexes as well as spontaneous C3b formation led to incipient vascular damage, because protective CRPs are diminished.

Classical morphea is characterized by the gradual appearance of indurated, often hyperpigmented patches with an erythematous border. Initially, when there is active progression of the disease, these so-called lilac rings contain superficial dermal mononuclear infiltrates. Later, the inflammatory component disappears and the end-result of dermal fibrosis is left. Spontaneous resolution may occur, both in limited and cutaneously disseminated cases of morphea. Clinical variants of morphea include linear morphea and morphea or scleroderma '*en coup de sabre*'. Treatment of morphea is generally not possible, spontaneous resolution has to be awaited.

See also: Autoimmune diseases; Rheumatological disorders; Skin, contribution to immunity.

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SKIN, CONTRIBUTION TO IMMUNITY

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The skin is the largest organ of the human body. Amongst other functions, it serves as a general defense system. Normal skin contains two functionally and anatomically different layers: the epidermis, which forms a physical barrier and primarily contains keratinocytes, and the dermis, which consists of connective tissue and contributes to the flexibility of the skin. During evolution the immune system has evolved as a complementary line of defense that efficiently copes not only with exogenous organisms that have crossed the corneal layer of the epidermis but also with endogenous hazardous elements such as newly formed malignant cells. Malignant cells are generated, with a high incidence in skin, presumably due to ultraviolet (UV) light exposure.

Various cell types are responsible for the immune reactivity in skin. They are involved in the homing of immune cells (endothelial cells, keratinocytes), in natural immunity (macrophages, neutrophils, natural killer cells) or in acquired immunity (antigen-presenting cells, T lymphocytes, mast cells). This complexity of cells, their products and their interactions in skin has been denominated the 'skin immune system'.

Skin immune system

In the epidermis, keratinocytes have an important role in nonspecific immune defense through the production of various soluble mediators. An important epidermal cell of the specific skin immune defense is the Langerhans cell. These cells form an almost closed network of dendrites within the prickle keratinocyte layer and function as the sentinels of the antigen-specific skin immune response. They trap antigen, transport it to the skin-draining lymphoid organs where they recruit antigen-specific T cells. Langerhans cells belong to the group of bone marrow-derived dendritic cells, and function purely to trap and present antigen to T lymphocytes. Antigen-trapping by Langerhans cells is facilitated by a high capacity of fluid phase and receptor-mediated endocytosis, the latter via various types of mannose receptors and Fc receptors. Furthermore, Langerhans cells constitutively express several molecules functional in antigen presentation, such as class II major histocompatibility complex (MHC) molecules, costimulatory molecules B7.1, B7.2 and interleukin 15 (IL-15), and

the adhesion molecules lymphocyte functional antigen 3 (LFA-3) and the intercellular adhesion molecule 1 (ICAM-1). Langerhans cells differ from other dendritic cells in that they contain structures in the cytoplasm referred to as Birbeck granules; these are rod- or racket-shaped membrane structures of unknown function.

Another remarkable feature of Langerhans cells is the high expression of CD1a. CD1a has a structural similarity with MHC molecules and is able to present certain bacterial lipids and glycolipids, as well as peptide antigens, to T cells. In normal human skin there is a slow circulation of Langerhans cells: they enter the epidermis as precursor cells, mature and subsequently emigrate from the skin after between a few days up to several weeks following their initial entry. In this way the specific immune system is continuously made aware of the antigenic microenvironment of the skin.

Human epidermis contains occasional T cells, mainly of the CD8⁺ type. In contrast to mouse epidermis, human epidermis contains low numbers of $\gamma\delta$ T cell receptor-bearing cells, that do not exceed their relative frequency in peripheral blood (5–10%). The papillary dermis, close to the epidermis, contains a capillary network and harbors various bone marrow-derived cell types involved as effector cells in natural and acquired immune reactivity of the skin. The human papillary dermis in normal skin contains modest numbers of extravascular neutrophils and eosinophils and relatively dense populations of macrophages and mast cells. A recently recognized aspect of potentially reactive connective dermal tissue is the occurrence of perivascularly located T cells. These T cells contact antigen-presenting cells scattered around the capillary vessels. A substantial proportion of these are CD4⁺ T cells of the memory phenotype with an $\alpha\beta$ T cell receptor and expressing the cutaneous lymphocyte-associated antigen (CLA), a molecule which directs homing into the skin.

Skin immune response

As yet, there is no direct evidence to consider skin itself as an independently operating immune organ. The inability to mount a separate local primary antigen-specific T cell response, and the virtual absence of B cells, B cell follicles and germinal center formation distinguish skin from connective tissues that

contain well-organized areas of lymphoid tissue, such as mucosal-associated lymphoid tissue (MALT). Clearly the elicitation of an immune response in skin, leading to immunity, sensitization or induction of tolerance, requires the participation of skin-draining lymph nodes. This consideration prompted Streilein to introduce the concept of a skin-associated lymphoid tissue (SALT), comprising the combined activity of constituents of skin and the draining lymphoid organs.

Skin provides an immunization site giving rise to generalized T and B cell priming, as mounted in the skin-draining lymphoid organs. Skin also participates in the efferent phases of both systems and local immune reactivities, providing a reliable reflection of the sensitivity status, evident from patch, scratch or prick tests. However, the occurrence of skin-specific diseases that cannot be explained by the local presence of antigen may provide arguments in favor of an additional skin-specific immune response. For instance, the occurrence of cutaneous T lymphomas may imply that subsets of T lymphocytes do exist that preferentially migrate into and home in skin, probably through the expression of CLAs.

The role of the skin immune response in the maintenance of physiological homeostasis is evident, especially in relation to infectious diseases and immunosurveillance. In addition, the skin immune system is involved in the pathogenesis of a large variety of inflammatory, autoimmune as well as allergic dermatoses.

Mechanisms of the skin immune response

A first line of skin immune defense follows from the induction of the expression of a large series of soluble and membrane-bound molecules by epidermal as well as dermal skin cells. These factors contribute to vasodilatation and the attraction, homing and activation of immune cells. A typical example of this nonspecific immune reaction is irritation reaction induced by small chemicals acting as haptens. Much information on the mechanisms in the antigen-specific skin immune response is obtained from animal model experiments with contact allergens that are able to bind protein, thus providing carrier (T cell) epitopes for the haptenic allergen. The contact allergic response is considered to be a classical example of delayed-type hypersensitivity.

Epicutaneous application of contact allergens is rapidly followed by penetration into the epidermis and trapping by Langerhans cells. Driven by the concerted action of IL-1 β and tumor necrosis factor α (TNF α), these antigen-loaded Langerhans cells leave

the epidermis at a relatively high rate and can be detected at high frequencies in draining lymph 6–24 h after allergen exposure. These Langerhans cells settle in the paracortical T cell areas of the draining lymph nodes, where they mature into interdigitating cells, which show a considerably higher expression of molecules involved in antigen presentation, such as MHC, adhesion and costimulatory molecules. Lymph node interdigitating cells, derived after 24 h of epicutaneous antigen exposure, are very efficient presenters of antigen to naive antigen-specific T cells *in vitro*, indicating their potential to induce selective expansion of allergen-specific T cells in lymph nodes *in vivo*. The important role of T cells in contact allergy is demonstrated by the fact that naive animals can be sensitized by adoptive transfer of T cells from syngeneic sensitized animals.

There is also evidence that bypassing Langerhans cells results in tolerance. Application of antigen to stripped epidermal skin sites or to areas with a low density of Langerhans cells (mouse tail) does not lead to sensitization but results in tolerance induction. Macrophage-like cells or dermal dendritic cells may be important in this tolerance induction. UV exposure of skin prior to sensitization may also induce tolerance, following from an UV-induced alteration of the Langerhans cell function.

The elicitation phase of the contact allergic response is less well understood. Probably, allergen is taken up by local antigen-presenting cells, such as epidermal or dermal dendritic cells, which activate allergen-specific T cells that have settled in the skin after sensitization. Upon activation these cells produce mediators, allowing the further entry of circulating contact allergen-specific T cells. Various chronic inflammatory skin diseases are associated with infiltrates of CD4⁺ T cells. Whereas allergen-specific CD4⁺ T cells have been identified in contact allergy and constitutional eczema, the antigen-specificity of the CD4⁺ T cells in diseases such as psoriasis and pityriasis rosea are unknown.

See also: Antigen-presenting cells; CD1; Contact hypersensitivity; Cutaneous anaphylaxis; Delayed-type hypersensitivity; Eczema; Innate immunity; Langerhans cells; Photoimmunology; Skin, autoimmune diseases.

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SOMATIC MUTATION

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Immunoglobulin heavy and light chains are encoded by a series of gene segments that undergo rearrangement and joining early in B cell ontogeny. The multiplicity of variable (V) region segments, the randomness of their recombination and the imprecision of their joining account for the diversity of the pre-immune immunoglobulin (Ig) repertoire. B cell exposure to antigen and specific B–T cell interactions result in the induction of V region somatic mutation. Somatic mutation is the predominant mechanism for generating postrecombination diversity of the humoral immune response and results in the *in vivo* evolution of high-affinity antibodies that provide protective immunity. Recent experimental data are yielding important insights into the site, mechanism and regulation of the somatic mutation process.

Ig gene mutation rates are estimated to be 10^{-4} – 10^{-3} per base pair per cell generation, approximately 10^6 times higher than in the rest of the mammalian genome. This rate appears to allow optimal B cell clonal diversity without introducing excessive numbers of stop codons. The analysis of Ig gene sequences from many hybridomas demonstrates that mutations are restricted to the V region and one kilobase of its flanking sequences. One hallmark of Ig hypermutation is that the constant region rarely undergoes mutation.

The histologic locale of Ig hypermutation is the germinal center of secondary lymphoid organs. Analysis of microdissected or fluorescence-activated cell sorter (FACS)-sorted murine and human germinal center B cells has enabled investigators to sequence V region genes from limited cell populations in order to define the timing and site of the mutation event. Ig mutations are first seen in germinal center B cells by day 7 of a primary immune response and continue to accumulate in a step-wise fashion to day 18. Hypermutating centroblasts in

germinal center dark zones exit into the light zones, where they encounter both antigen, presented by follicular dendritic cells, and antigen-specific helper T cells. Antigen-responsive B cells probably undergo repeated rounds of germinal center hypermutation and selection. Germinal center B cells that are not selected undergo apoptotic death. Memory B cells also appear to be generated at this time and may not continue to mutate following further antigen exposure. Although hypermutation is a process largely restricted to Ig genes, one study has reported a small number of mutations in the T cell receptor α chain of T cells derived from the identical histologic site as mutating B cells. This suggests that the hypermutation event is highly dependent on the local microenvironment.

Independent of selection, all V region bases may not be equally likely to mutate. Analysis of heavy and light chain genes of hapten-binding antibodies have led to the conclusion that the nucleotide motif AGY (where Y = pyrimidine) is an innate 'hot spot' for mutation. Analysis of the serine codons AGY or TCN in human V genes revealed that the hot spot AGY is much more prevalent in complementarity-determining regions (CDRs), whereas AGY and TCN are found in equal proportions in frameworks. These findings suggest that evolution has selected the more mutable AGY for positions within the Ig where mutation would be favorable for enhanced plasticity of the humoral response.

Ig hypermutation is primarily a T cell-dependent process. T-independent immune responses rarely include mutated antibodies, while T-dependent responses can generate V regions with up to 30 mutations. In patients with X-linked hyper-IgM syndrome who have deficient T–B cell signaling due to the absence of CD40 ligand, Ig genes exhibit fewer mutations. It must be noted, however, that in some

species Ig genes may mutate despite little or no T cell help.

Relatively little is known about the molecular basis of V region hypermutation. V region mutations are nearly always single base substitutions; insertions, deletions or gene conversion are rare in human and mouse V genes, although they can be a major mechanism for the generation of immunoglobulin diversity in other species. The mechanism may involve error-prone replication, transcription or repair, active mutagenesis or even reverse transcription. Most of the critical Ig sequences and all of the proteins involved in both the process and the regulation of hypermutation have yet to be determined. More information may soon become available now that *in vivo* and *in vitro* models for the study of somatic mutation are available. Recent studies have defined rearranged κ light chain transgenes that undergo hypermutation at frequencies similar to those of endogenous Ig genes. Requirements for the 3' κ enhancer and the intronic κ enhancer with its adjacent matrix attachment regions have been demonstrated for κ transgene hypermutation. Furthermore, mutations were found even when the Ig promoter was replaced by a human globin promoter. Non-Ig sequences replacing the V coding region in a κ transgene acted as substrates for hypermutation. These results suggest that V regions may mutate because of their Ig context rather than their innate sequence, and that Ig flanking sequences outside of the V region are responsible for the regulation of Ig hypermutation. The simultaneous mutation of both heavy and light chain genes, as well as the reported occurrence of mutations in Ig transgenes integrated into non-Ig sites suggest that *trans*-activating mechanisms are also operative. V region transcription and possibly transcription initiation are required for mutation to occur, even in nonproductive alleles.

Murine B cell lines can accumulate spontaneous point mutations in endogenous Ig alleles 100-fold less frequently than observed *in vivo*. Recently, B cell culture systems with hypermutating transfected Ig constructs, as well as *ex vivo* cultures of splenic B cells mutating an endogenous Ig gene, have been reported. These cell cultures may achieve *in vivo* mutation rates and do so by cumulative base substitutions. The process appears to require primed B cells and, for spleen cells, both surface Ig cross-linking and T cell-derived signals.

Dysregulated Ig hypermutation may contribute to the development of autoimmunity as pathogenic autoantibodies are often encoded by mutated sequences. Use of a bcl-2 expressing fusion partner that has apoptosis-sparing effects has recently allowed for the capture and characterization of autoantibodies that have arisen *in vivo* following immunization with a bacterial antigen. This work demonstrates that autoantibodies are often generated during an immune response and that B cells bearing such antibodies are normally tolerized or deleted.

See also: Affinity maturation; Diversity, generation of; Germinal center; Hyper-IgM syndrome; Immunoglobulin genes; Specificity.

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SOUTHERN BLOTTING

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Southern blotting is the transfer of DNA from an electrophoresis gel on to a dimensionally stable membrane support resulting in an hybridizable 'image' of the DNA banding pattern. Typically, restriction enzyme-digested DNA is size-fractionated by agarose gel electrophoresis. The double-stranded DNA is then denatured *in situ* by soaking the gel in alkali and transferred by capillary action to the membrane (usually nylon or nitrocellulose). The now single-stranded DNA is fixed to the membrane and may act as a target for hybridization with a specific nucleic acid probe. The probe, which is also single-stranded, will form hybrids with target DNA sequences on the filter to which it is complementary and, following extensive washing of the filter to remove unbound probe, specific hybridization is detected by a method dependent on the means by which the probe was labeled.

Autoradiography is used for the detection of radiolabeled probes. The sizes of the detected DNA fragments may then be determined by reference to DNA size markers on the original electrophoresis gel. These sizes may be of use in determining restriction enzyme maps of genes, revealing variations known as restriction fragment length polymorphisms (RFLPs). Southern blotting may also reveal alterations in gene structure such as insertions, deletions and rearrangements arising from recombination events.

Historical background

The ability to hybridize DNA strands to one another derives from the complementary nature of DNA so elegantly demonstrated in Watson and Crick's model of the structure of DNA. The earliest applications, carried out in solution, concerned the renaturation (annealing) of DNA that had been made single stranded by denaturation, and demonstrated the complex nature of genomic DNA by defining components that renatured at different rates. Similar studies involving the formation of DNA/RNA hybrids demonstrated tissue- and cell-specific differences in RNA expression. Hybridization later evolved to become more the process which we recognize today, with the development of the concepts of unlabeled target sequences (usually a heterogeneous mixture of sequences) and a labeled probe capable of detecting specific sequences within the target. A further development was the immobilization of the

target sequences on an inert matrix – initially nitrocellulose powder, and later nitrocellulose membrane.

The major breakthrough in hybridization came with the demonstration by Southern in 1975 that DNA fragments separated by gel electrophoresis could be easily transferred to a sheet of nitrocellulose membrane. This allowed characterization of DNA sequences not only by their hybridization behavior but also by the simultaneous determination of their sizes. The basic concept of blotting was later adapted to many other situations where spatial identification of a specific target was required. Blotting of bacterial colonies or phage plaques allows identification of specific clones in gene libraries and specific RNAs or proteins may be identified by blotting procedures known as northern and western blotting respectively.

The DNA to be analyzed

Southern blotting can be applied to the detection of specific DNA fragments both in simple and complex analysis situations, as typified by individual gene clones and total genomic DNA. In the latter case, adequate amounts of genomic DNA may be readily isolated from peripheral blood but, where much larger amounts are required, for example to act as reference DNA samples, placentas provide an essentially limitless source. Generally, around 5–10 µg of DNA is required for each analysis and it is cleaved with restriction enzymes prior to gel electrophoresis.

Restriction enzyme digestion

The choice of restriction enzymes is dictated by a number of factors. Often, the choice will be guided by a knowledge of the restriction enzyme sites in and around the gene to be analyzed. Where no such prior knowledge exists, three further considerations must be made. First, the frequency with which a restriction enzyme cuts DNA is a function of the length of the cut-site recognition sequence; the longer the sequence, the less frequently the enzyme will cut. Secondly, in humans, this frequency is reduced if the recognition sequence contains a CpG dinucleotide which occurs less frequently than expected due to a secondary consequence of DNA methylation. Finally, some restriction enzymes are inhibited depending on whether or not the recognition sequence is methylated. Such methylation is often tissue-specific in nature. For most applications,

DNAs are cut with restriction enzymes producing fragments in the size range 0.5–15 kb.

Electrophoresis of DNA

The cut DNA is size-fractionated by gel electrophoresis and the choice of separation matrix is determined by the sizes of the DNA fragments. For most applications, agarose is the matrix of choice because of its ease of use. For fragments smaller than a few hundred base pairs, acrylamide has been used because of its greater resolving power but acrylamide gels pose practical problems at the blotting stage. In recent years, specialist agaroses have been developed with much improved resolution of small DNA fragments. Conventional agarose gel electrophoresis is incapable of resolving DNA fragments larger than ~15 kb and for such fragments it is often necessary to use field inversion gel electrophoresis (FIGE) or pulsed field gel electrophoresis (PFGE). The latter is capable in some instances of separating intact chromosomes.

Preparing the gel for blotting

Following electrophoresis, the gel is usually photographed to provide a permanent record. Next, it is soaked in dilute hydrochloric acid to partially depurinate the DNA. When the gel is later soaked in sodium hydroxide to denature the DNA, single-stranded nicks are introduced at depurinated sites in the DNA the effect of which is to fragment larger DNA segments which would otherwise blot with a much reduced efficiency. Such treatment is mandatory for the blotting of FIGE and PFGE gels.

The choice of membrane

As originally described by Southern, the blotting technique involved the transfer of DNA from the gel on to a sheet of nitrocellulose. This material has

many drawbacks, including fragility and the limited number of times that a blot can be rehybridized due to leaching of the target DNA from the membrane. Nowadays, nitrocellulose has almost been entirely replaced by neutral and positively charged membranes based on nylon. As well as alleviating the problems associated with nitrocellulose, they have an increased efficiency and capacity to bind DNA. This has resulted in being able to carry out Southern blot analyses with less target DNA than was previously possible.

The blotting procedure

Most people remain faithful to the basic transfer scheme originally devised by Southern. In essence, all that is required is that there be a membrane in contact with one surface of the gel and absorbent material (dry paper towels) to draw buffer (typically 20× or 10× SSC, standard saline citrate) and DNA toward the membrane. The process is aided if there is a reservoir of buffer so that blotting does not diminish as the gel becomes dehydrated. An exploded view of a typical Southern blot transfer is shown in Figure 1. The choice of the blotting buffer is somewhat dependent on the membrane being used, but 20× SSC is common for purely historical reasons as that was what was required to achieve binding of DNA to nitrocellulose. With the advent of nylon membranes, there has been considerable experimentation with the blotting buffer. Many advocate lower concentrations of salt and some methods even dispense with the neutralization step, blotting the gel in the alkaline denaturation solution.

A number of alternatives to the blotting apparatus depicted in Figure 1 have been devised. These include downward blotting with the membrane and absorbent material below the gel and a reservoir of buffer above. This is said to improve the efficiency of transfer by avoiding dehydration and compression of the gel. Where efficiency of transfer is of lesser

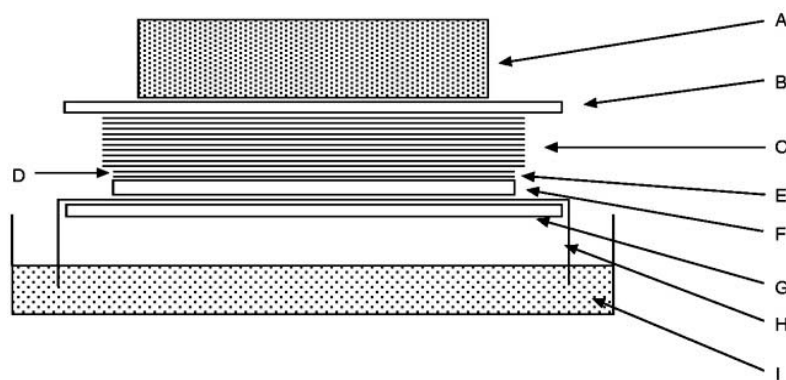


Figure 1 Schematic view of a typical Southern blot apparatus. (A) weight; (B, G) glass plates; (C) paper towels; (D) filter paper; (E) hybridization membrane; (F) gel; (H) filter paper wick; (I) buffer.

importance, it is possible to make two blots simultaneously from a single gel which is sandwiched either side by a membrane and absorbent material and the transfer is dependent entirely on the buffer in the gel. For each of these methods the use of paper towels is usually entirely adequate, though a number of manufacturers offer highly absorbent blotting pads which are said to improve the efficiency of blotting. The length of time required to blot most of the DNA from a gel on to the membrane is the subject of much discussion but, for most applications, 2 hours appears to be adequate.

Alternative techniques, including vacuum blotting and electrophoretic blotting have been devised but are only used rarely, the latter usually being necessary for the blotting of acrylamide gels. The final step of the blotting procedure is the permanent fixing of the DNA to the membrane. This is achieved with exposure to UV light for nylon membranes and by baking at 80°C for nitrocellulose.

Choice of probes

The most commonly used probes are cloned segments of genomic DNA or complementary DNA (cDNA). Both have advantages and disadvantages which stem from the complex organization of higher eukaryotic genes and genomes. In general, more satisfactory results are obtained with genomic DNA probes because they contain all the DNA sequences of a gene – both the introns and exons. Consequently, such probes correspond exactly in sequence with the target to which they are to be hybridized. However, care must be taken in the selection of genomic DNA probes to ensure that they do not contain dispersed repeat DNA sequences such as Alu which would cause them to hybridize to virtually all DNA fragments in a genomic DNA blot. Probes derived from cDNA are often judged subjectively poorer because the target sequences to which they are complementary are often dispersed over many DNA fragments resulting in relatively lengthy detection times. Paradoxically, cDNA probes, if full-length, do have the advantage of being ideal for the simultaneous detection of all genomic DNA fragments of an entire gene. Though infrequent, some cDNA clones harbor repetitive DNA sequences and so are unsuitable as hybridization probes.

The problem of probes which contain repetitive DNA sequences can be approached in two ways. The first is to subclone regions that are free of such sequences and the second is to prehybridize the blot with unlabeled repetitive DNA to block all repetitive target DNA sequences.

Synthetic oligonucleotides of defined sequence

may be used as probes as they can be designed to be allele-specific. However, they have major disadvantages in that hybridization and posthybridization washing conditions need to be critically controlled and the specific activity of probe that can be achieved is relatively low (see below).

Labeling of probes

The most common method for the labeling of probes uses random deoxynucleotide hexamer primers. The DNA to be labeled is made single stranded by heating, the primers are allowed to anneal and they direct the synthesis of complementary DNA using the Klenow fragment of DNA polymerase I. Labeling of the newly synthesized DNA is achieved by including labeled precursors of DNA synthesis in the reaction. The label can be radioactive or chemiluminescent, with the latter becoming more widely used in recent years.

Strand-specific probes are most easily generated by *in vitro* transcription of probe sequences cloned into vectors containing viral promoters upstream of the cloned probe. Such probes are labeled during transcription by the incorporation of labeled precursors.

End-labeling of probes with T4 polynucleotide kinase or terminal transferase is sometimes used but results in a low specific activity probe due to there being only one label per probe molecule. This method is usually reserved for the labeling of oligonucleotide probes.

Hybridization and stringency

A probe has the ability to hybridize to targets to which it is identical in sequence or targets which are not identical but which have considerable sequence similarity. The extent to which a probe will hybridize to targets to which it is not identical in sequence is determined by a parameter known as 'stringency'. Stringency is defined by a combination of ionic strength and temperature with high stringency being characterized by low ionic strength and high temperature. Hybridization is typically carried out at a temperature approximately 25°C below the expected melting temperature of the DNA duplex to be formed and at high ionic strength (65°C in 3 × SSC) because such low stringency conditions favor a rapid rate of hybridization. As an alternative to 3 × SSC, 0.5 M sodium phosphate, 7% SDS is commonly used. The length of hybridization is dependent on a number of factors but is typically between a few hours and overnight. During hybridization, both perfect and mismatched hybrids are formed between the probe and target, with stringency being achieved dur-

ing the posthybridization washing of the filter which is commonly carried out at 65°C but at much lower ionic strength (typically $0.1 \times \text{SSC}$ or $0.5 \times \text{SSC}$). Mismatched hybrids are relatively less stable than perfect matches and will be disrupted by the low ionic strength. If a series of blots are to be washed at different stringencies, it is much more convenient to vary the stringency with changes in the ionic strength than with temperature changes.

Although the rate of hybridization is determined primarily by the probe concentration, the ionic strength and the temperature, increases in the rate can be achieved by the addition of chemical accelerators to the hybridization solution. These include dextran sulfate and high molecular weight polyethylene glycol which are thought to work by increasing the effective probe concentration in the vicinity of the membrane. Proprietary rapid hybridization solutions are available from some manufacturers.

Many methods have been devised for the incubation of blots with probes. The main problems are evaporation of the hybridization solution and its containment, especially if the probe is radiolabeled. Solutions to the problem have included custom-made hybridization chambers and the use of disposable heat-sealed plastic bags. Currently, the favored method is incubation in glass hybridization bottles which are rotated around their long axis in an oven equipped with a rotisserie.

Probe detection

The method used to detect the regions to which the probe has hybridized to a membrane is dependent on the nature of the label used in generating the probe. Frequently, the label is radioactive (e.g. ^{32}P , ^{33}P or ^{35}S) and detection is by autoradiography using X-ray film. Detection of single-copy genomic DNA sequences usually requires the use of intensifying screens at low temperatures (-70°C). Exposure times will depend on many factors and is best determined empirically – it may range from a few minutes to several weeks. Chemiluminescent probes are also detected with X-ray film and, in general, detection times can be shorter than with radio-labeled probes.

A relatively recent advance in detection has been the introduction of the use of electronic imagers which are capable of detecting radioactivity and chemiluminescence as well as direct fluorescence. Their major advantages are increased sensitivity and linearity of response compared with X-ray film.

Re-use of blots

It is often desirable to re-use a blot when there are limited amounts of the target DNA available, which may be the case in clinical analyses. A probe may be easily stripped from a filter by a short wash in warm alkali; after a neutralization step the filter may then be hybridized with another probe. Southern blots on nylon membranes can be re-used many times.

Applications

Southern blotting has application in instances where the structure of a gene (cloned or uncloned) is to be investigated. It may be used to determine a restriction enzyme map of a gene or to investigate natural variation or mutational events in a gene such as insertions deletions or rearrangements resulting from recombination. If appropriate controls are included and the procedure is carried out with care, Southern blotting may be used in a quantitative fashion to investigate phenomena such as gene amplification or loss of heterozygosity. Dimorphism of restriction enzyme sites within the population which results in restriction fragment length polymorphisms (RFLPs) was first discovered by Southern blotting and this later led to the discovery of other forms of variation in genomes that have been crucial to the mapping of genes and to our understanding of mutational processes.

With regard to immunology, the principal application of Southern blotting remains the investigation of the clonal rearrangements of the immunoglobulin and T cell receptor genes involved in the immune response. This can be illustrated by considering B lymphocytes, each of which carry a productively rearranged immunoglobulin allele as well as an allele with the germline structure or one that has undergone a nonproductive rearrangement. Each rearrangement event will be specific to a single cell in the population of B cells and Southern blot analysis of total B cell DNA would reveal only the germline arrangement of immunoglobulin genes as any individual rearranged alleles would be represented negligibly. As a result, bands from rearranged alleles fall far below detection limits. The structure of rearranged genes only becomes apparent by Southern blotting where there has been clonal expansion of a single progenitor B cell such as occurs in lymphoproliferative disorders. In such instances, Southern blotting may be used to characterize the structure of the rearranged gene in the expanded clone.

Southern blotting versus PCR

In many applications the use of Southern blotting has now been superseded by polymerase chain reaction (PCR) amplification which has the advantages of greater speed and sensitivity. However, PCR remains dependent on the need for precise DNA sequence data to allow the design of primers, whereas Southern blotting may be performed in the absence of such data. Depending on the design of the PCR primers and the particular gene rearrangement being investigated, PCR may fail to produce any amplification product where Southern blotting would be likely to provide some information concerning the rearrangement. Where PCR really scores, in addition to its speed and sensitivity, is in its ability to amplify genes in an allele-specific manner. Faced with such advantages from PCR, Southern blotting risks becoming a lost art.

See also: Diversity, generation of; Genetic analysis at the molecular level; Northern blotting; Polymerase chain reaction (PCR) amplification; Western blotting.

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SPECIFICITY

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The immune antibodies all have in common the property of specificity, that is, they react as a rule only with the antigens that were used for immunizing and with closely similar ones, for instance with proteins or blood cells of one species, or particular bacteria, and related varieties.

The specificity of antibodies, whose range of activity was found to extend far beyond the proteins and to include simple chemical substances, underlies the practical applications of serology and constitutes one of the two chief theoretical problems, the other being formation of antibodies. It is known, however, that the selectivity is not absolute when proteins or cells of related origin are tested with the immune serum; indeed, using chemically well defined compounds, overlapping reactions occur regularly, provided the substances are sufficiently similar in chemical constitution. The word *specificity* then signifies

that the reaction with one of the antigens, namely, that used for immunization, is stronger than with all the others.

Historical background

The above citation, from Karl Landsteiner's classical writings *Die Spezifität der serologischen Reaktionen* in 1933, summarizes experimental manifestations of specificity. The concept of specificity itself was first clearly formulated in 1906 by Paul Ehrlich who adapted Emil Fisher's earlier (1884) 'lock and key' theory of enzyme substrate interaction to immune reactions. Ehrlich stated that the affinity of an antigen for the corresponding specific antibody depended on complementarity of surface structure, analogous to that of a lock and its key. Many chemists devoted much effort to analyzing the equilibria

involved in neutralization of toxins, and in the precipitation of antigens, by antibodies. As early as 1907, Svante Arrhenius described antibody-antigen reactions in terms of simple chemical equilibria, akin to biomolecular chemical reactions.

Obermeyer and Pick in 1907 and Landsteiner and Lampl in 1917 observed that simple chemical compounds (haptens) – for example, nitrobenzene and azido groups, are capable of eliciting specific antibodies when attached to ‘carrier’ proteins. Introduction of organic chemistry into immunology broadened the research considerably and allowed relative specificities of a series of chemical derivatives to be compared. With the realization of biological importance of oligosaccharides (most importantly, the blood group glycoproteins, cf., for example, Michael Heidelberger’s work in 1935), sugar derivatives gained importance as haptens. O. T. Avery, F. Karush and E. A. Kabat carried out studies on anti-hapten antibody affinities, the role of multivalence, and the relative importance of oligosaccharide structure and length to specificity and cross-reactivity.

Peptides were used as immunogens to mimic protein antigenic epitopes in model studies aimed at the basis of antibody specificity; Crumpton and Wilkinson and Atassi were among the pioneers in this field. In 1966, Michael Sela introduced novel synthetic methods of peptide chemistry into immunochemistry, and investigated antigenicity of nonnatural D-amino acid polymers. Among his discoveries were that antibodies to peptides are often specific towards the particular spacial shape of the peptide (conformational epitopes) rather than its amino acid sequence, and that production of specific antibodies is under genetic control. Another popular hapten, 2,4-dinitrophenol, was introduced, and its antibody response thoroughly studied, by Herman Eisen in 1954. David Pressman and his colleagues assembled valuable data on stereoisomers in homologous series of organic compounds, such as arsonates and benzoyl amino acid derivatives.

In 1940, Linus Pauling proposed that antibodies are protein molecules whose polypeptide chains are able to fold around, and thereby adopt shapes complementary to, a variety of chemical structures. This hypothesis was put in doubt by experiments carried out by Christian Anfinsen and coworkers in the early 1960s. They showed that the amino acid sequence alone carries the complete information for protein three-dimensional structure. Edgar Haber and Whitney and Tanford demonstrated independently, in 1964 and 1965, that denatured antibody Fab fragments regain, when refolded, their original specificity. It thus seemed that the different antibody specificities have their origin in differences in amino

acid sequences of antibody molecules. In 1965, Hilschmann and Craig and Frank Putnam’s group were able to demonstrate sequence differences among different immunoglobulin light chains (Bence Jones proteins). Amino acid and gene sequence data that accumulated since proved beyond doubt that different amino acid sequences of immunoglobulin light and heavy chain variable regions give rise to different antibody specificities.

If elicited antibodies are polyclonal mixtures of molecules each carrying a different binding site, a question arises of whether the specificity observed in serological reactions is due to many partially overlapping but different specificities of the chemically distinct molecules. This now seems unlikely, as specificity profiles of single antibody molecules were shown not to be qualitatively different from those of polyclonal preparations.

In the last decades, atomic coordinates of several antigen-antibody complexes have become available from X-ray crystallography. These include Fab fragments complexed with small haptens (phosphorylcholine, fluorescein), medium-sized antigens (nonadecapeptide) as well as protein antigens (monoclonal Fab fragments in complex with avian lysozymes, antibody-anti-idiotypic antibody Fab complexes, Fab complexes with influenza neuraminidase and hemagglutinin, bacterial phosphocarrier protein HPr, staphylococcal nuclease). These achievements make it possible, in principle, to understand the physical basis of antibody specificity.

Essential theoretical concepts related to specificity

Specificity of protein-protein, protein-ligand, antibody-antigen, etc. interaction comes from a large difference between binding constants characterizing binding of specific and nonspecific ligands. Thermodynamically, the higher binding constants for specific ligands arise from stronger overall ‘forces’ (Gibbs free energy differences) between the antibody and the antigen.

Quantitatively, the notion of binding specificity derives from comparison of association constants, K_{AS} , characterizing the reaction



Molar concentrations of the three molecular species are measured at equilibrium, and the strength (specificity) of the complex is estimated from its relative concentration, i.e. the ratio $K_{AS} = [\text{complex}]/[\text{antibody}][\text{antigen}]$. Related expressions involve the dissociation constant, $K_{DS} = 1/K_{AS}$, affin-

ity (K_{DS} expressed in molar units) and $pK_A = \log K_{AS}$. The experimentally measured K_{DS} relates to the Gibbs free energy of complex formation, ΔG , as $\Delta G = RT \log K_{DS}$ (R , the gas constant, is the product of the Boltzmann constant, k , and the Avogadro constant, L : $R = 8.314 \text{ kJ mol}^{-1} \text{ K}^{-1}$; T is temperature in degrees kelvin). Changes in Gibbs free energy, a thermodynamic quantity, can in principle be obtained from atomic structures of all the molecules involved in the reaction, provided all the physical forces responsible for complex formation are accurately known. Traditionally, the Gibbs free energy is partitioned into two components, the enthalpy, H (the internal potential energy of the system), and the entropy, S (indicative of distribution of energy among all the different states available to the system): $\Delta G = \Delta H - T\Delta S$.

Physical origins of specificity

Biological specificity arises from a cumulation of weak noncovalent interactions between complementary molecular surfaces. According to Linus Pauling, David Pressman and others, the principal enthalpic components of Gibbs free energy of antigen antibody reactions are van der Waals interactions, hydrogen bonds and electrostatic interactions among charged protein atoms. Among the entropic effects, hydrophobic force is of utmost importance. Diminution of water-exposed nonpolar molecular surface (in, for example, antigen-antibody complex formation) releases surface-bound water molecules into solution, increasing solvent entropy as well as the free energy of the reaction. In the first approximation, the magnitude of hydrophobic force is directly proportional to the contact surface between the two molecules of the complex.

The noncovalent forces responsible for binding are short range: van der Waals forces, hydrophobic force and hydrogen bonds have vanishingly small values at distances greater than 2–4 Å. While the first two types of forces are isotropic (spherically symmetrical), hydrogen bonds are strictly directional and require orientation of the participating groups. Electrostatic forces act at a distance but the high dielectric constant of water limits their reach effectively. Thus, the trapping of charged atoms at a protein-protein interface distinctly increases their effective electrostatic field. A consequence of all this is that the two molecular surfaces, antigenic epitope and antibody paratope, will enter into a stable complex only if they have complementary molecular shapes over a large area.

Although quantitative aspects of the above biophysical components to binding are still debated,

current estimates may be summarized as follows. 1) Van der Waals or dispersion/repulsion (London) interactions probably constitute most of what is termed shape complementarity in binding, in that they would penalize intermolecular contacts that provide either overlap of atoms, or cavities, either directly or through induced strain. Well-packed contact regions at antigen-antibody interfaces, though, are probably not much more favorable than the antibody/solvent and antigen/solvent contacts experienced by the free component of the complex. Surface tension data from organic liquid/water systems shows that the work of adhesion between hydrocarbon and water interfaces is essentially the same as that between two hydrocarbon interfaces. Furthermore, dispersion forces are relatively small in magnitude, and their possible differences amount to even smaller magnitudes. 2) The hydrophobic effect is the major stabilization factor of complex formation contributing, according to various estimates, between 25 and 72 cal (6–17 J) of Gibbs free energy stabilization per 1 Å² of protein-protein contact area. 3) Electrostatics of binding involves both desolvation of surface charges and creation of new charge-charge (coulombic) interactions at the (low dielectric) protein-protein interface. Recent electrostatics calculations, e.g. the finite difference solutions of the Poisson equation of the electrostatic fields generated by the antibody and the antigen, showed the desolvation to be energetically so costly that the total electrostatic binding energy disfavors complex formation by a significant amount (e.g. ≈20 to 40 kcal in the free energy difference). 4) On complex formation, immobilization of a freely rotatable torsional degree of freedom (a side-chain exposed to solvent) carries a free energy penalty of about 0.6–0.7 kcal. If many degrees of conformational freedom are lost on complex formation, sizeable amounts of free energy need to be expended on immobilization of flexible surface groups. Hence an excessive conformational freedom of antigens and antibodies acts against the strength (and specificity) of binding. Indeed, the highest binding constants (in the picomolar range) have been observed in antibodies directed against small, rigid organic molecules such as fluorescein or digoxin, while antibodies against molecules endowed with many degrees of freedom (oligosaccharides, peptides) have binding constants which are significantly lower (typically in micromolar range).

Specificity, affinity and molecular structure

From the above discussion, a direct connection between antibody affinity and specificity emerges:

specificity is merely another manifestation of dramatic differences in antibody affinity towards the 'specific' and 'nonspecific' antigens. Surface shape and electric charge distribution of antibodies are the likely molecular determinants of specificity. Opposing electrostatic charges must approximately match each other across the protein-protein interface for the complex to be possible at all, given the large charge desolvation penalty. Although surface charge distribution (including hydrogen bond donor/acceptor pairs) can be said to be an important determinant of specificity, a substantial price, in kilocalories of Gibbs free energy difference, has to be paid for desolvation of (full and partial) atomic charges when the antigen and the antibody associate. The hydrophobic effect (i.e. nonpolar surface desolvation), and molecular surface complementarity consequential to it, then emerges as the major stabilizing force of the complex.

If antibody affinity and specificity are intimately related, it is relevant to ask whether the molecular mechanisms of specificity in low- and high-affinity antibodies are essentially the same, or whether they are significantly different. As immune response matures, the specificity spectrum of antibodies often changes from broad specificity and low affinity to narrow specificity and high affinity. Most of the detailed atomic structures of antibodies and antibody-antigen complexes available to us from X-ray crystallography are those of monoclonal antibodies selected for high affinity. Highlights of those structures are large complementary contact areas between antigen and antibody (Figure 1), attractive charge-charge interactions at the antigen-antibody interface (Figure 2) and numerous hydrogen bonds spanning the antibody-antigen interface.

In virtually all the known protein structures, binding sites have the shape of cavities or grooves. In antibodies, the antibody binding site is located at the generally concave interface between the light and heavy chain variable domains. In one case, a small hapten (phosphorylcholine) enters deeply into the interdomain pocket; in another case the principal residue of a protein antigen (glutamine 121 of the hen egg-white lysozyme) is buried in the interface, while the rest of antigen-antibody contact area is rather flat, if irregularly undulated. It is natural for the curvature of binding-site cavities to match that of the antigens: binding sites that accommodate small ligands have high curvatures and appear as pockets; those directed towards large protein antigens have a low curvature, being more akin to valleys and grooves than to deep crevices (Figure 3).

Why should the concave surface be favored as a specific combining site? Although no definitive

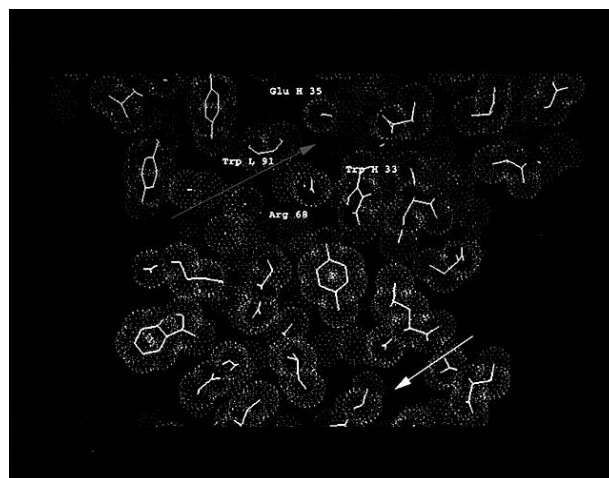


Figure 1 Surface complementarity between the antigen, hen egg-white lysozyme and the antibody HyHEL-5 binding site. In this cross-section through the antigen-antibody complex, 5 Å thick, centers of atoms are connected by sticks and atomic volumes are shown as Conolly dot surfaces. Atom color-coding on the color plate: oxygen, red; nitrogen, blue; sulfur, yellow; carbon in lysozyme, white; carbon in V_L domain, magenta; carbon in V_H domain, brown. The intermolecular interface, approximately traced by a line dividing the antigen atoms (lower part) from those of the antibody (upper part), consists of tightly packed regions, and occasional loosely packed regions, 'cavities'. The intermolecular cavities, for example the one delimited by lysozyme residue Arg 68 and the antibody residues L 91, H 33 and H 35 (blue arrow, on the top), are not any larger, however, than the occasional intramolecular cavities. Crystallographic coordinates by Sheriff *et al.* (1987). (See also color Plate 6A.)

answer is available at present, at least four good reasons can be cited. First, diffusion away from cavities is significantly slower than from flat surfaces, or through the solvent. Primitive 'binding' properties of simple concave organic molecules such as crown ethers or cavitands demonstrate this very clearly. Second, electrostatic fields are enhanced or focused in cavities, even though solvent quenches the field at other, flat or convex parts of protein surface. Third, hydrophobicity of a surface is a function of its curvature relative to the size and curvature of a water molecule, and concave surfaces are more hydrophobic than flat or convex surfaces. Fourth, a hydrodynamic drag that develops at concave sites as a ligand molecule approaches may give rise to a steering torque that forces the ligand into the pocket. The magnitude of this torque force has been as significant, perhaps even larger than, the electrostatic torque.

Induced fit and crossreactivity

Do antigens and/or antibodies change structure on binding ('induced fit hypothesis', first explicitly proposed for antibodies by Pauling in 1940) or is the binding essentially an association of two rigid bodies

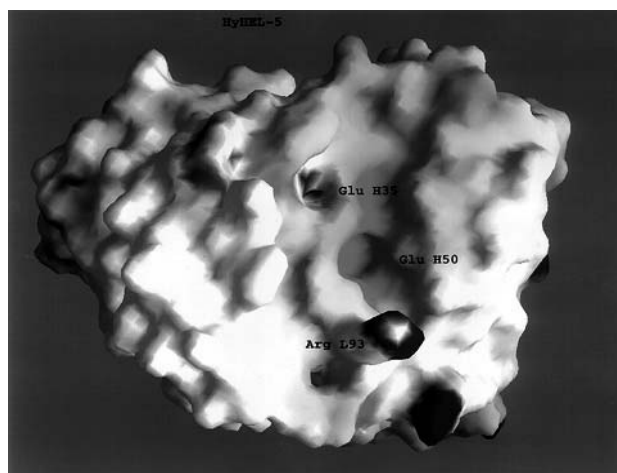


Figure 2 Electrostatic fields at the surface of the HyHEL-5 antibody binding site. This monoclonal antibody is specific to lysozyme, a protein with an exceptionally high positive charge. Using the crystallographic coordinates of the HyHEL-5 V_H and V_L domains (Sheriff *et al.*, 1987), molecular surface envelope was generated by the program GRASP (A Nicholls, Columbia University, 1991) and was color-coded by the electrostatic field produced by all the formally charged residues in the molecule (cf. the color plate: red, negative potential; blue, positive potential; color intensity proportional to the strength of the field). At the center of the binding site there is a deep, electronegative cavity 'below' which is a protruding side-chain of the Arg residue L93, with a weaker, positive, electrostatic field. In the HyHEL-5-lysozyme complex, the central negatively charged cavity becomes occupied by the two positively charged lysozyme side-chains, Arg 45 and Arg 68. The positive field generated by the antibody residue Arg L93 gives rise to a dipole, close to the antibody surface, perhaps helping to preorient the antigen at its approach to the binding site. (See also color **Plate 6B**.)

(the 'lock and key paradigm' of Fischer)? The X-ray crystallographic structures in fact reveal both types of binding modes. Examples of lock and key complexes, currently much more numerous, include the McPC 603 antiphosphorylcholine myeloma, D1.3 antilysozyme and the NC41 and NC10 antineuraminidase monoclonal antibodies, and the 26-10 antidi-oxin antibody. The best examples of large induced-fits on complexation are the anti-peptide antibodies 17/9 and 50.1 where the whole H3 loop pivots as if on two anchor points. Given the relatively high cost of conformational entropy (≈ 0.6 kcal per aliphatic side-chain torsion or 0.4 kcal per backbone torsion, see above), it may well be that those antigen-antibody systems where tight binding is required evolved towards rigid (lock and key) binding.

May the induced fits be operational in cross-reactivity? The only structurally well-documented case of cross-reactivity among haptens is that of the high-affinity ($\approx$ nM) mouse monoclonal antiprogestosterone DB3 antibody, which binds four different progesterone-like steroids with comparable affinities. X-ray

crystallography of these cross-reacting antigen-antibody complexes showed no changes to the gross (i.e. backbone) structure of the binding site on engaging the various ligands. Instead, cross-reactivity was accompanied by two distinct modes of hapten orientation (i.e. the steroid β ring with two methyl groups) with respect to the Trp side chain H50, one of the major hapten-contacting residues (**Figure 4**). Incomplete binding surface complementarity between the haptens and the antibody, and limited side-chain rearrangement(s), seemed to be the key structural feature that facilitated cross-reactivity.

Cross-reactivity among macromolecules (proteins) presents more difficult issues, if only because the molecular sizes and surfaces involved are much larger than those of small haptens. The fact that a delimited patch of protein surface can support several overlapping and different epitopes has, by now, been well established. There are two cases now where cross-reactive epitopes could be studied in atomic detail: 1) a degenerate binding of the same antigenic motif in the influenza N9 neuraminidase by two different monoclonals, NC41 and NC10; and 2) the two different antigens, the hen and guinea fowl lysozymes, engaged by the same monoclonal antibody F9.13.7. Neither case disclosed any obvious chemical features, or similarities, between the different epitopes, or the different binding sites, that would predispose them for cross-reactive binding. In the N9 neuraminidase complexes, $\approx 80\%$ of the NC41 and NC10 epitopes overlapped and one might have expected about two-thirds to three-quarters of the 'energetic' (see below) neuraminidase residues to be identical, and to contribute comparable binding energies, in the two complexes. This was clearly not the case, however, as established both by experiment and by calculations. The two antibodies, NC41 and NC10, engaged different side-chains of the same neuraminidase surface to create stable complexes (cf. **Figure 5**). A question thus arises of whether an area of a protein surface constitutes a single antigenic epitope, or whether it is a multitude of different overlapping epitopes? The term 'epitope' being an operational one, the answer to this question may also be formulated operationally. Some 50–60% of protein surfaces is made of polar atoms, including those that are formally charged. The charged atoms mostly occur at prominent surface convexities so that, naturally protrusions and their polar atoms constitute the best anchor points for a firm attachment of the antibody-binding sites (see below). Depending on topography of the larger, adjacent surface area, however, and relative dispositions of the multitude of surrounding polar atoms (both capable of and, when in the complex, required to, make hydrogen bonds),

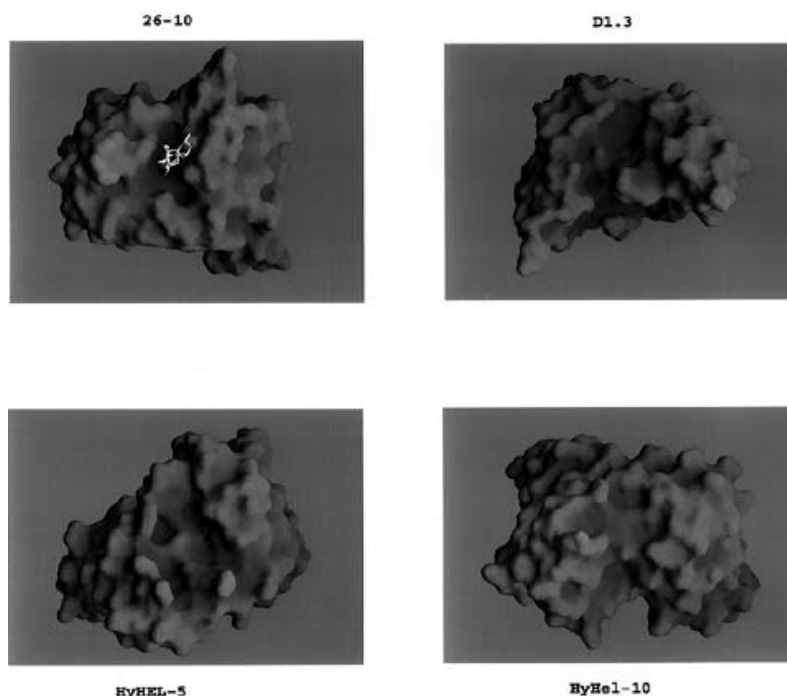


Figure 3 Comparison of antidigoxin and antilysozyme binding sites. In these 'elevation plots', points of the molecular surfaces, generated by the program GRASP, have been color-coded according to its distance normal to a three-dimensional envelope of the molecule. The envelope was defined, and constructed, as a smooth, curved plane connecting the 'highest' peaks of the molecular surface (M. Davis, manuscript in preparation). On the attached color plate, and reproduced here in black and white, the deepest protrusions on the surface of the molecule are shown in blue, the most elevated points of the surface are shown in red. The binding site of the 26-10 antibody directed against the hapten digoxin is a deep, narrow well (high 'global' curvature); the binding sites of the antilysozyme antibodies D1.3 (crystallographic coordinates by Bhat *et al.*, 1990), HyHEL-5 and HyHEL-10 (coordinates by Padlan *et al.*, 1989) are all larger and more shallow (low 'global' curvature). The D1.3 binding site contains a smaller, deep 'local' hole in its surface (complementary to the lysozyme residue Gln 121), the HyHEL-5 binding site contains a similar but shallower hole (complementary to Arg 68, cf. **Figure 1**). The HyHEL-10 elevation plot shows virtually no local depressions. (See also color **Plate 6C**.)

very many alternative solutions may exist for approximately complementary binding-site surfaces.

Functional (energetic) epitopes and cross-reactivity

The availability of X-ray crystallographic structures on the one hand and gene mutation techniques on the other makes it possible to probe into the role of individual amino acid residues in antibody specificity: a protein antigen can be mutated at a single amino acid position, three-dimensional structure of the mutant can be determined, and its affinity towards an antibody can be compared with that of the 'wild-type' antigen. Conversely, a single-residue mutation can be introduced into an antibody combining site and its effect on antigen binding can be measured. This mutational analysis of antigen-antibody interaction has been complemented with theoretical structural analysis, e.g. the rigorous free energy perturbation calculations or the semiquantitative, empirical estimates of Gibbs free energy changes accompanying antigen-antibody complex formation.

One of the major concepts emerging from the combination of the experimental and theoretical techniques has been that of an energetic (functional) epitope.

Immunochemists often observed that a small portion of an antigenic determinant was of crucial importance in defining its specificity. The term 'immuno-dominant' (Sela, 1969, quoting Heidelberger) has commonly been used to describe this phenomenon. Does 'immunodominance' have an identifiable molecular basis? The empirical Gibbs free energy calculations on antibody-antigen (and also enzyme-inhibitor) complexes consistently indicated that only a small number of amino acids, approximately 30% of the total contact surface area, contributed actively to binding energetics. In the antibodies, the bottom part of the antigen-binding cavity often dominated the energetics of binding, whereas in lysozyme, the energetically most important residues defined small (2.5–3 nm²) 'energetic', or 'functional', epitopes. Thus, a concept of protein antigenicity emerged that invoked the active, attractive contributions mediated by the energetic antigenic epitopes and the passive surface comp-

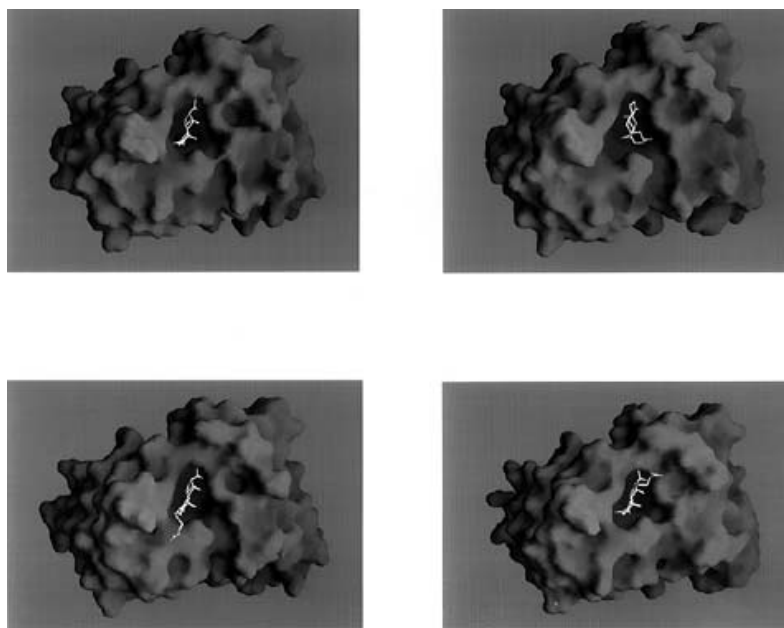


Figure 4 DB3 antibody binding site in complex with four different cross-reacting steroid haptens. The elevation plots shown here and on the attached color plate were prepared as described in the legend to **Figure 3**. Surface color-coding is from red (most elevated) to blue (the deepest). Crystallographic coordinates by Arevalo *et al* (1994). Upper left, DB3 with progesterone; upper right, DB3 with 3-aetiocholanolone (5- β -androstane-3,17-dione); lower left, DB3 with 5- α -pregnane-3- β -hemisuccinate; lower right, DB3 with 3-progesterone-11- α -OI-succinate. Haptens are shown in stick representations; carbons are color-coded white, oxygens red. It can be seen that the overall shape of the binding site does not change on engaging the cross-reacting ligands; rather, the steroids orient themselves differently with respect to the binding site cavity. At the periphery of the binding site, however, changes to the shape of the surface can be seen, due to surface side-chain rearrangements. (See also color **Plate 7**.)

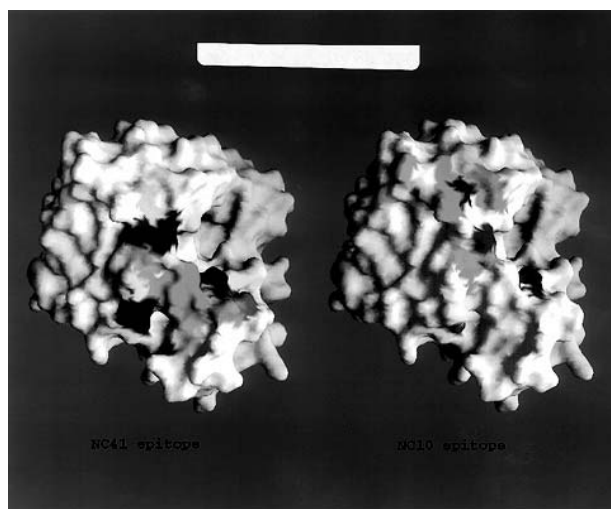


Figure 5 The two energetic epitopes (NC41 and NC10) on the surface of the influenza N9 neuraminidase. The calculated 'energetic' epitopes (Tulip *et al*, 1994) were color-coded, on the color plate, from green (residues with free energy contributions stabilizing the complex), through white ('neutral' residues), to red (residues whose contributions to the free energy of binding destabilize the complex). Although the two epitopes overlap by about 80%, their binding free energy attribution is strikingly different. (See also color **Plate 8**.)

lementarity contributed by the surrounding contact area. The concept offered a resolution of an apparent paradox: on the one hand, a multitude of side-chain-side-chain interactions at the interface (≈ 16 side-chains in the antibody as well as in the antigen) and, on the other, the experimentally derived size of the binding site no larger than 4–6 amino acids. A number of experimental data consistent with the hypothesis of an energetic epitope have accumulated, including those obtained on antibodies to the human growth hormone, human placental lactogen, the λ repressor, hen egg-white lysozyme, N9 neuraminidase, cytochrome *c* and cyclosporine.

The concept of a 'functional (energetic)' epitope has also given us an additional perspective on cross-reactivity (cf, for example, the NC41 and NC10 neuraminidase epitopes, **Figure 5**) and antigenicity. Antigenic analysis of the human growth hormone, the most extensive study of this kind currently at hand, showed that the functional epitopes correlated best with the most protruding regions of the molecule. Both the experimental data and the computer-assisted analysis of frequency of side-chains on protein surfaces accessible to large spherical probes (antibody domains) showed prominence of long, formally charged or dipolar side chains, Arg, Lys, Glu,

Gln, Asp and Asn, in the functional epitopes. The conjecture that antigenicity is mostly determined by surface protrusion provided a natural link between the two extreme antigenicity theories ('distinct antigenic epitopes exist' versus 'the whole surface is antigenic') by introducing the concept of antigenic probability that varies along the surface.

See also: Affinity; Antibodies, specificity; Antibody-antigen intermolecular forces; Antigen-binding site; Antigens; Epitopes; Hapten; Immunoglobulin structure; Monoclonal antibodies (mAbs); Antibody-antigen complexes, three-dimensional structures.

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SPLEEN

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The human spleen is a highly vascular organ which receives blood from the splenic artery and drains into the portal venous system. It is a discriminatory filter which removes aged erythrocytes and platelets from the circulation through phagocytic cells (macrophages) in a specialized section of the splenic venous compartment, the venous sinuses of the red pulp. This is a system of highly anastomosing vascular channels separated by cellular cords consisting of macrophages, lymphocytes and plasma cells with additional cells derived from the circulation, i.e. red cells, granulocytes and platelets. Another major component of the splenic parenchyma is the white pulp, which consists of lymphoid sheaths organized

around branches of the splenic artery (periarteriolar lymphoid sheaths, PALS). As in other peripheral lymphoid tissues, the PALS are organized into T-dependent and B-dependent zones. The latter are occupied by secondary lymphoid follicles with germinal centers. The periphery of the PALS, at the boundaries between the white and the red pulp, is termed the marginal zone. This displays a unique vascular organization and is important to the function of the white pulp.

Besides providing a blood cell reservoir capable of releasing erythrocytes, granulocytes and platelets into the blood when needed, the spleen functions as the site for destruction of red cells and platelets

which are phagocytosed by macrophages. The lymphoid tissue of the white pulp provides the environment for specific humoral and cellular immune responses mediated by B and T cells, predominantly against antigens circulating in the bloodstream.

Structure of the spleen

The human spleen is surrounded by a capsule of dense connective tissue containing relatively little muscle and therefore incapable of the extensive contraction exhibited by the muscular capsule of the spleen in dogs and cats. A rich branching network of trabeculae from the internal capsular surface subdivides the organ into communicating compartments. The capsule is deeply indented in one part of the spleen to form the hilus where blood vessels, lymphatic vessels and nerves enter and exit. Arterial vessels branch into the trabeculae and from there enter the pulp or parenchyma of the organ. Veins also run in the trabeculae, entering from the pulp. Lymphatics are found in the capsule and trabeculae but not in the pulp.

The splenic pulp is supported by reticular connective tissue and is mostly red due to the presence of blood, hence red pulp. This is made up by thin-walled blood vessels, the splenic sinuses (or sinusoids), and thin plates of cells which lie between the sinuses, the splenic cords. Clusters of lymphocytes are grossly visible as gray-white zones surrounded by the red pulp. This is the white pulp. These lymphoid clusters are seen around arterial branches forming the PALS. The splenic pulp, at the junction between the white pulp and the red pulp forms the marginal zone (Figure 1).

At the hilus, the splenic artery branches into trabecular arteries which leave the trabeculae and enter the PALS, where they are termed central arteries. The central artery runs out into the red pulp and usually terminates in the cords ('open' circulation) or into the sinuses ('closed' circulation) of the red pulp. Other branches of the central artery reach the marginal zone where they open into a marginal sinus and a perimarginal cavernous sinus which drain into venous sinuses. Veins of the pulp enter the trabeculae as trabecular veins which are continuous with the splenic vein which lies outside the organ.

The white pulp of the spleen is lymphoid tissue consisting of lymphocytes, macrophages and other free cells lying in a reticular meshwork and surrounding arterial vessels to form the PALS (Figure 2). These are lymphoid aggregates of cylindrical form and contain secondary follicles with germinal centers and mantle zones, similar to those found in the

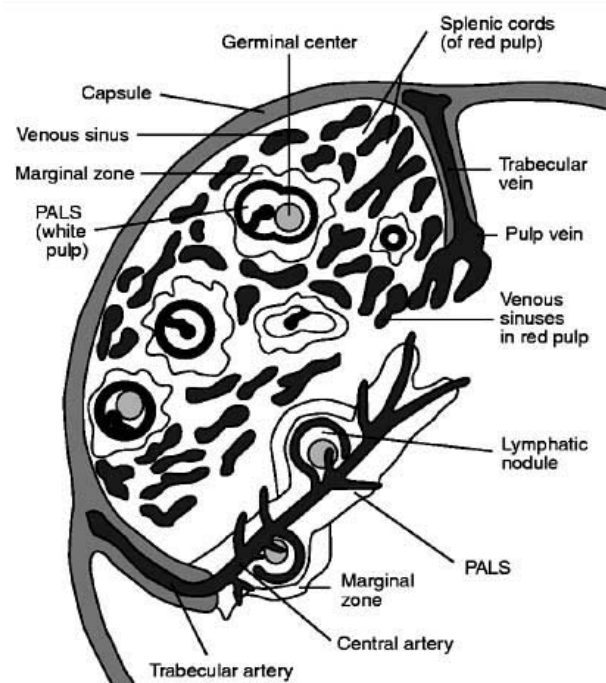


Figure 1 Organization of lymphoid tissue in the spleen. The white pulp is composed of the periarteriolar lymphatic sheaths (PALS) frequently containing germinal centers with mantle zones. The white pulp is surrounded by the marginal zone. (Marginal zones contain specialized antigen-presenting cells, macrophages and a special subset of B cells.) The red pulp contains venous sinuses separated by splenic cords. Blood enters the tissues via the trabecular artery and becomes the central artery which gives rise to many branches; some end in the white pulp, supplying the germinal centers and mantle zones, but most empty into or near the marginal zones. Some arterial branches run directly into the red pulp, mainly terminating in the cords. The venous sinuses drain blood into pulp veins and then trabecular veins. (Reproduced with permission from Roitt IM, Brostoff J and Male D (eds) (1998) *Immunology*, 5th edn. London: Mosby.)

lymph node cortex. The lymphoid follicles are also supplied with blood by branches of the central artery.

The marginal zone consists of a fine meshed spongework of reticular fibers. Its cells are arranged to form a structure similar to that of the cellular cords in the red pulp. A marginal sinus is located directly adjacent to lymphatic follicles. It consists of a series of flattened vascular spaces that lie between the white pulp and the marginal zone. A perimarginal cavernous sinus is situated outside the marginal zone or directly adjacent to the lymphoid follicles, in areas where the marginal sinus is absent.

The red pulp is a system of cellular cords lying between the venous sinuses. After distributing branches to the white pulp and marginal zone, the central artery branches into straight, slender vessels

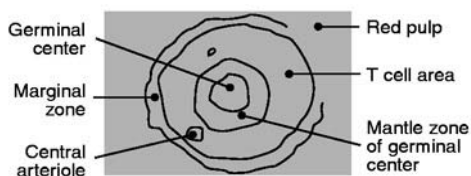
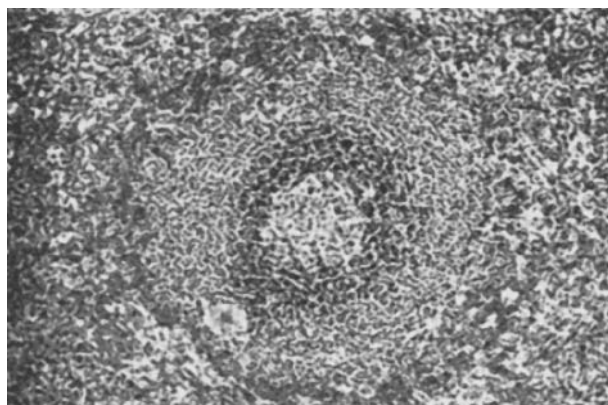


Figure 2 Spleen section showing the periaarteriolar lymphoid sheath (PALS). This view shows the lymphoid tissue arranged around an arteriole. The T cells are found close to the central arteriole and the B cell area has a germinal center. In the 'unstimulated' state the B cell area consists of a primary follicle. The lymphoid tissue is separated from the red pulp by the marginal zone. This contains blood vessels and is the site of entry of bloodborne lymphocytes into the splenic lymphoid areas. H & E stain, $\times 125$. (Reproduced with permission from Roitt IM, Brostoff J and Male D (eds) (1998) *Immunology*, 5th edn. London: Mosby.)

called penicilli which may terminate as such or become finer arterial capillaries. These terminal arterial capillaries may be surrounded by a sheath of closely packed macrophages (sheathed capillaries). A few arterial capillaries may connect with the venous sinuses, but many of them open directly in the reticular meshwork of the cellular cords. The venous sinuses are long vascular channels with a unique endothelium and basement membrane. Endothelial cells are elongated and lie parallel to the long axis of the vessel. They lie side by side with slit-like spaces separating them. The endothelium is perforated by large, regularly arranged, polygonal fenestrae. This arrangement is traditionally likened to a barrel in which the wooden staves correspond to the endothelium and the hoops to the reticular fibers that support the endothelial wall without a basement membrane. Sinuses are tributaries of the veins of the pulp, which in turn drain into the trabecular veins (Figure 1).

The cellular cords are supported by a reticular meshwork. The interstices of this cordal meshwork are typically crowded with erythrocytes, macrophages, platelets, plasma cells and granulocytes.

Macrophages, which differentiate from circulating monocytes, are often the predominant cells.

Functional anatomy of the spleen

Many arterial capillaries open freely into the cellular cords of the red pulp, consequently, plasma is separated from cells in the splenic circulation. Concentration of blood cells in the spleen enhances its storage function. Approximately one-third of the platelets of the body are normally stored in the spleen, in ready reserve. The spleen monitors circulating erythrocytes and platelets, permitting intact cells a through passage but detaining and destroying aged or damaged cells. This role is carried out in the sinuses and cords of the red pulp and is a function of macrophages. Since these cells express IgG Fc receptors, red cells or platelets coated with IgG (auto)antibodies are avidly phagocytosed in the spleen. Aged erythrocytes appear to lose sialic acid on their surface, exposing mannose and galactose residues which may be recognized in the spleen and cause such erythrocytes to be phagocytosed.

Another distinctive function of the spleen is that of clearing particulate materials from the circulation. Particles are phagocytosed in the marginal zone, cords or sheathed capillaries.

The spleen also functions as a peripheral lymphoid tissue where effector T or B cells differentiate in response to antigens. Recirculating T and B cells take a distinctive pathway through the spleen. They enter the white pulp from the marginal zone, where they are selectively released from the circulation. Within the white pulp, T cells segregate predominantly in the periaarteriolar sheaths whereas B cells home into the lymphoid follicles. Lymphocytes leave the spleen white pulp via the venous sinuses. The lymphatic vessel system does not extend to the splenic pulp but remains confined to the capsule envelope and the major trabeculae. Bloodborne antigens are taken up by the spleen and reach the white pulp via macrophages and antigen-presenting cells found in the marginal zone. It thus appears that the marginal zone is the major route of entry to the white pulp for lymphocytes seeding into the spleen and for antigens and antigen-presenting cells. It is of note that the marginal zone also harbors the majority of natural killer (NK) cells found in the spleen (5–15% of the lymphoid cells). The reason for this selective localization and the function of splenic NK cells is presently unknown. Cells in the marginal zone are predominantly macrophages. However, it has been shown that a particular subset of B cells resides in the marginal zone and these cells respond to type II thymus-independent antigens. Antibody production against

circulating antigens is a major function of the spleen. Activated B cells primed in the white pulp migrate to the red pulp where the majority of the mature plasma cells are found.

See also: Bone marrow and hematopoiesis; Lymph nodes; Lymphocyte trafficking.

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STAPHYLOCOCCUS, INFECTION AND IMMUNITY

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Staphylococci are ubiquitous organisms living on the skin and mucous membranes of humans and other mammals, on birds, certain plants, and in food. They have a spherical shape, stain gram-positive, form grape-like (Gr. *staphylos*, grape) clusters since they divide in more than one plane, produce catalase, and grow as yellow-white colonies. The cell wall, containing teichoic acids, is rigid and confers tolerance to drying, high salt and sucrose concentrations, and to certain disinfectants. About 30 different species are now recognized (Table 1). *Staphylococcus aureus* is by far the most pathogenic of these species, despite the fact that it, like other members of the genus, forms part of the normal microflora of human skin and mucous membranes. Staphylococci can produce a variety of toxins, enzymes and cell surface proteins

mediating binding to different extracellular matrix and plasma proteins, several of which have been claimed to act as virulence factors. The majority of these are antigenic and elicit production of antibodies which may confer protection to the host. Production of these substances is regulated by super-regulatory genes, such as *agr*, which in turn is controlled by environmental factors, such as the concentration of magnesium ions.

Characteristics of the organisms and their antigens

Coagulase and fibrinogen binding

An important character of *S. aureus* and a few other species is their ability to coagulate plasma and to

Table 1 Selected staphylococcal species and disease spectrum

Species	Coagulase	Characteristic	Disease spectrum
<i>S. aureus</i>	+	Human	Furuncles, wound infections Septicemia Endocarditis, osteomyelitis, arthritis, abscesses Toxin-related disorders Postinfluenzal pneumonia
<i>S. intermedius</i>	+	Animal	Furunculosis, abscesses
<i>S. schleiferi</i>	+	Animal	Furunculosis, abscesses
<i>S. hyicus</i>	Variable	Animal	Furunculosis, abscesses
<i>S. lugdunensis</i>	–	Human	Endocarditis, wound infections, arthritis
<i>S. epidermidis</i>	–	Neutropenia Biomaterial	Septicemia Localized infection with subsequent spread
<i>S. hemolyticus</i>	–	As for <i>S. epidermidis</i>	
<i>S. simulans</i>	–	As for <i>S. epidermidis</i>	
<i>S. hominis</i>	–	As for <i>S. epidermidis</i>	
<i>S. saprophyticus</i>	–	Novobiocin-resistant	Urinary tract infection
<i>S. cohnii</i>	–	Novobiocin-resistant	Biomaterial infections, urinary tract infections
<i>S. xylosus</i>	–	Novobiocin-resistant	Opportunistic pathogen

form a fibrin barrier around localized infectious foci. This is mediated by production of one enzyme, coagulase, and by three specific surface-exposed proteins which bind fibrinogen, thereby inducing polymerization of fibrinogen to fibrin.

Hemolysins

At least four different extracellular proteins have been described which lyse erythrocytes and are cytotoxic to a number of other cells. Human strains of *S. aureus* commonly produce α -lysin whereas strains isolated from bovine and other animals produce β -lysin. The δ -lysin has a broad cell specificity with amphipathic properties (detergent-like).

Leukocidin

Although α -lysin is toxic to leukocytes, *S. aureus* strains also produce a separate toxin, the two Pantón-Valentine factors, exerting synergistic toxic activity to human leukocytes.

Enterotoxins

These belong to a family of proteins of which five immunologically distinct toxins, staphylococcal enterotoxins (SE) A–E, are recognized. They cause staphylococcal food poisoning, presenting with vomiting and diarrhea (Table 2). These toxins differ substantially from enterotoxins of gram-negative pathogens, such as *Vibrio cholerae* and *Escherichia coli* which bind to a specific receptor on intestinal cells. Instead, staphylococcal enterotoxins have been suggested to bind to neurons in the gastrointestinal tract, and may act as neurotoxins. They are also immunomodulating toxins, like TSST (see below).

Toxic shock syndrome (TSS)

In the early 1980s this syndrome of fever, hypotension, a skin rash with subsequent desquamation, and symptoms from at least three organ systems was defined. It was initially related to tampon usage in young women, but since then TSS has been described in postoperative wounds and burns. One toxin, TSST-1 has been identified but also SEB and SEC can be involved (Table 2). TSST-1 has structural similarity with enterotoxins and with pyrogenic streptococcal toxins. It acts as a superantigen, like the

enterotoxins, has mitogenic properties, and induces production of specific cytokines such as tumor necrosis factor.

Staphylococcal scalded skin syndrome (SSSS)

This syndrome is an uncommon cause of epidermolysis, painful desquamation of the skin surface, mainly in infants. It is caused by phage group II strains of *S. aureus* which produce toxin A and/or B (synonyms: epidermolytic toxin, exfoliatin) (Table 2).

Extracellular enzymes

Staphylococcal strains can produce a range of enzymes, such as proteases, various nucleases and lipase which may act as spreading factors. One other enzyme, staphylokinase, activates plasminogen in a similar way to urokinase and a streptococcal kinase, forming plasmin, a potent proteolytic substance. Staphylokinase has a broad host specificity and acts also on plasmin from a number of animal species. It is produced only by *S. aureus* strains, and acts as a spreading factor by breaking down the fibrin barrier and blood clots.

Protein A

This surface exposed protein is produced only by *S. aureus* strains. When the *agr* gene is not activated, protein A is produced. It binds to the Fc part of immunoglobulin molecules, and probably confers resistance to phagocytosis. Furthermore, it mediates activation of complement and of the coagulation cascade.

Binding of fibronectin and other extracellular matrix proteins

In the late 1970s fibronectin binding by bacteria was first described for *S. aureus*. This launched intense research in the area of microbial interaction with various other extracellular matrix and plasma proteins, and expression of specific binding of fibronectin, collagen, vitronectin and laminin is now recognized as common properties of a number of different bacterial, fungal and parasitic pathogens. In *S. aureus* separate proteins have been identified

Table 2 Staphylococcal toxin-related disorders

Organism	Disease	Virulence factor(s)	Immune control
<i>S. aureus</i>	Food poisoning	Enterotoxins, SE A–E	Antibody blocks toxin
<i>S. aureus</i>	Scalded skin syndrome	Toxin A and/or toxin B	Antibody blocks toxin
Staphylococci	Toxic shock syndrome	TSST-1, SEB, SEC	Antibody blocks toxin

mediating binding of these different proteins. Fibronectin binding has been proposed to enhance tissue colonization, collagen binding to target pathogens to collagenous tissues, like joints and bone, and expression of laminin binding to enhance targeting to basal membranes, e.g. in the kidney. Vitronectin binding by microbes is of great interest since it may enhance tissue colonization, like fibronectin binding, and may enhance complement activation by blocking the inhibitory activity of vitronectin on the terminal step in the complement cascade.

Glycosaminoglycan binding

Of the glycosaminoglycan (GAG) family of molecules, heparin is present in blood whereas others, such as heparan sulfate, chondroitin sulfate and dextran sulfate, form part of the extracellular matrix (ECM). Bacteria, such as *S. aureus* and the causative agent of gastritis, *Helicobacter pylori*, bind heparan sulfate and heparin, and the cell receptor for the viral pathogen herpes simplex is heparan sulfate. Since GAGs are present in the ECM, binding to GAG molecules may enhance tissue colonization. Several growth factors, such as basic and acidic fibroblast growth factors and platelet-derived growth factor, bind heparin. Microbial binding of heparin may delay wound healing by sequestration and inactivation of growth factors.

Hemagglutination

One surface-exposed hemagglutinin has been identified in uropathogenic strains of *S. saprophyticus*. It recognizes *N*-acetyl lactosamine, and may act as an adhesin for the human urinary epithelium.

Species differentiation

The ability to coagulate plasma from various animal species clearly differentiates *S. aureus* and a few other staphylococcal species from other staphylococci. Based on biochemical tests and DNA homology studies about 30 coagulase-negative species have been identified. Of these, *S. epidermidis* is the most frequently isolated species. Strains resistant to novobiocin, an antibiotic which is not used for treatment, form a separate group consisting of *S. saprophyticus* and a few other species. Sensitivity to bacteriophages by *S. aureus* (phage typing) is used as an epidemiologic marker to follow, for example, nosocomial spread. To verify identity between strains, however, molecular biology methods such as restriction enzyme analysis of isolated DNA are required. Such methods are commonly replacing phage typing.

Antibiotic sensitivity

Staphylococci frequently acquire resistance to various antibiotics. In hospital environments the vast majority of *S. aureus* strains produce β -lactamase, and are resistant to various penicillins. Strains may acquire resistance to penicillinase-stable penicillins, such as methicillin, by incorporation of *mec* gene(s). These methicillin-resistant *S. aureus* strains, MRSA, may be resistant to several other antibiotics and to heavy metals, and constitute a serious problem in hospital hygiene. Methicillin-resistant coagulase-negative staphylococci, MRSE, may likewise be resistant only to penicillins or also to several other antibiotics. MRSE constitute an increasing problem in hospital hygiene.

Disease spectrum

S. aureus acts as a primary human pathogen and can cause localized superficial skin infections, like furuncles and wound infections. It is also an important agent of tissue-invasive infections (Table 1). After spread to the bloodstream they may cause endocarditis, e.g. in intravenous drug addicts, osteomyelitis and other deep infections. *S. lugdunensis*, though coagulase-negative, has a spectrum of disease similar to that of *S. aureus*. It was first recognized as a cause of endocarditis in the Lyon area of France, and was therefore named 'lugdunensis' (meaning 'from Lyon'). *S. epidermidis*, *S. hemolyticus* and several other coagulase-negative species are so-called opportunistic pathogens which can cause tissue-invasive infection in immunocompromised hosts. However, with the increasing use of foreign materials for temporary (e.g. intravascular and intraventricular catheters) or permanent implantation (e.g. orthopedic prostheses, heart valves and intraocular lenses), the *S. epidermidis* group has emerged as the most important agent of biomaterial-associated infections. *S. saprophyticus* is a common cause of urinary tract infection, particularly in younger women. Staphylococcal strains producing enterotoxins, epidermolytic toxins and TSST may cause different toxin-related infections (Table 2). Of these, TSST can also be produced by coagulase-negative staphylococci.

Immune responses of the host

Since staphylococci form a large part of our normal microbial flora, most people produce antibodies to various staphylococcal antigens, such as the cell wall-associated teichoic acid and the extracellular protein α -hemolysin. They do not appear to confer resistance to staphylococcal infections. The use of detection of antibodies to these antigens as a diagnostic marker

is of limited value, and requires demonstration of a significant rise in antibody titer. However, high levels of antibodies to TSST, enterotoxins and exfoliatins seem to be protective. Since these toxins are recognized as immunomodulatory, with mitogenic activity and inducing cytokine production, they may modulate the immune response. In biomaterial-associated infections staphylococci are embedded in a polysaccharide matrix on the biomaterial surface, the so-called biofilm, which prevents presentation of antigens to the host. Hence, no diagnostic antibody test is available for these kinds of infections, and no protective antibodies have been identified.

Vaccines

Research activity to develop a vaccine against *S. aureus* infections up to the 1960s was in vain, and interest in this field was fading during the 1970s. However, with the emerging spread of antibiotic resistance among staphylococci we encounter a new interest to pursue this nonantibiotic approach to pre-

vent staphylococcal infections. So far, no promising vaccine candidate has appeared.

See also: Bacteria, Immunity to; Toxins.

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STATISTICS, USE IN IMMUNOLOGY

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Immune response data are usually analyzed in one of two basic forms: the individual concentrations or titers themselves (often subsequent to data transformation) or the proportion of subjects responding to a stimulus in a prescribed manner.

Analysis of concentrations or titers

Measures of antibody responses to vaccines and other immune stimuli are typically highly skewed. Thus, they tend to violate the normality requirement for applying statistical methods based on the normal distribution. However, these data are frequently log-normal, which means that they tend to be approximately normally distributed on the log scale. Frequently used transformations are the natural log, log to the base 2, or the common log (base 10). Statistical analyses requiring normally distributed data are then applied to the log-transformed data.

Commonly used methods

Geometric mean (GM) Because of skewness, the arithmetic mean is usually not an accurate measure

of central tendency. Other ways of characterizing skewed data are the median and its confidence interval and various other percentiles.

One of the simplest ways to summarize immune response data is by the geometric mean and its confidence interval. The GM of a group of n concentrations is the n -th root of the product of the n concentrations. It is usually calculated, however, as the antilog of the arithmetic mean of the log-transformed data. For untransformed concentrations $C_1, C_2, C_3, \dots, C_n$, the GM is calculated as

$$GM = \text{antilog} (\Sigma \log C_i / n)$$

Confidence limits are obtained by finding the antilogs of the lower and upper limits calculated from the log-transformed concentrations. Confidence intervals are informative in virtually all evaluations, but are especially recommended for assessing equivalence or similarity of groups being compared.

When a few immune responses are recorded as zero and thus cannot be log transformed, it is usually

possible to find a constant which, when added to all data values prior to transformation, produces data that are approximately normal. This constant is subtracted from the calculated GM and both confidence limits to compensate for being added before transformation.

***t*-Test and ANOVA (analysis of variance)** Student's *t*-test is used when two independent groups are compared, while the ANOVA extends the *t*-test to more than two groups. Both methods are parametric and assume normality of the data and equality of variances across comparison groups. Both analyses are performed on log-transformed data and compare the means of the groups. The variable analyzed may be either the log fold increase (baseline log concentration subtracted from poststimulation log concentration) or the log concentration.

The ANOVA is also appropriate when there is more than one explanatory factor, in which case a multifactor ANOVA may be used. ANOVA models may be parameterized in several different ways.

ANCOVA (analysis of covariance) If adjustment for a continuous baseline variable is desired in conjunction with a *t*-test or ANOVA, then ANCOVA may be appropriate. Baseline concentrations or subject characteristics, such as age, are frequently controlled baseline covariates in immune response analysis.

Regression This method is used when interest is in the association between immune response and one or more explanatory factors. The regression model assumes that such an association exists and attempts to describe the nature of the relationship quantitatively. This method is also useful if interest is in predicting immune response based on some associated factor, such as age, maternal antibody level, preimmunization antibody level, etc.

Nonparametric methods There are various nonparametric methods of analysis available, which do not require normality and in general make fewer assumptions about the data than do the above methods. The Mann-Whitney-Wilcoxon test, and Kruskal-Wallis test are nonparametric methods commonly used to analyze immune response data. These methods test for differences in central tendency between groups being compared. When there are only two groups being compared, the Kruskal-Wallis test is equivalent to the Mann-Whitney-Wilcoxon test. When differences between only two groups are assessed, these methods are the rank analogs of the two-sample *t*-test. When there are more than two

groups, the Kruskal-Wallis test is the nonparametric analog of the ANOVA.

Advanced methods

Repeated measures analyses If immune response data are collected longitudinally (over time) from the same individuals, the data may be modeled through repeated measures analysis. Such methods may be useful for examining the association between one or more covariates and immune response over time. This method differs from the usual multiple regression (which assumes no correlation among measurements) in that the correlations inherent in measurements collected on the same individuals at multiple time points are accounted for in the analysis. These models may contain fixed or random effects, or both, depending on the desired scope of inference. A type of repeated measures analysis, a Bayesian random-effects regression model for longitudinal data, has been used to estimate the appropriate timing of revaccination with hepatitis B vaccine, based on postbooster antihepatitis B virus surface antigen titers measured over time. There are other types of longitudinal methods, such as generalized estimating equations (GEE), which also may be applied to immune response data measured over time.

Percentile regression This is another methodology which has been applied to longitudinal immune response data. It is a repeated measures type of analysis which allows the estimation of specific percentiles of the immune response distribution for given covariate values. This method of analysis may be informative when interest is in effects on different levels of the distribution of immune responses.

Mixture models Often when log transformation of immune response measurements fails to produce data that are approximately normally distributed, it is because a larger-than-expected number of the responses are left-censored (lie below the lower limit of assay detection). In such cases, there may be a mixture of two different distributions characterizing the data – one for responses above the detection limit and another for those below. A Bernoulli/lognormal mixture model has been applied to the analysis of antibody data from a high-titer measles vaccine, the Bernoulli representing a point mass distribution characterizing the nonresponders, and the lognormal assumed for the remaining censored parametric continuous distribution. Response distributions other than the lognormal also may be appropriate.

Analysis of proportions responding

Another widely used approach to evaluating immune response is through the proportion of individuals responding in some specified manner. For a single group of subjects, a meaningful analysis is simply the point estimate of the proportion responding and its confidence interval. The following methods are for comparing two or more groups, or for evaluating the effects of covariates.

Commonly used methods

Chi-square and Fisher's exact test Both these methods compare proportions responding in a specified manner to immune stimuli. Fisher's exact test is used alternatively to the chi-square.

Advanced methods

Logistic regression Logistic regression differs from simple or multiple regression in that the response variable is discrete and typically dichotomous rather than continuous, and also in that the distribution of the errors is binomial rather than normal. Thus, this method is adaptable to the study of binary immune response status (e.g. positive or negative). It may be used to assess the association between immune response and one or more explanatory factors, or it may be employed for classifying subjects as does discriminant analysis (see below).

Proportional hazards (Cox) regression This method, a type of survival or time-to-event analysis, has been used to evaluate the effect of covariates on time to seroconversion. Survival methods are especially useful when individual subjects in a study are not followed for equal periods of time, or when there is censoring (which occurs when an individual is lost to follow-up during the study). In order for this analysis to be informative about serological data, blood samples must be drawn at numerous time points. However, it may not be possible to know precisely when seroconversion actually occurs – only when it is discovered through a laboratory test.

Discriminant analysis This technique has been used to assess the effects of covariates on seroconversion status in response to vaccination. The basic purpose of discriminant analysis is to classify an individual with specified covariate characteristics into one of two or more population groups. In serological analysis, these groups are usually only two, responders and nonresponders. The linear discriminant function is appropriate when the explanatory variables in

each comparison group are approximately multivariately normally distributed with equal variances/covariances. If explanatory factors are qualitative rather than continuous, then logistic regression, which does not require normality, may be preferable.

Assay comparison studies

Frequently, immune responses of the same group of individuals are measured in different laboratories, perhaps using different assay techniques. Or a new assay may be compared to an established assay. In both cases, it is of interest to compare the different assay methods.

Correlation coefficient Pearson's correlation coefficient is commonly used in assay comparison studies. It is often, however, misinterpreted. The correlation coefficient is properly interpreted as a measure of association only. It is not a measure of agreement. Thus, high correlation cannot be taken as evidence that two assays yield the same results; it is a necessary, but not sufficient, condition for concluding that two sets of values are equivalent.

Error-in-variables model If assays of immune response could be measured with great accuracy, then two assays could be compared via a simple linear regression model. Since biological assays are rarely very precise, a model which incorporates this imprecision, or error in both measured variables, is usually recommended. The error-in-variables model is of the form

$$Y_i = \beta_0 + \beta_1 X_i + (\epsilon_i - \beta_1 \cdot \delta_i)$$

where X and Y are the results observed using assays X and Y , respectively, and δ and ϵ represent random error components of the observed X and Y values, respectively. The parameters to be estimated, β_0 and β_1 , represent the constant and proportional biases, respectively, inherent in estimating values of assay Y from those of assay X . β_0 and β_1 are estimated by the method of maximum likelihood, given that an estimate of the error variance ratio $\lambda = \sigma_\epsilon^2 / \sigma_\delta^2$ is known.

Graphical methods

Data analysis is enhanced by visual displays of the data. Although there is a large repertory of graphical techniques available, the following methods are those commonly used and informative in the understanding of immune response data. Examples of

these graphs can be found among the references suggested for further reading.

Histogram (frequency distribution, bar diagram) This graph is a simple, well-known tool for visualizing many types of data. Histograms are especially useful as one of several ways of assessing the normality of data when parametric methods requiring normality are used.

Pre- versus postimmunization concentrations In vaccine studies, prevaccination antibody concentrations are sometimes plotted on the horizontal axis against postvaccination concentrations on the vertical axis, usually on the logarithmic scale. A person's prevaccination and postvaccination concentrations are represented on the graph by a single point. A 45-degree line drawn from the origin of the graph represents nonresponse, and other lines drawn parallel to it indicate n -fold responses. This type of graph allows visual detection of unusual pre- versus post-immunization associations and distributions.

Cumulative distribution plot (also called ogive) This graph is based on the cumulative distribution function (CDF):

$$C_x(x) = \Pr(X \leq x)$$

where X is a random variable, and the expression on the right means 'the probability that X is equal to or less than a specific value x '. The horizontal axis represents the range of immune response values of interest, often on the logarithmic scale, while the vertical axis indicates percentages, from 0 at the bottom to 100 at the top, of subjects having equal or lesser response values. If two groups of responders are being compared, the difference between their CDFs may be statistically analyzed via the nonparametric Kolmogorov-Smirnov test. The Kolmogorov-Smirnov statistic also may be obtained through another graphical technique called the pair chart.

Reverse cumulative distribution (RCD) plot This graph is based on the function

$$R_x(x) = \Pr(X \geq x)$$

where now the expression on the right means 'the probability that X is at least a specific value x '. RCD plots are especially useful in vaccine studies, because interest is mainly in determining the proportion of subjects who meet or exceed some threshold level of response. The horizontal and vertical axes are the same as those of the CDF plot.

Note that $R_x(x)$ is similar to the well-known survival function $S_x(x)$ given by

$$S_x(x) = \Pr(X > x)$$

differing only in the equality sign on the right-hand side of the equation. When immune response is measured on a continuous scale, $R_x(x) = S_x(x)$, and survival analysis techniques, such as a logrank test, may be used to compare two RCDs statistically, if the curves do not cross significantly. Alternatively, when the scale is continuous $R_x(x) = 1 - C_x(x)$, so that the RCDs also may be analyzed by the Kolmogorov-Smirnov test. When response data are discrete, $R_x(x)$ and $S_x(x)$ may differ enough that a logrank test should be applied to the $S_x(x)$ functions instead.

See also: **Enzyme-linked immunosorbent assay (ELISA); Immunoassays; Limiting dilution analysis.**

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STREPTOBACILLUS, INFECTION AND IMMUNITY

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The genus *Streptobacillus* appears little related taxonomically to other bacteria. There is a single, reasonably well-characterized species, *S. moniliformis*. Other, ill-defined though probably related organisms have also been recovered from pathological material, including pneumonia in calves (*S. actinoides*). These *S. moniliformis*-like agents are heterogeneous with protein patterns quite distinct from those of typical *S. moniliformis* and their taxonomic position, distribution and pathogenicity is obscure.

The genus contains Gram-negative, facultatively anaerobic, nonmotile, nonacid-fast, typically pleomorphic rods, whose appearance depends on, and varies with, their age and environment. Modally the slender cell (up to 1.5 µm long) has pointed or rounded ends and may possess mid-length a fusiform or bulbous swelling. Long, wave chains or filaments, often also with the swellings and up to 150 µm long, produce floccules and impart a 'breadcrumb' appearance to growth in broth. In culture the organism is fastidious and requires for growth (which is slow even at the optimum temperature 35–37°C) rich media liberally supplemented by native protein, a humid environment and possibly enhanced amounts of CO₂; strictly anaerobic isolates are known. There is no growth in milk. During growth the bacteria possess the striking property of reversibly producing L-forms or transitional-phase variants *in vivo* and *in*

vitro. This behavior might be related to the low content of glucosamine and muramic acid in the wall of the modal cell. The bacillary form is sensitive *in vitro* to the antimicrobial drugs in common use clinically, sulfonamides apart.

Despite their moderate importance as pathogens of animals and their zoonotic potential, streptobacilli are relatively unexplored in all respects. Most aspects of their taxonomy, nutrition, biochemical behavior, antigenic structure, epidemiology and the molecular basis of their pathogenicity await detailed investigation.

The antigenic structure of *S. moniliformis* has been little studied by modern procedures. Antisera reacting with the whole cell have been described and a high-titered humoral response, recognized by agglutination or complement fixation, is mounted by clinical cases in humans or in experimentally infected animals. The superficial structures of the bacilli contain about 64% protein, 28% lipid and 6% carbohydrate and there is at least one antigen present which is absent from the L-phase. The protection conferred experimentally on mice against infection with the bacillary phase might be attributable to this antigen(s), as vaccine prepared from the L-form is unprotective. Scans of whole-cell lysates of strains of *S. moniliformis* by SDS-PAGE indicate the presence of some 40–50 proteins ranging from 18 to 100 kDa.

All strains share four major components accounting for 20–30% of the total protein and which are about 60–67 kDa. Variability among 31 isolates from various species was apparently greatest in two regions, namely that encompassing those proteins >67 kDa and that involving those about 42–44 kDa. The role of these components in pathogenicity, pathogenesis and immunity needs detailed examination.

Streptobacillus moniliformis is distributed worldwide and apparently has its principal habitat in the oronasopharynx of rodents, particularly the laboratory rat, which appear clinically healthy. The prevalence of the organism in the wild or the laboratory rat is not precisely known; the agent may be present in half the latter. *Streptobacillus moniliformis* is accorded significance mostly because it is naturally pathogenic for humans. The disease it produces takes two forms. One is streptobacillary fever, which is acquired through a rat bite. The other is Haverhill fever, which occurs in small, common-source epidemics. Clinically, either form of the disease in humans exhibits protracted pyrexia, skin rashes and arthralgia. Before antibiotic treatment was available the disease tended to run a prolonged course and had as sequelae endocarditis, pneumonia, abscesses in various organs and a persistent, nonsuppurative arthritis. A putative association between sporadic streptobacillary fever and the weasel, squirrel, dog, cat and pig has been recorded. The possibility of such species constituting reservoirs of the infection for humans is uninvestigated but seems unlikely in at least some instances. For example, dog and cat bites in humans are common but rarely if ever have as a sequel the distinctive clinical syndrome associated with infection by *S. moniliformis*. Whereas the epidemiology of streptobacillary fever is clear, with infection being established in the right circumstances by the bite of a rat, the epidemiology of the rare Haverhill fever is more obscure. Haverhill fever is often attributed to the consumption of contaminated milk or water but there is no clear-cut evidence that rodents shed the agent in urine or feces. The circumstantial association between the disease and the consumption of raw milk or untreated or inadequately treated water, presumably contaminated with rodent dejecta, is certain, but the direct microbiological link was incomplete in the two clearly established outbreaks investigated and reported.

Streptobacillus moniliformis is no mere commensal of rodents. Spontaneous epizootics of disease have been recorded in the laboratory rat (taking the forms of bronchopneumonia, middle-ear disease and conjunctivitis), the laboratory mouse (fetal absorption, abortion, septicemic disease, polyarthritis) and the guinea pig (cervical adenitis). Spontaneous dis-

ease (tenosynovitis, arthritis) in turkeys also occurs and outbreaks have been traced to the bites of wild rats, the agent in the turkeys and trapped rats being bacteriologically and antigenically the same, as determined by agar-gel diffusion tests.

Immune responses of host

The course of the disease in human beings and other animals, the possibility of persistent sequelae and the relative high mortality rate before the antibiotic era all suggest that the immune response to infection with *S. moniliformis* is imperfect. Humans and other animals obviously mount an immune response to the infection but which components are important in its curtailment is uncertain and whether the resolution of an established infection depends upon antibody production alone is untested. Experimentally, antibody (passive or active) also incompletely diminishes the consequences of challenge with the organism in the mouse.

Evasive strategies by the organism

In the susceptible host episodes of bacteremia occur and the organism establishes itself in, for example, the central nervous system, endocardium, other serosae and bones and joints (particularly in younger animals), where it is possibly sheltered from immune mechanisms to some extent. Macrophages readily phagocytose even the unopsonized organism but are destroyed by it through unknown processes. Thus the host counters the infection inadequately or slowly, partly for such anatomical or physiological reasons.

Vaccines

Experimentally in mice even simple inactivated preparations of *S. moniliformis* induce a measurable but incomplete protection against challenge. However, the apparent rarity of clinical disease in humans or other animals from infection with *S. moniliformis* has meant that little attention is paid to immunization and vaccination against it. This position is unlikely to change. Hence studies of the molecular biology or immunology of *S. moniliformis* and the disease it produces lack impulsion. The one possible exception exists in colonies of experimental rodents. In that case processes more certain than immunological procedures can be used to eliminate the infection and the disease through the establishment of barrier-maintained, pathogen-free stock.

See also: Bacteria, immunity to; Bacterial cell walls; Circulatory system infections.

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STREPTOCOCCUS, INFECTION AND IMMUNITY

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The genus *Streptococcus* is composed of a number of species that cause disease by means of a wide array of pathogenetic properties, including stimulation of an intense inflammatory response by activating the complement cascade, production of toxins and tissue-damaging enzymes, and initiation of immune-mediated diseases such as rheumatic fever or glomerulonephritis (Table 1).

Various components of the streptococci are important for determining virulence and stimulating an immune response, as well as for classification by serologic means. When present, the outermost

component of many streptococci is a hyaluronic acid capsule which interferes with ingestion by phagocytic cells. This capsular material resembles ground substance in the connective tissue of the host, and is, therefore, essentially nonantigenic. The outermost part of the cell wall contains two antigenically distinct major protein fractions, M and T proteins; M proteins are located on fimbriae that extend radially from the cell wall through the capsule. Based on differences in these proteins, more than 80 serotypes of group A streptococci have been identified, with little serologic cross-reactivity among them. The M

Table 1 Examples of mechanisms by which streptococci cause disease

Organism	Disease	Pathogenesis	Immune control
<i>S. pyogenes</i>	Cellulitis, fasciitis	Enzymes (hyaluronidase) destroy tissues; streptococcal pyrogenic exotoxin β degrades extracellular matrix proteins	Antibody to M protein promotes phagocytosis
<i>S. pyogenes</i>	Scarlet fever	Toxins (pyrogenic exotoxins) damage capillary integrity	Antibodies block toxin (neutralize?)
<i>S. pyogenes</i>	Acute glomerulonephritis	Circulating immune complexes deposit in glomeruli of kidney	
<i>S. pyogenes</i>	Acute rheumatic fever	Antibodies to M proteins are reactive with cardiac antigens	
<i>S. pneumoniae</i>	Pneumonia	Intense inflammatory response fills alveolar spaces	Antibody to capsular polysaccharide promotes phagocytosis

protein is a virulence factor impairing phagocytosis by preventing the interaction of the organism with complement components, an effect that is enhanced by the ability of M protein to precipitate fibrinogen on the surface of the organism. Strains that are deficient in M protein are avirulent; they are phagocytosed and killed by polymorphonuclear leukocytes (PMNs) in the absence of type-specific antibody. Type-specific antibody to M protein facilitates phagocytosis, is protective against reinfection and imparts longlasting immunity. The T proteins are useful for typing of organisms, as in epidemiologic studies, but appear to play no role in immunity. In close association with M proteins in the fimbriae of streptococci are lipoteichoic acids which interact with fibronectin on the surface of mammalian cells and determine bacterial adherence.

Interior to the protein layer is the group-specific carbohydrate, which enables β -hemolytic streptococci to be distinguished, by immunologic tests, into approximately 20 groups designated A through H and K through V (Lancefield groups). Although antibodies to group carbohydrates do not appear to contribute to immunity, they may have a role in nonsuppurative immunologic sequelae of streptococcal infection because of cross-reaction with mucoproteins in human tissues.

The inner layer of the cell wall consists of the basic mucopeptide structural unit common to all bacteria. This peptidoglycan forms the rigid skeleton of the cell wall, giving the bacteria their shape. When injected into rabbits, peptidoglycan produces effects much like endotoxin as well as causing a generalized Shwartzman reaction. Antibody to peptidoglycan is cross-reactive with a number of different gram-positive organisms. Peptidoglycan has been said to be the cell wall component that most vigorously activates the alternative complement pathway; streptococci produce disease in part by evading phagocytic defenses and then activating complement.

Streptococci elaborate a number of toxins and extracellular enzymes which also contribute to the clinical manifestations of disease. Streptococcal pyrogenic exotoxins (erythrogenic toxins), elaborated by bacteriophage-infected strains, are responsible for the characteristic rash seen in scarlet fever. Antibody produced in response to pyrogenic exotoxin is protective against reinfection, accounting for the rarity of this syndrome in adults. The streptococcal toxic shock syndrome may be due to one or several superantigens elaborated by streptococci, including pyrogenic exotoxins A, B, C and streptococcal superantigen. These molecules have significant amino acid homology with the staphylococcal enterotoxins A through F and toxic shock syndrome

toxin 1 and share several biologic functions, such as pyrogenicity, enhancement of susceptibility to endotoxin shock and suppression of immunoglobulin production. Subsequent stimulation of T cells results in elaboration of the cytokines interleukin-1, interleukin-2, interleukin-6, interleukin-8, tumor necrosis factor α , and interferon γ which may be responsible for many of the signs and symptoms observed in the streptococcal toxic shock syndrome.

Streptococci infect skin and soft tissues, causing erysipelas, cellulitis and/or fasciitis. Pharyngitis and tonsillitis occur when soft tissues in the pharynx are infected, and peritonsillar abscess may result. These organisms also cause pneumonia, pericarditis, meningitis and urinary tract infection. Disease results from intense stimulation of inflammation and damage to tissues by extracellular toxins and enzymes. Streptococcal pyrogenic exotoxin B, for instance, is a cysteine protease which is well conserved among most strains of *S. pyogenes*, and cleaves fibronectin and degrades vitronectin, two extracellular matrix proteins present in most tissues. Streptococci may also resist efforts at control by the host by producing a C5a peptidase that cleaves surface-bound C5a and, consequently, delays migration of PMNs into infected foci, or a leukocidin that may kill PMNs following ingestion of streptococci.

Antibodies to more than 25 extracellular products appear after streptococcal infection. Five of these antibodies have been especially useful in serodiagnosis: 1) antistreptolysin (ASO); 2) anti-DNase B; 3) antihyaluronidase; 4) anti-NADase; and 5) anti-streptokinase. Immune responses to these extracellular products differ somewhat, depending on the focus of infection. After an episode of pharyngitis, 95% of individuals develop antibodies to multiple antigens and 80% have a fourfold increase in ASO titers. Conversely, ASO responses are slight or absent after skin infection, probably because streptolysin O becomes inactivated by skin lipids. Anti-DNase B responses, on the other hand, are brisker after pyoderma than after pharyngitis.

Group A streptococcus

Several immunologic diseases result from infection with *S. pyogenes*, the group A streptococcus. Acute glomerulonephritis, characterized by hematuria, proteinuria, hypertension and edema, may appear 6–14 days after infection caused by a limited number of group A streptococcal serotypes. Attack rates are related to the magnitude of the immune response, with high levels of antibodies to streptococcal cell membrane antigens being found in the serum of affected patients. Electron microscopic demon-

stration of subepithelial lumps in renal biopsy specimens, along with hypocomplementemia, suggests that the renal injury is due to deposition of preformed immune complexes. The identity of the streptococcal components involved in this disorder remain unknown.

Acute rheumatic fever, a syndrome of migratory arthritis, carditis and/or chorea appears about 18 days after pharyngitis due to a group A streptococcus. Several theories regarding the pathogenesis of this disorder exist, but most widely accepted is that autoimmune phenomena are responsible, based on the similarity of streptococcal antigens to various human tissue antigens. Strains of group A streptococci that cause rheumatic fever appear to have in common M proteins with an antigenically conserved surface-exposed domain. Epitopes of these M proteins have been shown to share antigenic determinants with cardiac muscle myosin or sarcolemmal membrane antigens.

Guttate psoriasis is a well-described form of psoriasis characterized by the abrupt appearance of small psoriatic lesions over much of the skin; it occurs primarily in children and young adults a few weeks after streptococcal pharyngitis. This disorder appears to be a result of superantigenic stimulation of T cells by streptococcal pyrogenic exotoxin C with subsequent selective accumulation of $V\beta 2^+$ T cells in the areas of the skin lesions.

Pneumococcus

Streptococcus pneumoniae (pneumococcus), the most frequent cause of bacterial pneumonia, can be singled out from the other streptococci. The surface layer of pneumococci consists of a polysaccharide coating or capsule which enables these bacteria to elude ingestion and killing by macrophages and PMNs; some 90 antigenically different capsular types have been recognized. Pneumococci commonly colonize the nasopharynx of healthy children and adults, generally persisting for 6 weeks to 6 months. Invasion may occur soon after colonization as a result of many poorly defined factors, including concurrent, nonbacterial upper respiratory infection and numerous other conditions that compromise immunologic capacity. Once tissue barriers have been penetrated, pneumococci readily proliferate. Cell wall constituents (especially teichoic acid) and capsular polysaccharide activate the complement cascade via the alternative pathway. Antibody to cell wall constituents is present in nearly all normal adults because common antigens are shared among gram-positive bacteria; the resulting antigen-antibody reaction also activates complement by the

classical pathway. This activation generates C5a, leading to an intense inflammatory response which, in pneumococcal pneumonia, is the principal component of the disease. Some pneumococcal toxins may also play a role. For example, pneumolysin damages ciliary action and stimulates ingress of PMNs, causing many of the histologic features of disease in experimental animals.

The most effective mechanism by which the normal host can resist pneumococcal infection is by producing immunoglobulin G (IgG) or IgM that reacts with capsular polysaccharide. Such antibody opsonizes these bacteria for ingestion and killing by so-called professional phagocytes. Anticapsular antibody appears after colonization or infection. Nevertheless, most adults lack antibody to the majority of commonly infecting pneumococcal serotypes, indicating a relatively low level of resistance in the community.

Normal adults have ample antibody to phosphocholine-containing determinants of pneumococcal cell wall. However, the reaction between this antibody and the bacterial wall takes place beneath the capsule, and the resulting complement fragments and bound Fc are not accessible to phagocytic cells. Despite demonstration of a protective effect of antiphosphocholine antibody in experimental animals, this mechanism is generally thought not to be protective in human subjects.

As a result of these considerations, it is not unexpected that individuals who have impaired capacity to produce immunoglobulin are especially susceptible to pneumococcal pneumonia. Included are those with congenital or acquired hypogammaglobulinemia, acquired immune deficiency syndrome, multiple myeloma, lymphoma, chronic renal insufficiency or chronic liver disease. Probably the greatest increase in pneumococcal pneumonia is seen in persons infected with the human immunodeficiency virus. Pneumococcal pneumonia occurs with greatly increased incidence in individuals who have pre-existing bronchopulmonary disease, either acute such as influenza, or chronic such as bronchitis or emphysema, because local factors that might allow clearance of inhaled pneumococci are not operating normally. Individuals who lack a normal spleen are at risk of overwhelming sepsis once pneumococcal infection occurs.

Vaccines

Development of vaccines to prevent streptococcal infection has been problematic because of the concerns regarding autoimmune responses. When sufficiently large doses of M protein have been given to

provoke type-specific antibody responses, local and systemic reactions have been frequent, possibly because M proteins have superantigen characteristics. Recently, M proteins derived from limited pepsin digestion of streptococci have been purified to homogeneity and the heart reactive epitopes have been separated. Immunization with these purified M protein preparations has generated type-specific opsonic antibodies without cross-reactive heart antibodies. Another approach to immunization, namely the stimulation of antibodies directed to the adherence moiety of streptococci, lipoteichoic acid, is limited by the poor immunogenicity of this constituent unless complexed to a protein molecule.

Vaccination with capsular polysaccharides has been shown to be effective in preventing *S. pneumoniae* infection in otherwise healthy adults who are exposed to epidemic pneumococcal infection, usually because of working and/or living in crowded conditions such as military camps. It has been difficult to demonstrate conclusively the benefits of vaccination in those individuals who are more susceptible because of chronic pulmonary disease and advancing

age. Although this prophylactic treatment is recommended for immunologically compromised individuals, these subjects are least likely to respond with adequate production of antibody.

See also: Autoimmunity; Bacteria, immunity to; Bacterial cell walls; Carbohydrate antigens; Circulatory system infections; Molecular mimicry; Opportunistic infections; Superantigens; Toxins.

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STRESS AND THE IMMUNE SYSTEM

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What is stress?

In order to explore the relationship between stress and the immune system we need first to examine what we mean by the term 'stress'. It is a word borrowed originally from physics and engineering where it denotes a force with the potential to deform or distort an object. In the context of our lives, stress is generally taken to refer to a situation that results in a challenge or threat to our adaptive capacity – something that disturbs our dynamic equilibrium or homeostatic balance. In other words, it is a perceptual or cognitive assessment made by an individual about the impact of a particular circumstance or constellation of events on his or her life.

How can stress be studied?

One of the difficulties therefore of studying the effects of stress in the lives of people or animals is that of evaluating the impact and comparability of

stressful situations on different individuals. In an attempt to circumvent this difficulty, many studies of stress and the immune system have been conducted by the controlled application of 'standard stressors' to populations of laboratory animals (Table 1). Here, a particular situation (usually involving some form of physical insult) is judged by the experimenter to be stressful on the basis of the subsequent behavior of the animals. Such studies have been valuable in delineating some of the pathways of interconnection between autonomic, neuroendocrine and immune system activities, as we shall see later. They have highlighted, for example, the distinction between the effects of acute and chronic stressors, the potential modulating and exacerbating effects of 'control' and 'helplessness' respectively, and the fact that corticosteroids, while clearly important immunomodulators, are not obligatory players in neuroimmune responses to stressors.

Although an analogous approach in human studies has been to submit volunteers to 'stressful' situations and to assess short-term effects on immune variables,

Table 1 Investigating stress*How can stress be studied?*

- Apply 'stressors' to animals or people and observe behavioral and physiological changes
- Compare groups of individuals living under circumstances that differ in their degree of 'stressfulness'
- Observe individuals at various times in their lives and correlate effects with their personal assessments of stressfulness

What questions can be asked?

- Are there correlations between stress-associated immune effects and disease or illness?
- What immune variables are affected by stress?
- Are there differences between the effects of short-term and chronic stressors?
- Is there a distinction between interpersonal and nonsocial situations?
- What are the mechanistic pathways within an individual's physical makeup through which stress might be acting on the immune system?

much human stress research has focused on 'naturally stressful' events in people's lives and selected subjects undergoing the same particular event. Accepting the notion that stress is an individual assessment, a further approach has sought to relate self-reported stress, usually accessed through standardized stress and anxiety questionnaires, to immune activity. Although there are many problems inherent in the use of questionnaires and self-reports, this approach has merit in that it relates stress to the context and meaning of individual lives and opens the possibility that altered perceptions might correspondingly alter physiological and immunological impact.

Stress and immunity in animal studies

Numerous animal studies have demonstrated that a wide variety of stressors can alter many aspects of the immune response. Much of the animal work has concentrated on applied, physical stressors (**Table 2**) but there have been some studies with more 'naturalistic' or ethologically relevant stressors such as maternal separation in young animals, social defeat and exposure to the odors emitted by littermate animals under stress. A wide variety of immune measures have been found to be affected by stress. These include nonspecific, mitogen-induced proliferative responses of lymphocytes, development of antigen-specific cytotoxic T cells, generation of specific antibody responses, macrophage activation, alteration of Th1/Th2 balance, secretion of cytokines and the expression of cytokine receptors. Stressors have also been shown to alter the migration patterns of immune cells between and into compartments of the

Table 2 What stressors have been studied?*Applied stressors**Physical*

Electric shocks
Overcrowding
Sleep deprivation
Noise
Rotation
Restraint
Immersion in cold water
Isolation

Mental

Maternal separation
Frustrating laboratory tasks
Conflict resolution
Public speaking
Examinations

*Naturalistic stressors**Interpersonal*

Social defeat
Separation
Marital conflict
Divorce
Bereavement
Job loss
Care-giving to chronically ill relatives
Examinations
Battle task vigilance
Natural disasters

immune system. Indeed it is difficult to find many measurable aspects of the immune system that cannot be altered by some stressor.

Although a variety of stressful behavioral manipulations that affect immunity also influence susceptibility to a variety of disease states, the stressors used do not all produce the same effects. The impact and direction of the effects of stress on immune behavior and on disease susceptibility depend on such factors as the quality, quantity and duration of the stressor (e.g. acute application of a particular stressor inhibits immune responsiveness, while more chronic exposure can sometimes enhance immunity); the temporal relationship between exposure to the stressor and the introduction of antigen or pathogenic stimulation; socioenvironmental conditions (e.g. whether the animals are housed individually or in groups, or how often the animals are handled during the neonatal period); and various host factors such as species, strain and sex. For example, stressors can accelerate the growth of transplanted tumors in animals, but such an effect could be mediated through nonimmune mechanisms, such as through stress-induced changes in blood flow to tumor sites. Nevertheless, immune effects are probably important here too because natural killer (NK) cell activity is also compromised, and if the stress effect on NK cells is pharmacologically blocked, there is no stress-related

increase in tumor growth. Conversely, if NK activity is reduced using a monoclonal antibody directed against NK cells, tumor growth rates increase in a manner similar to the stressful situation.

Many of the stressors in animal research have involved physical 'insults', but the impact of stress on immunity cannot be explained simply in physical terms. What the stressor means for the animal and the attitude which it adopts towards the situation can also be profoundly influential. For example, being placed into the established territory of another rat, even for a brief period of time, severely inhibits the production of antibodies to antigens administered before the intruder is introduced into the new territory. Moreover, in such confrontational situations it is the adoption of submissive behaviors by the introduced animal which correlates most closely with reduction of antibody production. Antibody responses in nonsubmissive animals are relatively unaffected. Other work has demonstrated that if an animal perceives a stressor to be controllable or escapable then effects on immune responses are much less than in inescapable situations.

Stress and immunity in human studies

In humans, some studies have been conducted into the effect of short-term, laboratory-based stressors on immune variables but most investigations have centered on more 'naturalistic' stressful situations (Table 2). Where perception of environmental stressors has been assessed using such indicators as daily mood, hassles, anxiety, social support or various coping measures, there has generally been a correlation between high perceived stress and depression of humoral and/or cell-mediated immunological measures. Often the most significant correlation has been with depression of NK cell activity. The notion that a critical parameter is the *perception* of stress clearly has important implications and illustrates how much the way we respond psychologically to our environment can affect the way we respond physiologically.

Four examples serve to illustrate this. In a 1-year study of a group of medical students, there was a strong association between examination stress, increased incidence of illness, and depression of various immunological measures. At times of examination stress, those students who were chronic carriers of latent Epstein-Barr virus (EBV) exhibited increased antibody titers against EBV, which is indicative of virus reactivation. In a further group of students who were immunized with hepatitis B virus vaccine at examination time, those who seroconverted early were significantly less stressed and anxi-

ous than those who did not, and the students who reported greater social support also gave a stronger immune response to the vaccine. In another study, volunteers completed questionnaires assessing degrees of perceived psychological stress and then were inoculated intranasally with rhinovirus under controlled conditions in an isolation unit. They were then monitored for the development of evidence of infection and common cold symptoms. The results showed that perceived psychological stress at the outset correlated strongly with increased risk of acute respiratory infection, increased viral shedding in the nasal mucosa and decreased antiviral antibody titers. Recent research on elderly people experiencing the chronic stress of long-term caring for relatives with dementia discovered that, compared with a suitably-matched control group, their inflammatory cytokine responses were diminished, they healed wounds more slowly, and their immune responses to influenza virus vaccination was down-regulated.

Importance of context

These examples emphasize that it is the context in which the event occurs for an individual rather than the event itself that constitutes the stress. Whether or not that context includes chronic stress may also modulate the immune effects of an acute stressor. For example, in a recent study, the effects on NK and T cell responses of acute psychological stressors was most marked in people reporting high levels of chronic stress at the time.

Thus, there will be conditions under which stressors interfere with immunity, have no effect or even enhance immune responses. For example, a stressor may interfere with immunity only if it occurs at or immediately before the time of antigen exposure, or may alter one immune variable but not another. This means that we cannot generalize about the effects of a particular stressor on immune function as a whole, particularly when one realises that most human stress studies assess blood-associated immune variables but blood contains less than 10% of the total lymphoid pool and is certainly not representative of the populations of cells in secondary lymphoid organs. Further, the immune system contains a high degree of redundancy, and so the fact that an event might alter a particular immune measure need not necessarily indicate any alteration of immune responsiveness within the whole individual.

Influence of the nervous system on the immune system

Until the last decade, a common view was that the

immune system is essentially autonomous – a closed system driven by challenges from foreign antigens and self-regulating, predominantly through the actions of cytokines. Yet the striking similarities in overall behavior between the immune system and the nervous system were also appreciated. For example, both are involved in the receipt and processing of information. In the case of the nervous system this is sensory information, while for the immune system it is molecular topological information. Both systems respond to a diverse range of stimuli and in each case the response is specific for stimulus. In each, there are a range of available responses and the system adapts its resources to provide what is appropriate in any situation. Neither the immune system nor the nervous system can know in advance exactly what stimuli are likely to occur and so each system has the capacity to respond to unexpected stimuli. Both systems have the ability to remember specific stimuli and to modify and tailor subsequent responsiveness. In essence, both systems are involved in the generation and maintenance of a distinct individual or 'self' in the context of an environment containing

many other individuals ('nonself') and this led people to wonder how coordinated and inter-related the activities of these two integrative systems might be.

It is now clear that there are extensive pathways of bidirectional communication between the immune and nervous systems, and the existence of neural-immune interactions allows us to make more sense of relationships between behavioral-psychological events and immune activity. For, if neural processes regulate immune processes, then there are pathways by which psychological factors such as stress could affect immunity (**Figure 1**). Conversely, if immune processes alter neural function, then they can also potentially have an impact on behavior, emotion and thought.

The brain has two ways of controlling peripheral organs and processes – a cabling network and a fluid-based communication hierarchy – and both are able to influence the operation of the immune system. The cabling network is called the autonomic nervous system and consists of sympathetic and parasympathetic branches which innervate visceral organs – heart, liver, stomach, kidneys, etc. Histochemical staining

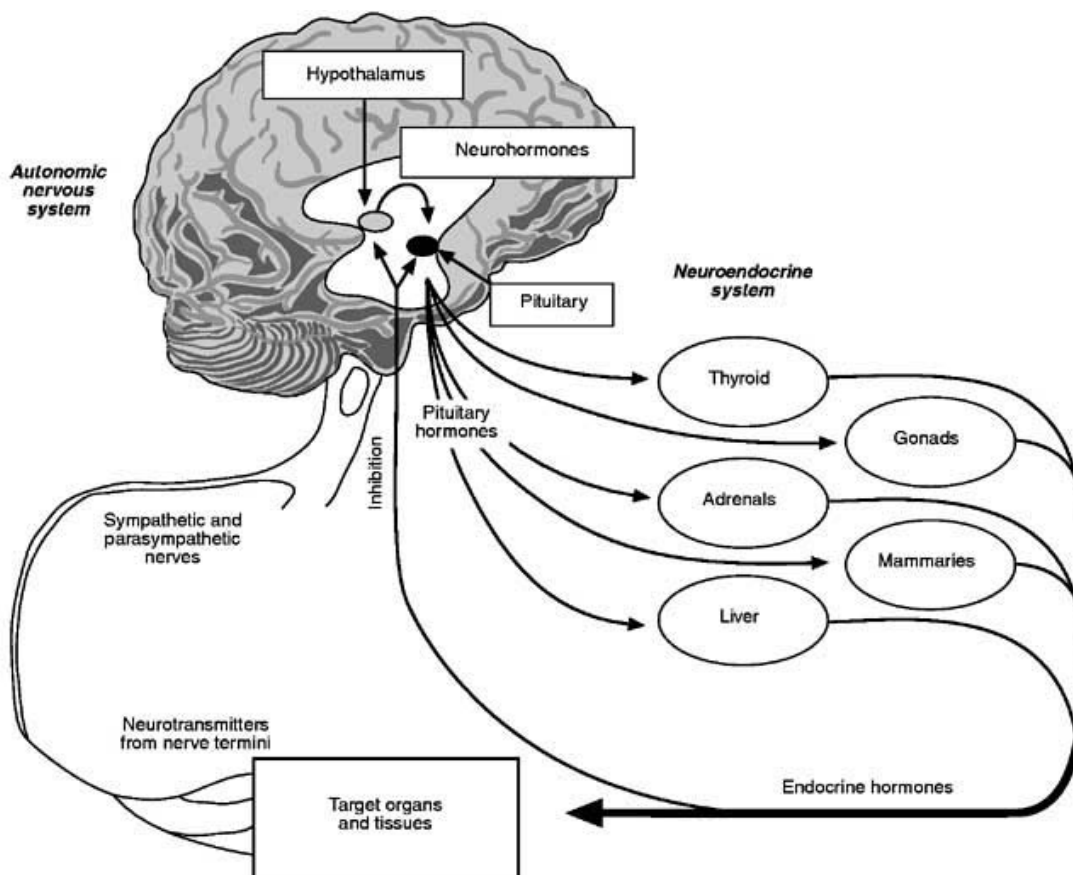


Figure 1 Autonomic and neuroendocrine systems. The nervous system operates to affect visceral processes through autonomic sympathetic and parasympathetic nerves, and through neuroendocrine hormones via the hypothalamus–pituitary axis controlling endocrine gland output.

techniques to identify small nerves have revealed extensive innervation of all lymphoid tissue (both primary and secondary lymphoid organs) by noradrenergic nerve fibres. Norepinephrine (noradrenaline) is the predominant neurotransmitter released by these nerves although epinephrine (adrenaline) and serotonin can also be used. In lymph nodes, the nerves appear to terminate predominantly in the T cell-rich regions, suggestive of possible links to immunoregulatory functions, particularly as receptors for norepinephrine and catecholamines have been found on lymphocytes, macrophages and cells of the granulocyte lineages.

The fluid-based communication hierarchy through which the brain can communicate with peripheral organs comprises the glands and hormones of the neuroendocrine system. This centers particularly on the hypothalamus, pituitary and adrenal glands. In the brain, the paraventricular nucleus of the hypothalamus is able to synthesize and release certain hormones into the portal blood system at the base of the brain. Some of these hormones act close by on the anterior lobe of the pituitary gland, causing it to synthesize and release other hormones, which, in turn, travel in the bloodstream to signal various peripheral endocrine organs. In times of stress, one of the important products of the hypothalamus is a substance called corticotropin-releasing hormone (CRH), which activates pituitary synthesis and release of adrenocorticotrophic hormone (ACTH). ACTH is released into the blood and ultimately arrives at the cortex of the adrenal gland, where it causes the release of steroid hormones called glucocorticoids.

Autonomic pathways to the immune system

The limbic system of the brain, which is pivotal in aspects of affective and cognitive behavior, directly regulates the neurohormonal and autonomic outflow of the nervous system. As the hypothalamus is central to this process, it is pertinent that perturbations of the hypothalamus also affect immune responses. For example, experimentally introducing lesions into the anterior lobe of the hypothalamus of rats depresses antibody responses, while electrically stimulating the hypothalamus at the time of immunization enhances antibody responses. Lesions in hypothalamus and hippocampus have also been found to alter such immune responses as NK activity and T cell functions. Focusing further down the autonomic nervous system, we find that the ultrastructural features of the contacts made between the sympathetic nerve termini in lymphoid tissues and

lymphocytes are similar to nerve synapses, indicating the likelihood of some sort of intimate control or regulatory function. So it is no surprise that if the nerves to a lymphoid organ are cut or chemically damaged, the immune responsiveness of that lymph node is altered. Further, using specific antagonists to block hormone receptors on lymphocytes also alters the course of immunity. Thus, elevation of norepinephrine and epinephrine levels, which accompanies stress, may also produce changes in lymphocyte, monocyte and leukocyte circulation patterns and functions. Both lymphocytes and macrophages have β -adrenergic receptors which are increased in density following acute stressors. Moreover, β -adrenergic agonists generally downregulate immune system function.

Neuroendocrine pathways to the immune system

Corticosteroids, produced by the adrenal cortex as a result of stress-induced signals from the hypothalamus through the pituitary, have profound immunosuppressive effects on a variety of immune parameters, as outlined in Table 3. They decrease the production of many cytokines and mediators of inflammation and decrease the effects of certain inflammatory molecules on various target tissues. This, together with the more general immunosuppressive characteristics of corticosteroids, is why they are used clinically to treat immunoproliferative disorders and some autoimmune conditions, and to control rejection of transplanted tissues. However, removal of adrenal function by adrenalectomy, or suppression of corticosteroid synthesis with drugs, eliminates many but not all of the immunosuppressive effects of stress, indicating that other molecules mediate stress-induced immunological effects as well.

In addition to corticosteroid effects, a number of the nonsteroidal hormones, produced by other neuroendocrine organs in response to hypothalamic-pituitary messages, also influence immune responses; for example, sex hormones diminish *in vivo* responses, while growth hormone, thyroxine and insulin elevate responses under certain conditions. Numerous neuropeptides and neurotransmitters,

Table 3 The physiological effects of corticosteroids

Generally depress <i>in vivo</i> responses
Depress phagocytosis
Inhibit macrophage activation
Inhibit <i>in vitro</i> B and T cell responses
Alter lymphocyte migratory patterns
Decrease cellularity of the thymus

which exert multiple functions as intercellular messengers within the nervous system, may also modify functional properties of cells of the immune system (Figure 2). For example, in addition to the elevation of CRH, corticosteroids and the catecholamines with stress, levels of β -endorphin, which is derived along with ACTH from cleavage of the pro-opiomelanocortin polypeptide, are also elevated, as are levels of several other opiates. Opiates function to alleviate pain during a fight-or-flight encounter but also inhibit immune responsiveness. In contrast, two anterior pituitary hormones, growth hormone and prolactin, are immunoenhancing and appear to act as a balance to corticosteroids and catecholamines.

Neurological mediators could act directly on cells of the immune system to produce their effects or they could act indirectly by inducing immunological mediator production from other cell populations. Receptors for many of the neuropeptides, neurotransmitters and endocrine hormones have been found on cells of the immune system and so at least some of their effects may be a consequence of direct binding to lymphocytes or monocytes.

Immune system pathways to the nervous system

If the immune system is also able to influence the nervous system then effects should be evident during

times of immune activity. The fact that when we have a cold or 'flu' we feel drowsy, feverish and unsociable alerts us to the possibility of immune activity affecting the nervous system and behavioral and cognitive patterns as a consequence. Indeed, hypothalamic electrical activity has been observed to increase at the time of peak B cell responsiveness to administered antigen, and neurotransmitters such as norepinephrine in the hypothalamus also show profound changes at this time. Potentially there are at least two ways in which this could occur. Lymphocytes themselves have only limited access to the brain and so responding lymphocytes could secrete neurological mediators or, alternatively, cytokines produced during the course of the immune response could directly affect neural tissues. In practice, both these pathways probably operate (Figure 3).

A variety of neuroendocrine mediators can be synthesized by cells of the immune system under various conditions of activation. In fact, in many ways it seems as if lymphoid tissues can behave rather like the pituitary gland itself. For example, CRH can stimulate macrophages to secrete interleukin 1 (IL-1), which in turn induces B cells to secrete ACTH and β -endorphin. Like pituitary production of ACTH and β -endorphin, this effect can also be downregulated by corticosteroids. In addition, some cytokines (e.g. IL-1, IL-2, IL-3, IL-6, IL-8, tumor necrosis factor α (TNF α) and interferon γ (IFN γ))

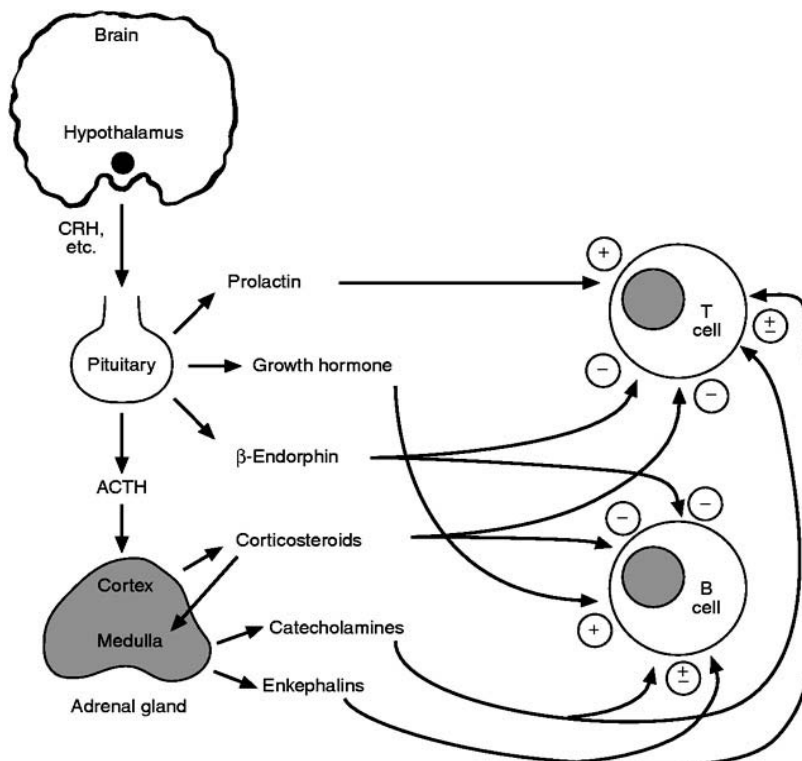


Figure 2 Some neuroendocrine pathways to the immune system. Many of the hormones released by the hypothalamus, pituitary and endocrine glands affect the activity of lymphocytes. In stress, hormones and neurotransmitters from the adrenal cortex (corticosteroids) and medulla (principally catecholamines and enkephalins) are especially important.

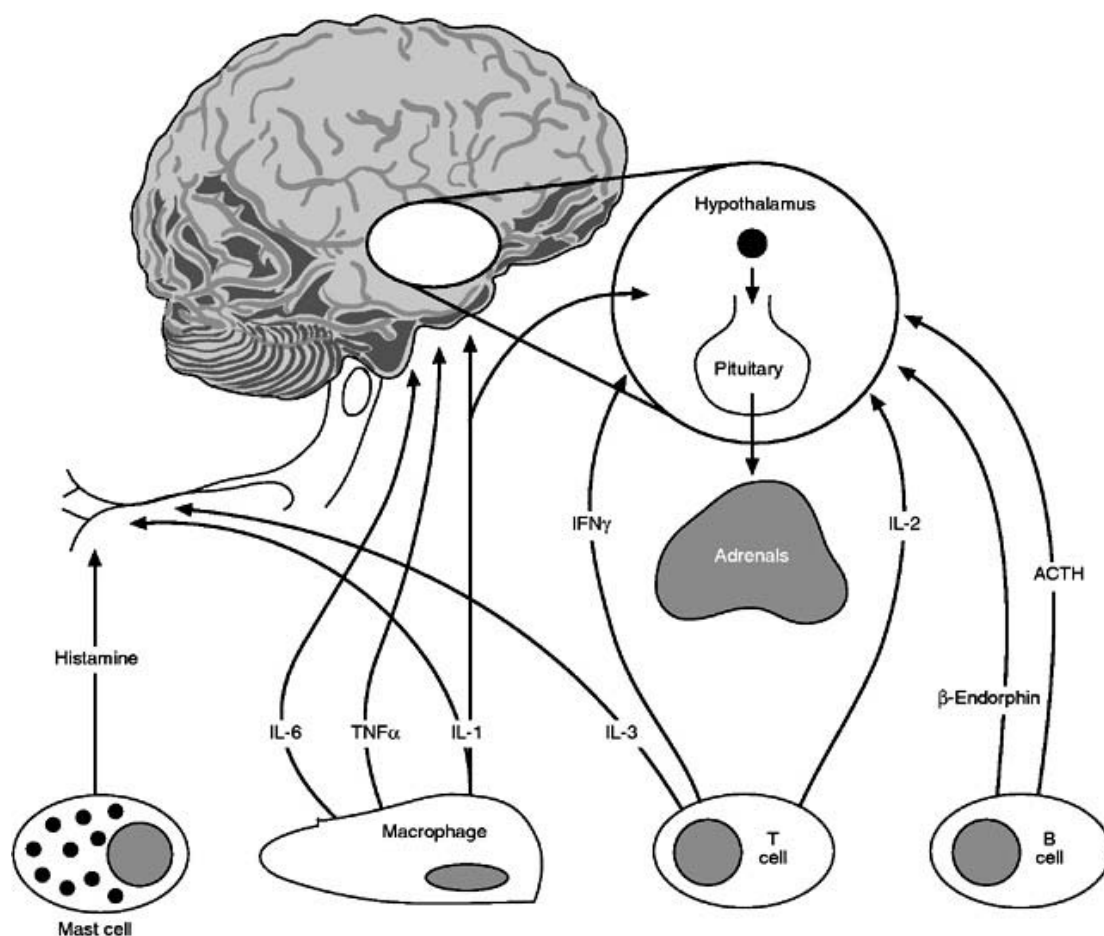


Figure 3 Some immune system pathways to the nervous system. Various cytokines released by activated cells of the immune system modulate the activities of the autonomic and neuroendocrine systems. In addition, lymphocytes and other immune cells release some neurotransmitters and neurohormones.

exert effects on the central nervous system and in some cases (notably IL-1 and IL-3) may act as intrinsic neuromodulators within the brain. However it is the neural effects of the cytokine IL-1 (and coordinately IL-6 and TNF α), released by macrophages during immune and inflammatory responses, which are the most well studied.

Activation of macrophages produces IL-1 and subsequently leads to altered electrical activity in the brain, and metabolism of the neurotransmitters norepinephrine, serotonin and dopamine in certain brain regions. These neural changes can also be produced by injected IL-1 and can be blocked by an IL-1 receptor antagonist. Does this mean then that immune-produced IL-1 acts directly on the brain? Certainly neurons in the preoptic nucleus have receptors for IL-1 (as well as IL-6 and TNF α) but because IL-1 is a relatively large, lipophobic protein it may have difficulty crossing the blood-brain barrier easily. An active transport mechanism could exist to carry IL-1 across the barrier, or alternatively, IL-1 might

cross the vascular endothelium in regions of the brain, such as the preoptic nucleus of the hypothalamus, where the barrier is weak or absent. Another possibility is that IL-1 stimulates peripheral nerves, such as the vagus, to send afferent input to the brain. This possibility was highlighted by recent findings that if the vagus nerve was cut just below the diaphragm, stress-induced, IL-1-mediated effects on the brain were considerably diminished.

Conclusions

Clearly then, there are extensive bidirectional pathways of communication between the immune system and the brain (Figure 4). Perceived stress, through this neuroimmune network, can influence immune responses. But stress is not a generic event, and different stressors, or stressors perceived differently, produce different mixes of autonomic activation and hormones. For example, one stressor might lead to intense autonomic activation and consequent plasma

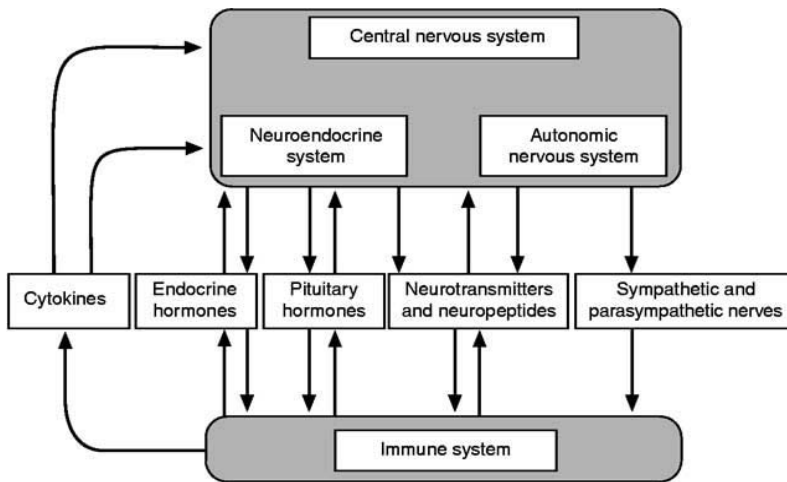


Figure 4 A summary of neuroimmune physiological interactions. The extensive set of hormones, cytokines and other modulatory processes shared between the immune and nervous systems illustrate the degree to which the two systems cannot be considered as operating independently of one another in living organisms.

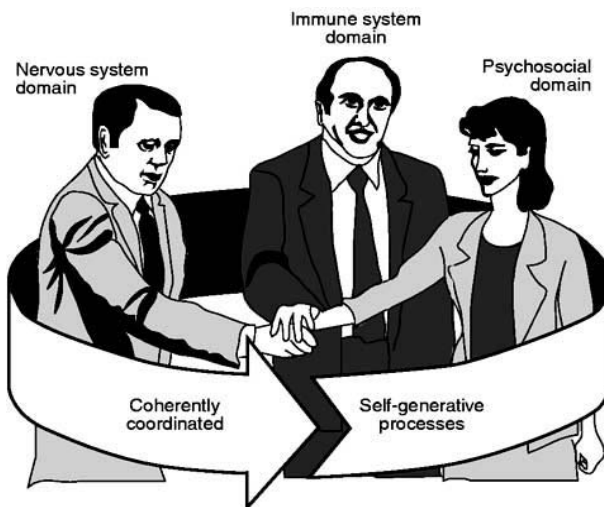


Figure 5 Coherence among neural, immune and psychosocial domains. Psychosocial processes (such as the perception of stress), acting through the neuroimmune network, clearly have the potential to modulate immune responsiveness. Conversely, immune activity influences how we think, feel and act. Thus, these three integrative systems – immune, neural and psychosocial – are coherently coordinated into the self-determination process of an individual.

catecholamine release but relatively little activation of the pituitary and adrenal glands and their hormones. Another might produce the opposite pattern. In addition, emotions, thoughts, personality, coping processes and other psychological and social variables will modulate autonomic and hormonal consequences of exposure to stressors and in turn the immune consequences.

Conversely, immune system-derived information acting on the nervous system, during the course of an infection for instance, could also be responsible for triggering changes in behavior patterns similar to stress-associated behaviors. For example, animals

exposed to stimuli which arouse fear or anxiety reduce social interactions, curiosity, food and water ingestion and sexual activity. Administration of IL-1 or substances that stimulate macrophages to secrete IL-1 produce very similar behavior changes.

All this suggests that the immune system may truly act as a sensory organ, conveying to the brain information about antigens through lymphocyte-derived hormones, while at the same time being modulated by neural factors (stress related or otherwise). As illustrated in Figure 5, therefore, the immune, neural and psychosocial domains are coordinated as coherent self-generative processes within an individual's life such that it is the context, interpretation and meaning of stress for the individual that determines its immune effects.

See also: Cytokines; Glucocorticoids; Neuroendocrine regulation of immunity; Stress proteins.

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STRESS PROTEINS

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The stress response

Cells respond to a variety of stressful stimuli by coordinately increasing the synthesis of up to two dozen specific proteins. The DNA loci involved in the stress response are the most highly conserved genetic regions known. Although the best studied of these stressful stimuli is an increase in temperature or 'heat shock', others include nutrient deprivation, oxygen radicals and metabolic disruption. Accordingly, the proteins which are induced in response to these stimuli are often called heat shock proteins (hsps) or more generally, stress proteins. Many higher eukaryotic cells have been shown to undergo a stress response during intracellular infection and also dur-

ing malignant transformation. Induction of transcription of stress proteins is coordinated by a shared nucleotide sequence in the 5' noncoding region which serves as the promoter for hsp mRNA synthesis.

Stress proteins

Stress proteins are grouped into major families (generally named according to their approximate molecular weight in kDa). Much of the current knowledge relating to these protein families is outlined in Table 1. Within a family, hsps show remarkable sequence homology across the phylogenetic spectrum and are indeed among the most highly con-

Table 1 Members of the major stress protein families

Family	Members	Organism	Localization	Normal function
hsp110	C23 nucleolar protein	Mammals	Fibrillar nucleolus	RNA binding, ribosome stabilization?
hsp90	HtpG (C6.25)	<i>E. coli</i>	Cytosol	ATP-dependent chaperone
	hsp82	<i>S. cerevisiae</i>	Cytosol	
	Hsc82		Cytosol	
	hsp83	<i>D. melanogaster</i>	Cytosol	
	hsp90	Mammals	Cytosol	ATP-dependent chaperone
	grp95	Mammals	ER	
hsp70	DnaK	<i>E. coli</i>	Cytosol	ATP-dependent molecular chaperone
	SSa1-4p	<i>S. cerevisiae</i>	Cytosol	
	SSb1/2p	<i>S. cerevisiae</i>	Cytosol	
	Kar2p	<i>S. cerevisiae</i>	ER	Required for protein translocation across lipid bilayers
	SSclp	<i>S. cerevisiae</i>	Mitochondria	
	hsp72 (hsp70)	Mammals	Cytosol	
	hsp73 (hsc70)	Mammals	Cytosol	
	BiP (grp78)	Mammals	ER	ATP-dependent molecular chaperone Binds hydrophobic protein surfaces bearing the general motif: X W/F X W/F X W/F/L/I X W/F/Y X
hsp60	mtp70	Mammals	Mitochondria	
	GroEL	<i>E. coli</i>	Cytosol	ATP-dependent protein foldase in conjunction with hsp10 as a large oligomeric complex
	Tcp1p	<i>S. cerevisiae</i>	Cytosol	
	hsp60 (mif4p)	<i>S. cerevisiae</i>	Mitochondria	
	TCP1	Mammals	Cytosol	
	hsp58 (hn cha60)	Mammals	Mitochondria	
Small hsps (14–50)	SecB (16 kDa monomer)	<i>E. coli</i>	Cytosol	SecB tetramer facilitates transport of partially folded proteins across cytoplasmic membrane. Binds positively charged stretches of amino acids
	hsp26	<i>S. cerevisiae</i>	Cytosol	Coalesce to form heat shock granules in stress
	hsp25–27	Mammals	Cytosol	
	a-crystallin	Mammals	Cytosol	
hsp10	GroES	<i>E. coli</i>	Cytosol	See hsp60
Ubiquitin (8 kDa)		All species	Cytosol	Targeting aberrantly synthesized proteins for cytosolic degradation by the 26S proteasome

served proteins in existence. For example, there is over 50% sequence homology between hsp70 from mycobacteria and humans. Yeast ubiquitin, another stress protein, differs from its mammalian counterpart by only three amino acids. Despite this homology, subtle differences exist in the apparent functions, inducibility and cellular location among related hsps for a given species.

The major stress proteins can accumulate to very

high levels of stressed cells, but occur at low to moderate levels in cells that have not been stressed, indicating that they play a role in normal cells. For example, in most mammalian cells hsp90 and hsp60 are abundant at normal temperatures whereas hsp70 is hardly detectable. Nevertheless, all are further induced by stress. For some proteins, the effect is dramatic: *Escherichia coli* hsp60 and hsp70 account for 1.5% of total cell protein at normal temperatures

whereas they account for 30% of total protein after heat shock.

In addition to increased synthesis, many hsp's alter their intracellular distribution in response to stress; an observation which may relate to their role in thermotolerance.

The function of stress proteins

A common feature of hsp's is their ability to function as molecular chaperones. This term describes their ability to bind to denatured proteins, preventing their aggregation, and provides the key to understanding the function of hsp's under normal conditions and in conditions of stress.

Normal function

The binding of hsp's to denatured proteins is regulated by the hydrolysis of ATP. The ATPase activity of hsp60, hsp70 and hsp90 is stimulated by their binding to unfolded proteins, with ATP hydrolysis promoting dissociation of the complex.

hsp70 has been implicated: 1) in the assembly of immunoglobulins, by binding to heavy chains in the endoplasmic reticulum (ER) prior to their assembly with light chains; 2) in the folding of influenza hemagglutinin; and 3) in the translocation of proteins across the mitochondrial membranes and possibly also the ER membrane. This is achieved by binding to unfolded protein substrate as it appears in the mitochondrial matrix (or ER lumen), thus pulling it in and stabilizing it in an unfolded state and preventing it from precipitating. A structure of the substrate binding unit of DnaK has been solved and shows a unique peptide-binding site comprising a channel which can be closed by a lid.

hsp90 may function in a similar way to hsp70 although it forms longer lived complexes with some substrates, displays a higher degree of substrate specificity and (perhaps consequently) is less effective in facilitating the experimental refolding of *in vitro* denatured substrates.

hsp60 has a characteristic oligomeric structure of 14 subunits arranged in a stack of two heptameric rings. Successive rounds of substrate binding, ATP hydrolysis and substrate release allows newly synthesized proteins to fold in a controlled way in the core of this stack. The hsp60 14mer normally functions in association with a heptameric ring of hsp10, which stimulates ATPase activity and acts as a lid on the stack. The proper folding of one protein substrate molecule requires several rounds of ATP hydrolysis by the hsp60/hsp10 oligomer. Newly synthesized proteins may therefore first bind to hsp70 or hsp90, before being passed to hsp60 for folding.

Ubiquitin, which has been included in this entry because of its upregulation during stress, is not a molecular chaperone, but is normally employed in the intracellular turnover of cytosolic proteins as a molecular 'tag' for unfolded proteins which are destined for degradation by the 26S proteasome. In some systems, the enzymes which attach ubiquitin to protein substrates via an isopeptide bond (E2s) are also heat inducible.

Function in thermotolerance

Abundant evidence indicates that hsp's play a vital role in thermotolerance. For example, mild heat shock (enough to induce hsp synthesis) protects cells against a subsequent, otherwise lethal sudden temperature increase. Microinjection of hsp-specific antibodies into cultured cells abrogates their ability to survive the effects of elevated temperatures. Clues to the role of hsp's in thermotolerance have been sought from their normal cellular roles. Thus, these proteins may stabilize partially unfolded states of proteins whose tertiary structure is disrupted by heat. This interaction would prevent heat-damaged proteins from aggregating and forming a toxic intracellular precipitate in the same way as for newly synthesized prefolded proteins. Heat shock proteins may also dissociate aggregates of denatured proteins. hsp60 in conjunction with hsp70 might even recover native proteins from these denatured states. In addition to simply stabilizing heat denatured proteins, hsp's are thought to maintain thermotolerance by promoting their degradation. This could be achieved by keeping an unfolded protein in a protease-sensitive conformation, or by stabilizing a more active conformation of the protease itself. Ubiquitin plays a key role in the degradation of abnormal proteins which would be expected to accumulate during stress. Indeed, ubiquitin may be induced by the intracellular accumulation of denatured proteins.

Stress proteins as target antigens

In infection

Heat shock proteins have been shown to be among the dominant antigens recognized by both the regulatory (helper T lymphocytes) and effector (B lymphocytes and cytotoxic T cells (CTLs)) arms of the adaptive immune system for a variety of pathogenic infections and experimentally induced immune reactions.

B cell responses In mice, immunization with a mycobacterial lysate elicits antibody responses to only a limited number of *Mycobacterium tubercu-*

losis or *M. leprae* antigens, including hsp70, hsp60, hsp18 and hsp12. In a World Health Organization sponsored workshop, a collection of 24 murine monoclonal antibodies raised against *M. leprae* were found to recognize six proteins, of which three were stress proteins. Seven of these antibodies identified six different epitopes of hsp60. Similar results were obtained with monoclonal antibodies raised against *M. tuberculosis*. Mycobacterial stress proteins are also recognized by the sera of patients suffering from tuberculosis (and tuberculoid leprosy). hsp60 is the dominant target antigen of serum antibodies in a wide variety of other bacterial infections including those due to *Coxiella*, *Legionella*, *Treponema* and *Borrelia*. In fact, an immunodominant antigen long known to be shared by most bacterial species called 'common antigen' turns out to be hsp60. Antibodies to hsp70 have been found in the sera of patients with diverse parasitic infections, for example malaria, trypanosomiasis, leishmaniasis and schistosomiasis. In addition, hsp90 is a target for antibodies in malaria and trypanosomiasis and a member of the small hsp family is recognized by sera from some patients with schistosomiasis.

T cell responses Twenty per cent of the CD4⁺ T cells cloned from the draining lymph nodes of mice immunized with *M. tuberculosis* recognize hsp60. Some of these have been shown to secrete interleukin 2 (IL-2). hsp60 is also a major target antigen in mycobacteria-infected humans. Typically 25–30% of the mycobacteria-reactive CD4⁺ T cell clones isolated from infected individuals react against *M. leprae* hsp60. Around 12 separate epitopes have been mapped in the hsp60 protein of mycobacteria which are recognized by MHC class II restricted CD4⁺ T cells. CD4⁺ T cells recognizing hsp60 have also been isolated from individuals immunized with heat-killed *M. tuberculosis*. Mice immunized with a tryptic digest of *M. tuberculosis* have been shown to yield CD8⁺ CTLs specific for epitopes on hsp70. In addition, CD8⁺ CTLs recognizing *M. tuberculosis* hsp70 have been isolated from the pleural effusion of patients with tuberculosis. Human $\gamma\delta$ receptor-bearing T cells which recognize hsp60 have been isolated from the peripheral blood of individuals immunized with BCG.

In autoimmune disease

Because hsps are ubiquitous and highly conserved, and have been shown to be dominant antigens in a range of infections (see above), they have been proposed to provide a link between anti-infectious and autoimmune responses. Thus, antibodies and T cells elicited to hsps encoded by a pathogen might cross-

react with self-hsps. Indeed, peptides derived from endogenous (self) hsps have been isolated from the binding grooves of both MHC class I and class II molecules. Anti stress protein reactive T cells have been implicated in two experimentally induced animal models of autoimmune disease and also in rheumatoid arthritis.

Adjuvant arthritis Intradermal injection of heat-killed *M. tuberculosis* in rats induces a disease similar in many respects to rheumatoid arthritis. An autoimmune process involving T lymphocytes is responsible for the generation of the disease, and transfer of T cells from arthritic rats to irradiated recipients can cause a transient form of the disease in the latter. One arthritogenic T cell clone was shown to recognize a mycobacterial hsp60 epitope. Also, immunization of mice with mycobacterial hsp60 in immunostimulating complexes (ISCOMS) will induce CD8⁺ $\alpha\beta$ CTLs which cross-react with murine hsp60 and are therefore autoreactive.

Streptococcal cell wall induced arthritis (SCIA) Similar results have been obtained for a second polyarthritic disease in rats, SCIA, which can be induced by intraperitoneal injection of *Streptococcus pyogenes* cell walls. Streptococcal cell wall preparations contain an antigen recognized by antimycobacterial hsp60 antibodies and T cells. Again, the disease condition can be transferred with purified T cells.

Rheumatoid arthritis Results with adjuvant arthritis prompted investigators to ask whether T cells from rheumatoid arthritis patients can also recognize mycobacterial hsps. In some studies, mycobacterial hsp60 and hsp70 have been shown to be among the antigens recognized by polyclonal synovial T cells, and there is evidence that these can cross-react with human hsp70. $\gamma\delta$ T cells have also been cloned from rheumatoid synovia which respond to mycobacterial hsps. Moreover, elevated levels of IgG and IgA antibodies that bind mycobacterial hsp60 and hsp70 have been detected in some patients with rheumatoid arthritis. However, any role for mycobacteria (or indeed any other infectious agent) in the development of rheumatoid arthritis in humans remains highly speculative.

Stress proteins and other chaperones in antigen-presentation pathways

hsp70

The ER-resident member of the hsp70 family, BiP, has been shown to associate with newly synthesized

human (but not mouse) MHC class I molecules. It is unclear whether this association is important for the assembly of class I molecules, or whether the BiP:heavy chain complex is an intermediate on the way to heavy chain degradation in the ER. Similarly, MHC class II molecules synthesized in the absence of invariant chain associate with BiP prior to their intracellular degradation.

Antibodies raised to a unique, constitutively expressed member of the hsp70 family (PBP72/74) block MHC class II restricted antigen presentation. Although it is not clear how PBP72/74 is involved, the well-documented ability of hsp70 to bind peptides in an ATP-dependent way suggests that one way may be to protect or transport peptides prior to their association with MHC class II. A similar role has been suggested for cytosolic hsp70 in the chaperoning of peptides from their site of production (the proteasome) to the TAP transporter, although the evidence is extremely indirect and is based on the ability of hsp70-peptide complexes to induce peptide-specific CTLs.

hsp90

Both hsp90 and grp95 (as well as hsp70 – see above) purified from meth-A induced tumors can elicit MHC class I restricted CTLs which are specific for the tumors from which the hsps were purified. These CTLs actually recognize peptides which copurify with the hsps, in a class I restricted way, and this observation has led to the suggestion that hsp90 serves to chaperone these immunogenic peptides to an intracellular site where they can bind to class I. By extension, it has been suggested that grp90 may normally play a role in chaperoning peptides arriving in the ER from the TAP transporter to awaiting MHC class I molecules.

Ubiquitin

The proteolytic events involved in generating antigenic peptides in the cytosol for presentation with MHC class I molecules involve the proteasome. This multicatalytic complex is responsible for disposing of ubiquitin-tagged proteins (see above), and the requirement for ubiquitination of cytosolic proteins destined for the MHC class I processing pathways has been implicated in a number of instances. For example, a temperature-sensitive Chinese hamster ovary (CHO) cell mutant of one of the ubiquitinating enzymes was able to present cytosolic antigens to

MHC class I restricted CTLs at the permissive, but not the nonpermissive temperature.

Calnexin and calreticulin

Although the ER-resident chaperone proteins calnexin and calreticulin are not stress proteins, they are included here for the sake of completeness. They bind to newly synthesized MHC class I heavy chains as they assemble with β_2 -microglobulin and retain them in the ER until they are fully assembled with antigenic peptides supplied by the TAP heterodimer.

Class II specific molecular chaperones: DM and Ii

Similarly, two proteins involved in the biogenesis of MHC class II-peptide complexes are included here even though they are not stress-induced. Invariant chain (Ii) prevents MHC class II molecules from aggregating in the ER; hinders their association with peptides in the ER; and specifically targets newly synthesized MHC class II molecules to the endosomal compartment. There it is degraded, leaving an Ii-derived peptide called CLIP bound in the MHC class II peptide-binding groove. The removal of this peptide, and its replacement with an antigenic peptide is facilitated by the MHC-like molecule DM which is itself encoded within the MHC class II locus.

See also: **Adjuvant arthritis; Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Autoimmunity; ISCOM (immuno-stimulating complex); Molecular mimicry; Mycobacteria, infection and immunity; Rheumatoid arthritis, human; T cell vaccination.**

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STROMAL CELLS

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One of the distinguishing features of lymphoid development is that it involves blood-borne multipotential precursor cells seeding to a selective site where their differentiation to phenotypically and functionally distinct mature cells is induced. This is regulated by a population of sessile cells, collectively termed stromal cells, which form an interconnected network providing not only the structural integrity of the organ but also the specific inductive microenvironment responsible for the differentiation and maturation of the incoming precursor cells. As distinct from hemopoiesis, the complete spectrum of lymphocyte development, particularly that of T cells, cannot be induced by a combination of cytokines – cell:cell plasma interactions are a critical requirement. Identification of the non-MHC (major histocompatibility complex) surface molecules involved in this is a central focus of research into the lymphoid microenvironments. Given that there are as many as 8–10 steps in T and B cell development lineages, each definable by a combination of surface molecules rather than single stage-specific markers, a logical prediction would be that the stromal cells controlling these pathways are equally complex. Despite the wide acceptance of the term ‘stromal cells’, historically based on the lack of histologically distinct features between these cells, more sophisticated phenotypic analyses now make it clear the stromal cells and their organization are equally as complex as the cells whose development they regulate. The thymus as the primary lymphoid organ for T cell genesis is the best studied for stromal cells, its composition and structure serving as a valuable model for other tissues (Figure 1).

Thymic stromal cells

Basic thymic structure

Thymus stromal cell heterogeneity is no doubt a direct reflection of the input of all three germ layers. The thymus is bounded by a connective tissue capsule. Between this and the underlying epithelium is a subcapsular space which contains some vasculature and is the site of entry of blood-borne precursors and macromolecules. It is thus very likely that the thymus is bathed in plasma constituents, which has very important implications for self tolerance. Fibroblast- and type 1 collagen-rich trabeculae branch off from the capsule and penetrate the cortex, terminating at

the corticomedullary junction, thereby lobulating the thymus and providing a structural link between the subcapsule and the medulla. These trabeculae are well vascularized and innervated and contain predominantly fibroblasts and macrophages; there are also minor populations of adipocytes, eosinophils, neutrophils, plasma cells, mast cells and granulocytes. Towards the internal base of the trabeculae are the perivascular spaces which effectively constitute the blood–thymus barrier. These connective tissue-rich regions are actually external to the thymic parenchyma, being separated from it by type 1 epithelium (see below) and from the vascular endothelial cells by basal laminae. They contain multiple leukocytes, including neutrophils and mature T and B cells and even germinal centers. Although there is no formal barrier dividing the cortex from the medulla, both of these areas are readily definable by their desmosomal-linked epithelium which have been extensively classified ultrastructurally and phenotypically.

Epithelium

Subcapsular/subtrabecular/perivascular (type 1 epithelium) Immediately underlying the capsule and lining the internal face of the trabeculae and perivascular spaces is a continuous layer of flattened epithelium which rests on a basal lamina, effectively separating cells of the connective tissue compartment from the lymphoepithelial compartment. This is termed type 1 or lining epithelium. It completely encases the cortex at the interface with the trabeculae and the capsule, but it is discontinuous at the base of the trabeculae. This latter feature may have important implications for cellular migration from the vasculature into the thymus. Phenotypic similarity with neuroendocrine tissue suggests this epithelium has its neurogens from the neural crest. Many antigens expressed in the subcapsular epithelium are shared with the medullary epithelium, indicating a potential lineage relationship, but they also express region-specific molecules. This is best exemplified in the chicken, where the subcapsular type 1 epithelium is exclusively defined phenotypically. Furthermore there is ultrastructural and phenotypic heterogeneity within the type 1 epithelium. Being such a discrete region, its functional significance is difficult to investigate but its potential importance stems from the finding that there are



Figure 1 The thymic stromal architecture. (Reproduced by permission from *Immunology Today* 14: centrefold. The author is very grateful to D Godfrey for the provision of the figure.)

abnormalities in type 1 epithelium prior to the onset of autoimmune disease in genetically prone chickens. It is unlikely to involve abnormal selection to self peptides, however, because the type 1 epithelium is very poor, if not negative, in MHC class II expression. Given that this is the site of intrathymic precursor entry and subsequent localization and many of these are dividing, the working hypothesis for the role of the type 1 epithelium is that it attracts blood-borne precursor cells and induces their activation/proliferation, which may be subsequently coupled to TcR gene rearrangement and commitment to the T cell lineage.

Cortical epithelium Over 85% of thymocytes are localized within the cortex. The cortical epithelium consists of an interconnected network of stellate-shaped cells characterized by relatively small cell bodies and long cytoplasmic processes which surround the tightly packed thymocytes. There is a remarkable degree of elasticity in the cortical epithelium, as it is able to rapidly expand and contract

in response to shifts in the thymocyte pool, essentially keeping the desmosomal contacts intact. Although most monoclonal antibodies (mAbs) which react with cortical epithelium label the entire network, there is evidence at the ultrastructural level of heterogeneity. Types 2 and 3 epithelial cells are located in the outer cortex, have large nuclei, are metabolically active and often form thymic nurse cells (TNCs). Type 4 epithelial cells are 'dark', electron dense with more oval/spindle-shaped nuclei and located in the deeper cortex/medullary regions.

Of the many structural features of the thymus, TNCs are arguably the most interesting. They are multicellular complexes consisting of a cortical epithelial cell (type 2 or 3) which has physically encompassed surrounding thymocytes varying in number from <5 to over 100, depending on the species. These T cells are not within the cytoplasm but rather encompassed within vacuoles formed by the extensive plasma membrane processes of the epithelial cell. Whether TNCs are fully enclosed structures *in situ* is debatable but the intimate association between the

enclosed thymocytes and the epithelial plasma membrane is indicative of a major role in thymopoiesis. The epithelial cell expresses high levels of MHC class I and II. In fact these structures may be microcosms of T cell development because they contain CD3⁺CD4⁺CD8⁻ precursors, CD3⁺ or CD31⁺ CD4⁺CD8⁺ immature T cells and phenotypically and functionally mature CD3^{hi} CD4⁺CD8⁻ and CD3^{hi} CD4⁺CD8⁺ cells. The level of mature T cells in TNCs is increased in TcR transgenic mice. Hence TNCs may be the primary site of positive selection, given the current dogma that this is the domain of cortical epithelium. Consistent with this is the presence in TNCs of acidic organelles necessary for antigen processing and presentation. There is also evidence of apoptosis in TNCs, which may reflect negative selection or death of T cells with a nonproductive TcR or one which fails to recognize self MHC-peptide complexes. Although there are relatively few TNCs per thymus, they may represent the most extreme form of dynamic opening and closing pockets of thymopoiesis in the cortex.

Medullary epithelium Unlike the stellate network of the cortex interconnected by long cytoplasmic processes, the medullary epithelium is more compact. The medulla is clearly distinguished from the cortex phenotypically, which may reflect different germ-layer origins, although the subcapsule and medulla share antigens. This relationship between the subcapsule (type 1 epithelium) and medulla is still unresolved, but since the former also lines the trabeculae, which extend into the corticomedullary junction, there is a continuous physical link between the outer and inner thymus. Hence precursor cells entering the thymus at either the subcapsule or corticomedullary junction will encounter the same epithelial cells. The profound phenotypic differences between the cortex and medulla no doubt reflect functional differences but these are still speculative. Medullary epithelium is not necessary for positive selection but it may contribute; it does have a role in tolerance at the level of inducing anergy rather than deletion. Given that T cell migration is thought to occur from the medulla, the epithelium in this region might provide the necessary differentiation signals. In addition to the interconnecting epithelium network in the medulla, there are also small clusters of swirling keratinized epithelial cells termed Hassall's corpuscles, particularly in the human, which typify the thymus. They are likely to be end-stage epithelium and may also be a site of removal of dying medullary thymocytes.

Origins of epithelium Although still unresolved, it is likely that both ectoderm and endoderm contribute

to the thymic epithelium and mesoderm to the leukocyte component. Whether the cortex and medulla derive from separate precursors or are different maturation stages of a common lineage is also unresolved. Until recently, the dogma was that there is a putative epithelial stem cell expressing determinants of the cortex or medulla, which develops into either of these regions, depending on the inductive signals it receives. It now appears likely that both regions develop autonomously and their maturation and expansion, at least in part, are dependent on the T cells themselves – immature thymocytes regulating cortical epithelium and mature T cells of either $\alpha\beta$ - or $\gamma\delta$ -TcR type inducing medullary epithelium expansion. This highlights an important symbiotic developmental relationship between the thymocytes and the stromal cells.

Nonepithelium

Despite its primary role as the central organ for inducing T lymphocyte development, but consistent with the need to present immature T cells with a broad spectrum of self antigens, there is a surprising heterogeneity in the stromal cell compartment.

While the epithelial cells constitute the specific microenvironment, they do not act alone but rather in concert with a heterogeneous group of nonepithelial stromal cells. Of these, the most important are the dendritic cells, which are the subject of separate entries in this encyclopedia. There are two types of dendritic cells in the thymus – those of so-called lymphoid origin and those of myeloid origin. Their relative intrathymic localization is not clearly known but, as a population, dendritic cells are spread throughout the thymus, with preferential localization at the corticomedullary junction where they are thought to be the major mediators of negative selection through their specialized ability to trap, process and present antigen. In addition, MHC-class II negative macrophages are also present throughout the thymus, including within the trabeculae; their major role is in the removal of the vast number of thymocytes which die *in situ*. One of the more unusual stromal cells in the thymus are myoid cells which are present in the medulla. These contain contractile proteins reminiscent of striated muscle. Their role is unknown but they may influence T cell migration, and the presence of acetylcholine receptors on them is no doubt important for establishing tolerance to neuroendocrine molecules. In thymic hyperplasia, these acetylcholine receptors may be involved in the pathogenesis of myasthenia gravis. Fibroblasts and reticular fibroblasts are a major part of the trabeculae but they also surround the vasculature and are present scattered throughout the cortex in particular.

While they clearly have a structural role in the thymus, undergoing activation following thymic damage, the fibroblasts also provide the differentiation-inducing ligand for CD44, which is expressed on intrathymic precursors.

Given the obvious importance of vascular endothelium cell adhesion molecules and/or their ligands in mediating leukocyte extravasation at sites of inflammation, and lymphocyte trafficking through high endothelial venules in lymph nodes, it is highly likely that they also play an important role in thymocyte migration. It is unclear precisely how and where precursors enter the thymus and mature cells emigrate, although it is likely to be complex because E-cadherin – a member of the cadherin superfamily of adhesion molecules – apparently mediates migration of prethymic but not intrathymic precursors.

How does the thymic stroma influence thymopoiesis?

Despite the obvious functional importance of the thymic stroma, precisely how it mediates T cell development is unclear. MHC–peptide complexes are essential for selection and while most cells can mediate negative selection, the current dogma is that cortical epithelium would appear to be unique in its ability to induce positive selection. There appear to be differences in MHC class II expression, peptide processing and antigen presentation properties between cortical and medullary epithelial cells, which may facilitate their preferential roles in positive selection and self tolerance, respectively. It is feasible, however, that medullary epithelium is potentially capable of inducing positive selection but, because of the structural restraints of the thymic architecture, do not contact the appropriate target thymocytes. The identity of the plasma membrane molecule(s) responsible for positive selection is one of the major unsolved questions of thymus function. While much of thymic function is organ autonomous, it is very much under the influence of neuroendocrine molecules.

Thymic epithelial cells produce many cytokines, particularly interleukin 7 (IL-7), which promotes precursor survival and TcR gene rearrangement, and transforming growth factor β (TGF β), a product of subcapsular and cortical epithelial cells, which also regulates the differentiation of early thymocyte subsets. While cytokines obviously have important roles, no combination to date, alone or in conjunction with nonthymic stromal cells, has been able to recapitulate complete T cell differentiation from precursor through to mature T lymphocyte. Extracellular matrix proteins are important in cellular differentiation and function in many systems including the

thymus. A wide variety of extracellular matrix constituents have been described in the thymus, such as laminin, fibronectin, merosin and type I and IV collagens, each localizing to discrete regions. These may influence thymopoiesis either directly through provision of ligands for specific T cell surface receptors or indirectly by trapping cytokines by acting as a reservoir. They may also facilitate thymocyte migration through the stromal milieu. Multiple cell adhesion receptor–counter-receptor pairs, such as LFA-1/ICAM-1, -2, VLA-4/VCAM-1 and $\alpha_E\beta_7$ /E-cadherin, have been proposed to be involved in thymic stromal cell–T cell interactions, and, while none appear to be imperative for thymopoiesis, their combined effects and coordinated expression patterns could be major constituents of the specific thymic microenvironment.

Bone marrow

As the primary lymphoid organ in mammals generating B lymphocytes, and the major site of hemopoiesis, it is not surprising that stromal cells also play an important role in the microenvironment of the bone marrow. In contrast to the thymus, bone marrow stromal cells are almost all mesenchymal in origin. The cellular architecture of this tissue is obviously much more difficult to study than that of the thymus, and there has not been a concerted phenotypic analysis of these cells with specifically targeted mAbs. Electron microscopy and, in particular, numerous *in vitro* culture systems of bone marrow stromal cells, either as heterogeneous mixtures or long-term cell lines (LTBMCs) and clones, have provided some insight into the nature of the bone marrow stroma. It is clearly a complex three-dimensional matrix consisting primarily of cells broadly defined as: adventitial reticular cells which are localized around venous sinuses and regulate blood cell flow; fibroblastic reticular cells present in the marrow hemopoietic cord and in close proximity to foci of granulopoiesis; macrophages dispersed throughout the marrow including the central erythroblastic islands and surrounding the sinus endothelium, adipocytes which form the majority of the marrow cavity, and the vascular endothelium. All of these cells and their associated extracellular matrices are proposed to be involved in various phases of haemopoiesis and/or B cell genesis. Cell culture has provided one approach to resolving the cellular basis to bone marrow function. Following the original 'Dexter' long-term cultures of bone marrow stroma for hemopoiesis, various modifications have been described, notably that of Wittlock and Witte, which skew the cultures towards B cell genesis. The identity

of the stromal cell(s) mediating B cell genesis is unclear but similar activity has been reported for some non-bone marrow stromal cells, including the thymus and fetal liver.

A variety of cytokines are produced by bone marrow stroma including G-CSF, M-CSF, GM-CSF, IL-1, IL-6, IL-7, IL-8, IL-11, SCF, TGF β , TNF; these are released into the immediate environment but may also selectively bind to components of the extracellular matrix, forming foci of cytokines. Bone marrow stromal cells also express many of the cell adhesion molecules important for induction of lympho- and hemopoiesis and, together with the cytokines, for maintaining the structural integrity of the microenvironment.

Not only are the bone marrow stromal cells vitally important for normal blood cell development, there is also evidence that they may be of etiological significance in some forms of leukemia development, although leukemia-associated abnormalities in bone marrow stromal cells may be a consequence rather than cause of leukemia.

In conclusion, the specific inductive microenvironments of the primary lymphoid organs are clearly encompassed within the stromal components. These cells, however, may not only be exclusive to the thymus or bone marrow. Some may be present in other sites throughout the body, such as the spleen, gastrointestinal tract and fetal liver, but it is their structural organization and proximity to the cells whose development they induce and regulate that normally provides the capacity for highly efficient lympho- or hemopoiesis. As a corollary, abnormalities in these cells at the cellular, molecular or structural levels could be of etiological significance in development of disease states such as leukemia or immunodeficiencies. The generic term 'stromal cells' clearly belies their phenotypic and functional complexity. Identification of the specific stromal cell genes which regulate lymphopoiesis and the factors determining the structural organization of the lymphoid tissues remain the key areas of research in this field, the ultimate goal being to target stromal cell products as a rational approach to immunotherapy.

See also: B lymphocyte differentiation; Bone marrow

and hematopoiesis; Dendritic cells; Thymic epithelium: potential role in regulatory T cell tolerance; Endothelium; Fibroblasts; Lymph nodes; Microenvironment; Spleen; Thymus; T lymphocyte differentiation.

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STRONGYLOIDIASIS

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Strongyloidiasis is caused by infection with any member of *Strongyloides*, a parasitic genus comprising 38 named species collectively known as threadworms. Humans, dogs and cats are infected by *Strongyloides stercoralis*. *S. ratti* is a parasite of wild rodents which has a worldwide distribution and can be easily maintained in laboratory rats and mice. The relationship of these two species with their respective hosts' immune system has been investigated most extensively, but other species are of considerable clinical and economic importance. These include *S. fülleborni*, primarily a parasite of primates, but known to cause human infections in Africa and New Guinea; *S. ransomi* and *S. westeri*, which infect swine and equines, respectively, on the American continent; and *S. papillosus*, found in ungulates throughout Europe and North America.

Life cycle

The adult females of *S. stercoralis* reside in the lamina propria of the small intestine, just underneath the epithelial layer. Their eggs hatch locally and liberate rhabditoid larvae. These slow-moving, plump larvae reach the intestinal lumen and pass into the external environment with the feces. In the soil, they molt into slender, fast-moving filariform larvae, the infective forms able to penetrate the skin of suitable hosts. From the skin, filariform larvae follow poorly understood migratory pathways, molt several times along the way, become adults and settle in their small intestinal niche.

S. stercoralis has the ability to reproduce within a single host, a phenomenon referred to as autoinfection. This occurs as some rhabditiform larvae molt into the infective filariform larvae while still in the host's intestine, penetrate the colonic wall, complete their cycle and return to the small intestine as adult parasites. Otherwise healthy hosts may harbor asymptomatic infections for decades while the total worm burden appears to remain relatively constant. In compromised hosts, particularly those receiving corticosteroids, the host-parasite balance may be altered, with a resulting amplification of the autoinfectious cycle (hyperinfection) that rapidly leads to the widespread dissemination of filariform larvae throughout the body and death.

Humoral immune responses

More than 90% of immunocompetent patients with strongyloidiasis have detectable serum levels of immunoglobulins of the IgG, IgA and IgE classes specifically directed against the antigens of *S. stercoralis* filariform larvae. The most prominent humoral response directed against *S. stercoralis* filariform larvae is represented by the IgG4 subclass. More than 50 different antigens, most between 15 and 100 kDa, are recognized by IgG4, while less than 20 antigens are recognized by the other IgG subclasses. A 40 kDa protein identified as zinc endopeptidase and two other unidentified proteins (31 and 28 kDa) are among the antigens that elicit the most consistent and specific responses in infected patients. There is no evidence, however, that any of the circulating antibodies directed against *S. stercoralis* antigens have a role in protecting the host against dissemination. Many patients without detectable humoral responses against parasite antigens have apparently well-controlled, asymptomatic infections. High titers of parasite-specific antibodies may be found in the serum of immunocompromised patients with disseminated infection. Immunologic studies in primate and canine models of steroid-induced disseminated strongyloidiasis have failed to demonstrate a relationship between humoral responses and the course of the parasitosis. Partial protection against primary infection, however, has been demonstrated in rats actively and passively immunized against *S. ratti* larval antigens.

Cellular immune responses

In published reports of patients with disseminated strongyloidiasis, some unspecified impairment of the cell-mediated immunity is often cited as the cause of the dissemination of the parasite. Though plausible, this concept is not supported by experimental or clinical data. In canine and primate models no correlation has been demonstrated between decreased lymphocyte responsiveness to *S. stercoralis* antigens *in vitro* and behavior of the infection. Human studies have been few and inconclusive, in part because immunocompromised patients with disseminated infection are frequently on drug regimens that greatly and nonspecifically depress their cellular immune response.

An argument against a central regulatory function of cellular immunity in the protection against disseminated strongyloidiasis is provided by the fact that disseminated *S. stercoralis* infection has not emerged as an opportunistic infection in patients with the acquired immune deficiency syndrome (AIDS), even in areas such as Central Africa and Brazil where both strongyloidiasis and AIDS are highly endemic. Thus, the host factors that regulate the autoinfection may not be affected by the destruction of the effector branch of the immune system occurring in AIDS.

Hypothetical regulatory mechanisms

Investigations of the relationship between *S. stercoralis* and its hosts have resulted in the characterization of a number of immune responses, some of which (IgG and IgE) have been exploited to develop sensitive and specific serodiagnostic tests. However, the regulatory mechanisms that appear to control the parasite population in competent hosts and fail to operate in the presence of immunosuppression still elude explanation.

A recent theory suggests that glucocorticosteroids themselves (but not their immunosuppressive effects) could cause the dysregulation of the infection that may lead to hyperinfection. According to this hypothetical model, *S. stercoralis* maintains the optimal size of its parasitic population in the human duodenum by regulating larval molting through the production of ecdysteroids (molting hormones found in most invertebrates, including parasitic worms). The host develops specific humoral and cellular immune responses which, although insufficient to eradicate all the parasites, may exert an additional control over the size of the population. Their absence may

allow the development of larger parasitic populations, but does not cause total dysregulation because, to a large extent, worms regulate themselves. Similarly, the presence of these host responses is not sufficient to prevent dissemination should the parasitic population's own regulatory mechanisms fail. The administration of exogenous — and in some cases, even an excess of endogenous — glucocorticosteroids results in increased amounts of ecdysteroid-like substances in the host's tissues, including in the intestinal wall, where adult females reside. These substances may compete with ecdysteroid receptors and send molting signals to the existing rhabditiform larvae, which transform intraluminally into filariform larvae. Once a population has become very large it may continue to expand rapidly even at low molting rates, and the discontinuation of steroids may not be sufficient to arrest the relentless growth process. The population has been irreparably dysregulated and forced to do what parasites have learned to avoid: kill their host.

See also: Immunosuppression; Parasites, immunity to; Sex hormones and immunity.

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SUPERANTIGENS

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Superantigens (SAGs) are proteins produced by bacteria and viruses that are able to stimulate a large proportion of T lymphocytes via interaction with the variable domain of the β chain of the T cell antigen receptor (TCR-V β). To be presented to the TCR, SAGs must bind to the major histocompatibility complex (MHC) class II molecules. In contrast to con-

ventional peptide antigens which generally activate <0.01% T cells, SAGs have the ability to stimulate 2–30% of the T cell repertoire. SAGs are extensively used in studies of T cell biology in humans and rodents. SAG-mediated immune activation is likely to be responsible for some of the characteristic symptoms associated with infections with SAG-producing

microbes, such as the toxic shock syndrome and food poisoning caused by staphylococci.

The superantigen family of bacterial and viral proteins

Proteins with SAg property are produced by evolutionary divergent microorganisms, including gram-positive bacteria, gram-negative bacteria, mycoplasma, rhabdoviruses and retroviruses and it is likely that SAGs confer a biological advantage in terms of survival and persistence of the microorganisms in the host. Proteins reported to have SAG properties are listed in Table 1. The prototype bacterial SAGs are the *Staphylococcus aureus* entero-

toxins (SEs). The SEs are single-chain polypeptides with high intrinsic stability and molecular masses ranging from 22 to 28 kDa. The amino acid sequence homologies among different SEs varies from 20 to 80% identity. The structure of several SEs (SEA, SEB, SEC₂, SED and TSST-1) has been determined by X-ray crystallography which revealed a highly similar topology in their three-dimensional structures, indicating a strong evolutionary pressure to preserve the structural integrity of the proteins. In general, they are monomeric two-domain structures (Figure 1). The two domains are made up of two β sheets capped by an α helix (domain I) and a β sheet packed against a central helix (domain II).

Mouse mammary tumor viruses (MMTV) are the

Table 1 TCR-V β recognition of superantigens

Superantigen	Mouse TCR-V β	Human TCR-V β
<i>Viral</i>		
Mtv-1 (Mls-4a)	3, 5	
Mtv-2	14	
Mtv-3	3, 5, 17	
Mtv-6 (Mls-3a)	3, 5	
Mtv-7 (Mls-1a)	6, 7, 8.1, 9	
Mtv-8 (Dvb11.1)	5, 11, 17	
Mtv-9 (Etc-1;Dvb 11.2)	5, 11, 12, 16	
Mtv-11 (Dvb 11.3)	5, 11	
Mtv-13 (Mls-2a)	3, 5	
Mtv-27	3, 5	
Mtv-29	16	
Mtv-43	6, 7, 9	
Mtv-44	3, 5, 8.1, 9	
Mtv-50	6, 8.1	
Rabies virus	6,7	8
<i>Bacterial</i>		
SEA	1, 3, 10, 11, 12, 17	1.1, 5, 6.3, 6.9, 7.3, 7.4, 9.1, 18
SEB	7, 8.1, 8.2, 8.3	3, 12, 14, 15, 17, 20
SEC1	7, 8.2, 8.3, 11	3, 6.4, 6.9, 12, 15
SEC2	8.2, 10	12, 13.1, 13.2, 14, 15, 17, 20
SEC3	7, 8.2	3, 5, 12, 13.2
SED	3, 7, 8.3, 11, 17	5, 12
SEE	11, 15, 17	5.1, 6.1, 6.2, 6.3, 8, 12, 18
TSST-1	15, 16	2
ExF-T	10, 11, 15	2
SPeA	8.2	2, 8, 12, 15
SPeB		2, 8
SPeC		1, 2, 5.1, 8, 10
SPeF		2, 4, 8, 15, 19
SSA		1, 3, 5.2, 15
MAM	6, 8.1, 8.2, 8.3	3, 17

The data has been collected from various reports. Some of the SAGs are not yet available in recombinant form and their classification as SAGs remains uncertain. Mtv, mouse mammary tumor virus; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin 1; ExF-T, exfoliative toxin; Spe, streptococcal pyrogenic toxin; SSA, streptococcal superantigen; MAM, mycoplasma arthritis mitogen.

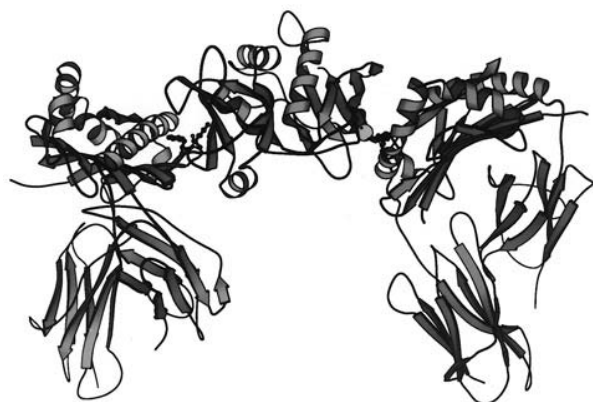


Figure 1 Molscript model of SEA interacting with two MHC class II molecules. SEA is colored red, MHC class II and bound peptide blue and purple, respectively. On the left-hand side MHC class II β -chain contacts the SEA coordinated zinc ion (yellow) through a histidine residue on the MHC class II β chain (His81, illustrated as ball-and-chain). The right-hand side shows the interaction between MHC class II α chain and SEA. This point of interaction is centered around a phenylalanine residue in SEA (Phe47, illustrated as ball-and-chain) and a lysine residue in MHC class II α chain (Lys39, illustrated as ball-and-chain). The TCR binding interface is located at the top of the SEA molecule. (See also color Plate 9.)

best characterized viral SAGs. They were originally discovered because of their ability to stimulate a primary mixed lymphocyte reaction between MHC-identical mice (Mls antigens). MMTV SAGs are encoded within the 3' long terminal repeat of the virus genome, either by retroviral cDNA incorporated in the mouse genome or from B lymphocytes infected with MMTV. They are type II membrane proteins of approximately 320 amino acid residues. Most likely, the MMTV SAG is a proteolytic cleavage fragment of the full length protein. The products of the SAG genes of the MMTV affect the T cell repertoire by causing thymic deletion of T cells expressing TCR encoded by V β segments reactive with the MMTV SAG. Since these deletions affect large populations of T cells with receptors of heterogeneous specificity, they serve as an important model for the study of T cell development in normal mice. A similar role of endogenous SAGs has so far not been demonstrated in humans.

MHC class II binding

SAGs do not activate T cells in solution, but generally require multivalent presentation on MHC class II-antigen-presenting cells (APCs). SAGs differ from conventional peptide antigens in that they bind as unprocessed proteins with high affinity to MHC class II molecules on APCs. In this way, SAGs are able to subvert the T cell antigen presentation pathway for their own benefit. The binding of SAGs to MHC

class II is promiscuous and allows each SAG to bind to a large number of different MHC class II alleles and isotypes, although with variable affinity. The binding of SAGs to MHC class II generally occurs outside the conventional peptide-binding groove, but the affinity is to some extent influenced by the nature of bound peptides.

Structural and functional studies have shown that SEA and SEB share a common binding site located in the amino-terminal domain, interacting with the nonpolymorphic α chain of MHC class II. In addition SEA (as well as SED, SEE and probably SEH) have a second binding site located in the carboxyl-terminal domain, which is dependent on a zinc ion for interaction with the β chain of MHC class II (Figure 1). Biochemical studies of SEA have shown that three amino acids in domain 2, His187, His225 and Asp227, are crucial for zinc coordination and interaction with MHC class II (Figure 1). Structural models suggest that SEA cannot engage the α and β binding sites on a single MHC class II molecule and indicate that SEA may cross-link separate MHC class II molecules. Cross-linking of MHC class II by SEA induces the expression of inflammatory cytokines in monocytes. SEA mutants or natural SE variants lacking either the MHC class II α or β chain binding sites are unable to stimulate monocytes, implicating that cross-linking of separate MHC class II molecules is a biologically important feature allowing the release of inflammatory cytokines.

Recent studies have demonstrated that SAGs targeted to cell surfaces, by conjugation of the SAG with monoclonal antibodies to cell surface structures, activate some but not all SAG responding T cells in the absence of MHC class II. Moreover, SAGs are able to activate T cells in mice lacking MHC class II. This suggests that SAGs may engage TCRs in a functional manner in the complete absence of MHC class II. A direct interaction between SAGs and the TCR is supported by biochemical and structural data implicating a binding affinity in the μ M to mM range. MHC class II molecules are abundantly expressed on all types of APC. It is therefore possible that MHC class II molecules were favored during evolution as a SAG receptor to allow multivalent presentation on a broad variety of APC. In addition, for certain SAG-TCR combinations, MHC class II molecules contribute to create a high avidity due to the intrinsic affinity between MHC and TCR complexes.

TCR interaction

The ability of SAGs to activate such a substantial fraction of the T cell repertoire is mainly due to the interaction of SAGs with the V β domain in the TCR.

The contribution of the other five, D β , J β , V α , D α and J α , variable parts of the TCR $\alpha\beta$ complex is relatively limited. A particular SAg activates a distinct pattern of TCR-V β expressing T cells, allowing each individual SAg to give an almost unique immunological 'V β fingerprint' (Table 1).

The TCR binding region in the SE proteins is located in the vicinity of the shallow cavity formed between the two domains (Figure 1). Recent structural studies of SEC2 and SEC3 in complex with a TCR β chain indicate that the key points of interactions, in the V β chain, are located in the CDR1, CDR2 and HV4 loops.

The large family of MMTV SAgS, probably containing more than 50 different SAgS, seem to primarily differ in the outermost C-terminal part. This region has been assigned as the TCR binding domain because of a remarkable correlation with homology between different MMTV and the V β repertoire of activated T cells. MMTV interactions with TCR-V β has primarily been mapped to the HV4 loop.

Superantigens and T cell biology

T cells require two signals to be activated to proliferate and produce cytokines. SAgS essentially activate T cells in a manner similar to conventional peptide antigens. SAgS bound to MHC class II complexes on APCs provide the first signal by ligation of the TCR, while a second signal is delivered by costimulatory molecules on the APC, such as CD80 or CD86. Exposure to bacterial SAgS *in vivo* results within hours to secretion of T cell-derived cytokines, including interleukin 2 (IL-2), tumor necrosis factor α (TNF α) and interferon γ (IFN γ). This is followed within days by expansion of CD4 and CD8 T cells bearing the SAg reactive TCR-V β and development of cytotoxic T cells. IL-2 production resides predominantly in the CD4 subset, while IFN γ is produced by both subsets. Production of substantial amounts of TNF α is seen in T cells as well as monocytes, emphasizing that multiple immune cells are activated after SAg exposure *in vivo*. Activated CD8 cytotoxic T cells efficiently kill cells with SAgS bound to their MHC class II molecules, a mechanism termed SAg-dependent cell-mediated cytotoxicity (SDCC). SDCC represents a powerful mechanism for targeted T cell cytotoxicity against APCs. It has been suggested to serve as a mechanism to inactivate APCs and thereby downregulate specific immunity against the SAg-producing pathogen. Later in the activation phase, extensive T cell deletions and functional inactivation (anergy), particularly among CD4⁺ T cells, limit the size and function of the expanded T cells.

Superantigens in disease

Bacterial superantigens have been implicated as a causative agent in several acute pathological conditions, including food poisoning, toxic shock associated with staphylococcal sepsis, the toxic shock syndrome, scalded skin syndrome and scarlet fever. A common feature of these diseases is massive systemic immunoactivation, as reflected by high serum levels of TNF α , IL-1, IL-6 and IFN γ . Associated toxic shock is likely, at least in part, to be due to excessive release of cytokines.

SAgS are believed to be involved in the pathogenesis of some viruses. The MMTV retrovirus is secreted in the milk of infected mice and infects B cells of lactating pups. MMTV SAgS presented on infected B cells induce V β -specific T cell activation. The T-B cell interactions support proliferation of infected B cells, facilitating perpetuation and spreading of the virus. The involvement of similar SAg encoding retroviruses in human disease remains to be shown. SAgS are also implicated in the pathogenesis of rabies virus. This is supported by the fact that T cells expressing TCR-V β 6 are expanded in rabies-infected mice, and mice lacking V β 6 T cells are difficult to infect. The immunostimulatory properties of SAgS may lead to a breakdown of tolerance and favor development of autoimmune responses. In Kawasaki syndrome, a cause of acquired heart disease in children, general immunoactivation with expansion of T cells expressing TCR-V β 2 and 8 and high systemic levels of IFN γ is frequently observed. IFN γ induces expression of MHC class II molecules on aortic endothelial cells, which is believed to facilitate SAg-dependent destruction of these cells. Staphylococcus strains producing TSST-1 have been implicated as a major causative agent. In a number of autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, Sjögren's syndrome, autoimmune thyroiditis and psoriasis, a skewed pattern of V β usage has been observed. Moreover, it has been shown that SEs and possibly *Mycoplasma arthritidis* mitogen induce and exacerbate autoimmune disease in various experimental models. This has suggested to a number of investigators that SAgS may be involved in modulating human autoimmune disease. However, no firm evidence is present to support a role for SAg in the initiation or perpetuation of chronic inflammatory diseases.

The potent immunostimulating properties of SAgS have recently been exploited for immunotherapy of cancer. Recombinant fusion proteins between tumor reactive monoclonal antibody fragments and SAgS mutated to reduce MHC class II binding have been used successfully to treat experimental tumors. Such

tumor-specific SAgS localized to the tumor and were able to induce massive infiltration in tumors of T cells actively producing TNF α and IFN γ , and resulted in apparent cure of animals in the absence of systemic toxicity.

See also: Antigen presentation via MHC class II molecules; Anergy, T cell; Clonal deletion; Cytokines; Septic shock; *Staphylococcus*, infection and immunity; T cell receptor, recognition by; T lymphocyte activation.

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SUPPRESSOR T LYMPHOCYTES

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From almost the beginning of immunology as a research discipline, there has been the realization that the immune system must have some way of controlling or preventing antiself (autoimmune) responses. Related to this issue is the question of how the immune response is turned off once a response to antigenic challenge is initiated. In the early 1970s, Gershon, among others, made a remarkable suggestion that there existed cells in the immune system whose purpose was to inhibit the capacity to mount an immune response. These cells he termed 'suppressor cells'. He hypothesized that such cells could explain many of the phenomena that immunologists had observed earlier, such as infectious tolerance, i.e. the ability of T cells tolerant to antigen A to transfer tolerance to antigen A from one host to another without affecting the response to antigen B. For the past 20 years a large number of researchers have been attempting to unravel the complexities of immune regulation and to try to determine whether the ingenious but nonetheless controversial proposal of Gershon, namely the existence of a specific subset of T suppressor (T_S) cells, is valid.

Historical background

In the late 1960s it was found that mice made tolerant to either a hapten or protein antigen contained

lymphocytes in the spleen and lymph nodes that could specifically transfer the tolerance to a recipient animal. Conversely, fresh T lymphocytes (antigen primed or unprimed) transferred into a tolerant recipient were unable to break the tolerant state and also became tolerant. These results had two consequences: first, it was demonstrated that tolerance could be both antigen specific and dependent on the presence of a specific population of lymphocytes, namely T cells; secondly, it laid to rest the notion that the shutdown or negative control of an immune response was solely the result of antibody-dependent feedback mechanisms. Other workers, at the same time, using syngeneic murine tumor models were demonstrating that tumor-bearing animals possessed fully immunocompetent antitumor cells. Nonetheless, these mice not only failed to respond to the tumor, but contained T cells that prevented the host from making an antitumor response. Yet another converging line of studies came from experiments using strains of mice that spontaneously developed autoimmune syndromes such as the NZB/NZW F1. It was shown that T cells from young NZB/NZW F1 that had not yet developed autoimmune disease were capable of delaying the full expression of the autoimmune syndrome when transferred to older mice already beginning to show clear signs of disease.

While these studies provided the phenomenological background for the emerging hypothesis concerning the existence of specific suppressor cell mechanisms, other lines of experimentation were developing that gave the concept a more mechanistic foundation. It had been noticed in the late 1960s that certain strains of guinea pigs and mice injected with synthetic homopolymers of selected amino acids (polylysine or polyglutamic acid) failed to mount an immune response to these molecules. In subsequent exhaustive studies of this phenomenon, particularly in the mouse, it was discovered that such nonresponsiveness to synthetic polypeptide antigens was directly linked to class II genes in the major histocompatibility complex (MHC). Furthermore, it was found that at the cellular level this lack of responsiveness was not due to lack of specific B cells or T helper cells but rather was because of the preferential activation of populations of antigen-specific suppressor T cells. These studies for the first time linked the phenomenon of suppression to specific sets of genes and it was assumed that from experiments such as these eventually would come complete genetic and molecular understanding of suppressor T cell biology and biochemistry.

Further support for the role of antigen-specific T_s cells in immune regulation came from studies that showed that cell extracts or supernatants obtained from cultures of such cells contained antigen-specific suppressor molecules that could substitute for the suppressor T cells. Such studies were in keeping with the emerging concept that all aspects of immune regulation were dependent on the production of soluble cytokines that served as growth and differentiation factors as well as inhibitors of immunity. Thus it was that antigen-specific suppressor cells were noted as being different from helper T cells or macrophages in their apparent capacity to down-regulate immune responses via soluble suppressor mediators rather than augmenting them.

Lastly, the soluble mediators of suppression were unique from most of the cytokines then being studied in that they showed exquisite antigen specificity. However, it is important to note that it has now been shown that much of what was taken as evidence of cell-mediated suppression in fact represents the interaction of specific populations of T cells termed T_{H1} and T_{H2} , each of which make unique sets of cytokines that have the capacity to suppress the activity of the reciprocal cell. These observations have now been broadened into the concept of type I and type II cytokine-producing cells in the immune system since it has been found that both $CD4^+$ and $CD8^+$ T cells as well as mast cells exhibit the capacity to

express either the type I or the type II cytokine phenotype.

Finally, although T_s cells could be distinguished functionally from other regulatory T cell subsets they were indistinguishable phenotypically from T cells involved in cytotoxicity (e.g. $CD8^+$) until several workers serologically defined an apparent new locus within the MHC class II region which they termed I-J. Alloantisera specific for this locus reacted with both T_s cells and T suppressor factors (as well as with a few $CD4^+$ T cells) and thus provided what was, at that time, the only reagent that could be used to generally distinguish T_s cells phenotypically from other $CD8^+$ T cells. Within the last couple of years, new serological markers have emerged that distinguish T suppressor cells in mice and humans from other T cells. In at least one case, the serological marker in mice has been biochemically defined and monoclonal antibodies specific for this structure made available.

Cell and molecular biological studies of suppressor cells

The studies mentioned above plus numerous others clearly established that the concept of cell-based negative regulation had merit. Furthermore, the phenomenology of suppression could be demonstrated in virtually all types of immune responses. The challenge became to establish the physiological, biochemical and molecular genetic basis of suppression. The major questions that required study were as follows: 1) Does there exist a phenotypically and biologically distinct population of lymphocytes whose sole function is to specifically suppress immune responses (i.e. 'professional' T_s cells)? 2) What is the precise role of such cells in immunity (e.g. do they play a role in tolerance, autoimmunity, regulation of normal immune responses and tumor immunity) and what is the interplay between T_s cells and other regulatory elements? 3) What is the biochemical and molecular basis of T_s cell function? 4) What is the nature of the antigen receptor on T_s cells and how is it related to the antigen-specific suppressor factors?

Initially, at least, the question of a phenotypically distinct population of T_s cells did not seem a major concern since the anti-I-J antisera seemed to preferentially distinguish T_s cells from other $CD8^+$ T cells. Then, it was discovered that in the region where I-J mapped genetically (between I-A and I-E), there was no I-J gene. Furthermore, when the two strains of congenic mice that were originally used to define the I-J gene location were examined, no difference was found at the DNA level in the region where genetic

mapping had placed I-J. Thus, researchers were found in the awkward position of having antibodies to an undefined cell surface determinant present on many T_S cells that had no known genetic origin, let alone a known function. This picture has been further complicated by the observations that interferon γ ($IFN\gamma$) produced by T_H1 cells can inhibit the differentiation and cytokine production by T_H2 cells (which make interleukins 4, 5 and 10). Conversely T_H2 cell-specific cytokines can block the activity of T_H1 cells. In certain *in vitro* and *in vivo* assays such activities read out as suppression and the cytokine-producing cells have been misrepresented as suppressor cells. It is also clear that both IL-10 and $IFN\gamma$ are capable of inducing monocytes, macrophages and B cells to produce additional cytokines that can negatively regulate immune responses. This has increased our sophistication in terms of understanding immune regulation but has only partially resolved the issue of antigen-specific suppression. Researchers working in both human and murine systems have also defined cell surface markers that denote specific subpopulations of $CD8^+$ T cells that act exclusively as suppressor cells. In the mouse these have been most clearly defined by a monoclonal antibody termed 984D4.6.2 which recognizes an $\alpha 2,6$ sialic acid-lined carbohydrate defined as LSTc, a carbohydrate moiety originally detected in human milk.

Finally, several laboratories have shown that T_S cells seem to be capable of recognizing nominal antigen in the absence of antigen-presenting cells; in fact, T_S cells could be shown to bind to immobilized antigens and selected much like B cells.

An additional challenge is that until recently T_S cells could not be cloned by growth on feeder layers in the presence of antigen and growth factors such as IL-2. Although T_S hybridomas could be constructed, such lines frequently exhibit both unstable phenotypic markers (e.g. variable expression of I-J or CD8) and a variable ability to produce antigen-specific suppressor factors. There has been a continuing effort to prepare stable T_S lines that has finally met with success. Several groups now report the preparation of both human and murine T_S clones that are antigen specific, bear the T cell receptor (TCR) complex and, in some cases, secrete or release antigen-specific T suppressor factors (TSF).

In terms of the precise role for suppressor cells in normal and pathological immune responses, evidence has continued to grow suggesting that the phenomenon of suppression is an important aspect of the homeostatic control mechanism in the immune system. As mentioned earlier, T_S cells could be demonstrated in MHC-controlled immune responses to

synthetic polypeptides, in response to protein and erythrocyte antigens, in contact sensitivity reactions, in delayed-type hypersensitivity, in antitumor immunity, autoimmune disease and in transplantation immunity. The common features of such studies were the existence of some type of inducer cell that produced an antigen-specific, non-MHC-restricted TSF, and effector T_S cells that produced either an antigen-specific, MHC-restricted TSF or a non-specific TSF (probably a cytokine) that blocked T helper function.

Indirect evidence suggests that for the most part, antigen-specific T_S cells act at the level of T helper cells. Thus, recent studies show a decrease in the production of T cell-specific cytokines (e.g. IL-2 or IL-4) following activation of T_S cells. Cloned T_S cells have now been shown directly to be capable of inhibiting the production of cytokines by T helper cells, providing the first biochemical evidence for a mechanism by which T_S cells regulate immune responses. This has been perhaps best studied in models of cellular immunity involving the response to alloantigen in mice and in humans.

Recently, it has become clear that many other cells besides the putative antigen-specific T_S cells can, at times, negatively regulate immunocompetent cell function. The interaction of T_H1 and T_H2 cells is one example cited earlier. The capability of T cells to respond to closely related peptides or proteins is directly linked to the expression of an appropriate MHC class II molecule and a given clone of T cells could fail to respond to an antigen due to failure to recognize that antigen in the context of an inappropriate class II protein. Experiments have shown that a given antigen-specific T cell clone can be rendered unresponsive to antigen if it is paralyzed by an antigen overdose. T cells that have not previously been exposed to antigen may also be rendered nonresponsive if they are presented with their cognate antigen by an inappropriate antigen-presenting cell such as a B cell. Related to this is the fact that immunocompetent cells exposed to other, nonimmune cells in the body could be rendered nonresponsive by such cells (veto cells) under certain circumstances, thus preventing a potential autoimmune response. Thus, immunologists have discovered several situations in which clear-cut evidence of unresponsiveness could be demonstrated not to be dependent on the presence of antigen-specific suppressor T cells.

Over the last few years it has become clear that T cell repertoire selection takes place in the thymus and is critically dependent on the expression of the T cell receptor and associated molecules such as CD4 and CD8. Thus, many potentially autoreactive T cells are eliminated before they ever leave the thymus. Like-

wise, T cells that are not self-responsive are selected in the thymus for migration out to the peripheral tissues. It has also been discovered that B cells can be rendered nonresponsive directly via mechanisms that are T cell independent.

The picture that is emerging suggests that the initial regulation of the immune system takes place in the thymus where positive and negative selection mechanisms operate. Despite the powerful selection processes that exist in the thymus, it has been repeatedly shown that autoreactive T cells and B cells exist in the periphery. The regulation of such cells to prevent their activation has been shown to involve T_S cells. There are now data to suggest that possibly this type of T_S cell-based regulation involves a subset of T cells that can recognize T cell antigen receptor idiotypes.

The issue that has most perplexed immunologists that study T_S cells has been the inability to understand the molecular basis of T_S cell antigen specificity and the nature of the antigen-specific TSF molecules. At the beginning of the 1980s, before the discovery of the molecular nature of the T cell antigen receptor, it was known that at least one form of TSF consisted of a disulfide-linked heterodimer, one chain of which was capable of binding to its inducing antigen and one chain of which contained the restriction element or serological determinant for MHC class II (e.g. I-J). By the mid-1980s, evidence had mounted that there were probably two major classes of TSF, one was the heterodimer and the other most likely a homodimer that is expressed by suppressor inducer cells.

All of the evidence concerning the structure of the TSFs came from experiments done using culture fluids or extracts of T_S cell hybridomas. While such experiments worked well at the protein level, the critical question about the nature of TSF genes remained elusive. With the discovery of the T cell receptor genes it became possible to analyze any T cell line for the presence of appropriately rearranged genes. Initially, there was some confusion about whether T_S cell hybridomas expressed normal T cell antigen receptors. That issue has now been resolved and a number of experiments have been performed at the cellular and molecular level to explore the role of T cell antigen receptor genes and proteins in antigen-specific suppression.

Taken as a whole, the experiments indicate that the TCR α chain protein forms the antigen recognition component of soluble TSF. In some T_S cells and lines it appears that the TCR β chain protein is also involved; however in most T_S cell lines studied the molecular and genetic character of the second chain of the TSF has not been unambiguously

defined. One exception is the recent study in immediate-type hypersensitivity which suggests that the non-antigen-binding chain of the TSF is a molecule termed 'glycosylation inhibitory factor'. This molecule has been cloned and characterized as being the biologically active component of a TSF that blocks the development of immediate-type hypersensitivity in tissue culture and *in vivo*. Suffice to say this area of research remains the most controversial aspect of suppressor cell biology and in the minds of most immunologists still remains to be fully explained.

Current prospects

At the phenomenological level (e.g. in tissue culture models and *in vivo*) antigen-specific suppression of immune responses mediated by specific subpopulations of T cells can be unequivocally demonstrated using monoclonal antibodies that recognize T_S cell-specific cell surface proteins. Such proteins have also been found on putative T_S cell hybridomas and more recently on T_S cell clones. Based on the use of monoclonal antibodies and experimental data from many laboratories, it is possible to construct a hypothetical scheme (see Figure 1) in which both nonspecific and antigen-specific T_S cells play a role in the regulation of immune responses. This scheme attempts to tie the activation and regulation of T_S cells with other cells

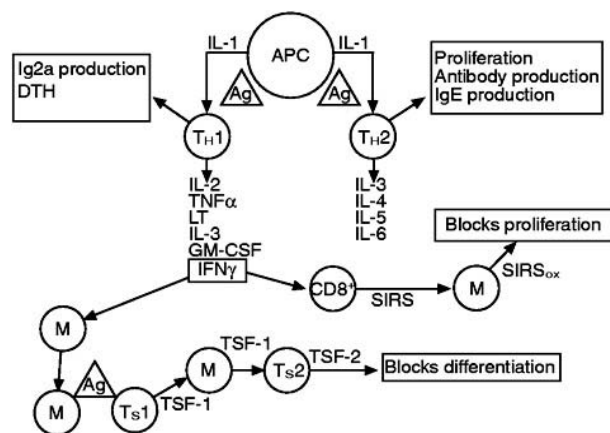


Figure 1 Hypothetical scheme of nonspecific and antigen-specific suppressor T cell regulation of immunity. The scheme attempts to integrate cytokine-mediated regulation by T helper cells (T_{H1} and T_{H2}) with regulation mediated by nonspecific and antigen-specific suppressor T cells. DTH, delayed-type hypersensitivity; APC, antigen-presenting cell; Ag, antigen; M, macrophage; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF, tumor necrosis factor; LT, lymphotoxin; IFN γ , interferon γ ; SIRS, soluble immune response suppressor; SIRS $_{ox}$, oxidized soluble immune response suppressor; T_{S1} , T suppressor inducer; TSF-1, suppressor inducer factor; T_{S2} , T suppressor effector; TSF-2, suppressor effector factor.

and cytokines. For example, it is proposed that interferon γ plays a key role in activating antigen-specific T_S cells via a mechanism similar to that involving induction of MHC class II expression. Other cytokines, such as IL-2, also play important roles in the growth and regulation of T_S cells of all types. With the relevant monoclonal antibodies, it is possible to test the validity of the scheme, at least in tissue culture models. Such antibodies also offer the chance along with appropriate cell lines to answer the fundamental questions posed earlier concerning the nature of the T cell antigen receptor on such cells and its relationship to the soluble factors produced by these cells. Only when the characterization of T_S cells and mediators at the molecular level takes place will immunologists be completely convinced that discrete antigen-specific T_S cells exist. Without such characterization, the phenomenon of antigen-specific T_S cells will remain to puzzle and perplex us.

See also: Cytokines; Effector lymphocytes; I-J; Immune response; T cell receptor, $\alpha\beta$; T lymphocytes.

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SURFACE PLASMON RESONANCE

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The use of biosensors in biomedical research has increased substantially in recent years. Many different types of biosensor have been developed, including electrochemical (amperometric, potentiometric or conductimetric), calorimetric, acoustic and

optical. Advances in the development of optical biosensors have been particularly fast and have resulted in the appearance of several commercially available instruments that are now used widely in academic, clinical and industrial laboratories. The

most commonly employed instruments have a detection system based on the phenomenon of surface plasmon resonance (SPR), which can be exploited to measure interactions between macromolecules in real time. This detection system essentially measures changes in the refractive index of the medium surrounding a 'receptor' immobilized on a solid support, which occur when a 'ligand' is allowed to bind. SPR has been particularly successful in the analysis of antibody-antigen interactions and can be used to obtain kinetic parameters (association and dissociation rate constants) and equilibrium binding constants, as well as measure concentrations, perform epitope mapping and determine antibody isotypes with experimental procedures that can be substantially more accurate, sensitive and reliable compared to conventional methodologies.

What is SPR?

When light traveling within an optically dense medium reaches the interface with a medium of lower refractive index it can be partially reflected or refracted depending on two parameters: the ratio of the refractive indices of the two media and the angle at which the incident light reaches the interface. Therefore, for a given refractive index ratio, there is a critical value for the angle of the incoming light above which total internal reflection occurs. Under these conditions, and even though the light is totally reflected, an electromagnetic field component, called the evanescent wave, penetrates the interface between the two media. As its name suggests, the evanescent wave is very weak and can only travel for approximately one wavelength within the medium of lower optical density. If the light is monochromatic and p-polarized (that is its electrical vector component is parallel to the plane of incidence) and the interface is coated with a thin layer of a metal, such as gold, then a resonance can occur between the evanescent wave and the delocalized surface of electrons (or plasmons) of the metal layer. This phenomenon, called SPR, results in amplification of the evanescent wave, taking up a considerable amount of the energy of the incident light and thus causing a measurable loss in the intensity of the reflected light. SPR only occurs at a distinct angle of the incident light, which in turn depends on the ratio of the refractive indices of the two media, the wavelength of the light and various physical properties of the metal surface. These latter parameters, as well as the refractive index of one medium can be kept constant and therefore by measuring the angle at which SPR occurs it is possible to monitor changes in the refractive index of the other medium. Since an increase in the amount

of a macromolecule causes a corresponding increase in the refractive index of a solution, SPR can be used to detect changes in mass close to a surface. For example, if an antibody is immobilized on that surface, binding of an antigen will cause an increase in the refractive index which can be measured by SPR.

SPR-based biosensors

Several optical biosensors are commercially available, all exploiting the evanescent wave and using SPR or other, wave-guiding techniques for detection. They include the BIAcore (Biacore AB, Uppsala, Sweden), IASYS (Affinity Sensors, Cambridge, UK), IBIS (Intersens Instruments, Utrecht, Netherlands) and BIOS-1 (Artificial Sensing Instruments, Zurich, Switzerland). The vast majority of published applications have used several different models of the BIAcore system and therefore the following descriptions are based on these instruments.

The BIAcore has a light source that produces a wedge of monochromatic light in order to provide a wide range of incident angles, all above the critical value for total internal reflection. The light passes through a prism and is focused on a small area (1.4×0.16 mm) on the surface of a disposable 'sensorchip'. The latter consists of a glass support on to which a thin gold film is attached. For most applications, a carboxymethylated dextran layer (100 nm thick) is fixed on to the gold surface in order to provide a hydrophilic environment for immobilization of one of the interacting components (such as an antibody, peptide or any other macromolecule). The sensorship is sealed against a micro-fluidic delivery cartridge, so that the dextran layer is present within a flowcell of precise dimensions (60 nl volume), through which solutions can be passed under conditions of constant flow and temperature. Several methods are available for immobilizing molecules on the dextran layer; the most commonly employed uses standard succinimide chemistry for the covalent immobilization of proteins through free amino groups. Indirect methods may also be used, such as immobilizing streptavidin to capture biotinylated molecules.

The principle of detection is demonstrated in **Figure 1** (as an example, antibody molecules are shown immobilized on the dextran layer). With buffer flowing through the flowcell, the medium close to the sensorchip surface has a certain refractive index which depends on the amount and size of the immobilized antibody and the composition of the buffer. SPR occurs at a certain angle, θ_1 , of incident light and is detected as an intensity 'dip' in the reflected light by

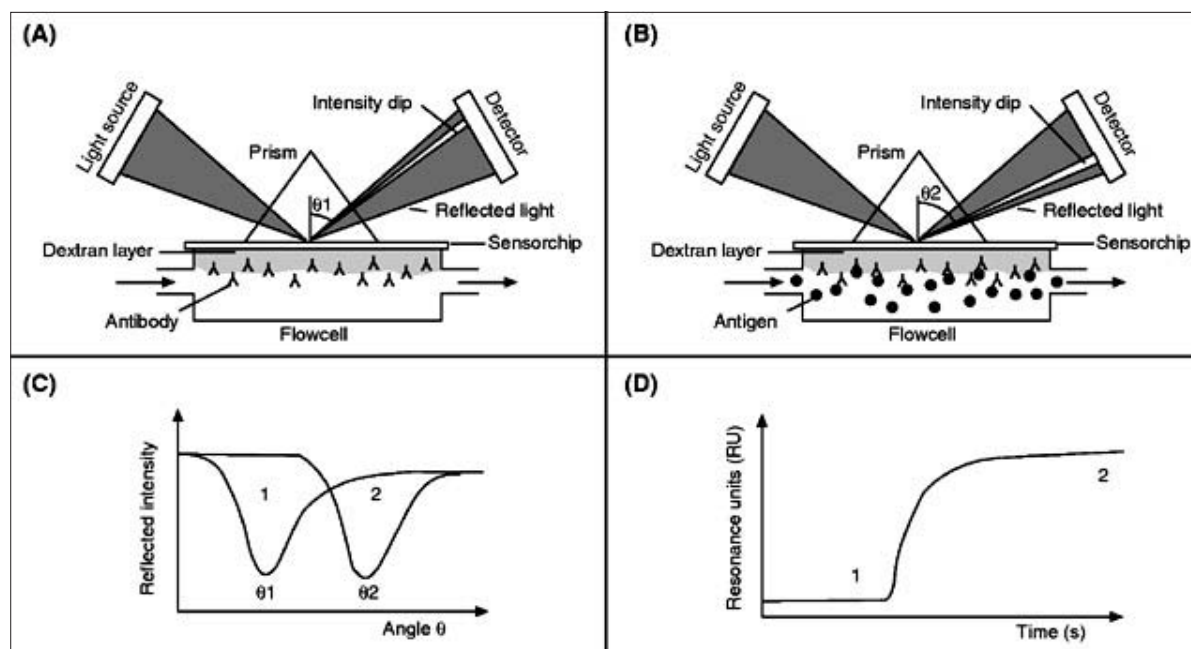


Figure 1 Schematic outline of the principle of SPR measurements using the BIAcore biosensor (not in scale). See text for further details.

a position-sensitive diode array detector (A). When antigen is injected through the flowcell, it interacts with the immobilized antibody, thus increasing the refractive index. Because of the very short distance the evanescent wave penetrates into the medium, only binding events very close to the sensorship surface are registered and the bulk of the protein solution contributes insignificantly to the signal (a 'bulk' effect can be seen at high concentrations and is constant throughout a protein injection). Upon binding, the angle of incident light at which SPR occurs changes (θ_2) and this is again registered by the detector (B). The changes in angle (C) are converted by the instrument's software into arbitrary resonance units which are plotted versus time to provide a binding curve called a sensorgram (D). The sampling rate can be set by the user and can be as high as 10 points per second, providing a real-time response. Quantitative analysis of the binding data is performed by suitable software.

Figure 2 shows examples of antigen binding to a panel of different monoclonal antibodies which are immobilized on the sensorchip. Following injection of the antigen, buffer flow is introduced resulting in dissociation of bound material and a corresponding drop in the signal. This part of the sensorgram can be used to measure the dissociation rate constant of an interaction, whereas the 'on' phase can be analyzed for quantitation of the association rate constant. Figure 2 demonstrates that different antibodies

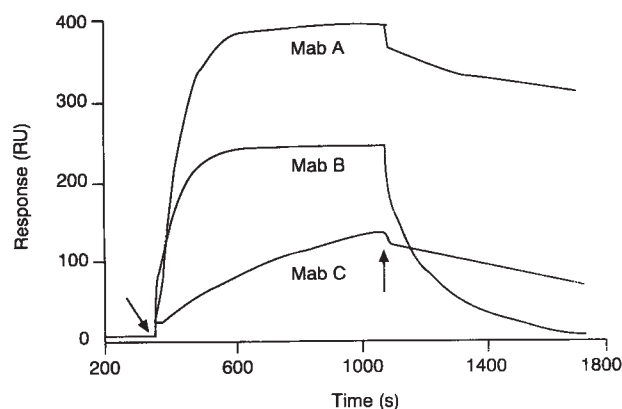


Figure 2 An example of antibody-antigen interaction measured by SPR. The same antigen was injected separately over three immobilized monoclonal antibodies. The arrows point to the beginning and the end of the antigen injection, which is followed by buffer flow. Note the differences between the antibodies in the association and dissociation rates. (Data kindly provided by Dr R. Karlsson, Biacore AB.)

have distinct on and off rates for the same antigen. The equilibrium binding constant of an interaction can also be determined by injecting a series of concentrations and allowing the reaction to proceed to equilibrium. The response can then be plotted versus the concentration and suitable fitting algorithms can be used for quantitation.

Advantages and limitations

The main advantage of SPR-based biosensor measurements is that macromolecular interactions can be monitored in real-time, providing a large number of high-quality data for analysis. There is no need to label one of the interacting components (with radioactive or other labels) and, most importantly, it is not necessary to devise experimental conditions for separating free from bound ligands, as is the case in many alternative techniques. This is particularly useful for interactions with fast dissociation rates. These factors have allowed the quantitation of interactions in a large number of different applications.

Apart from the high cost of the instruments, the most important limitation of SPR biosensors derives from the need to immobilize one of the interacting components. The immobilization process itself may alter the properties of the ligand, necessitating the development of indirect coupling methods. Moreover, the immobilized ligand must be stable to regeneration conditions which are commonly employed in order to remove bound analytes and thus prepare the ligand for subsequent experiments. Additionally, if the amount of immobilized material is not carefully controlled, mass transport limited interactions may occur which make impossible the correct quantitative analysis of the binding curves. A further limitation regards the size of the analyte which is allowed to interact with an immobilized ligand: Since SPR measures changes in refractive index, molecules with a small mass (up to 5 kDa) will give weak signals which may not be suitable for accurate analysis.

Applications

Recent years have seen an exponential growth in the number of studies that have employed SPR to address a plethora of biological questions, too numerous to list here. Applications relevant to immunology have been particularly successful due to the high affinity, large size and stability of antibodies. Approximate ranges for the various parameters that can be measured include binding constants of 10^4 – 10^{12} M⁻¹, association and dissociation rate constants of 10^3 – 10^7 M⁻¹/s and 10^{-5} – 10^{-1} s⁻¹, respectively and concentration measures of 10^{-3} – 10^{-11} M. The estimation of kinetic parameters has allowed very precise 'ranking' of panels of antibodies. Epitope mapping has also been successfully undertaken with SPR, especially using instruments with automated injection systems that allow unattended operation for a large number of cycles. The

same is true for determining the isotype of different antibodies, using specifically developed kits.

See also: Affinity; Antibody-antigen intermolecular forces; Epitopes; Immunoassays.

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SYSTEMIC LUPUS ERYTHEMATOSUS, ANIMAL MODELS

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As in any disease entity, animal models with spontaneous incidence of systemic autoimmunity have been sought, and a few have been identified. Of these models, the inbred murine strains of lupus are the best suited for study because their serologic, cellular and histopathologic characteristics closely match the corresponding human disorder.

There are three main types of mice with lupus predisposition (Table 1). The New Zealand (NZ) mice (NZB, NZW and their F1 hybrid), the BXSB mouse and the MRL mouse. Crosses of SWR with NZB also develop a lupus-like disease, as do certain F1 mice undergoing a graft-versus-host reaction upon injection of parental lymphoid cells, and mice with the motheaten (*me*) mutation. The NZB (H-2^d) and the NZW (H-2^z) mice are of unknown background, the BXSB mouse (H-2^b) was derived from crosses of C57BL/6 females with SB/Le males, and the MRL mouse has a complex genome composed of LG/J (75%), AKR (12.6%), C3H (12.1%) and C57BL/6 (0.3%). For (NZB × W)F1 mice, the females develop disease earlier than males, but, in contrast, BXSB males are much more severely affected. During inbreeding, some of the MRL offspring developed massive lymph node enlargement, splenomegaly and early-life severe lupus, while others remained free of this lymphoid cell accumulation and developed a mild, late-life, lupus disease, thereby creating the MRL-*lpr/lpr* (lymphoproliferation) and the MRL-*+/+* sublines, respectively. The incidence and severity of disease is almost equal for both MRL-*lpr* sexes. The *lpr* mutation is inherited as a single autosomal recessive gene. Since its original description, the *lpr*

mutation has been transferred to a number of other standard inbred strains. These normal background *lpr* homozygous mice similarly develop various grades of lymphadenopathy and autoantibodies but, in contrast to lupus-background *lpr* homozygous mice, exhibit minimal histopathologic manifestations, thereby indicating the requirement of additional genes for lupus pathology.

Two additional single autosomal recessive mutations, termed *gld* (generalized lymphoproliferative disease) and *lpr^{cg}* (*lpr*-complementing gene) have been described. Normal background mice with these mutations also develop early-onset humoral autoimmune responses and lymphoid hyperplasia, but histopathologic manifestations such as nephritis and vasculitis are minimal. The *lpr* mutation (mapped on chromosome 19) and the *gld* mutation (mapped on chromosome 1) are nonallelic and non-complementary, while the *lpr^{cg}* mutation is complementary to both the *lpr* and the *gld* mutation.

Histologic and serologic manifestations

Lupus disease-manifesting mice develop severe glomerulonephritis and early-life thymic atrophy. Lymphoid hyperplasia is minor in (NZB × W)F1, moderate in BXSB, and severe in MRL-*lpr* mice. Arteritis and arthritis are uniquely present in MRL-*lpr* mice, while degenerative vascular disease and myocardial infarcts characterize all these types of mice. The incidence of myocardial infarcts is greatly enhanced in crosses of BSXB males with NZB or NZW female mice. Lymphoid infiltrates in several

Table 1 Derivation, H-2, accelerating factors and survival rate of lupus mice

Strains	Derivation	H-2	Accelerators	50% Survival
NZB	Unknown	d	—	15 months
NZW	Unknown	z	—	22 months
(NZB × W)F1		d/z	Female hormones	Females, 9 months Males, 13 months
MRL	LG (75%) AKR (12.6%) C3H (12.1%) C57BL/6 (0.3%)	k	<i>lpr</i> gene	<i>lpr/lpr</i> , 4–5 months +/+, 16–18 months
BXSB	SB/Le (50%) C57BL/6 (50%)	b	Y chromosome-associated	Males, 5 months Females, 21 months

organs, including salivary and lacrimal glands, are observed, particularly in MRL-*lpr* mice.

Serologically, all of the above systemic autoimmunity-prone mice develop hypergammaglobulinemia, a variety of anticellular autoantibodies (predominantly antibodies to chromatin components), and antibodies against retroviral glycoproteins, but some strain-specific responses are noted. For example, anti-Sm autoantibodies appear only in MRL (both *+/+* and *lpr/lpr*) mice, and high titers of IgM and IgG rheumatoid factors are present primarily in the *lpr* homozygous mice, while anti-erythrocyte and antithymocyte autoantibodies mainly characterize the NZB and (NZB $\times$ W)F1, and antiplatelet autoantibodies are found in BXSB mice.

Cellular abnormalities

Transplantation experiments have documented that the genetic defects of lupus are associated with the lymphoid cell compartment. An increased number of activated/memory phenotype T cells is observed in all lupus strains, B1 (Ly-1⁻) cells are elevated in NZ mice, and there is a dramatic increase with age in BXSB of atypical monocytes. In *lpr* and *gld* homozygous mice, there is a massive accumulation of a CD3⁺, CD4⁻, B220⁺ T cell subset that appears to originate from the CD8⁺ compartment. The T cells of all lupus mice manifest defective responses to *in vitro* activation signals, perhaps as a reflection of their previous activation *in vivo*. Several disturbances in cytokine production have been observed, particularly low production and response to interleukin 2 (IL-2), and increased production of interferon γ (IFN γ) and IL-10.

Major histocompatibility complex (MHC) genes

Although lupus susceptibility is not restricted to a single MHC haplotype (Table 1), the major contribution of certain MHC alleles to lupus susceptibility has been clearly demonstrated. In BXSB mice there is a dominant role for H-2^b, while for NZ mice, heterozygosity of the MHC (H-2^{d/z}) appears to confer higher susceptibility. Yet, H-2^{d/z} heterozygosity is not the only factor contributing to accelerated autoimmunity in the F1 hybrid, as crosses of NZB with SWR (H-2^a) or BXSB (H-2^b) also develop a disease similar to that of (NZB $\times$ W)F1 and crosses of NZB with congenic BALB/c.H-2^e are devoid of disease. Additional studies of H-2 congenic NZB mice revealed that the H-2^{bm12} haplotype predisposes to early onset lupus similar in severity to that in (NZB

$\times$ W)F1, whereas the H-2^b haplotype, which differs from the H-2^{bm12} I-A β gene only in three amino acids in the peptide-binding groove, predisposes to a mild autoimmune phenotype similar to that of the wild-type NZB (H-2^d). This finding, together with the absence of lupus in MRL-*lpr* mice in which the class II MHC genes have been deleted by homologous recombination, strongly implies that the class II genes are responsible for MHC-associated lupus susceptibility. Overall, however, a predisposing MHC in itself is insufficient, and additional genes are required for disease susceptibility.

Apoptosis genes

Apoptosis, a major form of programmed cell death, refers to an active, gene-directed process associated with internucleosomal degradation. Recent advances in the molecular definition of apoptosis have indicated its regulation by a complex web of genes, some of which promote and others that inhibit this process. Apoptosis is the major mechanism by which self-reactive cells are eliminated centrally or peripherally.

The *lpr* mutation has been shown to be associated with an intronic insertion of an early retroviral transposon between exons 2 and 3 of the apoptosis-promoting Fas gene, leading to abnormal splicing and termination of transcription. The *lpr*^{ca} mutation is caused by a single point mutation within the intracytoplasmic domain of the Fas gene that modifies an amino acid essential for signal transduction and function. The *gld* mutation is a single point mutation in the C-terminal extracellular domain of the gene for Fas ligand (FasL), which abolishes its binding to Fas.

Despite these findings, *lpr* and *gld* mice do not exhibit defects in central (intrathymic) deletions of T cells recognizing endogenous superantigens or conventional peptide antigens. Nevertheless, in such mice, defects in peripheral mature T cell deletions have been observed, consistent with the primary role of Fas and FasL in activation-induced apoptosis. Activation-induced cell death is initiated by antigen receptor engagement, which upregulates Fas and induces FasL primarily in CD8⁺ cells and the T_H0 and T_H1 CD4⁺ subsets. B cells from *lpr* and *gld* mice also exhibit resistance to apoptosis. Although the discovery of the Fas/FasL gene defect is of major importance, these defects obviously do not provide a universal explanation for murine lupus. Moreover, no defects in the structure, expression and function of the Fas gene have been uncovered in the vast majority of lupus patients, although rare human correlates to the mouse *lpr* disease have been described.

Overall, however, identification of the Fas/FasL defects strongly suggests that lupus pathogenesis may be related to other abnormalities within the apoptosis machinery.

The Y-chromosome accelerator of autoimmunity

In contrast to the female predilection for lupus found in humans and most susceptible mouse strains, male BXSB mice develop a much more accelerated and severe disease than females. This striking sexual dimorphism is not hormonally-mediated, but results from a Y-chromosome autoimmune-accelerating (*Yaa*) gene that has not yet been cloned. The genetic contribution of *Yaa* to lupus susceptibility has been shown to be additive and dependent on other background genes. Non-autoimmune strains remain largely unaffected by the *Yaa* gene, whereas all lupus-susceptible strains develop an earlier and more severe disease that generally maintains the clinical characteristics of the background strain. For example, male NZW.*Yaa* congenics develop accelerated renal and cardiac disease and early death, while male NZB.*Yaa* mice primarily show enhanced anti-red blood cell autoantibody production. Regarding the mechanisms responsible for the *Yaa* phenotype, double bone marrow chimera experiments using a mixture of *Yaa*⁺ and *Yaa*⁻ cells of different Igh allotypes revealed selective production of antibodies to DNA by *Yaa*⁺ B cells. These and other findings have led to the postulation that the *Yaa* gene acts to increase expression of an 'intracellular adhesion molecule' on B cells, thereby promoting low-avidity T helper-B cell interactions.

Motheaten mice and the protein tyrosine phosphatase gene

Mice homozygous for the recessive allele 'motheaten' (*me*), and its milder variant 'viable motheaten' (*me*^v) express multiple hematopoietic and immunologic abnormalities, including developmental or functional defects in macrophages, granulocytes, T cells, B cells and natural killer (NK) cells. These mice develop systemic autoimmunity with hypergammaglobulinemia and antinuclear autoantibodies, and the majority of B cells are of the B1 type (Ly-1⁺). The *me* mutation was initially mapped to the distal end of chromosome 6. Subsequent linkage analysis positioned the *me* locus at the same site on chromosome 6 to which the protein tyrosine phosphatase 1C (PTP1C) gene (hematopoietic cell protein tyrosine phosphatase (Hcph) gene) had been previously mapped. Cloning and sequencing of the PTP1C gene from *me*^v mice

revealed two types of transcripts that differed from the wild-type catalytic domain by a 15 bp in-frame deletion or a 69 bp in-frame insertion. In contrast, the PTP1C catalytic domains from *me* and wild-type mice were identical in sequence. On the other hand, the majority of *me* PTP1C transcripts have a 101 bp frameshift deletion within the signal transducing SH2 (src homology 2) domain. There is also a second, minor transcript that instead has a single C nucleotide deletion in the SH2 domain. In contrast, no abnormalities were detected in the SH2 domain of the *me*^v PTP1C transcripts. These transcript changes, the result of single-point mutations and modification in the splicing process, lead to absence of PTP1C expression in the more clinically-severe *me* mutation (but not the less severe *me*^v mutation) and reduced catalytic activity in both mutations. How these hematopoiesis-affecting PTP1C mutations lead to autoantibody production is not clear. Recently, however, PTP1C was found to associate with FcγRIIB1 and CD22 and to inhibit B cell receptor (BCR) signaling. Thus, the lack of PTP1C could, upon BCR engagement, result in the uninhibited activation of Lyn and Syk kinases. B cell activation would be promoted, resulting in hypergammaglobulinemia and apoptosis primarily of B1 cells (Ly-1⁺) because of the normally poor response of B1 cells to BCR signaling.

Lupus susceptibility loci

A new avenue of inquiry for broadly defining the genetic defects of lupus is that of genome-wide searches using dense chromosomal maps derived from polymorphic microsatellite markers. Microsatellites or simple-sequence length polymorphisms (SSLP) are repeats of usually dinucleotides that exhibit a high degree of polymorphism in the number of repeats at a given chromosomal site.

Studies of the interspecific MRL-*lpr* and *Mus castaneus* (CAST/EI)F1 hybrid backcrossed to MRL-*lpr* identified two loci associated with renal disease, termed *Lrdm1* and *Lrdm2*. *Lrdm1* is located at the centromeric end of chromosome 7 and *Lrdm2* in the middle of chromosome 12.

Several groups have used microsatellite markers to identify susceptibility loci contributing to disease in the (NZB × W)F1 cross (Table 2). A study of (NZB × W)F1 × NZW backcross mice identified a single dominant locus (termed *nba1*) on chromosome 4 associated with proteinuria and early mortality. In another study, backcrosses of a new NZB × W recombinant strain (NZM/Aeg 2410) to the F1 with C57BL/6, was analyzed and four major loci linked to glomerulonephritis (GN) were identified, includ-

Table 2 Lupus susceptibility genes and loci^a

Genes	Alleles	Chr.	Mechanism ^b
<i>FasL</i>	<i>gld</i>	1	Apoptosis defect
<i>PTP1C</i>	<i>me</i> , <i>me</i> ^v	6	Signal transduction defect
MHC class II	Several	17	Antigen presentation
<i>Fas</i>	<i>lpr</i> , <i>lpr</i> ^{ca}	19	Apoptosis defect
Unknown	<i>Yaa</i>	Y	Enhanced T-B cell interaction
Locic	Strain ^d	Chr.	Phenotype
<i>Sle1</i>	NZW	1	Glomerulonephritis (GN)
<i>Lbw7</i>	NZB	1	Chromatin-specific antibody/splenomegaly
<i>nba1</i>	NZB	4	GN/mortality
<i>Sle2</i>	NZW	4	GN
<i>Lbw1</i>		17	GN/mortality/chromatin-specific antibody
<i>Lbw2</i>	NZB	4	GN/mortality/splenomegaly
<i>Lbw3</i>	NZW	5	Mortality
<i>Lbw4</i>	NZB	6	Mortality
<i>Lrdm1</i>	MRL	7	GN/mortality
<i>Sle3</i>	NZW	7	GN
<i>Lbw5</i>	NZW	7	Mortality
<i>Lbw8</i>	NZB	11	Chromatin-specific antibody
<i>Lrdm2</i>	MRL	12	GN/mortality
<i>Lbw6</i>	NZW	18	GN/mortality

^aOnly named susceptibility loci included in this table.^bPresumed mechanism.^cLoci on a given chromosome with different name designation may constitute the same locus.^dStrain associated with susceptibility allele.

ing the MHC and three loci designated *Sle1*, *Sle2* and *Sle3* on chromosomes 1, 4 and 7, respectively. The incidence of GN increased with the number of susceptibility alleles inherited regardless of the combination, suggesting a threshold liability model of inheritance with a simple additive contribution from each locus. Finally, a third study with (NZB × W)F₂ intercrosses identified eight susceptibility loci, designated *Lbw1* to *Lbw8* on chromosomes 17, 4, 5, 6, 7, 18, 1 and 11, respectively, and two loci that predisposed to splenomegaly (designated *Sbw1* and *Sbw2*) were mapped to chromosomes 1 and 4. Production of antibodies to chromatin was linked to three loci (*Lbw1*, 7 and 8). GN was linked to three loci (*Lbw1*, 2 and 6), all of which were also linked to mortality, consistent with GN being the major contributing factor to early mortality. The two non-MHC loci had no linkage to autoantibody production, suggesting that their contribution to GN involves a stage(s) after autoantibody production. Six loci, *Lbw1* to *Lbw6*, were linked to early mortality, but, of these, three were not linked to GN and presumably contributed to other pathologic manifestations that adversely affect mortality (vascular disease, neoplasia). The *Lbw2* locus showed strong epistasis with other loci and appeared to be required

for early mortality. Further mapping has revealed that the *Sbw1* and *Sbw2* loci map to the same regions as *Lbw7* and *Lbw2*, respectively, and are probably pleiotropic alleles, therefore the *Sbw* designations have been dropped.

Based on the overall findings to date, it can be concluded that the genetics of murine lupus follow a multiplicative model of inheritance in which specific combinations of genes acting additively are required for phenotype expression. Moreover, it appears that each susceptibility locus contributes to specific stages of lupus immunopathology, suggesting that separate thresholds for each stage are influenced by different sets of susceptibility genes. With loci predisposing to murine lupus susceptibility already mapped, the next step, identification and characterization of the actual predisposing genes will undoubtedly be forthcoming in the foreseeable future.

See also: Apoptosis; Autoimmune disease, spontaneous animal models; Fas (CD95) and fas ligand; Systemic lupus erythematosus (SLE), human.

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SYSTEMIC LUPUS ERYTHEMATOSUS (SLE), HUMAN

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It seems likely that the condition described by Hippocrates as herpes esthiomenos was a synonym for what is now known as systemic lupus erythematosus (SLE). In modern times this was first described by Cazenave and Clausit in 1852. In 1875 Hebra and Kaposi differentiated discoid lupus from the systemic form. By the beginning of the present century, Sir William Osler and others had comprehensively described many of the clinical features of the disease including involvement of the joints, kidney and central nervous system. The involvement of the heart in SLE was first recognized by Libman and Sachs in 1923 who described a classic form of endocarditis. In the mid-1930s Baehr, Klemperer and Schiffrin stressed the structural changes in the glomeruli of lupus patients. In 1948 Hargreaves first identified the so-called LE cell but the modern era of antinuclear antibody detection was ushered in by Friou and colleagues when using immunofluorescence in 1957–1958. The first mouse model of human lupus was described in the New Zealand black mouse in 1959.

Clinical features

As the name implies, systemic lupus is characterized by a very broad range of clinical manifestations. It is principally a disease affecting women during the child-bearing years. Invariably arthralgia or arthritis is involved (90%) but myalgia (50%) and tenosynovitis (20%) are also common features in the musculoskeletal system. Involvement of the heart and lungs is also common with shortness of breath, pleurisy and pleural effusions being present in at least a quarter of the patients. Current pulmonary function tests will detect abnormalities in up to 85% of lupus patients. Skin involvement is common in systemic lupus and up to 30% of patients have the so-called butterfly rash (Figure 1), essentially a vasculitis over the malar bones and bridge of the nose. A wide variety of other dermatological manifestations may be observed including alopecia, livedo reticularis, an erythematous maculopapular eruption and discoid lupus.

Involvement of the central nervous system (CNS)



Figure 1 The 'butterfly rash' seen in up to 40% of SLE patients. (See also color **Plate 10**.)

can manifest as an enormous range of features from single migraines or depression to frank psychosis, hemiplegia, or seizures. Recently a variety of CNS manifestations, together with a predisposition for venous or arterial thrombosis and spontaneous abortions has been recognized. These features are often linked to the presence of antiphospholipid antibodies.

Kidney involvement is the most important feature of lupus, especially as significant kidney damage is

the major threat to long-term survival. Whilst proteinuria is found in some 60% of patients overall, probably less than one-third of patients actually have serious renal disease. This may take a variety of forms ranging from minimal or mesangial involvement to severe diffuse proliferative glomerulonephritis. Gastrointestinal involvement is relatively uncommon. Anorexia occurs at some time in up to 40% of the patients and vague abdominal pains occur in approximately one-third but these often settle spontaneously.

Nonspecific but very troublesome features of lupus include lymphadenopathy (which may on occasion be severe enough to warrant biopsy to exclude an accompanying malignancy), weight loss and fatigue. Fatigue is frequently a major problem for patients with lupus. It is important to recognize that with time the cumulative clinical features in adult and childhood onset cases varies, as is shown in **Table 1**. There are also differences amongst different ethnic groups; for example, black patients are more likely to have renal and central nervous system problems.

Investigations

Hematological abnormalities

A normochromic normocytic anemia is a frequent feature of lupus. Hemolytic anemia, as evidenced by a positive Coombs' test, is rarely found but iron-deficiency anemia may be induced by the non-steroidal anti-inflammatory drugs sometimes prescribed. Thrombocytopenia occurs in three main forms: acute, chronic or antecedent. In this last case patients may initially be diagnosed as having idiopathic thrombocytopenia but within months or years other features evolve making it clear that the thrombocytopenia was merely the first sign of SLE. Persistent and sometimes profound leukopenia ($<4.0 \times 10^9 \text{ l}^{-1}$), often accompanied by lymphopenia ($<1.5 \times 10^9 \text{ l}^{-1}$), is frequently found.

Table 1 Cumulative clinical features in adult and childhood onset groups (as percentages)

	By 2 years		By 5 years		By 10 years
	Adult	Pediatric	Adult	Pediatric	Adult
Alive	163	37	113	28	50
Dead	2	2	10	2	13
Rash	89	83	82	82	82
Arthritis	96	78	98	89	98
Cardiopulmonary	33	19	54	36	64
Renal	17	27	25	32	72
CNS	16	19	28	21	38
Major hematological	16	38	20	39	24

Serological abnormalities

Antinuclear antibodies, as detected using rat liver or human epithelial cell substrates, are present in over 95% of lupus patients. Antigens within the nucleus which these antibodies recognize include double-stranded DNA (40–90%) single-stranded DNA (up to 70%) poly (ADP ribose) (up to 70%) RNP (35%) and Sm (30% in the USA, 5% in Europe). Antibodies to double-stranded DNA, notably of the immunoglobulin G (IgG) isotype in high titers, are essentially disease specific as are antibodies to Sm. In addition, antibodies to cytoplasmic antigens notably Ro (up to 35%) and La (15%) as well as those binding to cell surfaces of red cells, white cells and platelets are frequently found. Antiphospholipid antibodies, notably anticardiolipin, have been identified in up to half of the patients with lupus, whilst the so-called 'lupus anticoagulant' is probably present in about 20%. Apart from anti-DNA antibodies and those binding poly (ADP) ribose, the remainder seem to fluctuate relatively little with disease activity.

Natural history of SLE

The prevalence of lupus varies according to ethnic group. Amongst black females it is approximately 1 in 250, amongst Chinese approximately 1 in 1000, amongst Caucasian about 1 in 4000. The female-to-male ratio in lupus is approximately 9:1 though some studies have indicated that there are families in which the disease occurs in mainly males and is transmitted through the fathers.

SLE in pregnancy

Pregnancy for the lupus patient entails far more danger for the offspring than the mother. Generally speaking patients with very severe active lupus will not become pregnant and the risk of developing a major relapse during pregnancy seems to be mild to modest at worst. In contrast, the fetus is threatened by an increased predisposition to spontaneous abortion (which is associated with anticardiolipin antibodies) and prematurity which may be due to vasculitis affecting the placental blood supply.

Approximately 1 in 20 of lupus mothers who have the anti-Ro or anti-La antibodies will give birth to children with a transient rash or a potentially permanent cardiac conduction defect – the neonatal lupus syndrome. It has recently been speculated that the target of the antibodies in the fetal heart may be laminin.

SLE in children

Children as young as 2 years old have been described as having lupus. As Table 1 indicates, cardiopulmonary disease is more uncommon in children with lupus, but major hematological manifestations are more common. In addition, anti-DNA, anti-Sm, anti-RNP and low C3 have also been reported more frequently in younger patients. Lupus in children may be particularly severe.

Outcome

There is a 10-year survival in SLE patients of around 90–95% but when the disease is associated with major renal involvement this falls to around 60%. Fifteen years survival has been estimated recently at around 70%.

Drug-induced lupus

Approximately 10% of patients with SLE are thought to have a variant induced by drugs. These patients resemble idiopathic lupus with respect to the musculoskeletal and dermatological features, but it is generally a milder condition with the kidneys and CNS very rarely if ever involved. In addition, antibodies to double-stranded DNA are most unusual, whereas antibodies to histones and single-stranded DNA are almost universally found. The commonest drugs giving rise to this condition are hydralazine, procainamide, a variety of β -blockers and anti-convulsants.

Genetic factors and etiological considerations

The contribution of HLA to relative risk for developing lupus depends upon the ethnic groups studied. It appears for example that HLA A1 B8 DR3 is associated with lupus in Caucasians though it has been postulated that this haplotype is in linkage disequilibrium with a C4 null allele. In contrast, lupus amongst other ethnic groups, including the Japanese, seems common amongst patients who carry the HLA-DR2 haplotype. Inherited complement deficiencies also predispose to lupus. Thus the majority of individuals who are homozygous for C1q, C1r, C1s, C2 or C4 deficiency develop lupus or a lupus-like syndrome. IgA deficiency also appears to predispose towards systemic lupus.

Family studies have indicated that amongst monozygotic twins the concordance rate for lupus is around 25% but amongst dizygotic twins it is around 3%. Where more than one case of lupus occurs in the family (around 3%) it is usually present in a sister.

Immunopathology

The immunopathology of SLE is complex, involving a network of multiple cellular defects associated with the overproduction of a range of autoantibodies.

Immune complexes

For many years it was thought that the presence of circulating immune complexes (CICs) comprising in part DNA and anti-DNA antibodies in the sera of lupus patients was an important immunopathological characteristic of lupus. More recently several authors have provided evidence that antibodies binding the nucleosome (DNA and histone) could bind directly to targets in the kidney, hence forming local immune complexes *in situ*. Immune complexes, whether deposited from the circulation or formed *in situ*, are likely to be involved in the damage caused to the kidneys, skin, choroid plexus of the brain and other tissues. From these sites they initiate a number of inflammatory mechanisms including 'bystander lysis' whereby tissues surrounding the deposited complexes are damaged by complement after it has been fixed to the complexes and also by the recruitment of inflammatory cells.

Because immune complex formation is part of the normal immunophysiological process directed at eliminating foreign antigens, their presence even in 'healthy' subjects is not unusual. Their pathogenicity in SLE may be due to a number of physicochemical and biological factors including the size of the complex, the class, subclass, valency and affinity of the antibody and the composition and size of the antigen.

Based on the observation that New Zealand lupus-prone mice have largely low-affinity antibodies to DNA, it has been suggested that such animals are particularly susceptible to immune complex disease as they have a genetic predisposition to produce low-affinity antibody. As a result, antigen is not complexed and eliminated in an efficient manner, and this favors the persistence of antigen excess immune complexes. Binding affinities of serum anti-DNA antibodies in lupus patients with and without renal lupus have been measured and it was found that those with nephritis had lower affinity autoantibodies. However, the anti-DNA antibodies obtained from glomerular eluates were of uniformly high affinity. It may be that the antibody that is bound in CICs differs from those deposited in tissues.

It also remains possible that antibodies in the circulation merely happen to cross-react with DNA and are deposited in the kidney when they recognize other cross-reactive epitopes.

Complement

The complement system is involved in the sequence of inflammatory events that occur in SLE. Numerous studies have demonstrated that complement is fixed or consumed by immune complexes and localized in the tissues, particularly the kidney. The cleavage fragments C3a and C5a cause the release of vasoactive amines from mast cells. Furthermore, vascular permeability can be increased by fragments of C2 with kinin-like activity. This allows localization of immune complexes as well as causing acute inflammation *per se*.

Low complement levels in the serum are associated with active lupus. Not only is the overall hemolytic ability (as usually measured by the CH₅₀ test) of the complement system decreased, but levels of C3 and C4 are also reduced. Breakdown products of complement, notably C3d and C4d, have been reported to reflect disease activity even better than the total C3 and C4 levels.

Cellular aspects

It is now clear that numerous other cellular defects exist in SLE which can be seen in the T, B and accessory cell compartments. The major cellular defects described in lupus are depicted in Figure 2.

T cells Evidence suggesting that the immunological imbalance seen in lupus can be partly attributed to malfunctioning suppressor cells is now considerable. B cells from the blood of patients spontaneously secrete larger amounts of immunoglobulin *in vitro* than cells from normal subjects. In addition, the ability of lymphocytes from patients with SLE to develop suppressor activity following con A or adenosine activation is restricted. Further evidence for a suppressor cell defect comes from other studies with mitogens. It is well recognized that the T cell mitogens (Con A or phytohemagglutinin (PHA)) will elicit a greater stimulation in lymphocyte cultures if added after 24 h. This effect is thought to be due to the loss of a population of short-lived suppressor cells. However, when lymphocytes from lupus patients have been utilized, there is little or no enhancement of the response which suggests that the short-lived population of suppressor cells is absent in these individuals.

Cytokines It is also evident that in SLE patients there is a defect in the production and response to interleukin 2 (IL-2). Perturbed IL-2 regulation seems to play a key role in the immunological imbalance of lupus-prone mice and in patients with lupus and other autoimmune rheumatic diseases. *In vitro*, the

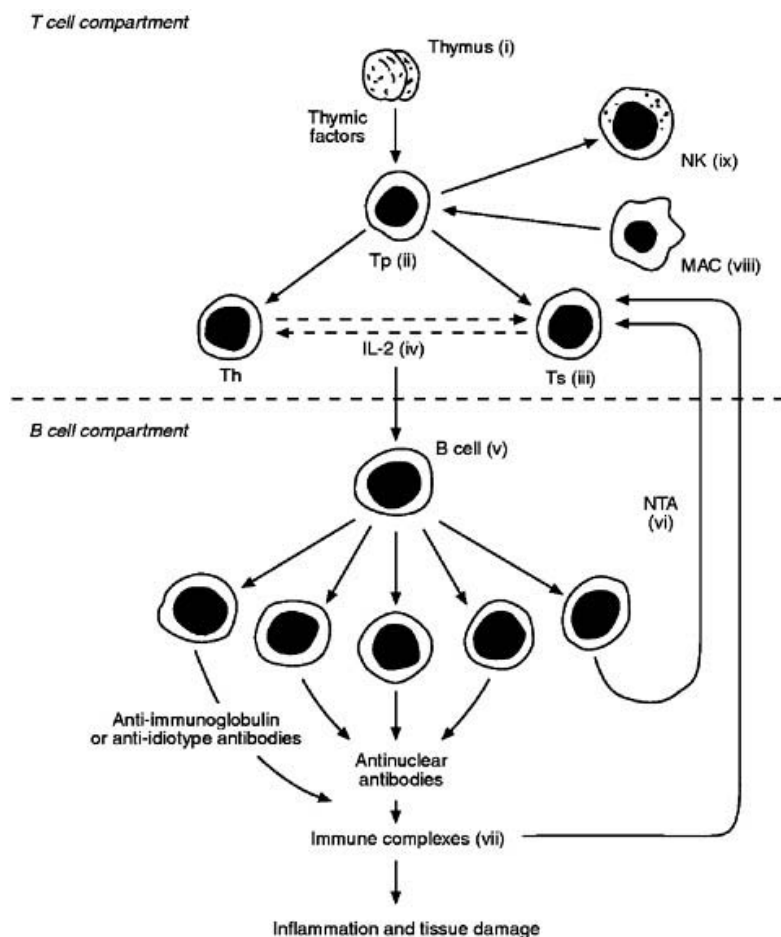


Figure 2 A summary of the major cellular defects in SLE. *T cell compartment:* (i) Defective thymic processing, decreased production of thymic factors. (ii) Decreased numbers and dysfunction of post-thymic precursor cells (Tp). (iii) Defective suppressor T cells (Ts) fail to regulate the B lymphocyte compartment, although induction of antibody production continues owing to impaired function of helper T cells (Th). (iv) Impaired interleukin 2 (IL-2) secretion and unresponsiveness causes further imbalances in the T cell populations. *B cell compartment:* (v) A primary B cell defect exists. (vi) Secretion of antilymphocyte or natural thymocytotoxic antibody (NTA) amplifies regulatory disorder. (vii) Immune complexes may further impair suppressor T cell function, as well as causing tissue damage. *Accessory cells:* (viii) Macrophages may contribute to the autoimmune process; various unusual characteristics have been observed (ix).

immunostimulatory agent isoprinosine increased PHA-induced IL-2 production by mononuclear cells, although it failed to stimulate IL-2 responsiveness.

Elevated serum levels of interferon γ (IFN γ) have been noted in patients with SLE, although others have reported that the production of both IFN γ and IFN α is decreased. Interestingly the IFN found in lupus is an unusual form in that it is acid-labile, a hallmark of IFN γ , but is neutralized with anti-IFN α antiserum.

Amongst other cytokine abnormalities it has been reported that IL-10 is increased in lupus patients. IL-10 has been shown to have a particular propensity for stimulating B cells to produce autoantibodies.

B cells Although B cell hyperresponsiveness has largely been attributed to lack of T cell regulation, there is now evidence to suggest that there is a B cell defect *per se* in lupus. Thus B cells from SLE patients have an intrinsic fault manifested by their inability to produce anti-influenza antibodies when sensitized *in vitro*. The antibodies produced by the B lymphocytes in lupus patients frequently carry common

idiotypes. It is evident that switching of both antibody isotype and idiotypes may be involved in the immunopathology of lupus. Thus IgG autoantibodies are evidently more pathogenic than IgM and certain idiotypes (e.g. Id 16/6) have been found in the serum of the majority of lupus patients and in many renal biopsies.

Accessory cells Dysregulated function of accessory cells may not be the most crucial immunological lesion in the SLE disease process, but nevertheless can amplify the inflammation in several ways.

Deficiencies in the reticuloendothelial system may influence the deposition of immune complexes. An *in vitro* test showing a neutrophil defect has been described in SLE.

Natural killer (NK) cells are normally associated with the killing of virus-infected or tumor cells and function in combination with interferon. Although the role of the NK population in SLE has yet to be fully determined, various abnormalities have been reported including reduced numbers and functional

immaturity and reduced killing capacity and insensitivity to interferon.

Apoptosis With the description of a transgenic mouse overproducing the proto-oncogene bcl-2 developing a lupus-like disease, anti-DNA and anti-Sm antibodies and an immune complex glomerulonephritis, much interest has focused on apoptosis in relation to SLE. Amongst a variety of hypotheses it has been suggested that some cells in lupus patients may undergo increased apoptosis, thus yielding a surfeit of nucleosomal material, but that other cells, notably the B cells, have a lower rate of apoptosis, the consequence of which is that they live longer and produce more autoantibodies. There has been considerable interest in the Fas molecule, a 43 kDa glycoprotein which is involved in inducing apoptosis in both B and T cells. In the MRL/lpr model, the lymphoproliferation mutation results in defective transcription of the gene that codes for Fas protein. However, an initial claim that 60% of lupus patients have elevated levels of the soluble Fas protein in their serum has not been confirmed by other studies and the role of this molecule in human lupus remains uncertain.

Treatment

The arthralgia, malaise and skin rashes of lupus can be treated with nonsteroidal anti-inflammatory drugs and a variety of antimalarial preparations. Patients with these problems alone rarely, if ever, need to go on any more major medication. When lupus involves the heart or lungs, modest doses of prednisolone, perhaps 30 mg a day initially, are invariably required. Profound hemolytic anemia or thrombocytopenia require large doses of prednisolone, perhaps 60–80 mg per day, often supplemented by another immunosuppressive drug such as azathio-

prine. Similar medication is required for severe renal and cerebral disease though studies from the National Institutes of Health in the USA have strongly supported the concept of intravenous monthly pulses of cyclophosphamide.

The use of plasma exchange in systemic lupus remains somewhat controversial though there is general agreement that it has no part to play in the treatment of mild or moderately severe disease. It may be of some value to patients with severe renal or CNS involvement.

Lymph node irradiation has not generally been adopted as a method of treatment in lupus. Dietary manipulation, notably reducing the total saturated fat content of the diet and increasing the level of fish oils, whilst simple and attractive, is unlikely to make a major impact on the disease.

See also: Antinuclear antibodies; Autoimmune diseases; Immune complexes; Plasmapheresis; Rheumatological disorders; Skin, autoimmune diseases; Systemic lupus erythematosus, experimental models.

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T

TARGETING OF IMMUNOLOGICAL AGENTS

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Although T cells represent the pinnacle of the host defense against cellular antigens, they can threaten the continued survival of a host. For example, T cells recognizing foreign or self antigens expressed on host tissues can cause autoimmune disease, reject donor organ grafts or, when infused into an allogeneic recipient, cause multiorgan system destruction termed graft-versus-host disease (GVHD). To combat the adverse effects of T cells in these situations, pharmacological reagents have been used to inhibit T cell responses and biological reagents have been synthesized to modify or eliminate T cells with undesired immune capabilities. GVHD will be used as the paradigm for discussion.

Pharmacological agents

Antiproliferative agents such as methotrexate and azathioprine, in combination with lympholytic agents such as glucocorticoids, have been used as the mainstay for GVHD prevention and therapy. Antiproliferative agents are broadly reactive because of their interference with basic cellular functions required for DNA synthesis, resulting in a wide range of toxicities, especially to organs with rapid turnover rates. Cyclosporine and FK506, agents commonly employed to suppress T cell responses for solid organ or bone marrow transplants, inhibit T cell activation earlier in the cell cycle than the antiproliferative agents. Although each binds to distinct intracellular proteins, they share common mechanisms of action by interfering with T cell receptor (TCR)-associated signaling events, leading to diminished cytokine synthesis which is required for productive T cell expansion. Rapamycin, structurally similar to FK506, inhibits lymphokine responses, and has been shown to prolong organ graft survival and inhibit GVH responses. Cyclosporine and rapamycin, used together, are additive or synergistic in suppressing T cell (including GVH) responses by inhibiting lymphokine production and responsiveness.

Antibody-directed approaches

The infusion of monoclonal antibodies (mAbs) with defined specificities offered high hopes that aberrant T cells could be eliminated and T cell-mediated disease processes subdued. Anti-CD3 (OKT3)-directed mAbs have been given to bone marrow transplant (BMT) patients with GVHD. However, because anti-CD3 mAbs are typically mitogenic, side-effects due to T cell activation are relatively common and may obscure the beneficial properties of this approach. Other problems for the use of mAbs in the BMT setting have included the need to administer large quantities, increasing the risk of allergic response and the fact that the recipient post-BMT may have an intact ability to remove mAb-coated T cells. Alternatively, drugs and radionuclides have been linked to antibodies, but drugs are mostly limited by their lack of potency and radionuclides have a notable cross-fire effect that affects adjoining tissue. Another solution is to use catalytic toxins from plants (ricin, abrin, ribosome inhibitory proteins) or bacteria (diphtheria toxin (DT), *Pseudomonas* exotoxin a) which are catalytic enzymes. These molecules share in common the fact that a single molecule in the cytosol destroys a cell by inhibiting protein synthesis in the 60S ribosome. First-generation immunotoxins (IT) were made by biochemically linking mAbs to toxins. Although *in vitro* they are highly potent in nanomolar concentrations, their *in vivo* efficacy is limited by instability and nonspecific side-effects due to localization in nontarget tissue. By constructing molecular ITs, the mAb or toxin moiety can be genetically engineered to reduce these undesired IT properties.

An example of these next-generation ITs are scFv (single-chain Fv) fusion immunotoxins. The scFv fragment consists of a single variable heavy (V_H) and a single variable light (V_L) domain (Figure 1) connected by a flexible linker. DNA elements from an scFv can be spliced to toxin cDNA such as truncated DT resulting in a protein that is small, configured in a single chain and produced in large quantities in bacteria. Such agents have been shown to give significant

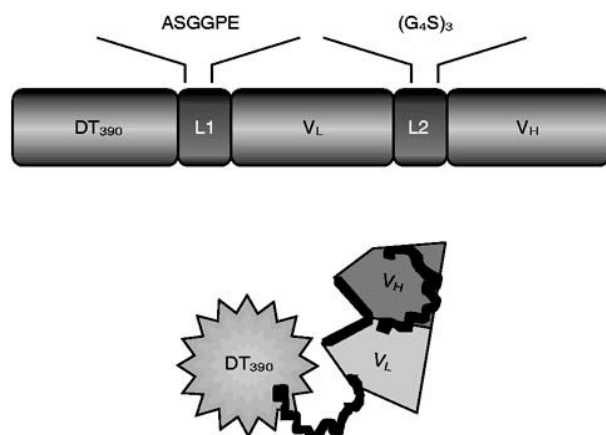


Figure 1 The DT₃₉₀anti-CD3sFv construct. Left to right (N-terminus to C-terminus): truncated diphtheria toxin, ASGGPE connector, V_L, flexible linker, and V_H. Below is a diagram of the folded protein with a function monovalent binding site.

antitumor and anti-GVHD effects in *in vivo* animal models.

The future of IT conjugates for *in vivo* use will depend upon solving the toxic side-effects of these potent reagents without hindering their biological efficacy.

Blockade of T cell costimulation

Additional T cell signals (termed costimulation), other than those delivered by CD3/TCR complex stimulation, are required to drive T cell expansion. A state of nonresponsiveness termed anergy is induced in T cells that have received signaling via their CD3/TCR complex in the absence of adequate costimulation. T Cells not receiving CD3/TCR complex signaling are unaffected. In theory, GVHD can be inhibited by temporarily precluding T cell costimulation during initial alloantigen exposure, leaving intact the majority of T cells to mediate leukemia- and viral-specific T cell responses upon antigen exposure later in the post-BMT course. Costimulator molecules include members of the immunoglobulin supergene family (e.g. CD28:B7), tumor necrosis factor/nerve growth factor receptor families (e.g. CD40 ligand: CD40, 4-1BB:4-1BB ligand; OX40:OX40 ligand), and adhesion molecules (e.g. LFA-1:ICAM-1, CD2:CD48). Balancing these positive signals are negative regulators which downregulate immune responses primarily by inducing cell death, compete for the same substrates as the positive regulators, or provide negative regulatory signals. The net balance of positive and negative regulators of T cell activation and expansion determine whether donor alloreactive T cells can reach the critical threshold number and stage of activation neces-

sary to cause GVHD lethality in a recipient. As an example of the requirement for costimulation, the CD28/B7 pathway, the most well-studied interaction, will be highlighted. CD28 is constitutively expressed on all murine T cells and its natural ligands, B7-1 or B7-2, are predominantly found on activated antigen-presenting cells. The infusion of CTLA4-Ig, a protein consisting of the high avidity B7 ligand-binding extracellular domain of CTLA-4 fused to the C_γ1 moiety of immunoglobulin, has been noted to suppress sheep red blood cell responses, xenogeneic islet cell and cardiac allograft rejection, immune responses to self antigens, and GVHD responses. The infusion of anti-B7 mAbs post-BMT allowed survival rates of $\geq 94\%$ in allogeneic recipients of either CD4⁺ or CD8⁺ T cells, in contrast to no survival of controls. In a human mixed lymphocyte reaction (MLR) system, blockade of CD28/B7 interaction was found to induce donor antihost alloantigen specific hyporesponsiveness. Thus, it may be possible to render donor T cells anergic to alloantigens *in vitro* prior to *in vivo* transfer into BMT recipients.

Cytokine regulation

CD4 T cells are distinguished as either T helper type 1 (T_H1) or T helper type 2 (T_H2) cells, based on the particular cytokines that are produced upon activation. Interferon γ is produced by T_H1 cells and is involved in cellular responses, whereas interleukin 4 (IL-4) is produced by T_H2 cells and is critical to humoral responses. T_H1 and T_H2 cells are cross-regulatory, and the balance of these cells determines the outcome of the immune response. T_H1-like inflammatory processes appear to be involved in the development of GVHD. T_H2 cytokines have been used during the GVHD induction phase to try to skew the CD4⁺ T cell response away from the deleterious T_H pathway. For example, T_H2-type alloreactive T cells can be generated *in vitro* or *in vivo* by coinubation of cells in the presence of IL-4 and antigen and the transfer of T_H2 cells can downregulate the GVHD potential of naive donor T cells. Interleukin 10 (IL-10: cytokine synthesis inhibitory factor) is a product of T_H2 clones and is an ideal candidate cytokine for inhibiting the acute GVHD response because it inhibits the MLR, downregulates major histocompatibility complex (MHC) class II expression, inhibits the production of inflammatory cytokines and nitric oxide synthesis from activated monocytes and macrophages which have been shown to be involved in acute GVHD generation, and downregulates B7 expression on accessory cells. High endogenous production of IL-10 has been

found to be associated with skin and cardiac allograft acceptance, the induction of neonatal tolerance and high endogenous levels of IL-10 mRNA were detected in circulating cells obtained from long-term BMT chimeric patients tolerant to host alloantigens *in vivo*. The addition of exogenous IL-10 to an MLR culture was shown to induce alloantigen-specific nonresponsiveness. Human CD4⁺ T cells activated by allogeneic monocytes specifically failed to proliferate after restimulation with the same alloantigens. The IL-10-induced anergic state was long-lasting, and could not be reversed upon restimulation of the cells through the CD3/TCR complex. Biochemical and cellular studies have shown that IL-10-induced anergy induction is distinct from that obtained by costimulation blockade as well as the immune regulatory approach achieved with manipulation of the T_H1/T_H2 balance.

CD4 immunotherapeutics

The CD4 molecule functions as a coreceptor for T cell receptor–antigen interactions and contributes to the intracellular signaling pathways required for CD4⁺ T cell activation. CD4⁺ cells are involved in the pathogenesis of several autoimmune diseases, e.g. multiple sclerosis, and participate in transplantation reactions. Over the last few years, several approaches have been developed to inhibit the responses of CD4⁺ T cells, including the use of mAbs and peptides which compete for the presentation of antigens, yet these may have broad effects on all CD4⁺ T cells. Recently, synthetic peptides that mimic structural features of the CD4 molecule itself have been designed to block the functional role of CD4 during antigen-specific T cell activation. It is thought that this approach can achieve inhibition of the CD4⁺ T cells that participate in autoimmunity or transplantation reactions, without impairing the ability of other T cells from later responding to other antigenic challenges.

Initially, a peptide was generated from the CDR3 region of the first immunoglobulin domain of the murine CD4 molecule, by computer-assisted structure-based design approaches. This CD4-CDR3 cyclized peptide was 13 amino acids long and was nonimmunogenic. The peptide was a potent inhibitor of CD4⁺ T cell responses in a murine autoimmune model and could prolong survival of MHC class II disparate skin allografts. It also inhibited the development of CD4⁺ T cell-mediated GVHD. Although equivalent human analogs of the CD4-CDR3 site were effective *in vitro*, their practical application was limited by the inability to reduce their size below 13 residues. As an alternative, computer analysis of the structural surface features of the first domain of

human CD4 revealed a potential pocket for intermolecular binding, formed between the CDR3 and the CC' loops. A heptameric cyclized peptide was generated to mimic the CC' loop and it was found to inhibit both human T cell proliferation and murine models for skin allograft rejection and GVHD. Computational analysis has recently been used to screen organic chemical libraries for potential compounds which can block the putative CD4-binding pocket formed between the CDR3 and CC' loops. Two small compounds (mol. wt <300) have been found in this way and have displayed specific inhibition of CD4⁺ T cells. Their application to the prevention of GVHD and autoimmunity is under current investigation. Although studies are needed to determine the precise mechanism of action of these compounds, it is clear that the computer screening approach will be an important tool for future drug discovery.

See also: Antibodies, bispecific; CD4; Cytokines; Effector lymphocytes; Graft-versus-host reaction; Monoclonal antibodies (mAbs); Second signals for lymphocyte activation; Toxins.

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T CELL RECEPTOR, $\alpha\beta$

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The importance of thymus-derived lymphocytes (T cells) in mediating the cellular basis of the immune response was recognized in the early 1960s. Independently, J. F. A. P. Miller, R. A. Good and B. Waksman and their colleagues observed that neonatal thymectomy of mice, or thymoma development in humans, led to a reduced ability to synthesize antibodies, reject grafts, and develop delayed-type hypersensitivity.

During the late 1960s and 1970s, many investigators built a vast body of evidence supporting the role of T cells in helping B lymphocytes to generate antibody responses (T–B cell cooperation). Certain T cells also provided similar helper–effector cell assistance to a different subset of T cells responsible for cell-mediated responses. The first population of T cells is known as the helper T lymphocytes (T_H) while the latter subset of T effectors are the cytotoxic (or killer) T lymphocytes (CTLs). It is now known that there are distinct subpopulations of helper T cells that can secrete specialized panels of cytokines.

The specificity of the helper and effector functions, as well as the finding that only a very few T lymphocytes could react to a given antigen, led to the conclusion that these thymus-derived cells (like their B lymphocyte counterparts) also carried antigen-specific receptors. The cell-bound antigen recognition structure on T lymphocytes is the T cell receptor (TCR). Upon specific interaction of the T cell receptor with its ligand(s), signals are transduced through the plasma membrane. This transduction results in activation of the lymphocyte, followed either by the initiation of cell division and the secretion of cytokines (T_H), or by lysis of the target cell (CTLs).

Thus, the specific recognition of antigens by the T cell receptor controls the activation and suppression of the specific immunity compartment. However, despite its importance, the identification of the genes for the T cell receptor, the elucidation of its molecular structure, and an understanding of the mechanism by which T cells generate their diversity, remained elusive throughout the 1970s and early 1980s.

Structure of the T cell receptor

Both helper and cytotoxic T cells recognize antigens in a specific manner and occur in diverse, clonally distributed repertoires. Unlike B cells, whose receptors (BCRs) are immunoglobulins (Igs) capable of interacting with free antigens, T lymphocytes only recognize antigens on the cell surface, and only in association with major histocompatibility complex (MHC) products, a phenomenon known as MHC restriction. Antigens recognized by T cells are now known to be oligopeptides bound to the grooves of the MHC class I or class II gene products. Most T_H recognize peptides associated with class II MHC products while the majority of TCRs of CTLs recognize peptides associated with class I MHC molecules. This MHC I/II discrimination is facilitated in part by the coreceptors CD4 and CD8, found principally on T_H and CTLs, respectively. It is the MHC restriction that enables T cells to survey the universe of antigens and to distinguish ‘self’ and ‘foreign’ molecules, aiding in the selection of thymocytes and the regulation of extrathymic responses against foreign antigens.

The specific recognition of these antigen–MHC

complexes is mediated by the T cell receptor. An understanding of the structure of the T cell receptor and the mechanism by which its diversity is generated was not reached until 1983, when the problem was resolved using two different approaches. First, several groups (Allison; Kappler and Marrack; Reinherz; Schwartz; and Trowbridge) reported that surface structures on T lymphocytes consisting of disulfide-linked heterodimers of an acidic α chain and a more basic β chain could be detected using monoclonal antibodies specific for individual T cell clones. Using the techniques of molecular biology, two groups (Davis; Mak) independently cloned the gene coding for the β chain of the TCR, thus defining its molecular structure. The deduced protein sequence of this message revealed a large extracellular portion linked to a short cytoplasmic tail by a transmembrane section. Like the immunoglobulin chains, the extracellular domain of the β chain is composed of variable (V) and constant (C) regions, linked by diversity (D) and joining (J) gene segments. Subsequently, several investigators isolated cDNAs encoding the α chain of the TCR. This polypeptide is similar to the β chain, being comprised of extracellular variable and constant domains, a transmembrane region and a cytoplasmic tail. Both the α and β chains of TCR are members of the immunoglobulin gene superfamily.

The greater portions of TCR chains are extracellular and form the antigen recognition structure. The extracellular portion of each TCR chain is composed of two domains. Each domain has an immunoglobulin-like structure of approximately 110 amino acids, with a central internal disulfide bond that spans approximately 65 amino acids. Several conserved residues are thought to be structurally important. The constant domain is essentially invariant, while the variable domains are distinct on different T cells. These variable domains are responsible for repertoire diversity and the recognition of peptide antigens in the context of either class I (for CTLs) or class II (for T_H) MHC gene products. This contention was directly supported by the finding that transfection of TCR α and TCR β chain coding sequences conferred the antigenic and MHC specificities of transferred receptors to recipient cells.

The transmembrane portions of the TCR anchor the receptor in the cell membrane as part of a large complex with the CD3 proteins that are believed to play a role in membrane transduction of the recognition signal. It has been proposed that, based on the conserved positively charged arginine and lysine residues in the transmembrane portion of the TCR α and β chains, and the negatively charged glutamic and aspartic acid residues in the CD3 δ , ϵ and γ

chains, interactions between these charged amino acids may be important for the association of TCR and CD3 in a multimeric structure.

X-ray crystallographic studies of fragments of the TCR α and β chains have confirmed that these T cell recognition proteins resemble the BCR immunoglobulin domains. However, the constant region of the C α and the interdomain pairing of C α with C β also show differences. In addition, it is likely that the complementarity-determining regions CDR1 and CDR2 of the TCR are less variable than their B cell counterparts. Crystallographic studies of a TCR-MHC-superantigen complex indicate that interaction of these proteins involves nonpolymorphic regions of TCR-V β .

The crystal structure of the TCR with peptide-MHC molecules indicates a flat combining site of the TCR except for a deep hydrophobic cavity between the CDR3 of the α and β chains. For this TCR, CDR1 and 2 of V α contacts the N-terminus of the peptide while CDR1 and 2 of V β binds the C-terminal region. CDR3 of V α and V β are positioned over the peptide between the helices at the middle region of the peptide.

Genomic organization

The diversity required for T cell recognition depends on the variable domain sequences, produced via somatic recombination of different noncontiguous variable (V), joining (J), and diversity (D) gene segments. Individual V, D and J segments used in a particular somatic recombination are believed to be chosen more or less at random. The genomic organization of the sequences encoding the TCR chains is similar to that of immunoglobulin gene clusters, in that multiple copies of V gene segments are located upstream of the segments encoding D, J and constant region sequences. However, the fine structures of the TCR α , TCR β and BCR gene clusters differ greatly in arrangement and number of gene segments, as well as in the size and complexity of the clusters as a whole (Figure 1).

β chain genes

The TCR β gene cluster, which spans several hundred kilobases of DNA (Figure 1) and includes two highly homologous C regions, is located on chromosome 7 in humans and chromosome 6 in mice. It has been estimated that there are approximately 23 TCR-V β gene segments in mice and about 47 in humans (Figure 2). Rearrangement studies with particular TCR-V β genes have indicated that many are located upstream of the two TCR-C β genes. At least one TCR-V β in the murine system is located about 10 kb

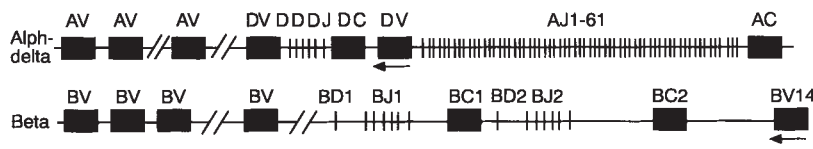


Figure 1 Genomic organizations of the T cell receptor $\alpha\delta$ and β chain genes. V, D and J denote variable, diversity and joining gene segments, respectively. C denotes constant region genes.

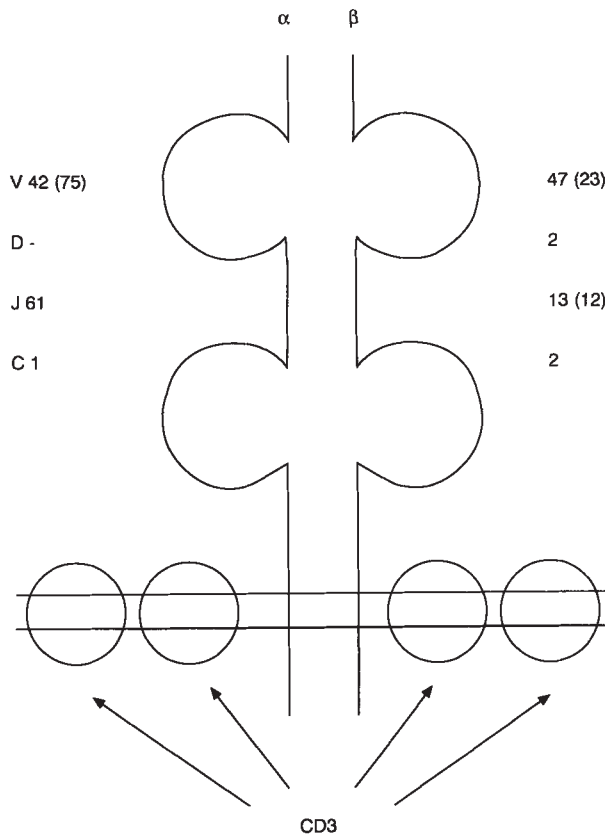


Figure 2 The T cell receptor $\alpha\beta$ heterodimer. The number of gene segments encoded within the germ line of human and mouse (in parenthesis where they differ) is illustrated. V, D and J denote variable, diversity and joining gene segments, respectively. C denotes constant region genes.

3' to the 3' TCR- C_β gene in an inverted transcriptional orientation. This TCR- V_β segment has been shown to be used in a functional T cell. Downstream of the TCR- V_β gene segments are two clonally related gene segment clusters. Each cluster consists of a TCR- D_β gene segment and six (in mice) and seven (in TCR- $J_\beta 2$ in humans) functional TCR- J_β gene segments (Figure 1) and is contained within a large stretch of several hundred kilobases of DNA (Figure 1).

Recombinant signals, similar to those in BCR genes, flank the TCR- V_β , TCR- D_β and TCR- J_β genes. Immediately proximal to the coding segment is a highly conserved heptamer, followed by a nonconserved spacer sequence, and then an A/T-rich nonamer. The spaces can be either long (23 nt) or short

(12 nt), with long spacers located 3' to the TCR- V_β and TCR- D_β segments, and short spacers located 5' to the TCR- D_β and TCR- J_β segments. This arrangement of recombinational signals precludes the direct recombination of TCR- V_β and TCR- J_β gene segments without the inclusion of TCR- D_β .

α chain genes

The organization of the TCR α chain locus stands in striking contrast to that of the TCR β chain and BCR loci. Large numbers of TCR- V_α gene segments have been identified in both human (42) and mouse (75), occupying several hundred kilobases on chromosome 14 in both species. The coding sequences of the TCR- J_α regions are several codons longer than those of BCR and TCR β chains and are also more numerous, spanning a distance of about 80 kb. The TCR- J_α genes are separated on average by 1 kb, with the first being located about 4 kb upstream of the single constant region. No TCR- D_α segment has been identified to date. Nested within the TCR α locus are the TCR δ genes. The products of these genes, TCR δ chains, are known to associate with the TCR γ chains to form the $\gamma\delta$ TCR heterodimers. Although TCR δ chains use their own TCR- D_δ and TCR- J_δ gene segments, some of the TCR- V_δ segments are shared with the TCR α chain genes.

Recombination signals, homologous to those of BCR and TCR β chains, are found flanking the TCR α chain segments, with long (23 nt) spacers 3' to the TCR- V_α genes and short (12 nt) ones 5' to the TCR- J_α gene segments. Since it has been shown that signals with short spacers only recombine with those with long spacers, it is possible for the TCR- V_α and TCR- J_α genes to join directly together without the inclusion of a D segment.

The majority of TCR rearrangement patterns can be explained by the looping/excision model, although patterns inconsistent with this model have also been described. In the case of the murine TCR- V_β segment situated 3' of the TCR- C_β region, functional rearrangement would require an inversion mechanism. The mechanism of allelic exclusion, in which the lymphocyte limits itself to one functionally expressed gene, has also been demonstrated in the TCR β chain locus. However, this phenomenon does not appear to be applied rigidly in the TCR α loci. Thus, multiple mechanisms, including gene inversion

and sister chromatoid exchange, may be used in the production of TCR genes.

TCR diversity

T lymphocytes, like their B cell counterparts, must handle a huge number of foreign antigens. Elegant mechanisms have evolved to generate a vast diversity while refraining from overburdening the genome with too many different sequences. An estimate of the total potential of TCR diversity before thymic selection of T cells is summarized in Table 1. For TCR α and TCR β chain genes, diversity is created in two key ways: through the germ line and through junctional and N-region diversity. Germline diversity is the summation of all the possible combinatorial joinings between the V, D and J gene segments of each gene locus, as well as the association between the products of the TCR α and TCR β genes. Junction diversity is the result of an imprecise joining between the V, D and J gene segments, leading to codon changes at the junctions. N-region diversity is generated by the addition of nucleotides (that are not coded by rearranging germline segments) to the V-D-J junctions during the rearrangement process. Assuming the insertion of three different codons at each junction, this process yields a potential 10^4 ($20 \times 20 \times 20$) different combinations of N-region diversity.

Both these processes substantially increase the repertoire of TCR sequences, but also produce a large number of nonfunctional genes by shifting the reading frame. Somatic hypermutation, which generates point mutations throughout the variable regions of BCR molecules, does not appear to occur in either the TCR α or TCR β genes, although there has been a single report that hypermutation occurs in TCR. One hypothesis is that, while somatic hypermutation can occur in T cells, it is a rare event because of the lack of selection pressure *in vivo* for the expansion of hypermutated cells. Such cells might exhibit a lower

dependence on affinity for the antigen/MHC complex for T cell activation than is the case for BCR affinity for antigen in B cells. Alternatively, somatic hypermutation may be prevented in T cells because it would allow the generation of potentially harmful (self) specificities. T cells undergo a rigorous selection procedure in the thymus that results in T cell recognition of antigen in the context of self MHC. The development of new, unselected specificities after thymic export could result in potentially dangerous autoreactive T cell clones.

Specificity of T cell receptors

The functional specificities of T cells is attributed mainly to the TCRs because the recognition of the antigenic peptides and MHC polypeptides ultimately depends on the final conformation and folding of the $\alpha\beta$ TCR heterodimer. Thus, a direct and simple structure–functional relationship between T cell specificity and primary TCR α and TCR β chain structure would be unexpected in most cases. Surprisingly, however, there are many examples in which a direct relationship exists between the specificity of the antigen and the primary sequence of the TCR. This correlation of structure and function breaks down into two categories: one including standard antigenic peptide–MHC complexes, and the other including ‘superantigens’, a class of antigens covering bacterial products (e.g. enterotoxins), minor lymphocyte-stimulating antigens MIs (e.g. endogenous retroviral products) and certain MHC products (e.g. I-E).

The most striking examples for the first category are the use of particular TCR-V α segments to recognize certain cytochrome *c* peptides in association with I-E products, and the use of specific TCR-V α J α , TCR-V β J β combinations to recognize a peptide from the myelin basic protein in association with I-A^b products. For superantigens, the correlation is often to a particular V gene (especially TCR-V β segments), with little structural contribution by the D and J gene segments or junctional sequences. It is likely that the conformation of these V gene segments possesses intrinsic affinity for these superantigens.

At this time, it is not known precisely how $\alpha\beta$ TCR heterodimers interact with their ligands (antigenic peptide–MHC product complexes). One hypothesis suggests that the V gene segments may recognize the MHC polypeptides while the V-D-J junctions make contact with the antigenic peptides. An understanding of the mechanism of this dual recognition may be gained when the three-dimensional conformational interactions between the $\alpha\beta$ TCR heterodimers and the peptide–MHC complex are defined.

Table 1 Estimate of human TCR repertoire

Gene segment	α chain	β chain
V	42	47
D	—	2
J	61	13
V $\times$ D $\times$ J	3×10^3	10^3
N-sequences	10^4	10^4
Total α or β	3×10^7	10^7
Total $\alpha\beta$ combinations	10^{14}	

Thymic selection of TCR $\alpha\beta$

Because the T cell receptor $\alpha\beta$ heterodimer is ultimately responsible for the specificity of the individual mature T cell, the development of functional TCRs providing an effective defense against infection by viruses, bacteria and parasites is essential. At the same time, these receptors must also be able to distinguish between 'self' and 'foreign' antigens, so as not to produce TCR reactivities against self antigens. Positive and negative selection and the participation of the coreceptors CD4 and CD8 are integral components of the thymic selection process.

It is believed that TCR $\alpha\beta$ combinations are generated randomly in the thymus by the association of the V, D and J gene segments, as well as by insertion of N-region sequences. Many of these TCRs will lack affinity for their own MHC molecules, and thymocytes bearing these TCRs die in the thymus during positive selection. Those TCRs with strong reactivities against self antigens (such as antigenic self peptide-MHC complexes, superantigen Mls or certain MHC) will also be clonally deleted (negative selection), thereby establishing central tolerance. Thus, only mature T cells which possess receptors having a weak affinity to self MHC products will be allowed to emerge from the thymus, resulting in a repertoire skewed towards the recognition of self MHC molecules in the presence of a foreign peptide.

TCR genes in malignancies

TCR and BCR genes provide useful tools for the evaluation of tissues from patients with lymphoproliferative disorders. Rearrangements of the TCR and BCR genes establish clonality of malignant cells in leukemia or lymphoma patients at the time of presentation or relapse, and are useful for establishing T or

B cell lineage, respectively. Chromosomal translocations involving the TCR genes are often found in T cell tumors. Using the genes of the TCR, several putative proto-oncogenes that may play a role in malignant transformation of T cells have been identified.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; CD3; CD4; CD8; Helper T lymphocytes; Chromosome translocations of immune genes; Cytotoxic T lymphocytes; Thymic epithelium: potential role in regulatory T cell tolerance; Immunoglobulin genes; MHC, functions of; MHC restriction; Superantigens; T cell receptor, $\gamma\delta$; T cell receptor, evolution of; T cell receptor, recognition by; T lymphocyte activation; T lymphocytes; T lymphocytes, autoreactive.

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T CELL RECEPTOR, $\gamma\delta$

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There is growing evidence that by comparison to cells bearing an $\alpha\beta$ T cell receptor (TCR), cells that bear a $\gamma\delta$ TCR recognize a broad and distinct spectrum of antigens, and contribute in unique ways to immune responses to pathogens, and to the development of autoimmunity.

The γ and δ chains can each be subdivided into variable (V) and constant (C) regions, the bodies of which comprise 'immunoglobulin (Ig)-like domains'.

As for other antigen receptors, the V regions of γ and δ chains are each encoded by genes assembled somatically from V, D (in the case of δ), and J gene segments that are noncontiguous in the germ line. Gene rearrangement is mediated between characteristic 'heptamer-nonamer' motifs that flank V, D and J, and depends on the recombinase-activating gene (RAG)-1 and -2 products. γ chain genes were first isolated in 1984 and were proposed to encode TCR

chains based on their capacity to rearrange like Ig genes, their organizational similarity to both Ig and TCR β chain genes, and their T cell-specific expression. δ genes were discovered 3 years later, during study of the TCR α chain locus, within which they are located. Between these two events, in 1986, human T cells were described that expressed a form of the TCR other than TCR $\alpha\beta$.

It now appears that $\gamma\delta$ cells, like B cells and $\alpha\beta$ T cells, are highly conserved components of vertebrate immune systems. Nonetheless, features of the $\gamma\delta$ repertoire can vary greatly among species. For example, $\gamma\delta$ cells in some species but not others are strikingly prominent within epithelia. Divergence of the γ chain genes may be one of the underlying causes of such differences.

γ gene families

Human γ chain genes are on chromosome 7, while the murine genes are on chromosome 13. Various nomenclatures have been adopted for γ genes. Table 1 lists two commonly used nomenclatures, adjacent to an official designation proposed in 1995 by a WHO-IUIS subcommittee. In BALB/c mice, there are seven potentially functional V_γ gene segments, three functional J_γ gene segments, and three functional C_γ gene segments. A fourth J_γ is a pseudo-(ψ) gene, as a result of which the proximal V_γ segment (GV5S3 ($V_{\gamma 3}/V_{\gamma 1.3}$), Table 1) is essentially unused. There are slight departures from the BALB/c γ gene organization in other strains of mice, but in all cases there seem to be six functionally employed V_γ genes, far

less than the ~ 23 V_β genes or ~ 75 V_α genes. Humans also have only $\sim$ six functional V_γ genes compared to ~ 47 V_β genes and ~ 42 V_α genes. However, V_γ gene divergence is such that it is difficult to establish human/mouse counterparts. Indeed, there are different genetic bases for the small size of the V_γ gene families in mice and humans. In mice, there are no overt $V_\gamma \psi$ -genes, whereas in humans, at least eight of ≥ 14 V_γ gene segments are ψ -genes (Figure 1). Table 2 compares commonly used human V_γ gene designations with the proposed designation.

δ gene families

TCR δ chain genes in mice and in humans are on the respective chromosomes 14. Like IgH and TCR β loci, TCR δ chain loci include D gene segments. The organization of the heptamer–nonamer motifs is such that D_δ segments may join to each other, and whereas V_δ - D_δ 2- J_δ is the most frequent rearrangement in murine fetal $\gamma\delta$ cells, most δ rearrangements in adult mice are V_δ - D_δ 1- D_δ 2- J_δ . Likewise, whereas early human fetal rearrangements join V_δ to J_δ via a single, J-proximal D (D_δ 3), rearrangements late in gestation include two or three D segments.

The sizes of the human and mouse δ gene families are difficult to assess, because V_δ gene segments are interspersed among V_α gene segments (Figure 1), and some V segments can be used as V_δ or V_α . For example, human V_δ 4, V_δ 5 and V_δ 6 have each been found rearranged to D_δ or J_α , as a result of which they are referred to by the new nomenclature as ADV6, ADV21 and ADV17, respectively (ADV denoting variable gene segments for both the alpha and delta loci). In the mouse, $\geq$ four murine V genes are transcribed as either V_δ or V_α mRNAs, and thus denoted ADV7S1, ADV7S2, ADV11SS5 and ADV17S2. Nonetheless, there are characteristic features of V segments commonly used in TCR δ chains (see below). As a result, it has been possible to estimate that there are ≥ 16 mouse V_δ genes. Of these, six are highly homologous members of a single (DV7/ADV7) family, reducing to 11 the number of distinct mouse V_δ segments, compared to $\sim$ eight distinct human V_δ genes. Although V_γ genes are highly diverged, it is possible to identify human/mouse counterparts, e.g. mouse V_δ 1 and human V_δ 2, that are more closely related to each other than they are to other Vs in the same species.

Gene structure

γ and δ chain genes comprise five and six exons, respectively. As in Ig genes, exon 1 of functionally rearranged $\gamma\delta$ genes encodes all but the last four

Table 1 Proposed official nomenclature for murine V_γ gene segments compared to two commonly used designations

Official designation ^a	Heilig and Tonegawa (1986) ^b	Garman et al (1986) ^c
GV1S1	$V_{\gamma 5}$	$V_{\gamma 3}$
GV2S1	$V_{\gamma 6}$	$V_{\gamma 4}$
GV3S1	$V_{\gamma 4}$	$V_{\gamma 2}$
GV4S1	$V_{\gamma 7}$	$V_{\gamma 5}$
GV5S1	$V_{\gamma 1}$	$V_{\gamma 1.1}$
GV5S2	$V_{\gamma 2}$	$V_{\gamma 1.2}$
GV5S3	$V_{\gamma 3}$	$V_{\gamma 1.3}$

In the proposed designation, G refers to γ ; V to variable; 1 to the allocated number for the particular V region family (in this case, numbers 1–4 are based on increasing distance from J_γ); S to the designation of a V segment as a member of a family of highly homologous V gene segments. GV1–V4 are not strongly homologous to any other V segments, thus they only have one member sets. By contrast, there are three closely related members (S1–S3) of the V5 family.

^aArden B, Clark SP, Kabelitz D and Mak TW (1995) *Immunogenetics* 42: 501–540.

^bHeilig JS and Tonegawa S (1986) *Nature* 322: 836–840.

^cGarman RD, Doherty PJ and Raulet DH (1986) *Cell* 45: 733–742.

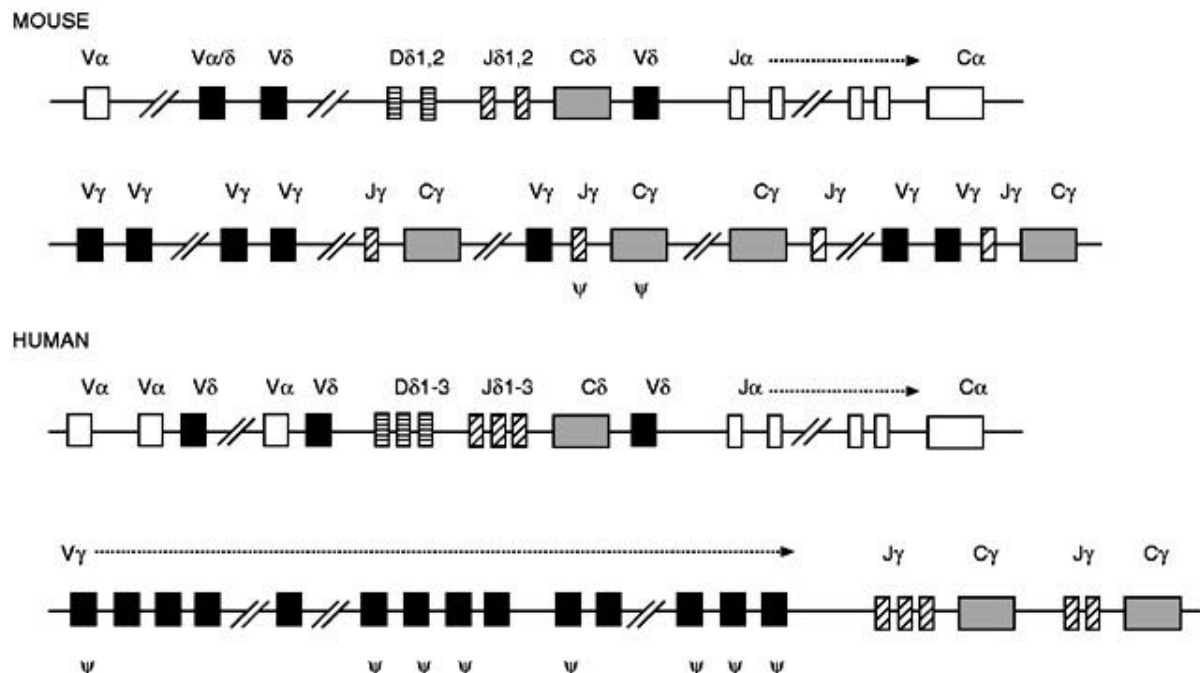


Figure 1 Schematic representation of the mouse and human $\gamma\delta$ gene families. TCR $\gamma\delta$ -V gene segments are shown as solid boxes, -D as striped boxes; -J as hatched boxes, and -C as stippled boxes; TCR α gene segments as open boxes; ψ indicates pseudogenes. Only representative V_δ and V_α gene segments are shown; some can be used as either (see text).

Table 2 Proposed nomenclature for human V_γ gene segments compared to commonly used designations

Official designation	Previous designations
GV1S1P	$V_\gamma 1$
GV1S2A1T	$V_\gamma 2$
	$V_\gamma 1.9$
GV1S2A2T	$V_\gamma 1.9$
GV1S3A1N1T	$V_\gamma 3$
	$V_\gamma 1.3$
GV1S3A1N2T	$V_\gamma 1.1$
GV1S4A1N1T	$V_\gamma 4$
GV1S4A1N2T	$V_\gamma 4$
GV1S5	$V_\gamma 5$
GV1S5P	$V_\gamma 5P$
GV1S6P	$V_\gamma 6$
GV1S7P	$V_\gamma 7$
GV1S8	$V_\gamma 8$
GV2S1A1	$V_\gamma 2.2$
	$V_\gamma 9$
GV2S1A2	$V_\gamma 9$
GV3S1P	$V_\gamma 10$
	$V_\gamma 3.1$
GV4S1P	$V_\gamma 11$
GV5S1P	ψVA
GV6S1P	ψVB

See footnote to **Table 1** for basis of designation, with the added use of: A1/A2 to denote alleles at a single locus; P, pseudogene; N, sequence substitutions that conserve the encoded polypeptide; T, allelic variants that are currently tentative.

amino acids of a hydrophobic leader. Exon 2 mostly encodes the Ig-like domain of the V region, fully formed by the somatic recombination of V, [D(δ)], and J. All murine J_γ segments are unusual in lacking 'glycine-X-glycine', a motif common to other Ig and TCR J regions. Exon 3 encodes the Ig-like domain of the C_γ/C_δ regions. Both V and C region Ig-like domains contain cysteine codons appropriately spaced to facilitate intradomain disulfide bonding, an important component of Ig-like structure.

In IgH genes, exon 4 encodes a protein hinge. Like that exon, the fourth murine and human δ gene exons are short and encode several prolines. But, proline codons are largely absent from the fourth γ gene exons, which are also noticeably diverged one from another: in murine $C_\gamma 1$, 2 and 3 genes, exon 4 encodes 10, 15 and 33 amino acids respectively; in human $C_\gamma 1$ it encodes 16 amino acids, while in $C_\gamma 2$, it is reiterated 2–3 times, depending on genotype. The short fourth exons can also encode Cys residues that mediate interchain $\gamma\delta$ disulfide bonding. For human $C_\gamma 2$, none of the reiterated copies contains a Cys codon, and $\gamma\delta$ TCRs encoded by $C_\gamma 2$ lack a $\gamma\delta$ interchain disulfide bond.

Exon 5 in all $\gamma\delta$ genes encodes a putative trans-membrane domain of uncharged, mostly hydrophobic amino acids. As in all TCR genes, these exons of $\gamma\delta$ genes each encode a basic (lysine) residue that may interact with a negatively charged residue in the

transmembrane domains of CD3. In mouse and human δ (and in TCR α and β) there is a potentially charged arginine, five residues N-terminal to the lysine. The coding potential of the γ genes ends in a hydrophilic, 12 amino acid, putative cytoplasmic tail, with a high charge density. In δ the putative cytoplasmic tail is very short (4–5 amino acids) and not highly charged. In δ genes, the 3' untranslated sequences and polyadenylation signals are within a sixth exon.

TCR $\gamma\delta$ protein

The sequence relatedness of $\gamma\delta$ genes to each other and to Ig genes is low. A γ cDNA is ~18–23% (depending on the regions being compared) similar to either TCR β cDNA or IgH cDNA; δ genes are only ~10–20% similar to other TCR and Ig genes, although parts of C_δ are claimed to be over 30% similar to IgG C regions. Despite these limited sequence similarities, the antigen receptor genes share high organizational similarities. Hence, based on the observation that within multigene families, protein folding is better conserved than primary amino acid sequence, TCR and Ig sequences have been intensively compared, in order to assess the likely resemblance of TCR protein structures to Ig structures that have been resolved by X-ray crystallography. The data suggest that $\gamma\delta$ TCR V and C regions can assume the characteristic structure of an Ig domain, termed the 'Ig fold' – seven β strands arranged in two antiparallel β sheets packed face to face. Furthermore, the Ig folds of V_γ and V_δ should associate, presenting at their surface a rather flat antigen-combining surface, perhaps of similar geometry to that of Ig.

When sequence data are used to create 'divergence trees', the human ADV regions (i.e. those rearranged as either V_α or V_δ genes) differ from other V_α regions in occupying their own branch, one that in mouse is occupied solely by V_δ regions. Such comparisons pinpoint structural signatures of TCR $\gamma\delta$, particularly in regions implicated in $\gamma\delta$ chain-pairing and in the putative complementarity-determining regions (CDR1, CDR2 and CDR3) that most likely bind antigen. For example, in all human and mouse V_α , residue 14 is glutamic acid, and residue 37 is glutamine, whereas neither is the case in any mouse or human V_δ . Residue 37 may be critical in restricting chain-pairing of V_α with V_β , and V_δ with V_γ . Indeed, residue 37 in human DV102 ($V_\delta 2$) is positively charged, while the only γ chain that is negatively charged in the equivalent position is GV2 ($V_\gamma 2/V_\gamma 9$) with which DV102 pairs almost exclusively.

Compared to most V_α regions, DV102 ($V_\delta 2$) is two

amino acids longer in CDR1, while DV103 ($V_\delta 3$) is two amino acids longer in CDR2. Likewise, the ADV genes have longer N-terminal sequences, that may extend the exposed, antigen-binding face occupied by the CDR loops. Interestingly, V_γ and V_δ chain genes are similar in many respects (e.g. both have CDR2 regions four amino acids larger than V_α), but compared to V_β , GV2 is two amino acids longer in CDR1, just as DV102, the δ chain commonly paired with GV2, is longer in CDR1. Similarly, GV1 has a two amino acid extension in CDR2, as does DV103, with which it commonly pairs.

CDR3 regions largely comprise amino acids encoded at the V(D)J join. Their diversity stems from variability in the joins and is heightened by the capacity of TCR D segments to be read in all three reading frames. CDR3 lengths are predicted to profoundly affect CDR shape and hence ligand specificity. Comparison of many sequences showed that murine and human CDR3 δ lengths are both highly variable and commonly longer than those in other TCR or Ig chains. This is in part because of D_δ – D_δ joining. CDR3 γ lengths are shorter, but more variable than CDR3 α or CDR3 β . The constraint on CDR3 α and CDR3 β may be imposed by the requirement of TCR $\alpha\beta$ to 'fit' on to major histocompatibility complex (MHC) molecules. Conversely, the IgH-like variability in CDR3 δ , and the imbalance between CDR3 γ and CDR3 δ (akin to that between IgH and IgL) indicates that $\gamma\delta$ TCRs are not restricted to recognition of a single class of antigen presentation molecule (e.g. MHC), and may recognize diverse antigens, as does Ig. Consistent with this is the high divergence of $\gamma\delta$ V gene segments. For example, two murine γ genes <1 kb apart are only ~30% similar. Hence, CDR1 and CDR2 would be very different in $\gamma\delta$ TCRs using these two V_γ s, predicting that the TCRs might bind dissimilar antigens.

Nonetheless, there are constraints on TCR $\gamma\delta$. Unlike Ig, there is no evidence that diversity is increased by somatic hypermutation, and the small size of $\gamma\delta$ gene families limits combinatorial diversity, obtained by recombination of any one of many V segments with any one of many D and/or J segments. There is also limited chain-pairing, and remarkably, certain epithelial $\gamma\delta$ populations show virtually no diversity; e.g. a single TCR ($V_\gamma 5$ – $J_\gamma 1$: $V_\delta 1$ – $D_\delta 2$ – $J_\delta 1$ (GV1–GJ1:DV101–DD2–DJ1)) is expressed by >95% of murine skin T cells; another TCR ($V_\gamma 6$ – $J_\gamma 1$: $V_\delta 1$ – $D_\delta 2$ – $J_\delta 1$ (GV2–GJ1:DV101–DD2–DJ1)) is expressed by most murine uterine T cells. Taken together, the data suggest that $\gamma\delta$ T cell repertoires may recognize a small number of highly diverse antigens.

Antigen specificity

Table 3 lists many of the antigen specificities reported for $\gamma\delta$ cells. Specificity is broad, and includes proteinaceous and nonproteinaceous ligands. By two criteria, TCR $\gamma\delta$ specificity can be distinguished from most TCR $\alpha\beta$ specificity. First, there is conspicuously little data for the recognition of peptides presented by conventional MHC (Table 3A). Second, recognition by TCR $\gamma\delta$ can be of native antigens, e.g. class II MHC (Table 3B), or herpes simplex virus glycoprotein I (Table 3I), without requirement for either antigen processing or presentation of the antigen on cells.

Tables 3C and 3D indicate that some $\gamma\delta$ cells may resemble a poorly understood set of $\alpha\beta$ T cells that can recognize murine nonclassical MHC and human CD1, molecules that may be functional analogs. Again, the direct target of $\gamma\delta$ cell recognition may be the molecules themselves, or bound antigens that can include lipids, glycolipids or hydrophobic peptides. Different CD1 and nonclassical MHC molecules show tissue-specific expression, perhaps compatible with the association of particular $\gamma\delta$ TCRs with certain tissues. CD1 responses may be a subset of those that participate in the immune response to mycobacteria: $\gamma\delta$ cells have been identified that react to mycobacterial antigens (Table 3E), that may be proteinaceous (Table 3E2) but that may not require presenting cells (Table 3E1, E2). Human $\gamma\delta$ cells can also react to low molecular weight phosphorylated, nonproteinaceous mycobacterial antigens (Table 3E3): currently no mouse $\gamma\delta$ cells have shown such specificity. Other specificities include less well-defined bacterial or protozoan antigens (Table 3F), staphylococcal enterotoxins in the absence of class II MHC (Table 3G), and cell-bound LPS (Table 3J). Although the specificities listed in Table 3 are highly diverse, it is not clear that $\gamma\delta$ cells *in vivo* can react to myriad foreign antigens in the same way that $\alpha\beta$ cells or B cells can. Thus, specific responses to challenging antigen are difficult to elicit in $\alpha\beta$ cell-deficient mice (see below).

Autoreactivity and selection

$\gamma\delta$ Cells recognizing mycobacterial heat shock protein (hsp) (Table 3E2) are cross-reactive to autologous hsp, provoking the idea that $\gamma\delta$ cells are primarily autoreactive. There are human $\gamma\delta$ cells that recognize Ig (Table 3H), possibly presented by hsp. Self-reactivity, e.g. to stress or activation antigens, may underlie $\gamma\delta$ cell reactivity to tumor cells or activated autologous cells of many types, in the absence of foreign antigen (Table 4). Likewise murine intra-

epithelial $\gamma\delta$ repertoires are commonly established in fetal/perinatal animals prior to foreign antigen exposure, and unlike $\alpha\beta$ repertoires, seem essentially constant in specific pathogen-free animals.

Several results suggest that the maturation of $\gamma\delta$ cells includes an interaction of the $\gamma\delta$ TCR with a stromal ligand, an equivalent of positive selection for $\alpha\beta$ cells. Since positive selection for $\alpha\beta$ cells may differ from negative selection that purges the repertoire of strongly autoreactive cells largely by a difference in affinity of the TCR for the ligand, one wonders whether autoreactive $\gamma\delta$ cells are similarly purged by high-affinity interactions with the stroma. This is suggested by studies of mice transgenic for an MHC-reactive $\gamma\delta$ TCR, but currently no data pertain to naturally arising $\gamma\delta$ cells.

Lymphoid and epithelial $\gamma\delta$ cells

The anatomical representation of $\gamma\delta$ cells varies strikingly. In adult mice, $\gamma\delta$ cells account for ~1–3% of T cells in spleen and lymph nodes, 10–50% of intestinal intraepithelial T cells, the majority of reproductive epithelial T cells, and $\geq 95\%$ of resident epidermal T cells. In humans, $\gamma\delta$ cells usually account for from 0.5% to 15% of T cells in peripheral blood, are variably represented in the gut epithelium, but are not obviously present in the skin. In chickens, $\gamma\delta$ cells frequently comprise 30% of peripheral T cells, and can constitute the majority of gut intraepithelial T cells. In ruminants, 30–80% of blood T cells are $\gamma\delta$ cells.

A high proportion of $\gamma\delta$ cells are double negative (lack surface CD4 and CD8) (see Tables 3 and 4), reflecting the fact that $\gamma\delta$ cells generally do not pass through positive selection by class I or class II MHC. Many gut $\gamma\delta$ cells are CD8⁺, but express the CD8 $\alpha\alpha$ homodimer that may be unrelated to recognition of class I MHC.

Murine epidermal $\gamma\delta$ cells can recognize keratinocytes, and gut $\gamma\delta$ cells may recognize gut epithelial cells. Epithelial cells acting as antigen-presenting cells would be relatively immobile, providing for few encounters with T cells. Thus, if the T cells are to be activated, those few encounters must have high chance of presenting a $\gamma\delta$ TCR ligand. This concept provoked the idea that intraepithelial $\gamma\delta$ T cells would have limited antigen specificity, strikingly borne out in the murine skin and reproductive epithelia.

Ontogeny of $\gamma\delta$ T cells

Considerable interest has been aroused by the ontogeny of $\gamma\delta$ cells. In mice and chickens, $\gamma\delta$ TCR

Table 3 $\gamma\delta$ T cell specificity: antigens

Origin	CD4/CD8	TCR usage	Antigen	Restriction element	Reference
A. MHC-restricted antigens					
Human	CD8 ⁺	V _δ 1	Tetanus toxoid	HLA-DR4 (using mismatched APCs)	Kozbor <i>et al</i> (1989) <i>J Exp Med</i> 169 : 1847
Human	Double negative	V _γ 9/V _δ 2	Tetanus toxoid, 12-mer of fragment C	HLA-DRw53 (blocked by α-DRw53 mAb)	Holoshitz <i>et al</i> (1992) <i>J Clin Invest</i> 89 : 308
B. MHC antigens					
Human	Double negative	V _δ 1 J _δ 1–2	n.d.	Class I (blocked by α-HLA-A, -B, -C mAb)	Rivas <i>et al</i> (1989) <i>J Immunol</i> 142 : 1840
Mouse	Double negative	V _γ 1 J _γ 4	n.d.	H-2D (using mismatched APCs)	Bluestone <i>et al</i> (1988) <i>J Exp Med</i> 168 : 1899
Human	Double negative	V _γ 3 J _γ 2	n.d.	HLA-DR7 (using mismatched APCs)	Jitsukawa <i>et al</i> (1988) <i>Eur J Immunol</i> 18 : 1671
Human	Double negative	n.d.	n.d.	HLA-DR (blocked by α-HLA-DR, but not α-HLA-A, -B, -C, CD1a, -c mAbs)	Takamizawa <i>et al</i> (1995) <i>J Clin Invest</i> 95 : 296
Human	CD4 ⁺ CD8 ^{+/–}	V _δ 1	n.d.	HLA-DQw6 (blocked by α-Cl II mAbs)	Vandekerckhove <i>et al</i> (1990) <i>J Immunol</i> 144 : 1288
Mouse	DN	V _γ 2 J _γ 2/V _δ 5 D _δ 2 J _δ 1	Mapped to site outside peptide-binding region, affected by glycosylation	I-E ^{b, k, s, r}	Matis <i>et al</i> (1989) <i>Science</i> 245 : 746 Schild <i>et al</i> (1994) <i>Cell</i> 79 : 29
C. Nonclassical MHC antigens					
Mouse	DN	n.d.	Poly(Glu50Tyr50)	Q a	Vidovic <i>et al</i> (1989) <i>Nature</i> 340 : 646
Mouse	DN	V _γ 4 J _γ 1/V _α 11.3D _β 1 D _β 2 J _β 1	n.d., but CI I/CI II processing not required	TL (T10 ^b , T10 ¹²⁹ , T27 ^b /T22); amino acid sequence differences in all 3 TLs do not affect specificity	Bluestone <i>et al</i> (1988) <i>J Exp Med</i> 168 : 1899 Schild <i>et al</i> (1994) <i>Cell</i> 76 : 29 Weintraub <i>et al</i> (1994) <i>J Immunol</i> 153 : 3051
Mouse	DN	V _γ 4 J _γ 1/V _δ 5 D _β 2 D _β 1 J _β 1	Unresolved	TL (T27 ^b /T22)	Ito <i>et al</i> (1990) <i>Cell</i> 62 : 549
D. MHC class I-related antigens					
Human	DN	V _γ 9 J _γ 2/V _δ 1 J _δ 1–2	n.d.	CD1c	Porcelli <i>et al</i> (1989) <i>Nature</i> 341 : 447
Human	DN	V _γ 9(–); V _δ 1 J _δ 1–2	n.d.	CD1c	Faure <i>et al</i> (1990) <i>Eur J Immunol</i> 20 : 703
Human	DN	V _γ 3 JP2/V _δ 1	TCT.1 (GPI-linked transmembrane protein on hematopoietic cells)	Encoded by Blast-1, a gene in CD1 region; not blocked by α-Cl I mAb	Del Porto <i>et al</i> (1991) <i>J Exp Med</i> 173 : 1339
E1. Mycobacterial antigens					
Human	DN	V _γ 9/V _δ 2	Acetone-precipitable fraction of mycobacteria	Requires APCs but not MHC-restricted (not blocked by α-Cl I/II mAbs)	Holoshitz <i>et al</i> (1989) <i>Nature</i> 339 : 226
Human	n.d.	n.d.	Mycobacterial cell wall antigens	Requires histocompatible APCs	Modlin <i>et al</i> (1989) <i>Nature</i> 339 : 544
Human	DN	V _γ 9/V _δ 2	Heat-killed mycobacteria	Requires APCs but not HLA-DR-restricted	Kabelitz (1990) <i>J Exp Med</i> 171 : 667
Mouse	DN	n.d.	Solubilized extracts of mycobacteria	CI II not required (not blocked by α-Cl II mAbs)	Janis <i>et al</i> (1989) <i>Science</i> 244 : 713

Table 3 Continued

Origin	CD4/CD8	TCR usage	Antigen	Restriction element	Reference
E2. Mycobacterial antigens related to self heat shock proteins (hsps)					
Human	DN	n.d.	hsp65	Requires histocompatible APCs	Haregewoin <i>et al</i> (1989) <i>Nature</i> 340 : 309
Human	n.d.	V γ 9/V δ 2	GroEL hsps (58 and 60) in Daudi B cells	Non-MHC-restricted, (using β_2m^- cell lines)	Fisch <i>et al</i> (1990) <i>Science</i> 250 : 1269
Mouse	n.d.	polyclonal V γ 1 J γ 4	Purified protein derivative (PPD); hsp65 (including 7- and 17-mer synthetic peptides)	Non-MHC-restricted (using mismatched APCs)	O'Brien <i>et al</i> (1989) <i>Cell</i> 57 : 667 Happ <i>et al</i> (1989) <i>Nature</i> 342 : 696 Born <i>et al</i> (1990) <i>Science</i> 249 : 64 Fu <i>et al</i> (1994) <i>J Immunol</i> 152 : 1578 Kalataradi <i>et al</i> (1994) <i>J Immunol</i> 153 : 1455
Mouse	Intestinal IELs	n.d.	hsp71	APCs not required	Beagley <i>et al</i> (1993) <i>Eur J Immunol</i> 23 : 2049
Rat	Peritoneal	n.d.	hsp60	n.d.	Kimura <i>et al</i> (1996) <i>Immunology</i> 87 : 21
E3. Nonpeptidic antigens derived from mycobacteria					
Human	n.d.	n.d.	LMW (1.5–3 kDa) protease-resistant ligands	n.d.	Pfeffer <i>et al</i> (1990) <i>Eur J Immunol</i> 20 : 1175
Human	n.d.	V γ 9/V δ 2	Nonproteinaceous, LMW, phosphocarbohydrates	n.d.	Schoel <i>et al</i> (1994) <i>Eur J Immunol</i> 24 : 1886
Human	n.d.	V γ 9/V δ 2	Monoalkyl phosphates (synthetic)	Non-MHC-restricted (not blocked by α -CI I/II mAbs)	Tanaka <i>et al</i> (1994) <i>Proc Natl Acad Sci USA</i> 91 : 8175
Human	n.d.	V γ 9/V δ 2	γ -derivatives of TTP	n.d.	Constant <i>et al</i> (1994) <i>Science</i> 264 : 2647
Human	n.d.	V γ 9/V δ 2	Isopentenyl pyrophosphate and related prenyl pyrophosphate derivatives	n.d.	Tanaka <i>et al</i> (1995) <i>Nature</i> 375 : 155
F. Other pathogen-associated antigens					
Human		V γ 9 V δ 2	Extracts from <i>L. monocytogenes</i> and <i>E. coli</i>	Optimal stimulation requires APCs, but non-MHC-restricted (using mismatched APCs, and not blocked by α -CI I/II or β_2m mAbs)	De Libero <i>et al</i> (1991) <i>J Exp Med</i> 173 : 1311
Human	n.d.	V γ 9 V δ 2	Heat-killed <i>L. monocytogenes</i> , listerial lysate, and listerial culture supernatant	Reactivity against lysates affected by proteinase K treatment, but not much by alkaline phosphatase treatment	Munk <i>et al</i> (1996) <i>Immunology</i> 87 : 230
Mouse	DN	V γ 7 and V δ 4 or -2	Extracts from <i>L. monocytogenes</i>	n.d.	Tsuji <i>et al</i> (1996) <i>Int Immunol</i> 8 : 359
Human	n.d.	V γ 9/V δ 2	<i>P. falciparum</i> -infected erythrocytes	Requires CI II (blocked by α -CI II mAbs)	Goodier <i>et al</i> (1992) <i>Int Immunol</i> 4 : 33
Human	DN	V γ 9/V δ 2	<i>P. falciparum</i> schizont lysate	Requires CI II (blocked by α -CI II mAbs)	Behr and Dubois (1992) <i>Int Immunol</i> 4 : 361
Human	n.d.	V γ 9	Live <i>Salmonella</i>	n.d.	Hara <i>et al</i> (1992) <i>J Clin Invest</i> 90 : 204

Table 3 Continued

Origin	CD4/CD8	TCR usage	Antigen	Restriction element	Reference
<i>G. Enterotoxins</i>					
Human		V γ 9/V δ 2	<i>Staphylococcus</i> enterotoxin A (SEA)	CI II not required (blocked by α -CI II mAbs; but use of CI II(-) mutants makes no difference)	Rust <i>et al</i> (1990) <i>Nature</i> 346 : 572
Human		V γ 9/V δ 1	SEB, SEC1, toxic shock syndrome toxin (TSST)	CI II not required (blocked by α -CI II mAbs; use of CI II(-) mutants makes no difference)	Rust <i>et al</i> (1990) <i>Nature</i> 346 : 572
<i>H. Other self antigens</i>					
Human	DN	n.d.	Immunoglobulin	Non-MHC-restricted (not blocked by α -CI I/II mAbs); may be presented by hsp	Wright <i>et al</i> (1989) <i>J Exp Med</i> 169 : 1557 Kim <i>et al</i> (1995) <i>J Immunol</i> 154 : 1614
<i>I. Native antigen</i>					
Mouse	DN	V γ 2 J γ 2/V δ 8(V α 2) D δ 1 D δ 2 J δ 1	HSV-1 glycoprotein I	No APCs required	Sciammas <i>et al</i> (1994) <i>J Immunol</i> 152 : 5392
<i>J. Carbohydrate antigen</i>					
Mouse	DN	V γ 7/V δ 4	Lipopolysaccharide (LPS)	Non-MHC-restricted (using mismatched APCs and APCs from CI I- and CI II-deficient mice); requires cell-cell contact	Tsuji <i>et al</i> (1996) <i>Int Immunol</i> 8 : 359

n.d., not defined; α -CI I, antibody against MHC class I; α -CI II, antibody against MHC class II.

is the first TCR expressed in the fetal thymus. Early fetal rearrangements are simple, using single D δ elements and no N nucleotides. Consecutive waves of early murine $\gamma\delta$ cells express successively V δ 1 (DV101) + V γ 5 (GV1) and V δ 1 + V γ 6 (GV2), and populate successively the skin, and reproductive tract. The schedule of V γ gene usage seems in part due to differential gene activation, although it is likely that important interactions occur between the expressed $\gamma\delta$ TCRs and ligands expressed by the thymic stroma in a developmentally programmed fashion (the intrathymic development of skin and uterine $\gamma\delta$ cells requires a fetal thymus). At later times, gut and lymphoid $\gamma\delta$ cells mature, expressing successively V δ 6 (ADV7)⁺ and V δ 5⁺TCRs with high junctional diversity.

Because humans lack obvious $\gamma\delta$ repertoires in skin or reproductive epithelia, it might have been predicted that there would be no equivalent of the murine early fetal thymic waves of V δ 1⁺ cells. Instead, the human thymus appears to produce similar waves of $\gamma\delta$ cells characterized by specific V δ expression. V δ 2 (DV102), the V gene most closely related to murine V δ 1 (early fetal), appears before V δ 1 (DV101), that most closely resembles mouse V δ 6 (neonatal), that appears prior to DV3, that resembles

mouse V δ 5 (postnatal). By contrast, the V γ chains paired with these V δ s are not as obviously related: for example there is no clear human equivalent of V γ 5 (GV1) that pairs with V δ 1 (DV101) in cells of the first thymic wave which populate the skin. Perhaps V γ divergence contributes to differences in ligand specificity (Table 3) and anatomical location between murine and human $\gamma\delta$ cells.

At least some cells that attempt and fail $\gamma\delta$ gene rearrangements can become $\alpha\beta$ cells, indicating a late common thymic precursor. Nonetheless, there may be multiple origins of $\gamma\delta$ cells: some epithelial $\gamma\delta$ cells may develop extrathymically, and the establishment of murine epithelial, but not lymphoid, $\gamma\delta$ populations, requires the syk tyrosine kinase, which may be involved in signal transduction from the TCR.

Functions of $\gamma\delta$ T cells

Although $\gamma\delta$ cells are probably heterogeneous in their functional capabilities, they can together seemingly perform all functions ascribed to $\alpha\beta$ cells. Thus, some protection against pathogens can be provided by $\gamma\delta$ cells in $\alpha\beta$ T cell-deficient mice, and $\gamma\delta$ cells can drive the production of autoantibodies,

Table 4 T cell specificity: target cells

Origin	CD4/CD8	TCR usage	Target	Restriction element	Reference
A. Cells of lymphoid origin					
Human	CD4 ⁺ CD8 ⁻	V γ 9	T: K562, Jurkat, KG1, MOLT-4. B: Daudi	Non-MHC-restricted (using mismatched APCs and not blocked by α -CI I/II mAbs)	Faure <i>et al</i> (1988) <i>J Immunol</i> 140 : 1372
Human	DN	V γ 3 or 4 J γ 2/V δ 1 and V γ 9/V δ 2	T: K562, CEM, MOLT- 4, HD-MAR	K562 is CD1 c(-); CEM is CD1 c(-); MOLT-4 is CD1 c ^{lo} ; HD-MAR is CD1 c ^{hi}	Faure <i>et al</i> (1990) <i>Eur J Immunol</i> 20 : 703
Human	CD4 ⁺ CD8 ⁻	V γ (9)(-)	T: K562, JM Histiocytic: U937	Non-MHC-restricted (using mismatched APCs)	Jitsukawa <i>et al</i> (1988) <i>Eur J Immunol</i> 18 : 1671
Human	DN	V δ 1 J δ 1-2	B: MSAB (EBV- transformed)	CI I-restricted (using mismatched APCs, and blocked by α -CI I mAbs)	Rivas <i>et al</i> (1989) <i>J Immunol</i> 142 : 1840
Human	n.d.	V γ 9/V δ 2 and V γ 9/V δ 1	B: Daudi	Non-MHC-restricted (using mismatched APCs)	Fisch <i>et al</i> (1992) <i>J Immunol</i> 148 : 2315
Human	n.d.	V δ 1	B: non- and EBV- transformed lines	n.d.	Orsini <i>et al</i> (1994) <i>Eur J Immunol</i> 24 : 3199
Human	n.d.	V δ 1	B: B-LCL (EBV- transformed)	Autologous and allogeneic but not resting B cells	Hacker and Wagner (1995) <i>Immunology</i> 84 : 105
Mouse	Intestinal IELs	V γ 1 J γ 4, V γ 7 J γ 1/V δ 6	B: A20	Does not require CI II (using CI II (-) cell lines)	Sano <i>et al</i> (1993) <i>Immunology</i> 80 : 388
Mouse	DN	V γ 4 J γ 1 V δ 5 D δ 2 D δ 1 J δ 1	T: EL-4	TL(?)	Ito <i>et al</i> (1990) <i>Cell</i> 62 : 549
Mouse	DN	V γ 5 J γ 1/V δ 1 D δ 2 J δ 2	CD4 ⁺ autoreactive T cells	Graft-vs-host reaction	Shiohara <i>et al</i> (1996) <i>J Exp Med</i> 183 : 1483
Mouse	n.d.	V γ 1 J γ 4	T: ALC (V β 1 2(+)), EL- 4, RBL-5 Myeloid; P388D Mastocytoma; P815 LPS-activated spleen cells, and macrophages	Does not require CI I (H-2K, D, Tla ^b , -II, TAP-2, β_2 m or hsp; linked with <i>Nromp</i> b/c react with Bcg ⁻ , not Bcg ⁺ -derived macrophages	Penninger <i>et al</i> (1995) <i>Nature</i> 375 : 241
Cow	n.d.	n.d.	Monocytes (only ruminant-derived; fresh or fixed)	Non-MHC-restricted (using mismatched APCs); enzymatic treatment of APCs destroys reactivity	Okragly <i>et al</i> (1996) <i>Immunology</i> 87 : 71
B. Cells of epithelial origin					
Human	n.d.	n.d.	Breast cancer line (MCF-7) transfected with hsp27	n.d.	Mahvi <i>et al</i> (1993) <i>Cancer Immunol Immunother</i> 37 : 181
Human	n.d.	V δ 1 D δ 3 J δ 1	Colorectal, pancreatic, renal, and Wilms' tumor cell lines, and early passage cells from 'normal intestine'	Non-MHC-restricted (not blocked by α -CI I, -II, or CD1a, -c, or hsp mAbs)	Maeurer <i>et al</i> (1996) <i>J Exp Med</i> 183 : 1681
Human	n.d.	V δ 1	Lung carcinoma	Requires autologous cells, but not blocked by anti-HLA CI I mAb	Zocchi <i>et al</i> (1990) <i>Eur J Immunol</i> 20 : 2685 Zocchi <i>et al</i> (1994) <i>Immunology</i> 81 : 234

Table 4 Continued

Origin	CD4/CD8	TCR usage	Target	Restriction element	Reference
Mouse	DN	V γ 4 J γ 1 V δ 5 D δ 2 D δ 1 J δ 1	PCC3 (embryonic carcinoma)	TL	Ito <i>et al</i> (1996) <i>Cell</i> 62 : 549
Mouse	DN	V γ 4 J γ 1 V γ 1 1.3 J δ 1	PCC3	TL	Schild <i>et al</i> (1994) <i>Cell</i> 76 : 29
Mouse	DN epidermal IELs	V γ 5 J γ 1 V δ 1 D δ 2 J δ 2	Heat-shocked keratinocytes	Not MHC-restricted (using mismatched APCs; not blocked by α -CI I, -II, -Qa, and -TL mAbs)	Havran <i>et al</i> (1991) <i>Science</i> 252 : 1430 Lewis and Tigelaar. pers. comm.
Mouse		V γ 1 J γ 4 (polyclonal)	Trophoblasts (mouse and human; fresh or fixed)	Not MHC-restricted (using $\beta_2m^{-/-}$ -derived cells)	Heyborne <i>et al</i> (1994) <i>J Immunol</i> 153 : 2918

thereby contributing to autoimmune disease. Both these effects may stem from generic interactions of $\gamma\delta$ cells with other lymphoid effector cells. However, most evidence suggests that antigen-specific responses cannot be efficiently induced in $\alpha\beta$ T cell-deficient mice, which are therefore highly immunocompromised toward most pathogens. Several non-exclusive reasons for this can be considered. $\gamma\delta$ Cells may not recognize a sufficient spectrum of foreign antigens. Thus, when TCR $\beta^{-/-}$ mice were repeatedly challenged with coccidia, $\gamma\delta$ B cell help was induced, but was not obviously directed at the pathogen. Nonetheless, given the reactivity of many $\gamma\delta$ cells to mycobacteria, it is difficult to understand how limited antigen specificity alone explains the immunodeficiency of TCR $\alpha/\beta^{-/-}$ mice toward mycobacteria. Alternatively, there may be inherent differences in the ways that $\alpha\beta$ cells and $\gamma\delta$ cells respond to antigen, even though $\gamma\delta$ cells reportedly can produce essentially all known effector molecules. Third, $\gamma\delta$ cells may be unable to interact efficiently and antigen specifically with professional antigen-presenting cells (APCs), and thereby coordinate a protective immune response. This inefficiency may be due to a failure of $\gamma\delta$ cells to recognize foreign antigens presented as peptides on MHC molecules possibly compounded by a failure of $\gamma\delta$ cells to express the surface and intracellular molecules that facilitate the efficient interaction of $\alpha\beta$ cells and APCs. The latter differences might underlie differences in the susceptibility of $\gamma\delta$ cells and $\alpha\beta$ cells to selection during development. They may also explain the lack of evidence for $\gamma\delta$ cells converting functionally to memory cells in response to foreign antigen.

A significant role for immunoregulation by $\gamma\delta$ cells is indicated by reports that $\gamma\delta$ cell-deficient mice show exaggerated lymphoid responses, both to pathogens (TCR $\delta^{-/-}$ mice show heightened immunopathology), and to self (TCR $\delta^{-/-}$ mice on particular

genetic backgrounds show increased autoimmune disease). This may again reflect recognition of activated effector cells, including $\alpha\beta$ cells, as indicated in several studies (Table 4).

In summary, there is overwhelming evidence that $\gamma\delta$ T cells have a distinct set of antigen specificities, facilitating their response to a variety of conditions. An important result of this in the healthy individual may be immunoregulation. In the immunocompromised, $\alpha\beta$ T cell-deficient individual, such as an AIDS (acquired immune deficiency syndrome) patient, $\gamma\delta$ T cells may activate other lymphoid effectors, such as B cells, but the targets of this combined action may more commonly be self than the challenging pathogen. Hence $\gamma\delta$ cells both contribute to and regulate immune responses to pathogens and to self.

See also: Antibodies, specificity; Antigen-binding site; Bacteria, immunity to; Mucosa-associated lymphoid tissue (MALT); Mycobacteria, infection and immunity; Stress proteins; T cell receptor, $\alpha\beta$; T cell receptor, evolution of; T cell receptor, recognition by; T lymphocyte differentiation.

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T CELL RECEPTOR, EVOLUTION OF

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T cell receptors (TCRs) comprise a subset of the vast immunoglobulin gene superfamily. Genes coding for four TCR polypeptides, designed α , β , γ and δ , have been well characterized in human and mouse. Transcripts for one or more of these polypeptides have been identified in numerous other vertebrates, including shark, salamander, chicken, sheep, cow, pig, rat and chimpanzee. It is believed that the TCR evolved during the origin of the vertebrate evolutionary lines, perhaps in conjunction with the major histocompatibility (MHC) locus. TCRs initiate the cellular immune response by responding to foreign antigens presented on the surface of antigen-presenting cells. Presented antigens take the form of small peptides nestled in the groove of receptors coded by the class I or class II genes located in the MHC locus. The repertoire of TCR α/β or γ/δ combinations is highly diverse, as required for an adaptive immune system. Diversity in the TCR repertoire of an individual organism is generated by a sophisticated immune cell-specific somatic recombination machinery that conjoins distantly-spaced DNA sequences in the chromosomes of T cells. An additional source of diversity from an evolutionary perspective is the expansion and contraction of gene segment numbers by nonhomologous recombination.

Generation of the T cell receptor repertoire in an individual

Two types of T cells are distinguished in humans and mice, according to their constituent TCR subunits – α/β or γ/δ . The predominant T cell type in humans and mice is the α/β form. The protein subunits, α , β , γ , δ , each contain a membrane-bound constant region and a variable region that recognizes the antigen–MHC complex. T cells originate in the thymus, where positive and negative selection ensures survival of only those T cells whose receptors can effectively recognize foreign antigens. Because thymic selection is tailored specifically to an individual's

MHC haplotypes, only a fraction of the potential diversity of the TCR repertoire is actually used.

Diversity in an individual's TCR repertoire is primarily generated as a result of two unique activities of the immune cell-specific recombination machinery: 1) rearrangement of chromosomal DNA so as to bring distantly spaced gene segments into conjunction (recombination), and 2) modification of DNA sequence at the junctions of gene segments by exonuclease nibbling and non-template-directed nucleotide addition (N-region addition). Polymorphism in variable gene segments contributes only a minor source of diversity to an individual's TCR repertoire, in contrast to the MHC. Diversity engendered by recombination is a function of the number of variable (V), diversity (D) and joining (J) gene segments in the germline TCR loci. Our current understanding of the organization of the TCR loci for human and mouse is schematized in Figure 1, and the relationship between a TCR gene, its transcript, and the polypeptide produced from the transcript is illustrated for the TCR β subunit in Figure 2. The α and γ loci each rearrange one V gene segment to one J gene segment; the β locus undergoes D–J, then V–DJ recombination; and the δ loci can rearrange V to DJ or V to DDJ. Several of the $V\alpha$ genes in human and mouse can rearrange to either a DJ segment of the δ locus or a J segment of the α locus. Once rearrangement of the TCR loci has occurred, permanently altering the chromosomal DNA of the T cell, a TCR of either the α/β or γ/δ subunits is produced from the mRNAs generated by transcription and splicing of the rearranged gene segments. Generally, only one α/β or γ/δ combination is allowed per T cell. Thus, the immune cell-specific recombination machinery ensures TCR uniqueness at the level of individual T cells and their descendants, and diversity in the TCR repertoire as a whole.

TCR loci differ from subunit to subunit and species to species in the number of germline gene segments capable of undergoing rearrangement. For

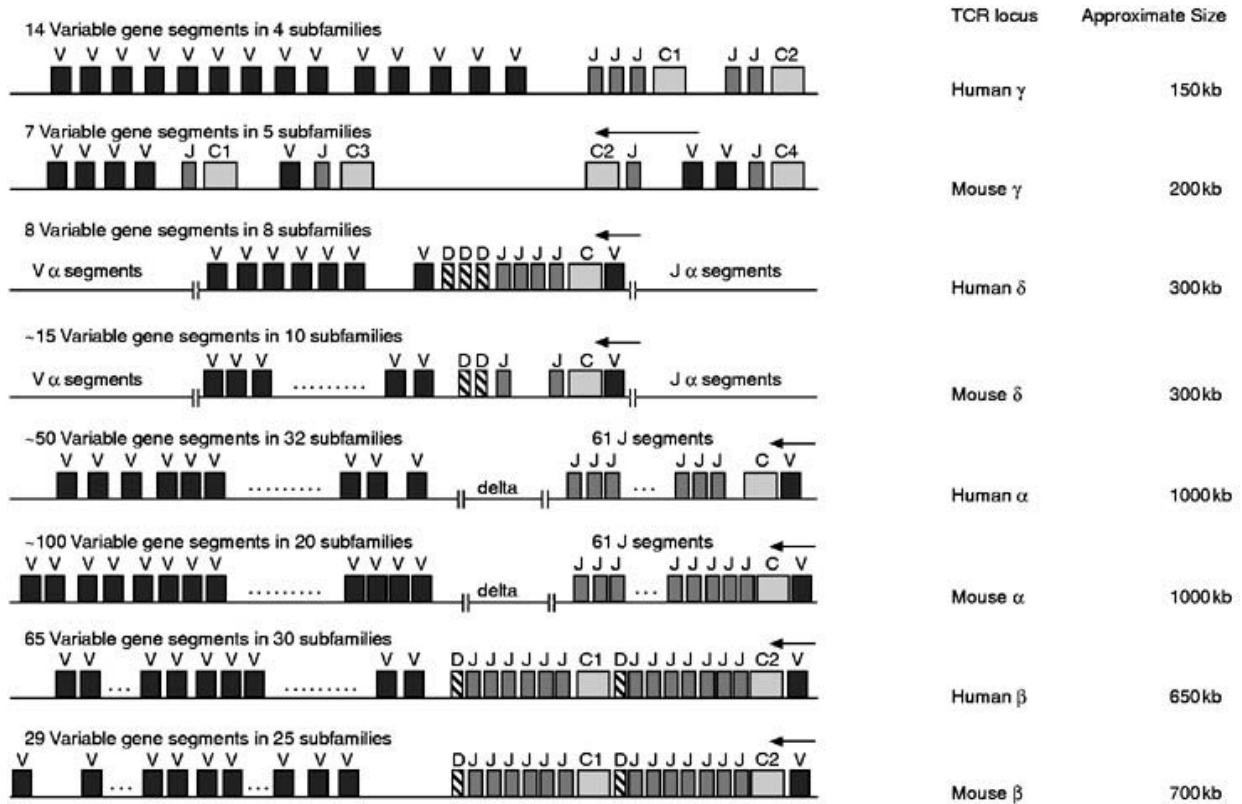


Figure 1 Genomic organization of the human and mouse TCR loci. Arrows indicate gene segments whose transcriptional orientation is inverted with respect to the other gene segments in the locus. kb, kilobases.

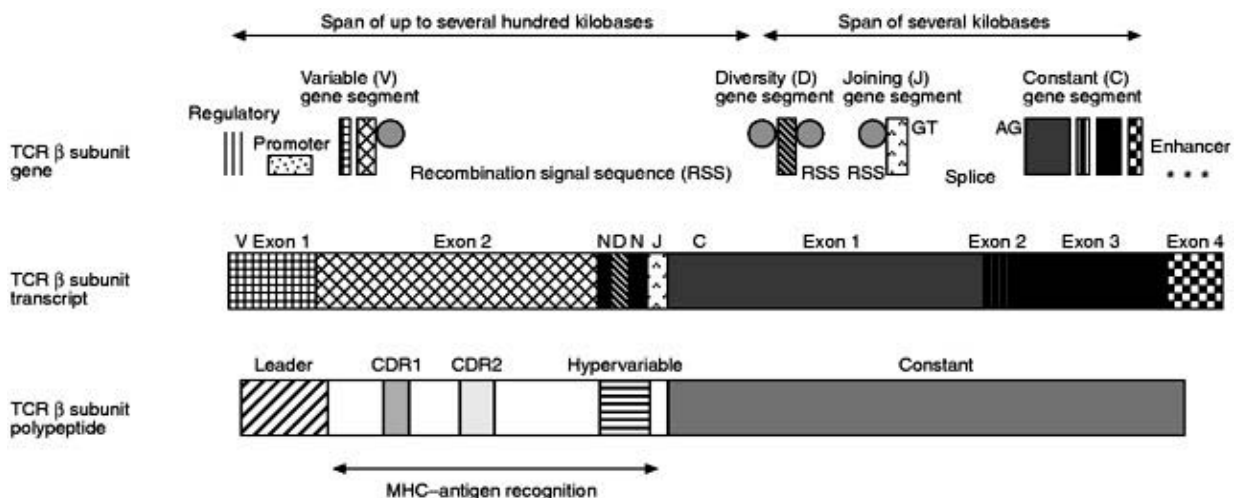


Figure 2 The TCR β subunit, from the perspective of gene, transcript and polypeptide. Genes for the TCR loci contain features not found in the transcript. The enhancer, promoter and other regulatory elements allow gene expression. Recombination signal sequences, indicated by a circle, facilitate alteration of the TCR chromosomal DNA through recombination and N-region addition, as described in the text. After transcription, introns are spliced to generate a mature transcript, which is translated into the polypeptide subunit. The VNDNJ region of the transcript provides the coding information for the third hypervariable region of the protein receptor, responsible for antigen-MHC recognition.

example, the human TCR β locus has twice the number of V gene segments as the mouse, whereas the converse is true for the α locus. The germline repertoire of γ/δ gene segments is more limited than that of α/β in human and mouse, but not necessarily so in other species, for example, chicken.

Diversity in the TCR repertoire is not, however, solely a function of the number of V, D and J gene segments in the loci. Indeed the most variable portion of the TCR transcript lies in the VJ or VDJ junctions. Codons from this region (VNJ or VNDNJ) translate into the third hypervariable region of the protein, which plays a key role in MHC-antigen recognition (Figure 2). During recombination, exonucleolytic nibbling of the 3' end of the V gene segment, both ends of the D segment, and the 5' end of the J segment is followed by the addition of an average of three to five nucleotides (N) in each junction, prior to the covalent joining of the gene segments. Due to nibbling and random N region addition, TCRs that share the same V, D, J and C gene segments can nonetheless differ in the length and amino acid sequence in the third hypervariable region. Thus, N region addition gives added value to the diversity potential inherent in the germline V, D and J gene segments.

Generation of the T cell receptor repertoire in a species

The number and overall organization of the V, D, J and C gene segments within the TCR loci are the result of evolutionary processes that lead to very different germline combinations in different species. In outbred populations within a species, there are variations in the TCR loci due to base substitution and insertion-deletion polymorphisms. These polymorphic variations provide the source material for potential evolutionary changes.

Across species, the organization of the TCR loci can differ greatly, even though the loci evolved from common ancestral TCR gene families. For a given TCR locus, species vary in 1) order of V, D, J and C gene segments, 2) number of V, J, D and C gene segments, 3) number of distinct subfamilies of V gene segments (V gene segments with sequence similarity less than 75% are in different subfamilies), and 4) the number of gene segments in each V subfamily. While most of the mapping and sequencing data come from the human and mouse TCR loci, some information, mostly in the form of cDNA sequences, has been obtained from other primates, other rodents, rabbit, artiodactyls, chicken, salamander, rainbow trout and shark. From the available data, inferences about mechanisms at work in the evolution of the TCR loci can be made.

Movement of large units of DNA

The complete DNA sequence of the human TCR β locus confirmed an organizational feature of the TCR loci that had been intimated by the physical maps, namely, the existence of multiple copies of locus-specific DNA sequence repeats. Most of the multimembered families of β variable gene segments are embedded in six repeats ranging in length from 0.8 to 31 kilobases. The number of copies of these repeats ranges from two to ten, indicating extensive duplication of some of the subfamilies. Blocks of repeated DNA sequence, or homology units, can be arranged in a tandem, dispersed or inverted orientation. Tandem arrays are particularly subject to increases or decreases in copy number due to recombination through unequal crossover. Tandem arrays can become dispersed, and thereby less subject to modification in copy number, by the intrusion of genome-wide interspersed repeats such as retroviral elements or LINE sequences. A comparison of the human and mouse γ loci illustrates how the movement of blocks of DNA can bring about differences in the organization of TCR gene segments between species (Figure 1). The mouse γ locus contains four copies of a 30–40 kilobase V–J–C homology unit, one of which is in an inverted orientation with respect to the other three. The human γ locus, by way of contrast, is organized along the lines of the β locus, with the V segments all 5' of the J–C elements.

Evolution within a species can take odd turns. For example, humans and closely related primates have two copies of a ~100 kilobase portion of the β locus containing several V gene segments, a functional copy on human chromosome 7 and a nonfunctional copy on chromosome 9. Presumably, a one-time duplication and translocation event occurred in the germline of a primate ancestor and subsequently became fixed in the population, but not necessarily because the duplication conferred a selective advantage. As a second example, humans today have a prevalent 20 kilobase insertion-deletion polymorphism involving three V β gene segments, two of which are functional, suggesting that the evolution of the β locus remains dynamic in our species.

Conservation of ancestral DNA units

A comparison of the physical maps and cDNA sequence data for the mouse and human TCR β loci shows that the order of orthologous gene segments is largely preserved for the single-membered subfamilies, suggesting the preservation of ancestral units of DNA containing several V gene segments. Sequences similar to some of these orthologous genes have been identified in artiodactyls and salamander, but the

maps for these species are not known. By way of contrast, chicken appears to have only two subfamilies of V β segments, each of which has been duplicated several times. The most striking example of preservation of an ancestral unit was obtained from a sequence comparison of 95 kilobases extending from the 3' most J δ to the 3' most V α in human and mouse (Figure 1). Not only was the order of the orthologous gene segments preserved in the two species, but much of the sequence was as well, exhibiting an overall 71% similarity, even in the noncoding regions. Interestingly, 32 J α segments have been identified in rainbow trout, suggesting that a large number of J α segments might have arisen early in vertebrate evolution. It would be fascinating to know how much sequence conservation, if any, has remained across the large evolutionary distance between fish and mammals.

Gene conversion

Gene conversion is a unidirectional exchange of sequence information between similar sequences, wherein one stretch of sequence is used as a template to 'repair' or 'correct' another stretch of sequence. Because the TCR loci are multigene families, with many regions of similar sequence due to duplications, gene conversion is likely to be common. Depending on the length of the converted region, which can vary from a few bases to many kilobases, gene conversion can be either a diversifying or a homogenizing force in evolution. The homogenizing effect of gene conversion has occurred independently in the constant gene segments of the β locus in several species (e.g. human, mouse and salamander). Although the D-J-C duplication in the β locus probably occurred early in tetrapod evolution (chicken lacks this duplication), the constant gene segments in each species investigated bear more intraspecies similarity than interspecies similarity. This suggests that there are species-specific constraints, such as a need to bind to accessory proteins, that exert selective pressure on the C β genes, leading to their concerted evolution.

Mutation

During DNA replication or recombination, DNA sequences can be altered by base substitution, insertion or deletion. Alterations in sequence can also occur by insertion of transposable elements. Because of mutation, functional gene segments can evolve into pseudogenes due to damage to the promoter, recombination signals, splice sites or coding sequence (frameshift, premature termination or deleterious amino acid substitution). Presumably, mildly damaged pseudogenes can evolve into functional genes

as well, through mutation or gene conversion. Badly damaged pseudogenes evolve into relics, gene segments identifiable primarily by sequence similarity rather than functional features. In the human TCR β locus, there are 45 or 46 functional genes, 19 or 20 pseudogenes and 22 relics. All but possibly one of the functional V β gene segments had been identified by sequencing cDNAs prior to the public release of the genomic sequence. Because pseudogenes are vastly underrepresented in cDNA libraries, genomic sequencing of other TCR loci will reveal heretofore unidentified pseudogenes and relics and will thus contribute to our understanding of the evolutionary dynamics of the TCR loci.

Because of their complexity, the TCR loci present a fascinating system for the study of molecular evolution. As the complete genomic sequences of TCR loci in different species become available, a detailed cross-species comparison of promoter and regulatory regions, recombination signal sequences and NDN and NJ diversity, will lead to a more complete understanding of this key component of the vertebrate immune system.

See also: T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T cell receptor, recognition by.

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T CELL RECEPTOR, RECOGNITION BY

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Unlike antibodies, T lymphocytes do not recognize antigens in solution. Instead, they recognize antigens on intact cells in the context of surface molecules encoded by the major histocompatibility complex (MHC). How cells could recognize both a foreign antigen and MHC proteins was the central problem in immunobiology for many years. The answers to this problem have come from elucidation of the structure of the T cell antigen receptor (TCR) and MHC molecules, and appreciation of the phenomenon of antigen processing by antigen-presenting cells (APCs).

Usually, T lymphocytes do not respond to intact protein antigens, but rather to peptide fragments. APCs such as macrophages or B lymphocytes take up intact antigen and process it into fragments which are recognized in the context of MHC molecules. This process also occurs for normal cellular constituents as well as viruses so that 'self' proteins are also processed and presented.

MHC molecules have a surface consisting of two parallel α helices supported on a β pleated sheet. A groove or pocket is formed which has an α helix on each side and the β pleated sheet as its floor. The T cell 'sees' antigenic peptides which are bound into this groove in the MHC molecule. From the T cell's perspective, the MHC and the central peptide probably form an undulating surface, the exact contours of which will be determined by the amino acid sequence of the MHC and the enfolded peptide.

There are two classes of TCR, sometimes referred to as TCR1 and TCR2 in view of their appearance during ontogeny. TCR1 is a heterodimer composed of polypeptides called γ and δ chains, which appears

at about day 14 in the mouse embryo. The TCR2 heterodimer is composed of polypeptides called α and β chains, and appears at about day 17 in the mouse embryo. At birth, TCR2 is the predominant form and 90–99% of T cells in the blood and lymphoid organs of mice and humans express this class of receptor. TCR2 mediates conventional MHC-restricted antigen recognition by T cells. The nature of the antigen recognized by TCR1 is not yet clear.

Both TCR2 α and β chain gene complexes encode variable (V), joining (J) and constant (C) region segments and, in addition, the β chain gene contains diversity (D) segments. Further heterogeneity at the V–J or V–D–J functions can result from junctional flexibility and the loss or addition of nucleotides during the process of recombination (N region diversity). Comparison of the structure of V gene sequences for TCR and Ig suggests that TCR V genes, like Ig V genes, encode two regions of 'hyper-variability' known as complementarity-determining regions (CDR1 and CDR2); these, together with CDR3, form the surface which interacts with the antigen. In both TCR and Ig the third hypervariable or complementarity-determining region (CDR3) is encoded by the extreme 3' end of the V gene, together with (D and) J gene segments. It appears that the TCR α and β chain V regions form two wing-like surfaces with CDR1 and CDR2 regions on the outside and the two CDR3 regions adjacent to each other in the middle. Modeling this structure on to the MHC–peptide complex described above suggests that the V region CDR1 and CDR2 loops interact with the α helices of the MHC molecule, while the CDR3 segment interacts with the peptide. TCR

CDR3 diversity would allow recognition of an exceedingly large array of antigenic peptides. Theoretical calculations suggest that there are at least 10^{14} TCR2 combinations.

X-ray crystallography of MHC-peptide-TCR complexes has revealed that two or three amino acids on the peptide are exposed for potential TCR interaction, with the rest of the amino acids buried in the MHC groove. While the broad prediction of CDR3 interaction with the peptide has been confirmed the model is more complicated than expected. In one complex the β chain only makes contact through 2 or 3 amino acids in CDR3, and the CDR1 and CDR2 loops appear to have no contact with antigen. Thus there appears to be considerable variability in the way the TCR can bind to the relevant antigens. No definitive model can be deduced until many complexes have been analysed.

Analysis of the interaction between TCR2 and MHC-peptide complexes reveals low-affinity binding, largely due to a tendency for the complex to dissociate rapidly. This implies that the interaction between T cell and APC is mediated by non-TCR adhesion molecules and the TCR itself functions only to detect the presence or absence of specific antigens. Quantitative studies of TCR occupancy suggest that the T cell 'counts' each interaction with specific antigen so that the cell is triggered to respond when a particular predetermined threshold is passed.

Structural studies of TCR2 suggest an important difference from Ig molecules in the way that the V and C regions interact. In Ig molecules the hinge between V and C allows V region interaction with antigen without altering the conformation of the C region. In TCR molecules V-C interaction is rigid and therefore TCR-antigen recognition can lead to signal transduction through an altered conformation of the C region.

Subtle differences in TCR-antigen interaction can have profound effects on TCR responses. Changing a single amino acid in the antigenic peptide can change the effect from agonist to antagonist. Single mutations in important viral antigens can lead to T cell inactivation instead of lysis. Other changes may produce partial agonist effects which result in altered patterns of cytokine secretion. Finally, some changes induce anergy.

Recent X-ray crystallography indicates that class II MHC molecules probably occur as dimers on the APC surface. Interaction between TCR dimerized MHC and CD4 molecules could lead to the formation of recognition zones where multiple recognition events occur simultaneously in close proximity.

TCR1 ($\gamma\delta$) have considerably less V region diversity than TCR2 but at least as much CDR3 diversity.

Some $\gamma\delta$ T cells recognize antigens in the context of nonclassical MHC-like molecules such as CD1. Further, $\gamma\delta$ cells specific for nonprotein lipid-like determinants appear to be an important component of the response to *Mycobacterium tuberculosis* infections. Important differences in the location of $\gamma\delta$ T cells exist between humans and mice where defined populations of $\gamma\delta$ cells are found in specific epithelial sites. Overall the data suggest that $\gamma\delta$ cells may recognize unconventional antigens associated with cell surface molecules that do not show the exceptional polymorphism found in MHC molecules. The antigen binding groove of CD1 is much deeper and lined with hydrophobic amino acids when compared with conventional MHC molecules. It appears to be adapted to binding lipid antigens and presenting lipid-derived moieties to both $\gamma\delta$ and $\alpha\beta$ TCR. The TCR probably recognizes exposed carbohydrate structures linked to the embedded lipid. $\gamma\delta$ cells may be an important early pathway to recognition of organisms which are capable of evading conventional immune responses. However, unlike TCR2 deletion of the TCR1 receptor does not lead to rapid death from infection.

Both TCR1 and TCR2 have a hydrophobic transmembrane segment and short cytoplasmic tails. Both are physically associated with the CD3 complex of proteins. Following recognition of antigen by TCR, the CD3 complex probably acts as a transducing element converting recognition into a signal which induces cytokine and receptor expression. Ultimately, this signal leads to cell replication or expression of effector functions such as cytotoxicity.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; CD1; CD3; MHC restriction; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T cell receptor, evolution of.

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T CELL VACCINATION

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T cell vaccination (TCV) is a term describing a method for modulating immune responses in which T cells are administered as immunogenic agents. Typically, an autoimmune response is the subject for modulation and the vaccine consists of T cells specifically reactive to the target autoantigen. The first example of TCV in an autoimmune disease was reported in 1981 by Ben-Nun, Wekerle and Cohen who treated Lewis rats with a line of Lewis rat CD4⁺8⁺ T cells specifically reactive to the dominant epitope of myelin basic protein (MBP). The anti-MBP T cells, which served as the vaccine, were irradiated (1500 R) to prevent them from attacking the recipients and producing experimental autoimmune encephalomyelitis (EAE). Vaccination with the attenuated anti-MBP T cells led to resistance to later attempts to induce EAE by active immunization to MBP in adjuvant. Subsequently it was shown that TCV could also induce resistance to EAE adoptively transferred by virulent anti-MBP T cells.

T cell vaccination was extended to other disease models (adjuvant arthritis (AA), collagen-induced arthritis, experimental autoimmune neuritis, experimental autoimmune thyroiditis and insulin-dependent diabetes mellitus (IDDM)). Moreover, TCV was found to abort established autoimmune disease (AA, IDDM) and spontaneous autoimmune disease (IDDM) and so can be viewed as therapy as well as prevention.

The term TCV was coined to describe manipulation of autoimmune responses using autoimmune T cell vaccines; however, in principle and in practice the procedure may be applied to modify undesirable T cell responses central to allograft rejection or to allergy.

The concept of vaccination was introduced by Jenner about two centuries ago to describe the use

of cowpox to induce immunity to smallpox. A century later, Pasteur extended the word to encompass the concept of using an attenuated infectious agent of disease to induce resistance (now understood to be immunological) to virulent forms of the agent. TCV represents a further extension of Pasteur's vaccination concept to include the individual's own lymphocytes as perpetrators of disease from which vaccines might be made.

T cell vaccination may be analyzed conveniently by dividing the subject into three parts: the T cell vaccine, the regulatory response to the vaccine, and the lymphocyte networks linking these T cells.

T cell vaccines

It seems that T cells do not perform well as vaccines unless the cells have been activated. To be activated, the T cells are incubated for a minimum of about 6–8 h with their specific antigen, or with a T cell mitogen together with antigen-presenting cells. The molecular changes induced by activation that are critical for TCV have not been identified, but their effect is marked. Treatment of whole T cells with chemical cross-linkers can also enhance the effectiveness of some but not all T cell vaccines.

T cell receptor TCV

The T cell receptor (TCR) is clearly an important actor in TCV. Peptides of two different domains of the TCR have been found to mimic some of the effects of whole T cells as vaccines: TCR V region peptides and TCR joining region peptides. The joining regions of the TCR (VDJβ and VJα) determine the epitopes that can be recognized by the particular TCR; the joining regions also create the T cell idotype. Thus, the response to vaccination with a TCR joining region peptide is an anti-idiotypic

response, by definition. Indeed, TCV using whole T cells was found to activate anti-idiotypic T cells of both CD4⁺ and CD8⁺ phenotypes specific for the vaccine. In contrast to TCR joining region peptides, TCR V region peptides are not idiotypic by definition because a given V gene segment can be common to different T cell clones. Thus, TCV using a TCR V region peptide should be viewed as a kind of class-specific stimulus, and not as an anti-idiotypic immunization. Nevertheless, TCV with V region peptides is effective, at least in rodents, possibly due to restrictions in the TCR V regions deployed in the dominant T cell clones that mediate autoimmune diseases. Recently, it has been shown that naked DNA vaccination using DNA encoding a V gene segment relevant to mouse EAE can vaccinate against the disease.

Ergotypic TCV Partial resistance to experimental autoimmune diseases has been induced using attenuated, activated T cells lacking a specific TCR relevant to the particular disease. TCR-independent TCV seems to be induced by cell membrane factors expressed by T cells primarily in the activated state. The term *ergotype* was coined to designate the molecules associated with T cell activation that were proposed to function in the T-T interaction between effector T cells and anti-ergotypic regulatory T cells. It is possible that anti-ergotypic T cells synergize with anti-idiotypic T cells in the T-T interactions involved in TCV. Indeed, the requirement for activation of the T cells for effective TCV may be explained by the augmenting effect of the resulting anti-ergotypic response.

Mechanisms of regulation

An open question is how the anti-idiotypic and anti-ergotypic regulatory cells arrest the autoimmune disease process. Specific autoimmune T cells persist in TCV-treated animals, suggesting that effective regulation of disease does not depend on total deletion of the autoimmune effector cells. Indeed, TCV was found to suppress inflammation without inhibiting the production of autoantibodies. The divergent effects of TCV on tissue damage and antibody production may be explained by the T_H1-T_H2 dichotomy. TCV has been found to enhance a switch from a proinflammatory T_H1-type cytokine response to an anti-inflammatory T_H2-type cytokine response. Following TCV, the treated animals still respond to the target self antigen; however, instead of producing large amounts of interferon γ and interleukin 2 (IL-2), the responding T cells produce low amounts of these cytokines and high amounts of IL-4, IL-10 and

transforming growth factor β (TGF β). The shift in cytokine profile induced by TCV leads, in mice, to the production of immunoglobulin G1 (IgG1) isotype antibodies to the target self antigens. This shift has been observed both in naked DNA TCV against mouse EAE and in TCR peptide (VDJ β) vaccination against spontaneous diabetes in the NOD mouse. It remains to be determined whether TCV works by inducing existent T_H1 clones to change their cytokine output, or by activating dormant clones of T_H2 T cells to suppress the pathogenic T_H1 clones. In either case, these findings call attention to the influence of T-T interactions on the cytokine profile of the immune response.

Lymphocyte networks

Study of the physiological basis of TCV suggests that its effectiveness in regulating autoimmune disease owes much to natural lymphocyte networks that already exist before intervention by TCV. Vaccination with autoimmune T cells is effective in controlling an autoimmune disease because the anti-idiotypic regulatory T cells are present and await activation. According to this view, TCV merely strengthens a natural network; it does not initiate a new response.

The evidence for the existence of natural lymphocyte networks is discussed at length in several reviews cited at the end of this article. In brief, such anti-idiotypic networks can be observed in animals responding to particularly dominant autoantigens such as MBP, heat shock protein 60 (hsp60) and insulin. The uniformity of T cell receptor genes and the dominance of the major autoantigens is another expression of these lymphocyte networks. For example, MBP is a dominant neuroantigen because a pre-existing network of T lymphocytes anticipates MBP. The dominance of MBP is encoded in the anti-idiotypic helper T cells that drive the response of the anti-MBP idiotypes. It is quite possible that these anti-idiotypic T cells select, and thereby restrict, the T cell receptor gene products used for recognizing MBP. EAE is expressed clinically when natural anti-idiotypic regulation is inadequate to control the vigor of the anti-MBP T cells; TCV induces resistance to MBP by strengthening the regulatory networks. Likewise, TCV seems to arrest the development of diabetes in the NOD mouse by enhancing the regulation of a critical autoimmune response to hsp60.

Generalizing from the networks revealed in EAE and diabetes, it is quite possible that the limited number of autoimmune diseases and the uniformity of autoantigens recognized in these diseases is the

product of a limited number of networks that focus the immune response to selected autoantigens.

Elsewhere I have defined the lymphocyte networks responsible for the dominance of certain self antigens as an immunological homunculus, the immune system's preformed picture of the body. The homunculus operationally determines the dominance of particular self antigens and regulates the immune response to these antigens so that overt disease is rare. Disease emerges when the regulatory T cells of the network falter. TCV aborts disease by augmenting natural regulation. One expression of network regulation would seem to be the cytokine profile of the autoimmune response.

Clinical applications

Specific TCV has been used clinically in patients suffering from relapsing multiple sclerosis. The vaccines were prepared using autologous clones of attenuated T cells responsive to MBP. Similar to the results in experimental models, the patients with multiple sclerosis were found to develop anti-idiotypic T cells whose appearance was associated with a sharp decline in the frequency of anti-MBP T cells in the peripheral blood, as detected by T cell proliferation. More patients and more time will be required to learn whether TCV can influence the long-term course of disease. In any case, the immunology of TCV staged in experimental animals is reflected in clinical trials of TCV in natural human disease.

See also: Adjuvant arthritis; Experimental autoimmune encephalomyelitis (EAE); Idiotypic network; Stress proteins; Vaccines.

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THEILERIOSIS

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Theileria species are tick-transmitted intracellular protozoan parasites of domestic ruminants and wild Bovidae and the diseases they cause are collectively referred to as theileriosis. Of the several *Theileria* species infecting domestic ruminants (Table 1), the two most economically important are *T. parva* and *T. annulata*. There are approximately 250 million cattle at risk from these two parasites, which together are responsible for inhibiting livestock development in many parts of the world (Figure 1). The distribution of *T. parva* is limited to eastern,

central and southern Africa where it is predominantly transmitted by the tick *Rhipicephalus appendiculatus*. *Theileria annulata*, transmitted by several species of *Hyalomma* ticks, is more widely distributed, extending from northern Sudan and the Mediterranean countries to the Middle East, India, southern Asia and China.

Theileriosis is mainly controlled by the application of acaricides to domestic ruminants. Apart from being expensive, these chemicals are toxic and hazardous to humans and the environment. Ticks also

Table 1 Important *Theileria* species of domestic ruminants

Species	Main hosts	Vector genus	Disease names	Distribution
<i>T. parva</i>	Cattle, African buffalo ^a	<i>Rhipicephalus</i>	East Coast fever Corridor disease January disease	Africa
<i>T. annulata</i>	Cattle, water buffalo	<i>Hyalomma</i>	Mediterranean/tropical theileriosis	Africa, Asia, Europe
<i>T. mutans</i>	Cattle, African buffalo ^a	<i>Amblyomma</i>	Benign theileriosis	Africa
<i>T. taurotragi</i>	Cattle, African eland ^a	<i>Rhipicephalus</i>	Benign theileriosis Tzaneen disease	Africa
<i>T. velifera</i>	Cattle, African buffalo	<i>Amblyomma</i>	—	Africa
<i>T. sergenti/orientalis/buffeli</i> complex	Cattle	<i>Haemaphysalis</i>	—	Asia, Europe, Africa, Australia
<i>T. hirci</i>	Sheep, goats	<i>Hyalomma</i>	Malignant theileriosis	Europe, Asia, Africa
<i>T. ovis</i>	Sheep, goats	??	—	Africa
<i>T. separata</i>	Sheep	<i>Rhipicephalus</i>	—	Africa

^a Reservoir host.

rapidly develop resistance to acaricides. Therefore, alternative methods of controlling theileriosis are sought, mainly focused towards the development of vaccines.

Characteristics of *Theileria* and their antigens

Sporozoites injected during tick feeding are the infective stage; these then invade the host mononuclear

leukocytes (see Figure 2). The target cells for *T. parva* sporozoites are mainly bovine T cells, while those for *T. annulata* are B lymphocytes and macrophages. The target cell types for other *Theileria* species have not been defined. Following entry, sporozoites differentiate into multinucleate schizonts. The

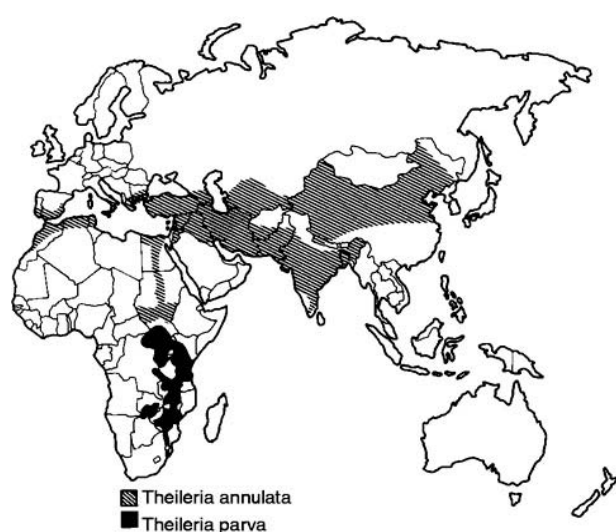


Figure 1 Distribution of the two most economically important *Theileria* species, *T. parva* and *T. annulata*. The distribution of the tick vectors is wider than these parasites and hence the potential for spread is high.

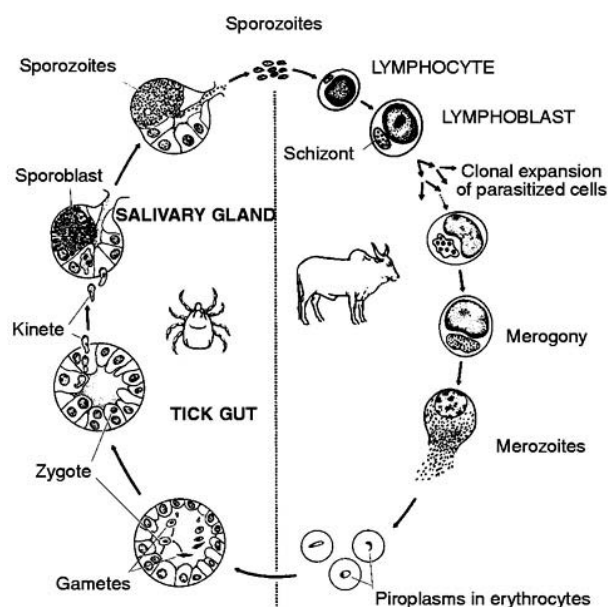


Figure 2 Life cycle of *Theileria parva*. Sexual cycle is obligatory and occurs in the tick. Transformation and clonal expansion of infected cells has only been described in *T. parva*, *T. annulata*, *T. taurotragi* and *T. hirci*. All stages except zygotes and kinetes are haploid. The transmission is trans-stadial, thus larvae and nymphs of three host ticks become infected when feeding on an infected host and the resultant nymphs and adults transmit the parasite.

schizonts of *T. parva*, *T. annulata*, *T. taurotragi* and *T. hirci* induce the host cells to transform and proliferate. Casein kinase II in the host cell and the parasite may be involved in the signaling of this proliferation. During host cell division schizonts associate closely with the mitotic spindle and divide synchronously with the cell, resulting in clonal expansion of infected cells. Between 3 and 4 days after infection, some schizonts undergo merogony to produce large numbers of merozoites. These escape following the rupture of the host cells and invade erythrocytes, where they develop into piroplasms.

Ticks become infected when taking a blood meal from infected animals. Within the ticks the parasites undergo a complicated development, including a sexual cycle, to produce motile kinetes, which migrate to the salivary glands where they undergo a process of maturation to produce infective sporozoites.

The clinical signs of theileriosis are predominantly associated with the schizont stage. Infected animals develop pyrexia and enlarged lymph nodes, coinciding with the appearance of intracellular schizonts. Infected cells rapidly spread to other lymphoid tissues causing generalized lymphadenopathy. With the emergence of host killer cells and rupture of infected cells a lymphodestructive phase follows

which results in severe leukopenia. The animals quickly lose condition, develop respiratory distress and frequently die within 10–15 days with massive pulmonary edema and severe gastroenteritis. Anemia, associated with erythrocytic merogony is an additional feature in animals infected with *T. annulata* and *T. hirci*.

A number of *Theileria*-specific antigens have been identified and characterized (Table 2). An antigen referred to as the polymorphic immunodominant molecule (PIM) of *T. parva* is present in all the stages of the parasite and varies considerably in size among the different stocks. The PIM gene shows highly conserved terminal sequences flanking a central variable region. Some antibodies specific for the PIM antigen neutralize sporozoite infectivity for lymphocytes *in vitro*. However, cattle immunized with recombinant PIM are not protected against *T. parva* challenge. The role of PIM in the induction of cell-mediated immune responses is currently being evaluated.

The 67 kDa antigen of *T. parva* is localized on the surface of sporozoites. The gene coding for this antigen has been sequenced and characterized. It is present as a single copy, has a small 29 bp intron and is highly conserved among different stocks of cattle-derived *T. parva*. Murine monoclonal antibodies

Table 2 Antigens of different *Theileria* species

Species	Molecular weight (kDa)	Method of identification	Localization	Identified function	Application
<i>T. parva</i>	104	Recovered sera/immunoblot	Sporozoites	Rhoptry associated	None
	85 (PIM)	Recovered sera/immunoblot	Sporozoites, schizonts, piroplasms	None	Diagnostic antigen for ELISA
	67	Recovered sera/immunoblot	Sporozoites	Role in sporozoite entry	Candidate vaccine antigen
	32	Recovered sera/immunoblot	Sporozoites, schizonts, piroplasms	Merogony	None
<i>T. annulata</i>	43, 12 and 4.2	T helper cells	Schizonts	None	None
	85, 72, 63, 54 family	mAb/immunoblot/immunoprecipitation	Sporozoites	Role in sporozoite entry	Candidate vaccine antigen
	99, 116, 125 family	Immunoprecipitation	Surface of schizont-infected cells	None	None
	32	mAb, immunoprecipitation	Sporozoites, schizonts, piroplasms	Merogony	Diagnostic antigen for ELISA
<i>T. mutans</i>	117	mAb	Schizonts, merozoites	Merogony	None
	32	Recovered sera/immunoblot	Piroplasms	None	Diagnostic antigen for ELISA
<i>T. sergenti/orientalis/buffeli</i>	32	Recovered sera/immunoblot	Piroplasms	None	Diagnostic antigen for ELISA

(mAb) and rat polyclonal sera raised against different stocks of *T. parva* and bovine sera from ECF-endemic areas neutralize the infectivity of sporozoites *in vitro*. The potential of this antigen as a subunit vaccine is currently being explored (see below).

In *T. annulata* a family of related proteins (85, 72, 63 and 56 kDa) referred to as SPAG1, and expressed on the surface of sporozoites, has been identified by a mAb. This mAb and bovine infection sera block sporozoite entry into leukocytes *in vitro*. The SPAG1 gene has a high degree of homology with the C-terminus of the 67 kDa gene of *T. parva*. The protective capacity of SPAG1 has yet to be fully evaluated.

Infection-specific proteins (95–120 kDa) on the surface of *T. annulata* schizont-infected cells have been identified and mAbs against these proteins mediate complement lysis of the infected cells. However, it is not clear if these proteins are parasite or host specific.

A family of 32 kDa antigens has been found in all *Theileria* species so far examined. This antigen has been more extensively characterized in *T. annulata*. It is expressed at low levels in the schizont stage but is upregulated during merogony and is localized on the surface of merozoites. The gene encoding the polypeptide has been sequenced and shows a high degree of homology with the 32 kDa merozoite genes of *T. parva*, *T. mutans*, *T. taurotragi* and *T. sergenti/orientalis/buffeli* complex. The recombinant forms of the Asian *Theileria* complex induce high levels of specific antibody in cattle but do not confer protection.

During the acute phases of infection, schizonts and piroplasms can be identified in Giemsa-stained smears of lymph nodes and blood, respectively. Immunofluorescence tests have low sensitivity and specificity. Improved antibody detection ELISAs based on recombinant antigens such as PIM for *T. parva* and p32 for other *Theileria* species are currently being validated. DNA probes and mAbs are available for the characterization of stocks and strains of *T. parva*, and polymerase chain reaction (PCR)-based tests have been developed for differentiation of *Theileria* species.

Hydroxynaphthoquinones parvaquone and buparvaquone, and halofuginone are highly effective anti-theilerial drugs but are relatively expensive and have to be administered early in the infection to be effective.

Immune responses of the host

The immune responses in cattle to *T. parva* and *T. annulata* have been more extensively characterized than for other *Theileria* species. Cattle exposed to

challenge in the field exhibit variation in severity of disease, which is largely dependent on the dose of sporozoites inoculated. Animals that recover are resistant to homologous challenge and in the absence of further challenge the immunity lasts between 3 and 5 years. Cattle immunized using live parasites (see below) may also exhibit a similar duration of immunity.

Cell-mediated immune responses play an important role in protection. The transfer of CD8⁺ lymphocytes from a *T. parva* immune calf to its naive twin protect the recipient against a homologous challenge. Parasite-specific major histocompatibility complex (MHC) class I restricted cytotoxic T cells (CTLs) directed against schizont-infected cells are the major effectors of protective responses in both *T. parva* and *T. annulata* infections.

Humoral responses directed against schizonts and piroplasms are unimportant in the generation of natural protective immunity. Sera from immune animals with high levels of schizont and piroplasm antibodies do not confer protection when passively transferred into naive cattle. In addition, animals that are immunized using lysates of schizonts or piroplasms are fully susceptible to infection with sporozoite challenge. Thus, infection with sporozoites and the development of schizonts appear to be essential for the development of natural immunity.

Evasive strategies by the organism

Although no specific evasive strategies such as antigenic variation in trypanosomes and malaria parasites are known to be deployed by *T. parva*, there are certain features of the parasite development that ensure its survival and transmission. The sporozoite stage enters very rapidly into the host cells at the tick bite site, thus evading the first line of host defense mechanisms. Following entry, sporozoites rapidly differentiate into schizonts that induce the host cells to transform, enabling the parasite to expand in numbers. Animals that ultimately recover from infection become life-long carriers, enabling successful transmission to the ticks. In some *T. parva* carrier animals, schizonts multiply in the anterior chamber of the eye, an immunologically privileged site.

Vaccines

T. annulata live vaccine

The establishment of immortalized schizont-infected cell lines in culture has been the significant breakthrough in the development of a tissue culture-based vaccine for *T. annulata*. Following several passages *in vitro* cell lines lose their virulence. Such

'attenuated' cell lines, when inoculated into cattle, establish patent infection without causing disease. They invoke protective immune responses and are now produced commercially as vaccines in Israel, India, Iran and China. The live vaccines are inoculated subcutaneously at a dose ranging between 10^6 and 10^7 infected cells per animal.

***T. parva* live vaccine**

Although *T. parva* can be attenuated *in vitro*, the tissue culture-based approach for vaccination has not been successful because schizonts of *T. parva* do not easily transfer from cell to cell and hence cattle cannot be reliably infected with attenuated cell lines. A method of immunization against *T. parva* has been developed that utilizes infection of cattle with a potentially lethal dose of sporozoites and simultaneous treatment with a long-acting oxytetracycline. This method induces a mild infection causing transient pyrexia and schizont parasitosis. Recovered animals are subsequently immune to homologous challenge. Wider adoption of this method has been slow, mainly because it has been difficult to apply in the field due to cold-chain requirement, and the immunity engendered is strain specific.

***T. parva* subunit vaccine**

A subunit vaccine based on the p67 antigen is currently under development. The recombinant form of this antigen protects approximately 70% of immunized cattle against experimental challenge. A number of studies are in progress to enhance the protective level before field testing. It is expected that a combination of antigens provoking responses directed at sporozoite and schizont stages will ultimately be used for vaccination against *T. parva*.

See also: **Cell-mediated immunity; Cytotoxic T lymphocytes; Parasites, immunity to; Ungulate immune systems.**

phocytes; Parasites, immunity to; Ungulate immune systems.

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THY-1

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The Thy-1 antigen was discovered by Reif and Allen in 1964 as a mouse alloantigen distinguishing between the AKR/J mouse strain and many other inbred strains of mice. The antigen was initially called the Θ (theta) antigen, and its alloantigenic variants were called Θ -AKR and Θ -C3H, respectively. It was shown to be expressed preferentially in the nervous tissue of the mouse and on some

lymphocytes, with a particular high level on thymocytes. When it became apparent that the AKR/Cum inbred strain possessed the Θ -C3H rather than the Θ -AKR alloantigen, and that rats possessed the Θ -AKR determinant, the name of the Θ antigen was changed into Thy-1 and its alloantigenic variants became Thy-1.1 and Thy-1.2.

In 1969 the Thy-1 antigen was shown to be

present on a distinct subset of mouse lymphocytes that became known as T lymphocytes. Schlesinger and Yron showed that the Thy-1 antigen was characteristic for those lymphocytes that disappeared from the peripheral lymphoid system upon neonatal thymectomy or following treatment with xenogeneic antilymphocyte serum, while Raff showed that the antigen was virtually absent from the lymphoid system of nude mice.

The Thy-1 antigen was subsequently demonstrated in many other species, including humans, rats, dogs, chickens, frogs and even squids and tunicates. The tissue distribution of the Thy-1 antigen in the various species is, however, somewhat different. In almost all species studied, the Thy-1 antigen is present in the nervous tissue. In mice and rats Thy-1 was shown to be absent from glial cells but was found on neurons, with the exception of the primary olfactory neurons.

On the whole, the Thy-1 antigen has proven to be a very useful reagent for the detection and selection of the T lymphocyte subpopulation among the white blood cells of mice. Thy-1 could, however, also be detected at low concentrations on pluripotent hematopoietic stem cells in mice and in rats. The antigen was expressed on progenitor cells of the erythroid, myeloid and B lymphocyte pathway, but was absent from more mature stages of development. In humans, the Thy-1 differentiation antigen (CD90) is expressed on CD34⁺ hematopoietic progenitor cells isolated either from the bone marrow or from the peripheral blood.

Biochemical analysis of Thy-1 by Williams and colleagues indicated that it is a glycoprotein with a molecular weight of 17.5 kDa and 18.7 kDa in the brain and thymus, respectively. The Thy-1 molecule contains three N-linked oligosaccharides, which constitute about one-third of the mass of the molecule. Two of the N-linked glycans consist of complex-type oligosaccharides, while the third glycan consists of high-mannose oligosaccharides. The overall structure of the Thy-1 molecule resembles that of other members of the immunoglobulin superfamily. The Thy-1 and immunoglobulin molecules are similar in the position of the disulfide bonds, and in the high content of β sheet secondary structure with no α helices. The protein moiety of the Thy-1 molecule expressed on the surface of cells was found to consist of 111 amino acids in human and rat Thy-1 and of 112 amino acids in mouse Thy-1. In contrast, Silver demonstrated that the cDNA of Thy-1 codes for a Thy-1 molecule that should consist of 141–142 amino acids. The 31 amino acids beyond the carboxyl-cysteine at position 111 were not expressed in most Thy-1 molecules. The missing stretch of amino acids contained a 26 amino acid sequence that is extremely

hydrophobic, resembling the transmembrane segment found in many other membrane proteins. This apparent discrepancy became resolved when it was shown by Low and Kincade that most of the Thy-1 molecules do not penetrate through the cell membrane but rather are attached to a glycolipid on the cell membrane. It now seems that the entire 141–142 amino acid Thy-1 molecule is synthesized in the endoplasmic reticulum of the cell, is then cleaved off in position 111, and an amide linkage is formed between the carboxyl group on cysteine 111 and an ethanolamine amino group on a glycolipid precursor. The Thy-1 molecule, attached to glycolipid by a phosphatidylinositol (GPI) link is subsequently transported to the cell surface membrane, where it displays a distinct sensitivity to cleavage by phosphatidylinositol-specific phospholipase C. Like other GPI-linked proteins, Thy-1 is localized in distinct membrane subdomains that are glycolipid enriched and detergent resistant. Immune precipitation of Thy-1 from nonionic detergent cell lysates of T cells coprecipitates the p56^{lck} and p60^{src} src family protein kinases.

What is the function of Thy-1? Although they lack a transmembrane domain, Thy-1 molecules are involved in the mitotic triggering of T lymphocytes. A brisk mitotic response can be elicited by xenogeneic antibodies against the Thy-1 antigen and by exposure to allogeneic monoclonal Thy-1 antibodies followed by xenogeneic anti-immunoglobulin antibodies. The crucial role of the Thy-1 molecule in the mitotic triggering by Thy-1 antibodies was established by the demonstration that transfection of lymphoid cells with the Thy-1 gene conferred upon them the capacity for a mitotic response to Thy-1 antibodies. Protein kinases and possibly other accessory signaling molecules which are associated with Thy-1 molecules seem to be involved in the activation of T cells by Thy-1 antibodies.

Analysis of T cell mutants that have lost the expression of T cell receptors (TCRs) and experiments on the transfection of lymphocytes with the Thy-1 gene indicate that the TCR and particularly their ζ chain, are necessary for effective mitotic stimulation of lymphocytes by Thy-1 antibodies. Indeed, stimulation via either Thy-1 or TCR may proceed through a shared signaling pathway.

Rock and colleagues showed that T lymphocytes failed to be stimulated when they were coated with Thy-1 antibody and then exposed to anti-immunoglobulin antibodies bound to plastic. In contrast, exposure of T cells to antibodies against either TCR or the CD3 antigen, followed by exposure to immobilized anti-immunoglobulin antibodies resulted in mitotic stimulation. Thus, whereas cross-linking of

the transmembrane molecular complex of the TCR suffices for mitotic stimulation, stimulation by Thy-1 may require entry of the molecules into the cell, possibly as a mechanism for communication with other components in the interior of the T cell.

In addition to regulating the mitotic triggering of T cells, Thy-1 molecules mediate intercellular attachment. Antibodies to Thy-1 inhibit the binding of mouse thymus cells to syngeneic erythrocytes, and interfere with the attachment of activated T lymphocytes to various nucleated target cells. He *et al* established that Thy-1 mediates the adhesion of thymocytes to thymic epithelial cells through a Ca^{2+} -independent mechanism. In the nervous system, Thy-1 stabilizes neuronal connections and inhibits neurite outgrowth on astrocytes. The prevention of neurite outgrowth by Thy-1 molecules may reflect their role in mediating the adhesion between various cells within the nervous system.

See also: Alloantigens; Immunoglobulin gene superfamily; Membrane-associated cytoskeleton: role in regulating immune cell function; Mouse inbred strains; Thymus; T lymphocyte activation; T lymphocytes.

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THYMIC EPITHELIUM: POTENTIAL ROLE IN REGULATORY T CELL TOLERANCE

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Two theoretical concepts on self tolerance

The most accepted view on tolerance is based on the Burnet/Lederberg theories (and developments thereof) which consider *all* autoreactivities as dangerous to the organism. *All* self reactive lymphocytes should therefore be physically eliminated (deletion) or irreversibly inactivated (anergy). Tolerance is thus established by 'default' (lack of self reactivities), that is, by 'ignorance' of what self is. For this concept, tolerance and (pathological) autoimmunity are problems which should be dealt with at the single cell or clonal level, and thus discards the notion of

interclonal regulation of lymphocyte populations and consequently the notion of system. Not surprisingly, mechanisms of tolerance based on 'suppression' or 'regulation' of self reactive cells that 'escaped' deletion have seldom been considered.

In sharp contrast, an alternative concept was developed, originally born from Jerne's network hypothesis on the *organization* of the immune system as a web of idiotypic interactions of diverse antibody populations. The key starting principle was that as a general rule of any *diverse* system of recognition, (self) recognition between components is unavoidable. In the case of the immune system of high vertebrates, the elimination of all self reactive lympho-

cytes would lead to the destruction of the system itself. Not surprisingly therefore, experimental evidence accumulated over the years indicating that functional self reactivities are rather frequent events in healthy individuals.

With these theoretical concerns in mind, a concept of T cell tolerance emerged based on observations obtained in several laboratories studying experimental systems of central (thymic) and peripheral T cell tolerance. The concept can be summarized as follows: while accepting deletional mechanisms as a way to regulate frequencies of self reactive cells, controlled autoreactivity is a natural phenomenon in physiology and, as paradoxical as it may seem, those self reactivities are fundamental for the induction and maintenance of the state of natural tolerance to organs and tissues. In other words, the immune system needs *functional* T cells, obviously committed to nondestructive activities, which should recognize at least part of the antigenic structures of the organism. The function of these cells should be the suppression or regulation of the activity of other T cells, namely those that can recognize other components of the body and capable of differentiating as aggressive effectors.

Tolerance to self is a developmental problem

In spite of the fact that many mechanisms supposed to be involved in tolerance have been identified and are currently under active investigation, the field still remains largely phenomenological. I will briefly summarize a number of observations that any theoretical concept on natural tolerance should account for, and that, in our view, the current dogma cannot bring together in a coherent scheme.

Medawar's phenomenon

In 1953, Medawar and colleagues made a fundamental demonstration in the field of transplantation tolerance: in mice, allogeneic tissue grafts (skin, heart, etc.) are accepted if an individual is exposed to donor tissues in the embryonic or perinatal life. The immune system can thus 'learn' to treat foreign tissues as 'self'. The time 'window' of tolerization for allogeneic antigens was shown to be short (less than a week) and restricted to the perinatal period of development. The immune system becomes refractory to tolerization to alloantigens after this period.

Later studies using transgenic mice for foreign antigens confirmed that tolerance operates efficiently in the embryonic/perinatal life while expression of tissue-specific antigens late in development leads to

massive tissue destruction, irrespective of the nature of antigen-presenting cells (APCs).

Hence, tolerance is a *learning* process and the immunological 'self' is composed by the set of structures that are available to immune recognition at critical developmental times.

Is antigen persistence enough to establish tolerance?

Medawar's experiments were based on the injection into newborn mice of live cells obtained from spleen and other tissues. Later in life, these animals permanently accepted skin transplants of the original donor strain.

It was subsequently shown that donor hematopoietic cells (HCs) were the cell type responsible of tolerance induction.

In contrast, adult or embryonic skin grafts failed to induce tolerance when grafted in embryos or newborn recipients. Even if grafted during embryonic life in age-matched recipients and before T cell development, the transplants which were allowed to developed normally with the host were rejected once the immune system reached full competence.

Although some cases of full tolerance or chronic/reversible rejection were described in other species (as for example extensively studied by Le Douarin and colleagues in birds), the principle that emerges from all these experiments is that allogeneic or xenogeneic tissues other than hematopoietic tissues do not tolerize to themselves.

Hence, antigen expression and persistence from early periods of development is not *per se*, in most cases, sufficient for life-long tolerance induction.

Le Douarin's phenomenon

Initially using an experimental system of chicken-quail chimeras, Le Douarin and colleagues made another fundamental demonstration in the field of tolerance: life-long tolerance is invariably obtained to a variety of embryonic tissue or organ grafts if pure thymic epithelium (TE) from the same donor is cotransplanted. In fact, the establishment of TE-induced tolerance could operate in the absence of those peripheral tissues, and TE-chimeras will accept adult tissues of TE-donor origin later in life. These observations, extensively confirmed in mice, 'broke' the dogma that in the thymus, the role of the epithelial component of the stroma was only (efficiently) ascribed to positive selection, while negative selection or tolerance was a property of the HC compartment.

Given that TE does not express the complete repertoire of antigens of the organism, an immediate

paradox of these observations is how tolerance to tissue-specific antigens is established.

Le Douarin's phenomenon can thus be phrased as follows: peripheral tissues are tolerized if TE from the same donor is functional in the recipient. Hence, tissue-specific tolerance is thymic dependent.

Developmental shift from tolerance to immunity: the time problem

Based on Medawar's window of development for tolerance induction, Burnet coherently postulated that all self reactive cells were deleted early in ontogeny, for lymphocyte production only happened during this period. This later statement was soon disproven: new lymphocytes are continuously produced throughout life. This led Lederberg to postulate that tolerance or immunity is directly related to the development of the cell: immature lymphocytes are sensitive to receptor ligation and die (tolerized by deletion or anergy), while at a later stage the cell will engage in effective immune responses upon antigen recognition. For T cells this made a great deal of sense, as they develop inside the thymus only exposed to self antigens, exogenous antigens being excluded from this organ. This new concept, however, discarded the fact that the establishment of self tolerance is restricted to the embryonic/perinatal life and, as discussed below, 'obliges' to extend even further Lederberg's 'immature window' of lymphocyte differentiation to peripheral T cells in order to cope with tissue-specific antigens.

Studies on population dynamics and repertoires of peripheral T cell pools during ontogeny revealed three additional interesting observations:

1. The peripheral T cell immune system grows exponentially by a factor of 500–1000 during the first 3–4 weeks of postnatal life; that is, the overwhelming majority of lymphocytes will be produced well after Medawar's window of tolerization is over.
2. The peripheral T cell compartment of a normal mouse is built up by accumulation of thymic emigrants, with negligible peripheral expansion and T cell lifespans of 1–2 months; that is, even if memory T cells may be capable of surviving in the organism for several months, perinatal lymphocytes will be diluted out by the exponential accumulation of new thymic emigrants.
3. Healthy adult individuals contain immunocompetent (potentially pathogenic) self reactive lymphocytes (e.g. T cells recognizing with high affinity myelin basic protein).

All these considerations led us to define one of the

central problems in the understanding of natural tolerance to tissues as the 'time' problem.

Self is only learned during development but new lymphocytes are produced throughout life and the overwhelming majority of lymphocytes are actually made after the developmental window for tolerization, that is, prone to immunity. Since lymphocyte receptor genes rearrange randomly, the adult thymus will continuously produce autoreactive T cells at a given frequency, capable of recognizing self ligands expressed only in peripheral tissues.

Since there is no way to structurally distinguish self from nonself antigens, the difference is, Medawar taught us, in the 'history' of the antigen rather than its origin (self or nonself); that is, their presence or absence in the organism during development.

Maintenance of tolerance throughout life therefore requires a state of memory of the developmental self that keeps newly-formed, immunocompetent autoreactive cells 'silent' while not interfering with the development of immune responses to foreign antigens.

What are these mechanisms, which cells are involved (and what specificities) and what dynamic properties of maintenance and renewal do they need to have?

TE-dependent selection of regulatory T cells

The thymus plays a fundamental role in the establishment of tolerance to self antigens expressed on its stroma through elimination (deletion) or inactivation (anergy) of many self reactive, differentiating T cells. The overlap between antigens expressed intrathymically and on peripheral tissues can obviously account for a major role of the thymus in tolerance induction to those antigens, without the need to invoke other mechanisms. Independent observations have shown the relative inefficiency of TE to mediate clonal deletion, and imply a differential functional capacity of TE and HCs in tolerance induction. 'Anergy' rather than deletion is the mechanism invoked in TE-induced tolerance.

In the establishment of tolerance to tissue-specific antigens, the role of the thymus is much less clear, as negative selection mechanisms of peripheral tolerance (deletion, anergy, ignorance, immunodeviation) can all dispense with thymic selection. Active suppression or 'infectious tolerance', on the other hand, can accommodate a role for the thymus in this process. Thymic-dependent specific suppression of aggressive responses to autoantigens that are not expressed intrathymically, however, is highly contro-

versal and the respective mechanisms difficult to envisage.

I shall now describe the observations obtained using a system of TE-dependent transplantation tolerance to alloantigens that led to 1) the implication of TE in the peripheral suppression of tissue-specific antigens, through the selection of a first wave of regulatory T cells; and 2) the ability of these to educate those nontolerant T cells into similar effector functions.

In mice, grafts of TE rudiments of E10 embryos, before colonization by hematopoietic cells, restore T cell development and function in allogeneic athymic mice. These TE-chimeras are specifically tolerant to grafts of peripheral tissues (e.g. skin and heart) from the TE donor strain, and tolerance in all cases is life-long. Initial analyses of the mechanisms underlying the acquired state of tolerance, based on transfer experiments, revealed that tolerant TE-chimeras harbor immunocompetent, tissue-reactive T cells that can readily reject the tolerized grafts. In support of the notion that TE-tolerance is mediated by regulatory mechanisms, CD4 T lymphocytes selected by TE were thereafter shown to specifically inhibit, in a quantitative manner, the rejection of the tolerized grafts by tissue-reactive T cells from immunocompetent donors. Because, in this experimental system, alloantigen expression is strictly limited to the TE, these results demonstrated that peripheral tolerance is centrally selected.

A number of postulates could be derived from these observations:

1. Differentiating T cells with high avidity to MHC-peptide ligands on TE are positively selected (rather than deleted), locally activated and committed to regulatory functions (rather than anergized) by the TE microenvironment.
2. Differentiating T cells positively selected on low avidity interactions with TE exit the thymus as naive, resting lymphocytes, and include the pool of tissue-specific T cells.

Recent experiments have indeed shown a dose-dependent positive selection of TCR transgenic thymocytes by 'high-avidity peptides', while deletion requires hematopoietic APCs. Furthermore, T cells selected by high avidity on TE seem refractory to proliferation upon restimulation by the selecting ligand, as in previous descriptions of 'anergy' for proliferation, that we interpret to indicate the distinct functional competence of these cells.

Figure 1 represents the theoretical selection curves describing these two postulates.

3. Most ligands recognized by TE-selected regulat-

ory T cells are ubiquitous peptides, that is, common to all tissues.

Specific activity of TE-selected regulatory T cells in the periphery requires a significant overlap between the peptide repertoires of TE and all peripheral tissues. Peripheral tissues, presenting ubiquitous peptides recognized by regulatory T cells, as well as the peptide targets for tissue-specific self-reactive T cells, would provide local conditions for the regulatory T-pathogenic T cell interactions.

Peripheral regulation of the class of T cell effector functions: the phenomenon of 'education'

T cells which were positively selected in the thymus following 'low' affinity interactions with TE cells, and passed the 'deleting HC filter', leave the thymus in a 'resting' immunocompetent state. They will constitute the vast majority of the peripheral T cell compartment, and are supposed to recirculate continuously through the body, 'chasing' possible invaders. Their lifespan in normal animals is of the order of 1–2 months and they will die after this period if they do not encounter antigen. This picture seems, however, not to be as monotonous as it looks for the notion has been that every naïve T cell reaches the periphery still in a critical state of sensitivity to TCR ligation. At this stage they are called 'recent thymic emigrants' (RTEs) as opposed to T cells that have already been in the periphery for longer. These we shall call peripheral, mature and resident (PMR) lymphocytes.

The reason to postulate this 'critical period', which could last for few hours or few days (no one knows), is directly linked to the necessity of tolerizing self reactive T cells to tissue-specific antigens not present in the thymus, and thus rescue the Lederberg concept of sensitivity to deletion/anergy of immature T cells for peripheral T cells. We know now that this is wrong: RTEs as well as mature T cells from the thymus can reject allogeneic skin transplants with the same efficiency as PMR T lymphocytes.

Interestingly, however, experimental evidence was obtained on the distinct sensitivity of RTE and PRM states to regulatory T cell-mediated suppression and 'education', thus supporting the existence of a post-thymic 'critical period' of lymphocyte functional commitment.

In TE-chimeras regulatory CD4 T cells are positively selected by the allogeneic TE, escape deletion because hematopoietic APCs express a distinct major histocompatibility complex (MHC), and can thus be produced throughout life. This contrasts with the

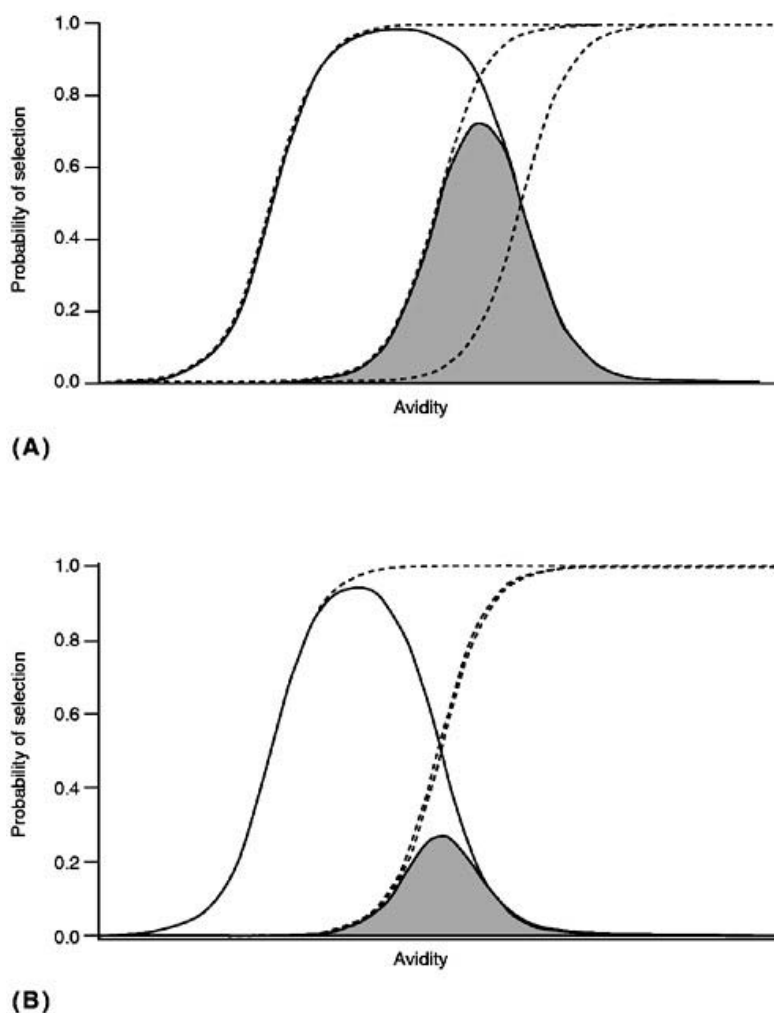


Figure 1 Thymic T cell selection is a function of the avidity of TCR-MHC-peptide interactions. For any given T cell the probabilities of positive selection, activation and deletion are described by logistic functions with thresholds A1, A2 and A3, respectively (dashed lines). For increasing levels of avidity, the products of these probabilities result in no selection, positive selection into resting state (naïve T cell), positive selection and activation (regulatory T cell) (shaded area in the plot), and deletion, respectively. The figure compares the selective properties of TE (A) and HC (B). It should be noted that T cells selected with a given phenotype (naïve or regulatory) on TE can be deleted by later interaction with thymic HC (as is the case for most regulatory cells in B, given the relative position of the deletion curves for the two types of stroma cells), but cannot be switched to another phenotype by a second interaction.

situation in normal mice, where the progressive colonization of the thymus by HCs is expected to restrict the putative generation of regulatory T cells to the perinatal period of thymic development. Transposing the observations on TE-chimeras to normal tissue-specific tolerance, other mechanisms have to be invoked. Thus, since the numbers of peripheral T cells increase by 1000-fold in the first 3–4 weeks of life by accumulation of RTEs, putative regulatory T cells produced around birth would be diluted out in the periphery by high numbers of tissue-specific T cells produced throughout life.

To answer these questions we have recently demonstrated that:

1. RTEs are more susceptible to suppression by TE-selected regulatory T cells than PRM T cells.
2. Both RTEs and PRM T cells display the ability to reject tissue grafts in the absence of TE-T cells.
3. The peripheral encounter of antigenic tissue *per se* does not tolerize RTEs, demonstrating both

the immunocompetence of cells at this differentiative stage, and immunogenic antigen-presentation by the grafted tissue.

4. RTEs are particularly sensitive to dominant tolerance mechanisms.
5. TE-selected, regulatory CD4 T cells are capable, together with specific antigen, of recruiting immunocompetent T lymphocytes into similar regulatory functions.
6. Only RTEs, not PRM T cells, seem susceptible to being 'educated' into regulatory functions.
7. Both CD4 and CD8 RTEs can be recruited into regulatory functions, each population mediating peripheral mechanisms of tolerance.

All these types of lymphocyte interactions are schematically represented in **Figure 2**.

T cells whose receptor happens to recognize tissue-specific antigens (not expressed in the thymus) will leave the thymus in a resting state, uncommitted to any effector function.

The functional pathway of differentiation of naïve

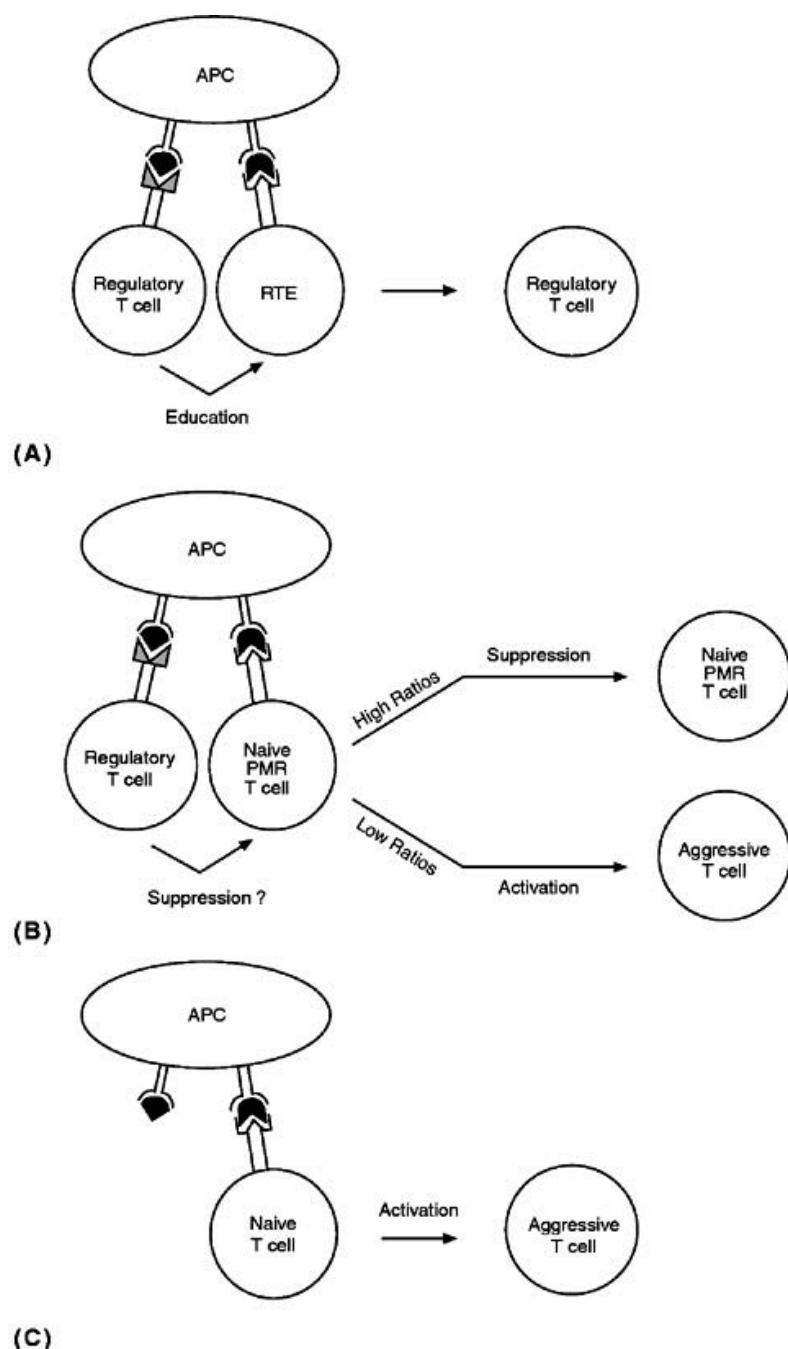


Figure 2 The three distinct fates of peripheral naive T cells recognizing specific antigen on an APC. Regulatory T cells recognize in peripheral tissues MHC-peptide complexes towards which they were selected and activated on TE (ubiquitous peptides). Naive T cells (RTEs or PMRs) respond to tissue peptides that are absent from the thymus (tissue specific). The specificities of the regulatory and naive T cells are thus unrelated. In (B), the variable 'ratio' applies to the numbers of regulatory versus PMR T cells interacting on the same APC.

T cells recognizing specific antigen on a peripheral APC ('professional' or otherwise) will be dictated by the local environmental conditions. As shown in Figure 2 three distinct situations can be envisaged.

1. The naive T cell is a RTE, and the APC it interacts with is concomitantly recognized by regulatory T cells. Under these circumstances the naive cell will be 'educated' such that it itself becomes a regulatory T cell. This is the situation that is expected to occur during the ontogenic development for self reactive cells.
2. The naive T cell is a PMR T cell, and the APC it interacts with is concomitantly recognized by regulatory T cells. In this case the outcome depends on the ratio of regulatory/PMR T cells at the surface of the APC. Thus, for high ratios, the PMR T cell is suppressed and remains naive. For low ratios, the regulatory T cells are too rare to control the naive PMR cells, which are therefore activated and become aggressive. The first case is the situation that again should occur for tissue-specific T cells that did not have the opportunity to encounter self antigen at the stage

of RTE. The second case would represent the local conditions to be set up for the development of an immune response against, for example, a viral antigen in the tissues.

3. The naïve T cell is either a RTE or a PMR T cell, and the APC it interacts with is not simultaneously recognized by regulatory T cells. In this case, the T cell is activated and displays an aggressive functional phenotype.

Finally, Figure 3 shows how the process of peripheral T cell education during development shifts the repertoire of regulatory T cells from TE selected to tissue specific.

From an artificial experimental system to normal immune systems

The transplantation system briefly described here has demonstrated that TE-induced tolerance to peripheral tissues operates in the presence of both non-tolerant and regulatory T cells with specificity to tissue antigens of the TE donor strain. Although direct evidence is still not available, these observations strongly suggest a role of TE in induction of regulatory T cell function. In turn, TE-selected regulatory T cells ensure peripheral tolerance of tissue-

specific antigens of the TE donor. The production of both aggressive and regulatory T cells by thymic selection in TE chimeras is explained on the basis of the established inefficiency of TE to mediate deletion of self reactive T cells. The regulatory activity generated in the thymus of allogeneic TE chimeras can be maintained in the periphery for long periods of time, even after decay of the initial pool of regulatory T cells.

These observations provided the basis for a strategy followed by the immune system to deal with potentially aggressive tissue-specific T cells produced throughout life, which is not based on their elimination or inactivation, but on their recruitment into the regulatory pool, thus progressively reinforcing peripheral tolerance. This strategy appears fully adapted to the T cell population dynamics in development.

A normal thymus is expected to delete (on hematopoietic APCs) most TE-selected regulatory T cells, such that their production and export must be predominantly restricted to the perinatal period, before full HC colonization.

A small number of TE-selected regulatory T cells produced early in ontogeny, can ensure the peripheral functional 'education' of all tissue-specific T

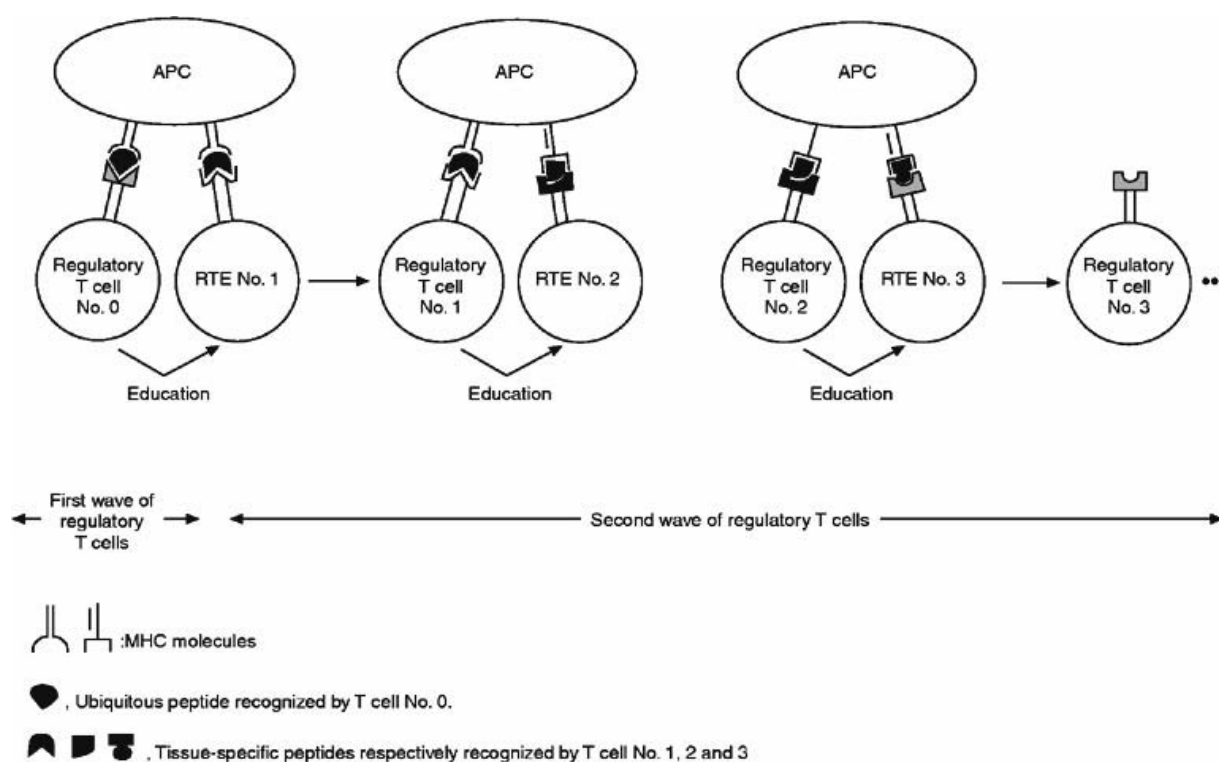


Figure 3 Diversification of the tissue-specific regulatory T cell pool and the corresponding 'repertoire spreading' of peptidic self markers by a chain reaction of peripheral RTE education. The chain is initiated by T cell No. 0, which is a regulatory T cell selected by TE, and, thus 1) specific for an ubiquitous peptide, and 2) the first to be produced and exported to the periphery.

cells, given that the whole T cell pool is postnatally built up by accumulation of RTEs. This process explains the establishment, maintenance and renewal of the peripheral pool of self-specific regulatory activities. Thus, it accounts for a fundamental aspect of natural self tolerance: in spite of the life-long production of newly-formed T cells in the thymus, acquisition of tolerance is restricted to the perinatal period, such that a developmental shift from tolerance to immunity occurs in normal animals.

The prediction is that the 'regulatory' T cell repertoire progressively shifts from TE-specific towards tissue-specific, through the local recruitment of tissue-specific RTEs by TE-selected T cells recognizing ubiquitous peptides, that can prevent destruction of a variety of tissues.

The finding that functional recruitment of RTEs requires antigen recognition also explains why newly formed T cells with specificities towards nonself antigens differentiate as naive resting cells to recruitment-resistant PRMs. It follows that antigens introduced in adult life will induce conventional immune responses that are no longer submitted to regulation, providing the basis for the developmental shift from tolerance to immunity.

A similar process of 'education' was previously described in mice tolerized to minor histocompatibility antigens, after *in vivo* treatment with nondepleting anti-CD4 and anti-CD8 monoclonal antibodies. This process, designated 'infectious tolerance', was shown to operate on CD4 T cells of thymectomized mice, hence on nonrecent thymic emigrants. This experimental system failed, however, to tolerize to major histocompatibility antigens.

Available observations suggest a major role of regulatory T cells in the establishment and maintenance of natural tolerance in normal animals. Neonatal thymectomy induces T cell-mediated, organ-specific autoimmune diseases that can be prevented by transfers of normal adult CD4 T cells, suggesting the operation of thymus-selected regulatory cells during normal development. Normal adult rats and mice contain subsets of T cells that can induce autoimmune disease if transferred into immunodeficient recipients, but also harbor other T cell subsets which confer protection from this autoaggressive T cell activity. The repeated findings of 'spontaneous' autoimmune disease in interleukin- or T cell-deficient mice is also most compatible with dominant tolerance mechanisms. Finally, Lafaille and colleagues

have recently shown that mice transgenic for an autoreactive tissue-specific TCR against myelin basic protein will only develop disease at high frequency if crossed into a RAG-1-deficient background; that is, in the complete absence of any other T or B cell population.

Concluding remarks

The alternative theoretical concept here described has major implications in therapeutic approaches to autoimmune diseases and organ transplantation, as well as in tumor immunology.

Nevertheless, I should again stress that regulatory T cells of the type described have so far not been isolated in any experimental system, and the corresponding molecular mechanisms are still largely unknown.

All the theoretical and experimental work here discussed is to be entirely shared with Antonio Coutinho and Yves Modigliani (Institut Pasteur) and Nicole Le Douarin and Josslyne Salaün (Institut & Embryologie Cellulaire et Moléculaire du CNRS et du Collège de France, Nogent-sur-Marne France).

See also: Autoimmunity; Anergy, T cell; Clonal deletion; Clonal selection; Idiotype network; T lymphocyte differentiation; T lymphocytes, autoreactive; Thymus; Tolerance, central; Tumors, immune response to; Tumors, immunological escape of; Transplantation; T cell vaccination.

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THYMIC HORMONES AND PEPTIDES

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Bone marrow-derived T cell precursors, termed prothymocytes, migrate to the thymus gland, undergo a stringent selection and a complex series of events leads them to the mature, major histocompatibility complex (MHC)-restricted, self tolerant T cell found in peripheral lymphoid organs. The thymus micro-environment represented by nonlymphoid cells (epithelial cells, macrophages, dendritic cells and fibroblasts) and extracellular matrix components, plays a central role.

In particular, the thymic cortical epithelial cells (TECs) control this sequential transformation, associated with the acquisition of antigenic markers and T cell function, in at least two distinct ways. First, in the early stages, by cell-cell interactions, the most important of which are mediated by the MHC products that are highly expressed on epithelial cell membranes. Second, in the final stages of maturation, by the secretion of a variety of polypeptides, such as thymic hormones, interleukin 1 (IL-1), -2, -3, -6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, classical hormones and neural innervation affect lymphoid development and function.

Thymic hormones and factors

Several polypeptides have been extracted, mainly from the thymus glands of young calves, and some of them have been successfully isolated and prepared synthetically (Table 1). A variety of these compounds exhibit immunobiological activity, although the precise role of many of the factors purported to have intrathymic effects is still unknown. They include thymulin, thymopoietin, thymic humoral factor (THF) and thymosins. So far, only thymulin is recognized as a hormone in accordance with the classical endocrine and physiological criteria, i.e. it is thymus restricted and regulated in its secretion. Thymectomy does not remove the presence of the other thymic factors from serum, and their genes are expressed in other nonthymic tissues. It must be remembered, however, that these criteria are under revision and, for example, in the same way, it is now realized that the lymphocytes produce serotonin and other neural peptides that once were thought to be confined to the nervous system. Other peptides may act as components of the extracellular matrix.

Another central question remaining about these

Table 1 Thymic peptides

Name	Chemical properties
Thymosin α 1 (T α 1)	Polypeptide of 28 residues. 3.1 kDa; pI 4.2. Sequence determined
Prothymosin α (ProT α)	Polypeptide of 109–111 amino acids, exhibiting T α 1 at N-terminal position. 13 kDa; pI 3.5. Sequence determined
Thymopoietin	Polypeptide of 49 residues. 5–6 kDa; pI 5.5. Sequence determined
Thymopentin (TP5)	Pentapeptide fragment of thymopoietin residues 32–36
Thymic humoral factor (THF)	Polypeptide of 31 residues. 3.2 kDa; pI 5.7. Sequence determined
THF γ 2	Octapeptide. 918 Da. Sequence determined
Thymulin (Zn-FTS)	Nonapeptide. 857 Da; pI 7.5. Sequence determined

factors is whether they only possess intrathymic activities or whether they are secreted to act on distal sites.

Thymulin

Initially called 'facteur thymique serique', thymulin was originally purified from porcine and human serum and from calf thymus. An important characteristic of thymulin is that it is biologically active only when coupled in an equimolar ratio with zinc; patients with Crohn's disease or acute lymphoblastic leukemia are zinc deficient and have a reduction in thymulin activity. The presence of an altered zinc status is also found in the elderly.

Thymulin appears to be synthesized and released by a subpopulation of TECs. It helps regulate the differentiation of an immature thymocyte subpopulation and also the function of mature T and natural killer (NK) cells by binding to a membrane receptor with high affinity. The production of thymulin is under pleiotropic control, involving the levels of thymulin itself and of other hormones such as prolactin, growth hormone (GH) through insulin-like growth factor I (IGF-I) secretion, adrenocorticotropin (ACTH), thyroxine (T₄), the opioids β -endorphin and leu-enkephalin, and cytokines IL-1 α and β .

Behavioral results support these physiological immunoneuroendocrine connections. The serum levels of this peptide decrease with age, coincidental with thymic atrophy. However, aged thymus retains the capacity to increase thymulin secretion in response to GH and T₄.

Thymic humoral factor

Trainin and colleagues isolated a crude extract of calf thymus, THF, and, by various chromatographic systems, subsequently the octapeptide THF γ 2. Both enhance lymphocyte proliferation and IL-2 production by spleen cells from neonatally thymectomized mice or from immunocompromised patient's lymphocytes.

However, THF γ 2 was not detected in TECs grown in culture, and its serum levels are not reduced by thymic involution or thymectomy.

Thymopoietin

G Goldstein and coworkers isolated thymopoietin from calf thymus by its neuromuscular effect. Later it was found that thymopoietin binds cell membrane receptors present on prothymocytes and on mature T cells and certain T cell lines, involving, respectively, stimulation of adenylate or guanylate cyclase.

Although immunoreactive material was detected in human TEC cultures, thymopoietin mRNA is present in primary and secondary lymphatic tissues as well as other sites, such as striated muscle, heart, small intestine and cerebellum, and is particularly abundant in isolated thymocytes. Moreover, serum levels of thymopoietin are not reduced by thymic involution or thymectomy, and it is localized in the cells intracellularly and no secretagogue has been reported, thus indicating a nonendocrine role.

Three distinct human thymopoietin mRNAs have been described with distinct structural domains and functional motifs, which suggests that the proteins have unique functions and may be directed to distinct subcellular localizations. A nuclear role has been suggested.

Thymopoietin from calf thymus enhances early T cell differentiation and the expression of specific alloantigens *in vitro*, perhaps by the same mechanism as the pentapeptide composed of residues 32–36, called thymopentin (TP5), which also has distinct biological properties.

Thymosins

Thymosins are a group of low molecular weight acidic peptides isolated from the original factor thymosin fraction 5 (TF5) extracted from the bovine

thymus by AL Goldstein and colleagues. At least 30 different peptides can be identified by isoelectric focusing of TF5, including thymosin α 1 (T α 1), β 4 (T β 4) and MB35. TF5 displays potent stimulatory effects on T cell-mediated immunity, and may be involved in maintaining the size of the thymus and of thymocyte subpopulations. T α 1 induces the expression of the T cell differentiation antigens Thy-1, CD5 and CD8 and increases murine lymphocyte mitogenic responses, antibody and cytokine production. T β 4, as T α 1, increases terminal deoxynucleotidyltransferase (TdT) expression. In addition, multiple effects of these preparations on neuroendocrine function have been identified. For instance, T β 4 is able to stimulate luteinizing hormone (LH) secretion from pituitary and LH-releasing hormone from the hypothalamus, MB35 stimulates the production of prolactin and GH, and T α 1 downregulates thyroid-stimulating hormone (TSH), ACTH and prolactin secretion, at least partially, through hypothalamic pathways.

A full-length cDNA of 1200 bp for T α 1 has been cloned and studied by Eschenfeldt and colleagues. This cDNA encodes a highly acidic protein of 111 amino acids called prothymosin α (ProT α), which contains T α 1 in positions 1–28. ProT α has no N-terminal signal peptide, and its mRNA is exclusively located on free polysomes. The ProT α gene belongs to a family of six members, one of which gives rise to two mRNAs. Sequence homologies with other mammalian species are high; it is found in multiple tissues and has a nuclear targeting sequence. T α 1 has been localized intracellularly in dense vacuoles within the cytoplasm of TECs but not in the endoplasmic reticulum or Golgi apparatus. These findings suggest a nuclear function for ProT α (which is most likely the active compound) in the regulation of cell proliferation, and T α 1 is probably a decay product, because a small constitutive production has been observed, although no secretagogue has been identified.

T α 1 has sequence homologies with the vasoactive intestinal peptide (VIP) family and it is able to function as a VIP receptor antagonist with weak intrinsic activity. Recently, cell surface receptors for ProT α were found and, while not yet fully characterized, they appear to be different to those for T α 1. ProT α also has immunopotentiating activities.

T β 4 is identical to Fx, an actin-sequestering peptide which is present in many different cell types. The sequence of MB35 is identical to a portion of histone H2A, a component of nuclear protein A24.

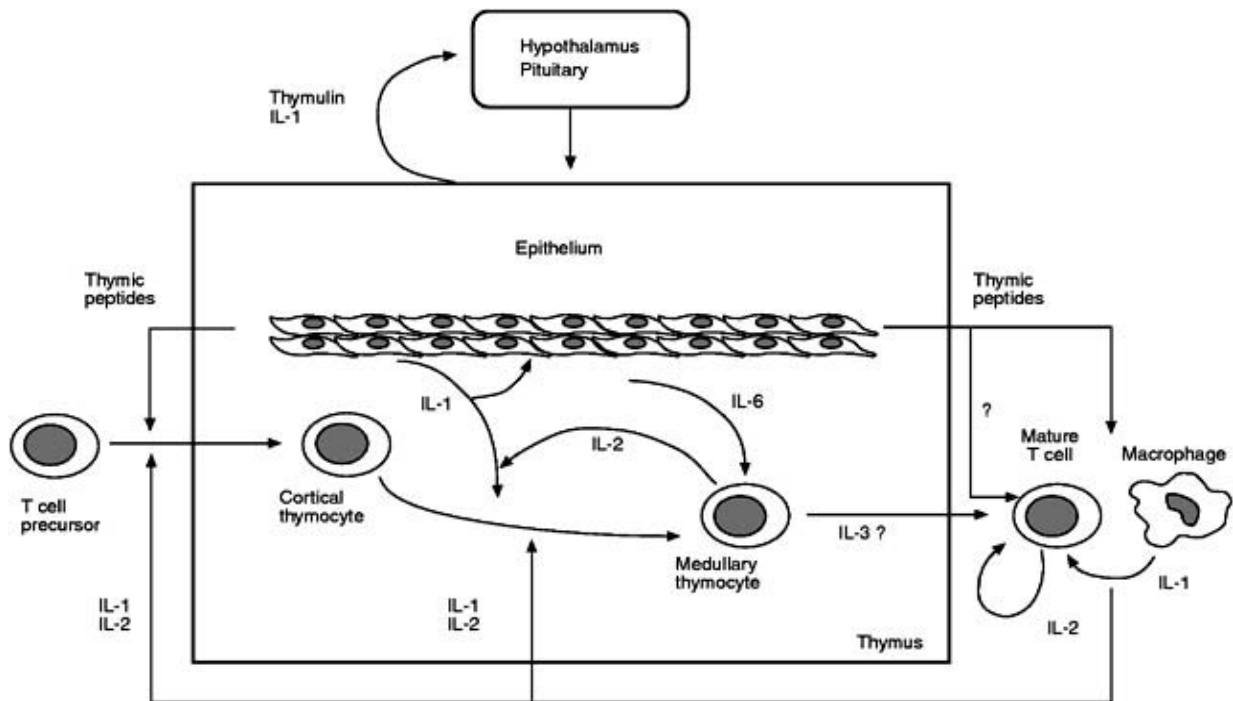


Figure 1 Thymic epithelial cells, interleukins and proposed thymic peptide functions. Thymic peptides of epithelial or lymphoid origin may act on the enhancement of interleukin-dependent intra- or extrathymic differentiation of T lymphocytes.

Thymic peptides as immunoregulators or biological response modifiers

As mentioned above, only thymulin can today be described as a classical hormone. Nevertheless, further investigations are required regarding the physiological relevance of the numerous data showing immunological effects of thymic peptides. 1) Between 400 and 800 pg/ml T α 1 (or ProT α , this fact remains to be elucidated), 300–500 ng/ml thymopoietin and 0.5–3 pg/ml thymulin are present in human plasma. THF has also been detected. Except for thymulin, these levels are not reduced by thymic involution, but T α 1 levels are decreased or augmented in some groups of sick patients or monkeys. 2) Thymic peptides, except for THF, were secreted specifically by some epithelial cells in the thymic stroma. 3) They bind to different receptors on T cells and other cells and tissues. So while some investigators believe that thymic peptides are a result of cellular damage or death, others have postulated that certain nucleoproteins may be targeted by proteases during cellular activation to produce 'nucleokines' that in turn migrate to the cytoplasm and may be secreted.

Although the discovery and the first studies on thymic peptides (TPs) are historically linked with their role in the developmental events of T lymphocytes within the thymus, more recent investigations have

focused upon the effects of TPs on mature effector cells (Figure 1). TPs have been extensively explored as potential regulators of T cell deficits which would allow specific immunotherapeutical approaches of clinical utility. Thus, whereas the involvement of TPs in the induction of markers of T cell differentiation is of minor significance compared with the role of cytokines, TPs appear to play a potentially important role in stimulating various T cell functions in normal and in partly T cell deficient rats, mice and humans. In general, TPs tend to normalize the immune balance, either by enhancing suppressed systems or by stimulating suppressor mechanisms in hyperresponsive states. Thus, TPs may act, outside of the thymus, as fine physiological immunoregulators contributing to the maintenance of T cell subset homeostasis.

In vitro effects

Thymulin restores basal NK activity in spleen cell cultures obtained from old, but not young mice. It increases the release of interferon γ (IFN γ), IL-2 and IL-1 α by mitogen-stimulated cells of normal individuals, whereas it inhibits the release of these cytokines and also of IL-6 in systemic lupus erythematosus (SLE) patients. Inhibition of tumor necrosis factor α (TNF α) production was found in both groups. Thus, thymulin may function as an immunomodulator by exerting control over cytokine production.

Peripheral blood mononuclear cells obtained from patients with chronic hepatitis B virus infection have an increased production of IL-2 and TNF α in response to THF γ 2, suggesting an antiviral role for this peptide *in vivo*. An effect on hematopoietic progenitor cell proliferation was recently documented in which THF γ 2 increased the number of myeloid colonies of bone marrow cells from normal mice. Human erythroid and myeloid hematopoietic progenitor cells are also stimulated to growth by THF γ 2.

Thymopentin (TP5), the synthetic pentapeptide corresponding to the active structure of thymopoietin, has shown impressive immunoregulatory activity. TP5 modulates T_H1 and T_H2 cytokine response; it enhances IFN γ , TNF α and IL-2 release and reduces the levels of IL-4 in different models. In addition, bone marrow T lymphocyte precursors treated with TP5 show increased thymus colonization.

T α 1 and ProT α increase the number of high-affinity IL-2 receptors, IL-2 production and IL-2/IL-2R internalization in stimulated lymphocytes. They enhance T lymphocyte proliferative responses and improve NK- and LAK-cell activity of normal and cancer patients. Moreover, ProT α enhances MHC class II expression and mRNA accumulation in human monocytes, murine splenocytes and tumor cell lines.

In vivo effects

Thymulin and thymopentin restore antibody avidity in aged or thymectomized animals, enhance antibody production in aging mice, stimulate the male-to-female skin graft rejection, the prolongation of skin graft survival and the abrogation of delayed-type hypersensitivity. There are positive correlations between zinc levels, active thymulin and peripheral immunological parameters in young cancer patients. Additionally, thymulin reduces induced hyperalgesia in rats and mice.

TP5 reduces the susceptibility of aged mice to cutaneous leishmaniasis and modulates cytokine production by splenocytes from aged mice.

THF γ 2 injections in neonatally thymectomized mice restore myeloid colony formation. THF γ 2 reduces the metastatic load and restores immunocompetence in 3LL tumor-bearing mice receiving anticancer chemotherapy, raises the T cell activities in immunodeficient aging mice, and enhances the responsiveness of both CD4⁺ and CD8⁺ T cells to murine cytomegalovirus infection.

T α 1 and ProT α protect mice against opportunistic infection by *Candida albicans*, increase NK- and LAK-cell activity in tumor-bearing mice and stimu-

late the responsiveness to IL-2 in immunosuppressed mice. T α 1 enhances the fertilizing capacity of human sperm. It can be used in combination with an antiviral drug in controlling influenza virus. ProT α increases the production and release of migration inhibitory factor by mononuclear cells.

Clinical research

TPs have been used clinically as antiviral agents and in the immunotherapy of cancer. TP5 is a highly effective drug as an antiviral therapy in recurrent herpes simplex, herpes zoster and human papilloma virus infection, reducing the relapse rate. It has also been shown to be a safe and effective adjunct to therapy in patients with severe atopic dermatitis, in which it decreases the release of polymorphonuclear leukocyte-derived inflammatory mediators. TP5 is able to produce consistent clinical and immunological effects in melanoma patients with cutaneous metastases, and is a potentially useful agent in the treatment of a subgroup of patients with Sézary syndrome.

Combination therapies with T α 1 are used in infections and tumors, for example, AZT, T α 1 and IFN α in HIV patients. T α 1 is being used in clinical trials of head and neck cancer, advanced non-small-cell lung cancer and metastatic melanoma. T α 1 is being used in hepatitis B and C trials worldwide. Other thymic preparations such as thymostimulin (TP1) are used in combination therapies, for example in metastatic colon cancer.

See also: Aging and the immune system; Immune response; Immunopotentiality; Immunotherapy of tumors; Neuroendocrine regulation of immunity; Thymus; T lymphocyte differentiation; T lymphocytes; Zinc and the immune system.

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THYMUS

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The thymus is a bilobed structure situated above the heart in the anterior mediastinum. This organ is conspicuously large in young life and undergoes atrophy at the time of puberty. The thymus is responsible for the production and differentiation of T lymphocytes and is thus essential for cellular immunity.

History

Until 1960 the function of the thymus was obscure. In fact even in the 1950s it was not appreciated that the thymus is normally a very large organ in infancy. With the availability of chest X-rays, pediatricians were impressed with the size of the thymus and invoked the condition of ‘status thymolymphaticus’ – chronic strangulation by an enlarged thymus – to explain minor respiratory ailments. To relieve ‘pressure’ on the trachea, the thymus was exposed to irradiation! Despite such bizarre reasoning, as early as 1900 Beard, working on the stingray, had proposed that the thymus is the principal source of ‘leucocytes’. This prescient suggestion – forgotten for the next 60 years – presaged the crucial finding of Miller in 1961 that removing the thymus from neonatal mice induced a wasting syndrome associated with impaired cellular immunity. Miller’s conclusion that the thymus is the source of ‘immunologically competent cells’ and that ‘lymphocytes leaving the thymus are specially selected cells’ began the modern age of thymology.

Thymic architecture

In sections, the thymus is divided into two zones: the cortex and medulla (**Figure 1**). The cortex is the dominant zone in healthy young animals and accounts for about 80% of thymic tissue. This region is packed with small lymphocytes and has a characteristic dark appearance in sections stained with hematoxylin, a reflection of the high nuclear-to-cytoplasmic ratio of cortical lymphocytes (**Figure 1A**). The vast majority of lymphoid cells in the cortex are immature thymocytes expressing high levels of CD4 and CD8 molecules. These ‘double-positive’ (DP) cells express low levels of the $\alpha\beta$ T cell receptor (TCR). Special stains reveal that DP thymocytes reside within a rich network of epithelial cells expressing major histocompatibility complex (MHC) molecules (**Figure 1B**). As discussed later, MHC expression on cortical epithelium plays a crucial role in positive selection, the process by which a proportion of DP cells are instructed to differentiate into mature T cells.

The medullary region of the thymus contains mature T cells. These cells comprise a mixture of ‘single-positive’ (SP) CD4⁺8[−] and CD4[−]8⁺ cells expressing the $\alpha\beta$ TCR. SP cells are intermingled with antigen-presenting cells, such as dendritic cells and macrophages. Like DP cells in the cortex, SP cells are enmeshed in a dense network of epithelial cells (**Figure 1B**). The typical pale appearance of the medulla in hematoxylin-stained sections probably reflects the greater density of epithelial cells in the medulla than the cortex. In humans, medullary epi-

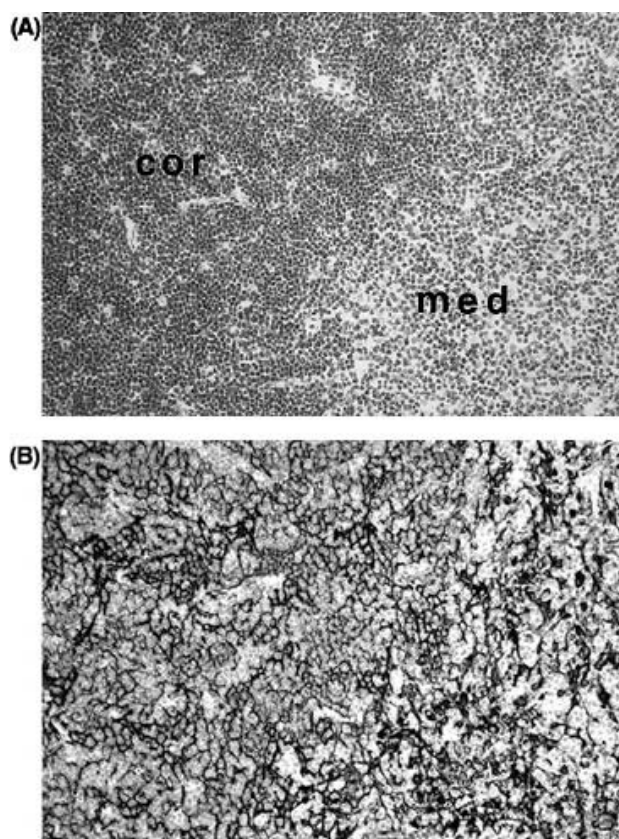


Figure 1 Histology of the mouse thymus. (A) Frozen thymus section stained with hematoxylin, a dye which stains the nucleus. Two zones of distinct cell density are visible: the cortex (cor) is densely packed with small lymphocytes, whereas the medulla (med) contains larger lymphocytes at a lower density. (B) A serial frozen thymus section stained with an antibody specific for epithelial cells and counterstained with hematoxylin. Note that a dense network of epithelial cells is apparent throughout the thymus: the epithelial cells in the cortex are dendritic in morphology with a thin cytoplasm, whereas the epithelial cells in the medulla show a more heterogeneous morphology.

thelial cells form cystic structures termed Hassal's corpuscles; these structures are inconspicuous in mice.

In addition to $\alpha\beta$ T cells, the thymus contains small numbers of T cells expressing $\gamma\delta$ TCRs. These cells are a minority population (<1% of total thymocytes) and are found scattered throughout the cortex and medulla.

Thymocytes, especially DP cells, are highly sensitive to the action of glucocorticoids and other steroids such as sex hormones. Because of this sensitivity to steroids, the thymus undergoes atrophy after the onset of puberty. Thymic atrophy is usually marked by mid-life, although remnants of thymic tissue are generally apparent even in old age. Thymic atrophy is also prominent in diseases associated with stress, e.g. chronic infection. With acute stress, thymic atrophy preferentially affects the cortex.

Surface markers on thymocytes

Although epithelial cells are a major component of the thymus in sections, thymic cell suspensions consist almost entirely of lymphoid cells. On the basis of CD4 and CD8 expression, thymocytes comprise four broad subsets of cells. The bulk of thymocytes (80–85%) consist of immature cortical CD4⁺8⁺ cells expressing variable levels of the $\alpha\beta$ TCR; most of these cells are small resting lymphocytes but 10–20% are blast cells. Mature SP cells account for about 15% of thymocytes and comprise a 2:1 ratio of resting CD4⁺8⁺ and CD4⁺8⁺ cells; most (though not all) of these cells display high levels of the $\alpha\beta$ TCR. The remaining cells (1–3%) are CD4⁺8⁺ and comprise a heterogeneous collection of cells, most of which act as immature precursors of CD4⁺8⁺ cells.

Effects of thymectomy

In some species, e.g. humans and sheep, the thymus develops early in ontogeny and releases substantial numbers of T cells before birth. In these species removing the thymus at birth causes only a mild decline in T cell numbers by the time of maturity. In other species, e.g. mice, the thymus develops late in ontogeny and does not produce mature T cells until just before birth. Neonatal thymectomy of mice thus precludes the formation of peripheral T cells and leads to a long-lasting state of profound T cell deficiency. Unless maintained in a pathogen-free environment, neonatally thymectomized mice fail to thrive and develop chronic infections associated with marked weight loss (runting) and death. This syndrome can be prevented by injecting the hosts with mature T cells taken from normal syngenic donors.

Ontogeny of thymic development

The epithelial components of the thymus descend into the anterior mediastinum from the third and fourth pharyngeal pouches and become colonized by stem cells derived from the yolk sac and fetal liver (depending on the species concerned). After proliferation and differentiation, these stem cells give rise to CD4⁺8⁺ cortical cells and then to CD4⁺8⁺ and CD4⁺8⁺ cells. In mice, mature SP cells are first apparent at about day 18 of gestation, i.e. at 2–3 days before birth. In humans, mature T cells begin to be produced at the end of the first trimester of pregnancy.

T cell differentiation and turnover in the mature thymus

In contrast to the peripheral lymphoid tissues, the vast majority of thymocytes turn over at a very rapid rate. Thymocyte differentiation begins with a population of stem cells which seed the thymus continuously from the bone marrow; these cells appear to be pluripotent but rapidly become committed to the T cell lineage.

The earliest stem cells in the thymus are characterized by a $CD4^{lo}8^{-} CD44^{+} CD25^{-}$ (IL-2R $^{-}$) phenotype. These cells proliferate extensively and give rise to $CD4^{-}8^{-} CD44^{+} CD25^{+}$ cells and then to $CD4^{-}8^{-} CD44^{-} CD25^{+}$ cells (Figure 2). During this transition, the cells cease to divide and then rearrange the TCR β gene and begin to upregulate both CD4 and CD8 molecules. The TCR β chain associates with a non-polymorphic α chain (gp33, pre-TCR α) and this heterodimer initiates a new wave of proliferation, perhaps by binding to a ligand on stromal cells. Proliferation is extensive at this stage and leads to the production of early $CD4^{+}8^{+}$ blast cells. Through rearrangement of polymorphic α chain genes, $CD4^{+}8^{+}$ blast cells rapidly express the typical $\alpha\beta$ TCR on the cell surface but at a low level. Proliferation ceases soon after $\alpha\beta$ TCR expression and the cells accumulate as typical small cortical $CD4^{+}8^{+} \alpha\beta TCR^{lo}$ cells; these cells are $CD44^{-} CD25^{-}$. The majority of small $CD4^{+}8^{+}$ cells survive for only a brief period (3–4 days) and die *in situ* from apoptosis. Only a small proportion of these cells undergo positive selection (see below).

The mature $\alpha\beta TCR^{hi} CD4^{+}8^{+}$ and $CD4^{-}8^{+}$ cells in the medulla are the direct descendants of $CD4^{+}8^{+}$

cells and display a relatively slow turnover. For obscure reasons, these cells remain in the medulla for up to 1–2 weeks before being released to the extrathymic environment.

Positive selection

Positive selection is a reflection of the fact that the specificity of $\alpha\beta$ T cells is not directed to native antigen but to peptides bound to MHC molecules. Since MHC molecules display extensive polymorphism, the range of peptides that associate with MHC molecules can vary considerably from one individual to another. Because of MHC polymorphism, the specificity of the T cell repertoire is unique in each individual – except in identical twins or in congenic strains of mice – and is tailored to give optimal immune responses to a broad array of foreign antigens (peptides) presented in association with 'self' MHC molecules. To prevent autoimmunity, however, mature T cells have to be rendered tolerant to peptides derived from autologous proteins. The T cell repertoire thus has to be finely tuned to be unresponsive to self (autologous antigens) but strongly reactive to slight perturbations of self (foreign antigens). This specificity is shaped in the thymus by a combination of positive and negative selection (Figure 3).

Positive selection is directed to self peptides bound to MHC class I and class II molecules expressed on cortical epithelial cells. $CD4^{+}8^{+} \alpha\beta TCR^{lo}$ cells with binding specificity for these peptide/MHC complexes receive a low-level signal which induces the cells to survive and differentiate into mature SP cells. The delivery of this survival/differentiation signal is

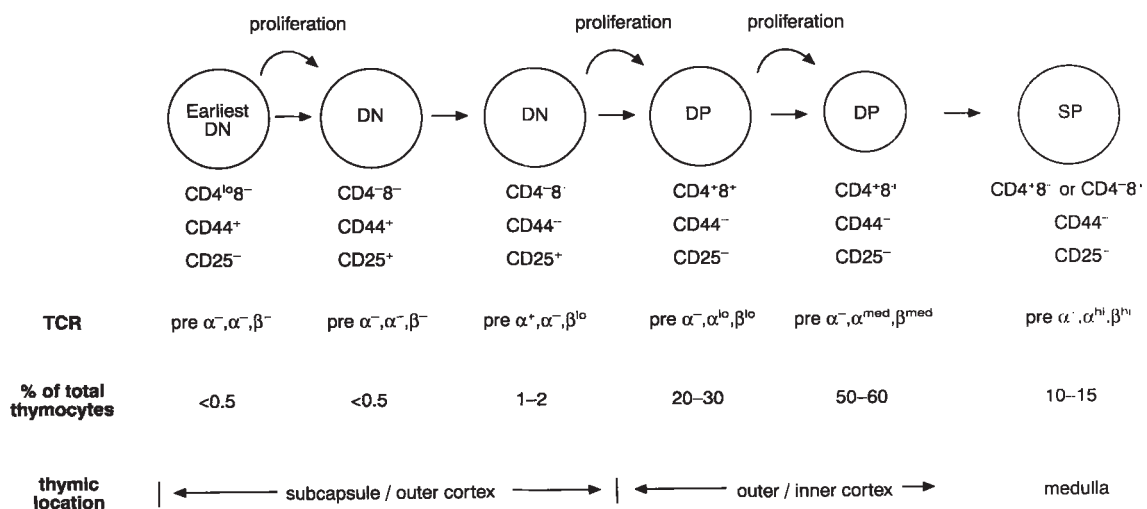


Figure 2 Schematic diagram of T cell developmental stages in the mouse thymus. Immature T cells undergo various maturation steps which can be followed by the expression of the cell surface markers listed in the diagram. Note the relative cell size at different stages and the transition steps which are associated with significant cell proliferation.

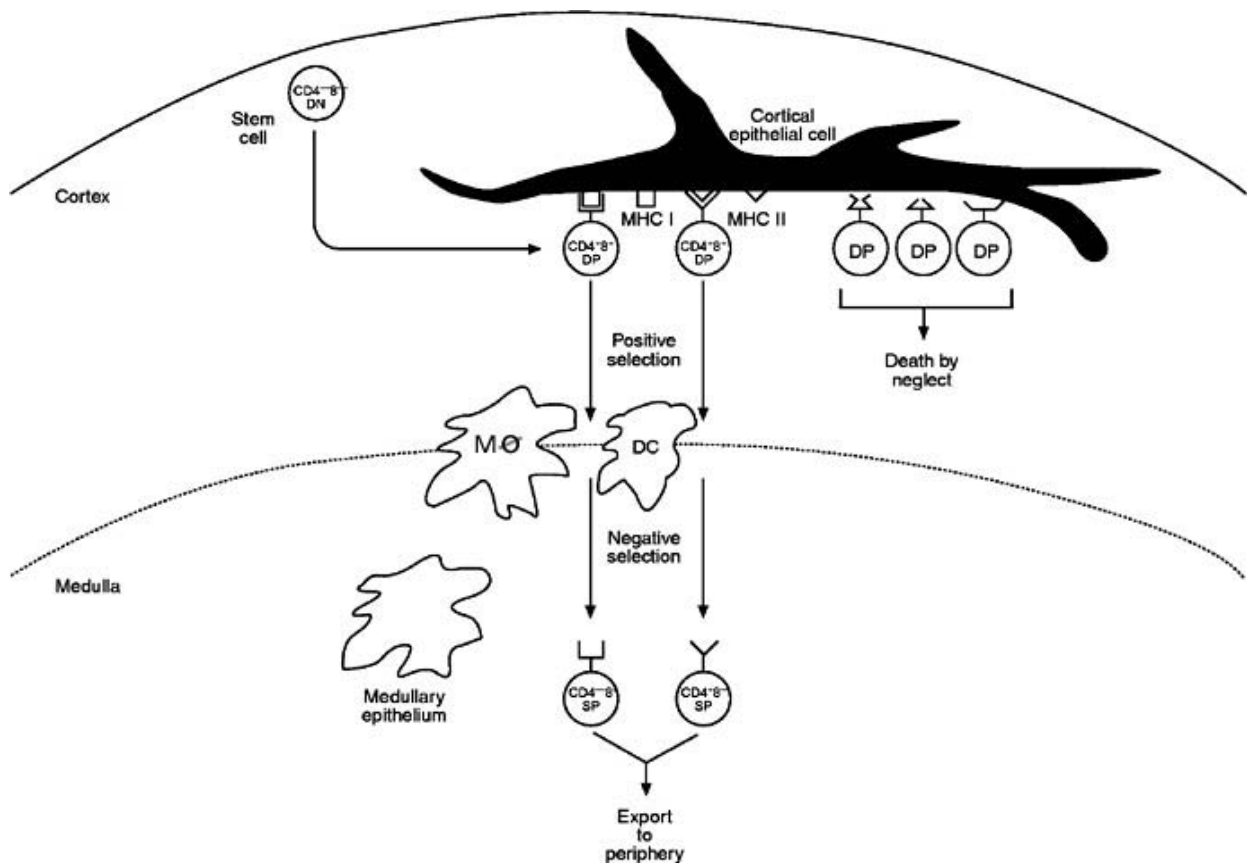


Figure 3 Positive and negative selection in the thymus. Immature CD4⁺8⁻ 'double-negative' (DN) cells under the capsule proliferate and differentiate into CD4⁺8⁺ 'double-positive' (DP) cells expressing a low density of the $\alpha\beta$ T cell receptor (TCR). A small fraction of DP cells express TCRs that have low but significant binding affinity for peptides bound to self MHC class I and II molecules displayed on cortical epithelial cells. These DP cells undergo positive selection. This process rescues DP cells from death and results in the cells migrating into the medulla and maturing into either CD4⁺8⁻ or CD4⁺8⁺ 'single-positive' (SP) cells. In the medulla, SP cells encounter antigen-presenting cells in the medulla, such as dendritic cells (DC) and macrophages (MΦ). These cells display MHC-bound self antigens entering from the bloodstream, and any T cells with overt reactivity for these antigens are deleted *in situ* (negative selection). As the result of positive and negative selection, only a very small proportion of total thymocytes (1–2%) are exported from the thymus; the remainder of the cells die within the thymus.

mediated by the $\alpha\beta$ TCR and is aided by CD8 or CD4 molecules. These two molecules act as coreceptors by binding to nonpolymorphic epitopes on MHC class I and class II molecules, respectively. Thus, if the T cell is recognizing a peptide bound to class I, this binding is aided by CD8 molecules. Such binding stabilizes CD8 expression but leads to down-regulation of CD4, with the result that the cell differentiates into a mature CD4⁺8⁺ cell. Conversely, positive selection to peptide/class II complexes is aided by the CD4 coreceptor and leads to differentiation into CD4⁺8⁻ cells.

The range of self peptides controlling positive selection is still unclear, but weak (antagonist) peptides seem to be particularly important. Most of these peptides are probably derived through breakdown of various intracellular proteins. Antagonist peptides may be particularly suited to provide the

weak signaling required for positive selection. This level of signaling is sufficient to induce differentiation but fails to cause cell division. As discussed later, strong TCR signaling of CD4⁺8⁺ cells is injurious and leads to rapid death (negative selection).

Positive selection involves only a very small fraction of CD4⁺8⁺ cells, i.e. 2–4%. The vast majority of DP thymocytes (>95%) have no binding specificity for the particular self MHC molecules expressed in the thymus and these cells die rapidly from 'neglect'. Death of these cells involves apoptosis, and special staining techniques reveal that apoptotic cells are rapidly cleared by adjacent macrophages. At face value the fact that the vast majority of thymocytes are doomed to die *in situ* seems wasteful and inefficient. However, this widescale destruction of immature T cells reflects the need to select only those cells that are equipped to function optimally in the

extrathymic environment, i.e. to respond to foreign peptides presented by the particular self MHC molecules of the individual. The TCR repertoire of CD4⁺8⁺ cells is presumed to be very broad and to encompass reactivity for all of the different MHC molecules expressed in the species as a whole. In any one individual, however, the range of MHC molecules is very small, with the result that specificity for these self MHC molecules is limited to a tiny fraction of DP cells. The fact that only a small number of DP cells undergo positive selection can thus be viewed as a reflection of the extent of MHC polymorphism.

Negative selection

Negative selection is designed to deplete the T cell repertoire of cells with overt reactivity for self components. Elimination of these cells is essential for self tolerance induction and the prevention of autoimmune disease. Since immature T cells cannot withstand strong TCR signaling, exposure to self antigens in the thymus deletes T cells with strong reactivity for these antigens via apoptosis. Since negative selection requires more intense TCR signaling than positive selection, negative selection is probably directed largely to agonist rather than antagonist peptides, i.e. to peptides that are immunogenic for mature T cells.

The site of negative selection in the thymus is controversial. Since most self components are conserved and expressed by all cell types, the majority of the self peptides that lead to negative selection are presumably expressed in the cortex, e.g. on epithelial cells. Indeed, negative selection in the cortex is apparent in models utilizing TCR transgenic mice. Whether negative selection occurs in the cortex under normal physiological conditions, however, is still unclear. By contrast, the medullary region of the thymus is a well-defined site for negative selection. In contrast to the cortex, the medulla is permeable to bloodborne proteins and is packed with bone marrow-derived cells with antigen-presenting function. The 'open' structure of the medulla means that any self antigens present in the bloodstream will be carried into the thymus and thus be positioned to tolerate immature T cells specific for these antigens. Conversely, self antigens that fail to enter the bloodstream, i.e. tissue-specific antigens, do not reach the thymus and thus do not induce negative selection. In this respect it is well accepted that the T cell repertoire retains reactivity for a variety of tissue-specific self antigens, e.g. myelin basic protein. When exposed to the immune system in immunogenic form, these antigens break tolerance and lead to

autoimmune disease in the tissue concerned, e.g. in the brain for myelin basic protein.

It should be emphasized that negative selection is quantitative and reflects the concentration of antigen reaching the thymus. In the case of abundant antigens such as albumin, the concentration of antigen in the thymus is high and deletes T cells with a spectrum of affinities for the antigen, including low-affinity cells. For this reason breaking self tolerance to albumin at the T cell level is difficult or impossible. Other self antigens, e.g. hormones such as insulin, reach the thymus in much lower concentrations. In this situation negative selection is incomplete: high-affinity T cells are deleted but low-affinity cells are spared. These latter cells are generally innocuous because the concentration of antigen encountered in the periphery is no higher than in the thymus. Under experimental conditions, however, self tolerance to these antigens can be broken, i.e. by injecting the antigen in very high concentrations in immunogenic form.

T cell outputs from the thymus

Because of the combined requirements for positive and negative selection, the total numbers of mature thymocytes exported to the peripheral lymphoid tissues is very limited. The vast majority of these cells are mature SP cells; DP cells are not exported. At present, quantitative information on the number of T cells released from the thymus is limited to mice. In this species, the rate of T cell export from the thymus amounts to about 1% of the total cellularity of the thymus. Thus, the large thymus of 4-week-old mice, which contains about 2×10^8 cells, releases in the order of 2×10^6 cells day⁻¹ into the periphery. With the onset of thymic atrophy, thymic outputs drop dramatically. Thus, by 6 months of age the release of T cells from the thymus is reduced to about 1×10^5 day⁻¹. Since small amounts of thymic tissue are detectable even in old age, production of new T cells from the thymus probably continues throughout life, but at a very limited rate.

Extrathymic sites of T cell production

Since the number of mature T cells in the secondary lymphoid organs remains high throughout life, it is often argued that maintenance of the T cell pool after the stage of thymic atrophy reflects *de novo* production of T cells in other organs, e.g. the gut, liver and bone marrow. Although some degree of T cell differentiation can occur in these organs, there is currently no clear evidence that such differentiation contributes to the formation of the typical $\alpha\beta$ T cells

found in the blood, spleen and lymph nodes. The most likely explanation for the maintenance of the T cell pool size into old age is that T cells are intrinsically long lived. The thymus is vital for the initial formation of the T cell pool, but once the pool reaches its maximal size in young adult life generation of new T cells by the thymus becomes relatively unimportant. By puberty the thymus is largely redundant and atrophy ensues.

See also: Apoptosis; Thymic epithelium: potential role in regulatory T cell tolerance; T cell receptor, $\alpha\beta$; Thymic hormones and peptides; T lymphocyte differentiation.

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THYROID AUTOIMMUNITY, ANIMAL MODELS

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That autoimmunity is a major cause of thyroid disease has been evident for over 35 years, but many critical questions about the immunopathogenesis remain unanswered. This entry focuses on the prototypic autoimmune thyroid disease, chronic thyroiditis or Hashimoto disease, and aims to answer these questions by reference to experimental models. The questions pertain primarily to the molecular and genetic basis of autosensitization, to the principal thyroid antigens and the immunological characteristics of pathogenetic autoimmunity.

Two types of models are available for study in the research laboratory: spontaneous and experimentally induced thyroiditis. Injection of thyroglobulin, usually with an adjuvant, induces thyroiditis in a variety of animals including rabbits, rats, mice, guinea pigs, dogs, rhesus monkeys and chickens. This method has the advantages of dealing with a well-characterized

antigen and beginning at a defined point in time and, therefore, lends itself to precise genetic and molecular investigations. Spontaneous models of thyroiditis can be found in the OS chickens, the BUF and BB strains of rats, NOD mice and the Argonne Laboratory colony of beagle dogs. Thyroiditis and a number of other organ-specific autoimmune diseases can be produced by carefully timed neonatal thymectomy of certain strains of mice, or by adult thymectomy combined with irradiation of rats. These manipulations alter the balance of T cell subsets. These forms of the disease more closely resemble human chronic thyroiditis and are especially useful for defining the complex interactions involved in the pathogenesis of thyroiditis. For purposes of discussion, we have divided the pathogenesis of autoimmune thyroid disease into three stages: induction, progression and expression.

Induction

The induction of autoimmune thyroid disease in humans depends upon the interaction of innate and environmental factors. The balance of these two varies from situation to situation, as will be described subsequently. Family studies and twin studies show that predisposition to thyroid autoimmunity is genetically acquired. Since concordance among identical twins is less than 50%, however, clinically relevant disease is probably precipitated by environmental triggers.

The genetic traits predisposing to thyroid autoimmunity may exert their effects primarily through the immune response or through the thyroid target organ.

Genetic factors influencing immune response

The genetic factors influencing the immune response are as follows:

Immunoglobulin variable (V) gene repertoire Studies show that the full range of heavy and light chain V region gene families is involved in the induced autoimmune response to thyroglobulin in mice. Yet, there are reports of a limited number of dominant idiotypes in spontaneous thyroiditis of the BUF rat and, recently, in human thyroiditis patients, suggesting preferential use of certain V gene families in spontaneous forms of the disease.

Major histocompatibility complex (MHC) class II genes These genes act either by controlling presentation of particular antigenic determinants or by shaping the T cell repertoire during the positive selection process in the thymus. A class II gene (referred to as Ir-Tg) is the most important single determinant of the vigor of the immune response to thyroglobulin in the mouse, but its importance is somewhat overshadowed by other genes in the rat. MHC class II genes are also highly significant in spontaneous thyroiditis in the OS chicken. In human autoimmune thyroid disease the picture is mixed since there is a clear but weak association of MHC class II genes with Graves disease, but only a questionable association with Hashimoto thyroiditis on a populational basis. Within a particular family, however, siblings who share both HLA haplotypes with the patient show a great increase in thyroid autoimmunity.

T cell receptor (TCR) genes In other experimental autoimmune diseases, notably allergic encephalomyelitis, a striking restriction of TCR-V β family usage has been reported. The situation with respect to experimental thyroiditis is still under investi-

gation, but there is some evidence of restricted TCR-V β usage during the early stages of autoimmunization.

Regulation by T cells and T cell products Despite many claims in the literature, the physiological role of regulatory T cells remains unclear in human autoimmunity. However, experiments in the rat, the mouse and the chicken all point to the presence of a thymus-derived regulatory lymphocyte population. In experimental thyroiditis of the mouse, a unique antigen specific thymus-derived suppressor cell has recently been described. It is a CD3⁺, CD8⁺, I-J⁺ large lymphocyte which is MHC restricted and age related; that is, it is readily detected in young animals of most strains, but greatly reduced in older (30 months of age or more) mice.

Sex Thyroiditis is four to five times more frequent in women than in men. Using experimental thyroiditis in mice, we showed that castrated male mice given estrogen were more susceptible to disease than controls. In BUF rats, repeatedly-bred females had a higher incidence of thyroiditis than age-matched virgin females or males.

Genetic factors affecting target organ susceptibility

A number of different genetic traits pertain to target organ susceptibility.

MHC class I genes These are influential in determining the degree of thyroid damage during experimentally induced thyroiditis in the mouse.

Thyroglobulin allotypes Subtle antigenic differences among thyroglobulins have received relatively little attention in the past, but such small differences may be critical in determining the binding of thyroglobulin fragments with MHC class II products of the antigen-presenting cells.

'Disease-associated' epitopes Studies in both the mouse and human have shown that the autoantibodies associated with disease react with a restricted portion of the thyroglobulin molecule. In both species these epitopes are species specific. It is reasonable to postulate that the presence of an immune response to these epitopes is a biomarker of pathogenic autoimmunity.

Adhesion molecules LFA-1 and its ligand ICAM-1 are highly expressed in foci of lymphocyte infiltration of the thyroid gland.

Environmental factors

A number of environmental factors have been implicated in the pathogenesis of autoimmune thyroid disease. These factors may operate by increasing the immunogenicity of thyroid antigens or by heightening the immune response. 1) Diet is clearly of importance with respect to the iodine content of the thyroglobulin. It is likely that the steady increase in thyroid autoimmunity in North America and Western Europe is related to the increasing consumption of dietary iodine. There is evidence that iodine enhances the immunogenicity of the thyroglobulin molecule through its recognition by helper T cells. 2) A number of other chemicals and drugs are known to increase thyroid autoimmunity in predisposed animals. A striking example is the role of methylcholanthrene in raising the prevalence and severity of autoimmune thyroiditis in BUF rats. 3) Infection has long been postulated as one of the triggers of thyroid autoimmunity. At least two well-studied epidemics of mumps and one local paramyxovirus epidemic have been followed by clusters of thyroiditis cases. Possible molecular mimicry between thyroglobulin and *Yersinia* has been described. 4) Finally, stress is known to precipitate clinical manifestations of thyroid autoimmunity, probably by affecting steroid or peptide hormones.

These examples of genetic and environmental factors that influence the induction of thyroid autoimmunity are cited not as an exhaustive list, but as illustrative of the fact that the induction of thyroid autoimmunity is both multifactorial and polygenic. None of the genes described above is 'abnormal'; rather they encode traits distributed widely in the general population. The conjunction of a number of normal genes together with appropriate environmental triggers is responsible for the initiation of autoimmune thyroid disease. The particular mix of genes varies from animal to animal. Thus, MHC class II genes are prominent risk factors in some species but not in others. Similarly, environmental factors may be more or less important depending on the circumstances. A powerful stimulus, like Freund's complete adjuvant, plus homologous thyroglobulin will induce thyroiditis in virtually every immunized rabbit regardless of its genetic constitution.

Progression

It has long been known that B cells binding autologous thyroglobulin are present in normal mice. These B cells are probably responsible for the low level of 'natural' autoantibodies found in normal sera. When they are immortalized by hybridization they produce low-affinity IgM antibody directed to the conserved

portions of the thyroglobulin molecule. These naturally occurring autoantibodies may well serve a physiological function in facilitating the removal of intact thyroglobulin that escapes the thyroid follicles and enters the bloodstream.

The critical requirement for the progression of an autoimmune response to autoimmune disease is the stimulation of self-reactive T cells. There is indirect but firm evidence that T cells reactive with autologous thyroglobulin are not entirely deleted in normal mice. One premise is that these thyroglobulin-reactive T cells are not fully eliminated in the thymus because they are of relatively low affinity. Since they are not easily triggered, an adjuvant, such as Freund's complete, lipopolysaccharide, or poly A:U, is mandatory to initiate their proliferation.

If self-reactive T cells are stimulated, there is a stronger possibility that an autoimmune response will progress to pathological autoimmunity. The expression of autoimmune disease depends upon the elaboration of cytokines emanating from the stimulated T cells. Very little information is available, as yet as to what determines the mix of cytokines produced by stimulated T cells.

Additional important factors influence pathological progression of autoimmunity to autoimmune disease, such as immunological escalation. Cytokines such as interleukin 1 (IL-1) lower the threshold of activation of self-reactive T cells and promote the expression of activation markers, such as IL-2 receptor, Ia and LFA-1. Under these conditions even the lower affinity T cells can be triggered.

Therefore, once autoimmune inflammation has begun, other organ-specific antigens of the thyroid add to the overall autoimmune response. Experiments have shown that primates immunized with purified thyroglobulin eventually develop autoantibodies to thyroid peroxidase (thyroid microsomal antigen). The initial thyroid inflammation serves as the equivalent of an adjuvant, permitting additional organ-specific antigens of the tissue to express themselves. It is significant that in all of the organ-specific autoimmunities studied to date more than one organ-specific reaction is demonstrable.

Expression

The expression of autoimmunity after stimulation of self-reactive T helper cells depends upon the mixture of lymphokines liberated. For example, IL-4 and IL-6 effect isotype switch of B cells and help to determine the affinity and biological properties of antibodies produced, whereas IL-2 and interferon γ (IFN γ) favor cell-mediated immunity.

In the case of autoimmune thyroid disease, at least

three antibody-mediated functions have been described. They include 1) complement-mediated lysis, 2) antibody-dependent cellular cytotoxicity (ADCC), and 3) immune complex deposition. Complement-mediated lysis entails action upon a thyroid cell membrane antigen. The antigen is lost rapidly during *in vitro* cultivation of the thyroid epithelium and, therefore, has not yet been characterized. ADCC has been demonstrated using thyroglobulin-coated erythrocytes or mastocytoma cells. The identity of the killer cell involved in these reactions is uncertain, but it appears to be a large granular lymphocyte, morphologically resembling the natural killer (NK) cell. Immune complex deposition of thyroglobulin antithyroglobulin has been found in the kidneys of rabbits with experimental thyroiditis (as well as patients with Hashimoto's thyroiditis). In addition, immune complexes have been found within the thyroid gland itself in OS chickens and contribute to the final pathological picture. It is important to recall that local production of antibody is a major feature of spontaneous autoimmune thyroid disease in the chicken and the rat, as evidenced by the presence of plasma cells and of germinal centers.

The production of cytotoxic T cells may depend primarily upon the elaboration of such lymphokines as IL-2 and IFN γ by the helper T cells. In the mouse, cytotoxic T cells are both H-2 restricted and thyroglobulin specific, showing that at least some antigenic determinants of thyroglobulin are expressed on the surface of the thyroid epithelial cell in culture.

Tumor necrosis factor (TNF) is another lymphokine produced by stimulated helper T cells and, experimentally, TNF α has been shown to injure rat thyroid tumor cells. The role of TNF α and TNF β in experimental or spontaneous thyroiditis, however, has not yet been established.

Finally, activated macrophages are prominent in experimental thyroiditis in the mouse, rat and rabbit, and spontaneous thyroiditis in the chicken and rat. Since they are an important source of cytokines, hydrolytic enzymes, nitric oxide, oxygen free radicals and prostaglandins, they may contribute significantly to the final pathologic picture.

Summary

Both genetic and environmental factors contribute to

the induction of autoimmune thyroid disease. A number of genes, acting by diverse mechanisms, converge to provide the genetic susceptibility to thyroid autoimmunity. MHC class II genes are the major genetic determinants of susceptibility in most species. The response to the principal thyroid antigen, thyroglobulin, is not clonally restricted on the B cell level, but clonal restriction of T cells is still a possibility. Autoimmunity begins as a response to a restricted region of the thyroglobulin molecule, but a number of different epitopes is represented in that region. The pathogenetic expression of autoimmunity differs from species to species. In the mouse, for example, experimental thyroiditis is produced mainly by cytotoxic T cells whereas antibody plays a significant pathogenetic role in spontaneous thyroiditis of the OS chicken.

See also: Adhesion molecules; Autoantigens; Autoimmune disease, induced animal models; Autoimmune disease, pathogenesis; Autoimmune disease, spontaneous animal models; Cytokines; MHC disease associations; Thyroid autoimmunity, animal models.

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THYROID AUTOIMMUNITY, HUMAN

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In areas of the world where dietary iodine intake is adequate, autoimmune mechanisms are the commonest cause of thyroid disease. In this situation autoimmune thyroid disease (AITD) occurs in up to 4% of the population and manifests itself clinically, predominantly as either thyroid overactivity (hyperthyroidism) with or without characteristic changes in the eyes (ophthalmopathy) or thyroid underactivity/failure (hypothyroidism). In both situations the thyroid may be enlarged (goitre). Clinical descriptions of the disease states long predate our understanding of their cause. Depending on where one's national loyalties lie and one's reading of the historical descriptions, hyperthyroidism with diffuse enlargement of the thyroid and ophthalmopathy is variously referred to as Graves' disease, von Basedow's or Parry's disease. In contrast, thyroid failure attributable to AITD is best referred to collectively as chronic autoimmune thyroiditis, in which lymphocytic infiltration is characteristic and can be subdivided, depending on the clinical presentation, into goitrous (Hashimoto's) thyroiditis and atrophic thyroiditis (primary myxedema) in which the thyroid is not palpable.

The turning point in our understanding of these disease states came in 1956. The establishment of an animal model of AITD by Rose and Witebsky and the demonstration by Roitt, Doniach and their colleagues of autoantibodies to thyroglobulin in the sera of patients with Hashimoto's thyroiditis opened an era of intense research activity. An equally momentous observation, also in 1956, was that by Adams and Purves, who demonstrated in the sera of patients with hyperthyroid Graves' disease an activity distinct from thyroid-stimulating hormone/thyrotropin (TSH), which stimulated the uptake of radioiodine into the guinea pig thyroid with a delayed time course of action as compared with TSH; thus was both the concept of the long-acting thyroid stimulator (LATS) which was shown by Kriss and colleagues in 1964 to be an immunoglobulin.

Etiology

Graves' disease and chronic autoimmune thyroiditis belong to the group of organ-specific autoimmune diseases, which includes insulin-dependent diabetes mellitus (IDDM), myasthenia gravis, pernicious anemia, Addison's disease, primary ovarian failure and

viteligo. These diseases, besides occurring together in the same individual more frequently than one would expect by chance, are also found commonly in the families of affected individuals though no clear pattern of inheritance is recognizable. Additionally, well-recognized HLA associations are known to occur with these diseases and they are commonly associated with chromosomal anomalies such as trisomy 21 and Turner's syndrome and in familial Alzheimer's disease. Concordance rates in Graves' disease in monozygotic (30–60%) and dizygotic (3–9%) twins further emphasize the importance of genetic factors. Historically, most effort has been directed at the contribution which genes in the HLA region on chromosome 6 make towards genetic susceptibility in patients and families with AITD. In Caucasians with Graves' disease a weak but consistent association with HLA-DR3 is well established. A stronger association with HLA-DQA1*0501 (which is in linkage disequilibrium with DR3) has been reported which is still present when DR3-positive individuals are excluded. The examination of associations of polymorphisms in other relevant, non-HLA genes with Graves' disease, including genes for immunoglobulins, cytokines, the T cell receptor and the TSH receptor, have not been rewarding.

Family studies to date have not contributed significantly towards characterizing the major genetic determinants contributing towards the development of AITD, primarily because of the small number of families studied and the small number of genetic markers used. The examination of a large number of multiplex families using automated technology and a large number of microsatellite markers spanning most of the human genome (as pioneered in studies of IDDM) offers exciting prospects for understanding the genetic contribution to the development of AITDs. The AITDs occur more commonly in females and are characterized by circulating autoantibodies and activated T cells and in addition lymphocytic infiltration of the target organ (thyroid). If we feel more confident now (as discussed below) in our understanding of the pathogenic mechanisms leading to AITDs the same cannot be said of their etiology. The consensus view would have to be that the organ-specific AITDs are multifactorial diseases which arise in genetically predisposed individuals in whom the onset of disease is triggered by an insult which may be constitutional or environmental.

In the context of AITDs a number of constitutional factors have been implicated which include the age, sex and race of the individual and their hormonal status; these diseases remit during pregnancy but commonly present at times of major hormonal alteration such as at puberty, in the postpartum period and at the menopause. Although less than 25% of patients presenting with Graves' disease are male, men who get the disease have much more severe disease with higher levels of antibody at presentation and higher relapse rates following antithyroid drug treatment, compared with women with the disease. Stressful stimuli can influence both thyroid function and the immune system. In seeking to define potential mechanisms considerable interest and activity has focused on examining the implications of the resulting induction of heat shock proteins by stressful stimuli on the subsequent development of AITDs. In supporting the potential role of stress in inducing AITDs a number of studies highlight the increased frequency of negative life events in the 12 months preceding the development of hyperthyroid Graves' disease in patients as compared with matched healthy controls. Such retrospective, subjective observations need to be extended by more robust data but the association observed does seem likely.

The establishment normally of a state of self-tolerance, particularly during development in the thymus when as part of T cell maturation, T cells with a high affinity for self antigens are deleted, protects against the induction of AITDs. These diseases follow the breakdown of self-tolerance. Breakdown is likely to occur in genetically susceptible individuals when target organ damage by an environmental insult (perhaps an infectious agent) damages a target organ, thus exposing previously sequestered self antigens to the immune system. The resulting autoimmune response is determined by factors such as the antigen dose, type of antigen presenting cell, major histocompatibility complex (MHC) class II haplotype and cytokines involved which direct the differentiation of previously naive CD4⁺ T cells into either T_H1 or T_H2 subsets. This latter process determines whether a humoral (antibody-mediated) or cell-mediated immune response is then generated. Cytokines themselves may be directly cytotoxic. Definition of the epitopes within target autoantigens against which the humoral and cell-mediated immune response are directed is a major focus of the research effort into AITDs.

Environmental factors have been extensively studied, particularly in the context of Graves' disease. The seasonal and geographic influences which are well recognized almost certainly reflect differences in dietary intake of iodine. Across Europe the frequency

with which Graves' disease is the commonest presenting cause of hyperthyroidism ranges from 45% of patients in Goettingen, Germany (an area of iodine deficiency) to 87% of patients in Athens, Greece (an area of higher than normal iodine intake). In the UK the seasonal variation in Graves' disease may well have a similar explanation. The major source of iodine in the UK is milk and its content of iodine almost doubles during the winter months when cattle are brought indoors and are fed on food supplements rich in iodine, particularly iodized salt licks. The pattern of Graves' disease seems to follow this pattern with the disease presenting most commonly immediately after winter and being most uncommon immediately before winter. Recurrence of Graves' disease, following thyroid surgery to cure it, is most likely in areas of iodine excess (Reykjavik, Iceland) compared with areas of lower iodine intake (Aberdeen, Scotland).

The other major potential environmental inducers of AITDs which have attracted attention are infectious agents. In this area results to date have been disappointing, though modern recombinant DNA technologies in conjunction with characterization of the target autoantigens will undoubtedly lead to previous seemingly inaccessible agents such as viruses being open to examination. Elegant work on *Yersinia enterocolitica* and common strains of gut commensals such as *Escherichia coli* have shown clear evidence of cell surface constituents which bind TSH with similar characteristics to that of the TSH receptor on thyroid cells. The concept that is beginning to emerge is that through molecular mimicry an immune response directed against these organisms could include the generation of immune mechanisms which, in a genetically susceptible individual, not only control the microorganisms' proliferation but also, because of the homology between their 'TSH receptor-like constituents' and the thyroid TSH receptor, allow the development of Graves' disease.

A further important environmental insult increasingly implicated in the development of AITDs is cigarette smoking. This is particularly relevant to the development and worsening of Graves' ophthalmopathy. The mechanism remains uncertain.

Whilst enormous advances have been made in the past 40 years the most exciting developments have come in the last few with the cloning of the thyroid enzyme, thyroid peroxidase (TPO) and the TSH receptor and the demonstration that these are the key target autoantigens in AITDs, against which the aberrant autoimmune response is directed.

Graves' disease

The diffusely enlarged thyroid gland detected clinically or by radioisotope scan in conjunction with the presence of ophthalmopathy, and less commonly changes in the skin on the front of the legs (pretibial myxedema) and in the nails (thyroid acropachy), allows the diagnosis of Graves' disease to be made confidently in a patient presenting with hyperthyroidism. It is the commonest cause of hyperthyroidism in the UK accounting for 75% of the cases seen.

The disease is due to autoantibodies to the TSH receptor. Historically these antibodies have proved a source of great confusion since a wide variety of assay systems for their detection have been developed and have resulted in a wide variety of terms being used to describe the activity detected. The antibodies are unique in that not only do they bind to the TSH receptor but they are also able to stimulate thyroid function and thus themselves induce hyperthyroidism, acting through the adenylate cyclase second messenger system. Assays to detect these activities are therefore able to detect binding of these autoantibodies to the TSH receptor (radioreceptor assay) or measure the ability of the antibodies to stimulate thyroid cell function (bioassay). In the radioreceptor assay measurement is made of the ability of antibodies to inhibit the binding of radio-labeled TSH to a thyroid membrane preparation. Bioassays, using predominantly human thyroid cells in primary culture or the rat FRTL5 thyroid cell line, measure end-points of cell activation which are mediated via the TSH receptor. With the increased sensitivity of the assay systems TSH receptor antibody activity is detectable in the vast majority of patients with Graves' disease when they first present with hyperthyroidism. In responses to treatment the antibody activity falls and failure of the antibody to disappear in response to treatment makes disease recurrence almost certain. The antibody does therefore seem to correlate fairly closely with disease activity.

The best evidence, however, that the antibody is indeed pathogenetic comes from studies in the offspring of mothers with Graves' disease. TSH receptor antibodies are of the immunoglobulin G1 (IgG1) class and therefore cross the placenta. When the level of antibody is high enough in the mother it will, by crossing the placenta, induce hyperthyroidism in the fetus and newborn which lasts as long as the antibody is present at sufficient concentration in the neonate (6–8 weeks).

In addition to autoantibodies which stimulate thyroid cell function, it is now also clear that autoanti-

bodies exist which, by binding to the TSH receptor, inhibit thyroid cell function. These TSH receptor blocking antibodies (TSHRBAb) can also be detected by the radioreceptor assay, but this assay does not itself distinguish between stimulating and blocking TSH receptor antibodies. However, a modification of the bioassay can be used to examine the ability of these antibodies to inhibit TSH-induced cyclic AMP production by thyroid cells.

Yet another group of antibodies have been described which rather than acting to stimulate or block thyroid function, are involved in inducing or inhibiting thyroid growth. Considerable controversy surrounds these antibody activities: Are the assay systems measuring parameters of growth reliable? Are the effects mediated via the TSH receptor? Is there any difference between TSH receptor-stimulating antibodies and thyroid growth stimulating antibodies? The answers are not yet available.

What is not in doubt is the fact that the spectrum of clinical presentations of AITDs with changes in both thyroid gland function and size occurring not only in different individuals but also in the same individual points to the presence of a spectrum of autoantibodies acting as agonists and antagonists and able to influence both thyroid cell function and growth. With the cloning of the TSH receptor clear characterization of the antibodies binding to it and the different epitopes of the receptor recognized by these antibodies will very soon confirm or refute the presence of antibodies which stimulate and block both thyroid function and growth.

The cloning in 1989 of the TSH receptor by the groups of Vassart and Rapoport has provided a major impetus to the work on Graves' disease. The receptor consists of a single polypeptide of 764 residues which, in keeping with other G protein-coupled receptors traverses the cell surface membrane seven times. The extracellular part of the receptor comprises 395 residues and that this is the region involved in the interaction with the glycoprotein hormone TSH and with TSH receptor antibodies has been confirmed with expression of the recombinant protein in eukaryotic cells. The field is moving rapidly; B cell epitopes are being defined and T cell epitopes will follow. Elucidation of the interaction of the spectrum of autoantibodies to the TSH receptor with the receptor is at hand.

Graves' ophthalmopathy

Clinically, the changes occurring in the eyes of patients with Graves' ophthalmopathy (GO) are, when overt, easily recognized and well described. In a significant number of patients the changes are more

subtle, not detectable clinically or only demonstrable with highly specialized investigations such as computerized tomography of the orbits. As a result the true frequency of GO and the frequency of its association with AITD remains controversial. Is it a distinct organ-specific autoimmune disease or is it an associated feature of the AITD? We do not yet know. It certainly occurs most frequently in patients with Graves' disease. Clinically, its importance lies in helping to define the cause of hyperthyroidism in a patient presenting with the disease but it is also important to recognize, since in a small group of patients with GO (perhaps no more than 5%) it can progress to threaten vision. Why this progression occurs and what causes it we do not know.

Examination of the orbits of patients with GO at postmortem reveals marked extraocular muscle hypertrophy with an increase in surrounding fat and connective tissue. Histology of these tissues reveals inflammatory and immune cell infiltrates. The pathogenesis remains as uncertain as the natural history. Are there autoantigens on orbital tissues which share epitopes with autoantigens in the thyroid? Data have accumulated to suggest that both antibodies and T lymphocytes may recognize determinants on extraocular muscles or orbital fibroblasts and lead to antibody-dependent or antibody-independent cell-mediated cytotoxicity. The area remains confused and controversial. In all of endocrinology GO remains one of the great enigmas and one of the least well understood and most poorly managed clinical problems. Until we can characterize confidently the autoantigen targets and obtain them in sufficient quantities naturally or through cDNA expression systems, prospects for understanding the disease are poor.

Thyroiditis

Patients with chronic autoimmune thyroiditis most commonly present with clinical and/or biochemical evidence of thyroid failure with or without goitre. Clinically they can be divided into those with goitrous (Hashimoto's) thyroiditis and those with atrophic thyroiditis (primary myxedema). Historically, establishment of their autoimmune basis has relied on the presence of autoantibodies to thyroglobulin (in the minority) and to the thyroid microsome (in the majority), detected by immunofluorescence or hemagglutination assays. More sensitive assays, particularly using ELISA techniques, have allowed accurate quantitation of the autoantibody activity and the production of monoclonal antibodies to the thyroid microsome (TMA).

Understanding of the pathogenesis of these dis-

eases, and particularly the significance of autoantibodies to the TMA, was limited by the recognition that the TMA was an intracellular constituent of the thyroid cell exocytotic vesicle carrying thyroglobulin (TG) to the apical cell surface of the cell for its processing and maturation to thyroid hormones. The demonstration, with highly purified polyclonal and monoclonal antibodies, that the TMA was in fact expressed on the cell surface (at the time that the exocytotic vesicle released TG) and was therefore accessible to the immune system, awakened interest in the TMA as a potential target autoantigen. With these autoantibodies it was rapidly shown to be a 105 kDa molecular weight protein and screening of thyroid cDNA λ gt11 libraries allowed detection of a protein of 933 amino acids which turned out to be thyroid peroxidase (TPO), the key thyroid enzyme involved in the generation of thyroid hormones from TG.

Considerable evidence has accumulated to demonstrate that TPO is the major autoantigenic determinant of TMA. DNA expression systems making use of the polymerase chain reaction have provided TPO recombinant proteins and allowed the rapid mapping of B cell epitopes. Whilst a spectrum of autoantibodies are now recognizable as being directed against the TPO molecule, their significance remains uncertain. Unlike the situation in Graves' disease, the passage of TPO antibodies across the placenta is not associated with any alteration in thyroid function in the offspring. Investigations as to the role of antibody-dependent or independent cell-mediated cytotoxic mechanisms directed against TPO in the induction of thyroid failure are now ongoing. T cell clones established from the thyroid lymphocytic infiltrates and recombinant TPO and peptides spanning the molecule will allow characterization of the T cell epitopes and evaluation of the significance of T cells in the pathogenesis of the thyroid cell destruction leading to hypothyroidism.

Whilst antibodies to TPO are detectable as frequently in patients with atrophic thyroiditis as in those with goitrous thyroiditis, an interesting additional or alternative explanation for the pathogenesis of atrophic thyroiditis has emerged with the demonstration in perhaps as many as half the patients of TSHRBAb. These antibodies, on crossing the placenta, cause transient neonatal hypothyroidism and therefore almost certainly represent a pathogenetic mechanism which would explain, by the blockage of access of TSH to its receptor, both the atrophy of the thyroid as well as its failure to synthesize thyroid hormones.

Postpartum thyroid dysfunction

An interesting variant of the group of AITDs are the syndromes of transient postpartum thyroid dysfunction (PPTD). Described initially in Japan by Amino and his colleagues, these syndromes occur in at least 5% of women postpartum. Women who are shown to have autoantibodies to TPO when they present to prenatal clinics and who are (if Caucasian) HLA-A1, -B8, -DR3 positive, are at particular risk of developing PPTD, irrespective of their past medical history. The syndromes are usually subclinical but biochemical monitoring of the postpartum period reveals changes in thyroid function which include hyperthyroidism, hypothyroidism or hyperthyroidism followed by hypothyroidism. The syndromes are important to recognize since they may well contribute to the etiology of the well-documented changes in mood and behavior that occur in the postpartum period and, more importantly, although initially transient they may be the first signals of subsequent permanent thyroid failure.

The pathogenesis of these syndromes remains to be fully elucidated. The hyperthyroid phase contrasts with Graves' disease in that the thyroidal radioisotope uptake is low or undetectable and antibodies to the TSH receptor are not detectable. The evidence to date suggests that the hyperthyroid phase results from immune-mediated thyroid cell destruction and therefore thyroid hormone release. If this process is severe enough it is followed by hypothyroidism, the duration of which is determined by the extent of destruction and the time it takes for thyroid tissue recovery.

Conclusions

The clinical syndromes of AITD are well established. The pathogenetic mechanisms are now also increasingly well understood. Recombinant DNA technology has provided the necessary tool for the elucidation and characterization of the target autoantigens. Establishment of B cell epitopes and, through cloning of target tissue T cells, the definition of T cell epitopes, will almost certainly tie up the pathogenesis story. Two issues remain: what is the etiology and in this context what does the environment contribute, and can we modulate or prevent AITD?

Cloning of the autoantigens will allow an answer to the first question through approaches such as examination of homologies between these autoantigens and infectious agents. The second question is more difficult. With the understanding we now have and the strategies available there seems little reason, once the relevant autoantigenic epitopes are established, why prospects for autoimmune disease should be any different from those of infectious diseases and why immunization against AITD should not become a reality. Studies in animal models of AITD using oral tolerance as a therapeutic option also offer an exciting alternative strategy.

See also: Autoantibodies, tests for; Autoantigens; Autoimmune disease, pathogenesis; Autoimmune disease, spontaneous animal models; Autoimmune diseases; Autoimmunity; Eye, autoimmune disease; Molecular mimicry; Polyendocrine autoimmunity; Thyroid autoimmunity, animal models.

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TISSUE TYPING

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What is tissue typing?

Most commonly, tissue typing refers to the testing of lymphocytes for their human leukocyte antigens (HLA). Although other cells or 'tissues' could be used, in actual practice lymphocytes are almost exclusively used as the targets. 'Types' can also mean many other polymorphisms, but again, commonly it is the HLA antigens which constitute the tissue type. Red blood cell types may also be on certain tissues, but historically, since they were first found on red cells, types such as type A, B and O are said to be red cell types.

Although red cell types were first discovered about 90 years ago, it has only been about 30 years since the tissue types came to be generally recognized. In this relatively recent past, very great strides have been made in defining over 300 'types'. Much of the development has been fueled by the parallel advances in the field of clinical transplantation, for the main clinical utility of tissue typing is transplantation. Today, tissue typing is performed on over 20 000 kidney transplants annually and about 10 000 other types of transplants, such as heart, liver, pancreas and bone marrow.

How is tissue typing done?

Similar to determining red cell types, it was recognized early that the best approach would be to find antibodies directed against cells other than red cells, namely white cells. The earliest attempts, therefore, used a similar agglutination reaction against white cells to that used for red cells. The first antibody to a type or specificity was found by Dausset in 1958 in sera from patients immunized by transfusions, using the leukoagglutination reaction. Patients immunized by pregnancies were also shown, by Payne, to have agglutinating antibodies to leukocytes. Because the reactions produced were so complex, van Rood introduced the use of computers to identify sera which were similar to each other. In this way, he first defined an allelic group of sera, 4a and 4b.

It was at this point in 1964 that the micro lymphocyte cytotoxicity method of Terasaki and McClelland was introduced. This method, together with some modifications, has been adopted and used universally for the past 30 years. The basic feature of

requiring an extremely small volume of antisera (0.001 ml per test) and a small number of lymphocytes was critical to its widespread adoption.

In 1969, a group of investigators met at the National Institutes of Health (NIH) and agreed to use identical incubation times (30 minutes with anti-serum and 60 minutes with complement) to standardize the test (often, since, called 'the NIH test').

Starting with the discovery of the molecular structure by Bjorkman in 1987, and through the subsequent establishment of the amino acid sequence of each of the HLA specificities and the development of the polymerase chain reaction (PCR) in recent years, it has become possible to determine HLA type by DNA-based methods. These methods have the advantage of not being dependent on rare, specific antisera, but rather on probes and primers which can be readily synthesized. In addition, they do not rely on living lymphocytes, as DNA can be isolated from any nonviable nucleated cell. The end-point of the tests is also not influenced by the viability of cells or the activity of complement. Perhaps most importantly, DNA typing can identify 'splits' or variants in the specificities which are impossible to detect serologically.

Currently, one drawback to the DNA tests is that they take a longer time to perform (3–6 hours). This time is further extended when all the specificities are typed. The expense is considerably higher, particularly for class I. Many of the amino acid substitutions detected by DNA typing may not be relevant for transplantation as they do not all form immunogenic epitopes against which the body reacts. When antibodies are formed against an epitope, it is clear that the target epitope is one to which the host responds immunologically. The relevance of each variant remains to be established, particularly for solid organ transplantation. For bone marrow transplants, the 'minor' mismatches appear to have some effect on the success of the transplant.

Methods

Serology

Lymphocytes are isolated from blood by immunomagnetic beads coated by monoclonal antibodies against T cells or B cells. Once the cells adhere to the beads, the beads are pulled to one surface of the

test tube with a strong magnet. After washing, the beads and the attached cells are reacted in the tissue typing tray. Alternatively, lymphocytes are isolated with a monoclonal antibody cocktail which contains antibodies to all unwanted cells, such as red cells, platelets, granulocytes and B lymphocytes, in order to obtain T lymphocytes for typing. The cells are then stained with carboxyfluorescein in the fluorescence test.

Next, lymphocytes are added to the predotted tissue typing tray. These trays contain highly selected antisera either from pregnant women or from monoclonal antibodies produced in mice. Each antibody reacts to an epitope which is unique to a certain HLA specificity. Typically, the tray will have 72 or 96 different antisera. Each of the valuable antisera are pre-dispensed on the tray in 0.001 ml volumes, and stored frozen until needed.

After incubation of lymphocytes with antibody for 30 minutes, rabbit complement is added (0.005 ml) and incubated for an additional hour. With monoclonal antibodies, rabbit complement can be incorporated into the first step, thus reducing the total incubation time to 1 hour or less.

After the addition of ethidium bromide or propidium iodide, the reactions are read by the fluorescence test. Living cells are stained green with carboxyfluorescein while dead cells are stained red with ethidium bromide or propidium iodide. In the eosin dye test, dead cells are stained red with eosin and living cells appear bright white under phase-contrast microscopy. In both tests, dead cells denote a positive reaction in which antibody and complement reacted. For example, if cells from a given individual are killed by antibodies against HLA-A2, the person is said to have A2.

An essential element in the micro cytotoxicity test is a drop of mineral oil which is placed in each well. This prevents the evaporation of the small quantities of reagents used. In addition, it allows for the repeated addition of reagents simply by 'injecting' them into the wells. These reagents automatically sink to the bottom and are resealed by the oil. In this way, it is not necessary to cap and uncapped the reaction 'vessel' for each new reagent.

DNA methods

DNA is extracted from nucleated cells, usually white blood cells, by phenolchloroform extraction or guanidine proteinase K precipitation followed by ethanol precipitation of DNA. One of the most common methods of identifying the HLA alleles is the sequence-specific oligonucleotide probe (SSOP) method. This consists of first amplifying all DNA common to a given HLA region, such as DR, by the

PCR reaction. Then, the reaction with various oligonucleotide probes is detected. Another common method is sequence specific primer (SSP), which consists of directly amplifying the specific HLA alleles and detecting them by gel electrophoresis or by a fluorescent dye.

The most accurate way of determining the alleles is by sequencing the nucleotides. Because of the expense, this method is usually only performed to type cells with questionable reactions and for standard reference typings.

The HLA antigens

The HLA antigens were established as a result of an unprecedented large-scale international effort over the past 25 years. Through 11 international Histocompatibility Workshops, hundreds of laboratories worldwide collaborated on defining the HLA antigens present in the human race. In the early stages, antisera discovered were shared among the laboratories to compare the reactions they produced in different populations. As the specificities became clearer, a World Health Organization nomenclature committee met after each workshop to assign internationally agreed names to each specificity. Over 300 laboratories have also collaborated in a monthly exercise of typing cells from unknown individuals for the purpose of assuring international standardization of test results.

The HLA specificities and alleles are listed in Table 1. Numbers given in parenthesis are the 'broad' specificities which include the narrower 'splits'. For example, A9 was the broad specificity first discovered. Two 'splits', A23 and A24, were later identified and included in A9. A23 and A24 are denoted as cross-reactive specificities of A9. This phenomenon, which was also discovered for many other specificities, is attributed to A23 and A24 molecules having a unique common site, A9.

Transplantation

Bone marrow transplants

HLA typing is essential to bone marrow transplantation. Most of the transplants performed are between HLA-identical siblings, which provides the highest rate of success. One-quarter of the siblings are HLA identical. Transplants are not all successful, in that graft-versus-host reactions often occur even with HLA-identical sibling donor grafts due to incompatibility for minor histocompatibility antigens. Recurrence of leukemia is also often a problem.

In recent years, volunteer bone marrow donor

Table 1 Complete listing of recognized HLA specificities and alleles

<i>HLA specificity</i>	<i>HLA alleles</i>
A1	A*0101, 0102
A2	A*0201, 0202, 0204-0209, 0211-0214, 0216-0222
A203	A*0203
A210	A*0210
A3	A*0301, 0302
A9	A*2410
A10	A*2502, A*6603
A11	A*1101-1104
A23(9)	A*2301
A24(9)	A*2402101, *2402102L, *2404-8, *2413-2414
A2403	A*2403
A25(10)	A*2501
A26(10)	A*2601-2608
A29(19)	A*2901-2903
A30(19)	A*3001-3004
A31(19)	A*31012
A32(19)	*A3201-3202
A33(19)	A*3301, *3303
A34(10)	A*3401-3402
A36	A*3601
A43	A*4301
A66(10)	A*6601-6602
A28	A*6803
A68(28)	A*68011-68012, *6802
A69(28)	A*6901
A19	A*7403
A74(19)	A*7401-7402
A80	A*8001
A'Blank'	A*0215N, A*0303N, A*2409N, A*2411N
B7	B*0702, *0704-0708
B703	B*0703
B8	B*0801-0804
B13	B*1301-1304
B15	B*1511, *1528-1529, *1533-1537
B18	B*1801-1805
B27	B*2701-2704, B*27052-53, B*2706-7, B*2709-2711
B2708	B*2708
B35	B*3501-3508, *35091-2, *3510-21
B37	B*3701-02
B38(16)	B*3801-2
B9(16)	B*3903-4, *39061-62, *3907-12
B3901	B*39011, *39013
B3902	B*39021-22
B16	B*3905
B40	B*4003-4
B4005	B*4005
B41	B*4101-2
B42	B*4201-2
B44(12)	B*4402, *44031-2, *4404-8
B12	B*4409-10
B45(12)	B*4501
B46	B*4601
B47	B*4701-2
B48	B*4801-3
B49(21)	B*4901
B50(21)	B*5001
B51(5)	B*51011-2, *5104-5, *5107-9
B5	B*5106
B5102	B*51021-2
B5103	B*5103
B52(5)	B*52011-2
B*53	B*5301-2

Table 1 Continued

<i>HLA specificity</i>	<i>HLA alleles</i>
B22	B*5505, *5603
B54(22)	B*5401
B55(22)	B*5501-4
B56(22)	B*5801-2
B57(17)	B*5701-4
B58(17)	B*5801-2
B59	B*5901
B60(40)	B*40011-12, *4010
B61(40)	B*4002, 4006-9
B62(15)	B*1501, 1504-8, 1515, 1520, 1524-5, 1527, 1532
B63(15)	B*1516-7
B64(14)	B*1401
B65(14)	B*1402
B67	B*67011-2
B70	B*1509
B71(70)	B*1510, 1518
B72(70)	B*1503
B73	B*7301
B75(15)	B*1502, 1521-3, 1530-1
B76(15)	B*1512, 1514, 1519
B77(15)	B*1513
B78	B*7801, *78021-2
B81	B*8101
B'Blank'	B*1526N
Cw1	Cw*0102-3
Cw2	Cw*02021-3
Cw9(w3)	Cw*0303
Cw10(w3)	Cw*0302, 0304
Cw4	Cw*0401-3
Cw5	Cw*0501
Cw6	Cw*0602
Cw7	Cw*0701-7
Cw8	Cw*0801-3
	Cw*12021-2, 1203-4, 1301, 1402-3, 1502-4, 15051-2, 1601-2, 1604, 1701-2, 1801-2
	E*0101-2, 01031-2, 0104
	G*01011-3, 0102-4, 0105N
DR1	DRB1*0101, 01021-2, 0104
DR103	DRB1*0103
DR15(2)	DRB1*15011-2, 15021-2, 1503-6
DR16(2)	DRB1*16011-2, 16021-2, 1604
DR17(3)	DRB1*03011-2, 0304-5
DR18(3)	DRB1*03021-2, 0303
DR3	DRB1*0306
DR4	DRB1*04011-2, 0402-4, 04051-2, 0406-24
DR11(5)	DRB1*11011-3, 1102-3, 11041-2, 1105-7, 11081-2, 1109-30
DR12(5)	DRB1*1201, 12021-2, 12031-2, 1204-5
DR13(6)	DRB1*1301-2, 13031-2, 1304-27
DR14(6)	DRB1*1401-29
DR7	DRB1*0701
DR8	DRB1*0801, 08021-2, 08031-2, 08041-2, 0805-16
DR9	DRB1*09012
DR10	DRB1*1001
DR51	DRB5*01011-2, 0102, 0201-3
DR52	DRB3*01011-3, 0201-3, 0301
DR53	DRB4*01, 0101101, 0102, 0103101, 0105
DR'Blank'	DRB4*0103102N, 0201N, 0301N
	DRB6*0101, 0201-2
	DRB7*01011-2
	DRA*0101-2

banks have been established throughout the world. The National Donor Program (NMDP) in the USA has two million donors registered and pretyped for HLA class I types. Similar but smaller registries have been formed, including those in England, Europe, Australia, Taiwan and Japan. Because of the high polymorphism of the HLA system, these large registries are necessary to enable most patients requiring a bone marrow transplant to find a compatible donor. To date, over 4000 bone marrow transplants have been performed from unrelated donors using the NMDP.

Although serologic typing is usually sufficient for identifying an HLA identical sibling within a family, DNA typing is an important means of assuring the most accurate typing and matching for unrelated donors.

Kidney and other solid organ transplants

Over 3000 kidneys have been shipped among transplant centers throughout the USA in order to provide transplants with zero HLA-A, -B and -DR specificity mismatch. Such matched grafts were shown to have an 88% 1-year graft survival and a 15-year half-life. In comparison, mismatched transplants had an 80% graft survival with 8-year half-life. Thus, due to this nationwide sharing of kidneys, a group of patients with outstanding graft survival now exist. Again because of the high polymorphism of HLA, only about 10% of the 30 000 patients waiting on dialysis in the USA were able to obtain such well-matched grafts. The matching was performed by serological methods. If matching were done for all the known specificities by DNA typing, the number of zero A, B, DR mismatched transplants would probably be less than 1%. Thus, not all the known specificities can be utilized on a practical level for selecting cadaver donors for kidney transplants.

A similar effect is expected for heart, liver and pancreas transplants once it is possible to share these organs on a large scale. Currently, heart and liver patients cannot be maintained for long periods of time on artificial support, although there have been considerable advances in cardiac assist devices. Also, the maximum cold ischemia times for these organs are shorter than for kidneys, thus making them more difficult to ship.

Disease association

It has been shown that the HLA is associated with susceptibility to disease. The most useful marker has proved to be the association of HLA-B27 with ankylosing spondylitis. Other disease associations are important for basic studies of the disease but, in

practice, none has sufficiently high relative risk to provide clinical utility. Certainly, HLA has been useful for genetic counseling in diseases such as juvenile diabetes, idiopathic hemochromatosis and 21-hydroxylase deficiency.

Studies on the immune response

It is generally assumed that the extremely diverse HLA system evolved in response to the increasing variety of microorganisms against which an immune response is required. Extensive studies are underway to define the peptides which 'fit' into the groove of the HLA molecule. Certain peptides are shown to fit into the groove of certain HLA alleles but not others. This contact is thought to be a critical step in the immune response, accounting for the fact that some individuals in one species are resistant to a microbe while others are not. Even the response to cancer antigens is thought to be controlled by the HLA specificity present in the patient.

Anthropological studies

Because of the extensive polymorphism of HLA, the system is a powerful tool for anthropological studies. A major survey of world distribution was made in 1972 at the Fifth International Histocompatibility Workshop. A similar survey with many more populations and with about four times the number of markers was completed in 1991 as part of the Eleventh International Histocompatibility Workshop. In the Twelfth Workshop of 1996, DNA-based alleles were first used in anthropological studies.

New DNA typing methods have increased the power of HLA in anthropological studies. The samples can be stored and studied more carefully by DNA typing because viable cells are not necessary. HLA-A2, for example, which is the most 'successful' HLA allele, in that it is most prevalent in the world, exists in 17 variants which Parham has shown to have a characteristic distribution. Eventually the frequencies of all HLA alleles throughout the world will be established. The occurrence of point mutations and the spread of gene conversions through migration for each HLA allele can be followed.

See also: Antiserum; Cell separation techniques; Cytotoxicity, assays for; Hematopoietic stem cell transplantation; HLA class I; HLA class II; MHC disease associations; Mixed lymphocyte reaction (MLR); Transplantation; Viability, methods of assessing leukocyte.

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T LYMPHOCYTE ACTIVATION

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T cells, unlike B cells, do not recognize antigens by themselves and instead require an antigen-presenting cell for T cell activation. Thus, the T cell has developed a more complex receptor for antigen recognition and activation. Significant progress continues to be made in understanding the cascades of biochemical events that occur during the first few seconds, minutes and hours of T cell activation. These biochemical signals culminate about 24 h later in the initiation of DNA synthesis and, soon thereafter, in cell division. The events of this period can be divided, conceptually, into three temporal phases: activation, proliferation and differentiation. The activation phase begins with the stimulation of the T cell receptor by cognate antigen and ends with the secretion of interleukin 2 (IL-2), an autocrine growth factor for T cells. The second, or proliferative phase, encompasses the replication of the cellular DNA, and the creation of a pair of daughter cells. The third, or differentiation phase, may require several rounds of proliferation before it is complete. The initial biochemical events involved in T cell activation are the focus of this entry.

The activation phase, during which T cells change

from quiescent (G_0) cells to blast transformed (G_1) cells, is a complex cell process. More than 70 genes are newly expressed during the first 4 h of T cell activation. To put the cascade of events leading to the secretion IL-2 into perspective, the activation period encompasses the initiation of several parallel, temporally staggered programs, each of which is an important part of the preparation for cell division. These programs include turning-on of the secretory, translational and transcriptional machinery or acceleration of their rate of product generation.

Binding of the T cell receptor by antigen/MHC is necessary but insufficient for optimal activation

T cell activation requires interactions between multiple T cell surface structures and their respective ligands on antigen-presenting cells. Among these surface structures are the specifically rearranged heterodimeric T cell receptor, T_i , and its associated invariant complex, CD3. Discovered in 1983, this complex is referred to as the T cell antigen receptor (TCR) and is comprised of eight protein chains. Under physiologic conditions the binding of antigen/MHC (major histocompatibility complex) to the TCR is necessary, but this interaction is insufficient to result in T cell proliferation. Instead, the induced coassociation of a number of accessory receptors, among them CD4 or CD8, and CD45 is required for immediate signaling events. Yet another set of sur-

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face structures appears to enhance T cell adhesion to the antigen-presenting cell, to amplify TCR-stimulated signal transduction, and may ensure the ability of the T cell to respond flexibly to antigen presented by different types of antigen-presenting cells.

The transduced signal

Protein tyrosine kinases and phosphatases

The principal structural unit that regulates coupling of the TCR to downstream signal transduction is termed the immunoreceptor tyrosine-based activation motif (ITAM) or alternatively, antigen recognition activation motif (ARAM) (Figure 1). Each ITAM consists of two YXXL/I repeats separated by 6–8 residues. There are one each in the cytoplasmic domains of CD3 γ , δ , ϵ and three in the TCR ζ chain (ζ 1, ζ 2 and ζ 3). Since each TCR can contain a TCR ζ dimer and two CD3 dimers ($\epsilon\delta$ and $\epsilon\gamma$), each TCR can contain a total of 10 ITAMS. This is many more than are present in other lymphocytes such as B cells, natural killer (NK) cells and mast cells. The first biochemical event that has been measured following the triggering of the human T cell receptor is the activation of protein tyrosine kinases (PTKs), enzymes that phosphorylate tyrosine residues in proteins. This is observed as an increase in the phosphate content of several tyrosine-containing proteins and occurs within 5 s of stimulation of the TCR. At least three families of PTKs are associated with the TCR. Two

of these kinases, p59^{fyn}, and p56^{lck}, are members of the Src family of nonreceptor protein tyrosine kinases. p59^{fyn} is associated with the TCR while p56^{lck} is also associated with CD4 and CD8, CD28 and the IL-2 receptor. A third kinase, ZAP-70 is a member of a distinct kinase family that also includes the Syk kinase expressed primarily in B cells. ITK, a member of still another family of PTKs is also associated with the TCR via poorly defined mechanisms. The fact that the proteins ZAP-70, Lck, Fyn and ITK exist as T cell-specific kinases underscores their central importance in T cell activation. A primary function of the Src family kinases is to phosphorylate key tyrosine residues within ITAMs. Recent work has suggested that at least two such tyrosine phosphorylated proteins may be the proto-oncogene Vav and the newly cloned SLP-76 which are expressed exclusively in hematopoietic cells and are both readily tyrosine phosphorylated in response to TCR ligation. Deletion of Vav leads to defective antigen receptor-mediated proliferation of mouse B and T cells. Other nonreceptor substrates of these PTKs are still being elucidated, and several are indicated in Figure 2.

In other systems, such as the metabolic cascade that results in the synthesis of glycogen and the concomitant inhibition of glycogenolysis, the action of kinases is opposed by specific phosphatases. In T lymphocytes, the tyrosine phosphatase CD45 and the serine/threonine phosphatase calcineurin have been identified as having important roles in TCR signal transduction. CD45 is an integral membrane protein tyrosine phosphatase that plays a critical, although apparently nonessential role in T cell activation, since T cells from mice with a genetically engineered absence of CD45 can still be activated. There is evidence to suggest that the phosphatase activity of CD45 is involved in the activation of the TCR-associated PTKs. It is thus possible that the CD45 tyrosine phosphatase is required to maintain activation of both the TCR and/or CD4-associated kinases. It is now recognized that downstream of the T cell receptor and the initial activation of PTKs are at least three essential biochemical pathways that convey activation signals: 1) calcium pathways; 2) protein kinase C pathways; and 3) Ras pathways (Figure 2).

hTCR ζ 1	QIYNELNLGRREHYDY
hTCR ζ 2	GLYNELQKDKMAIAYS
hTCR ζ 3	GLYQGLSTATKDTYDA
hCD3 γ	QIYQPLKDREDDQYSH
hCD3 δ	QVYQPLRDRDDAQYSE
mCD3 ϵ	PDYEPKRGQRDLYSGL
mIg α	NIYEGNLNDDCSMIYEC
mIg β	HTYEGLNIDQTATYEDI
hIgE FcR γ	GVYTGSLSTRNQETYETI
hIgE FcR β	RVYEEELNIYSATYSEL
BLV gp30	SDYQALLPSAPEIYSHL
EBV-LMP2A	IYSHLSPVKPDYENL

Figure 1 Immunoreceptor tyrosine-based activation motifs (ITAMs) are expressed in T and B cell antigen receptors, the mast cell receptor for IgE, and in the viral glycoproteins of bovine leukemia virus (BLV) and Epstein-Barr virus (EBV) latent membrane protein 2A.

The calcium pathway

The activation of the TCR-associated tyrosine kinases is followed within 15 s by the activation of another enzyme, phospholipase C (PLC), which may be activated by phosphorylation on tyrosine residues. Activation of PLC is accompanied by binding of a 36 kDa protein which may have a role as an adapter protein in localizing PLC to the cell membrane. PLC

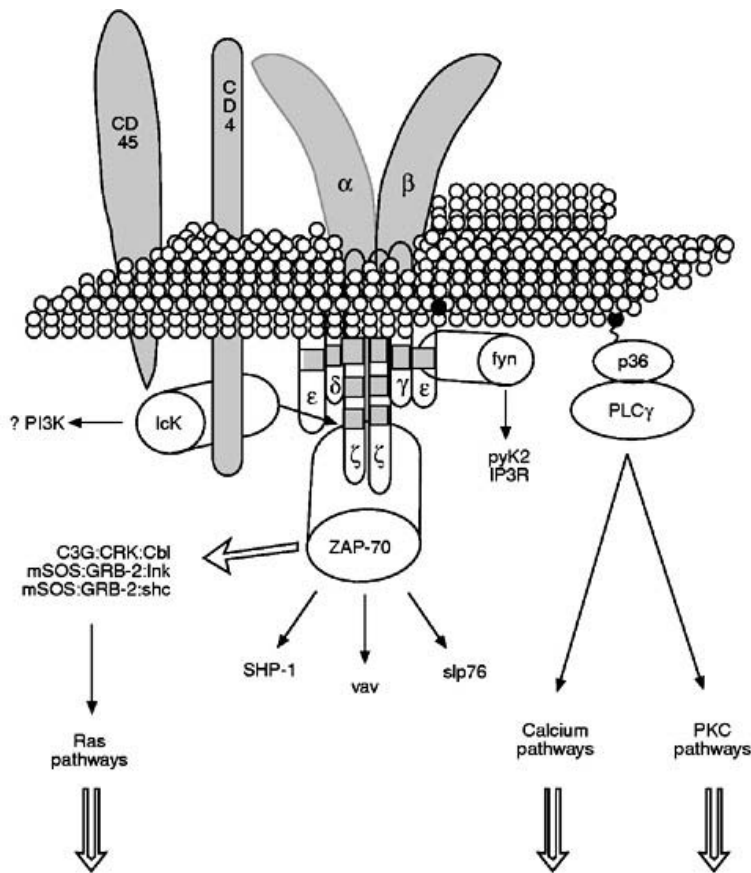


Figure 2 Signaling complexes and pathways associated with the T cell antigen receptor (TCR). The eight chain TCR and the CD4 and CD45 coreceptors are shown with associated kinases Ick, fyn and ZAP-70. There are 10 ITAM motifs encoded in the TCR subunits. Tyrosine kinase activation results in the phosphorylation of a number of substrates, including phospholipase C (PLC). Activation of PLC results in increased calcium concentration and protein kinase C (PKC) activity. Tyrosine kinase activation also leads to activation of Ras via several mechanisms. mSOS, mammalian Son of Sevenless; GRB-2, growth factor receptor-bound protein 2; IP3R, inositol triphosphate receptor; SHP-1, SH2-containing tyrosine phosphatase 1; pyK2, proline-rich tyrosine kinase 2.

cleaves a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) into a cyclitol polyphosphate, inositol, 1,4,5-triphosphate (IP_3) and a diacylglycerol, (DAG). Both of these enzymatic products function in the subsequent steps of the signal transduction cascade. IP_3 is the ligand which operates a receptor, located in the endoplasmic reticulum and plasma membrane, to release a nonmitochondrial, ATP-dependent store of ionized calcium. In some T cells, the IP_3 receptor is a substrate of the TCR-associated p59^{fyn} tyrosine kinase. As a result of the actions of IP_3 the concentration of intracellular calcium ion rises briefly from resting levels of $0.1 \mu\text{M}$ to $0.5 \mu\text{M}$ or higher. Calcium activates the serine/threonine phosphatase calcineurin. One target of calcineurin is NFATp, a transcription factor that has a role in IL-2 gene expression. Calcium has many roles in cellular homeostasis and cell activation, and it is clear that calcium-mediated signaling pathways that are both calcineurin dependent and independent, exist in T cells. Several reports have indicated that isolated cytosolic free calcium elevations in T cells tend to provoke apoptosis or anergy. This can occur by apparently independent mechanisms involving alterations of TCR signaling, by the

absence of costimulatory signaling or by treatment of cells with calcium ionophore.

The PKC pathway

DAG, the other product of PLC activation, along with cofactors such as Ca^{2+} and phospholipids, activates protein kinase C (PKC), a family of serine/threonine kinases that phosphorylate a number of substrates, initiating the PKC pathway. PKC has been implicated in the control of many signaling pathways, including p21^{ras} and the MAP kinases such as ERK, and c-JUN N-terminal kinases (JNK-1/2). The role of specific PKC isozymes is poorly understood, although $\text{PKC}\epsilon$ and $\text{PKC}\theta$ can regulate the transcription factors AP-1 and NFAT in leukemic T cells, and a recent study indicates that $\text{PKC}\theta$ may have a special role during antigen activation of T cells. Phorbol esters activate many forms of PKC, however it is now clear that many effects of phorbol esters on T cells that were previously attributed to PKC are in fact Ras mediated. *In vitro* studies have shown that the PKC isozymes δ , ϵ , η , λ and ζ are activated by D-3 phosphoinositide lipids. $\text{PKC}\zeta$ has also been suggested as a direct target for ceramide

and thus may play a critical role in sphingomyelinase signaling.

Ras pathways

The 21 kDa guanine nucleotide-binding Ras protein (p21^{ras}) family has been implicated in TCR signaling. Triggering of the TCR perturbs the guanine nucleotide-binding cycle of p21^{ras} and results in the rapid and prolonged accumulation of active p21^{ras}-GTP complexes. The coupling of the TCR to p21^{ras} activation occurs via a PTK-dependent mechanism. The level of active p21^{ras}-GTP complexes is determined by a balance of the rate of hydrolysis of bound GTP and the rate of exchange of bound GDP for cytosolic GTP. The importance of Ras activation in T cell signal transduction has been demonstrated most clearly in studies using mutant forms of the Ras protein that are either dominantly negative or constitutively active. Transfection of a dominant negative Ras inhibited the induction of transcriptional events including the induction of the IL-2 gene and induction of the nuclear factor NFAT. In contrast, transfection of the constitutively active Ras could, in conjunction with a calcium signal, induce NFAT and IL-2 gene transcription. There are both positive and negative regulators of p21^{ras} activity: the GTPase-activating proteins (GAPs) such as p120-GAP can increase the intrinsic GTPase activity of p21^{ras} leading to inactivation, whereas the Cdc25-like homologs help in the dissociation of GDP leading to p21^{ras} activation. The inhibition of Ras-GAP proteins is almost certainly one mechanism used by the TCR to regulate the Ras guanine nucleotide-binding cycle.

Role of adapter molecules in TCR coupling to p21^{ras}

In a variety of cell types many receptors regulate p21^{ras} by stimulating the guanine nucleotide exchange protein SOS, which is associated with the adapter protein GRB-2 (Figure 2). GRB-2 is composed of two SH3 domains that bind to a guanine nucleotide exchange protein that activates p21^{ras}, and an SH2 domain that interacts with tyrosine phosphoproteins that regulate cell localization/function of this p21^{ras}-exchange protein. In T cells, at least two tyrosine phosphoproteins can potentially interact with GRB-2 SH2 domains, namely SHC and the membrane-located tyrosine phosphoprotein p36, which may be related to the Lnk protein recently cloned from rat lymph node. Indeed, the major tyrosine phosphoprotein to associate with GRB-2 SH2 domains in TCR-activated cells is a 36 kDa molecule (p36). In TCR-activated T cells, both SHC and p36

are tyrosine phosphorylated and *in vitro* can be shown to bind GRB-2 SH2 domains. SHC has an SH2 domain that can bind to the tyrosine phosphorylated TCR ζ chain, hence tyrosine phosphorylated SHC could potentially recruit GRB-2 and SOS to the TCR complex in activated cells. However, reports disagree about the formation of SHC-GRB-2-SOS complexes after TCR ligation, having only been demonstrated in murine T cell hybridomas but not TCR-activated peripheral blood derived T cells. This illustrates that there may be considerable heterogeneity of TCR responses in different cell types. In addition, it appears that the SHC interaction with TCR ζ chains is of relatively low affinity as compared with the binding of tyrosine kinases such as ZAP-70 or p59^{lyn}. This may ensure that SHC interaction with ζ is transient and only sufficient to bring SHC to the TCR complex where it can be tyrosine phosphorylated, but is insufficient for the formation of stable TCR-SHC complexes. The formation of p36-GRB-2-SOS complexes is a rapid response to TCR ligation that correlates well with p21^{ras} regulation. The failure to find tyrosine-phosphorylated SHC binding to GRB-2 and SOS in TCR-activated cells may be due to competition between p36 and SHC for the SH2 domain of GRB-2.

In addition to GRB-2, it is clear that there are several other adapter molecules that may be involved in coupling the TCR to the p21^{ras} pathway. For instance, T cells express another protein termed C3G which can catalyze guanine nucleotide exchange on p21^{ras} and which binds to the SH3 domains of the adapter molecule CRK. Three forms of CRK are found in T cells and CRK has a single SH2 domain and one or two SH3 domains. TCR triggering results in tyrosine phosphorylation of CRK-binding proteins such as a Cbl, analogous to the TCR regulation of GRB-2 binding complexes. A constitutively active Cbl mutant has been demonstrated to stimulate Ras-dependent and calcium-stimulated activation of NFAT. In fact, there is evidence that GRB-2 is constitutively associated with Cbl regardless of the activation of Jurkat cells. Another report has demonstrated that TCR ligation stimulates tyrosine phosphorylation of Cbl which correlates with the dissociation of the GRB-2-Cbl complex and the formation of CRK-Cbl complexes via the CRK SH2 domain. The reasons for the redundancy in the coupling of the TCR to p21^{ras} remain unclear. One possibility is that these multiple mechanisms provide several contingency routes in the event of malfunction of one or more of these mechanisms. A leading alternative hypothesis is that subtle differences exist in the pathways downstream of Ras.

Role of PKC in p21^{ras} activation

There is an alternative p21^{ras} pathway activation mediated by PKC which, unlike Ras expression, is not ubiquitous but is restricted to T lymphocytes. Hence, phorbol esters inhibit GAP activity in T lymphocytes allowing GTP to remain bound to p21^{ras}, biasing the GTP/GDP balance on p21^{ras} toward GTP. This suggested role for PKC in the activation of p21^{ras} is not involved in mediating TCR-driven p21^{ras} activation which is entirely PTK dependent. It is possible that there is a PKC-sensitive, T lymphocyte-specific Ras exchange protein and one candidate molecule is the proto-oncogene Vav which is expressed only in hematopoietic cells. This protein possesses a region homologous to guanine nucleotide-releasing factors such as yeast Cdc24 or rodent Cdc25Mn. Vav is rapidly tyrosine phosphorylated upon CD3 ligation, but Vav cannot explain the apparent lymphoid specificity of the PKC–p21^{ras} link because Vav is expressed in all hematopoietic cells and mast cells or myeloid cells that do express Vav do not express a PKC-mediated route for p21^{ras} regulation. Moreover, the role of Vav as a Ras guanine nucleotide exchange protein is controversial and structurally Vav is a more likely candidate for an exchange protein for the Ras-related Rho/Rac proteins.

The MAP/ERK pathways

The best-defined signaling cascade initiated by Ras in T cells is the activation of the extracellular signal-regulated kinases (ERKs) which are members of a family of mitogen-activated kinases (MAPKs) (Figure 3). MAPKs are a group of protein serine/threonine kinases that have been termed 'dual specificity kinases' because they are activated by phosphorylation on conserved threonine and tyrosine residues. T cells express ERK-1 and ERK-2, both of which are activated in response to TCR ligation. Two intracellular pathways for ERK-2 regulation coexist in T cells: one mediated by Ras and the other by PKC. The TCR activates both p21^{ras} and PKC but p21^{ras} and not PKC couples the TCR to the regulation of MAP kinases. Activation of ERKs is achieved by a kinase cascade which is regulated by a balance of specific kinase and phosphatase activity (Figure 3). An MAP kinase kinase (MEK or MKK-1) phosphorylates on both tyrosine and threonine residues and stimulates the ERK-1 and -2 kinases directly. MEK activity is itself controlled by phosphorylation and hence an MAP kinase kinase (MEKK) plays a crucial role in the regulation of ERK-1 and -2. The serine/threonine kinase Raf-1 has been identified as an MEKK and the

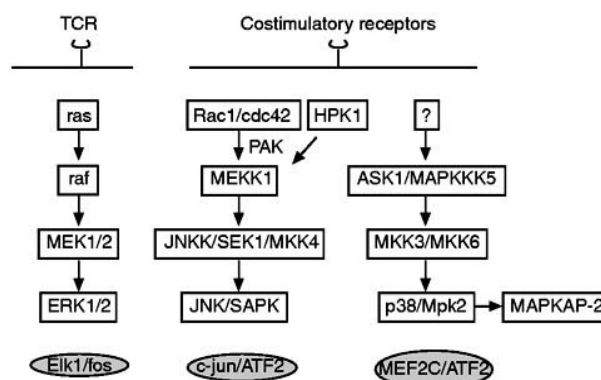


Figure 3 Mitogen-activated protein kinase (MAPK) pathways serve as a major cascade to connect cell surface receptors to specific transcription factors. Signals from the TCR are sufficient to activate the Ras pathway, leading to ERK activation. In contrast, costimulatory signals are required for activation of JNK, and perhaps the p38 kinase. ERK, extracellular signal-regulated kinase; HPK1, hematopoietic progenitor kinase 1; PAK, p21-activated kinase; JNK, c-JUN N-terminal kinase; SAPK, stress-activated protein kinase; ATF2, activated transcription factor 2; MEF2C, myocyte enhancer factor 2C.

N-terminal regulatory domain of Raf-1 can interact directly with activated GTP-bound p21^{ras}. The role of Ras may be to recruit Raf-1 to the membrane where it is activated. In T cells, several observations collectively indicate that Raf-1 is one effector molecule for p21^{ras}: 1) constitutively active Raf-1 can mimic the effect of activated Ras and synergize with calcium signals to induce the IL-2 gene; 2) activated Raf-1 can also mimic the TCR and stimulate ERK-2; 3) a dominant negative Raf-1 prevents TCR stimulation of ERK-2.

ERK-2 is reported to regulate the Elk1 transcription factor and AP-1 activity. Phosphorylation of Elk1 by ERK appears necessary for the induction of the *c-fos* gene. Accordingly, the role of Ras in coupling the TCR to ERK-2 and the ability of ERK-2 to translocate to the nucleus where it can directly modulate transcriptional factors could explain the role of p21^{ras} in TCR signal transduction. Fos proteins are components of the AP-1 complex that is clearly important for NFAT activity and cytokine gene induction. However, not all RAS functions are mediated by the MAP kinase ERK-1/2 cascade. For example, in transgenic mice experiments, a dominant negative MKK can mimic inhibitory p21^{ras} mutants and suppress positive selection of thymocytes. However, inhibitory Ras mutants prevent TCR-induced proliferation of thymocytes, whereas an inhibitory MKK mutant does not. Also, in transfected T cell lines, expression of a dominant negative MKK can abrogate NFAT induction analogous to the effects of dominant negative Ras, but expression of a constitutively active MKK cannot substitute for constitutively

acute Ras and induce AP-1 or synergize with calcium dependent signals to induce NFAT.

Two additional MAP kinase signaling pathways have been characterized which allow parallel regulation of the kinases JNK-1/2 and p38 MAPK. These pathways appear to mediate cell survival or death in response to some forms of receptor activation. The role of the p38 MAPK in T cell activation remains unclear, although regulation of the JUN kinases 1 and 2 has been reported to be a point of convergence for the TCR and costimulatory CD28 signaling pathways. JNK-1/2 are activated by dual phosphorylation on threonine and tyrosine by SEK, a kinase homologous to MEK in the MAPK pathway. SEK is itself regulated by a serine/threonine kinase termed MEKK forming a cascade analogous to, but distinct from, that which links Raf-1 and MAPK. SEK conveys survival signals as studies with cells with genetically deleted SEK indicate that it protects thymocytes and T cells from TCR- and Fas-induced apoptosis. The activation of JNK-1 in T cells seems to require calcium/calcineurin signals, CD28 signals and other TCR-derived signals.

Costimulatory signals

Induction of cell cycle progression requires the sustained activation of PTKs, PLC and the Ras pathway. The affinity of peptide/MHC ligand to TCR, and especially the off rate, has important consequences for the nature and duration of the transduced signal, as does the receipt of one or more costimulatory signals delivered by the interaction between accessory receptors on the T cell and the antigen-presenting cell. Among the most well-studied of these accessory receptors are CD2, CD4/8, CD28 and CD152 (CTLA4).

CD2 is a single-chain glycoprotein of 47–55 kDa that is expressed on thymocytes, all T cells and on large granular lymphocytes. It plays roles in T lymphocyte adhesion and in signal transduction. A physiologic ligand for the CD2 receptor is CD58 (LFA-3), a glycoprotein found on the surface of many hematopoietic and nonhematopoietic cells. Select pairs of monoclonal antibodies to CD2 have been shown to be capable of activating PTKs and PLC, and of inducing a proliferative response, suggesting either that CD2 might be an antigen-independent signal transduction molecule, or that CD2 amplifies the TCR-mediated signal. In mice, injections of CD2 antibody induce a state of antigen unresponsiveness.

CD4 and CD8 function in MHC-restricted antigen presentation by binding to invariant regions of the MHC molecules. CD4 and CD8 transmit signals important for T cell activation and growth via the

associated protein tyrosine kinase, p56^{lck}. These coreceptor molecules provide an essential contribution for the TCR supramolecular complex (Figure 2). Recent studies suggest that the efficiency of CD4 recruitment to the TCR is a key factor in determining whether peptide–MHC complexes will display agonistic or antagonistic functions for CD4 cells.

The CD28 receptor family is the best understood of the costimulatory T cell receptors. CD28 is a 44 kDa homodimer and a member of the immunoglobulin gene family. It is an example of a heterophilic cell adhesion complex, in which its cognate receptors are the ligands CD80 (B7-1) and CD86 (B7-2). CD28 appears to regulate a signal transduction pathway that is distinct from the TCR in some aspects and has a major role in the induction of cytokine secretion and the expression of the cell survival gene Bcl-x_L. The CD28 cytoplasmic tail has no obvious enzymatic activity and unlike the TCR, it does not encode for ITAM motifs. The intracellular domain of CD28 does encode a (p)YXXM binding motif that can bind to the p85 regulatory subunit of phosphatidylinositol 3-kinase. It may be of clinical importance that CD28 signal transduction is resistant to a number of immunosuppressive agents. CD152 (CTLA4) is related to CD28 and shares the same ligands, CD80 and CD86. However, the signal transduction pathways used by CTLA4 appear to be distinct from CD28 and to primarily induce downregulation of T cell activation. *In vivo*, it is likely that CTLA4 has a major role in maintaining tolerance to self antigens.

CD154 (CD40L) is a type II membrane protein that is expressed on T cells. CD40L is not constitutively expressed on T cells and expression is induced by TCR signals, and in some cases, by costimulatory signals. It appears that CD40L has a role as a master regulator of the immune system for both humoral and cellular immunity. The role of CD40L is to signal antigen-presenting cells. This occurs by ligation of CD40 on antigen-presenting cells and precedes upregulation of the CD28 ligands CD80/CD86 on the antigen-presenting cell.

Anergic, agonistic and antagonistic signal transduction

T cells activated by the TCR in the absence of costimulation signals lose the capacity to secrete interleukin 2, a state called clonal anergy. Anergy induction appears to involve the calcium/calcineurin pathway as it is blocked by cyclosporine and can be partially mimicked by the calcium ionophore ionomycin. Analysis of CD3- and CD28-induced signal transduction has revealed that p21^{ras} remained unac-

tivated and found reduced ERK and JNK enzyme activities in murine anergic T cells. Thus a block in Ras activation may lead to defective transactivation of AP-1 sites in anergic cells and may enable T cells to shut down IL-2 production selectively during anergy.

Initial predictions were that the TCR would function as a simple 'on-off' switch for the T cell. Recent studies have indicated that T cell activation is not an all-or-none event, but rather involves a spectrum of biochemical events depending on the precise structure of the peptide-MHC ligand. Altered peptide ligands are analogs of immunogenic peptides in which the TCR contact sites have been substituted. These analogs may have agonistic, partial agonistic or antagonistic effects on T cells. Altered patterns of tyrosine substrate phosphorylation and selective activation of the calcium-signaling pathway are induced by altered peptide ligands. The mechanistic basis for altered ligand signaling remains unknown. The simplest notion to resolve the marked differences in cellular outcome between antigen ligands of differing affinity is to propose a time lag for the full assembly of the TCR signaling complex. Thus, the dissociation rate of the ligand from the TCR most likely determines whether antigen recognition will result in partial or full cellular activation. McKeithan has proposed a kinetic model of TCR signaling in which the complexity inherent in the TCR pathways serves to permit TCRs to finely discriminate between ligands. Some partial peptide agonists induce a state of T cell anergy while others induce altered patterns of cytokine secretion and fail to induce T cell proliferation. It is likely that signal transduction induced by altered peptide ligands has substantial physiologic importance as the pool of altered ligands *in vivo* is likely very large compared with the number of specific ligands that have selected the T cell repertoire.

See also: Anergy, B cell; CD2; CD4; CD5; CD8; CD28; CD40 and its ligand; CD45 (the leukocyte common antigen); Effector lymphocytes; Immunoglobulin gene superfamily; Integrins; Interleukin 2; Lymphocyte function-associated antigen 3 (LFA-3); Phorbol esters; Proliferation, lymphocyte; Protein kinases; Second signals for lymphocyte activation; Superantigens; T cell receptor, $\alpha\beta$.

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T LYMPHOCYTE CLONAL EXPANSION

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The most remarkable feature of the immune system is its ability to respond specifically to a potentially vast number of foreign pathogens. How can such a diverse repertoire of potential responses be generated and maintained in an organism? In 1957, Burnet and his colleagues proposed the clonal selection hypothesis, suggesting that individuals generate a multitude of lymphocytes that each can recognize and respond to a distinct antigenic determinant. If an antigen derived from a pathogen activates one of these pre-existing clones, only then does that cell proliferate to a high frequency (a clonal expansion). Then this expanded clone can efficiently eliminate the pathogen.

This theory has largely been proven for T lymphocytes and accepted as a cornerstone of immunology. When antigen is presented to a unique naive T lymphocyte with appropriate costimulatory signals, the T cell will rapidly proliferate and differentiate into an effector cell population. In addition, it is now known that the immune system tightly regulates both the extent of clonal expansion, as well as the functional activity of the differentiated T cell population.

Naive T cell expansion

T cells first mature in the thymus, and then emerge as CD4⁺ or CD8⁺ naive lymphocytes. The ability of these mature T lymphocytes to respond to a pathogen is dependent on the expression of a heterodimeric T cell antigen receptor (TCR). During their development in the thymus, TCR α and β genes each undergo an ordered and specific DNA recombination event that results in the generation of a unique TCR on each T cell. This enables each T cell to recognize only a specific antigenic peptide bound in the groove of a major histocompatibility complex (MHC) molecule which is expressed on the surface of an antigen-presenting cell (APC).

An immune response is initiated when a naive T cell recognizes and responds to a peptide bound to the MHC molecules of an APC within lymphoid tissue. Naive T cells express the homing receptor L-selectin enabling them to circulate between the blood, spleen and peripheral lymph nodes. Antigenic peptide derived from a pathogen enters the lymphoid tissue either through afferent lymph or via APC migration. Within the periarteriolar lymphoid sheaths of the spleen or paracortex of the lymph

node, naive T cells interacting with antigen and APC are induced to secrete the autocrine growth factor interleukin 2 (IL-2), resulting in a rapid expansion. The extent and duration of this expansion depends on both antigen dose, and on the affinity of the TCR for the peptide/MHC complex. The frequency of a naive clone of any given specificity is approximately 1 in 10⁶–10⁸, and expansions of 1200-fold over 6 days have been observed in the primary response *in vivo* (Figure 1).

To be fully activated, naive lymphocytes must receive costimulatory signals from the APC in addition to TCR ligation. These costimulatory signals result largely from the interaction of the CD28 molecule on the T cell and a B7 counter-receptor (B7-1 or B7-2) on the APC. The insult of infection induces APCs to express high levels of B7, and B7-derived costimulatory signals allow the presented peptides to be immunogenic. CD28 signaling in naive T cells promotes IL-2 production by enhancing both the activity of transcription factors (e.g. AP-1, NF κ B) at the IL-2 promoter and by increasing the stability of the IL-2 mRNA. This CD28 signal transduction remains poorly understood; however, it appears to be biochemically distinct from TCR signaling and may involve phosphatidylinositol 3-kinase, as well as the Jun N-terminal kinase (JNK).

Effector cell generation

During the course of a primary immune response, most antigen-stimulated T cells differentiate into an effector cell phenotype. Unlike naive cells that only produce IL-2, differentiated T cells develop the capacity to produce effector cytokines such as IL-4, IL-5 and/or interferon γ (IFN γ). The type of APC, the amount of antigen available, the affinity of the TCR for the antigen, and the lymphokine milieu all influence the T cell's subsequent cytokine production profile. Two principal effector phenotypes have been observed in both human and murine CD4⁺ cells (T_H1 or T_H2). T_H1 cells produce mostly IL-2 and IFN γ and are important in cell-mediated immunity by helping, for example, in both the differentiation and expansion of cytolytic precursors (CD8⁺) and in the generation of delayed-type hypersensitivity responses. In the presence of IFN γ and IL-12 (but in the absence of IL-4) differentiating lymphocytes tend to become T_H1 cells. On the other hand, T_H2 cell

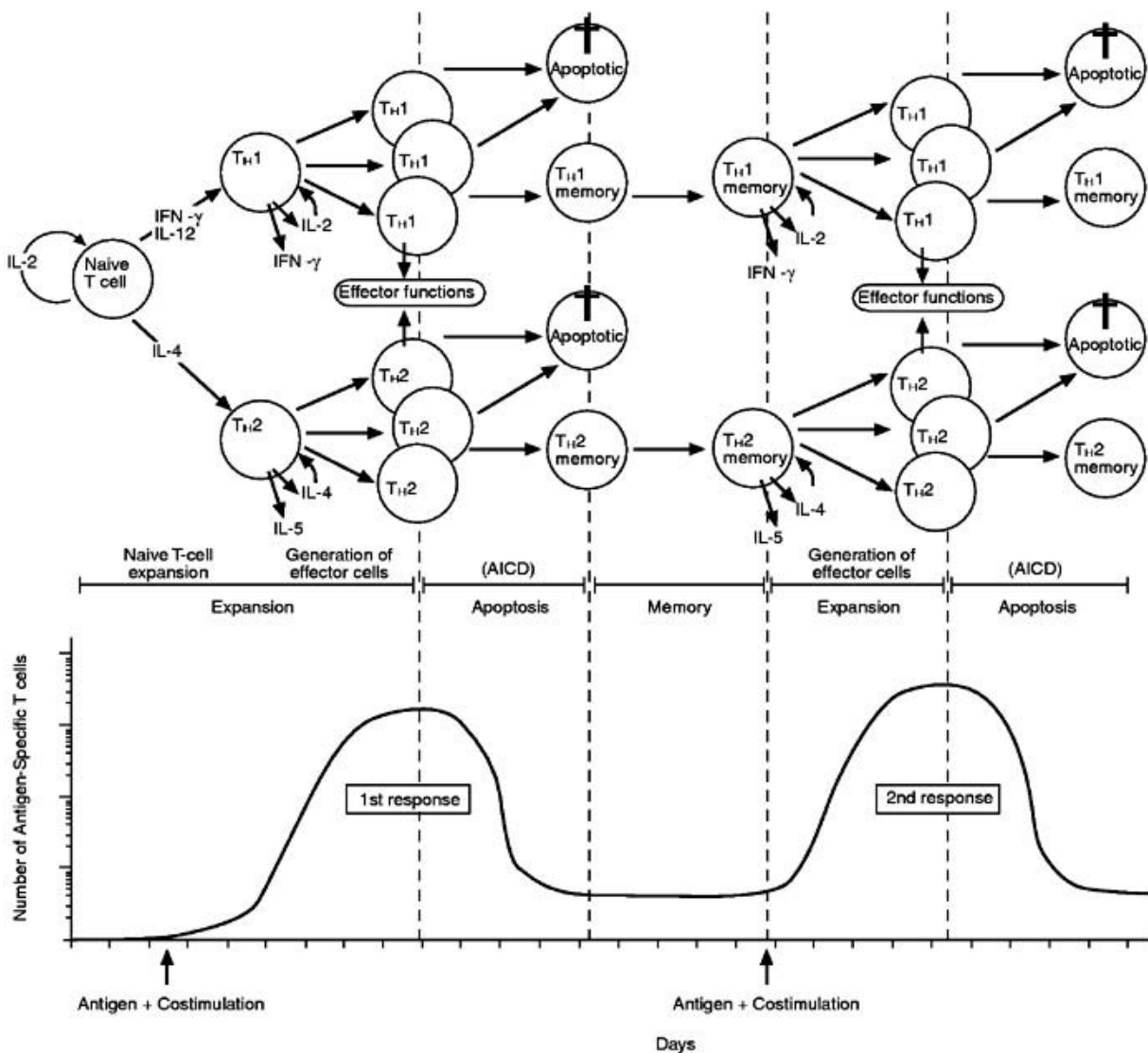


Figure 1 CD4⁺ T cell activation, expansion and differentiation that occurs during primary and secondary immune responses. The graph indicates the relative numbers of T cells that occur at each of the stages represented in the cartoon above. After exposure to the antigen and costimulatory signals, the cells first undergo differentiation to the TH1 or TH2 phenotype influenced by the cytokines IL-4, IFN γ , or IL-12. The cells continue to rapidly expand during the first generation of effector cells. Most of the effector cells then undergo an activation-induced cell death that appears to be mediated by the Fas and FasL molecules. A few T cells survive and are considered the memory cells and thought to be long-lived. The secondary response demonstrates many of the same properties of the primary response except that the T cells expand more quickly after exposure to antigen and costimulation and the peak expansion occurs more quickly as well.

development is enhanced by IL-4, and these cells produce IL-4 and IL-5 and are potent helpers for antibody production. CD8⁺ helper cells have also been found to fall into the same pattern of cytokine production after differentiation (TC1 or TC2 cells).

These differentiated T cells continue to expand when exposed to antigen, and this effector population then carries out its function to eliminate the antigen/pathogen. At the end of the primary T cell immune response there is tremendous cell death. Many studies *in vivo* indicate that the majority of

these effector cells exist for as few as 5 days. Nevertheless, it is clear that there remains a significant population of long-lived 'memory' cells following the response. These cells lack L-selectin and spend little time in the lymphoid structures. As a result of upregulation of integrin adhesion molecules (e.g. LFA-1, VLA-4) on their surface, they quickly leave the circulation and percolate through the parenchymal tissues to provide surveillance against infection.

Compared to the primary response, memory cells expand more rapidly upon activation and also reach

their peak expansion much more quickly. This allows for a more robust immune response following a second exposure to antigen. This gain of function is for two reasons: 1) the antigen-specific memory clone has an increased frequency within the lymphocyte repertoire (up to 30–40-fold) even after the contraction of the effector population; 2) these cells have enhanced responsiveness to APC due to increased expression of adhesion molecules and growth factor receptors.

Like naive cells, T_H1 - and T_H2 -type memory cells require costimulation for proliferation and may depend on it for continued survival. CD28 costimulation upregulates the expression of the death repressor Bcl- x_L , a molecule that enhances the cell's resistance to apoptosis. T_H1 cells also require CD28 signaling for maximum IL-2 production, whereas costimulation allows T_H2 cells to respond and proliferate better to their autocrine growth factor, IL-4. In contrast, costimulation does not appear as important for the effector functions of these highly differentiated cells, such as cytotoxicity, or IFN γ secretion. This may be important to allow them to target pathogen-infected cells that do not express any costimulatory ligands.

Negative regulation of expansion

Effector cells exist at high frequency only transiently during an immune response. Once the antigen/pathogen has been cleared (or in the event that it cannot be cleared), effector cells eventually stop proliferating and die. This negative regulation may prevent exceedingly strong and persistent immune responses that could be damaging to the host. In addition, the apoptosis of no-longer-needed effector cells may prevent the buildup of T cells that could dilute out naive responders and take up valuable space in the immune system.

The CTLA4 molecule appears to play a critical role in this negative regulation of clonal expansion. Unlike CD28, this receptor for B7 has been found to exert a negative effect on lymphocyte proliferation. CTLA4 expression is delayed following activation, corresponding to the time when the immune system begins to limit further lymphocyte expansion. Remarkably, CTLA4 homozygous-mutant mice (that do not express this molecule on their T cells) are found to have both an expansion of T lymphocytes and lymphoproliferative disease. Thus, ligation of CTLA4 by B7 may serve as an important control mechanism in limiting immune responses.

The principal mechanism for the eventual clearance of effector cells appears to be an activation-induced cell death (AICD). Persistent TCR signaling

causes effector T cells to slowly upregulate the death receptor Fas and its counter-receptor Fas-ligand (FasL). This expression results in death of the T cell and other Fas⁺ cells, as Fas signaling triggers apoptosis. Interestingly, the mutant mouse strains *lpr/lpr* and *gld/gld* have defective Fas and FasL, respectively, and their effector lymphocytes are resistant to AICD. This results in the accumulation of lymphocytes in these strains, which in turn appears to cause autoimmune disease. Thus, AICD may be essential to control the expansion of potentially deleterious effector cells.

Prevention of expansion

Although negative selection of T cell precursors in the thymus is effective in eliminating cells with strong reactivity to self antigens expressed in the thymus, not all self antigens are expressed there, and some are expressed only at later times in an individual's life (e.g. the human chorionic gonadotropin protein in pregnancy). Therefore, the immune system needs mechanisms to prevent expansion of potentially deleterious T cells that are responsive to these self antigens.

Like naive cells, memory cells that receive only the TCR signal without costimulation (CD28) fail to proliferate. More importantly, these cells can be induced into a functionally unresponsive state called clonal anergy, as a result of the stimulation. Anergic T cells are incapable of proliferating in response to antigen unless they are also exposed to an exogenous growth factor (IL-2). Proliferative anergy in T_H1 -like cells is caused by a defect in the production of IL-2. In mouse helper T cells, this appears to be related to poor activation of the AP-1 enhancer sequences within the IL-2 promoter. Recent studies further suggest that clonal anergy blocks Ras-dependent signaling through the extracellular signal-regulated kinase (ERK) and JNK protein kinases, preventing the induction of an AP-1 response. Anergic T_H2 cells, on the other hand, still produce their autocrine growth factor IL-4; however they are unable to grow in response to it. Finally, the failure to produce growth factors or respond to them probably reduces the capacity of anergic T cells to resist apoptosis, leading in some cases to the physical elimination of deleterious clones (Figure 2).

Clonal anergy may occur *in vivo* when a clone specific for a pathogen is also cross-reactive with a self antigen, so that after the pathogen is cleared, the immune system turns off the clone (anergy) perhaps until it may be needed again. Most nonhematopoietic tissues do not express costimulatory molecules even though they may be induced to express MHC mol-

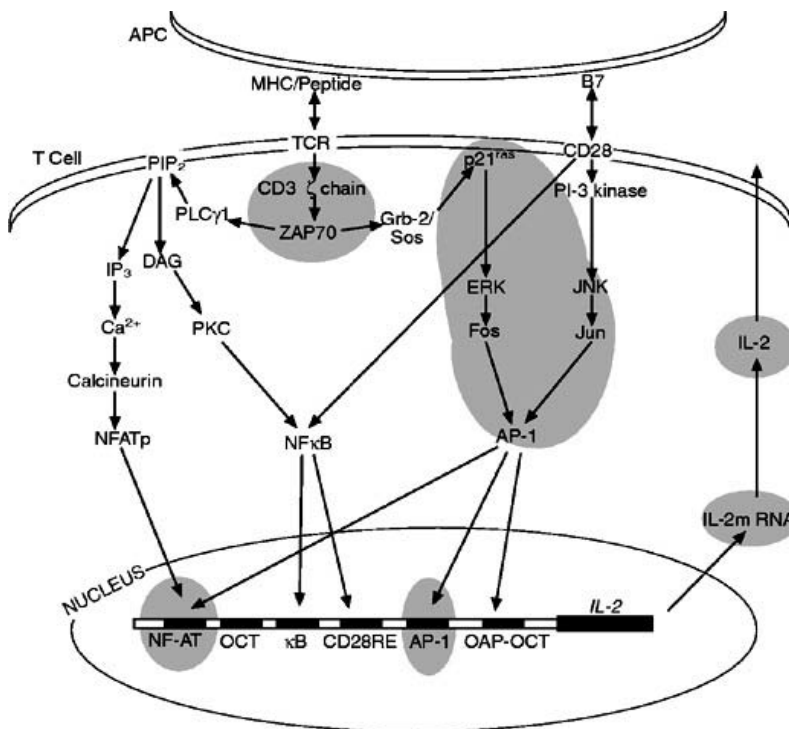


Figure 2 This model illustrates intracellular signaling pathways during T cell activation. T cell receptor (TCR) interacts with peptide on the MHC molecule of the antigen presenting cell (APC) to initiate signaling. Costimulator signals arise from CD28 on the T cell interacting with its counter-receptor B7 on the APC. These signals in the naive T cell result in production of the growth factor interleukin 2 (IL-2). Arrows indicate direction of activation signals, and gray-shaded areas are molecules that have been reported to be defective in anergic T_H1 T cells. TCR signaling involves the following molecules: ζ chain of CD3, phosphatidylinositol biphosphate (PIP_2), diacylglycerol (DAG), $p21^{ras}$, ZAP70, protein kinase C (PKC), Grb-2/son of sevenless (Sos), calcium ions, calcineurin, nuclear factor of activated T cells (NFATp), extracellular signal-regulated kinase (ERK), Jun-N-terminal kinase (JNK), Fos, Jun, AP-1 (heterodimer of Fos and Jun), nuclear factor- κ B (NF κ B). IL-2 upstream transcription sites include: NF-AT, OCT, κ B, CD28RE, AP-1, and OAP-OCT

ecules during an inflammatory response. Therefore, self antigens expressed on these tissues will tend to induce the anergic state.

Conclusion

The effector arm of the immune system is very dependent on the clonal expansion of T lymphocytes after their first interaction with antigen. It is through this clonal selection mechanism that the immune system is poised to respond to such a wide variety of foreign pathogens. Recent studies also demonstrate that the immune system tightly regulates the expansion of these clonal populations to prevent inappropriate responses. A better understanding of these regulatory mechanisms in the future should allow the immune response in human disease states to be modulated 1) to increase the kinetics or magnitude of the clonal expansion early during the course of infection, or 2) to blunt the growth and/or survival of harmful T cells responding against self peptides in the setting of autoimmune disease.

See also: Adhesion molecules; CD28; Cytokine genes, regulation of; Cytokines; Thymic epithelium: potential role in regulatory T cell tolerance; Effector lymphocytes; Lymphocyte trafficking; Memory, immunological; T lymphocyte activation; T lymphocyte differentiation; Tolerance, peripheral.

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T LYMPHOCYTE DIFFERENTIATION

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Two characteristic conditions distinguish the development of B lymphocytes from that of T lymphocytes. First, although both T and B cells develop from hematopoietic stem cells, B cells are generated in the bone marrow, while T cell differentiation occurs, at least early in ontogeny, most efficiently in the thymus. Although other prominent sites of T cell development have been described (most notably, the gut-associated epithelial tissues), intrathymic development is the focus of this review because much of our detailed knowledge of the many different stages T cells pass through before becoming an end-stage mature T cell has been acquired by studying that organ. The second feature that distinguishes T cells from B cells is determined by the distinct way in which T cells recognize antigens: B cells can recognize antigens in solution, while T cells can recognize foreign antigens only when they are presented, in the form of short peptides, by the body's own major histocompatibility complex (MHC) molecules. The thymus in fact has a dual role in establishing the particular features that distinguish T cells from B cells: it functions not only as a microenvironment supporting early T cell commitment steps, but also as a sieve which selects, out of the broad repertoire of randomly generated antigen receptors that developing T cells express, the ones with the appropriate MHC restriction specificity.

Many membrane proteins on T lymphocytes are involved in ultimately generating an immune response against the foreign antigenic peptides that are presented by the MHC molecules on the surface of antigen-presenting cells (APCs). These include many cell adhesion molecules and so-called costimulatory receptors, the coreceptors CD4 or CD8, and receptors for cytokines. It is, however, the clonally distributed T cell antigen receptor (TCR) which actually recognizes the antigenic peptides presented by a

given set of MHC molecules, and which therefore dictates the specificity of the response. This TCR forms a complex with nonpolymorphic membrane proteins (collectively referred to as CD3) which are noncovalently associated with the TCR and provide the TCR with signaling capacity. On most T cells, the TCR consists of two disulfide-linked chains (Figure 1) termed α and β , and cloning of the α and β genes revealed that generation of diversity among these chains is accomplished through a process of rearrangements of multiple variable (V) gene regions to diversity (D) and joining (J) segments associated with either one of two constant (C) region genes, in a process similar to that used during immunoglobulin gene rearrangements in developing B cells. A comparable type of TCR has been identified on small subsets of T cells: the chains composing this TCR are termed γ and δ , are also disulfide linked, generate diversity through rearrangements, are associated with the same set of nonpolymorphic CD3 proteins as the $\alpha\beta$ TCR, and can display similar effector functions upon TCR-mediated signaling as $\alpha\beta$ TCR-expressing T cells.

During the past 10 years, our understanding of the molecular mechanisms of T cell development has been dramatically increased through many key advances:

1. After the TCR α , β , γ and δ genes had been cloned, the order of appearance of TCR-gene rearrangements and transcription during thymic development could be determined, and from that information, studies on the regulation of these T cell lineage-determining events have been initiated.
2. Monoclonal antibodies (mAbs) against numerous T cell surface proteins represent another set of tools that proved invaluable: these allowed a detailed analysis of the order of appearance of

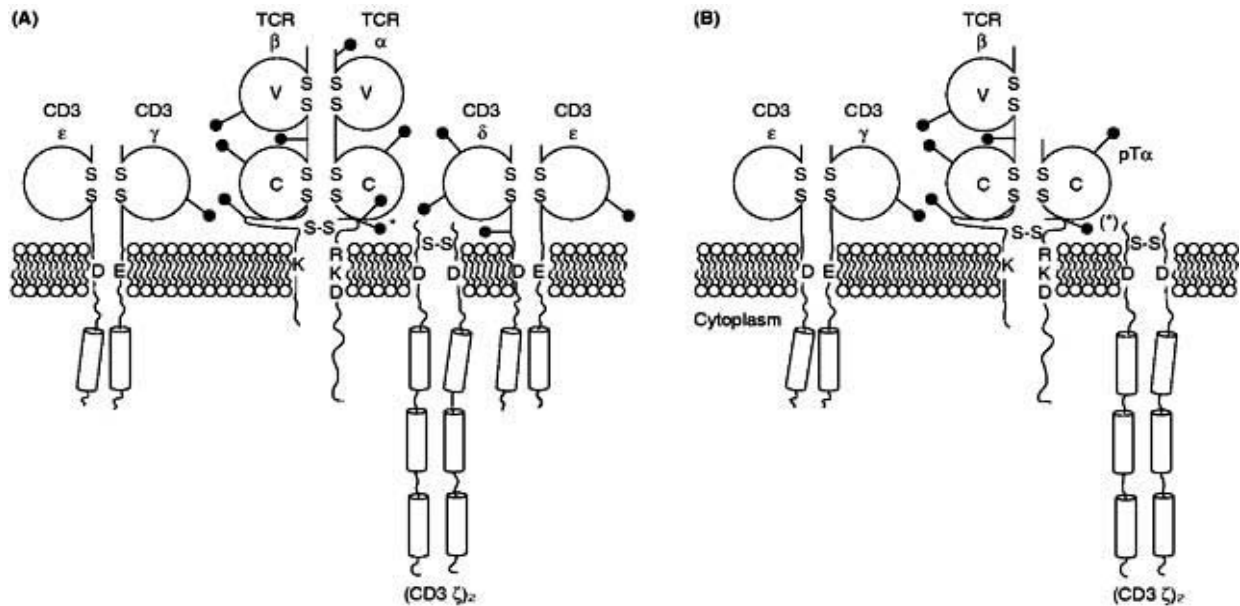


Figure 1 Models of the TCR (left) and pre-TCR (right).

surface structures critically involved in antigen recognition, cell adhesion and growth during development; this has resulted in the definition of an increasing number of intermediate differentiative stages amongst developing T cells (Table 1), with better prospects for gaining insights into the regulatory mechanisms a clear result.

- Once transgenic mice in which all T cells express the same TCR became available, direct visualization (by a combination of functional analysis and flow cytometry) of both the positive and the

negative selection process was achieved; also, transgenic introduction of model self antigens to specific sites, through the use of suitable regulatory elements, has allowed a better definition of the many different ways in which self tolerance can be acquired in the immune system.

- Perhaps the greatest contribution to our understanding of T cell development has been made by the application of gene-targeting experiments; molecules critically involved in defined T cell differentiative stages have been identified by

Table 1 Stages of intrathymic mouse and human T cell development

	Triple negative thymocytes (CD4 ⁻ CD8 ⁻ CD3 ⁻)				ISP	DP	SP
	Lymphoid progenitor	Pro-T	Early pre-T	Late pre-T	→	→	→
mCD117	++	+	-	-			
mCD44	+	+	-	-			
mCD25	-	+	+	-			
huCD34	++	+	+		±		
huCD38	-	+	+		+		
huCD1	-	-	+		+		
Progeny	B/T/NK/DC	T/DC	T	T $\alpha\beta$ /T $\gamma\delta$	T $\alpha\beta$		
RAG-I/II	±	+	++	+	+	++	-
TCR β	-	-	+	+	+		
TCR γ , δ	-	-	+	+	+		
pT α	-	-	+	+	+	+	-
TCR α	-	-	-	-	±	+	

ISP, immature single positive (CD4⁺ or CD8⁺ cells that do not yet express a mature TCR); DP, double positive (CD4⁺CD8⁺); SP, single positive (CD4⁺CD8⁻ or CD4⁻CD8⁺); DC, dendritic cells.

documenting at which stage T cell development was arrested in mice lacking such molecules (see **Figure 2**), and these studies have marked the 'checkpoints' that developing T cells go through to ensure that useless T cells do not complete differentiation.

Definition of developmental stages

In the mouse, the embryonic thymus is seeded by stem cells from the fetal liver around days 11–12 of gestation, while the adult thymus is repopulated by stem cells from the bone marrow. The human thymus is repopulated by stem cells from fetal liver around 8 weeks of gestation, while the bone marrow cells can function as a source of stem cells from 16 weeks of gestation onwards. Mature functional T cells can be identified in the developing mouse thymus around the day of birth (days 19–20 of gestation), and in the human thymus around 20 weeks of gestation. Although it is clear that the developmental pathways of fetal and adult lymphopoiesis are not identical, they follow by and large similar successive steps. The cell surface markers currently available allow, together with a few other essential features (**Table 1**), an overall distinction into two stages during embryonic thymocyte development: 1) an early pro- and pre-T cells stage during which commitment to T cell development is imposed, and 2) a later phase during which committed precursors further expand and differentiate.

Early phase

This major expansion phase, during which all thymocytes express a CD3⁺CD4⁺CD8⁺ (triple positive, TP) phenotype, marks the time that progenitors gradually lose their multipotent developmental potential, and become irreversibly committed to T cell development. The TP thymocyte subset is not homogeneous, as further subdivisions can be made on the

basis of size and expression of the CD44 glycoprotein, and the receptors for stem cell factor (CD117) and interleukin 2 (IL-2, CD25 (in the mouse)). Such cytokine receptors represent a crucial role for these types of growth factors in early lymphoid development.

In the human thymus, the markers CD34, CD38 and CD1 mark distinct progenitor populations within the TN compartment. Importantly, the earliest progenitors in the TN population are not yet exclusively committed to the T cell lineage, and progenitors that do not express CD25 (for the mouse) or CD1 (for the human) can still develop into a special subset of dendritic cells, and into natural killer (NK) and B cells. At the same time, these progenitors appear to have lost the ability to develop into the myeloid or erythroid lineages. For the mouse, irreversible commitment to T cell lineage development occurs sometime during the CD25⁺ stage and coincides with initiation of TCR β chain gene rearrangements: CD25⁺ cells that express CD44 still have the TCR β chain genes in germline configuration and can still become dendritic cells, while loss of CD44 coincides with appearance of extensive rearrangements. In the human, it appears to be the CD34⁺CD1⁺ stage that marks the committed T cell precursors, while a stage corresponding to the uncommitted CD25⁺ lymphoid progenitors in the mouse, with potential for development into dendritic cells, B cells and NK cells, is represented by the CD34⁺CD1⁺ population. What is important to realize in the type of precursor-progeny studies that have led to these nominations of developmental stages (**Table 1**), is that they have yet to be performed at the clonal level or with clonal markers. It is still possible that the studies that have led to the concept of a multipotential lymphoid progenitor for B, NK, T and dendritic cells, are in reality due to the circumstance that the cell populations used contain a mixture of committed precursors for dendritic, B, NK and T cells. Introduction of suitable marker genes in early progenitors should help resolve this issue in future studies.

The most pertinent feature of the early TN period of thymocyte development is, from the point of T cell commitment, the beginning of rearrangements and transcription of TCR gene loci. It is not clear, however, whether these rearrangements themselves determine T cell commitment, or whether they are linked to some yet to be defined control mechanism. When rearrangements are prevented, such as in mice lacking the recombinase-activating genes (RAG), T cell development is arrested at the early pre-T cell stage (**Table 1**). TCR gene loci are in germline configuration among the earliest progenitors, while the

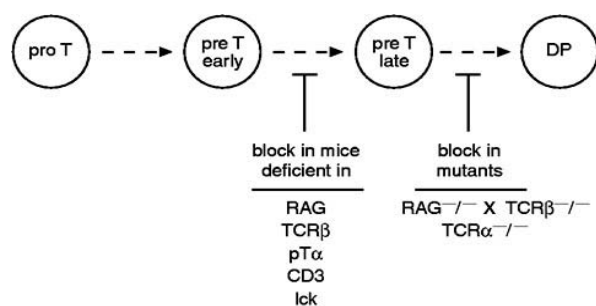


Figure 2 Scheme of T cell differentiation, summarizing the phases discussed in this article, and indicating the block in development observed in mice carrying the indicated mutations.

proportion of cells undergoing γ , δ and β chain rearrangements can be seen to progressively increase following RAG gene expression. Thymocytes with rearrangements in one TCR locus tend to have rearrangements in the other loci, such that a given cell apparently undergoes synchronous rearrangements of all TCRT loci. Yet, rearrangements do not occur simultaneously for all TCR loci: completion of certain defined V-D-J and V-J rearrangements in the δ and γ loci, respectively, is achieved earlier than that in the TCR β loci, while the β chain rearrangements occur later, and V-J α chain rearrangements are not even initiated until TCR β chain rearrangements have been completed. This ordered appearance of rearrangements is also reflected at the level of transcription: full-length γ and δ gene mRNA can be detected first, followed by β gene transcripts, and α chain mRNA coming last. Interestingly, preferential usage of particular V_γ and V_δ segments has been observed during early and late days of fetal ontogeny, fuelling suggestions that specific $\gamma\delta$ cells exhibit defined functions in early T cell differentiation.

The complete TCR β chain forms a heterodimeric pre-TCR complex with a nonpolymorphic glycoprotein termed 'pre-T α ' (see Figure 1), which becomes expressed at the cell surface and employs the CD3 chains for signal transduction. While the precise order of appearance of the different components of the CD3 complex (consisting of ϵ , δ , and γ chains, and $\zeta\zeta$ dimers; see Figure 1) is not known, it is clear that they are available for formation of the pre-TCR complex as soon as the TCR β chain and the pre-T α chain are expressed. There is some debate as to which CD3 components are expressed in the pre-TCR as compared to the mature TCR (Figure 1), but CD3 ζ , CD3 γ and CD3 ϵ have been positively identified in the pre-TCR. It remains to be established whether the different CD3 chains have unique or overlapping functions in T cell development, and other nonpolymorphic proteins associated with the pre-TCR are being identified.

In the adult thymus, a TN subset can also be found; it constitutes a minor (<5%) portion of all thymocyte subsets, and precursors of all T lineage cells (both CD4⁺CD8⁺, double positive (DP), and CD4⁺CD8⁻ or CD4⁻CD8⁺, single positive (SP) cells) can be found among the TN cells of both fetal and adult origin. Such TN cells have, upon i.v. transfer into irradiated recipients, the capacity to home back to the thymus where they differentiate in a single wave, indicating their limited self-renewal capacity. Both homing and precursor activity are contained within a subset of adult TN cells with a CD44⁺CD25⁻ phenotype, and these adult TN cells,

like their fetal counterparts, pass through a CD25⁺ stage before they lose CD25 and CD44 and give rise to DP cells. Again, this stage marks the loss of ability to generate NK and dendritic cells, and true commitment to T cell development.

Later phase

Completion of TCR α gene rearrangements, appearance of mature T cell surface proteins such as CD4 and CD8 at the cell surface, and upregulation of the TCR-CD3 complex at the cell surface mark the subsequent phase of thymocyte development. $\alpha\beta$ T cell precursors in fact do not enter this stage if they have not completed their TCR β gene rearrangements. Small numbers of thymocytes begin to express CD8 first (the so-called 'immature single positive stage', ISP in Table 1), and this is followed by appearance of CD4 expression, such that after both relative and absolute increases, the majority of thymocytes exhibit the double positive or DP (CD4⁺CD8⁺) phenotype. Early DP thymocytes express only low levels of the TCR-CD3 complex, but this gradually increases. The proportion of DP T cells expressing low levels of the $\alpha\beta$ TCR-CD3 complex progressively increases, and single positive (SP) CD4 or CD8 cells with notably higher levels of the $\alpha\beta$ TCR-CD3 complex finally appear. The precise function of DP cells was for many years unclear, but it has been conclusively demonstrated that these DP cells contain precursors for the SP thymocytes. T Cell functions such as cytotoxicity and cytokine production can be induced through receptor-mediated activation in a subpopulation of SP cells, so these cells have apparently differentiated to a mature stage.

During the final stage of T cell development, selection of the T cell repertoire has to occur (see also below). Potentially harmful cells, such as autoreactive cells, must be eliminated or inactivated (a process termed 'negative selection'), and events favoring preferential differentiation of T cells capable of recognizing foreign antigens in association with self MHC products (a process termed 'positive selection') must allow for the generation of useful T cells. It has been recognized for many years that final T cell selection for export is a consequence of interactions between surface proteins on immature thymocytes and the nonlymphoid elements in the thymus. Thymic selection must logically involve the MHC gene products (class I and class II), the $\alpha\beta$ TCR, and the CD4 and CD8 molecules, as antigen recognition by the $\alpha\beta$ TCR is facilitated by the CD4 and CD8 coreceptors. These coreceptors not only increase the affinity of interactions between T cells and MHC (CD4 molecules can bind to MHC class II, and CD8 molecules can bind to MHC class I), but also func-

tion as signaling molecules in mature T cells and immature thymocytes. Thymic selection can only begin after expression of the CD4, CD8 and $\alpha\beta$ TCR-CD3 complex has occurred, and consequences of its effects are therefore first observed in these late stages of thymic development. The contribution of adhesion and costimulatory receptors is only beginning to be addressed, as is the identification of the signaling pathways involved. Features yet to be accounted for include the fact that the T cell repertoire is nonrandomly expressed in the two main subsets of T cells, i.e. most MHC class II-restricted T cells are CD4⁺CD8⁻, and most MHC class I-restricted T cells are CD4⁻CD8⁺.

The nonlymphoid (stromal) cells that present the MHC-peptide complexes to developing T cells can be divided into two main cell populations: bone marrow-derived cells, such as dendritic cells, and non-bone marrow-derived cells, such as epithelial cells. Many experimental models have attempted to define whether positive and negative selection can be compartmentalized, i.e. whether specialized stromal cells can determine the fate of TCR-expressing thymocytes interacting with the MHC antigens expressed on the surface of stromal cells. Both types of stromal cells can express class II and class I MHC antigens, and earlier studies established some basic rules about the role of epithelial and hematopoietic cells in T cell selection.

Hematopoietic cells are responsible for the induction of clonal deletion in potentially self-reactive DP thymocytes, as a result of presumably high-affinity interactions between such DP cells and self MHC-peptide complexes. Since hematopoietic cells can also effectively function as APCs for activation of mature T cells, induction of clonal deletion is clearly not a consequence of intrinsic properties of such APCs. Rather, signals delivered by hematopoietic cells must be perceived differently by TCR-expressing DP thymocytes than by SP mature T cells, and it is clear that the genetic make-up of DP cells poises them for death. It has long been known that DP thymocytes do not display the same set of functions as mature T cells do upon TCR-mediated signaling, and death may be as pertinent a function for immature T cells as 'conventional' hallmarks of activation, such as cytokine production and cytolysis, for mature T cells. Nevertheless, signals delivered to DP cells by epithelial cells result in a different outcome: preferential differentiation (positive selection), if their TCR fits the MHC-peptide complexes presented with a presumably lower level of affinity. It is not clear which factors determine the difference between positive and negative selection, nor have any differences between signals delivered by epithelial cells and dendritic cells

been identified. Among the more likely possibilities currently studied is differential participation of costimulatory signals, and it will be a true revelation in immunobiology indeed when this fascinating puzzle is solved.

Crucial selection events in T cell development are determined by actions of the pre-TCR and TCR

Out of the diverse repertoire of TCR specificities generated through rearrangement events in the thymus, each individual selects those that are useful, i.e. able to recognize foreign peptides when presented by his or her own MHC, and not harmful, i.e. unable to see self peptides. T cells with an $\alpha\beta$ TCR learn, somewhere during their developmental pathway, how to recognize MHC molecules as presenters of foreign peptides, to be able to adequately fight off infections once the T cells have migrated out of the thymus. At the same time, T cells have to be able to 'see' the difference between MHC molecules presenting foreign peptides and those presenting self peptides: if they did not, harmful autoreactivity would be the result. The Nobel Prize for Physiology and Medicine 1996 went to Rolf Zinkernagel and Peter Doherty who, together with others, contributed much to our understanding of this learning process.

The ability to 'learn' the difference between self and nonself MHC is acquired in the thymus, and we call this screening process 'positive selection': it is thought that differentiating thymocytes are tested for their MHC restriction specificity by interactions between their TCRs with MHC molecules expressed on the thymic epithelium, and only T cells with a TCR that recognizes the MHC molecules expressed in the thymus (and thus elsewhere in the body) are allowed to mature further and migrate to the peripheral lymphoid organs. Insights into how the thymus allows for selective differentiation of self MHC-restricted cells were particularly well-visualized in studies with TCR-transgenic mice. In mice with either class I- or class II-restricted receptors, mature thymocytes develop only when the thymus expresses the MHC product for which the transgenic receptor has restriction specificity. The concept that interactions between TCR and MHC are crucial for positive selection to occur has been further extended with the finding that the appropriate MHC has to be expressed on thymic epithelial cells rather than hematopoietic cells (see above). Generation of DP thymocytes is not affected by the MHC, indicating that positive selection occurs late in the DP stage. The MHC restriction specificity of a given transgenic TCR determines the phenotype of mature T cells gen-

erated also in other ways: T cells with a transgenic class I-restricted TCR express mostly the CD8 phenotype, while CD4 T cells preferentially develop in transgenic mice with a class II-restricted TCR. Whether the TCR determines expression of CD4 or CD8 by instruction or selection is not clear. The requirements for TCR-MHC interactions in positive selection have meanwhile also been observed in normal mice, while antibody-blocking studies and gene targeting experiments designated the CD4 and CD8 coreceptors as additional participants in the interactions leading to positive selection. Much current research focuses on the identification of the signaling pathways that may be involved, and components of the MAP kinase pathway have already been identified as important mediators.

Before migrating to the periphery, developing thymocytes have to be screened for a second feature: they must not recognize thymic MHC molecules too eagerly, because then they would, if allowed to mature and migrate out of the thymus, cause harmful autoimmunity. Self MHC plus self peptide-reactive T cells are therefore eliminated in the thymus by clonal deletion, and this 'negative selection' process plays a major role in the acquisition of self tolerance, at least for those antigens that are expressed in the thymus, or for circulating native self proteins that have access to the thymus. The first major breakthrough in the understanding of acquisition of tolerance was made in 1987 by Kappler, Roehm and Marrack, when it became clear that T cells expressing an $\alpha\beta$ TCR with $V_{\beta}17a$ (identified with a specific mAb) exhibit preferential reactivity for a given class II MHC-peptide combination, regardless of the α TCR chain or J, N and D region sequences in their TCR. T Cells with such a $V_{\beta}17a$ TCR were found to be eliminated in mouse strains that possess a functional $V_{\beta}17a$ in their germline but express the particular MHC-peptide complex recognized by $V_{\beta}17a$. The elimination was evident among peripheral T cells, as well as among the $\alpha\beta$ TCR-expressing single positive thymocytes, but not much among the DP thymocytes, implying that deletion occurred at or after the DP stage of T cell development in the thymus. Subsequent studies generated additional correlations between expression of particular class II MHC-peptide combinations and deletions of T cells (both thymocytes and peripheral T cells) with defined V_{β} chains. Eventually, formal evidence for clonal deletion of developing thymocytes and T cells came from studies in TCR-transgenic mice expressing the appropriate antigens intrathymically. Clonal deletion originating in the thymus has thus been documented as a major mechanism for acquisition of self tolerance, although it clearly is not the only mechanism.

During the past few years, it has become clear that T cells are not only subject to selection processes late in their development, based on the specificity of their TCR, but also early on, at the time they have not even begun rearrangements in the TCR α locus. TCR β gene rearrangements are necessary for further maturation of $\alpha\beta$ T cells, which stops at the early pre-T cell stage (Figure 2) in mice that have been unable to produce a TCR β chain protein. How the TCR β chain may direct further expansion and differentiation has been suggested by recent data: at the surface of early pre-T cells, TCR β is disulfide linked to a nonpolymorphic protein termed 'pre-T α ' (pT α). This chain has potential signaling motifs, which, together with the signaling motifs of the CD3 proteins also associated with the TCR β /pT α dimer, endows this 'pre-TCR' with signaling capacity. Which types of ligand, if any, needs to be recognized by the pre-TCR has not been elucidated, but it does use the p56^{lck} tyrosine kinase to act as the mediator of what we could call the checkpoint of 'β-selection': no further proliferation and differentiation occurs when this pre-TCR cannot be formed or signal because crucial components are missing. A human counterpart of this checkpoint presumably exists, since human pT α has been cloned and its expression pattern corresponds to that in the mouse.

Summarizing, we now know that T cells with an $\alpha\beta$ TCR are subject to three selection steps during their maturation: β -selection (which checks whether further differentiation is useful; there is no point in initiating TCR α chain rearrangements and subsequent differentiation events if there is no TCR β chain), positive selection (which checks for ability to see thymic MHC once the mature $\alpha\beta$ TCR has been formed), and negative selection (removing potentially harmful T cells with an autoreactive $\alpha\beta$ TCR). Perhaps additional checkpoints will be defined in the future, and one putative checkpoint that is the subject of much research (and summarized below) is the point where the $\alpha\beta$ and $\gamma\delta$ lineages diverge.

Lineage commitments

A small percentage of fetal thymocytes (<5%) express a TCR-CD3 complex at an early stage of development when thymocytes still express a TN phenotype, but the TCR portions of these receptors are entirely composed of γ and δ chains (not to be confused with the invariant γ and δ chains of the CD3 portion of the TCR-CD3 complex). This is not surprising, since the TCR γ and δ loci are the only TCR gene loci having completed rearrangements by this time. This $\gamma\delta$ TCR-expressing subset remains a minor population of thymocytes throughout fetal

development and in adult life. Yet, it is not a homogeneous subset: it undergoes changes with respect to the V_δ and V_γ chains used, as a consequence of a developmentally regulated order of rearrangements in the γ and δ genes, and subsets of defined V_γ and V_δ -expressing cells home to certain tissues, such as skin, spleen, lung, intestinal epithelium and reproductive organs. It should be noted that such fetal $\gamma\delta$ TCR-CD3-expressing cells are already functional: both cytokine production and cytotoxicity can be induced through CD3-mediated signaling, and by these criteria, $\gamma\delta$ T cells represent the earliest mature T cells in the thymus, appearing several days before T cell function can be detected among the $\alpha\beta$ TCR-CD3-expressing SP cells.

Since $\gamma\delta$ T cells are expressed before $\alpha\beta$ T cells during fetal thymic ontogeny, the question arises whether $\gamma\delta$ cells might include precursors for $\alpha\beta$ cells. Could individual cells sequentially rearrange their γ and δ loci first and their α and β loci later? Or are $\alpha\beta$ lineage commitments largely independent of the status of prior γ and δ gene rearrangements? TCR γ , α and β genes are all unlinked, but the δ gene is located between the V_α and J_α sequences of the α locus. How commitment to either $\gamma\delta$ or $\alpha\beta$ development is regulated is a matter of much debate. Expression of a TCR β chain is an absolute determinant of T cell commitment, but not of $\alpha\beta$ versus $\gamma\delta$ diversification: V-DJ β rearrangements are frequently in-frame in $\gamma\delta$ cells. Compatible with a successive rearrangement model, V-JC γ rearrangements are often nonproductive in $\alpha\beta$ cells, suggesting that commitment is related to whichever rearrangement has been completed first. On the other hand, the frequency of productive V-DJ δ rearrangements in $\alpha\beta$ T cells has been found to be either random or nonrandom, making it difficult to decide whether the nature of the initial successful rearrangement dictates lineage commitment. It therefore remains to be determined at what point the $\alpha\beta$ and $\gamma\delta$ lineages diverge, and how the nature of the initial rearrangements is regulated.

Conclusions

The consequences of the positive and negative selection process have finally been clearly defined, most notably using TCR-transgenic mice and monoclonal antibodies that identify distinct TCR. Also, consensus has been reached with respect to the order of events during initiation of TCR gene transcription and rearrangements and appearance of the most critical T cell surface proteins during fetal thymocyte development. Yet, many questions remain to be answered, and those that are the subject of the most

active areas of research include those regarding selection and commitment:

1. What is the molecular nature of the signals leading to clonal deletion of immature thymocytes, and how are these different from signals leading to activation of mature T cells?
2. What is the nature of the signals involved in selection of the T cell repertoire that dictate the difference between positive and negative selection?
3. What is the nature of the signals leading to initiation and termination of TCR gene rearrangements?

Clearly, future research will be focused on identification of components of signaling pathways and regulatory molecules, and identification of lineage determining genes. These studies will no doubt reveal connections to developmental biology in other systems, a prediction illustrated nicely by the 'winged helix' class of gene regulators, which determine cell fate in *Drosophila*, while regulating development of the thymic stroma in mice.

See also: CD3; CD4; CD8; Clonal deletion; Cytokines; Thymic epithelium: potential role in regulatory T cell tolerance; Ontogeny of the immune response; Superantigens; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; Thymus; T lymphocyte activation; T lymphocyte clonal expansion; T lymphocytes; Tolerance, peripheral.

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T LYMPHOCYTES

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To define the term 'T lymphocyte' it is first necessary to provide a definition for the word 'lymphocyte'. These cells were originally defined by morphological criteria to have a large nucleus to cytoplasm ratio, with cytoplasm rich in free ribosomes, containing a number of mitochondria, a small Golgi area and few lysosomes. Lymphocytes are found in high proportions in the bloodstream, lymphoid organs and the lymphatics. They have been divided into small, medium and large, although their size distribution is continuous rather than trimodal.

The term T lymphocytes (or T cells) was originally given to 'thymus-derived lymphocytes' by Roitt and colleagues (1969) several years after the discovery by Miller (1961) of a population of circulating lymphocytes that was produced in this organ. This definition was used to refer to those lymphocytes that matured in, and were exported from, the thymus rather than cells that simply occupied this organ. Host cells found in the thymus are referred to as 'thymocytes'. These consist of T lymphocyte progenitors at various stages of differentiation.

Phenotype of the T lymphocyte

Defining the T lymphocyte as a 'thymus-derived lymphocyte' would be satisfactory if we were only interested in providing a semantic definition. But this does nothing for our ability to identify such cells once they leave the thymus. Since the discovery of a thymus-derived population of lymphocytes, there has been an explosion of immunohistologic reagents available for the characterization of different blood cell types.

This has allowed immunologists to phenotype various lymphocyte subsets. In this process, several cell surface markers have been found to be relatively specifically expressed on T lymphocytes. The primary marker of a T lymphocyte is its receptor for antigen; this is referred to as the T cell receptor (TCR). Most T lymphocytes express T cell receptors of the $\alpha\beta$ type, which function by recognizing antigen in the context of major histocompatibility complex (MHC) class I and class II molecules. A smaller subset of T lymphocytes express $\gamma\delta$ T cell receptors, the specificity of which is unclear. When we describe the T lymphocyte as a lymphocyte with a receptor for antigen, it is important to distinguish these cells from B lymphocytes, which also express receptors for antigen. There are, however, major functional differences between these populations of lymphocytes: B lymphocyte antigen receptors recognize antigen in its native state, and when these lymphocytes are activated they secrete their receptors as soluble molecules known as 'antibodies' or 'immunoglobulin'. In contrast, the receptors on T lymphocytes generally recognize processed peptide antigens bound to molecules encoded by the MHC. This is known as MHC restriction. Furthermore, activated T lymphocytes do not secrete their receptors but rather use them to recognize cell-associated antigens. At a phenotypic level, all T lymphocytes express the CD3 complex, which is associated with the T cell receptor and is required for signaling during antigen recognition. The CD3 complex is not expressed by B lymphocytes.

Most T lymphocytes in the mouse express Thy-1

(CD90) on their surface, which distinguishes them from B lymphocytes and is often used as a general marker for these cells. Thy-1 can be expressed by other cell types, however, and is not expressed by a minority of T lymphocytes. The other classical molecules expressed by T lymphocytes are the coreceptor molecules CD4 and CD8. These molecules are involved in recognition of relatively nonpolymorphic regions of MHC-encoded molecules and are expressed on mutually exclusive T lymphocyte subsets. CD4 is usually expressed by cells bearing MHC class II-restricted T cell receptors, whereas CD8 is expressed on MHC class I-restricted T lymphocytes. Once again, however, these markers can be expressed by a minority of other cell types and are not found on all T lymphocytes.

T lymphocyte development

T lymphocytes develop from bone marrow-derived cells that migrate to the thymus. The earliest thymic progenitor is the CD4^{lo}CD8⁻CD3⁻ thymocyte. This progenitor passes through many stages of maturation, including a CD3⁻CD4⁻CD8⁻ stage and then a CD3^{lo}CD4⁻CD8⁺ stage, before becoming a fully mature CD3^{hi}CD4⁺CD8⁻ or CD3^{hi}CD4⁻CD8⁺ T lymphocyte. Early in this process, before the CD3^{lo}CD4⁺CD8⁺ stage, there is a branch in this pathway towards development of a subset of $\gamma\delta$ T lymphocytes. Other subsets of $\gamma\delta$ T lymphocytes develop in the thymus during fetal but not later life.

T lymphocyte function

Functionally, T and B lymphocytes perform the role of cells that, upon specific encounter with antigen, are activated to provide immunological functions usually associated with fighting infections. These cells are also responsible for the specific memory aspect to immune responses, such that secondary encounter with the same antigen results in a more rapid and aggressive immune response. While B lymphocytes are responsible for the secretion of antibody molecules, T lymphocytes are the central players in cell-mediated immune responses. CD4⁺ $\alpha\beta$ ⁺ T lymphocytes are the major subset of T lymphocytes and are classically referred to as helper T cells because they provide 'help' to B cells for the production of T-dependent antibodies. This subset of T lymphocytes recognizes class II-restricted antigens and plays a pivotal role in most types of immunity – especially in inflammatory cell-mediated responses. They are responsible for production of many different cytokines and, when stimulated by antigen, develop into activated cells that can be loosely divided into two

broad subsets, T_H1 and T_H2, based on the types of cytokines they secrete. T_H1 cells produce interferon γ (IFN γ), interleukin 2 (IL-2) and lymphotoxin (tumor necrosis factor β , TNF β), whereas T_H2 cells produce IL-4, IL-5, IL-6, IL-9 and IL-10. CD4⁻ T lymphocytes can also be cytotoxic, but this function is primarily the responsibility of the other major subset of T lymphocytes, the CD8⁺ $\alpha\beta$ ⁺ T lymphocytes. These class I-restricted cells are generally referred to as cytotoxic T lymphocytes or killer T cells and are primarily responsible for the killing of viral-infected or tumor cells. CD8⁺ T lymphocytes also secrete various cytokines and have been loosely divided into T_C1 and T_C2 cells on the same basis as described for T_H1 and T_H2 cells. Induction of effective cytotoxic T lymphocyte responses may require 'help' from CD4⁻ T lymphocytes.

There are also a number of $\gamma\delta$ T lymphocyte subsets, but the function of these cells is less well defined and beyond the scope of this description.

Some problems with this definition

As with all definitions, it can be somewhat problematic when taken too literally. Lymphocytes can be broadly divided into three groups: the B lymphocytes, the T lymphocytes and natural killer (NK) cells. NK cells also have receptors for antigens, but the antigens these receptors recognize appear to be the MHC molecules themselves. In contrast to T lymphocytes, these cells are activated when their receptors fail to encounter such molecules on target cells. Thus, NK cells kill cells that do not express MHC molecules. NK cells are not classically encompassed by the definition of the T lymphocyte, but they can be generated in the thymus, thus fulfilling the definition of a thymus-derived lymphocyte. However, they do not express the CD3 complex, which is expressed by $\gamma\delta$ and $\alpha\beta$ T lymphocytes. This criterion becomes more relevant when we consider that small numbers of lymphocytes with indistinguishable properties to those of T lymphocytes can be generated extrathymically. For example, in nude mice, which do not possess a thymus, there are some T lymphocytes present in the circulation.

Thus, while the classical definition of the T lymphocyte is a 'lymphocyte that is derived from the thymus', such cells may be more accurately described as 'lymphocytes that express the T cell receptor and CD3 complex and are generally derived from the thymus'.

See also: B lymphocytes; CD3; CD4; CD8; H2 class I; H2 class II; Lymphocytes; T cell receptor, $\alpha\beta$; T cell receptor, $\gamma\delta$; T cell receptor, recognition by; Thy-1; T lymphocyte differentiation.

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T LYMPHOCYTES, AUTOREACTIVE

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Diverse mechanisms contribute to immune tolerance of self antigens. Because of the importance of this immune function the appearance of autoreactive T cells in various diseases is regarded as a failure of normal regulation. While this is certainly true in the case of pathological manifestations, there is substantial evidence that autoreactive T cells are a normal and even necessary part of the immune system. An important distinction to make here is between the 'inappropriate' activation of resting T cells by self and the continued stimulation and expansion of T cells that have previously and appropriately been activated by foreign antigen. Cells of the former type, specific for well-defined tissue antigens, have been described in certain disease models. This entry focuses on the latter population, normal T cells specific for foreign antigens that can also give rise to autoreactive progeny.

Origin

The origin and persistence of autoreactive T cells is most readily understood from a consideration of the requirements for T cell maturation. The mature peripheral T cell population is restricted to recognize foreign antigens in association with only those major histocompatibility complex (MHC)-encoded molecules expressed in the thymic differentiation environment. Since foreign antigens are not, in general, present in that environment, this clearly means that at an early stage of differentiation T cell precursors are self reactive. It is presumed that some process of negative selection prevents seeding any very high

affinity self reactive clones in the mature population. Nevertheless, antigen-specific, MHC-restricted peripheral T cells must derive from thymic precursors that express a functional affinity for self MHC, possibly in association with other self molecules. The basis for this transition from an autoreactive thymic precursor to an antigen-dependent, MHC-restricted peripheral T cell is not understood. Present evidence suggests that this transition cannot be explained by somatic mutation of rearranged T cell receptor genes. It appears, therefore, that T cell maturation must be accompanied by physiological changes that modulate T cell activation signals or activation thresholds such that self MHC recognition does not, in general, suffice to induce clonal expansion of resting peripheral T cells. Such changes could reflect properties of mature T cells relative to thymic precursors as well as properties of the stimulatory environment in secondary lymphoid tissues relative to the thymus. The nature of these physiological changes is not known and their stability cannot be predicted. A considerable body of evidence suggests, however, that some mature T cells either have retained or reacquired the ability to be stimulated by self MHC in the absence of foreign antigens.

Autoreactive T cells and T cell lines derived from normal individuals have been independently described in many laboratories. Such T cells are activated in the absence of any identifiable foreign antigen by class II MHC syngeneic but not MHC allogeneic stimulators. In particular, autoreactive responses have been demonstrated in the absence of

xenogeneic serum components. It has been suggested that such T cells manifest the autoreactive specificity that was the basis for positive selection in the thymus. This is supported by evidence that autoreactive T cells can derive from antigen-specific precursors. It has been shown that, under well-defined conditions, the specificity of autoreactive T cell clones selected from antigen-primed populations reflects the predominant MHC restriction specificity of T cells specific for the immunogen. Thus, I-E subregion specific autoreactive T cells are detected at a much higher frequency in populations primed with a foreign antigen whose presentation is I-E-restricted than in populations primed with antigen whose presentation is predominantly I-A-restricted. A striking confirmation and extension of these observations has been noted in the analysis of T cell receptor gene expression in autoreactive T cell clones selected from pigeon cytochrome *c*-stimulated populations. The diversity of germ-line T cell receptor variable region genes expressed in the pigeon cytochrome *c*-specific T cell repertoire is relatively limited. It has been observed that autoreactive T cells also arise in pigeon cytochrome *c*-stimulated T cell lines and that the same T cell receptor genes predominate in these autoreactive T cells as in the antigen-specific T cells. These results strongly support the conclusion that autoreactive T cells can derive from antigen-stimulated precursors.

Such 'normal' autoreactivity may represent a particular physiological state that recapitulates the conditions of thymic selection and is induced in many antigen-specific, MHC-restricted peripheral T cells as a result of normal antigen-dependent activation. In keeping with the suggested physiological difference, there is evidence that autoreactive T cells have unique activation requirements distinct from the activation requirements of antigen-specific, MHC-restricted T cells. In particular, it has been shown that autoreactive T cells are more dependent on interleukin 1 (IL-1) and on signals transduced through recognition of MHC class II on the stimulator cell than are antigen-specific T cells.

Significance

Even if autoreactivity is a transient physiological state in activated normal peripheral T cells, it could make a substantial contribution to clonal expansion and amplification of immune functions. The functional capabilities of autoreactive T cells have been investigated in a number of laboratories. In general, they have been found to provide the same physiologi-

cal signals as other CD4⁺ T cells. Both lymphotoxin and interferon γ (IFN γ) producing autoreactive T_{H1} and IL-4 secreting autoreactive T_{H2} have been isolated. Examination of the helper functions of both murine and human autoreactive T cell clones has clearly demonstrated that these T cells can provide helper signals that serve to enhance B cell proliferation and maturation of immunoglobulin secretion. Autoreactive T cells that secrete other lymphokines, including lymphotoxin and IFN γ , may play an important role in inflammatory responses. *In vivo* injection of autoreactive T cell clones in footpads of syngeneic mice induced delayed-type hypersensitivity reactions in the dermis. In addition, autoreactive T cell clones with cytotoxic activity (lymphotoxin secretion) induced skin lesions with infiltration of lymphocytes in the epidermis and epidermal cell damage.

As suggested by these observations, autoreactive T cells could play a role in both normal and pathological immune responses. Their contributions *in vivo* cannot be fully evaluated until the mechanisms that must limit their expansion have been identified. The available evidence suggests that autoreactive T cells derive from mature antigen-dependent precursors which have undergone a physiological transition that restores their thymic selected ability to respond to self. This could be a normal reversible transition which frequently accompanies specific T cell activation. In this case any mechanism that returns T cells to a resting state would eliminate autoreactive expansion until another cycle of stimulation is induced by a specific foreign antigen. Under conditions of chronic stimulation, when high levels of MHC class II are continuously expressed and/or immune regulation is abnormal, autoreactive T cells could give rise to severe and persistent inflammation.

See also: Thymic epithelium: potential role in regulatory T cell tolerance; Effector lymphocytes; H2 class II; T lymphocyte clonal expansion; T lymphocytes.

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TNF RECEPTORS

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In multicellular organisms immune defense mechanisms are coordinated, as, in fact, are all other cellular functions, by receptors – proteins that are capable of binding specific extracellular ligands. This regulation occurs by activation of signaling cascades, a series of allosteric modulations initiated by the binding of the ligand to its receptors and terminating in activation of intracellular enzymes that affect specific mechanisms of defense.

The activities regulated by the tumor necrosis factors (TNFs) and their receptors are multitudinous. Some of the signaling pathways involved in their function already existed very early in evolution (e.g. the stress-activated protein kinase 2 (p38/RK kinase) cascade and its yeast analog, the Hog kinase pathway). The TNFs themselves and their receptors have so far been fully characterized only in mam-

mals. However, there is some evidence that they occur in various other organisms as well, and also appeared rather early in evolution. Understanding of the role of the TNFs in immune defense is still limited. Nevertheless, our knowledge of the mechanisms by which they act has been greatly advanced through studies of the TNF receptors and their mode of signaling.

The TNFs are produced mainly by cells of the immune system. Their receptors, however, are of wide occurrence. Although they are expressed by various professional immune cells and regulate their functions, they are much better known for their action in cells that are not considered as part of the immune system, such as endothelial cells and fibroblasts. They are best known for their ability to induce activities of cells that participate in inflam-

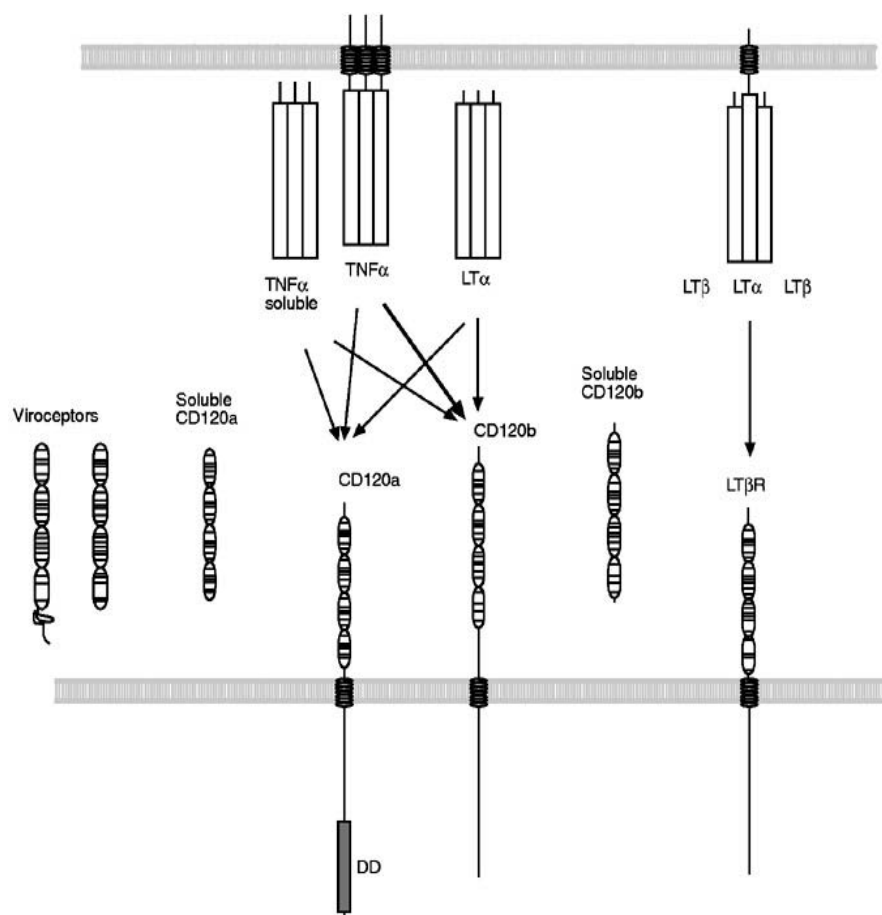


Figure 1 Interactions of the known cell bound and soluble TNF receptors and ligands. DD, death domain.

matory processes, as well as for the various destructive effects, particularly the cytotoxic effects, that they can trigger.

The receptors and their binding properties

The terms TNF and TNF receptors relate respectively to a number of ligands and receptor molecules that can interact in different ways (see Figure 1). Three structurally related ligands, TNF α , lymphotoxin α (LT α) (or TNF β) and LT β , and three receptors with structurally related extracellular domains are currently known to operate in this response system. Two of the receptors have received CD designations, though other names are also still in use. One of these receptors is designated CD120a (or the type I, the p55 or the p60 receptor) and the other CD120b (or the type II, the p75 or the p80 receptor). The third is termed the LT β receptor (LT β -R), the type III TNF receptor or TNFR-RP. Both the ligands and the receptors belong to growing families of structurally related molecules, known as the TNF-ligand and the TNF/NGF receptor families.

TNF α and LT α have been shown to act as homotrimers, and this is apparently true for all other members of the TNF-ligand family. Another common feature of most of these ligands, including TNF α and LT β , is that they are functionally active not only as soluble molecules (like most of the known cytokines) but also as cell surface, type II transmembrane proteins, from which the soluble forms are derived proteolytically. LT α is the only known ligand of the TNF family which is released by cells as a secreted protein. It has the ability to bind to LT β molecules and thus, even though formed as a soluble molecule, it also occurs in a cell-bound form, comprised of heterotrimers of LT α and LT β (see Figure 1). As discussed below, both the ligands and the TNF receptors occur in cell-bound as well as in soluble forms, the latter being derived from the cell surface forms following cleavage by cell bound metalloprotease(s).

The known interactions between the three ligands and the three receptors are illustrated in Figure 1. Both CD120a and CD120b can bind to the cell-bound as well as to the soluble forms of homotrimers of TNF α and LT α . CD120b, however, binds more effectively to the cell-bound form of TNF α and is preferentially activated by it. The LT β -R binds only to heterotrimers of LT α and LT β .

Location

Most of our knowledge of the cellular expression of the TNF receptors is based on their occurrence in

cultured cells; little has been done so far to assess their expression *in vivo*. From the cell culture studies it appears that almost all cells express TNF receptors, though the proportion of the different receptors on the cells varies. CD120a is constitutively expressed in almost all cells; indeed, its promoter displays house-keeping features. In contrast, the expression of CD120b is quite restricted. It is constitutively expressed in certain hematopoietic cells, such as lymphocytes and mononuclear phagocytes, where its amounts may greatly exceed the amounts of CD120a. Many other cells express little of this receptor, but can be induced to express it by certain stimuli (e.g. TNF itself and interleukin 1). Almost nothing is known about the cellular pattern of expression of the LT β -R.

Structure

CD120a, CD120b and the LT β -R are single transmembrane type I proteins. The three receptors bind to their ligands via a shared extracellular repetitive cysteine-rich motif that characteristically occurs also in the other members of the TNF/NGF receptor family. Their intracellular domains are structurally unrelated. Several functionally distinct regions have been defined in these domains. Of these, the only one found so far to show sequence homology with regions in other proteins is the 'death domain' (DD), a protein-binding motif of about 80 amino acids, found in CD120a. This region is involved in self-association of CD120a as well as in its association with other proteins, including some that also contain DDs and bind through their DDs to the DD in CD120a (see below).

Function

CD120a

Selective triggering of CD120a in cultured cells by antibodies to the receptor results in the induction of a wide range of TNF effects, including cytotoxic, growth stimulatory, antiviral, antichlamydial, prostaglandin-inducing actions and others. In fact it appears from these experiments that most of the known effects of TNF are triggered by this receptor. Accordingly, knockout mice for CD120a are deficient in several of the most pronounced *in vivo* activities of TNF. For example, these mice are resistant to the lethal effect of bacterial lipopolysaccharide (measured under conditions of sensitization to this effect, i.e. when hepatic RNA synthesis is blocked). They also display a highly increased vulnerability to certain intracellular pathogens, such as *Listeria*.

CD120b

As the prevalent TNF receptor in professional immune cells such as lymphocytes and monocytes, and apparently the only receptor expressed by them in certain differentiation states, CD120b is likely to play an important role in the immunoregulatory activities of the TNFs. However, present knowledge of its function lags significantly behind knowledge of the function of CD120a. Studies of CD120b function in cultured cells indicate that this receptor 1) assists the binding of TNF to CD120a; 2) signals, in certain cells, for effects identical to those triggered by CD120a, e.g. cytotoxicity, activation of the transcriptional factor NF κ B, and growth enhancement. As mentioned above, CD120b was recently shown to be preferentially triggered by the cell-bound form of TNF, suggesting that it acts primarily as a mediator of paracrine TNF effects (effects induced in the close vicinity of the TNF-producing cell).

LT β receptor

Because this receptor has been known only for a relatively short time, knowledge of its function is still very limited. Only two *in vitro* effects of its triggering have been observed so far: activation of NF κ B and, in some cells, induction of a cytotoxic effect. Although at the time of writing LT β -R^{-/-} mice had not yet been produced, indirect information on the *in vivo* function of the receptor could be gained by comparing the phenotype of LT α ^{-/-} mice to those of CD120a^{-/-} and CD120b^{-/-} mice. The data point to a unique role of LT β -R *in vivo*, which is distinct from those of the two other TNF receptors, namely, signaling for effects which are needed for the embryonic development of peripheral lymph nodes.

Signaling mechanisms

The TNF receptors signal for both gene activation-dependent and -independent effects. Knowledge of the signaling mechanisms for both kinds of effects, though still fragmentary at present, is increasing rapidly. Several transcription factors involved in the gene activation-dependent effects of the receptors have been identified. The most extensively studied is NF κ B, a transcription factor (or rather a family of related factors) which participates in the transcriptional regulation of many genes related to defense. Studies of the cytotoxic effect, the best characterized of gene activation-independent TNF effects, revealed a critical role of several ICE/CED3 proteases in this death induction, resembling in that sense other programmed cell death processes. Several protein kinases whose activities change rapidly after receptor

triggering have been identified, including those comprising the three currently known main mitogen-activated protein (MAP) kinase cascades (the stress-activated protein kinase 1 (JUN N-terminal kinase, JNK), stress-activated protein kinase 2 (p38 kinase), and the extracellular signal-regulated kinase (ERK) cascades). In addition, several lipases, including a phosphatidylcholine specific phospholipase C and the cellular phospholipase A₂, are activated by the receptors. Signaling by the TNF receptors is initiated as a consequence of interaction of two or three receptor molecules, caused by their binding to the trimeric ligand molecules (or by cross-linking with TNF receptor-specific antibodies). Triggering results in recruitment of several adapter and signaling molecules.

The known receptor-associated adapter proteins and some of the signaling molecules that they activate are illustrated in Figure 2. They include a group of structurally related adapter proteins called the TRAFs (TRAF1, 2, 3 and 5), which bind through a shared C-terminal sequence motif (termed the TRAF domain) to distinct regions in CD120b and LT β -R (though not to CD120a). They also include three adapter proteins, TRADD, MORT1/FADD and RIP, which contain DDs and bind through them to the DDs in CD120a and in the structurally and functionally related receptor CD95 (Fas/Apo1). These three proteins do not bind to CD120b or to the LT β -R. Both TRAF2 (which binds to CD120a and to the LT β -R, as well as to some other members of the TNF/NGF family, such as CD40) and TRAF5 (which binds to the LT β -R) are involved in the activation of NF κ B by these receptors. TRADD, a DD-containing adapter protein that binds to CD120a, is involved in cell death induction, which most probably occurs through the binding of its DD to that of MORT1/FADD, a DD-containing adapter protein that binds directly to Fas/Apo1. TRADD is also involved in the activation of NF κ B by CD120a, most likely through interaction of the region upstream of its DD with TRAF2.

Events further downstream in the death-inducing cascade include recruitment of a CED3/ICE protease called MACH (or FLICE or Mch5 or caspase-8) to MORT1/FADD, and its consequent activation. They also include activation of several other ICE/CED3 proteases, including CPP32/Apopain/Yama (caspase-3) and Mch3/ICE-LAP3 (caspase-7), which is most probably a consequence of activation of MACH/FLICE. Activation of the acid sphingomyelinase, phospholipase A₂ and JUN kinase by the TNF receptors may also participate in the death-induction process. The most crucial events further downstream in activation of NF κ B by the receptors

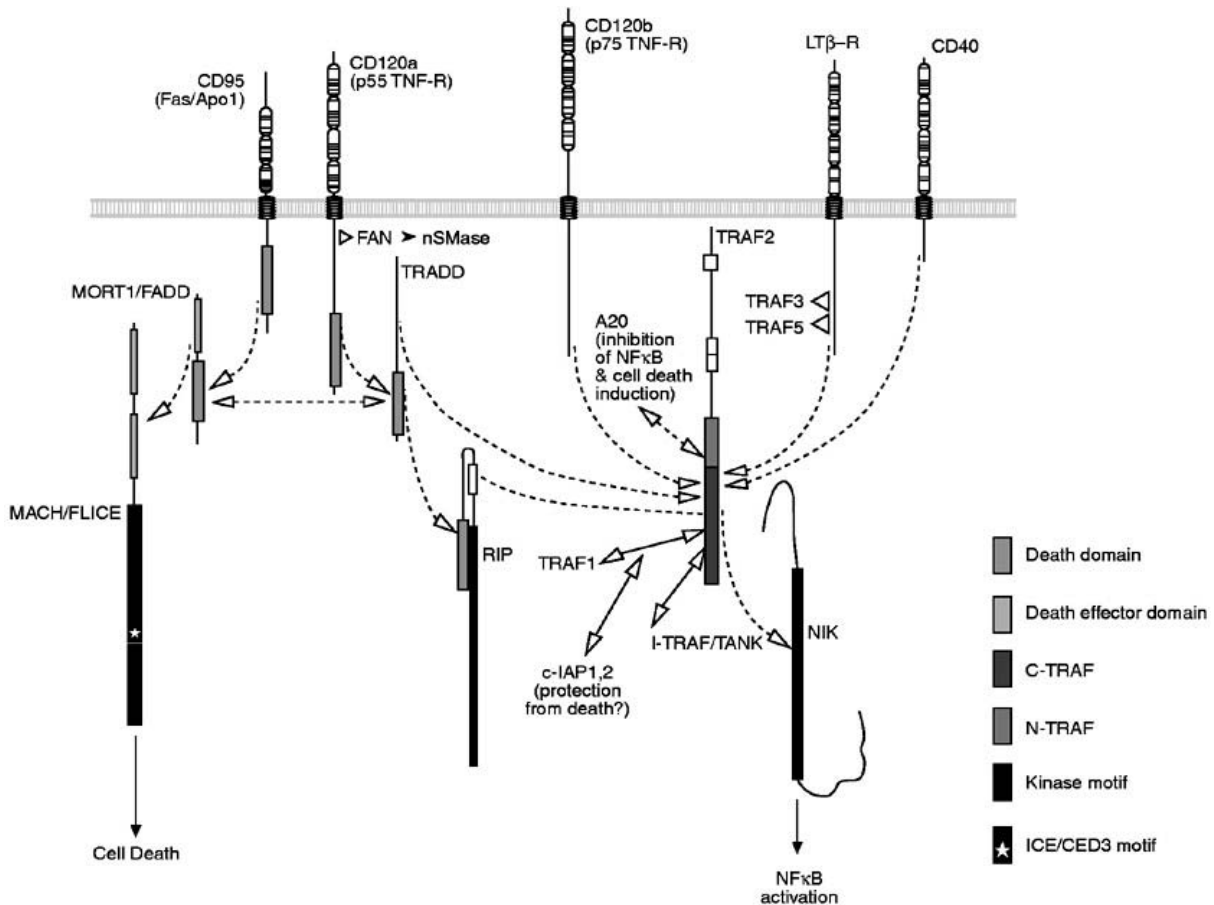


Figure 2 Signaling molecules that bind to the intracellular domains of the TNF receptors and some of their functions.

are the induced phosphorylation and consequently the proteolytic degradation of I κ B. The exact sequence of molecular events leading to this phosphorylation is not yet known, except that it appears to involve the participation of TRAF2 and a protein called TANK or I-TRAF, which binds to TRAF2. A MAP kinase kinase kinase called NIK, which binds to TRAF2 and apparently participates in NF κ B activation, was recently identified.

A few TNF receptor-associated adapter proteins that signal for other TNF effects have also been identified. One of these is FAN, an adapter protein that is apparently involved in the TNF-induced activation of the neutral sphingomyelinase. FAN binds to a distinct region in the CD120a intracellular domain upstream of the DD.

Taken together, the emerging molecular picture reveals a rather wide diversity of receptor-activated signaling cascades, consistent with the wide range of activities that these receptors are able to trigger. Another interesting feature is an extensive interac-

tion among the molecules that participate in the signaling activities of the three TNF receptors, as well as between these molecules and those that participate in the signaling for effects of other receptors of the TNF/NGF family. Such interactions apparently account for the ability of these different receptors to induce many common effects.

Soluble TNF receptors and their therapeutic uses

There is a growing list of pathological situations in which much harm is caused by the effects of TNF, and where blocking of TNF function can be therefore beneficial. One of the current approaches in the attempt to inhibit TNF functioning in disease involves injection of soluble TNF receptors. This approach reproduces a naturally occurring control mechanism. In various inflammatory processes, serum concentrations of the soluble TNF receptors are greatly increased as a result of their proteolytic

cleavage triggered by various stimuli associated with inflammatory processes, such as TNF itself. The soluble TNF receptors bind to TNF. They are capable both of competing with the cell surface TNF receptors for TNF and of stabilizing TNF by preventing its spontaneous dissociation into inactive monomers, and thus may affect TNF function in a complex manner. When present in sufficiently high concentrations, however, they act purely as TNF inhibitors.

Ongoing clinical trials with recombinant preparations of soluble receptors, as well as with high molecular weight derivatives of these molecules with lower rates of clearance from the blood circulation (such as soluble receptors fused to the Fab portion of immunoglobulin G (IgG) or linked to polyethyleneglycol), demonstrate their significant therapeutic effects in diseases that involve deleterious action of TNF, including rheumatoid arthritis, the inflammatory bowel diseases, and septic shock.

TNF viroceptors

An impressive example of the functional significance of the soluble TNF receptors is provided by the action of their virus-produced analogs. Several viruses, all of the poxvirus family, have been found to encode such 'TNF viroceptors'. These molecules are comprised of the same cysteine-rich repeated motifs that occur in the extracellular domains of the cellular TNF receptors. They also contain a unique C-terminal region, which in some of these receptors imposes formation of receptor dimers. Some of those viroceptors bind TNF α only, whereas others bind both TNF α and TNF β . Deletion of the DNA regions encoding these viroceptors from the genome of the viruses does not affect their replication in cultured cells. However, it significantly compromises their pathogenicity in infected animals, indicating that the viroceptors counteract a rather effective antiviral function of TNF.

See also: **Apoptosis; Cytokine inhibitors; Cytokine**

receptors, soluble; Cytokines; Cytokine receptors; Cytotoxicity, mechanisms of; Fas (CD95) and fas ligand; Inflammatory bowel disease; Interleukin 1 and its receptors; Lymph nodes; Rheumatoid arthritis, human; Septic shock; Tumor necrosis factor α ; Lymphotoxin.

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TOGAVIRUS, INFECTION AND IMMUNITY

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The Togaviridae family of small positive-strand RNA viruses are responsible for a large number of diseases of domestic animals and humans. Illness varies from mild inapparent infection to acute fevers and in many cases severe fulminating diseases with high morbidity and mortality. Many of the infections reach epidemic proportions, are arthropod borne (usually mosquitoes) and, for some of the viruses, reservoirs of infection exist within wild vertebrates such as birds, rodents and primates, where infection is inconsequential. In other cases the major source of infection is human. The viruses cause their characteristic diseases on transmission to humans and domestic animals which can be tangential to the wildlife cycle. Togaviruses are important zoonoses with humans becoming infected as a consequence of vector transmission from domestic livestock (e.g. pigs, horses). Studies on infection and immunity with these viruses have revealed particular facets of immune responses to viruses in terms of mechanisms of immunity, immunopathology and immunological tolerance.

Togaviridae are small (60–65 nm diameter) positive-stranded RNA viruses with a characteristic icosahedral capsid and 'close fitting' envelope (hence the original description as TOGA-viridae). Many of the viruses of the various genera have now been cloned and sequenced (Table 1). Prior to molecular data the flaviviruses were regarded as a genus within the

Togaviridae, but they are now considered a separate family.

The Togaviridae are amongst the most simple enveloped viruses and comprise three genera and two members: alphaviruses (e.g. Sindbis virus, Semliki Forest virus, eastern equine encephalitis); rubivirus (e.g. rubella); pestiviruses (e.g. hog cholera or swine fever virus, bovine viral diarrhoea virus – BVDV – and border disease virus of sheep – BD). Also included in the Togaviridae are equine arteritis virus (arterivirus) and lactate dehydrogenase (LDH) virus of mice. The latter is nonpathogenic but represents a good model for studying factors involved in control of viremia *in vivo* as it grows to extremely high titers in infected mice (10^9 infectious particles ml^{-1} of serum) which can persist for the lifetime of the animal without causing significant disease. Not all viral infections with these viruses are transmitted by vectors. Rubella and pestiviruses are directly transmitted between individuals by droplet infection and transplacental transmission.

The small nonsegmented RNA genome (10–11 kb 4×10^6 mol.wt) functions as mRNA and encodes three to four nonstructural (NS) proteins, a core protein closely associated with the nucleocapsid and one or two envelope proteins designated E1 and E2. The Togaviridae 49S RNA genome has a 7-methylguanosine cap and is polyadenylated at the 3' end with the order of the genes being nonstructural at the 5' end

Table 1 Some properties of Togaviridae

Genus (no. of spp.)	Disease	Main host(s)	Vector	Virus
Togaviridae				
Alphavirus (26)	Fever, encephalitis	Horse/human	Mosquito	Eastern equine encephalitis Western equine encephalitis Venezuelan equine encephalitis
	Myositis, encephalitis	Mouse/human	—	Sindbis ^a Semliki Forest
	Fever, arthritis, rash	Human	Mosquito	O'Nyong-nyong Chikungunya
Rubivirus (1)	Epidemic polyarthritis	Human	Mosquito	Ross River
	Fever, rash, arthritis, congenital infection	Human	None	Rubella
Pestivirus (3)	Viral diarrhoea mucosal disease	Cattle	None	Bovine viral diarrhoea
	Swine fever	Pigs	None	Hog cholera
	Congenital disease of skin and nervous system	Sheep	None	Border disease
Arterivirus (1)	Arteritis, abortion	Horse	Not known	Equine arteritis

^aSpecies type.

(four genes) and structural at the 3' end (four genes). The nonstructural proteins are translated directly from the full length RNA but the structural proteins are derived from a subgenomic 26S mRNA which is identical in sequence to one-third of genomic RNA at the 3' end. The Flaviviridae genome comprises a single uninterrupted reading frame which is not polyadenylated at the 3' end. Molecular cloning of one of the pestiviruses (BVDV) has revealed that the bovine pestivirus is closer to the Flaviviridae family than the Togaviridae. A strong case has been made for the reclassification of pestiviruses as members of the Flaviviridae family.

Rubella in humans, and BVDV and BD in ruminants can be inapparent infections in young individuals but severe problems arise if infections occur during pregnancy. Infection of the fetus results either in abortion or congenital malformations, or birth of animals which are immunologically tolerant to the infecting virus. Rubella in adults can induce an acute arthritis and skin lesions, and in 0.06% of cases a viral-induced encephalopathy can be a serious complication.

Immunity

Infections with many of the viruses can result in life-long immunity. In rubella the success of vaccination programmes in nonpregnant women has resulted in a significant drop in the number of rubella-induced congenital diseases. Epizootics of Venezuelan equine encephalitis virus in horses have been successfully controlled by attenuated vaccines. Establishment of vaccinal immunity in domesticated livestock can break the cycle of transmission from wild reservoir → domestic animal → human and thereby indirectly protect humans by vaccination of the intermediate host.

There is clear evidence that antibodies to the E1 protein (usually the hemagglutinin) as well as the E2 protein can both neutralize and passively protect in mouse and monkey models of alphavirus infection. Antibodies to the nonstructural proteins (e.g. NS-1) are also recognized on the surface of infected cells. By comparison with anti-E1 antibodies, anti-NS-1 antibodies are extremely efficient at triggering complement activation on cell surfaces and causing lysis of infected cells. Since these viruses mature from infected cells by budding through cytoplasmic membranes it is also extremely likely that T cell recognition of nonstructural proteins on the surface of infected cells represents an important mechanism of immunity in these infections. In view of the importance of nonstructural antigens and the need for vaccination with live virus rather than recombinant

proteins, vaccinia virus constructs expressing non-structural proteins have been constructed and used as vaccines experimentally. The recent evidence that epitopes on the nonstructural proteins may be more highly conserved between virus strains indicates that these immunogens could be extremely useful in stimulating broadly cross-reactive immunity.

In the alphaviruses the E1 proteins are involved in cell surface adsorption and fusion. The E2 proteins contain important virulence determinants and changes in either can affect host range and virulence. A single amino acid substitution in the E2 of Sindbis virus of an arginine for a serine at position 114 results in a strain with reduced neurovirulence for young mice. Studies with recombinant proteins reveal that in general virulence determinants map to the N-terminal half of the E2 glycoprotein. The E1 gene is more conserved than the E2 in the alphaviruses. Now that infectious clones of many of the viruses have been constructed it will be possible through site-directed mutagenesis to pinpoint virulence determinants and to use this information in the generation of attenuated clones for vaccination purposes.

Enhancement

Antibody-mediated enhancement of viral replication is thought to be mediated by Fc receptor (FcR) uptake of virus as immune complexes by FcR⁺ cells of the macrophage-monocyte series. Enhancement is mediated in a dose-dependent manner by subneutralizing concentrations of antibodies that bind to the E proteins of the virus. It is not observed with antibodies to the nonstructural proteins as these are not exposed to the virus surface. Viral immune complexes may exacerbate the disease in rubella where congenital infection gives rise to viremic children.

Tolerance

In the pestivirus group, transplacental infections of the fetus can cause congenital disease and induction of immunological unresponsiveness to the virus in the fetus. In the ruminant pestiviruses (BVDV, BD), there are two virus biotypes – cytopathic and noncytopathic. This difference correlates with distinct differences in viral polypeptides between the two biotypes. In the first 3 months of pregnancy BVDV infection of pregnant cattle with the noncytopathic BVDV virus may result either in fetal death or, as is more common, the birth of apparently normal animals which are virus positive but antibody negative. The same applies to BD infection of sheep. If infection occurs early in pregnancy during the develop-

ment of the immune repertoire, virus antigen is seen as self and animals become immunologically tolerant to the virus following *in utero* infection. These virus-positive, antibody-negative animals represent an important reservoir of infectious virus. On infection of these tolerant animals with the more virulent cytopathic virus, a fulminating infection results and animals die from a mucosal disease syndrome associated with destruction of the gut epithelium, inflammatory lesions of the alimentary tract and depletion of lymphoid tissue in the Peyer's patches.

Pestivirus infection in ruminants has analogies with rubella in humans. Congenital infection of the fetus with rubella is a chronic nonlytic infection associated with little inflammation but severe multi-system congenital abnormalities; 80% of congenitally infected children shed the virus and can secrete virus for up to 20 months after birth. The mechanism of rubella teratogenesis is unknown; nor has it been established how rubella escapes immune elimination in these children. It is possible that this could arise either because of some impairment in the capacity to make high titer antirubella immunoglobulin G (IgG) perhaps through T cell tolerance. The presence of antibodies (either host or maternal) may also exacerbate the disease and cause immune-mediated damage. The latter is not seen in the congenital pestivirus infections in ruminants as there is no transplacental passage of antibody during fetal life. The ruminant pestiviruses provide a valuable animal model for congenital rubella as well as being challenging diseases of veterinary importance.

See also: **Filoviruses (Marburg virus and Ebola virus) infection and immunity; Viruses, immunity to.**

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TOLERANCE, CENTRAL

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Self-tolerance, a natural state of an organism characterized by the inability to mount an immune attack against the body's own tissue components, is achieved by many different 'central' and 'peripheral' mechanisms which supplement and complement each other. In this way imperfections of one mechanism may be corrected by another. Traditionally, the term 'central tolerance', as opposed to 'peripheral tolerance', has been used to describe a state which is established by mechanisms that operate during development of lymphocytes in primary lymphoid organs

(i.e. thymus and bone marrow) and prevent immature cells expressing self antigen-specific receptors from becoming functionally mature autoreactive T and B lymphocytes. However, recent results suggest that the mechanisms thought to operate only during selection of the primary repertoire of antigen receptor specificities are also active in germinal centers during selection of the secondary repertoire of antigen specific receptors. What do we know about these mechanisms?

Almost 40 years ago Joshua Lederberg suggested

that 'The suppression of maturation [of self antigen specific lymphocytes] is a sufficient attribute to account for tolerance and this need not involve so drastic an event as the destruction of the cell. However, the destruction by premature reaction can safely be invoked as the means of the suppression.' Experiments performed during the last 10 years, which used antigen receptor-specific monoclonal antibodies and T cell receptor (TCR) and B cell receptor (BCR) transgenic mice to trace the development of lymphocytes with defined specificity for self antigens, fully substantiated this prediction. Direct evidence was obtained that self antigen-induced death of immature T and B lymphocytes represents the main mechanism purging the immune system of autoreactivity but that other, more gentle ways of establishing central tolerance, for example anergy and 'receptor editing', are also involved (Figure 1). Studies of lymphocyte development in transgenic and gene-deficient 'knockout' mice carrying targeted mutations in genes of choice continue to have a great impact on our present understanding of the mechanisms responsible for the establishment of central tolerance. This understanding is however far from being complete and despite impressive progress in identifying cells, proteins and genes involved in preventing the maturation of self antigen-specific lymphocytes, the ability to induce central tolerance to

transplanted tissue or to self antigens causing auto-immunity, remains a desired but as yet unattainable goal.

The two key issues in studies concerned with the mechanisms of induction of central tolerance are: 1) the role of antigen-specific receptors (and other cell surface molecules involved in intercellular communication) in triggering the biochemical reactions, which prevent the development of immature self antigen-specific lymphocytes into functionally mature cells and 2) the identification and characterization of the molecular pathways triggered by antigen receptors at different stages of lymphocyte development, which underlie deletional and nondeletional mechanisms responsible for central tolerance (Figure 2).

Numerous investigations have shown that the outcome of TCR or BCR engagement on developing lymphocytes, i.e. death, anergy, developmental arrest, survival or induction of differentiation, depends on a multiplicity of factors which include: 1) the developmental stage at which the lymphocyte encounters self antigen, 2) the nature of the antigen-presenting cell, 3) the concentration and mode of presentation of self antigen, 4) the affinity and avidity of antigen-specific receptors and 5) involvement of coreceptors and other auxiliary molecules influencing the function of TCR and/or BCR.

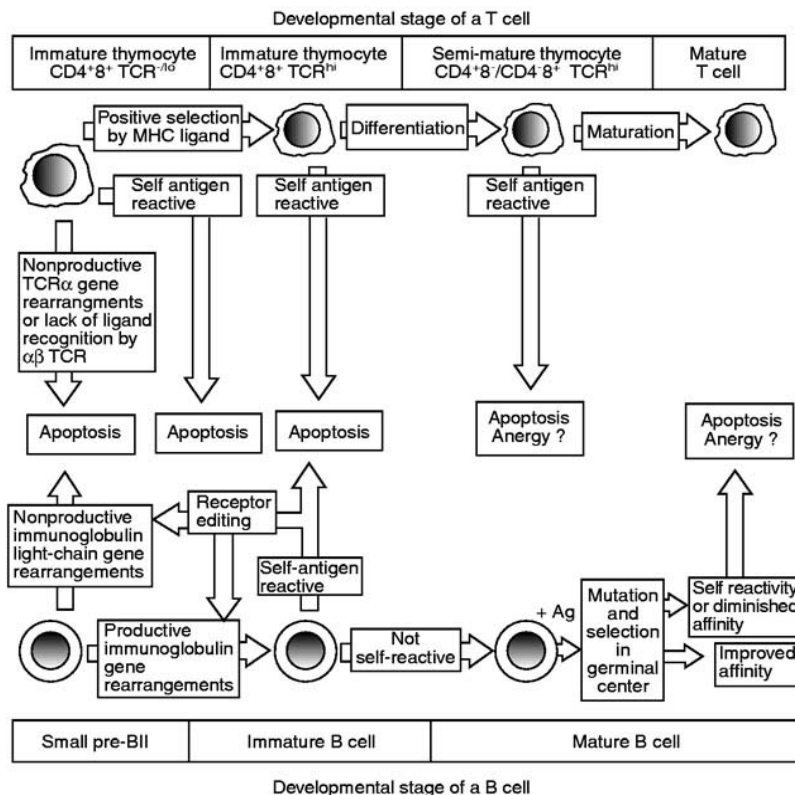


Figure 1 Mechanisms of central tolerance. Central tolerance is established by negative selection which prevents immature self antigen-reactive lymphocytes from developing into mature autoreactive T and B cells. The main mechanism of negative selection is apoptosis but developmental arrest, receptor editing and anergy also play a role.

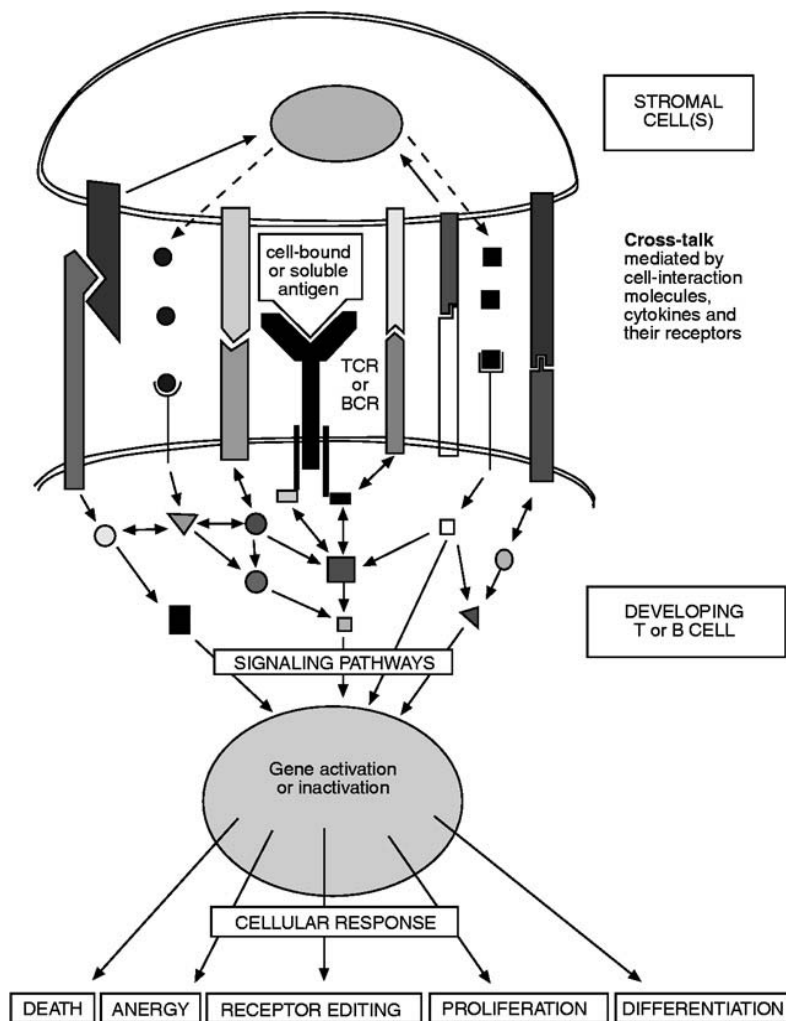


Figure 2 How immature lymphocytes expressing self antigen-specific TCRs or BCRs are prevented from maturation into functionally competent autoreactive T or B cells. This schematic, which purposely ignores the actual data, is intended to illustrate the complexity of intercellular and intermolecular interactions which are involved in deciding about the fate of immature autoreactive lymphocytes. Better understanding of the molecular mechanisms of central tolerance requires elucidation of the role of the everincreasing number of individual components of inter- and intracellular communication systems.

Central T cell tolerance

The bulk of the information on central tolerance comes from studies of intrathymic development of CD4⁺8⁺ and CD4⁺8⁻ T cells expressing $\alpha\beta$ TCR. By comparison, very little is known about tolerance induction during development of $\gamma\delta$ and CD4⁺8⁻ $\alpha\beta$ T cells.

Role of developmental stage

The $\alpha\beta$ TCR is first expressed at low levels on the surface of CD4⁺8⁺ (double positive, DP) thymocytes. DP thymocytes either die by apoptosis within 3 days from neglect (i.e. from the lack of TCR engagement) or are actively selected (either positively or negatively) by TCR ligands which consist of class I or class II major histocompatibility complex (MHC) molecules binding peptides derived from intracellular thymic proteins or from bloodborne antigens. Positive selection of DP thymocytes, a multistage process which involves rescue from programmed cell death

and induction of differentiation into single positive (SP) CD4⁺8⁻ and CD4⁺8⁺ cells, is accompanied by upregulation of TCR expression and by other phenotypic and functional changes. Several *in vitro* and *in vivo* experiments with TCR transgenic mice have shown that the same antigen which activates mature (HSA^{lo}, TCR^{hi}) SP thymocytes or T cells to perform their function, induces rapid apoptotic death of DP thymocytes expressing the same TCR. These results demonstrated that, as postulated by Lederberg, the developmental stage at which antigen is encountered is an important determinant of the lymphocyte fate.

Several approaches were used to identify more precisely the stage at which the developing cells become refractory to antigen-induced deletion. The important question was whether signals for positive selection induce resistance of DP thymocytes to antigen-mediated deletion. The results led to the conclusion that thymocytes undergoing positive selection remain susceptible to the rapid antigen-induced deletion until the 'semi-mature' SP (i.e. HSA^{hi},

TCR^{hi}) stage. However, the conditions and mechanisms responsible for deletion of DP and SP thymocytes seem to differ with regard to the dependency on Fas/FasL interaction and CD28-mediated costimulation. Since DP thymocytes reside in the cortex while SP thymocytes are found in the medulla, it is clear that negative selection can occur in both areas of the thymic architecture.

Recent studies by Kelsoe and colleagues suggest that in contrast to the majority of peripheral T cells, germinal center CD4⁺ T cells may undergo negative selection by mechanisms which usually affect immature thymocytes; i.e. they are very sensitive to the anti-TCR antibody-induced apoptosis and express the Nur77 transcription factor.

Role of antigen-presenting cells

The *in vitro* and *in vivo* studies demonstrated that not only bone marrow-derived cells, including thymocytes themselves, but also thymic epithelial cells, both cortical and medullary, have the capacity to induce negative selection of immature self antigen-reactive thymocytes. Thus, it seems that the ability to prevent maturation of self antigen-specific thymocytes is not restricted to the 'professional' bone marrow-derived antigen-presenting cells. However, the physiological relevance of the findings indicating the role of other cell types is not always clear.

Most evidence indicates that recognition of self antigen on different cells (dendritic cells, B cells, epithelial cells, thymocytes and fibroblasts) induces apoptosis and results in clonal deletion of thymocytes. However, induction of anergy in developing T cells, recognizing self antigens on thymic epithelial cells, was also reported. Despite intensive experimentation it remains unclear which factors determine whether deletion or anergy will be the outcome of antigen recognition by developing thymocytes.

Role of antigen concentration and mode of presentation

The concentration of antigen reaching the thymus is an important parameter. It was shown in several systems using different antigens (e.g. liver F protein, hen egg lysozyme, alloantigens or viral antigens) that suboptimal concentrations fail to induce full tolerance in the thymus and may be responsible for some forms of 'split tolerance'.

The role of the mode of antigen presentation in the process of negative selection is well exemplified by the results of two recent studies. One, analyzing T cell tolerance to C5, a complement component, demonstrated that exposure to circulating C5 completely deleted CD4⁺ SP thymocytes but caused only weak deletion of DP thymocytes. In contrast,

exposure to C5 in peptide form resulted in rapid elimination of DP cells. In another study it was shown that nuclear protein (β -gal) was able to induce tolerance in CD4⁺ T cells when expressed in medullary thymic epithelium which can use an MHC class II endogenous presentation pathway but not when expressed in bone marrow-derived cells which are unable to use this pathway. Tolerance in CD8⁺ T cells could be induced by both types of antigen-presenting cells.

Role of TCR affinity and avidity

According to the presently prevailing view, the most important factors determining the outcome of an interaction between immature thymocyte and stromal cells in the thymus are the affinity of the TCR for MHC-peptide complex and the number of TCRs engaged, both of which determine the 'strength' and kinetics of the interaction. In view of this so-called 'affinity/avidity model' of intrathymic T cell maturation, the complete lack of interaction or interactions below a certain threshold results in apoptosis from neglect; low-affinity/avidity interactions generate survival signals and result in positive selection, while high-affinity/avidity interactions generate death signals responsible for negative selection (Figure 3). Strong support for this model comes from investigations of the effects of altered peptide ligands (APLs) which indicate that TCR can interpret subtle structural changes in its ligand and selectively engage some parts, but not others, of its signaling machinery. Recent studies have assessed the role of different amounts of antagonist or agonist peptides in positive and negative selection of thymocytes and in mature T cell activation. The general conclusion from these studies seems to be that raising the concentration of peptides on MHC molecules above a certain threshold leads to negative rather than positive selection of thymocytes and that negative selection can be induced by T cell receptor interactions of a lower affinity than those required for full T cell activation. In agreement with the notion that the 'strength' of the TCR-ligand interaction plays the important role in determining the fate of developing thymocytes it was recently demonstrated that reduced expression levels of the male (H-Y) antigen-specific TCR in TCR transgenic CD3 ζ -deficient mice resulted in a shift from negative to positive selection of autoreactive T cells.

Role of other cell-interaction molecules

In addition to the TCR/MHC-peptide complex, several other pairs of molecules e.g. CD4/MHC-II, CD8/MHC-I, CD28/B7-2, CTLA-4/B7-1, LFA-1/CD54, CD2/CD58, Fas/FasL, gp39/CD40 and

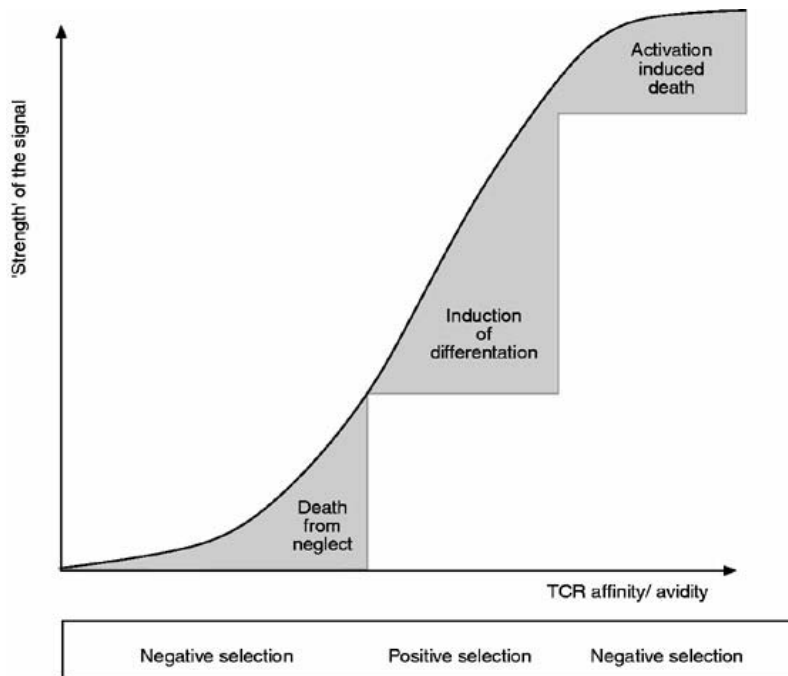


Figure 3 Affinity/avidity model of negative and positive selection of immature thymocytes.

others, may be involved in interaction of thymocytes with antigen-presenting cells and may influence the outcome of antigen recognition. Thus far, the analysis of the role of cell-interaction molecules in negative selection of thymocytes has generated inconclusive and sometimes conflicting results. Studies in CD2, CD4, CD8, CD28, Fas, FasL, or gp39-deficient mice indicated that although some inhibition of antigen- or superantigen-mediated negative selection was observed in several cases (e.g. in CD4^{-/-}, CD8^{-/-} or gp39^{-/-} mice), none of these molecules appears to be absolutely required for the induction of central T cell tolerance. On the other hand, the requirement for a costimulatory signal from antigen-presenting cells was clearly demonstrated in *in vitro* studies, in which ligation of TCR alone was insufficient to induce apoptosis of DP thymocytes. Inconsistent results were obtained with regard to the role of CD28 molecules and the search for the molecules generating the secondary signal(s) required for negative selection of immature thymocytes continues.

Signals for negative selection of thymocytes

The biochemical reactions triggered by the engagement of TCR at different stages of thymocyte development hold the clue to understanding the nature of signals that prevent self antigen-reactive immature thymocytes from becoming functionally competent self-destructive T cells. The important questions are: 1) Are reactions triggered by ligands inducing different responses of developing thymocytes qualitatively or quantitatively different? 2) What is the role of the

individual components of the TCR/CD3 complex in sensing the difference between various negatively selecting and positively selecting ligands and in translating those differences into biochemical signals that activate appropriate cellular responses? 3) What is the role of signals mediated by other cell-interaction molecules or by glucocorticoid and cytokine receptors in modifying the TCR-mediated signals? 4) What genes mediate apoptosis and nondeletional forms of negative selection of thymocytes? Although the complete answers to these questions are lacking, the progress in providing partial answers is breathtaking and can be summarized here only briefly.

Three types of approach have been used to investigate the role of various intracellular proteins in transducing signals for negative selection: pharmacological inhibition, targeted inactivation of genes and transgenic expression of mutated, dominant negative proteins. As a result, the evidence is accumulating that negatively selecting ligands may induce qualitatively different biochemical reactions from those induced by positively selecting ligands. It was shown for example, that a transgene encoding a dominant-negative p21^{ras} protein substantially blocked TCR-mediated positive selection without compromising the negative selection of thymocytes. Moreover, the results of another study suggest that the JAK3 Janus kinase plays a crucial role in negative but not in positive selection of thymocytes. Recent experiments suggest also that cyclosporine-sensitive signaling pathways mediated by calcineurin are important for positive but not negative selection.

It is now clear that the TCR/CD3 complex consists of more than one signal transducing 'modules' but it is unclear whether there are any functional differences between signals mediated by distinct CD3 components. The notion that selective engagement of different signal-transducing modules by positively or negatively selecting ligands could play a role in determining the fate of developing thymocytes remains an attractive but as yet unverified possibility.

Recently, an interesting new insight was obtained into the role of glucocorticoids. The observations suggest that endogenously produced steroids can antagonize TCR-mediated deletion. It was proposed that glucocorticoids kill thymocytes whose TCRs are not engaged but protect those which interact with MHC ligands with moderate avidity. According to this scenario, deletion of thymocytes that engage TCRs at high avidity would result from overriding the steroid/TCR antagonism by strong TCR-mediated signals or from a synergistic effect of glucocorticoids above a certain threshold of TCR avidity.

A synergistic role of stromal cytokines released in response to TCR-mediated activation of immature thymocytes has been suggested by a recent study by Rheinherz and colleagues. They found that prior to undergoing apoptosis DP thymocytes secrete cytokines which activate the surrounding thymic stroma to produce factors (e.g. IL-1 and Mig) which may be required for apoptosis. This observation provides an interesting example for the involvement of thymocyte-stroma cross-talk during the negative selection process.

The products of several genes have been proposed to act as intracellular mediators of lymphocyte apoptosis including p53, nur77, *c-myc* and interleukin-1B-converting enzyme (ICE) but their role in mediating the physiological process of negative selection is poorly understood. The list of other genes and gene products which have been implicated in the intracellular propagation or inhibition of signals leading to lymphocyte death is presented in Table 1. Currently there is much interest in the proteins belonging to the Bcl-2 family but despite rapid progress in clarifying the intricacies of the interactions between Bcl-2, Bax, Bad, Bcl-x_L and Bcl-x_S molecules, the mechanism by which this family of proteins regulate the balance between death and survival is not yet understood. The results obtained so far suggest that multiple death pathways regulated by distinct molecular mechanisms may be responsible for elimination of immature self antigen-reactive T and B cells.

Table 1 An incomplete alphabetical list of genes and gene products implicated in the regulation of apoptosis in lymphocytes, which may play a role in establishing central tolerance

<i>Positive regulators</i>	<i>Negative regulators</i>
Bad	A1
Bax	A20
Bcl-x _S	ALG-2
FADD/MORT	Bcl-2
Fas	Bcl-x _L
FAST	BHRF1
Glucocorticoid receptor	<i>c-myc</i>
Granzyme B	CrmA
HCP	FAP-1 (PTP-BAS)
HS1	pim-1
ICE-LAP3	
ICE/ced3	
Ich-2/Tx	
ILCME	
MA-3	
Mch-2	
nur77	
p53	
PITSLRE	
RIP	
Sphingomyelinase	
TIA-1	
TRADD	
TRAMP	
YAMA/ CPP32 (apopain)	

Adapted from reviews by Rothstein (1996) and by Osborne (1996).

Central B cell tolerance

Although there were suggestions in the literature that there may be no need for the central B cell tolerance because without T cell help B cells cannot respond to the majority of antigens, the results of studies, which became possible mainly due to the introduction of BCR transgenic models, provided clear evidence that immature self antigen-reactive B cells are usually prevented from maturation into autoreactive cells.

Since, as in the case of T cells, normal development of immature B cells depends on the unperturbed expression of BCRs and the molecular mechanisms responsible for the generation of diversity of TCRs and BCRs are essentially similar, the basic questions concerning central B cell tolerance are very much the same as those discussed above in relation to central T cell tolerance. The developmental stage of the B lymphocyte, BCR affinity, the antigen valency, concentration and mode of presentation, all have been shown to play a role in determining the outcome of self antigen recognition, i.e. whether developmental arrest, anergy or deletion will ensue. In fact, the

intracellular molecular mechanisms preventing maturation of self reactive B and T lymphocytes may be to a large extent identical as suggested by the observation that many molecules implicated in the regulation of signals mediating negative selection (e.g. *c-myc*, Bcl- x_s , Bad, p53 and others) are active during development of both lymphocyte lineages. The main differences in the mechanisms leading to central tolerance of B cells and T cells seem to exist at the level of antigen recognition and reflect differences in the structure of the antigen receptor, the nature of the ligand and the nature of accessory molecules involved during interactions between developing B cells and stromal cells.

An interesting example of a mechanism which seems to be specifically responsible for central B cell tolerance is the phenomenon of receptor editing. It has been observed in some systems that self-reactive B cells may undergo additional immunoglobulin gene rearrangements in response to encountering self antigen in the bone marrow. Such secondary rearrangements, which usually occur at the light chain locus, rescue immature autoreactive B cells from death by replacing the original receptor with a new receptor of different specificity. Thus, receptor editing may provide an alternative to antigen-induced apoptosis, which is believed to represent the main mechanism of central tolerance.

It has been recently demonstrated that not only immature B cells in the bone marrow but also germinal center B cells can be negatively selected by ligands which bind to their BCRs. Interestingly, there is a striking similarity between deletion-sensitive germinal center B cells and immature bone marrow B cells, which is reflected by the expression of high levels of HSA molecules and re-expression of recombination activating (RAG) genes. This indicates that mechanisms responsible for central tolerance are not confined to the immature stages of lymphocyte development but may also affect naive mature lymphocytes, which as a result of antigen recognition change or refine the specificity of their receptors and in so doing increase the risk of autoreactivity.

See also: Anergy, B cell; Apoptosis; Autoantigens; Autoimmunity; B lymphocyte activation; B lymphocyte differentiation; Anergy, T cell; Clonal deletion; Clonal selection; Thymic epithelium: potential role in regulatory T cell tolerance; Idiotypic network; Ontogeny of the immune response; Oral tolerance; Suppressor T lymphocytes; T lymphocyte activation; T lymphocyte differentiation; T lymphocytes, autoreactive; Tolerance, peripheral; Tolerance, models; Transplantation.

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TOLERANCE, MODELS

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Immunological tolerance defines a number of phenomena characterized by an antigen-specific unresponsive state. These unresponsive states are antigen driven in that they require exposure to antigen for induction and continuous presence of antigen for their persistence. Tolerance may be characterized by anergy (T or B cell), deletion or suppressor mechanisms. The latter results from regulatory mechanisms within the system rather than an inherent tolerant state and will not be discussed in this entry. Tolerance, in the form of immunological ignorance, may also apply to sequestered antigens which never contact the immune system in either a tolerogenic or immunogenic fashion. Models of primary importance are those induced in neonatal animals, mature (both conventional and transgenic) animals and *in vitro*. Induction of tolerance is most readily achieved in neonatal animals because of the lack of an environment which supports immune responses. That neonates lend themselves to the induction of tolerance to more complicated antigens such as cells is best seen following the injection of living hematopoietic cells, which results in the establishment of a chimeric state. Neonates are also readily tolerized with serum protein antigens and probably with any antigen that tolerizes mature animals. It has also been long known that neonatal lymphocytes are competent when presented with antigen in an adequate environment. Tolerance can be readily induced under the proper conditions in mature animals using either protein preparations that do not activate antigen-presenting cells or peptides that do not require antigen processing.

Induction in mature animals

Although it is well-established that a system of negative and positive selection occurs in the thymus, resulting in shaping of a nonself-reactive T cell repertoire, tolerance can also be induced to foreign antigens either in early or late life under the appropriate conditions. The finding that certain V_{β} specificities of the T cell receptors that are reactive *in vitro* to self antigens are absent in mice possessing these antigens has led to a number of recent and important studies that have given considerable insight to self-nonself recognition. Later studies using a variety of superantigens which activate an entire T cell population expressing the same V_{β} T cell receptor, irres-

pective of antigenic specificity, also demonstrated that *in vivo* exposure to these superantigens results in the elimination of these cells. The models resulting from such studies yield valuable information concerning immunogenetics of tolerance induction, possible mechanisms of negative and positive selection within the thymus and T cell anergy.

These latter models, however, do not readily lend themselves to understanding the mechanisms of tolerance induction involving the cytokine cascade, antigen presentation, roles of activation events and intracellular biochemical processes as well as the role of antigen processing. Another model, the unresponsive state induced in adult mice to monomeric forms of mammalian gamma globulin, more readily lends itself to the investigation of these latter events. An *in vivo* model of a central unresponsive state is presented in this review to delineate the conditions required for the induction and maintenance of self tolerance and the role of such induction and maintenance on susceptibility to autoimmunity.

A classical *in vivo* model of tolerance which mimics a number of aspects of self tolerance has been studied in the past by several groups of workers using monomeric preparations of mammalian gamma globulin. In the model to be discussed below, tolerance is induced to human gamma globulin (HGG) by a single injection of adult mice with deaggregated (monomeric) HGG (DHGG). Furthermore, tolerance to HGG is also readily induced in neonatal mice. Adult treated mice fail to respond to a subsequent injection of aggregated HGG (AHGG) at both the T helper and B cell levels. However, as much as 2–3 logs less HGG are required to induce tolerance in T helper cells than in B cells (Figure 1). This observation suggests that tolerance to self antigens present in limited concentration may exist in only the T cell compartment, and, thus, has implications in antibody-induced autoimmunity. Although it is not known whether tolerance in this model is the result of anergy or deletion, it is independent of both suppressor cell activity and Fc γ receptor expression. Of particular interest is that antigen-specific T cell proliferation, T helper function, antibody production and cytokine release are tolerized by prior injection of DHGG. This tolerance is independent of the thymus, since it can be induced in adult thymectomized as well as normal mice. Fol-

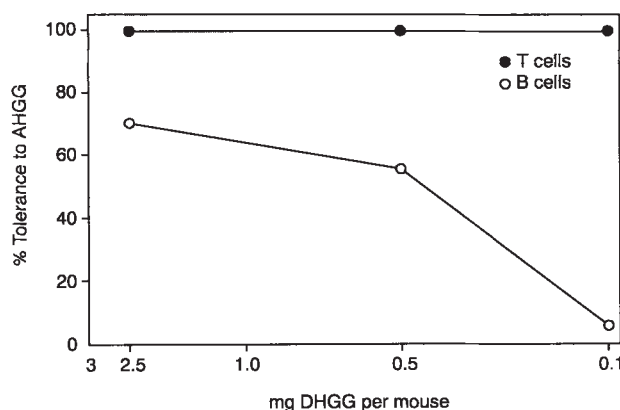


Figure 1 Effect of deaggregated human gamma globulin (DHGG) dose on the induction of tolerance to aggregated HGG (AHGG) in mouse T helper cells and B cells.

lowing a single injection of the tolerogen, tolerance to immunogenic challenge is induced in B cells and all subsets of T cells capable of releasing cytokines upon *in vitro* stimulation. The down side of this model is that purified preparations of HGG (immunoglobulin G1, IgG1) rendered monomeric by ultracentrifugation (at 4°C) are required. These preparations are relatively unstable and aggregate over a relatively short period of time.

Antigen presentation in tolerance induction

It is most likely that the nature of the antigen-presenting cells plays a critical role in the induction of tolerance as it does in the immune response. It is well accepted that resting B cells are very poor antigen-presenting cells (APCs) and readily lend themselves to the induction of tolerance in both *in vivo* and *in vitro* models. The targeting of antigen to B cells with Fab fragments specific for B cell receptors leads to a sound induction of tolerance in the T cell compartment. However, it is unlikely that resting B cells are obligate APCs, while macrophages and/or dendritic cells (and antigen-activated B cells) can present antigen only in an immunogenic fashion. It has been recently demonstrated that tolerance can readily be produced in all T cell subsets in B cell-deficient (knockout) mice tolerized with DHGG or superantigens. These findings argue that every APC has the ability to present antigen either in a tolerogenic or immunogenic fashion depending on such factors as form of the antigen, route of antigen administration and the presence of cytokines released by activated APCs. It is likely that the APCs that initiate T cell activation are dendritic cells with macrophages and B cells participating in the expansion of T cell subsets.

Role of antigen (tolerogen) dose on the immune status of T helper and B cells

The differential effect of tolerogen dose on T helper and B cells is reflected in the duration of tolerance induced with a given dose of DHGG. Thus, T cells remain tolerant for a considerably longer period of time than do B cells, probably because of the dependency of the tolerant state on the continued presence of DHGG tolerogen in both cell types. Following injection, DHGG equilibrates in equal concentrations between the intra- and extravascular fluid spaces of mice and is catabolized with a half-life of approximately 8 days. After an appropriate decrease of DHGG resulting from catabolism, there is a period of time when tolerance exists in the T helper cell after competent B cells have reappeared. This suggestion is supported by the ability to induce an anti-HGG response in mice late in tolerance at a time when T helper cells are tolerant but competent B cells are present, with injections of lipopolysaccharide along with aggregated HGG (AHGG). Using multiple injections of bovine serum albumin (BSA), a dual dose tolerance has been reported in which low doses of BSA induce a state of tolerance, an intermediate dose induces immunity, with tolerance reappearing at higher doses. This phenomenon has been explained by the following mechanism: At low doses of antigen the specific B cells would preferentially pick up antigen through high-affinity binding by antigen-specific surface Ig. Because resting B cells do not express appropriate costimulatory signals, any interaction with an antigen-specific virgin T cell would be tolerogenic. At an increased antigen concentration uptake by other MHC class II-positive cells would occur. These cells of the so-called professional antigen-presenting cells would present antigen to the virgin T cells in an immunogenic fashion. At high antigen concentration all B cells would take up antigen by pinocytosis. Since B cells far outnumber other MHC class II-positive cell types, the results would again be tolerance of the virgin T cells. This explanation presumes that 1) only B cells are able to induce T cell tolerance; 2) B cells are tolerogenic even in the presence of specific antigen; and 3) all non-B cell antigen-presenting cells activated virgin T cells constitutively. These assumptions provide a firm framework explaining the midrange, immunogenic dose of antigen and imply that a B cell-deficient animal would not be able to induce T cell tolerance. However, the fact that T cells from B cell-deficient mice given DHGG are tolerized rather than activated indicates that tolerance cannot be explained by the above hypothesis. Actually there is no evidence for two zones of tolerance to HGG over a wide range

of tolerogen doses (10 mg per mouse to 5 µg per mouse).

Modulation of tolerance by cytokines

It is well-established that lipopolysaccharide (LPS) and other activators of interleukin-1 (IL-1) production injected along with the DHGG convert a tolerogenic signal into an immunogenic signal as well as inhibit the induction of tolerance, resulting in both T helper cell activity and antibody production. Similar modulation of tolerance with LPS has been suggested with superantigens. Furthermore, with the HGG-mouse model, *in vitro* exposure of antigen-presenting cells with AHGG (immunogen) results in aggressive processing and secretion of IL-1, whereas *in vitro* presentation of monomeric HGG fails to result in aggressive processing and cytokines are not released. Thus, it seems reasonable to suggest for the HGG-mouse model that tolerance results in a failure to activate APCs and cytokines are not released and the lymphokine cascade is not activated. Whether inhibition of induction of tolerance with IL-1 (and tumor necrosis factor α , TNF α) results from direct interaction with T cells or their precursors or whether such cytokines upregulate costimulatory molecules is unknown at the present time.

Clonal deletion versus anergy

Although it is firmly established that anergy occurs *in vitro* in both B cells and antigen-specific T cell lines, the nature of the tolerant state induced *in vitro* is still debatable. Original studies using double transgenic mice, transgenic for both the antigen and the antigen-specific antibody (targeted to the B cells), suggested anergy as a mechanism of B cell tolerance. However, in later experiments in which the endogenous/transgenic ratio of B cells was increased, transgenic B cells were competitively deleted in the T cell area of the follicles. Similarly it was demonstrated that in transgenic mice in which a large (70%) portion of the B cells carried IgM rheumatoid factor (IgM-RF) specific for human IgG, injection of DHGG resulted in deletion of the IgM-RF⁺ B cells with the same kinetics as for antigen-specific B cell function deleted in conventional mice tolerized with DHGG. Antigen-specific T cell receptor (TCR) transgenic mice have also lent themselves to the question of deletion or anergy. Although some of these models have not been adequate to answer this question, others have given considerable insight into the roles of anergy and deletion in tolerance induced to peptide antigens. Although it is not clear whether

deletion is the only mechanism in tolerance, the most recent studies suggest that following injection of peptide, deletion occurs in the thymus without T cell activation, whereas T cell activation may precede deletion observed in peripheral T cells. Although deletion appears to be a major form of tolerance induced to superantigens, such deletion may be preceded by T cell expansion.

See also: Autoimmunity; Clonal deletion; Thymic epithelium: potential role in regulatory T cell tolerance; Effector lymphocytes; Helper T lymphocytes; Suppressor T lymphocytes; Tolerance, central; Tolerance, peripheral.

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TOLERANCE, PERIPHERAL

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Generation of effective immune responsiveness requires professional antigen presentation to T cells and cross-talk between helper cells and B cells. In each of these steps, the interacting cells both deliver signals and receive back-stimulation. Simply stated, this view of the immune system provides an overview not only for the generation of positive immune responsiveness (antibody formation, delayed hypersensitivity), but also for unresponsiveness or tolerance. As summarized in current versions of the two-signal hypothesis of Bretscher and Cohn, both recognition of antigen (signal one) and a second, costimulatory signal (signal two) are required for a positive immune response. Lacking such a second signal, the antigen-recognizing cells are rendered unresponsive. This may reflect the deletion of these cells via programmed cell death (apoptosis) or their anergy (functionally unresponsive but still alive).

Moreover, lymphocytes must be both positively and negatively selected in the thymus in terms of self-reactivity. Purging of the immune repertoire in the thymus is the first step in the process of tolerance. Thus, during the development of the immune system both *in utero* and after birth, T cells in the thymus are exposed to a variety of (self) peptides in conjunction with self histocompatibility (MHC) antigens. Peptides that do not appear in the thymus or fail to bind to self MHC do not participate in this process. Such peptides are either ignored by the immune system, stimulate an immune response if costimulation is provided, or induce tolerance peripherally if signal two is lacking. The ultimate result of this process also may be apoptosis or anergy.

History of tolerance

The ability to distinguish self from nonself (tolerance) is one of the basic tenets of immunology. As early as in the 1900s, Paul Ehrlich recognized the importance of this concept when he coined the term 'horror autotoxicus' (fear of poisoning oneself) to indicate the potential results of making an immune response against self components. However, it was not until four decades later that an experiment of nature provided a theoretical basis and explanation for this 'self-tolerance'. Thus, Ray Owen demonstrated that dizygotic (fraternal) cattle twins, which shared a blood supply *in utero*, possessed and tolerated red cells from their nonidentical twin. Sub-

sequently, it was shown that skin grafts could be reciprocally exchanged between these twins. It was suggested that the critical exposure to 'nonself' antigens during development (and their persistence as a chimeric state) led to tolerance to these antigens. Indeed, Burnet originally proposed in 1949 that any clones exposed to their specific antigen during this time would be eliminated.

Experimental verification of these predictions came in 1953 through the classic experiments of Billingham, Brent and Medawar. They demonstrated that perinatal exposure of mice to allogeneic lymphoid cells led to subsequent acceptance (tolerance) of skin grafts from the same strains that the mice were exposed to at birth; these animals still rejected skin grafts from other strains. Thus, this tolerance is *learned* and is *specific*. This phenomenon has been repeatedly confirmed and has led to a definition of tolerance as 'a specific unresponsive state induced by prior exposure to an antigen'.

Properties of unresponsiveness

Further analysis of experimental tolerance systems with a variety of antigens has verified that tolerance is not only specific, but is also dose-dependent and of finite duration. Indeed, early attempts to induce tolerance with nonreplicating antigens failed in part because the initial dose of antigen did not establish a persistent state of chimerism as in the Medawar experiments or the dose of antigens was insufficient to render the host unresponsive.

Tolerance, as such, generally represents a central failure of the immune system (with the exception of active suppression) and can be transferred with lymphocytes from unresponsive hosts to irradiated recipients just as secondary responsiveness can be adoptively transferred with primed lymphocytes. This unresponsive state is manifested in both T and B lymphocytes; indeed, Chiller and Weigle in now classic experiments showed that the kinetics, dose requirements and persistence of tolerance for T and B cells were different. For example, T cell unresponsiveness is more readily induced than B cell tolerance in terms of more rapid induction kinetics and lower dose requirements. Moreover, T cell tolerance lasts longer. This may, in part, be due to the relatively shorter half-life of B cells versus the time requirements to generate mature T cells from the thymus.

We will return briefly to (central) tolerance induction within the thymus later.

In addition, in experimental systems of tolerance induction, a small, detectable response is often obtained. When examined carefully, this response is often of low affinity for the tolerated antigen. Such a result is not surprising since the cells which would be most likely to be rendered tolerant would be those with the highest binding activity for antigen; thus, only the least avid cells would be spared. Therefore, it is not surprising that low-affinity autoantibodies can often be detected in normal human serum. These are usually of no pathological consequence and probably represent stimulation of cross-reactive clones against some extrinsic antigen that leads to a response with a low affinity for self.

It is assumed from the foregoing that tolerance represents the functional, if not physical, deletion of specific lymphocytes and that the waning of tolerance reflects the generation of new B and T cells in the absence of sufficient antigen to tolerize newly developing lymphocytes. Evidence supporting this notion has come from manipulation of either the amount of available antigen or the ability to generate new cells via the thymus.

Factors affecting tolerance

From the foregoing, it is clear that tolerance is a process that continues throughout life but begins during fetal development. Hence, experimental induction of unresponsiveness is easiest in fetal, neonatal or perinatal animals. Why? One theory is that these cells are the most immature and do not possess the appropriate costimulatory signals to become activated for a productive immune response. Any antigen to which these immature cells express receptors, upon exposure, will cause them to become tolerant. Thus, in some views, this is an obligate stage for tolerance induction. An alternate view is that, while highly sensitive to tolerance at this stage, there is little chance to receive 'signal two' in this milieu. The nature of these signals in the thymus is a source of great debate, but recent developments suggest that some of the normal signal-transducing mechanisms are involved (see below).

The conditions which favor tolerance not only shed light on the manipulation of responsiveness in disease states, but also provide insight into the multiple pathways employed in avoiding the horrors of 'autotoxicus' and regulating the level of responsiveness to extrinsic antigens *per se*. For example, while tolerance as stated above is more readily induced in immature lymphocytes, it can be induced experimentally in adult animals. It is most effectively induced

with systemic exposure to an antigen; this allows sufficient antigen to reach all antigen-specific B and T cells rapidly. Local administration, as in a natural exposure via the skin to the draining lymph nodes, instead stimulates priming for an immune response perhaps due to the nature and strength of costimulation on the antigen-presenting cells and the available inflammatory cytokines. Similarly, deaggregated antigens and those epitopes coupled to known self carriers, especially gamma globulins, also favor tolerance induction, even in adults. Whether these conditions also preclude costimulatory signals or promote inhibitory pathways like Fc receptor feedback remains to be resolved.

Mechanisms of responsiveness

Four broad-spectrum mechanisms have been proposed over the years to account for T and B cell tolerance (Figure 1): *deletion* of reactive clones; *clonal anergy*, in which specific lymphocytes are present but functionally inactive; T cell-mediated *suppression* of T or B cell responsiveness; and finally, *ignorance* of certain antigens that may be sequestered from the immune system. These mechanisms need not be mutually exclusive; however, no direct evidence for any of these was available for self-tolerance until recently. Transgenic models have been critical in unraveling these pathways.

In order to understand these mechanisms, one must first appreciate the developmental stages in lymphocyte maturation, especially since immature cells appear to be the most tolerance susceptible. Both B and T cells arise from pluripotential stem cells in the fetal liver and later from stem cells in the bone marrow. Stem cells (prethymocytes) migrate to the thymus (Figure 1) where they undergo a series of maturational steps, the most important of which is the rearrangement of the α and β chains, for example, of the T cell receptor (TCR). These T cells must be positively selected for self-MHC restriction and negatively selected for tolerance.

What happens to these cells as they are 'negatively selected'? Evidence from both normal and transgenic mice suggests that clonal deletion is one of the primary mechanisms for immature T cell tolerance. For example, in mice lacking the class II MHC antigen I-E, approximately 15% of the T cells in the thymus express an $\alpha\beta$ TCR using a specific V_β gene segment (designated $V_{\beta 17a}$). Strains of mice that express the I-E antigen lack the idiotype formed by this $V_{\beta 17a}$ in their mature T cells, but express it at low density in their immature $CD4^+$, $CD8^+$ thymocytes. Interestingly, F1 hybrids of I-A⁺ and I-E⁻ strains, which will codominantly express I-E, are also $V_{\beta 17a}$

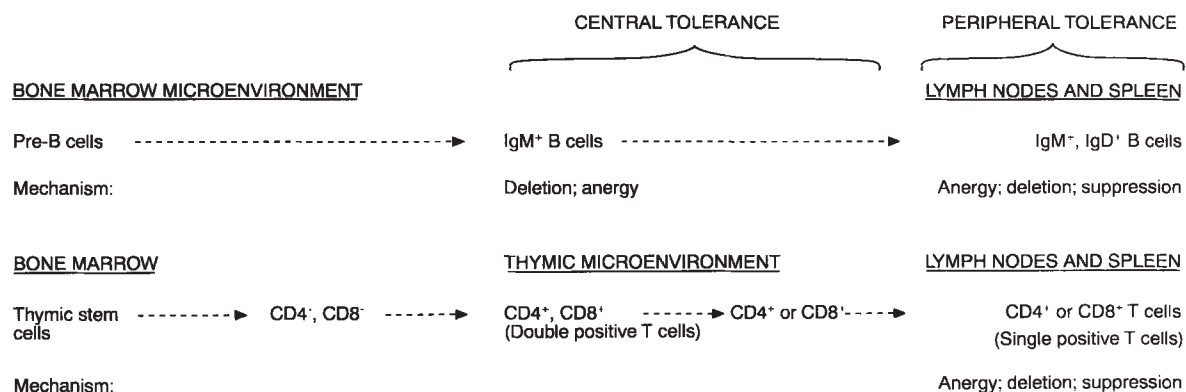


Figure 1 Schematic representation of the pathways of B and T cell tolerance. Once rearrangement of Ig genes occurs in pre-B cells in the bone marrow and IgM is expressed, these early IgM-positive B cells are sensitive to tolerance via both deletional and anergic mechanisms, depending on the nature of the antigen. Following maturation, lymphocytes migrate to the periphery (lymph node and spleen), where anergy, deletion and perhaps suppression act as tolerance mechanisms. In both cases, cognate interaction with T cells and the deliverance of costimulation and lymphokine signals may overcome tolerance induction and lead to antibody formation, including autoantibodies. Similarly for T cells, maturing CD4⁺, CD8⁻ immature T cells are deleted in the thymus, but peripheral T cells may undergo a tolerogenic mechanism leading to an anergic state of apoptosis when exposed to peptides in the absence or costimulation.

negative in the mature T cell pool, but possess V_{β17a}-positive immature lymphocytes. This suggests that expression of I-E leads to deletion of T cells bearing a particular TCR-V_β as they transit to a mature stage.

In analogy to this process in T cells, the B cell repertoire is generated by V gene arrangements during development. Thus, immunoglobulin H and L chains rearrange in pre-B cells in the marrow to give clones of antigen-specific B cells expressing, initially, immunoglobulin M (IgM). In B cells, however, it has been harder to obtain direct evidence for a deletional event for tolerance since there is no predominant usage of a particular V region in these cells. Nonetheless, it has been assumed that immature B cells with rearranged receptors binding self antigens with high affinity are clonally deleted based on data from three systems: the elimination of B cells by anti-IgM treatment of neonatal mice, the antireceptor-induced death of immature B cell lymphomas and a loss of specific B cells in transgenic mice expressing IgM receptors for self MHC antigen. In the last system, Nemazee and colleagues created transgenic mice in which a large number of the B cells bore IgM receptors which were specific for the membrane class I histocompatibility antigen, H-2K^k. When bred to an H-2^k mouse to induce tolerance in these anti-H-2K^k-specific B cells, the IgM-transgenic cells were deleted at an immature stage. It is worth noting that this deletional mechanism may depend on the nature of the antigen (membrane versus soluble) or on the maturity of the B cells when first exposed to tolerogen (see below).

Further data from the laboratories of Nossal and Scott and their colleagues showed that mice rendered

tolerant as adults still possessed B cells with free receptors for the tolerated antigen. These were not cells of low affinity, as is often observed with anti-self-reactive B cells in humans. Rather, these were high-affinity cells which were simply anergic to stimulation by antigen and appeared to be blocked at the G₁/S boundary of the cell cycle. More definitive evidence came from another transgenic model, this time one in which all B cells expressed IgM and IgD receptors for a peptide from hen egg lysozyme (HEL). When these mice are bred to lysozyme-transgenic animals, tolerance to HEL is produced. Interestingly, normal numbers of lysozyme-reactive B cells were still present in these double transgenic mice. However, their now anergic (tolerant) B cells had an altered phenotype: they had lost most of their IgM yet retained membrane IgD. This system differs from the deletional model above in terms of both the nature of the antigen and the fact that target B cells express both IgM and IgD, that is, they are mature B cells. More recent experiments suggest that a membrane form of lysozyme causes deletion in anti-HEL transgenics, regardless of IgM/IgD isotype expression, and that this can occur as anergic B cells traverse into the follicles of the nodes. Additional data with another transgenic model suggest that supercrosslinking of transitional cells with IgM and little or no IgD led to deletion. Thus, it appears that multiple pathways for B cell tolerance are probably due to the diversity of antigens that can be considered 'self' and the fact that cells may be exposed to tolerogens at different maturational stages.

Furthermore, there must be mechanisms to assure that potential antiself activities that arise from

somatic mutation during an immune response are silenced. Evidence from Klinman's laboratory suggests that secondary lineage B cells indeed pass through a 'tolerance susceptible window' after the initiation of an immune response. This would ensure that potentially high-affinity cells were eliminated from the repertoire, either functionally or physically.

Additional experimental data suggest that a form of clonal anergy may also be induced in T, as well as B, cells. Schwartz and his collaborators demonstrated that interleukin 2 (IL-2)-producing CD4⁺ T cell helper (T_H1) clones were rendered anergic if exposed to an 'inert' antigen-MHC complex such as fixed macrophages, presumably due to the lack of costimulatory second signals provided by viable, professional antigen-presenting cells (APCs). These anergic T cells fail to produce IL-2 when subsequently appropriately stimulated. Similar results have been reported by Gilbert and Weigle for T_H2 helper cells treated in the same way and later measured for their functional capacity to help B cells produce antibody.

How do we explain these disparate results in terms of anergy versus deletion? As stated above, one possibility with respect to B cell tolerance is that different antigens were involved, some soluble and others membrane bound. Alternatively, the maturity of the target B cell (IgM expressing B cells are deleted versus IgM + IgD-bearing cells are rendered anergic) could be an explanation. Indeed, with respect to T cell tolerance, there are clear differences in mechanisms and maturational states between double-positive immature thymocytes and mature T_H1 clones, the former being 'deletable', the latter becoming anergic. Moreover, further control pathways for clonal expansion may result in deletion of reactive clones via a Fas-dependent pathway. Thus, the mechanism of tolerance may depend both on the maturity or subset of target cells, as well as the nature of the antigen.

In all cases, therefore, one unifying tenet occurs: exposure to antigen at an inappropriate stage of development or in the absence of costimulation leads to tolerance. Provision of a positive costimulatory signal often overcomes the initial negative signal.

Where does suppression fit into all of this? Fifteen years ago, the activity of suppressor T cells in experimental tolerance systems was under intense investigation. The difficulty in cloning these T cells, as well as the lack of strong molecular evidence on the nature of their TCR, has made it difficult to analyze the activity of these cells. Recent data suggest, however, that at least some cells with suppressive properties bear a normal CD3-linked TCR complex. Indeed, it appears that under certain circumstances normal T

cell clones can effect suppression of B cell differentiation or other T cell activity. Thus, T_H1 cells secreting interferon γ or lymphotoxin (TNF β) may inhibit B cell differentiation whereas T_H2 clones may be directly cytotoxic for antigen-presenting cells displaying peptide + class II MHC on their surface. However, it is now clear that not all self antigens are tolerated via the same mechanism, a conclusion which makes teleological sense due to the variety of antigens and their distribution and concentration in the body.

Sites of peripheral tolerance induction

It is clear that the B cell repertoire is generated in two phases and that tolerance induction must occur at both levels: that of the immature B cell in the bone marrow and later in the periphery in the lymph nodes and spleen. Indeed, this can occur in the latter case both at initial antigen contact (in the absence of costimulation), as well as in the germinal centers during somatic hypermutation before a secondary response (see below). Similarly, while T cell repertoire development goes through positive and negative selection in the thymus, there must be some degree of regulation as peripheral tolerance in various nodes in the body.

The lack of costimulatory molecules on certain cells in the periphery that may be induced to express class II MHC antigens (e.g. by viral infection and interferon production) and present endogenous peptides makes these cells excellent candidates for tolerogenic APCs. Such 'nonprofessional' APCs could present antigenic peptides to T cells virtually anywhere they arise in the body, assuming a reasonable lymphatic or capillary access to those tissues. It is most likely, however, that this presentation occurs in the lymph nodes especially if resting B cells are the presenting APCs. In some cases, immature (less differentiated) dendritic cells could subserve this same function in the white pulp periarteriolar lymphatic sheaths (PALS) in the spleen or the paracortex of the peripheral nodes. It is not clear whether this process occurs elsewhere (such as in mucosal tissues). Suffice it to say that oral exposure to antigens leads to presentation in the gut and preferential skewing of responsiveness to T_H2, due for example to local production of transforming growth factor β (TGF β). At the systemic level, this may be expressed as peripheral T_H1 tolerance or even suppression. It is likely that this reflects differential cytokine profiles as a consequence of alternative antigen presentation pathways, although the mechanism is not known. In addition, the potential for peripheral deletion to occur in tissues with high levels of Fas ligand (FasL;

such as in privileged sites such as the eye or testis) needs to be considered.

An elegant model system to examine specific niches in which peripheral tolerance occurs was described by Goodnow and colleagues, using the HEL/anti-HEL transgenic model system. In one situation, they used heavy-chain-only transgenic mice so that the rearranged heavy chain could pair with endogenous light chains in the peripheral pool. This gave rise to HEL-binding B cells of various affinities but at a high enough frequency to be detected *in vivo*. By crossing these Ig receptor transgenic animals with HEL-producing mice, they could determine the relationship between affinity, antigen concentration and the fate of these HEL-specific B cells in the periphery. Their studies not only confirmed this relationship, they also showed that there was a competition between the transgenic 'autoreactive' B cells and the endogenous repertoire for space. That is, if mice contained 100% transgenic B cells, their tissue distribution was similar to B cells in normal mice even when the autoantigen (soluble HEL) was plentiful. But if normal B cells were in excess and antigen was present, these cells failed to enter the follicles in the lymph nodes. Thus, there is a gate, so to speak, that prevents autoreactive B cells (exposed to antigen) from entering the follicles and forming germinal centers. This makes sense since the germinal centers are sites of extensive proliferation and the potential to undergo somatic hypermutation, would be a dangerous event for the host in terms of forming high-affinity autoantibodies.

How does the 'gatekeeping' occur? One likely mechanism is via Fas and Fas ligand. Data from the same group show that FasL on T cells may be acting to delete those B cells that have upregulated their surface expression of Fas, for example by CD40 interaction. Curiously, binding to membrane immunoglobulin M has been shown by Rothstein's group to block susceptibility of those B cells in terms of their susceptibility to undergo a FasL-mediated death signal. This creates a dynamic battle in which antigen-specific and nonantigen-specific signals determine the fate of B cells to survive in a peripheral tolerance pathway depending on which signals predominate, as well as when and where they are delivered as antigen-specific cells sojourn in a lymph node.

Signals for anergy and apoptosis

What is the nature of the negative signals for tolerance? Initial stimulation of both T and B cells involve receptor coupling via Src family protein tyrosine kinases (PTKs), e.g. to phospholipase C and the production of inositol phospholipids, some of which

lead to calcium mobilization, and other products cause activation of protein kinase C and subsequent phosphorylation of critical substrates leading to gene transcription. Other downstream substrates involve MAP kinase activation and both common and alternative transcriptional events. Studies in T cell clones suggest that if the initial calcium signal is given alone or abortively, then the T cell is rendered anergic. Whether this process can be mimicked in B cells remains to be determined although the idea that abortive stimulation of B cells in the absence of costimulation via T cell help is not only appealing, but consistent with several models. One paradigm that has developed is that there is great commonality between negative signals for tolerance (growth arrest, anergy, apoptosis) and the initial positive signals in lymphocyte stimulation. The two-signal hypothesis indeed proposes that it is the second signal that is the determining factor.

Therefore, what is the nature of positive costimulation? As summarized in Figure 1, T cells recognize costimulatory molecules of the B-7 family via the CD28 and CTLA-4 receptors. Blocking these interactions can modulate a stimulatory response towards tolerance; indeed, clinical trials in this direction are in progress. For B cells, the CD40 receptor can positively signal for their growth and differentiation via T cell CD40L and can overcome tolerogenic (signal 1) stimuli. The precise nature of the biochemical signals is unclear, but is likely to involve kinase activation and a balance of interacting transcription factors. Understanding this balance will provide new approaches for clinical tolerance induction.

Loss of unresponsiveness: role in autoimmunity

It is important to consider that tolerance, once induced, may not persist forever and can be broken. Some source of persisting antigen must continue to render newly emerging B and T cells tolerant. Indeed, it is likely that T cell tolerance is more critical since most protein antigens require T cell help in order to activate a humoral immune response. Nonetheless, as already mentioned, it would be undesirable for B cells not to be tolerant and to mutate toward high-affinity antiself receptors. It is also conceivable, under conditions of acute or persistent viral infection, that nonspecific T cell help may be generated that could activate any nontolerant antiself B cells.

One possibility is that tolerance to self antigens is broken because available antigen is modified (e.g. via viral infection that creates new epitopes or induces class II MHC markers via interferon γ or other inflammatory cytokines). A novel concept recently

put forth by Matzinger and her colleagues is that there is no real distinction between 'self' and 'non-self', but only a response to 'danger', which is clearly present during inflammation or at other sites of non-apoptotic cell death. Any self peptide presented (with costimulation) in the presence of inflammatory cytokines would then be reacted against as violently as would a viral invader. This has been referred to as 'signal three'. It is also possible that autoreactive B cells may be generated in the absence of sufficient self antigen to tolerize. Alternatively, a mutation to a high-affinity antiself receptor could occur in developing B or T cells in the absence of tolerizing signals, especially in later life. Note that these hypotheses are not mutually exclusive.

Induction of class II MHC antigens on cells which normally do not express these determinants, can lead to T cell recognition (since they may never have been rendered tolerant to tissue-specific antigens if they are not expressed on class II-positive APCs). This inappropriate activation may lead to tissue-specific autoimmunity, but it is not clear whether class II expression in these diseases is the result rather than the cause of autoimmunity. Indeed, experiments in transgenic mice with class II expression under the control of the insulin promoter, so that I-E is expressed on pancreatic β cells, showed that these β cells were tolerogenic rather than immunogenic, arguing against aberrant expression of MHC class II as a trigger of autoimmunity.

See also: Affinity; Apoptosis; Autoimmunity; B lymphocytes; CD40 and its ligand; Clonal deletion; Fas (CD95) and fas ligand; Superantigens; Suppressor T lymphocytes; Thymus; T lymphocyte differentiation; Tolerance, peripheral; Tolerance, models; Transgenic animals.

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TOXINS

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The development of antitoxin therapy stemmed from observations by Loeffler, Roux and Yersin on diphtheria, Behring and Kitasato on tetanus, and Ermengen on botulism. These diseases were demonstrably caused by potent toxins that could be produced *in vitro* and which, when introduced into

experimental animals, reproduced the main features of the disease caused by live organisms. By 1892, Behring had discovered antitoxins. In his search for antibacterial substances, he injected tetanus-infected animals with iodoform. This spared the animals but did not kill the organisms. He reasoned that the

effect, therefore, must be on the toxin. Transfer of serum from animals so treated to fresh animals rendered the recipients resistant to the effects of toxin and led to the discovery of antitoxins, later recognized as proteins belonging to the immunoglobulin class of serum proteins.

The impact of these discoveries was enormous and stimulated much toxin-oriented research during the next 25 years. Serum therapy for diphtheria and tetanus was successfully developed using mainly horses for large-scale production of antitoxin. Such success prompted further searches for similar substances to explain the principal features of other bacterial infections, and against which antitoxins could be generated for therapeutic use; but, there were many fruitless searches and progress was less dramatic. During the First World War gas gangrene was added to the small number of diseases in which a toxin played a significant if not overriding role. Another development was the production in the 1920s, by Glennie in Britain and Ramon in France, of toxoids that are stable, chemically detoxified toxins safe for use in human prophylaxis.

During the late 1920s to the late 1930s several species of *Clostridium*, important in veterinary as well as human disease, were shown to produce a range of soluble antigens, some of which were toxic to experimental animals, and successful vaccines were developed. Serological analysis led to the recognition of additional extracellular antigens, some of which were of potential relevance in the establishment and/or spread of infection. It is important to note that many bacterial toxins (and other soluble antigens) were first recognized either by their biological activity or as serological entities. In many cases they were arbitrarily assigned a letter of the Greek alphabet. Thus we have α toxins of *Clostridium perfringens*, *Clostridium novyi*, and staphylococci, which are completely unrelated to each other in any way other than that they are designated α . Even more confusing is the fact that some toxins bearing different Greek symbols were subsequently shown to be serologically and/or biochemically related.

The onset of the Second World War stimulated further research into toxigenic anaerobes. The α toxin of *C. perfringens* – important in gas gangrene – became the first toxin for which a biochemical mode of action was recognized in 1941; Macfarlane and Knight showed it to be a phospholipase C enzyme (lecithinase in older literature). However an intriguing observation was the fact that not every bacterial species that produced phospholipase C was necessarily pathogenic, nor were all such enzymes equally toxic. Explanations for this can now be given (see below).

In the early 1950s Smith and Keppie and their

numerous colleagues at the Microbiological Research Establishment, Porton, UK launched a series of studies on pathogenic bacteria and their products derived from *in vivo* sources. The prevailing climate was the threat of biological warfare and how to combat it. One organism with perceived military potential was *Bacillus anthracis*, an aerobic sporer. The Porton group discovered anthrax toxin(s). Another very important observation was made in 1953 by De and his collaborators. They injected cell-free filtrates of *Vibrio cholerae* into rabbit ileal loops and demonstrated the accumulation of fluid. The active factor was a heat-labile protein, fittingly described as an enterotoxin.

During the 1960s the mode of action of diphtheria toxin was elucidated by several groups. It was the first of several A–B toxins shown to consist of two types of fragment: fragment B ('binding') somehow facilitates entry of fragment A ('active') into the cytoplasm of susceptible cells.

The 1970s were a period of intense activity, during which enterotoxins were examined in great detail. The initial surge came from the need to develop more effective vaccines to control outbreaks of cholera in areas where it was (and still is) endemic or in areas where natural disasters or the ravages of war create conditions conducive to the spread of the disease. Attention was focused on the enterotoxin discovered by De in 1953. This led to the evolution of the currently widely held view that cholera toxin acts by biochemical perturbation of transport processes in epithelial cells of the small intestine giving rise to a cAMP-mediated watery diarrhea. This became the paradigm used to explain other toxin-mediated water diarrheas.

From the 1980s to the present, several developments have occurred. First, work on toxin A of *Clostridium difficile* (the causative agent of pseudomembranous colitis) disproves totally that the cholera toxin-based paradigm for watery diarrhea is of universal applicability: *C. difficile* toxin A is a histotoxic hemorrhagic toxin (i.e. it destroys tissue architecture and induces hemorrhage) which causes bloody fluid secretion in ileal loops but clear 'cholera-like' fluid from the colon of experimental animals; in the latter case, the hemorrhage remains tissue-localized as the basement membrane underlying the colonic epithelium is not breached. Second, the cAMP-mediated mode of action of cholera toxin has been shown by Lundgren's group in Sweden to be too simplistic; any satisfactory model must embrace a role for the enteric nervous system and (almost certainly) a colonic component.

The third development concerns the applications of modern molecular genetics to solve some extant problems in pathogenicity and in the development of

attenuated vaccine strains. Deciding the role played by a toxin in the causation of disease by organisms where virulence is manifestly multifactorial is sometimes very difficult. The construction of mutants in which toxin genes have been mutated or deleted has greatly helped in solving such problems; one example is the demonstration that Shiga toxin only exacerbates but is not necessary for the induction of dysentery caused by *Shigella dysenteriae*. Alternatively, the transfer of toxin (and other) genes to isogenic non-virulent strains is being used. Molecular genetical approaches are also being used in the construction of live vaccine strains.

Finally, many toxins have been cloned, sequenced and mutated. Three-dimensional structures have been inferred from modelling or, in the case of some toxins, by resolution of crystal structures. These approaches have led to the recognition of functional domains in toxin molecules. Knowledge of such structure/function relationships have in turn led to exciting therapeutic developments, such as the exploitation of botulinum toxin in the treatment of muscle dystonias, or the synthesis of chimeric toxins (e.g. diphtheria toxin/IL-2 fusion proteins) for the treatment of interleukin 2 receptor (IL-2R)-expressing cancers.

Toxins that act on noncellular/extracellular elements

Pseudomonas aeruginosa elastase and one (only) of at least six proteases of *Legionella pneumophila* both induce fibrinopurulent exudation (influx of fibroblasts and leukocytes) in the rat lung (a model for *P. aeruginosa*-induced pneumonia in human cystic fibrosis) and the guinea pig lung (a model for legionnaires' disease) respectively. These characteristic pathological features almost certainly arise from the release of oligopeptides (from extracellular matrix substances) which are chemotactic for leukocytes and fibroblasts. The hemorrhagic aspects of these diseases may be interpreted in terms of the effect of such proteases on protein components of type I alveolar epithelial and endothelial cells. *L. pneumophila* protease, the 'major secretory protein' (38 kDa zinc metalloprotease) is also involved in survival within macrophages.

The proteolytic activities of the gas gangrene group of *Clostridium* species are not primary virulence determinants but a priori one must regard them as aggressins or auxiliary factors. Similar considerations apply to the hyaluronidase family of enzymes produced by streptococcal and clostridial species which act on the hyaluronic acid component of extracellular tissue matrix. *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Streptococcus mitis* and

Streptococcus sanguis produce immunoglobulin A (IgA) proteases, which could be important in establishing infections of mucosal surfaces.

Staphylococcal exfoliatin(s) – a family of toxins important in staphylococcal scalded skin syndrome – cause cleavage of desmosomes in the stratum granulosum. Whether the primary site of action is external, i.e. on desmosomal proteins, or internal on cytoskeletal proteins is not clear. The toxins have demonstrable esterase activity and amino acid sequence analyses predict 'active serine' protease activity. They bind selectively to an intracellular skin protein, profilin, but there is no evidence of ability to enter intact skin cells. Purified exfoliatin will split the skin but does not *per se* cause the erythema associated with the disease. This suggests a role for other factors such as staphylococcal δ -hemolysin.

Toxins that act on membranes without internalization

Enterotoxigenic *Escherichia coli* elaborates families of low molecular weight heat-stable (ST-I_a (porcine), ST-I_b (human), ST-2 (porcine)) as well as the heat-labile cholera-like toxins (LTs; see below). ST-1 binds to its receptor – membrane-bound guanylate cyclase – which is then activated, thereby elevating cGMP levels; ST-2 (active only in weaned pigs) acts via a second messenger which is not cGMP, cAMP or Ca²⁺. The result in each case is efflux of ions, and hence water, into the gut lumen.

Toxins that traverse cell membranes and either kill or deregulate cells

We know that these toxins are able to traverse membranes since their targets are intracellular. These proteins have common features: one component (usually described as the B fragment or subunit) binds to and interacts with a cell receptor and promotes the uptake of an active component (A fragment or subunit) in which resides the biological activity which confers toxicity. There are several ways in which one can subgroup these toxins.

1. Biologically, some kill cells (cytotoxic toxins) whereas others (sometimes called cytotoxic toxins) 'deregulate' cells.
2. Some belong to a well-defined biochemical group recognized by their ability to cleave NAD⁺ into nicotinamide and ADP-ribose moieties. They are designated ADP-ribosyl (ADPR) transferases since they transfer ADPR to different target proteins.
3. The third possibility reflects the genetic origin of

these toxins: (a) production from a single gene of a single peptide which undergoes post-translational modification into A and B fragments that are covalently linked; (b) production from separate genes of A and B subunits which non-covalently associate into stable complexes, and (c) production from separate genes of different proteins which do not associate into stable complexes but which must act in concert to express toxicity. These are known as binary toxins.

Cytotoxic toxins

The classic example is diphtheria toxin (DT) made by *Corynebacterium diphtheriae*. This is the product of the *tox* gene (a constituent gene of β -phage) whose expression is regulated by [Fe]; the latter is a co-repressor of the *tox* gene. The processing, internalization and mode of action of DT is outlined in Figures 1–3.

A new and very exciting development is just beginning. For decades attempts have been made to make immunotoxins by coupling to native toxins antibodies specific to some surface antigen on tumor cells with as yet little practical success. However, Murphy's group in Boston, USA, have genetically engineered DT by substituting that portion of the DT structural gene encoding the native toxin receptor-binding domain with modified cDNA encoding one of a variety of cytokines and growth factors including IL-2, IL-4, IL-6, epidermal growth factor (EGF)

and α -melanocyte stimulating hormone (α -MSH). The resultant fusion toxin bears a new 'cellular address', but retains all of the other biological properties of the native DT molecule as well as a three-dimensional structure nearly identical with native DT. The IL-2 receptor fusion toxin has undergone phase I/II clinical trials for the treatment of IL-2 receptor-positive hematological malignancies (and other IL-2-related conditions) and has been shown to be safe and well-tolerated. A new era of 'magic bullets'?

Pseudomonas exotoxin A (PsA) acts in a near identical manner to DT on NAD^+ and EF2, despite the fact that there are no sequence homologies or serological cross-reactivities between DT and PsA. For DT, HeLa cells are more susceptible than mouse cells; the converse is true for PsA.

Other ADP-ribosylating toxins and their targets are: *Pseudomonas* exotoxin S (erythrocyte proteins – not necessarily the main target); cholera and *E. coli* LT toxins (α_s subunits of G-regulator proteins); pertussis toxin (not the invasive adenylate cyclase described below – α_i subunit of G-regulator proteins); botulinum C2 toxin (not a neurotoxin; nonmuscle actin). Shiga toxin (*Shigella dysenteriae*) has an AB_5 (B_5 is a homopolymer)-type structural arrangement similar to cholera toxin. Certain biotypes of *E. coli* make Vero toxins (VTs; or Shiga-like toxins (SLTs)) which are identical (SLT1) or near identical (SLT2) with Shiga toxin. The role of Shiga

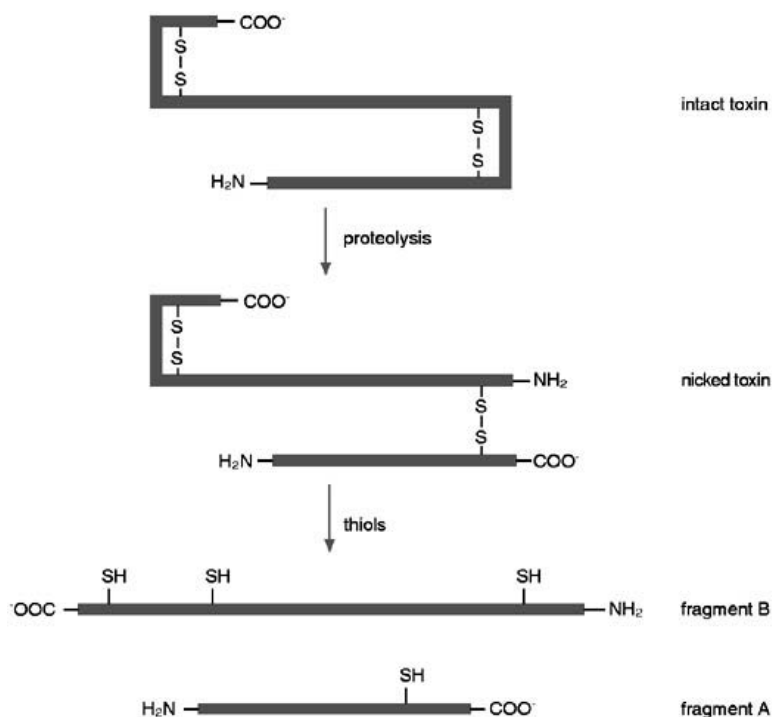


Figure 1 Diphtheria toxin production and post-translational modification. The toxin is synthesized as a single polypeptide but is cleaved (nicked) by proteases into two fragments designated A and B, held together by a disulfide bond. The latter is reduced probably during translocation of the A fragment into the cytosol. (Reproduced with permission from Mims *et al*, 1995.)

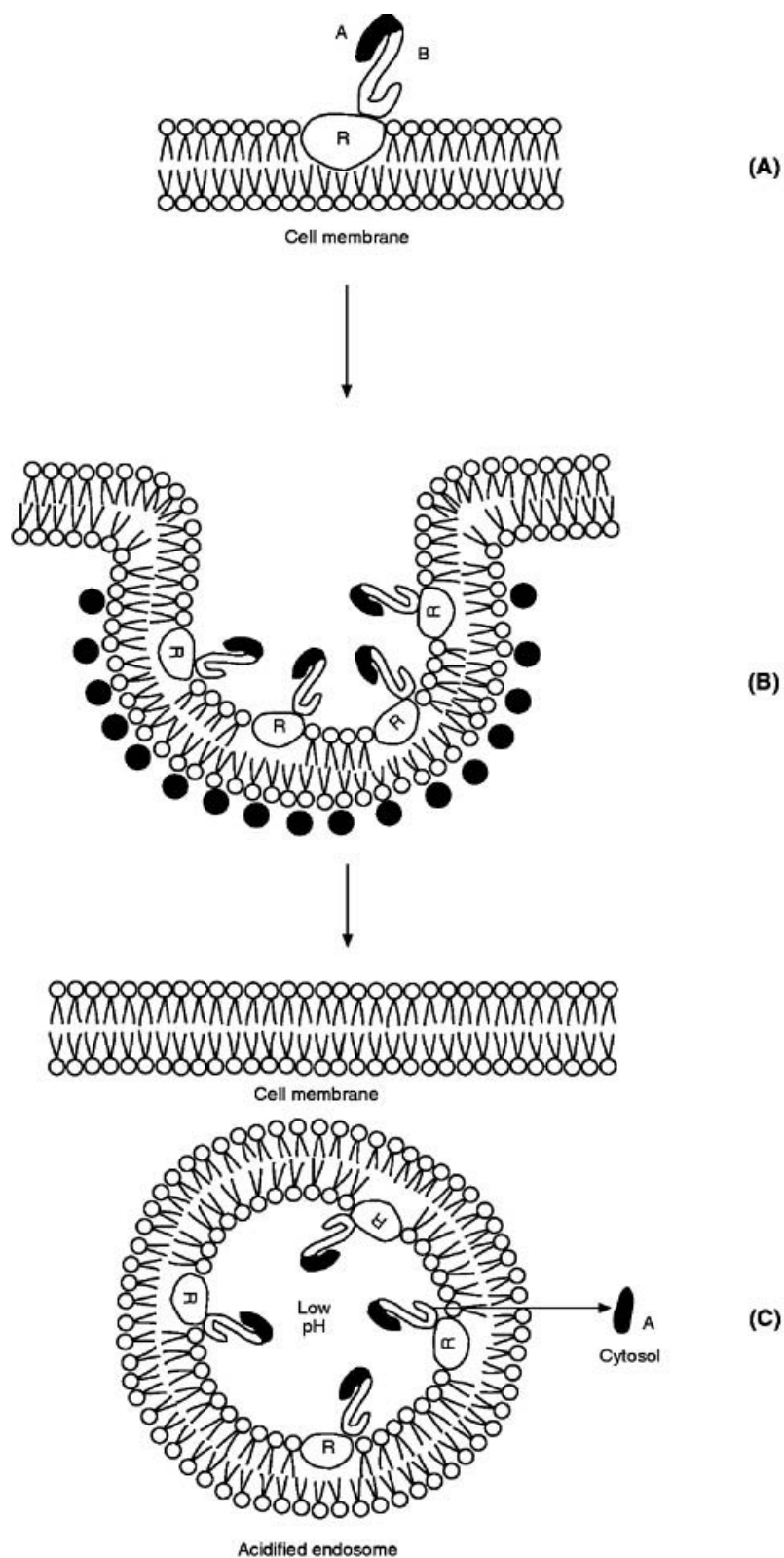


Figure 2 DTA fragment entry. (A) Toxin binds via B fragment to a glycoprotein receptor in the cell membrane. (B) These complexes migrate to clathrin-coated pits. (C) This gives rise to acidified endosomes which facilitate the escape of A fragment into the cytosol. (Reproduced with permission from Mims *et al*, 1995.)

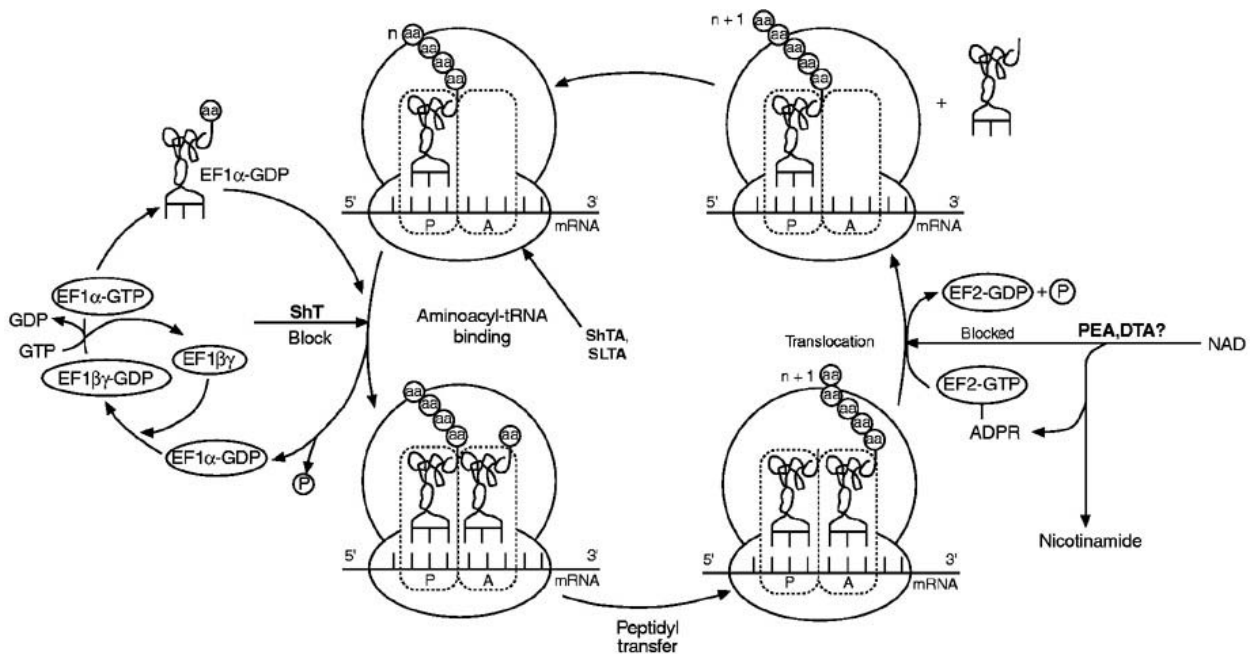


Figure 3 Protein synthesis: inhibition by DT, *Pseudomonas aeruginosa* exotoxin A (PEA), Shiga toxin (ShT), *E. coli* Shiga-like toxins (SLTs). The schema shows a round of peptide elongation and illustrates the key role played by two enzymes EF1 and EF2. DTA and PEA each ADP-ribosylates diphthamide (a modified histidine) in EF2-GTP which can no longer translocate the newly elongated peptide from the A site to P site. ShT A fragment is a specific *N*-glycosidase which cleaves an adenine residue from near the 3' end of the 28S ribosomal RNA. This depurination results in failure of EF1-dependent binding of aminoacyl-tRNA to site A and hence inhibits protein synthesis. EF1 α , nucleotide-binding protein; EF1 $\beta\gamma$, nucleotide exchange protein (Reproduced with permission from Mims *et al*, 1995.)

toxin in bacillary dysentery is to exacerbate but not to initiate disease. SLT-producing strains of *E. coli* are responsible for hemorrhagic colitis and hemolytic uremic syndrome. Crystallographic studies have shown a remarkable similarity in organizational structure of the B subunits of Shiga toxin and those of the cholera toxin despite the near complete absence of sequence homology. The B subunits attach to a ganglioside structure Gb3 or 4 (cholera toxin attaches to GM1) on susceptible cell surfaces. Shiga toxin A fragment is a specific *N*-glycosidase which cleaves an adenine residue from near the 3' end of 28S ribosomal RNA. This depurination results in failure of EF1-dependent binding of aminoacyl-tRNA to site A and hence inhibits protein synthesis; this perception comes from studies on cultured cells. How such cytotoxic activity gives rise to the enterotoxic activity – watery diarrhea seen in cases of dysentery preceding the bloody phase of the disease and attributed to the action of Shiga toxin on the small bowel – is not at all clear. Neither is there a clear explanation of the neurotoxic effects seen in severe dysentery (caused by *Shigella dysenteriae*) which definitely can be produced by administration of Shiga toxin.

Cytotoxic toxins

Vibrio cholerae attaches to enterocytes of the proximal small bowel and is capable of producing at least three toxins: classical cholera toxin (CT); a toxin that disrupts the zonula occludens tight junction (designated ZOT); and another less well-defined, auxiliary cholera enterotoxin (designated ACE). CT (an AB₅ toxin; B₅ is a homopolymer) is the classic example of a cytotoxic toxin and activates membrane-bound adenylate cyclase. The enzyme complex consists of several components: specific receptors for stimulatory (R_s) and inhibitory (R_i) hormones; the catalytic protein (C) responsible for the conversion of ATP to cAMP; and regulators of stimulation (G_s) and inhibition (G_i) comprising $\alpha_s\beta\gamma$ and $\alpha_i\beta\gamma$ subunits, respectively. See Figure 4 for mode of action of CT, LT and pertussis toxin (PTx). Phosphorylation by cAMP-dependent kinases of membrane proteins then occurs which perturbs ion transport – inhibiting absorption and stimulating secretion – and hence water. The biochemical data were derived mainly from studies on cultured cells (or isolated cell membranes) and the transport data from gut mucosal epithelia in Ussing chambers.

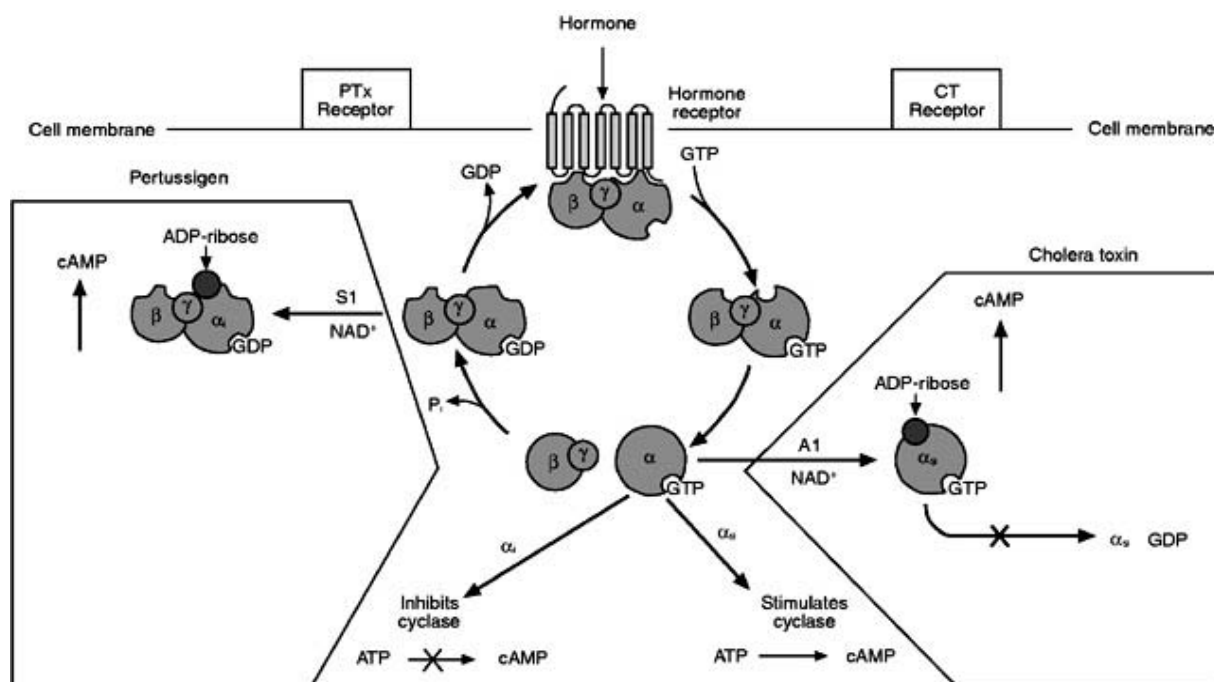


Figure 4 Mode of action of cholera toxin, LT toxins and pertussigen. There are five main features in this diagram:

1. cAMP is an important second messenger involved in the intracellular amplification mechanism of many cellular responses to external signals including hormones. The nature of the physiological response reflects the biochemical differentiation of the cell responding to the stimulus. For example, in gut cells the response would be altered ion transport and hence fluid secretion; in muscle cells it would be glycogen breakdown in response to the call for more energy. The production of cAMP is controlled both positively and negatively at two different levels. The central cycle represents a normal membrane-bound hormone receptor and the heterotrimeric ($\alpha\beta\gamma$) G protein regulator complex which is activated upon binding of hormone to the receptor. There are two receptors, each with regulatory G proteins: one system responds to stimulatory and the other responds to inhibitory stimuli; only one system is shown. In gut cells these receptors would be on the basolateral (nonluminal) side of enterocytes enabling responses to stimuli from the circulation or neuroactive hormone-like substances of which there are quite a few.
2. The endogenous GTPase properties of both stimulatory (α_s) and inhibitory (α_i) subunits of the G protein regulator may be outlined as follows. *Stimulation*: Stimulation of receptor results in the dissociation of the $\alpha_s\beta\gamma$ G protein from the receptor and of the subunits from each other, and the binding of GTP to α_s . α_s -GTP will productively interact with the catalytic unit of adenylate cyclase (not shown) and stimulate the production of cAMP from ATP. Endogenous GTPase activity in α_s results in a delayed conversion of α_s -GTP to inactive α_s -GDP which can no longer stimulate the cyclase but allows the reassociation of the trimeric G protein and loss of GDP. *Inhibition*: Stimulation of receptor results in an analogous situation for inhibiting cyclase activity via α_i ; inhibitory α_i -GTP inactivates the cyclase; α_i -GDP does not inactivate the cyclase.
3. The level of cAMP may be affected by physiological stimuli (which stimulates removal and modification of the G protein complex) or by intoxication which results in the perturbation of the normal regulatory cycle as illustrated for cholera toxin (CT) or pertussis toxin (PTx, pertussigen).
4. CT acts first by interacting (via CTB) with its receptor resulting in the internalization of the active A1 subunit. A1 ADP-ribosylates α_s -GTP which promotes continued dissociation of the heterodimer; also the endogenous GTPase is no longer functional hence the stimulation of the cyclase continues. LT toxins act in a similar manner. In the case of the enterotoxins the receptors are on the brush border membranes, i.e. on the luminal apex, of enterocytes.
5. PTx acts first by interacting (via PTxB) with its receptor resulting in the internalization of the active S1 subunit. S1 ADP-ribosylates the α_i -GDP $\beta\gamma$ heterodimer, which can no longer associate with the receptor (or lose GDP) to undergo another cycle of GTP activation; activated cyclase can no longer be turned off. (Reproduced with permission from Mims *et al*, 1995.)

This description (Figure 4) is correct but too simplistic. Studies by Lundgren's group in Sweden using gut preparations *in situ* have shown that up to 60% of CT-induced secretion is dependent on a functional enteric nervous system (ENS). Earlier studies by American workers (which have never been refuted) had shown that up to 60% of CT-induced secretion in ligated rabbit ileal loops was suppressed by cycloheximide, which inhibited protein synthesis and

mitotic activity (presumably in crypt cells), without affecting the elevation of levels of adenylate cyclase and cAMP. More recently, the colon has been implicated in CT-induced secretion via the ENS. The effect of ZOT has been demonstrated by electron microscopy in human biopsies but the relative importance of ZOT and ACE in human disease has not been quantified.

E. coli produces LT-I, and LT-II whose structure and mode of action is similar to CT. LT_h (human),

LTp (porcine) are minor variants of LT-I differing only by one amino acid in the A subunit and four amino acids in the B subunit. LT-I is plasmid encoded. LT-II (isolated from water buffalo) is chromosomally encoded, exists as IIa or IIb (which show partial serological identity), is a major variant of LT-I and is not neutralized by antisera to LT-I or CT. LT-II shows biological activity similar to LT-I, except that it does not cause secretion in ligated rabbit ileal loops, and (unlike LT-I) does not bind to ganglioside GM1. LT-IIb requires trypsin treatment for Y-1 adrenal cell assay.

CT and LT toxins are among the most potent antigens known and are also powerful adjuvants.

Bordetella pertussis produces several toxins including a heat-labile toxin, tracheal cytotoxin, endotoxin, adenylate cyclase-hemolysin (AC-Hly), and pertussis toxin (pertussigen). The latter has many biological activities (histamine sensitization, leukocytosis promotion, islets of Langerhans activations and adjuvanticity), is believed to be the most important but by no means the only virulence determinant of *B. pertussis*, and forms the basis of the vaccine against whooping cough. It is an AB₅ toxin in which the B₅ pentamer consists of four peptides comprising two heterodimers (S2, S4; S3, S4) held together by a fourth peptide (S5); A subunit is designated S1. The mode of action of A subunit is to ADP-ribosylate the α_1 -GTP complex of adenylate cyclase; this can no longer inhibit cyclase activity, thereby elevating cAMP. It is not yet clear how such biochemical activity relates to the clinical syndrome.

Clostridium tetani and *C. botulinum* both produce neurotoxins. Recently huge strides have been made in our knowledge of the structure and mode of action of these toxins (see Figures 5 and 6). There is very little overall sequence homology between BoNTs and TeTx; this could account for their immunological characteristics and the animal types normally affected. However, there is one structural feature common to all these neurotoxins located in the middle of the light chain and that is a zinc-binding motif. In fact it has now been formally proven that these neurotoxins are zinc endopeptidases. Their targets are protein constituents which comprise the synaptic fusion machine responsible for the exocytotic release of neurotransmitters. The intracellular target cleaved by BoNTs B, D, and F and TeTx is synaptobrevin (VAMP, vesicle-associated membrane protein) present in small synaptic vesicles. BoNTs A and E cleave SNAP-25 (synaptosomal-associated protein of 25 kDa) a highly conserved protein known to be involved in exocytosis. BoNT C acts on syntaxin, a synaptic membrane protein also involved in exocytosis.

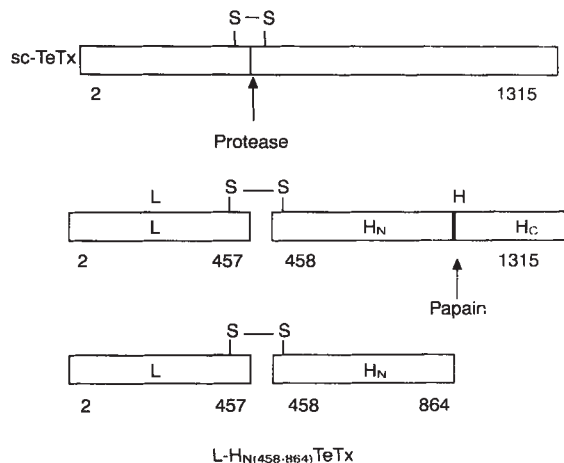


Figure 5 Structure/nomenclature of tetanus and botulinum toxins. sc-TeTx, single chain non-cleaved tetanus toxin; TeTx, tetanus toxin after proteolytic activation; L and H, intact light and heavy chains respectively; L-H_{N(458-864)}TeTx, intact L chain linked to a fragment of H chain (from residue 458 at the N-terminal end to residue 864); H_N and H_C, N-terminal and C-terminal parts respectively of the H chain. The corresponding nomenclature for botulinum toxin type A is BoNT/A, and for type B, BoNT/B, etc. (Reproduced with permission from Mims *et al.*, 1995.)

Binary toxins

Bacillus anthracis produces two binary toxins: edema factor (EF) and lethal factor (LF). For both toxins, protective antigen (PA) is required for activity by promoting entry of either EF or LF (but not both simultaneously) into the cytosol of susceptible cells. (Historically, these proteins were first described as factors I (EF), II (PA) and III (LF) and perceived as forming a tripartite synergistically acting toxin. (Current usage favours EF, PA, LF and a duality of binary toxins: PA+EF; PA+LF.) Current perceptions on the mode of action of these binary toxins are based on effects seen in cultured cells. PA binds to cell membranes, undergoes proteolytic activation by furin and internalization into an acidified endosome. Activated PA oligomerizes into a heptameric ring structure to which either EF or LF will bind in a competitive manner. The heptamer acts as a pore-forming complex aiding the translocation of EF or LF into the cytosol. EF is an inactive adenylate cyclase which becomes activated by calmodulin, leading to supraphysiological levels of cAMP. The known secretagogue activity of cAMP (can induce fluid secretion) could explain both the edematous reaction evoked in skin tests and the lethal systemic effects of anthrax. In guinea pigs, lethality was shown to be due to secondary oligemic shock. The action of anti-PA (PA is a protective immunogen) may be interpreted in terms of antibodies preventing the initial binding of PA, or the binding of EF or LF to PA. The binding of EF is

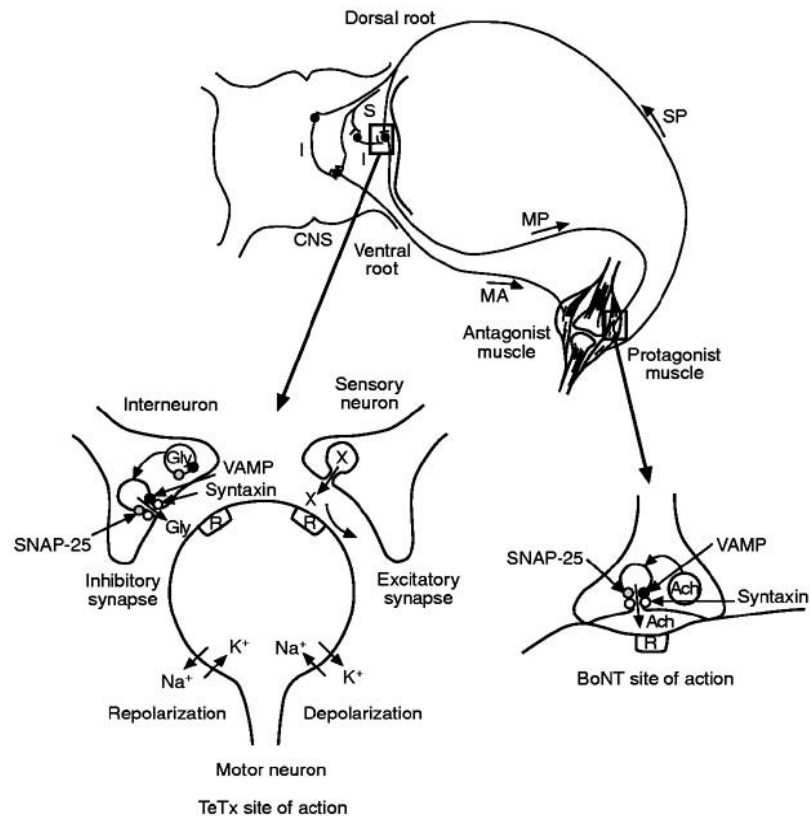


Figure 6 Sites of action of TeTx and BoNTs. This figure has three main features:

1. Reflex arc. Mechanism for inhibiting the antagonists to a muscle contracting in response to stretch. Muscles are reciprocally innervated with sensory and motor neurons, although for clarity this is shown only for the protagonist muscle. On stretch, the stretch receptors generate an impulse which is transmitted along the afferent sensory (S) neuron (SP) of the protagonist muscle. This SP enters the spinal cord by the dorsal root and synapses with the motor neuron (MP) supplying the protagonist muscle and with an interneuron (I) which in turn synapses with the motor neuron (MA) supplying the antagonist muscle. At the SP/MP synapse an excitatory transmitter is released that induces an impulse in MP which leads to contraction of protagonist muscle. However, excitation of I causes release of an inhibitory transmitter at the I/MA synapse which leads to relaxation of the antagonist muscle. Note that the basic reflex arc has been shown for simplicity but TeTx acts mainly on voluntary muscles.
2. A simplified version of the biochemical events occurring in synapses. Excitatory and inhibitory synapses, neurotransmitter release and action. Gly, glycine; R, receptors of neurotransmitters; X, hitherto uncharacterized (candidates include glutamate, dopamine, ATP, substance P, and somatostatin).
3. Sites of neurotoxin action. The predominant site of action of TeTx is the intermotor neuron synapse; the exocytotic machine is interfered with by the endopeptidase action of TeTx on VAMP. BoNT acts at the neuromuscular junction inhibiting the release of acetylcholine (ACh) by its proteolytic action on VAMP (types B, D and F) or SNAP (types A and E) or syntaxin (type C). (Reproduced with permission from Mims *et al*, 1995.)

depressed by prior binding of LF and this could explain the depression of edema produced by LF when added to EF and PA. The cell model does not explain or predict the increase in lethality resulting from addition of LF to EF and PA. However, it is now known that in high doses PA+LF is cytolytic to murine macrophages but that in very small sublytic doses it will stimulate expression of IL-1 and tumor necrosis factor (TNF). Protection of mice against LF is possible using passive transfer with anti-IL-1 or injection of IL-1 receptors, implying that, in mice at least, death could be due to the overstimulation of cytokines, particularly IL-1. LF is a putative Zn metalloprotease.

Other binary toxins

Clostridium botulinum C2 toxin (Figure 7) is not a neurotoxin but belongs to a family of cytotoxins. (*C. perfringens* iota toxin, *C. spiroforme* toxin, *C. difficile* ADP-ribosyltransferase) whose common features are that they are binary toxins and that they target cytoskeletal actin. Of these *C. botulinum* C2 is the best studied at present. Component C2I is the equivalent of the A subunit and C2II, the B subunit. Both are produced in parallel although different strains produce different ratios. C2 is produced more during sporulation than in the vegetative phase of growth.

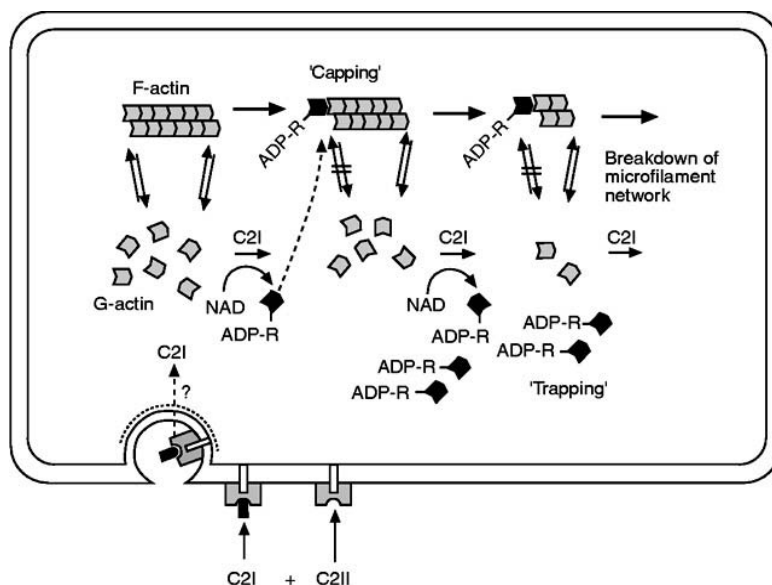


Figure 7 *C. botulinum* C2 toxin component 2II is activated by proteolysis and binds to the cell membrane followed by C2I. The latter is internalized and upsets the equilibrium between polymerization and depolymerization of actin. ADP-ribosylation of monomeric actin inhibits its polymerization and turns G-actin into a capping protein which binds to the fast-growing (barbed) ends of actin filaments. Capping of the barbed ends increases the critical concentration for actin polymerization. Since the slow-growing (pointed) ends of actin filaments are free, depolymerization of actin occurs at these ends. Released actin is substrate for the toxin and will be withdrawn from the treadmilling pool of actin by ADP-ribosylation, i.e. trapped. Both reactions will finally induce the breakdown of the microfilament network. The substrate specificity is high. The toxin will modify a specific arginine residue (177, present in all isoforms) in β and γ nonmuscle actin and γ smooth muscle actin but not in α isoforms of actin, which is surprising in view of the high degree of homology between the various isoforms of this protein. Neither component is toxic on its own but together the LD₅₀ dose for a mouse is about 5–50 ng. The two components may be injected into different sites or by different routes but toxic effects always occur at the site of injection of C2II. (Reproduced with permission from Mims *et al*, 1995.)

The other three form an iota subgroup in that antibodies will cross-react within this group but not with *C. botulinum* C2 toxin. Only within the iota group are the binding components interchangeable. The iota group will also modify all mammalian actin isoforms.

Other examples of binary toxins are staphylococcal leukocidin and staphylococcal γ -hemolysin.

Membrane-damaging toxins

Proteins, lipids and sterols are all known to be involved as targets for membrane-damaging toxins. Perhaps the best example of a toxin whose target is a membrane protein is the protease of *L. pneumophila* described earlier: this enzyme also induces the hemorrhagic response seen in experimental infections. Since the membranes of endothelial and type 1 alveolar epithelial cells undergo pathological changes, it is reasonable to ascribe these to the proteolytic activity of this toxin.

Phospholipases

There are many bacterial enzymes that act on phospholipids, of which the best known is the α -toxin of *Clostridium perfringens*. *C. perfringens* α -toxin is a

phospholipase C (PLC) which removes the head group, phosphorylcholine, from phosphatidylcholine and from sphingomyelin. It is of undoubted importance in gas gangrene. There are several reasons why all enzymes with such biochemical specificity are not equally toxic or important in determining virulence. By the combined use of monoclonal antibodies and molecular genetic analyses we now know that there are at least two functional domains in *C. perfringens* α -toxin. If one compares *C. perfringens* α -toxin with the phosphatidylcholine-preferring, nontoxic phospholipase of *Bacillus cereus*, one finds that two-thirds of the N-terminal sequence shows homology with the entire sequence of *B. cereus* PLC. This portion of *C. perfringens* α -toxin retains its PLC activity but not its hemolytic and lethal activities. The C-terminal part is not hemolytic, not enzymatically active and not cytotoxic for mouse lymphocytes but is necessary for conferring toxicity on the N-terminal part of the protein. In fact, the C-terminus is a potent immunogen that will solidly protect mice – and hopefully humans – against experimental infection with *C. perfringens*. Surprisingly, the C-terminal domain of the non-toxic *C. bifermentans* enzyme shows sequence similarity with that of its *C. perfringens* α -toxin

counterpart. The nontoxicity of this enzyme is ascribed to its comparatively much lower turnover rate, i.e. it is a much less efficient enzyme.

Staphylococcal β -hemolysin (a sphingomyelinase) exhibits the narrowest of phospholipid specificities and kills neutrophils.

Pore-forming toxins

SH-activated cytolysins A family of thiol-activated cytolysins exists produced by at least 17 bacterial species belonging to several genera. Sterol is the primary target in susceptible membranes to which such proteins, initially secreted as water-soluble entities, bind. Arc/ring complexes are formed in membranes which do not contain cholesterol, are extractable, and comprise conformationally altered proteins which will bind nonspecifically to other membrane lipids; sterol is only a primary anchor. Gross amino acid compositions of these proteins differ widely but serological cross-reactivity indicates at least some conserved epitopes. The majority are known not to be primary determinants of virulence. However, streptolysin-O (*Streptococcus pyogenes*) and listeriolysin (*Listeria monocytogenes*) are definitely produced *in vivo* and damage/kill neutrophils, causing their granules to discharge their degradative enzymes to kill the pathogen (or harm the host). Listeriolysin is also important in mediating escape of *L. monocytogenes* from vacuoles in nonphagocytic cells. Pneumolysin (*Streptococcus pneumoniae*) has definitely been implicated in experimental murine infections. Immunization of mice with pneumolysin toxoid prolongs the life of mice in an otherwise rapidly lethal respiratory infection with *S. pneumoniae*. Pneumolysin is also the primary ototoxic agent causing deafness in experimental meningitis in guinea pigs and rabbits.

RTX toxins These are a group of toxins whose common feature is the presence of an array of nine amino acid repeats; they have been designated RTX (repeats in toxin) toxins. The prototype is the hemolysin of *E. coli* which is important in extraintestinal infections caused by this organism. Other toxins of this group include hemolysins from *Proteus vulgaris*, *Proteus mirabilis*, *Morganella morganii* and *Actinobacillus pleuropneumoniae*, the leukotoxins from *Pasteurella haemolytica* and *Actinobacillus actinomycetemcomitans* and the adenylate cyclase-hemolysin (AC-Hly) of *Bordetella pertussis*. *E. coli* hemolysin is synthesized as an inactive precursor and actively secreted. The repeat domain is a Ca^{2+} -binding site needed for membrane recognition. *B. pertussis* AC-Hly, which is known to be produced early in infection in children (based on antibody detection), protects mice against colonization by

B. pertussis, and is a fascinating molecule having two functional domains. One confers adenylate cyclase activity and another hemolytic activity. It is possible to obtain Hly as a separate fragment from AC. It is 'anthrax-like' as far as being an adenylate cyclase is concerned. Its hemolytic activity arises from its pore-forming properties in cell membranes, the latter probably being responsible for the translocation of the AC portion of the complex. AC-Hly is also known as cyclolysin (or invasive adenylate cyclase).

Other pore-forming toxins Staphylococcal α - and δ -toxins act in a manner similar to that described for thiol-activated cytolysins with the exception that no known specificity for initial binding has been demonstrated. Based on studies using liposomes (artificial membranes), the size and orientation of the protein-lipid micelles generated by staphylococcal δ -toxin is concentration dependent: in larger concentration the effect is detergent-like. The genesis of pore-like structures is similar to what happens when the terminal C5b-9 complex of complement binds to membranes. It is also similar to the perforin-mediated killing mechanism whereby cytotoxic T lymphocytes kill target cells. The pore diameters for streptolysin-O, C5b-9, and staphylococcal α -toxin are 30, 5–10 and 2–3 nm respectively. These water-soluble entities undergo conformational changes and oligomerization on binding to membranes to form supra-molecular channels with the hydrophobic surfaces of the constituent proteins facing outwards and the hydrophilic surfaces facing inwards.

Toxic structural components of gram-negative bacteria: endotoxin

Endotoxins are well-recognized structural entities possessing microbial functions; they are lipopolysaccharide (LPS) in nature and are located in the outer membrane of gram-negative organisms (Figure 8). (The only lethal membrane-associated protein toxin is the complex guinea pig toxin of *Yersinia pestis*.)

Endotoxins are not primary determinants in initiating infection. The guts of healthy animals, including humans, are full of gram-negative organisms and their components which are normally not absorbed in quantity; small amounts are detoxified by liver Kupffer cells. But they are important in diseases caused by invasive pathogens: typhoid, tularemia, plague, brucellosis, and a variety of hospital-acquired infections caused by opportunistic gram-negative pathogens which include *E. coli*, *Proteus*, *Pseudomonas*, *Enterobacter*, *Serratia* and *Klebsiella* spp. In addition, endotoxin has been extensively studied as a possible causative agent of shock arising from

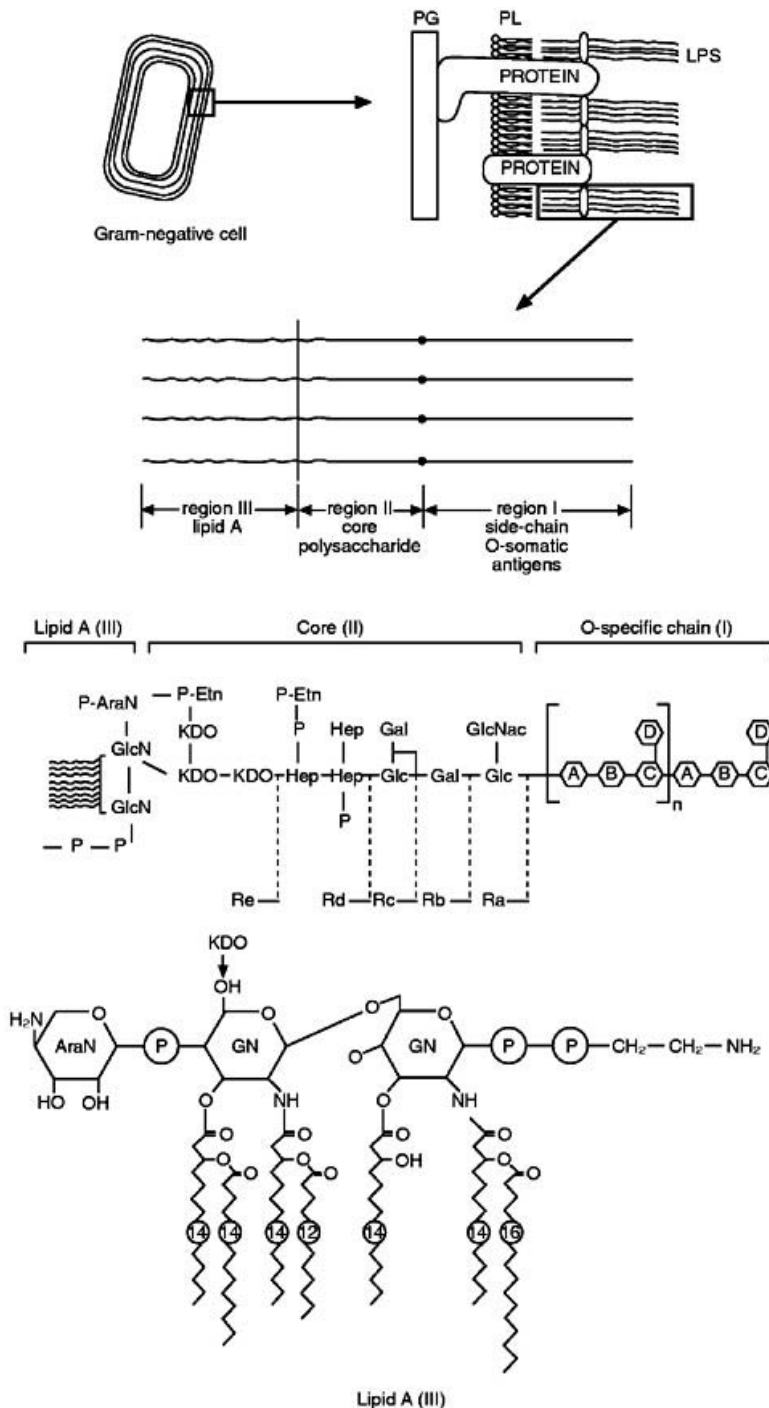


Figure 8 General structure of *Salmonella* lipopolysaccharide. PG, peptidoglycan; PL, phospholipid; A–D, sugar residues; Glc, D-glucose; Gal, D-galactose; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycerol-D-manno-heptose; KDO, 2-keto-3-deoxy-D-manno-octonate; AraN, 4-amino-L-arabinose; P, phosphate; EtN, ethanolamine; $\backslash\backslash\backslash\backslash$ hydroxy and nonhydroxy fatty acids. Ra–e, are incomplete forms of lipopolysaccharides. The structures indicated are typical of the Enterobacteriaceae and the Pseudomonadaceae. *Haemophilus influenzae*, *Neisseria meningitidis*, *Bordetella pertussis*, *Acinetobacter calcoaceticus* and *Bacteroides fragilis* have less complicated LPS structures in that they do not possess the equivalent of the O-somatic side-chains. Chlamydiae possess only lipid A and the inner core region comprising lipid A and KDO. (Reproduced with permission from Mims *et al*, 1995.)

postoperative sepsis or other forms of traumatic injury, in which the normal gut flora is regarded (at least by some) as the source of endotoxin. It is thought that accompanying changes in gut permeability allow absorption of higher levels of endotoxin.

The diversity and range of biological properties associated with endotoxin is quite bewildering and include pyrogenicity, induction of tolerance (totally unrelated to immunological tolerance), abortion (in

pregnant ungulates), the generalized Schwartzman reaction (giving rise to disseminated intravascular coagulopathy), hypotension, shock, effects on lymphoid cells, etc. Lethality is almost certainly the cumulative effect of many aspects of the endotoxin pathophysiological cascade. Lipid A is the primary toxiphore, but polysaccharide confers solubility and optimizes the size of micellar aggregates of LPS which affects biological activity. However, the

immune status of the test animal may affect toxicity: normal animals produce antibodies to epitopes on the surface of normal gut organisms (including O-somatic antigens). In such circumstances, the biological effects of endotoxin may be mediated by hypersensitivity mechanisms.

Strategies for preventing or neutralizing the effects of LPS with reagents other than expensive partially effective anti-lipid A antibodies are being developed and include exploitation of LPS-binding protein, the bactericidal/permeability increasing protein, the CD14 LPS receptor, nontoxic lipid A and lipid A analogs.

See also: **Bacillus**, infection and immunity; **Bacterial cell walls**; **Bordetella**, infection and immunity; **Clostridium**, infection and immunity; **Coryneform bacteria**, infection and immunity; **Endotoxin (lipopolysaccharide (LPS))**; **Escherichia coli**, infection and immunity; **Fever**; **Klebsiella**, infection and immunity; **Legionella**, infection and immunity; **Liposomes**; **Neisseria**, infection and immunity; **Perforin**; **Proteus**, infection and immunity; **Pseudomonas aeruginosa**, infection and immunity; **Shigella**, infection

and immunity; **Staphylococcus**, infection and immunity; **Streptococcus**, infection and immunity; **Venoms**; **Vibrio cholerae**, infection and immunity; **Yersinia**, infection and immunity.

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TOXOCARIASIS

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Characteristics of the organism and its antigens

The causative agent of toxocariasis is the nematode, or 'roundworm', parasite *Toxocara canis*. Infective eggs are released by sexually reproducing adult worms which parasitize the intestines of domestic dogs and related carnivores. The eggs are not initially infective, but require a period of 2–3 weeks for the development of infective larvae. These are contained within characteristically pitted eggs which are extremely resilient, and viable eggs can persist in soil for many years. Infection is initiated by ingestion of these eggs, and the release of the tissue-invasive larvae. This can occur in almost every species of mammal tested, and even some birds. The parasite will only develop to maturity in canids, but in other species will persist in the body, and remain infective to the definitive host through carnivory. The larvae remain dormant in the tissues of female dogs until pregnancy, whereupon they migrate to the uterus and invade the fetus. It has been reported that this results in a near 100% rate of infection in newborn pups, which can

also be infected by the transcolostral route. This complex and opportunistic life cycle is detailed in **Figure 1**.

Toxocariasis is not a significant veterinary problem and the main interest lies in its impact on humans. The tissue-invasive larvae of the parasite migrate throughout the soft tissues of the body, including the liver, musculature, brain and the eye, and give rise to symptoms which have been used to divide the condition into two main syndromes (**Figure 2**). The first of these is visceral larva migrans (VLM) which can present with high eosinophilia, high specific and nonspecific immunoglobulin E (IgE) responses, leukocytosis, hypergammaglobulinemia, hepatomegaly, pulmonary infiltration and pyrexia. The first two of these are characteristic of nematode infections. VLM is a chronic condition since the larva can persist in humans for up to 7 years, although it will gradually fade so long as new infections are prevented. The second syndrome is ocular larva migrans (OLM), in which the eye is invaded. This is associated with few systemic symptoms, and is thought to occur at lower levels of infection than VLM, and patients can be seronegative. OLM is, however, often the occasion upon which toxocariasis is first suspected. The granuloma produced in the

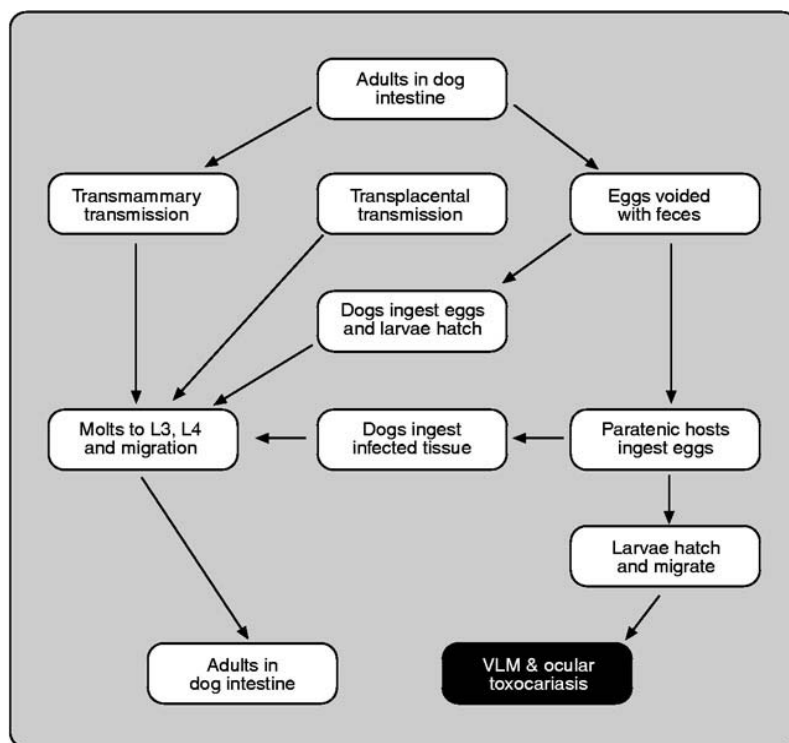


Figure 1 The life cycle of *Toxocara canis*, showing the various means by which the parasitic larvae can gain entry to the canine final host, and in which the human becomes an accidental host. *T. canis*, like all nematodes, undergoes a series of molts during its development to adulthood. These define four different larval stages, termed L1 to L4, which occur as detailed in the figure, beginning with the L2 stage which is in the egg and is infective to mammals. (Courtesy of Professor RM Maizels, University of Edinburgh and © Taylor & Francis.)

retina is sometimes misdiagnosed as retinoblastoma, which is also commonest in childhood, and unnecessary surgical removal of the eye can result. It can, however, be distinguished from the neoplasm by careful ophthalmoscopy or biopsy. The latter is clearly not always an option, and methods for the detection of parasite antigen in vitreous fluid are probably the only immunological means of diagnosis, in view of the lack of a detectable serological response in OLM, which would not be definitive anyway.

The antigens of *Toxocara* are amongst the best characterized among nematode parasites. This is chiefly because of the remarkable ease with which the infective larvae can be maintained *in vitro*; they can survive for up to a year in defined, serum-free medium, during which time they remain under developmental arrest, and release copious quantities of antigens into the medium. These are presumptively termed excretory/secretory (ES) antigens, and have become the most widely used preparation for the immunodiagnosis of toxocarasis. ES antigens are heavily glycosylated proteins, some of which are associated with enzymatic activity, e.g. proteinases, and have been detected in the circulation of infected humans and experimental animals. ES contains about eleven major components, ranging from 32 to 400 KDa. Monoclonal antibody analysis of ES has shown that there are both carbohydrate and peptide epitopes present, and that some of the former can be distributed among several otherwise unrelated

components. Moreover, certain of the glycoproteins bear epitopes similar to human ABO blood group substances. Recent analysis of the carbohydrate moieties in *T. canis* ES has revealed some structures which might explain the ABO blood group cross-reactions. Most monoclonal antibodies raised to *T. canis* also recognize the antigens of the closely related (and potentially hazardous) parasite of cats, *Toxocara cati*. However, one antibody is specific to

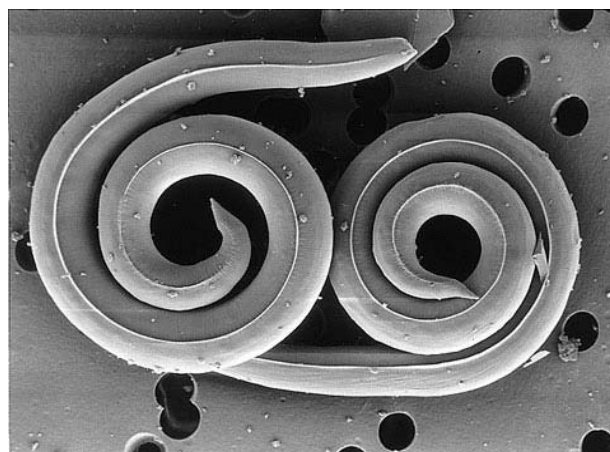


Figure 2 Scanning electron microscope picture of two infective larvae of *Toxocara canis*. The parasites were mounted on a membrane with pores of approximately 10 µm in diameter. (Courtesy of Dr L Tetley, University of Glasgow and Dr HV Smith, Scottish Parasite Diagnostic Laboratory and © Taylor & Francis.)

T. canis and is of some interest since it represents an example of a species-related carbohydrate epitope.

In common with a number of other nematode parasites, *Toxocara* is known to shed antigens rapidly from its surface and thereby contribute to ES. Utilization of monoclonal antibodies to surface and ES antigens has provided strong indications that they derive from the oesophageal gland which is ducted to the buccal cavity of the parasite, and the mid-body secretory gland which is directly connected to the cuticle by a duct opening at the secretory pore. Monoclonal antibodies to ES will bind to detergent-solubilized surface antigens, but few of them bind to the surface of intact, living, larvae. There are, therefore, cryptic epitopes, probably indicative of the orientation of glycoproteins in the surface layer of the parasite. The genes encoding two surface and/or ES antigens of the parasite have been cloned and sequenced; one encodes a phosphatidylethanolamine-binding protein and another a mucin-like protein. The latter might relate to the rapid shedding of surface antigens by *T. canis* and to the parasite's migratory propensity.

Humans are subject to infection with a number of nematodes that are antigenically cross-reactive with *Toxocara*, the most important of which is *Ascaris lumbricoides*. This could compromise specific serodiagnosis, but the significance of this problem remains to be established. However, with the availability of species-specific monoclonal antibodies and molecular cloning of ES antigens, which is now in progress, there is the potential for the development of specific serodiagnostic methods.

Immune responses of the host

Toxocariasis is a covert parasitism in humans, and immunodiagnosis remains the principal means by which infection can be detected. Seroprevalence rates in the general population are 2.8% and 2.0% among healthy adults or blood donors in certain areas of the USA and UK, respectively. Children have higher rates, for example, 23.1% and 14.3% in children under 10 years old in the USA and UK, respectively. A recent study in the Republic of Ireland also revealed alarmingly high seroprevalence rates amongst children. This increased prevalence among the young also occurs in less developed countries in which levels as high as 86.6% have been recorded in children under 6 years old in the Caribbean.

The other major aspect of the immune response in toxocariasis is eosinophilic granulomata which can occur throughout the body, with the apparent exception of the brain. Larvae can be found entrapped within liver granulomata, and there is some experi-

mental evidence that larvae can be killed within them. Granulomata can be found without larvae, and it seems that the parasite can escape entrapment, or deposited antigens stimulate a reaction to migratory tracks. ES materials have been shown to contain factors that stimulate eosinophilia *in vitro*, and at least one allergen whose activity is lost after treatment with a reducing agent.

T. canis infection elicits a strong T_H2-dominated response in experimental animals, and it was the analysis of the response of T cells to *T. canis* antigens which provided early evidence for the T_H1/T_H2 divide in humans.

Evasive strategies by the organism

T. canis clearly evades the immune response very successfully, but the means by which it does this are a matter of debate, and there are an increasing number of possible explanations. First must be the active shedding of surface antigens which has been observed *par excellence* in this parasite. Such a process could lead to a loss of bound antibody, complement and cells. Second, the release of enzymes. *T. canis* larvae are known to secrete proteases which could cleave bound antibody and complement, or aid in escape from granulomata. Acetylcholinesterase is also secreted, and this might serve to deplete local levels of acetylcholine which is known to enhance mediator release by mast cells, neutrophils, lymphocytes, and the release of interferon γ by T cells. *Toxocara* would be unusual amongst helminth parasites if it did not release enzymes capable of disarming the host's reactive oxygen defense mechanism, but these have not yet been described. Lastly, ES activates complement, which could lead to local depletion. The larval surface will, however, also activate human complement by both pathways, culminating in the deposition of C9, although this occurs without apparent damage to the parasite *in vitro*.

Vaccines

There is no immediate prospect of a vaccine. ES antigens have been shown to contain protective antigens in mice, and molecular cloning of some of the parasite's major ES components is encouraging. In common with other nematodiasis, most effort in vaccine development is aimed at surface and/or secreted antigens, and this is particularly appropriate to toxocariasis in which there is a vigorous response to surface and ES, but a poor response to internal components.

See also: Ascariasis; Filariasis, lymphatic; Parasites, immunity to; Skin, contribution to immunity.

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TOXOPLASMOSIS

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Toxoplasmosis is a disease of mammals and birds caused by the coccidian protozoan *Toxoplasma gondii*. It can be transmitted to humans via undercooked meat, contaminated soil and food, and by organ transplantation. The organism exists in three forms: tachyzoites, tissue cysts (which contain bradyzoites) and oocysts (which contain sporozoites). Tachyzoites are crescentic or oval shaped, 2–4 µm in width and 4–8 µm in length. They are seen during the acute stage of the infection and invade all types of mammalian cells except non-nucleated red blood cells. The tissue cyst and oocyst are the principal forms of the parasite involved in transmission. The tissue cyst is formed within the host cell cytoplasm and may vary in size from those containing relatively few organisms to those that are more than 100 µm in size and contain several thousand bradyzoites. Cysts are demonstrable as early as the first week of infection in experimental animals and probably persist throughout the life of the host. Although cysts may persist in virtually every organ, the brain and skeletal and heart muscle appear to be the most common sites of latent infection. Oocysts have been described only in members of the cat family. Schizogonic and gametogonic stages are found in the epithelium of the small intestine and culminate in the development of oocysts which are excreted in the feces of the cat.

Twenty-eight genes of *T. gondii* have been cloned. Based on the pattern of restriction fragment length polymorphisms of certain of these genes, strains of *T. gondii* have been classified into genotypes. The

highly virulent strains were found to have an essentially identical genotype whereas the less virulent strains were moderately polymorphic.

The presence of antigens specific for each form of the parasite has been reported, although each form shares common antigens. Tachyzoite antigens have been used for serodiagnosis of toxoplasmosis. Recently, tachyzoite antigens were reported to have a superantigen activity on murine lymphocytes.

Immune responses of the host

Infection with *T. gondii* induces both humoral and cell-mediated immune responses. The latter plays the major role in resistance to infection, although both are responsible for resistance.

Antibodies activate the classical pathway of complement to lyse extracellular parasites. Tachyzoites survive within normal macrophages, but are killed if coated with specific immunoglobulin G (IgG) antibody. Monocytes and neutrophils can rapidly kill tachyzoites. The protective effect of antibodies is only partial against challenge infection in laboratory animals. The cell-mediated immune response appears to be essential for survival of the host during the acute infection (Figure 1). Activation of macrophages by interferon γ (IFNγ), is a major effector mechanism against *Toxoplasma* infection. Macrophage killing of *T. gondii* is by both oxidative and nonoxidative means. Neutralization of endogenous IFNγ in mice by IFNγ-specific antibody resulted in a lack of macrophage activation during acute toxoplasma

infection; the mice died when infected with an avirulent strain which does not kill untreated mice. Tumor necrosis factor α (TNF α) plays an important role in IFN γ -mediated activation of macrophages. Natural killer (NK) cells and T cells are responsible for the efferent limb of the protective cell-mediated immune response. CD8 $^{+}$ T cells appear to be most important for induction of the protective effect. A major role of CD8 $^{+}$ T cells in this resistance appears to be production of IFN γ since an IFN γ -specific monoclonal antibody (mAb) completely ablated their protective activity in the acute infection. The role of CD4 $^{+}$ T cells is probably a production of interleukin 2 (IL-2) which augments IFN γ production by CD8 $^{+}$ T cells. NK cells are another important source of IFN γ . Production of IFN γ by NK cells appears to be important to activate macrophages in the early stage of infection prior to production of this cytokine by T cells. IFN γ produced by NK cells may play a critical role in induction of a T_H1-type response (IL-2 and IFN γ

production) of CD4 $^{+}$ T cells and augmentation of CD8 $^{+}$ T cell activity. IL-12, IL-15, TNF α and IL-1 β are important for activation of NK cells to produce IFN γ . *In vitro* production of IFN γ by spleen cells of SCID (severe combined immunodeficiency) mice (which lack T and B cells but have NK cells) following stimulation with *T. gondii* tachyzoites or antigens was enhanced by addition of antibodies against transforming growth factor β (TGF β) or IL-10. Thus, TGF β and IL-10 appear to play a role in downregulation of IFN γ production by NK cells during infection. IL-10 also downregulates IFN γ production by CD4 $^{+}$ T cells and inhibits IFN γ -mediated parasite killing by macrophages. IL-7 has enhanced the protective activity of NK and CD8 $^{+}$ T cells in a murine model. $\gamma\delta$ T cells are also involved in resistance against the acute infection and human $\gamma\delta$ T cells have cytotoxic activity against *T. gondii*-infected target cells and produce IFN γ , IL-2 and TNF α following stimulation with the infected cells.

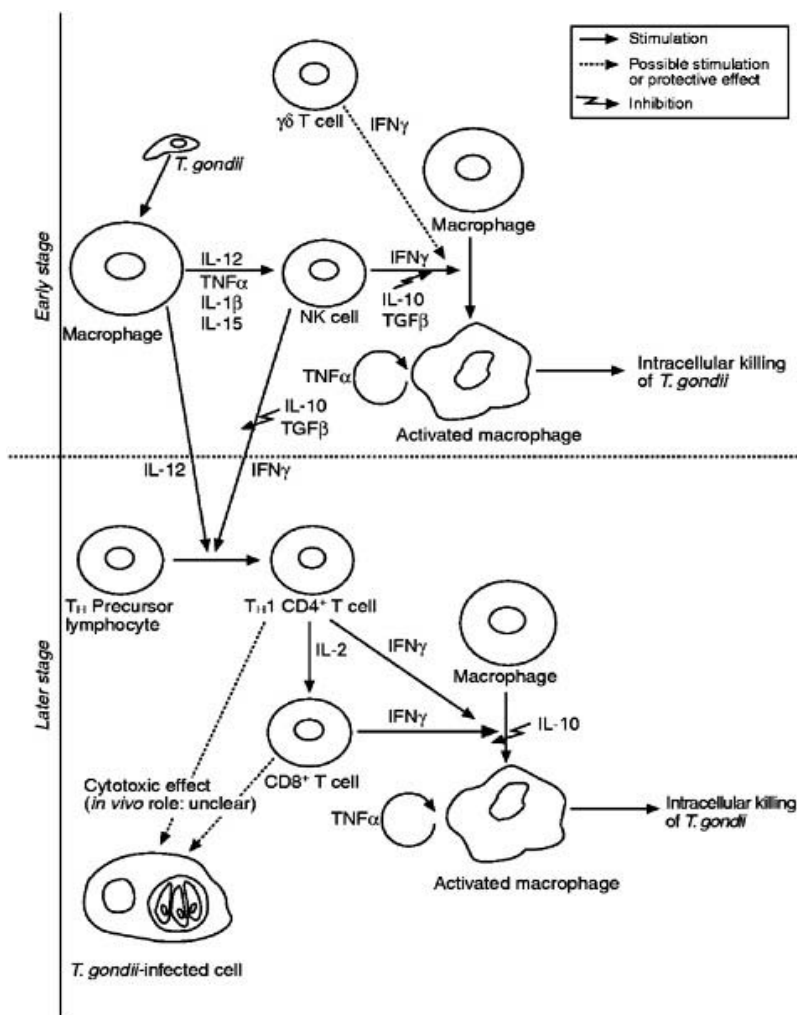


Figure 1 During the early stage of infection with *T. gondii*, macrophages are stimulated to secrete IL-12, IL-15, TNF α and IL-1 β . These cytokines activate NK cells to produce IFN γ which then activates macrophages. Activated macrophages phagocytose tachyzoites and effectively kill the organisms by both oxidative and nonoxidative means. Activation of macrophages through this mechanism appears to be earlier than through production of IFN γ by $\alpha\beta$ T cells. Production of IFN γ by $\gamma\delta$ T cells may be involved in activation of macrophages in this early stage of infection.

During the later stage of infection, IL-12 produced by macrophages and IFN γ produced by NK cells induce differentiation of T_H precursor lymphocytes into T_H1 CD4 $^{+}$ T cells. T_H1 CD4 $^{+}$ T cells secrete IL-2 which enhances IFN γ production by CD8 $^{+}$ T cells and also produce IFN γ . However, CD8 $^{+}$ T cells appear to be more important than the CD4 $^{+}$ T cells as the source of this cytokine *in vivo*. Macrophages activated by IFN γ kill tachyzoites through the mechanisms described above.

Downregulatory cytokines: TGF β and IL-10 appear to play a role in downregulating IFN γ production by NK cells. IL-10 also inhibits IFN γ -mediated killing of tachyzoites by macrophages. IL-7 is not included in this figure but has been reported to enhance the protective activity of NK and CD8 $^{+}$ cells. However, the sources and target cells for this cytokine in *T. gondii* infection are unclear.

The frequent occurrence of toxoplasmic encephalitis (TE) in patients with the acquired immune deficiency syndrome (AIDS), due to recrudescence of the previously latent (cyst form) infection, indicates the importance of cellular immunity in maintaining the infection in its quiescent form. Injection of chronically infected mice with mAb to IFN γ induced remarkable encephalitis along with evidence of cyst disruption. Impaired production of IFN γ in AIDS patients is probably a major reason for the occurrence of the encephalitis and dissemination of the organisms to other tissues. Genetic regulation associated with the major histocompatibility complex (MHC) is operative in resistance/susceptibility against development of TE in humans and mice. HLA-DQ3 was found to be significantly more frequent in North American Caucasian AIDS patients with TE than in the general Caucasian population or randomly selected control AIDS patients who had not developed TE. HLA-DQ3 thus appears to be a genetic marker of susceptibility to development of TE in AIDS patients.

Evasive strategies by the organism

Cysts persist in tissues of immunologically normal hosts without any evidence of inflammation around them. The immune response seems to be only partially effective in removal of parasites before they can form cysts. Tachyzoites, present in the acute stage of infection, are the main target for the protective immune response. Following invasion of tachyzoites into host cells, the organisms form parasitophorous vacuoles that act as a protective interphase which prevents fusion of the vacuole with lysosomes or acidification of its contents. However, when murine macrophages or human monocyte-derived macrophages are activated by IFN γ , the parasite is efficiently killed by those cells after its invasion. It has been reported that tachyzoites induce both antigen-specific and nonspecific suppressor cells to inhibit induction of the immune responses to the parasites. The effector cells of the antigen-specific suppression are CD4⁺ and CD8⁺ T cells; the cells inhibit proliferation of lymphocytes to toxoplasma antigens *in vitro*. The activity of the suppressor cells was remarkable only in symptomatic patients with acute infection. The effector cell of the antigen-nonspecific suppression is the macrophage. The activity of the suppressor macrophages was much stronger in the susceptible than in the resistant strain of mice.

Vaccines

There is no vaccine against *T. gondii*. Recent studies demonstrate that administration of mAbs to *T. gondii* confers partial protection against mortality in mice. The antigens recognized by such protective mAbs may be candidates for vaccine development. The genes of certain of these antigens have been cloned, and the recombinant antigens are being examined for their efficacy in animals. The antigens which stimulate T cells to produce IFN γ would also be candidates for vaccine development. Use of live organisms of a mutant strain is also in trial. The mutant organisms can infect cats, but do not produce oocysts which is a principal form of the parasite involved in transmission. Immunization of cats with this mutant strain confers marked resistance against oocyst production following challenge infection.

See also: Acquired immune deficiency syndrome (AIDS); Interferon γ ; Macrophage activation; Natural killer (NK) cells; Parasites, immunity to.

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TRANSFER FACTORS

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Transfer factors are proteinaceous molecules that 'transfer' the ability to express cell-mediated immunity from sensitized donors to unimmunized recipients. Administration of a transfer factor does not activate antibody production by the recipient and does not prime the recipients for anamnestic responses when the appropriate antigen is administered. The immunologic activities of transfer factors are antigen specific.

Transfer factors may have applications in clinical medicine as a mechanism for inducing effective cell-mediated immune responses against infectious microorganisms and, possibly, tumor cells.

The phenomenon of transfer factors was first described by H. S. Lawrence in the mid-1950s when he reported that lysates of peripheral blood lymphocytes from donors who had delayed-type hypersensitivity to antigens such as tuberculin, streptococcal M protein or diphtheria toxoid could transfer delayed-type hypersensitivity to recipients who had been shown to be unresponsive to these antigens. Later, Lawrence and associates showed that the substance that was responsible for transferring the reactivities was dialyzable through membranes with a cut-off value of 12 kDa.

There were several reasons why Lawrence's experiments were questioned by other investigators. First, in his studies in humans the recipients were tested for delayed hypersensitivity to identify candidates for the study; there was concern that these skin tests could prime recipients to respond to the antigen after treatment with the transfer factor. Second, at the time, it was difficult to comprehend immunologic specificity of molecules with molecular weights of ≤ 12 kDa. Third, Lawrence's observations were in conflict with results of similar experiments in guinea pigs.

Although the mechanisms of action of transfer factors have not yet been defined, most of Lawrence's findings, including specificity, have been confirmed independently by subsequent studies.

Nature of transfer factors

A number of molecular models have been proposed through the years. On the basis of sedimentation properties, Gottlieb and coworkers proposed that transfer factors were nucleopeptides. Subsequent reports from Burger's group and by Padlock, Wilson and Fudenberg led to models in which

a peptide moiety was linked to an oligonucleotide by a phosphodiester bond. The presence of a nucleic acid or polynucleotide component in molecules with transfer factor activity has not been established conclusively. In contrast, the evidence for polypeptide is very good.

A major step toward definition of the molecular nature of transfer factor molecules was the discovery that there was a specific interaction between a transfer factor molecule and the antigen for which it was specific. This has allowed development of affinity purification techniques for isolation of individual transfer factors from mixtures of several transfer factors. The affinity purified materials have been processed further by high-performance liquid chromatography and gel filtration; the final products were virtually homogeneous. These materials had molecular weights of 4.9–5.4 kDa and retained the antigen specificity of the starting materials. The entire mass of the molecules could be accounted for by the amino acid composition; there were no detectable nucleic acid components. Subsequent studies of amino acid sequences have shown that a portion of transfer factor molecules is conserved irrespective of the specificity of the transfer factor or the species of origin of the transfer factor. Peptide mapping experiments have shown peptides that are identical in all transfer factor molecules (that have been studied so far) and other peptides that are unique to each transfer factor. The latter peptides are tentatively believed to be related to the specificity of the transfer factors.

Comparison of the sequence of the 'conserved' peptide with other sequences in the database did not reveal a relationship to any previously reported peptides.

Immunological activities of transfer factors

Specificity

A major concern in transfer factor research is how molecules with molecular weights of 4.9–5.4 kDa can transfer antigen-specific immune responses. The mechanism of this specificity has not been defined, but the evidence for specificity is so strong that it must be considered in formulation of models for transfer factor activity. For example, Maurer prepared lysates of leukocytes from human donors who had received ethylene oxide-treated human serum.

These lysates would transfer delayed-type hypersensitivity to recipients that had not received the modified serum. Burger and associates examined transfer factor activity in blood leukocytes of donors both before and after they were sensitized with keyhole limpet hemocyanin (KLH). The preimmunization specimens did not contain KLH transfer factor activity, while the postimmunization materials transferred delayed hypersensitivity to 25 of 26 recipients. Petersen and associates and Kirkpatrick and coworkers used a murine model to examine specificity of transfer factors, including preparations from donors that were immunized with synthetic antigens composed of amino acid random copolymers. Their results are summarized in Table 1. Taken together, the findings of these reports make it essential to consider specificity in formulating hypotheses for the mechanisms of action of transfer factors.

Interactions between transfer factors and antigens

At one time it was proposed that transfer factors were composed of highly immunogenic forms of antigen. This model would explain the specificity of transfer factors. To test this hypothesis, Petersen and colleagues mixed transfer factors with the immunizing antigen and tested the ability of the mixtures to transfer delayed-type hypersensitivity to unimmunized recipients. Their results disclosed that mixing

the transfer factor with the homologous antigen 'neutralized' the immunologic effects of the transfer factor. On the other hand, mixing the same transfer factor with a different antigen did not alter the activity of the transfer factor.

This observation provided evidence against antigenic units in the transfer factors. Of greater significance, it provided the basis for the affinity purification of individual transfer factors.

Transfer factors and delayed-type hypersensitivity

There are no *in vitro* assays for transfer factors. Therefore, the activity must be measured by *in vivo* assays such as transfer of delayed-type hypersensitivity to nonimmune recipients. In a murine system, the footpad swelling assay has been quantitated so that it can be used to follow changes in specific activity during purification.

Transfer factors and cytokine production

The immunologic consequences of administration of transfer factors have been described in clinical and experimental settings. The earliest reports concerned patients with the Wiskott-Aldrich syndrome, chronic coccidioidomycosis or chronic mucocutaneous candidiasis. Similar results were observed in each group of patients. The recipients acquired the ability to express the delayed-type hypersensitivity responses

Table 1 Specificity of transfer factors in assays of delayed hypersensitivity

Experiment	Donor sensitivity	Recipient		
		Antigen used	Footpad response (Δ mm $\times 10^{-2}$)	P
A	HRPO	HBSS	7.17 $\pm$ 3.87	
		HRPO	35.00 $\pm$ 6.09	<0.01
		Ferritin	5.50 $\pm$ 2.46	NS
B	Cytochrome c	HBSS	0.33 $\pm$ 2.22	
		Cytochrome c	22.00 $\pm$ 4.20	<0.001
		Ferritin	2.00 $\pm$ 3.08	NS
C	Ferritin	HBSS	3.50 $\pm$ 1.12	
		Ferritin	18.33 $\pm$ 1.84	<0.001
		Cytochrome c	1.67 $\pm$ 1.99	NS
D	GAT ¹⁰	HBSS	0.20 $\pm$ 2.27	
		GAT ¹⁰	34.40 $\pm$ 4.01	<0.001
		Cytochrome c	1.50 $\pm$ 1.31	NS
		GLA ⁵	4.20 $\pm$ 0.58	NS
E	GLA ⁵	HBSS	1.17 $\pm$ 2.36	
		GLA ⁵	67.33 $\pm$ 2.29	<0.001
		Cytochrome c	-0.67 $\pm$ 2.54	NS
		GAT ¹⁰	4.67 $\pm$ 0.95	NS

HBSS, Hanks' balanced salt solution; HRPO, horseradish peroxidase; GAT¹⁰, terpolymer of glutamic acid, alanine, and tyrosine; GLA⁵, terpolymer of glutamic acid, lysine and alanine; NS, not significant.

Data from Kirkpatrick (1988).

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that were present in the transfer factor donors. Moreover, when blood lymphocytes from the recipients were examined for production of the lymphokine, macrophage migration inhibitory factor (MIF) *in vitro*, it was found that, in contrast to pretreatment results, the cells responded to antigen activation by secreting this lymphokine.

As with the studies of delayed-type hypersensitivity, acquisition of MIF production by recipients was closely correlated with the delayed hypersensitivity responses of the transfer factor donors.

Subsequent studies in mice have shown that recipients' spleen cells respond to antigen activation by secreting interferon γ (IFN γ).

Transfer factors and lymphocyte transformation responses

In normal subjects, there is a close correlation between antigens that evoke positive delayed hypersensitivity responses and *in vitro* lymphocyte transformation responses. However, this correlation is not common in recipients of transfer factors. Even though the recipients acquire strong delayed-type hypersensitivity, they usually do not have cells in their peripheral blood that proliferate *in vitro* in the presence of the same antigen (Figure 1).

The observations may indicate that the primary targets of transfer factors are IFN γ -producing effector T cells, and that transfer factors may not induce a population of antigen-specific memory T cells. This would be consistent with the observations that the best clinical results are seen in patients who are treated with repeated doses of the transfer factor.

Transfer factors and cytotoxic T cell activity

There have been fewer studies of cytotoxic T lymphocyte (CTL) activity in clinical or experimental situations. However, there are data in patients with malignant tumors and persistent viral infections that indicate that specific transfer factor therapy may activate CTLs.

Genetic studies of transfer factor production and activity

High- and low-responder mice were used to study genetic regulation of production of transfer factors. Donors were immunized with antigens such as amino acid terpolymers (GLA⁵ and GAT¹⁰) and ovalbumin in Freund's complete adjuvant. Spleen cells from high-responder donors were excellent sources of specific transfer factors. Low-responder donors did not express delayed-type hypersensitivity to the immunizing antigen, and their spleen cells did not contain detectable transfer factor activity. However, when the transfer factor from a high-responder donor was given to a low-responder recipient, the recipient acquired the ability to express delayed hypersensitivity to the test antigen that was comparable to the high-responder donor (Figure 2).

Thus, in this model transfer factor treatment changed the phenotype of the low-responder recipient to that of a high responder. This effect is similar to the immunological changes in patients with chronic or recurrent infectious diseases who are treated with specific transfer factors.

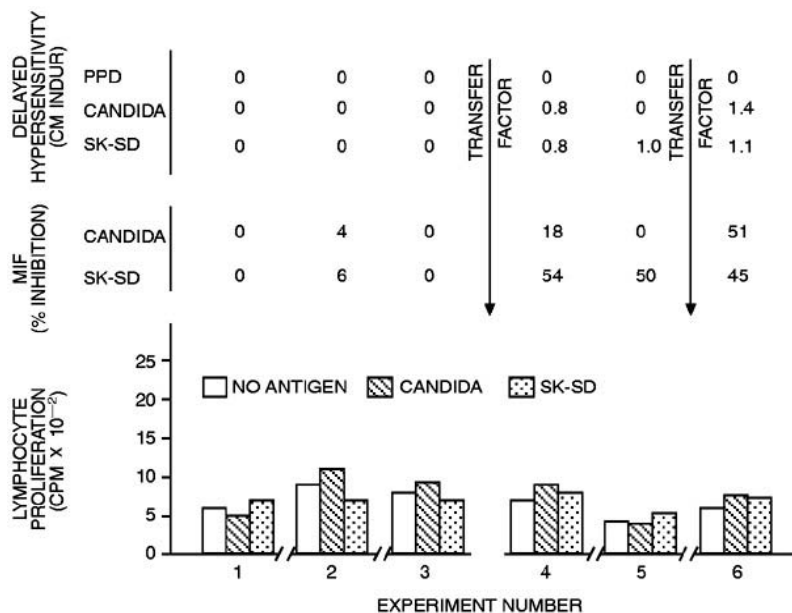


Figure 1 Effects of transfer factors on immune responses of recipients. These studies were conducted in patients with chronic mucocutaneous candidiasis. The transfer factor donor had positive delayed-type hypersensitivity (DTH) responses to candida and streptokinase-streptodornase (SK-SD) and a negative test with tuberculin (PPD). Note that before treatment the recipients were anergic, did not produce macrophage migration inhibitory factor (MIF) and did not have lymphocyte transformation responses in the proliferation assay. After treatment, the recipients could express DTH to candida and SK-SD and when their blood leukocytes were exposed to these antigens *in vitro* they secreted MIF. The treatment did not change the results of the lymphocyte proliferation test. The effect of the candida transfer factor was lost after 6 months but was restored by retreatment with the transfer factor.

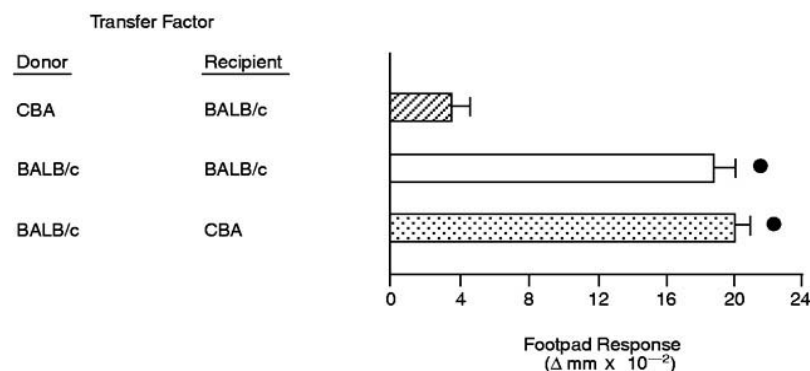


Figure 2 Effects of transfer factors on genetically controlled immune responses. CBA and BALB/c mice were immunized with ovalbumin (OVA) in Freund's complete adjuvant. After 3 weeks some animals were tested for DTH with the footpad swelling assay; others were euthanized and the spleens were collected for isolation of the ova-specific transfer factor. The CBA mice did not develop DTH to ovalbumin and their spleen cell extracts did not contain OVA-transfer activity that would transfer DTH to CBA (low-responder) or BALB/c (high-responder) recipients. The CBA donors produced a transfer factor that was specific for tuberculin (PPD) indicating that failure to produce the OVA transfer factor was related to the low responder phenotype. BALB/c mice expressed DTH to OVA and their spleen cell extracts produced an OVA-specific transfer factor. Moreover, the same transfer factor could transfer the ability to express DTH to OVA to low-responder CBA recipients. (Reproduced with permission from Kirkpatrick CH (1988) Transfer factor. *Journal of Allergy and Clinical Immunology* 81: 803–813.)

Clinical studies with transfer factors

Several facts must be considered in discussing possible clinical uses of transfer factors. Many of the reports describe one or two patients in which transfer factor treatment was used in desperation; in most instances the long-term outcomes are not described. Second, all of the clinical trials have used leukocyte dialysates; no studies have used materials that were highly purified and it is possible that the immunological and clinical benefits that were observed were due to components other than the transfer factors. The recent demonstration of immunological specificity in highly purified transfer factors, and the close concordance of clinical responses to immunological reconstitution make this explanation less likely.

Several placebo-controlled clinical trials have shown that treatment with specific transfer factors may endow recipients with immune responses that are beneficial. In patients with frequent recurrences of herpes simplex type 1 or type 2, many patients have had complete or nearly complete remissions while on treatment with a herpes-specific transfer factor. Relapses occurred when treatment was stopped or when patients were treated with a similar preparation from a donor who lacked cellular immunity to herpes simplex. Remissions occurred when the patients were retreated with the specific transfer factor.

A placebo-controlled study of a transfer factor preparation from leukocytes of coccidia-immune calves has shown benefits in AIDS (acquired immune deficiency syndrome) patients with intestinal cryptosporidiosis. Long-term remissions of chronic muco-

cutaneous candidiasis have been reported when the patients' immunological defects were corrected by administration of candida-specific transfer factor. A varicella zoster-specific transfer factor was active in preventing chickenpox in children with acute leukemia.

The studies indicate a need for further controlled clinical trials to define the diseases in which transfer factor therapy may be efficacious.

Future research

Important projects include completion of determination of the structures of molecules with transfer factor activity. This would be a significant step toward definition of the mechanisms of their effects on the immune system and why the effects appear to be confined to cell-mediated immunity. In addition, isolation of the appropriate cDNA and production of specific transfer factors *in vitro* will allow clinical studies with purified transfer factors.

See also: Cell-mediated immunity; Cytokines; Delayed-type hypersensitivity; Genetic analysis at the phenotypic level; Interferon γ ; Lymphocyte transformation test.

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TRANSFERRIN RECEPTOR (CD71)

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The first evidence for the existence of the transferrin receptor (TfR) was found in 1963 when Jandl and Katz showed that binding of transferrin to immature red cells was a specific and saturable process. Since then it has been shown that the TfR is present in virtually all dividing cells, which require iron for heme synthesis as well as for the activity of enzymes involved in DNA replication. Independently, monoclonal antibodies (mAbs), such as OKT9 and B3/25, raised against activated T lymphocytes, were found to recognize an immunodominant proliferation-associated cell surface antigen in activated hematopoietic cells. This surface antigen was shown to be the TfR because the mAbs were able to coprecipitate transferrin, although they did not react directly with it. Although the TfR was not initially considered as a cluster of differentiation antigen due to its wide cell distribution, it was finally assigned as CD71 at the 4th International Workshop on Leukocyte Typing.

Structure

The availability of anti-TfR mAbs made possible the biochemical characterization of the TfR and the identification of its coding cDNA sequence. The TfR gene maps to a region of human chromosome 3 containing several genes involved in metal binding. It encodes for a type II membrane protein anchored via a stretch of 26 hydrophobic amino acids (Figure 1).

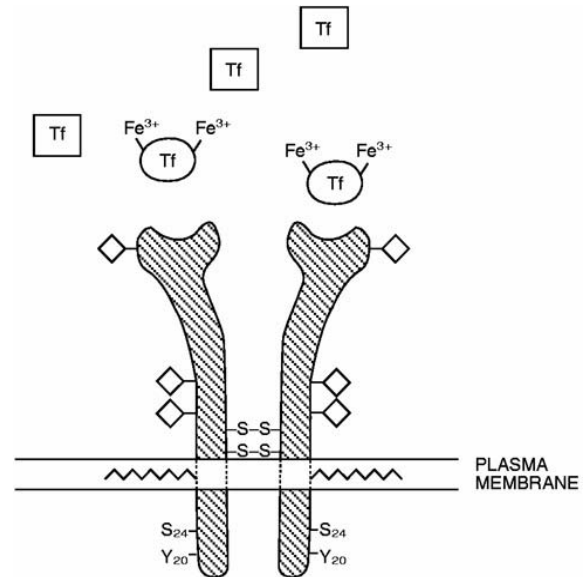


Figure 1 Structure of the transferrin receptor. The cartoon shows the positions of the phosphorylatable cytoplasmic tyrosine and serine residues, of a fatty acid residue, of the two disulfide bridges, as well as the positions of the three *N*-linked glycosylation sites (◊). At standard extracellular pH conditions, the TfR binds holotransferrin (iron-loaded) but not apotransferrin.

Human TfR has 760 amino acids, of which 672 are extracellular and the first 62 N-terminal ones are cytoplasmic. It forms a homodimer of two 95 kDa subunits which are disulfide-linked through cysteines

89 and 98. Each subunit is able to bind a molecule of transferrin. The protein has three sites for N-linked glycosylation but has no known O-linked sugars. Other post-translational modifications include the addition of palmitic acid at cysteine residue 62 and phosphorylation on serine 24, which appears to be the only site for protein kinase C (PKC). More recently, it has been shown that the TfR also becomes tyrosine phosphorylated.

Tissue expression

TfR-specific mAbs react with many cell types. Although it was originally thought that the TfR was a marker for proliferating cells because most express it, some resting cells can also express the TfR. Indeed, its expression is rather related to the intracellular iron levels, and it is because iron is essential for the activity of ribonucleotide reductase, which is required for DNA synthesis, that the TfR plays an important regulatory role in cell proliferation.

The TfR is broadly expressed in cells of hematopoietic origin (Table 1). In lymphoid tissues, it is present on germinal center cells and macrophages. Resting peripheral blood lymphocytes and monocytes do not express it, but its expression is induced upon activation.

During ontogeny, the TfR is expressed in the populations of cell precursors which are more actively proliferating and is downregulated as cells differentiate to a resting state. Thus, during T cell maturation, the TfR is only expressed in large-sized immature CD4⁺CD8⁻CD3⁻, CD4⁺CD8⁺CD3⁻ and CD4⁺CD8⁺CD3⁺ thymocytes of fetal as well as of

newborn and adult mice, and is subsequently gradually downregulated in the CD4⁺CD8⁺CD3⁻ population. Similarly, the TfR is also downregulated during granulocyte maturation.

Because the TfR is expressed by proliferating cells, it is not, therefore, surprising that it is broadly expressed on carcinomas and sarcomas of different origin as well as on hematopoietic cell tumors. Nevertheless, TfR expression does not seem to correlate with malignant phenotype because it is found, for instance, in low- as well as in high-grade lymphomas, although they may express qualitatively different TfR proteins. Thus, the epitope recognized by mAb Trump is expressed in high-grade but not in low-grade lymphomas.

TfR expression in rapidly proliferating cells makes it a possible target for antitumor therapy. For instance, it has been shown that treatment of bone marrow cells with an anti-TfR/toxin complex kills committed progenitors to CFU-GM and BFU-E but spares immature noncycling, TfR-negative progenitors. Such treatment has been proposed as a purging method for bone marrow autotransplants in leukemic patients.

Regulation of TfR expression

The mechanisms underlying endocytosis and recycling of the TfR have been extensively studied and indeed, the TfR constitutes the most accepted marker for recycling endosomes. Diferric transferrin binds to the TfR and this complex is rapidly endocytosed via coated pits and from there it is transferred via early endosomes to recycling endosomes (Figure 2). Here,

Table 1 TfR cell surface expression on nontransformed cells

Cell type	Expression	
	Unstimulated	Stimulated (stimulus)
Bone marrow		
CD34 ^{high}	++++ ^a	
CD34 ^{med}	+++	
CD34 ^{low}	+	
Cultured endothelial cells	++	+++ (TNFα) ^b
Peripheral blood T cells	+/-	++++ (PHA)
Peripheral blood B cells	+/-	+++ (SAC)
Peripheral blood NK cells	+	+ (IL-2)
Peripheral blood monocytes	+/-	
Peripheral blood eosinophils	-	
Peripheral blood neutrophils	-	- (fMLP)
Platelets	+/-	+/- (thrombin)
Cultured fibroblasts	++	+++ (IL-1)

^aExpression ranged from none (-) to all cells brightly stained with anti-TfR (++++).

^bStimuli used were: tumor necrosis factor α (TNFα), phytohemagglutinin (PHA), *Staphylococcus aureus* Cowan strain (SAC), interleukin 2 or 1 (IL-2, IL-1), formyl-methionyl-leucyl-phenylalanine (fMLP).

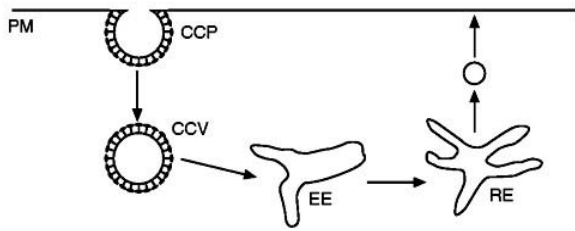


Figure 2 Endocytosis of the TfR. Upon binding, the holotransferrin/TfR complex is internalized in clathrin-coated pits (CCP) which detach completely from the plasma membrane (PM) as clathrin-coated vesicles (CCV), and then, become uncoated and fuse with a tubulovesicular compartment, early endosomes (EE). From early endosomes the complex is transferred to another tubulated compartment called recycling endosomes (RE) where under the low intraluminal pH conditions, iron is released to the cytosol to be captured by ferritin. The resulting apotransferrin/TfR complex remains associated and is recycled to the plasma membrane in exocytic vesicles. On the cell surface, under neutral pH conditions, apotransferrin dissociates from the TfR and both become ready to initiate a new cycle.

under low pH conditions, transferrin releases iron but remains associated to the TfR. The TfR/apotransferrin complex is recycled to the cell surface where, at neutral pH, apotransferrin dissociates from the receptor. Endocytosis of the TfR depends on a tetrapeptide sequence, YTRF, in its cytoplasmic tail. This sequence forms a tight turn and promotes endocytosis by binding to a complex of proteins known as adaptins which link the TfR tail to clathrin triskelions.

The expression of the TfR is also controlled at the translational level by the intracellular concentrations of iron. The 3' untranslated region of the TfR mRNA contains an iron-responsive element (IRE) which is also found in the 5' region of the ferritin mRNA. Under low intracellular iron concentrations, an IRE-binding protein (IRP) interacts with TfR's mRNA and inhibits its degradation, whereas the interaction with the 5' element in ferritin inhibits its translation. Both effects tend to increase the concentration of intracellular iron. Under high iron concentrations, IRP dissociates from IREs and the system is set for increased storage and reduced uptake of iron. In addition to iron concentration, other factors affect the level of TfR expression. In hematopoietic cells, interleukin 2 (IL-2), IL-6, cyclic nucleotides, phorbol esters, vitamin D and interferons regulate it. Thus, interferon γ (IFN γ) decreases TfR expression in macrophages; and IFN β , but not IFN γ , has been described to inhibit TfR expression during *in vitro* differentiation of monocytes to macrophages. Some of these cytokines (tumor necrosis factor α (TNF α), IFN γ) increase nitric oxide (NO) production that in turn may regulate TfR expression by increasing the

affinity of IRP for IRE, independently of intracellular iron concentrations.

Role of the TfR

Role in cell proliferation

The depletion of TfR membrane levels or the blockade of transferrin binding with TfR-specific antibodies inhibit cell proliferation probably due to inhibition of iron uptake. In addition, it has been shown that antisense oligonucleotides inhibited progression of mitogen-activated lymphocytes into the cell cycle, synchronizing the cells in G₁. Similar effects were produced by the serum immunosuppressive factor from patients with Crohn's disease that has been shown to inhibit progression of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) beyond G₀/G₁. This inhibition correlated with downregulation of TfR from the cell surface, although IL-2 receptor expression and IL-2 secretion were not affected.

In addition to the role in iron uptake, several reports suggest that the TfR could have some role in cell proliferation independently of its ability to bind iron, probably involving some form of signal transduction. This effect has been shown especially in T cells which seem to be more sensitive to inhibition of proliferation by TfR-specific antibodies than B cells.

Role on T cell activation and T cell differentiation

Regarding the effect of TfR on T cell activation different results have been obtained depending on the nature of the stimulus used, holo(iron-loaded)-transferrin versus mAbs; and among these, on the mAb used. However, all these data point to a signaling capability by the TfR. Holotransferrin has been shown to activate PKC in the T cell leukemic line CEM, whereas it did not induce increases of intracellular Ca²⁺. However, the TfR-specific mAb J64 was shown to induce IL-2 production by the T cell leukemic line HUT-78 without apparent translocation of PKC. A different TfR-specific mAb, FG1/6, was shown to induce IL-2 secretion by PBMCs and the T cell leukemic line Jurkat, and to costimulate proliferation of peripheral T cells together with PKC activators. Interestingly, J64 was unable to induce Ca²⁺ fluxes in HUT-78, whereas in Jurkat, FG1/6 produced significant increases of intracellular Ca²⁺ of about one-third of those obtained with mAbs against CD3. FG1/6 has also been shown to stimulate the tyrosine phosphorylation of several protein substrates including the ζ subunit of the TCR/CD3 complex and vice versa, CD3-specific antibodies cause the tyrosine phosphorylation of the TfR.

Indeed, it has been suggested that the TfR is functionally and physically associated to CD3- ζ . Nevertheless, most of these experiments were performed with leukemic T cell lines and have to be contrasted with nontransformed T cells.

Consistent with the expression of the TfR in large immature thymocytes, incubation of fetal thymic organ cultures with TfR-specific mAbs blocks differentiation of CD44-CD25⁺ CD4-CD8-CD3⁻ to CD4⁺CD8⁺CD3⁻ whereas it did not affect the differentiation of earlier thymic precursors to the CD44-CD25⁺ population. Most interesting, anti-TfR did not affect development of γ/δ T cells, suggesting a differential susceptibility to iron depletion of both T cell lineages.

Additional roles for the TfR have been claimed: as a putative target molecule for natural killer (NK) cells, as a receptor that mediates uptake and presentation of hepatitis B virus by T cells, and as a receptor that can mediate locomotion of adherent cells.

See also: B lymphocyte activation; CD3; Exocytosis; Immunotherapy of tumors; Iron and the immune system; Thymus; T lymphocyte activation; T lymphocyte differentiation.

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TRANSFORMING GROWTH FACTOR β (TGF β)

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Transforming growth factor β (TGF β) is a cytokine produced by a multitude of cells, including T and B lymphocytes and macrophages. T cells predominantly producing TGF β are both CD4⁺ and CD8⁺ T lymphocytes, and are sometimes termed T_H3 cells. The pleiotropic effects of TGF β include strong growth-enhancing as well as growth-inhibiting properties, depending upon the presence of other growth factors and the type of cells that it acts upon. TGF β is an important immunomodulator. The immunosuppressive property of TGF β was dis-

covered on characterization of a glioblastoma-derived T cell suppressor factor which, after cloning, was termed TGF β_2 . Mice deficient in the gene for TGF β_1 , generated by knockout technology, die from a wasting syndrome accompanied by inflammation in multiple organs. Although TGF β belongs to the group of cytokines with anti-inflammatory effects, it may under certain circumstances also exhibit proinflammatory activities. TGF β participates critically in mucosal tolerance to experimental autoimmune encephalomyelitis (EAE) and myasthenia gravis

(EAMG) induced by oral or nasal administration of autoantigens, and could represent a powerful tool in the future abrogation and prevention of autoimmune diseases.

Molecular structure

The TGF β superfamily consists of a number of structurally related regulatory proteins which, besides TGF β_1 to TGF β_5 , include the inhibins, activins, bone morphogenic proteins and related morphogenic proteins, which are all of importance in many areas of cell biology. The five isoforms of TGF β share biological activities. The prototype of this family is TGF β_1 , originally purified from human platelets. These represent the most concentrated natural source of TGF β . Only TGF β_1 , TGF β_2 and TGF β_3 have been identified and cloned in mammalian species, whereas TGF β_4 and TGF β_5 have been found only in chick and *Xenopus*, respectively. The genes for each of the mammalian isoforms are located on different chromosomes: TGF β_1 on chromosome 19, TGF β_2 on chromosome 1, and TGF β_3 on chromosome 14.

TGF β_1 is a disulfide-linked homodimer of the C-terminal 112 amino acids (12.5 kDa) derived from a 390 amino acid precursor peptide (Table 1). The processed mature TGF β homodimer (25 kDa) is biologically inactive or latent when noncovalently associated with the N-terminal part of the dimeric precursor, the latency-associated peptide (β -LAP), which is linked by disulfide bond(s) to a third molecule termed the latent TGF β -binding protein (LTBP). LTBP is a 125–160 kDa protein containing epidermal growth factor (EGF)-like repeats.

Activation of TGF β occurs by dissociation of the mature dimer from the β -LAP by exposure *in vitro* to pH changes, heat, urea or SDS. The mechanisms for activation *in vivo*, e.g. in the context of inflammation, are incompletely understood. There is evi-

dence suggesting that degradation with plasmin is involved. Retinoids also show a capacity to activate latent TGF β , possibly by increasing the levels of plasmin. Only activated TGF β binds to membrane receptors specific for TGF β . The latent form of the molecule circulates with a half-life of 90 min, whereas active TGF β_1 is removed from the circulation via the liver in 2–3 min.

The three mammalian TGF β isoforms are 25 kDa homodimers in their biologically active form, with 70–80% sequence identity. They exert similar but not identical biological activities *in vitro*, although certain cells respond selectively. The three isoforms are controlled by unique transcriptional regulatory elements and therefore are likely to have different functions *in vivo*. Recent studies using TGF β_1 and TGF β_3 knockout mice have, in fact, revealed functional specificities for each isoform. There are also quantitative differences in that TGF β_1 and TGF β_3 generally elicit their effects at about a 100-fold lower concentration than does TGF β_2 . Most studies to date have involved TGF β_1 .

Receptors and other binding proteins for TGF β

Low numbers of high-affinity receptors for TGF β are found on most normal resting and transformed mammalian cells. Two major receptor species (TGF β RI, 53 kDa, and TGF β RII, 70–80 kDa) are both serine/threonine kinases. Each has a small extracellular region, a single transmembrane domain, and an intracytoplasmic serine/threonine kinase domain. Almost all cells, regardless of their origin, express TGF β receptors and bind TGF β with high affinity. Signaling has been shown to require the formation of a trimeric complex consisting of both TGF β receptors I and II and TGF β . Betaglycan (type III receptor) is a third major cell surface protein with high binding affinity to TGF β . Other binding proteins for TGF β include α_2 -macroglobulin, fibronectin and β -amyloid precursor.

TGF β and the cytokine network

The role of TGF β in immune surveillance and immunotherapy must be conceptualized in the context of its role as a cytokine, participating in the cytokine network (Figure 1). This integrated network includes contributions made through all cytokines involved in inflammation and immunity. The outcome of an immune response is the result of the balance between synergy and antagonism of several cytokines, and dysregulation at any level can contribute to disease pathogenesis. Like other cytokines, TGF β is highly

Table 1 Physicochemical properties of human TGF β_1 , TGF β_2 and TGF β_3

	TGF β_1	TGF β_2	TGF β_3
Molecular weight (active)	25 kDa	25 kDa	25 kDa
Amino acids			
precursor	390	414	412
mature	112	112	112
Disulfide bonds	1	1	1
N-terminal part of precursor			
cysteine residues	3	6	5
glycosylation sites	3	3	4
Half-life			
latent	90 min	—	—
active	2–3 min	—	—

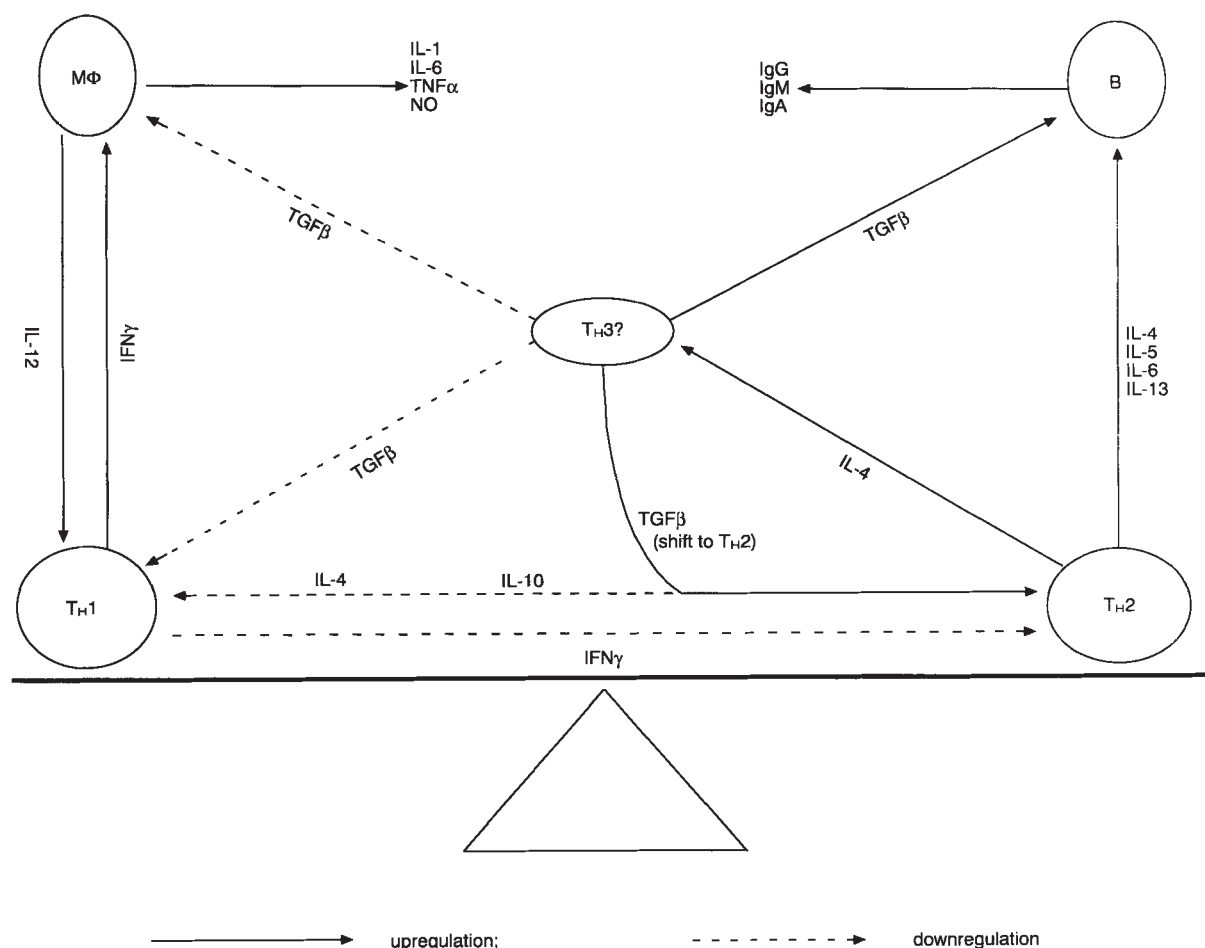


Figure 1 TGF β and the cytokine network. T_H1 cell-derived IFN γ suppresses the activation of T_H2 cells. T_H2-derived IL-4 induces T_H3 cells to secrete TGF β which, in turn, has effects on T_H1 cells, B cells and macrophages. Up- and downregulatory influences are indicated by continuous and noncontinuous arrows, respectively.

potent, acting on cell surface receptors at femtomolar concentrations (10^{-15} M). Since different cell types possess receptors for the same cytokine, TGF β like other individual cytokines may have multiple effects on the growth, differentiation and function of many cell types.

Detection of TGF β

To detect the involvement and quantitative changes of TGF β in experimental and clinical situations, either mRNA or total TGF β activity obtained after acid activation is measured. Bioassays for TGF β are based on inhibiting growth of mink lung cell line MV-1-Lu. *In situ* hybridization to detect TGF β transcripts in, for example, blood mononuclear cells, is a useful approach. It bypasses many of the obstacles inherent to conventional determinations of the concentrations of circulating TGF β by, for example, ELISA. TGF β secreted around diseased

tissue or by circulating lymphocytes is probably quickly captured by receptors on nearby cells as a result of its paracrine actions. The half-life of TGF β is short (Table 1), and its clearance from the systemic circulation is rapid. These features explain why TGF β measurements (as with many other cytokines) by, for example, ELISA, performed on human body fluids in disease states, frequently give inconsistent or negative results. There are, however, some reports on the successful evaluation of TGF β , as in serum from psoriasis patients, who showed elevated TGF β levels compared to healthy subjects, and even a significant correlation with clinical severity.

Measurements of mRNA or total TGF β activity tell nothing about the relative production of active versus latent forms of TGF β by, for example, blood mononuclear cells. Furthermore, the intracellular localization of cytokine transcripts does not necessarily equate with patterns of cytokine secretion. A discrepancy may be due to post-transcriptional

regulation of cytokine mRNA, or post-transcriptional regulation of cytokine secretion. Translational control has been reported for TGF β .

TGF β and immune privilege

Recent evidence supports the view that immune privilege is an active process and that its creation results from local tissue factors that interact with and alter the functional programs of cells of the immune system. Locally produced and released immunosuppressive cytokines constitute, besides neuropeptides, factors which maintain immune privilege. Intraocular fluid contains significant amounts of TGF β , which accounts in part for the immunosuppressive activities of the fluid.

Immunoreactive TGF β has been identified in CSF and in amniotic fluid. As these fluids are also considered to represent immune privileged sites, the possibility exists that TGF β contributes to a common

strategy designed to modulate immunity at such sites. In culture, microglia express the immunosuppressive cytokines TGF β_1 and interleukin 10 (IL-10) to a greater degree than proinflammatory cytokines. High TGF β levels could reflect one mechanism for immune privilege within the brain. Stimulation with LPS or IFN γ led to shift toward proinflammatory cytokine mRNA profiles in microglia, providing insight into the mechanism by which LPS and IFN γ act in inflammatory diseases of the CNS.

TGF β and the immune response

TGF β exhibits bifunctional properties, depending on the type and state of cells as well as the presence of other cytokines (Figure 2). TGF β_2 has been shown to completely suppress the proliferative response of CD4 $^+$ or CD8 $^+$ T cell clones to IL-2 or phorbol ester/ionophore. TGF β blocks the generation of both cytotoxic T cells in allogeneic mixed lymphocyte cul-

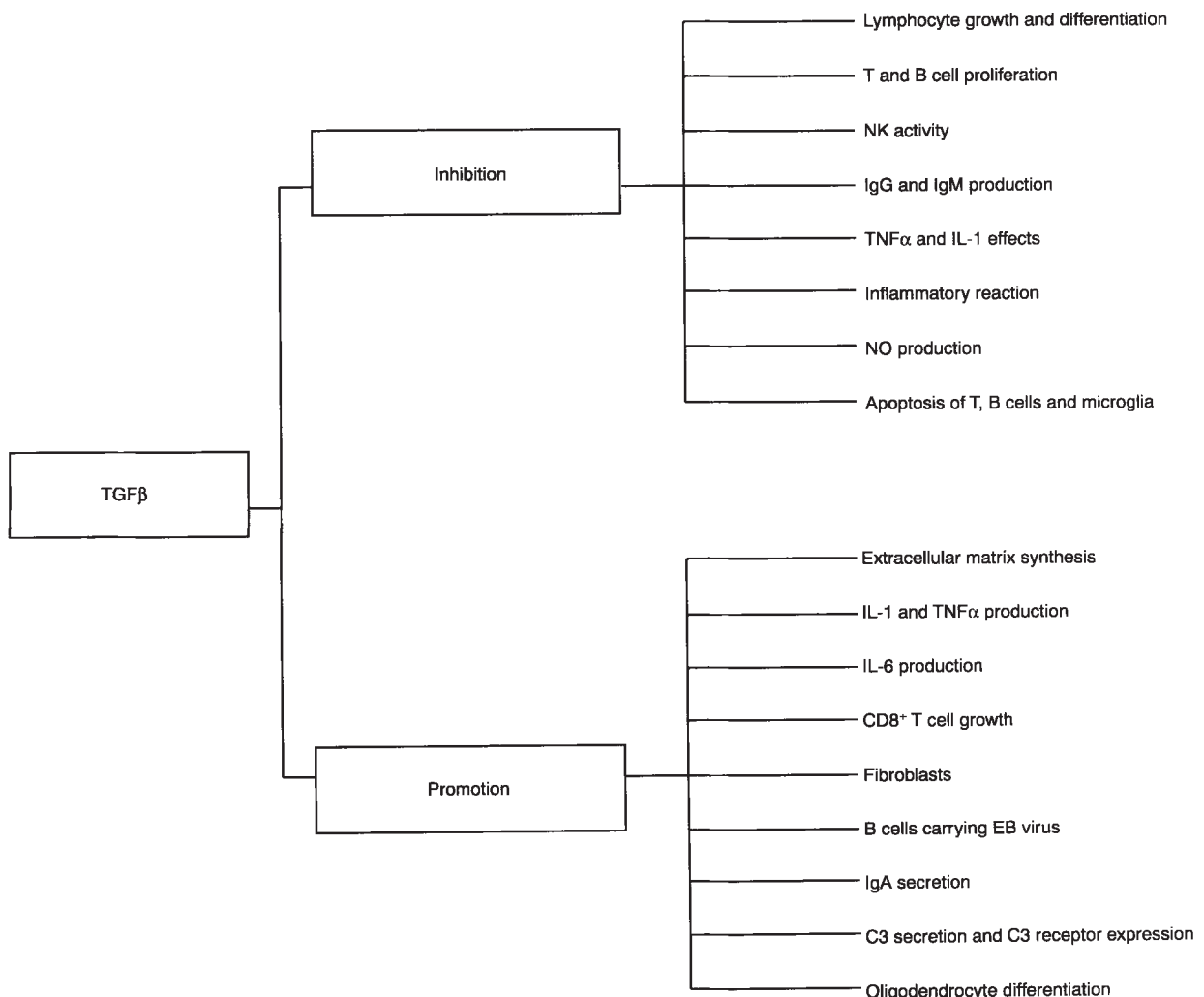


Figure 2 The bifunctional properties of TGF β .

tures and IL-2-induced cytokine-activated killer cells and natural killer (NK) cells. It has become clear that TGF β downregulates both cell-mediated and humoral immunity. TGF β probably exerts its immunosuppressive effects by regulating the expression of other cytokines, e.g. interferon γ (IFN γ). TGF β inhibits the production of both IFN γ and IL-4 in mitogen-activated human blood mononuclear cells.

TGF β administered systemically has profound effects on models of organ-specific inflammatory diseases, including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis. The pronounced suppression of the localized immune responses is not associated with any significant generalized immune suppression. These observations could reflect resistance to suppression of resting lymphocytes, but increased susceptibility of activated T lymphocytes. This may also explain the apparently opposing effects of TGF β on cells at different times in their life cycle: TGF β is released early and functions in many capacities to promote inflammation based on its stimulating effects on immature monocytes and T cells. Once these cells differentiate and become activated, they become susceptible to phenotypic modulation and functional inhibition by TGF β . Under normal conditions, this transition from pro- to anti-inflammatory responsiveness occurs with minimal disruption of host cell and tissue homeostasis.

TGF β -dependent suppression of antigen-induced T cell growth involves several distinct pathways which include 1) the direct inhibition of mitosis in the late G₁ phase of the cell cycle, and 2) regulation of transcription of the early growth factor-dependent *c-myc* gene and of the transferrin receptor. TGF β may also suppress clonal expansion indirectly by inducing synthesis of IL-1 receptor antagonist that has the capacity to abort IL-1-dependent responses.

TGF β also influences maturation of T helper cells in different ways. TGF β suppresses the development of IL-4-producing T lymphocytes and stimulates the generation of IFN γ -secreting T cells *in vitro*. In contrast, in an *in vivo* murine model, administration of TGF β at the time of infection with *Leishmania amazonensis* or *L. braziliensis* induced increased mRNA levels for IL-4 and decreased levels for IFN γ .

CD4⁺ and CD8⁺ T lymphocytes are differentially susceptible to TGF β : inhibition of CD4⁺ T cells is nearly complete, whereas TGF β is considerably less inhibitory and may even promote CD8⁺ cell growth. Resistant CD8⁺ T cells synthesize cytokines including IL-4 and IL-10 which have additional immunosuppressive properties. Under appropriate conditions, TGF β appears to foster the generation of inhibitory feedback molecules and apparently works in concert

with them to reverse immune processes. TGF β contributes to the shift toward T_H2-type responses through direct and IL-10-mediated pathways. TGF β can induce apoptosis in cultured uterine epithelial cells, hepatoma cells, gastric carcinoma cells, myeloid leukemia cells, resting B cells and hepatocytes *in vitro* and *in vivo*.

TGF β can inhibit proliferation and immunoglobulin (Ig) production by B cells. However, in the presence of an adequate combination of activators, e.g. antigen and T cell help, the inhibitory effects of TGF β are abrogated and large numbers of IgA⁺ cells can be produced in Peyer's patches along the small intestine. Which cells produce the TGF β involved in regulation of IgA switching by inducing germline α -transcripts and how it is regulated remains to be settled.

TGF β is known to induce its own production and secretion. The autoinduction of TGF β ₁ transcription is mediated by AP-1 (Jun-Fos) binding sites in the TGF β ₁ promoter. By this mechanism, TGF β activity might become persistent, resulting in deleterious effects.

An inflammatory process in the brain requires bidirectional interactions of the immune and nervous system. Expression of TGF β is highly regulated in T cells and is probably an important component in the molecular interaction between the immune and nervous systems. Glucocorticoids cause a significant decrease of TGF β ₁ mRNA levels in glial cells but not T cells, indicating that TGF β ₁-regulatory pathways can be utilized to affect the functions of neural and immune cells. Microglia, the macrophages of the central nervous system (CNS), are positive for TGF β ₁ mRNA and release TGF β ₁.

TGF β has immunosuppressive functions within the CNS. The target cells of this action are still unknown. TGF β ₁ selectively induced apoptosis of microglia, but not astrocytes and oligodendrocytes. Bcl-2 oncoprotein was mainly expressed in microglia, indicating that TGF β ₁-mediated microglial apoptosis was regulated by a bcl-2-independent mechanism.

Overproduction of nitric oxide (NO) by glial cells, but not by infiltrating mononuclear cells, can be a major cause of target tissue damage in autoimmune diseases such as EAE and multiple sclerosis (MS). TGF β has been shown to inhibit NO production by microglia. Astrocytes secrete both the active and latent form of TGF β ₂. Exogenous administration of TGF β could beneficially influence the course of such diseases. TGF β has also been shown to suppress rat astrocyte autoantigen presentation, and antagonizes hyperinduction of major histocompatibility complex (MHC) class II antigen induced by IFN γ or TNF α .

TGF β and experimental infectious diseases

The immunoregulatory effects of TGF β_1 and TGF β_2 have been assessed in mice infected with lymphocytic choriomeningitis virus (LCMV). Intraperitoneal injections of 1 μ g per day of recombinant TGF β_1 and TGF β_2 prolonged the survival interval after LCMV infection, inhibited the footpad swelling after local LCMV infection and impaired clearance of LCMV in infected mice. In Borna disease, a sporadically occurring progressive encephalopathy of sheep and horses that can be induced in a wide range of animal species by infection with Borna virus, a single-stranded RNA virus, TGF β inhibited clinical disease and reduced the inflammatory reaction and MHC class II expression in the brain and the production of antibodies to the virus.

TGF β and experimental autoimmune diseases

The influence of TGF β on EAE as a prototype of organ-specific CD4 $^+$ T cell-mediated autoimmune disease has been extensively studied. TGF β strongly inhibits *in vitro* activation of T cells from Lewis rats with EAE. During recovery from EAE, CD4 $^+$ spleen cells produce TGF β and thereby regulate both the production of IFN γ and the encephalitogenicity of effector cells. Systemic administration of TGF β reduces the incidence and severity of EAE induced in the SJL mouse, without generalized immunosuppression. This observation is in line with the known resistance of resting lymphocytes, but increased susceptibility of activated T lymphocytes, to suppression. *In vivo* administration of antibody to TGF β worsened the clinical signs of monophasic EAE. Taken together, these observations suggest that endogenous TGF β has an important role in the natural recovery from EAE. The mechanisms of how TGF β acts in EAE are not yet fully understood but it can be assumed that the disease-limiting effects are at least in part mediated through suppression of autoantigen presentation. In addition, TGF β_1 inhibited MBP-stimulated glial cell proliferation, and down-regulated the expression of ICAM-1 on MBP-stimulated glial cells. TGF β_1 -mediated inhibition of proliferation and down-regulation of ICAM-1 on glial cells were interrupted by IFN γ , indicating that biological effects of TGF β_1 vary depending on the local microenvironment.

TGF β also has protective effects on other experimental models for human autoimmune diseases, such as T cell-dependent arthritis induced by immunization with collagen. However, the injection of

TGF β into the ankle joint cavity of Lewis rats induced synovial erythema and swelling similar to that observed in rheumatoid arthritis, although the process was self-limiting. Besides the different consequences between systemic and local administration of TGF β , several other factors determine its biological effects. The wide-ranging biological effects of TGF β are illustrated in Figure 3. Local administration of TGF β_1 accelerates the healing of various cutaneous wounds. An excess of TGF β_1 causes scar tissue formation. TGF β can actively regulate the rate at which CD4 $^+$ CD8 $^+$ thymocytes are generated from precursor cells. TGF β can counteract the rejection process and could be useful for new therapeutic approaches of allograft rejection. TGF β is also a switch factor for the IgA class.

Tolerance induction and TGF β

Oral administration of myelin basic protein (MBP) to rats effectively prevents EAE induced by immunization with MBP in Freund's complete adjuvant. Evidence has been presented that such tolerance is mediated by CD8 $^+$ T cells producing TGF β . TGF β is also strongly upregulated in DA strain rats treated with low doses of MBP by the nasal route. Nasal tolerization with MBP efficiently prevents the induction of protracted-relapsing EAE in DA rats. In the acute Lewis rat EAE model, nasal administration of MBP or MBP peptides 63–88 or 89–101 resulted in increased levels of TGF β mRNA-expressing immune cells. TGF β -mediated bystander regulatory mechanisms are thought to be important for nasal tolerance induction. High numbers of TGF β mRNA-positive mononuclear cells are also found in lymph nodes of rats tolerized to the mainly B cell-mediated experimental autoimmune myasthenia gravis (EAMG) by oral or nasal administration of the target antigen acetylcholine receptor (AChR). Such findings suggest that a nonspecific bystander regulation dominated by upregulation of TGF β is pivotal for induction of tolerance to autoimmune diseases.

Human autoimmune and infectious diseases

Patients with multiple sclerosis (MS) have dramatically elevated levels of TGF β mRNA-expressing mononuclear cells in blood and CSF. MS patients also have elevated numbers of circulating MBP- and proteolipid protein (PLP)-reactive TGF β mRNA-expressing cells. Interestingly, patients with severe MS had fewer cells expressing TGF β mRNA compared to patients with mild MS. In another recent study, patients with stable MS had higher levels of

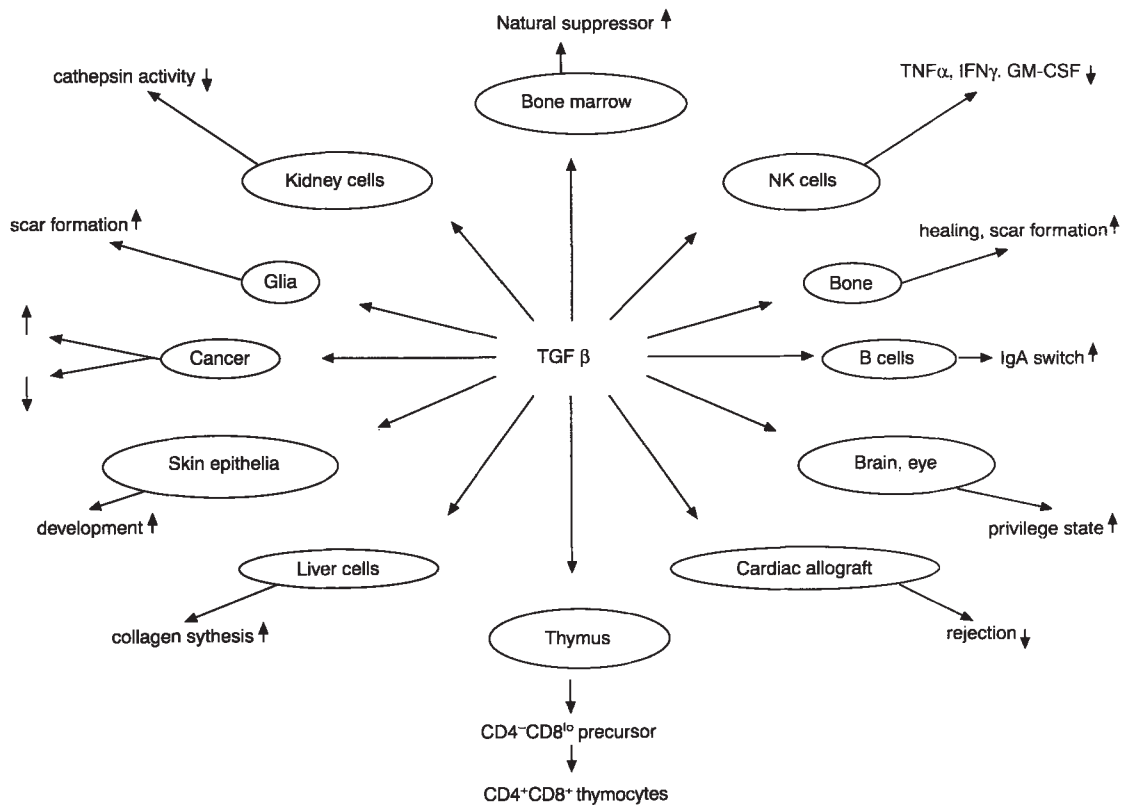


Figure 3 Local effects of TGF β .

TGF β mRNA in blood mononuclear cells than patients with relapsing MS. The data support a disease-limiting role for TGF β in MS.

Patients with myasthenia gravis have elevated levels of AChR-reactive TGF β mRNA-expressing blood mononuclear cells. Levels of circulating TGF β mRNA-expressing cells increase after thymectomy. The relevance of TGF β in myasthenia gravis is unclear, but it may have therapeutic potential in this disease.

Elevated TGF β levels have been reported in serum in HIV infection and in cell supernatants after culture of blood mononuclear cells from human immunodeficiency virus (HIV)-infected patients. Levels of TGF β mRNA-expressing mononuclear cells are elevated in HIV-infected patients' blood. TGF β has been identified by immunohistochemistry in brain tissue of patients with the acquired immune deficiency syndrome (AIDS). It has been suggested that TGF β may play an important role in the spread of infection and/or disease progression by amplifying the existing viral load.

Femtogram concentrations of TGF β_1 suppresses *in vitro* myelin antigen (MBP, PLP)-induced upregulation of $\text{IFN}\gamma$, IL-4, IL-6, $\text{TNF}\alpha$ and lymphotoxin, and of perforin, in MS. TGF β also suppresses AChR-induced upregulation of the same proinflammatory

cytokines in myasthenia gravis. In contrast, TGF β has no corresponding effects on IL-10 or on TGF β itself. These selective suppressive effects of TGF β_1 on autoantigen-induced upregulation of proinflammatory cytokines makes TGF β attractive as a potential therapeutic agent in MS and myasthenia gravis.

See also: Cytokines; Cytokine receptors; Experimental autoimmune encephalomyelitis (EAE); Immunosuppression; Mucosal immunity; Oral tolerance.

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TRANSFUSION

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Transfusion medicine relates to substitution therapies with human blood in cytopenias, protein deficiencies, and blood loss during surgery. Peripheral blood cell and plasma collection, processing, storage, compatibility matching and transfusion are large scale procedures in blood centers and hospitals today. Initially, humoral alloimmunizations presented a serious obstacle to allogeneic transfusion. This was overcome by the discovery of red cell blood groups and the development of modern blood group serology and immunohematology covering the clinical aspects of allo- and autoantibodies to red cells. Later, bloodborne viruses were recognized as the pre-eminent risk of transfusion. In the last 15 years, major progress has been made in limiting the transmission rate of viral infections. The possible immunologic side-effects of transfusion are a major and still largely unsolved topic in transfusion medicine.

Blood components

Whole blood is separated into its different cellular and plasmatic components and processed within several hours of donation. Processing is being continuously improved in order to reduce contaminating cells and plasma. Numerous processing steps are applied which influence the purity, side-effects, storage time, etc. Preparation procedures, standard blood components and indications vary considerably among and within different countries. Because of considerable disadvantages associated with the transfusion of whole blood, its use has almost been aban-

doned. Blood components are often administered on empirical grounds, when clinical studies to validate their use are lacking, to substitute specifically those constituents of the patients' blood which fall below the minimal tolerable threshold. During hemorrhage or hemolysis the oxygen-carrying capacity of erythrocytes must be replaced first, in addition to intravascular volume. After further blood loss the substitution of plasma and platelets to restore plasmatic and thrombocytic coagulation activity may also be required (Table 1).

Hematopoietic stem cell therapy

After suitable stimulation by cytokines, such as granulocyte colony-stimulating factor (G-CSF), hematopoietic stem cells can be harvested from peripheral blood by cytapheresis in sufficient quantities for stem cell transplantation. This may supplant, in many clinical situations, the collection of stem cells from bone marrow. Applications are autologous stem cell therapy to promote rapid hematopoietic recovery after intensive chemo- and radiotherapy, and allogeneic bone marrow transplantation in hematologic failure and malignancy.

Autologous blood

The term allogeneic blood refers to that used in transfusion from donor to recipient. However, the patient's own (autologous) blood can be collected before an expected transfusion need and transfused during, for example, planned surgery. Many but not

Table 1 Blood components used in current transfusion and bone marrow transplantation practice

<i>Blood components used in substitution therapies</i>	
Erythrocytes	
	Buffly coat depleted with additive solution (standard)
	Buffly coat depleted (standard in some countries)
	Leukocyte and thrombocyte reduced (filtered)
	Washed (rarely indicated)
	Stored frozen at -80°C or in liquid nitrogen (rarely indicated)
Plasma	
	Quarantine stored (standard)
	Fresh frozen (standard in some countries)
	Virus inactivated (alternative to quarantined plasma)
	Lyophilized and virus inactivated
Platelets (thrombocytes)	
	Buffly coat-derived platelet concentrates (standard)
	Platelet-rich plasma (standard in some countries)
	Leukocyte reduced (filtered)
	HLA-matched single donor thrombocytophoresis preparation
Granulocytes	
	HLA-matched single donor granulocytophoresis preparation
Whole blood (obsolete in some countries)	
	Fresh blood
	Warm blood
	Pure blood
<i>Hematopoietic active tissue used in bone marrow transplantation</i>	
Stem cell preparations (CD34⁺ cells)	
	Peripheral blood stem cells induced by growth factors (G-CSF, GM-CSF)
	Bone marrow stem cells prepared from bone marrow tissue with/without T cell depletion
Bone marrow tissue	
	Without further processing
	T cell depleted
	Purged for malignant cells (in autologous transplantation)

all immunologic problems of transfusions may be avoided by this strategy. Endogenous and exogenous materials in autologous blood may still cause adverse effects. It is generally accepted that autologous blood should be used in all groups of patients and indications for which benefit and cost-effectiveness is established. Other options reducing for allogeneic transfusion are perioperative blood salvage, increase of endogenous red cell production by erythropoietin and control of blood loss by surgical/anesthetic techniques.

Screening for infectious agents

Since the 1970s donors have been screened for hepatitis B virus infection and for syphilis. Alanine aminotransferase is used as a surrogate test for early viral hepatitis A, B and C or non A, non B, non C hepatitis. Major improvements were brought forth by the introduction of testing for human immuno-

deficiency virus (HIV) in 1985 and for hepatitis C virus in 1990, rendering the blood supply today safer than ever. The rate of infectious units in the blood supply varies considerably among different populations and over time within a population. It depends on the endemic diseases, the selection of the donor populations and their epidemiologies, which need to be monitored on a continuous basis. Because infectious donors may be missed during the diagnostic window, both the incidence of infections and their prevalence are important parameters. Removal of white blood cells, which serve as a shuttle for some viruses, may limit viral transmission. Plasma is often quarantined for 6 months until the donor is retested, once the window period of virus infections has expired (Table 2).

Immunohematology

The natural occurrence and lifelong persistence of alloantibodies (isoagglutinins) to the ABO blood group antigens preclude all ABO-incompatible transfusions. For this reason transfusion became feasible only after the discovery of the ABO blood group system in 1900 using the erythrocyte agglutination technique. Clinically relevant alloantibodies to most other blood group antigens hardly occur before immunization by transfusions or pregnancies. Besides ABO, a few other antigens such as Rh D and Kell are always considered in transfusion practice in Caucasians, because of their prevalence and immunogenicity. The relative immunogenicity of antigens is determined by comparing the actual antibody frequency and the calculated frequency of the probability of exposure. The genes of almost all blood groups are cloned, allowing the molecular

Table 2 Infectious marker screening

<i>Routinely tested in many countries</i>	
HIV-1/HIV-2	HIV-specific antibodies, sometimes p24 antigen
Hepatitis B virus	HBsAg, sometimes HBc-specific antibodies
Hepatitis C virus	HCV-specific antibodies
HTLV-1	HTLV-1-specific antibodies
Syphilis	<i>Treponema pallidum</i> -specific antibodies (TPHA)
Alanine aminotransferase	Enzymatic activity

Other pathogens and diseases commonly transmissible by blood

Cytomegalovirus, parvovirus B19, Epstein-Barr virus, yellow fever, malaria, Chagas disease, toxoplasmosis, leishmaniasis, onchocerciasis

HTLV-1, human T cell lymphoma virus.

characterization of the allelic diversity. Recently, all authenticated blood group antigens were revised according to their genetic basis and may fall into one of four classifications: systems, collections, low incidence antigens (700 series), and high incidence antigens (901 series). A numerical terminology was devised. Systems are genetically discrete from each other and are represented by antigens controlled by one or more very closely linked homologous gene loci. Collections comprise antigens, which are related but do not meet system status criteria. Low and high incidence antigens that do not belong to systems or collections are grouped into series. The antigen structure is generally constituted either by carbohydrates or by proteins (Table 3).

Since about 1960, efficient antibody detection and cross-matching (compatibility testing between donor and recipient) have been routinely applied using the antiglobulin (Coombs) test. A serologic incompatibility causing immediate or delayed hemolytic transfusion should rarely be a serious threat today. The recent introduction of matrix (gel) and solid phase tests increased the sensitivity of antierythrocyte antibody detection and holds promise in respect to standardization and automation.

Immunomodulatory effect of transfusion

The transfusion of various allogeneic blood components is circumstantially linked with subtle immunosuppressive effects. Whole blood transfusions may enhance renal allograft survival and reduce relapse rates of Crohn's disease. In some murine models tumor growth and bacterial infection can be

increased by transfusion of fresh allogeneic whole blood, but not by that of prestorage leukocyte-depleted blood. Many observational studies in humans reported considerable increases in serious short- and long-term sequelae of allogeneic transfusions, but are known to lack control of important confounding factors (Table 4).

Current clinical studies

The combined results of the prospective, randomized, controlled clinical trials conducted to date do not prove a significant difference in cancer recurrence nor in postoperative infection. While the sizable effects reported in many retrospective studies may be incorrect, smaller clinical differences of great public health interest cannot be excluded. Buffy coat-depleted erythrocytes were mainly used in the controlled studies; they are assumed to have fewer immunosuppressive effects than whole blood. Deleterious transfusion effects are also missed if they occur equally with allogeneic and autologous blood, because some undesired compounds like cell debris and cytokines may not differ.

Possible mechanisms

While the clinical relevance of a possible transfusion-associated immunosuppression have not been shown unequivocally, several mechanisms affecting the recipients' cellular immunity have been reported, including T cell anergy and decreases in helper:suppressor T cell ratio, natural killer (NK) cell function or antigen presentation. Any immunosuppressive effect based on tissue type mismatches of the donor-recipient pair is expected to depend on the HLA

Table 3 Blood group systems, genes, structure and antigens

Common name	ISBT terminology ^a	Gene names	Null alleles	Chromosomal location	Important antigens ^b	Year of discovery
<i>Carbohydrates</i>						
ABO	001	ABO	O	9q34.1–2	A A ₁ B	1900
P	003	P1		22q11.2–ter	P ₁	1927
Lewis	007	FUT3	le	19p13.3	Le(a) Le(b)	1946
H	018	FUT1	h	19q13.3	H	1952
<i>Proteins</i>						
MNSs	002	GYPA, GYPB		4q28–31	M N S s	1927
Rhesus	004	RHD, RHCE		1p34–36.2	C c D E e	1939
Lutheran	005	LU		19q13.2	Lu(a) Lu(b)	1945
Kell	006	KEL		7q33	K k Kp(b)	1946
Duffy	008	FY		1q22–23	Fy(a) Fy(b) Fy6	1950
Kidd	009	JK		18q11–12	Jk(a) Jk(b)	1951

^aGenetically based numerical terminology devised by the International Society of Blood Transfusion.

^bExamples for receptor functions of blood group antigens are: Le(b), *Helicobacter pylori*; Fy6, *Plasmodium vivax*, interleukin 8; P₁, parvovirus B19 (ISBT 209 GLOBO collection of antigens); AnWj, *Haemophilus influenzae* (ISBT 901 series of high incidence antigens). With the exception of Lewis antigens, all antigens are present on fetal red cells, albeit some antigens may be expressed with a reduced number of antigens per cell.

Table 4 Parameters relevant for the immunological consequences of blood transfusion

Blood component preparation technique
Content of therapeutically intended cellular and plasmatic components
Erythrocytes, thrombocytes, granulocytes and plasma
Contamination by constituents not intended for therapy
White blood cells (WBC) (in all blood components)
Plasma (in all cellular components)
Erythrocytes (in platelet and plasma components)
Number and viability of donor WBC
Preparation technique
Storage conditions (time, temperature)
Gamma or ultraviolet irradiation
Content of stem cells and T cells
Ratio of T cells/stem cells
Immunocompetence of recipient
Age of patient (fetus, newborn, elderly)
Underlying disease (lymphoma, immunodeficiency diseases)
Concurrent therapies (chemotherapy, bone marrow depletion, immunosuppressive therapies)
HLA-relationship between donor and recipient
Accidental similarity dependent on ethnic backgrounds
Intended similarity in organ transplants contaminated by WBC and HLA-matched blood components
Immunohematological differences between donor and recipient
Dependent on the degree of blood group matching
Accidental similarity dependent on ethnic backgrounds
Allo- and autoantibodies in plasma
Endogenous materials released after blood donation
Cytokines (IL-1, IL-6, IL-8, TNF α , etc.) from WBC
Proteolytic enzymes (elastase, cathepsins, etc.) from WBC
Metabolic products from erythrocytes and platelets
Soluble antigens from erythrocytes, platelets and WBC
Cellular debris from erythrocytes, platelets and WBC
Exogenous materials, processing steps
Bacterial and viral contamination
Storage conditions (time, temperature, agitation)
Stabilizers, anticoagulants and preservatives (adenine, glucose, sodium citrate, sodium dihydrogen phosphate, citric acid, mannitol)
Leaching of plasticizer from the plastic containers
Chemicals used for viral inactivation (methylene blue)
Cellular damage by gamma or ultraviolet irradiation

haplotype profiles and thus may vary between different populations.

Mononuclear cell microchimerism

The fate of transfused leukocytes is essentially determined by the balance of three factors: 1) the dose of allogeneic leukocytes; 2) the immunocompetence of the recipient; and 3) the antigenic differences between donor and recipient, which are mostly due to HLA mismatches. Generally leukocytes transfused by buffy coat-depleted erythrocyte preparations are rapidly cleared, with almost no persistence beyond 1 week, if donor and recipient were HLA unmatched.

HLA immunization usually occurs only after repeated or high-dose leukocyte exposure. However, leukocytes of fetal origin can circulate in the mother for months after delivery, and maternal leukocytes for years in the child. Lasting microchimerism is found in some HLA-matched solid organ transplant recipients, who may develop donor specific HLA tolerance. In the immunosuppressed patient prolonged leukocyte chimerism and life-threatening graft-versus-host disease (GVHD) may ensue, particularly if the HLA antigens are closely matched. In less well characterized instances microchimerism with lack of or subclinical GVHD occurs, possibly following induction of anergy or peripheral tolerance.

Transfusion-associated graft-versus-host disease

Transfusion-associated GVHD (TA-GVHD) in immunocompetent recipients may occur in transfusions of blood from HLA-homozygous donors to HLA-heterozygous patients who share an HLA haplotype. Because of a linkage disequilibrium, a few combinations of certain HLA antigens represent a large fraction of HLA haplotypes. Estimates for TA-GVHD-prone HLA constellations are calculated as a function of the HLA haplotype frequencies, which vary considerably among different populations (Table 5).

The actual occurrence of TA-GVHD is lower probably because of subtle HLA differences and because of HLA-independent elimination of T cells from the graft (for example by reduced viability after prolonged storage or prestorage leukocyte removal), which are not taken account of in current models. In transfusions between blood relatives the risk of TA-GVHD is considerably increased. In directed transfusions (i.e. blood donated for use in a specific recipient such as between blood relatives) gamma irradiation with a dose of 30 Gy (3000 rad) is mandatory for all blood components. The potential risk of TA-GVHD to the local population should be considered in directed blood donation programs.

Mediators in blood components

Mediators are produced and released during storage of blood components. Clinically relevant concentrations of pyrogenic cytokines (interleukin 1 (IL-1), IL-6 and tumor necrosis factor α (TNF α)) and histamine may occur. These cytokines – and not, as previously thought, the leukocytes themselves – are the cause of the high rate of febrile transfusion reactions experienced with platelet-rich plasma. Other blood components are less prone to mediator induction

Table 5 Frequency estimates for TA-GVHD-prone HLA constellations

Population	Estimate of frequency ^a		Relative increase in directed transfusions ^b
	Minimal	Maximal	
USA (Whites)	1:39 034	1:17 682	21-fold
Spain	1:175 296	1:10 595	23-fold
France	1:68 742	1:6 365	17-fold
Germany	1:48 583	1:6 903	18-fold
Japan	1:7 981	1:1 612	11-fold

^aIn nondirected transfusions.^bBetween parents and children or vice versa as compared to nondirected transfusions.

because of their smaller leukocyte content and lower storage temperatures. Evidence of clinical effects is lacking for the amounts of mediators occurring in most blood components.

Blood substitutes

The efficacy of synthetic blood substitutes as oxygen carriers has been known since the 1960s. Because of severe adverse effects, it is the improvement of biocompatibility rather than of oxygen-carrying capacities that is needed. Pharmacologic requirements are absorption and release of oxygen and carbon dioxide in physiologic conditions; oncotic, osmotic, rheologic and immunologic compatibility; lack of cell and tissue toxicity; limited resorption by the reticuloendothelial system; no effect on hematopoiesis; and sufficient but limited intravascular retention time.

Perfluorocarbon emulsions and modified hemoglobin solutions

Perfluorocarbons, which dissolve oxygen and carbon dioxide physically, are biologically inert fluorinated polycarbonates and can be excreted via the lung. They are oily liquids and need to be emulsified by tensids, which are often the cause of adverse effects. Because free hemoglobin is toxic, several modifications of hemoglobin, such as polymerization, conjugation, microencapsulation and S-nitrosylation, have been developed. After promising results in phase I studies, phase II trials are currently being conducted with both kinds of blood substitute.

Future prospects

Immunomodulation and blood component quality

Despite the large-scale use of blood substitution therapies for more than four decades, the immunologic sequelae of allogeneic transfusions are not fully understood. Improvement in transfusion medicine

will be achieved by enhanced preparation techniques, purer blood components, avoiding cytokine and leukocyte contamination, and consideration of the donor-recipient immunologic relationship. This development requires particular emphasis on quality assurance and transfusion management, which allows the better definition of trigger factors and exact indications for the different blood components. The characterization of mononuclear cell microchimerism may contribute to the understanding of peripheral tolerance induction. As our knowledge of the transfusion effects on the immune system increases, the importance of a more precisely defined blood component therapy will become more evident.

Stem cells, blood safety and substitutes

Major advances are currently being made in hematopoietic stem cell therapy. Many aspects of cell recovery by cytophoresis, progenitor cell processing and expansion, handling and storage are under investigation. Further advances in the safety of the blood supply are to be expected from improved screening methods for infectious agents, such as the polymerase chain reaction technique, and by the discovery of additional hepatitis viruses. Inactivation of viruses in cellular blood components is also being investigated. If the biocompatibility of artificial blood substitutes can be enhanced sufficiently, they may become useful in applications for which blood transfusion is not feasible.

Genotyping and recombinant technology

Blood group genotyping is already practised in prenatal diagnostics and may find much broader application in the future. Use of recombinant blood group proteins could improve antibody detection techniques. The large blood group databases may be utilized in the molecular genetic analysis of null mutations, silent mutations and gene hybridizations, elucidating the genetic diversity of alleles in natural populations.

See also: ABO blood group system; Agglutination; Alloantigens; Anemia, drug induced immune hemolytic; Antiglobulin (Coombs') test; Blood transfusion reactions; Cold agglutinins; Erythrocytes; Graft-versus-host reaction; Hematopoietic stem cell transplantation; Hemolytic disease of the newborn; Idiopathic thrombocytopenic purpura; Leukemia; Plasma; Rh antigens; Transplantation.

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TRANSGENIC ANIMALS

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Transgenic animals are those that, as a consequence of experimental DNA transfer application, have exogenous DNA integrated in their germ line. The integrated DNA may or may not derive from the same species as the host genome; it may or may not encode for a functional gene; and it may or may not be targeted to an intended site of incorporation in the genome. Although the term 'transgenic animal' fully represents all such cases, more specific terms such as 'knockout animals' or 'mutant animals' are used to notify the targeted disruption or mutagenesis of selected endogenous gene sequences in the germ line of animals. Most of what we know of transgenesis in animal species comes from experimental work on the mouse but transgenic technology has been also successfully applied in insects, domestic livestock, poultry and fish.

Historically, the first transgenic mouse was created by Jaenisch in 1976, by infection of preimplantation embryos with murine leukemia virus which resulted

in germline integration of the virus. Today, almost all transgenic mice are produced by pronuclear microinjection of DNA into fertilized eggs, a technique introduced in 1980 by Gordon and colleagues (Figure 1). One of the most important properties of transgenesis in animals is that the introduced transgenes usually retain their natural cell-type specificity of expression but show perturbed quantitative regulation. This selective perturbation of gene expression in whole animals allows us to interfere with physiological processes and thereby to reveal gene function. The use of transgenic animals in biological and biomedical research has increased dramatically during the last decade and has advanced greatly our understanding of gene structure and function in physiological and pathological processes. In addition, biotechnological applications of transgenesis in large animals have provided a potential for its commercial exploitation in agriculture and biotechnology.

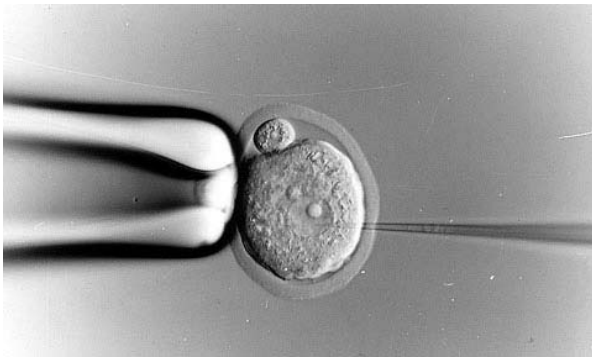


Figure 1 Microinjection of DNA into one of the pronuclei of a mouse zygote. (Photo taken by Sally Hill, Department of Molecular Genetics, Hellenic Pasteur Institute).

Production techniques

Direct microinjection of DNA into one of the pronuclei of a zygote (Figure 1), has been the most rewarding technique for the production of transgenic mice, rats, rabbits and large domestic livestock such as sheep, pigs and cows. The technique (Figure 2) requires special equipment and technical skills but in comparison to other methods it remains the most straightforward and efficient way to produce transgenic animals. Briefly, the procedure as it applies in the mouse, consists of collecting a number of fertilized mouse eggs, visualizing their male and female pronuclei under appropriate microscope optics and injecting one of them with a DNA solution

(containing a few hundred copies of the gene of interest) using a fine glass needle prepared on specialized equipment (pipette puller). Injected eggs are then surgically transplanted into the oviducts of 'pseudo-pregnant' female mice (mice that have been mated with a vasectomized male) and left to develop to term. In general, it is expected that in skilled hands, as many as 90% of the injected eggs survive the microinjection, approximately 20% of these develop to term and approximately 20–50% of the latter are found to carry the injected DNA in their tissues. Integration of the transgene occurs apparently at a random site in the genome, either as a single copy or as a tandem head to tail array. In rarer cases, head to head arrangements and multiple integration sites should also be expected. In most cases transgene integration occurs in 100% of the animal cells, however, in as high as 30% of transgenic founder mice genetic mosaicism has been observed which means that only a subset of cells in the different tissues, including the germ line, carries the transgene. Subsequent derivation of transgenic progeny from such transgenic animals leads to the generation of fully transgenic animals that transmit their transgenes in a Mendelian fashion.

In addition to the microinjection procedure, there have been several alternative methods for introducing foreign DNA into the germ line of mice. Infection of preimplantation embryos with recombinant retroviruses offers a highly efficient route of single copy

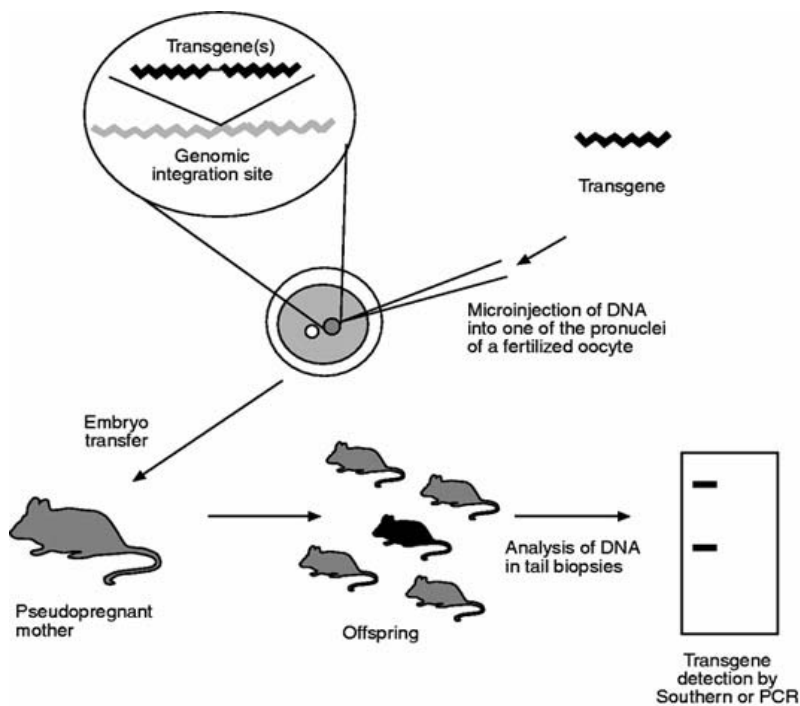


Figure 2 Experimental scheme for the production of transgenic mice by the pronuclear microinjection technique. (Computer graphics by Katerina Akassoglou, Department of Molecular Genetics, Hellenic Pasteur Institute).

integrations into the mammalian genome; however, a main disadvantage of this procedure is that retroviral vector DNA usually interferes with the expression patterns of the genes that it carries. Yet another route to transgenesis employs the introduction of exogenous DNA into totipotent mouse embryonic stem (ES) cells and their consequent incorporation into the blastocyst of a developing mouse embryo. This is technically a very demanding procedure and it does not offer advantages over the direct pronuclear microinjection for transgenesis; however it has been so far a uniquely applied technique for the targeted disruption ('knockout') or mutagenesis of endogenous genes in mice.

Optimal transgene design

It is well known that transcriptional efficiency and tissue specificity of gene expression is mainly influenced by proximity to the gene *cis*-acting elements such as promoters and enhancers, and when desired such elements should be included in the transgene construct. To discriminate the transgene and the protein that it encodes, from that of the endogenous counterpart, the injected gene must differ in some way from the respective endogenous gene and this is accomplished either by using transgenes from different species or by using transgenes that carry diagnostic modifications. Since it has been shown that prokaryotic sequences inhibit the expression of some transgenes, inclusion of large prokaryotic DNA sequences such as plasmid vector sequences in the linear DNA fragment to be injected, should be avoided. When desired, 'minigenes', that is genes with some introns deleted (e.g. constructs containing cDNA sequences) may be used, but the presence of introns seems to facilitate expression and they are usually included in the transgene construct.

A major issue in attempting to generate correctly regulated gene expression in transgenic mice is the influence of the so-called 'integration position effects' on transgene expression. For example transgenes containing all proximal control regions are rarely expressed at the same level as their endogenous counterparts and almost always there is no strict correlation between transgene copy number and level of expression. Moreover, expression of the transgene also at ectopic sites is a common phenomenon. An additional phenomenon which seems to interfere with the correct expression of transgenes is what is called 'position effect variegation' of transgene expression. This phenomenon leads to the generation of a variable and mosaic pattern of transgene expression within cells of the same tissue. All this is found to be mainly due to the absence from the

transgene construct of elements which control the tissue-specific and developmentally regulated activation status of chromatin and which insulate such genetic loci from the effects of neighboring chromatin. When available, inclusion of such locus control regions (LCRs) in transgene constructs, guarantees a strictly tissue-specific, developmentally regulated, nonvariegating, position-independent and copy number-dependent transgene expression.

Transgenic mice have been routinely generated with DNA fragments ranging from a few to several tens of kilobase pairs. For some time this limitation reflected technical difficulties in isolating and preserving the structure of larger DNA fragments for microinjection. However, advances in the use of vector systems such as those based on P1 bacteriophages and yeast artificial chromosomes (YACs) which may carry cloned DNA fragments of several hundred kilobase pairs, have allowed the efficient generation of transgenic mice carrying and expressing such large DNA constructs.

Modulation of transgene expression

Manipulation of the developmental timing and the cell specificity of transgene expression is particularly valuable in the analysis of gene function. There are currently several approaches which are used successfully for the inducible control of transgene expression *in vivo*. One of these makes use of the antibiotic tetracycline and its property to bind to a repressor protein that sits on control sequences at the promoter of bacterial genes. When tetracycline is administered, it binds and removes this protein from the promoter, switching the gene on. A modification in this system allows its use in mammalian cells as a way to turn genes either on or off. Additional systems are also being developed that transfer hormone responsiveness to mammalian cells by the stable expression of modified receptors that regulate a responsive promoter. Modified receptors are necessary in these systems to confer a specificity for an externally applied hormone, avoiding at the same time background activities from homologous endogenous hormones.

A new generation of tools for temporal control of transgene expression was introduced by the use of the bacteriophage P1 Cre/loxP site-specific recombination system. Expression of a Cre recombinase transgene in animal tissues mediates the excision of DNA segments flanked by loxP sites (floxed DNA). Such Cre-mediated excision can function to activate or inactivate transgenes in a tissue-specific manner (Figure 3). In principle, inducible activation or inactivation of transgene expression may also be

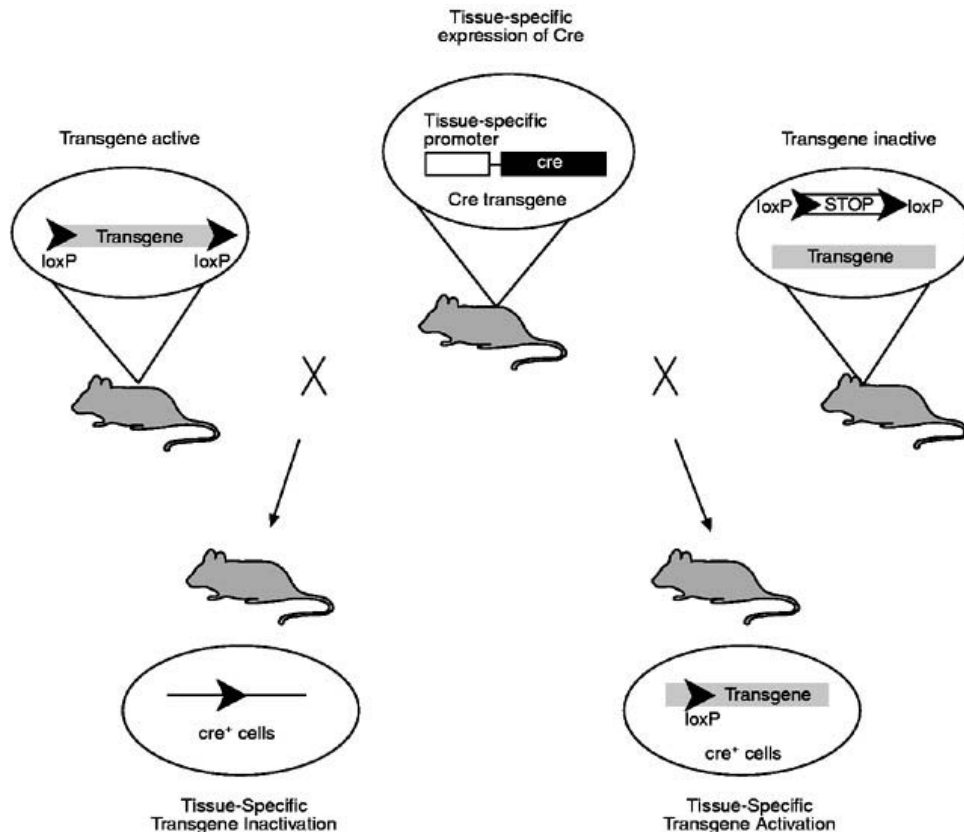


Figure 3 Tissue-specific activation or inactivation of transgenes by the use of Cre-mediated recombination of engineered loxP sites. (Computer graphics by Katerina Akassoglou, Department of Molecular Genetics, Hellenic Pasteur Institute.)

engineered when a tetracycline or hormone-inducible system drives the activation of Cre recombinase. Transgene activation may thus be achieved by Cre-mediated excision of a floxed DNA fragment which is engineered to reversibly interrupt the coding sequence of the transgene (e.g. when it is inserted in its 5' untranslated region). Conversely transgene inactivation is achieved when loxP sites are engineered to flank an essential part of an active transgene.

Applications of the transgenic technology

Transgenic technologies form today the core of 'interventional genetics', a key methodology for analyzing and interfering with gene function in whole animals. In agriculture, transgenic animals are engineered to achieve better genetic characteristics such as increased production of dairy proteins for food, enhanced production of wool or enhanced disease resistance. In biotechnology, the use of large transgenic animals or 'bioreactors' has been attempted, e.g. for the cost-effective production of high-value pharmaceuticals in the blood or milk of transgenic sheep or cows. Another promising area for the

exploitation of the transgenic technology has been in the field of xenotransplantation, where transgenic pigs are engineered to express immunological characteristics that would make their organs well tolerated after transplantation into humans.

However, so far, the major contribution of transgenic technology has been in basic research, in studies of gene expression, gene function and disease processes. Targeting of genes encoding toxins to specific cell types in the mouse (specific cell ablation) has been a helpful tool to address questions of mammalian development and cell lineage analysis. Mutagenesis or disruption of the functioning of endogenous genes by insertion of transgenes (insertional mutagenesis) has also been used not only to identify but also to clone developmentally important genetic loci. Animal modeling of human disease is certainly the area where transgenic technology has made a serious impact. Applications in these fields have allowed research into pathophysiological mechanisms, the long-term consequences and possible deleterious side-effects of therapeutic approaches including gene therapy, and the pathological interactions between multiple genetic components and the environment.

See also: Autoimmune disease, spontaneous experimental models; Gene therapy; Genetic analysis at the molecular level; Genetic analysis at the phenotypic level; Immunodeficiency, animal models; Knockout, genetic; Rheumatoid arthritis, animal models; Tolerance, models; Xenotransplantation.

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TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES, INFECTION AND IMMUNITY

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Scrapie, the most studied transmissible spongiform encephalopathy (TSE), is a fatal neurodegenerative disease of sheep and goats. Cuille and Chelle reported the first experimental transmission of scrapie, by intracerebral inoculation of healthy sheep with suspensions of brain from affected cases, in 1936. Human TSEs were recognized in 1959 with Hadlow's observation of similar central nervous system (CNS) pathology in cases of kuru, and confirmed by Gajdusek with his transmissions of kuru to non-human primates. Other human variants are Creutzfeldt-Jakob disease (CJD), transmitted in 1968, and a number of familial forms, for example Gerstmann-Sträussler syndrome (GSS) and fatal familial insomnia (FFI). TSEs are characterized by long, largely asymptomatic, incubation periods which may last for months or years. Pathological changes are restricted to the CNS and include vacuolation, degeneration of neurons and gliosis in the absence of demyelination or inflammation. Public awareness of, and anxiety about, TSEs has increased since the mid-1980s with the outbreak of bovine spongiform encephalopathy (BSE) in UK cattle, an apparent result of their consumption of scrapie-contaminated feed, and the inadvertent transmission of CJD in humans by the use of contaminated growth hormone, dura mater, cornea or EEG electrodes. We now face the probability that BSE has crossed the 'species barrier' into the human population with the emergence of a new variant of CJD presenting in younger adults and having a different clinical onset and pathology.

The pathogen: slow virus, virino or prion?

In the late 1940s, Wilson at the Moredun Institute, Edinburgh, classified the scrapie agent as a virus, based on filtration studies through membranes, and, in 1954, Sigurdsson included rida (Icelandic scrapie) with maedi/visna and infectious adenomatosis in his group of slow virus diseases. These diseases were shown to be caused by conventional viruses but the molecular structure of the TSE pathogen remains a subject for debate. While it has some of the properties of a conventional virus, such as strain variation and mutation, others, such as surviving virucidal

procedures, lend credence to the hypothesis that a host protein, PrP or prion protein, may be transformed into the infectious particle. Prion, slow virus and virino are all used as synonyms for the causative agent of TSE diseases: a virino is a composite structure of host protein (possibly PrP) and a host-independent molecule (possibly nucleic acid) which determines the strain of scrapie; a slow virus is a conventional structure of host-independent protein and nucleic acid; and one version of the prion structure has some form of PrP as the sole component of the scrapie pathogen. In all of these diseases PrP accumulates abnormally in the brain and this isoform (PrP^{sc} or PrP-res) copurifies with infectivity. In some TSEs, PrP^{sc} can also be identified in extracts from peripheral tissues (e.g. spleen, lymph nodes). Anti-PrP antibodies do not neutralize infectivity and no autoantibodies to PrP have been detected in the sera or cerebrospinal fluid of affected animals or man.

Host genes control infection

The overall rate of replication of infectivity, and hence the incubation period of the disease, depends primarily on the interaction between the strain(s) of pathogen and the genotype of the affected animal. The mouse has a single genetic locus, *Sinc*, with two alleles s7 and p7, which is the major determinant of its survival time when challenged by a standard dose of scrapie. Depending on the *Sinc* genotype, the incubation period can vary from a few months to beyond the lifespan of the mouse (>2 years). PrP is

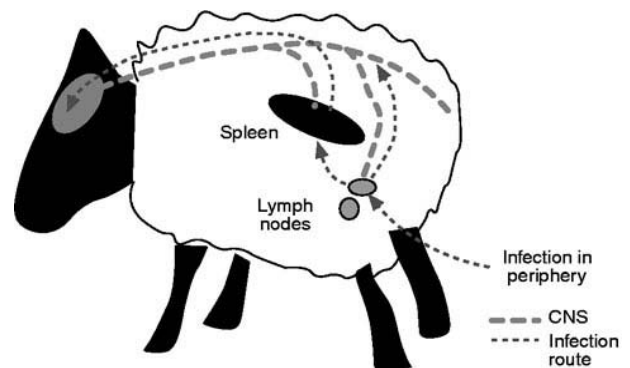


Figure 1 Scrapie spread to the CNS.

likely to be the *Sinc* gene product. There are several similar linkages of the PrP gene with susceptibility to GSS and other diseases in humans and susceptibility and resistance to natural and experimental scrapie in sheep. Disease does not develop in mice in which the PrP gene is ablated.

Other loci have minor effects on incubation period, including the major histocompatibility complex (MHC) of mouse (in the region of H2D) and sheep (various OLA loci, although the linkage is disputed).

Spread of infection

Extensive studies by Hadlow, Eklund and colleagues from the late 1950s, and more recently by Kimberlin and Walker, have helped define the spread of pathogen within an animal following natural or experimental infection with scrapie. The dynamics of spread in the mouse are dependent primarily on the strain of pathogen and *Sinc* genotype. After peripheral infection in the mouse a very short viremic phase is followed by titer rising first in the spleen and lymph nodes followed by the thymus and submaxillary glands prior to neuroinvasion of the thoracic region of the spinal cord and thence into the brain. There is also evidence in other models of routes which bypass the spinal cord and these may be very important with TSEs such as BSE, which seems more neuroinvasive after oral infection than does scrapie. That spleen is an important, but not unique, early replication site has been established by splenectomy, which delays the incubation period in mice (but not in hamsters) although it does not affect the estimate of end-point titer. The cell type(s) of spleen in which the pathogen multiplies have not been identified, although several lines of evidence point to a central role for the follicular dendritic cell. SCID (severe combined immunodeficient) mice are relatively refractory to infection by a peripheral route and PrP^{Sc} does not accumulate in their spleens. Engraftment with bone marrow from immunocompetent donors renders them susceptible to infection. Evidence also exists that spread across a species barrier requires an intact immune system and that infection cannot spread from peripheral tissues into the CNS without PrP expression. Circulating lymphocytes express low levels of PrP.

Once established within the CNS, infection spreads via neurons to specific brain areas, depending on the strain of scrapie and route of infection. The host genotype appears to have a minor role in this targeting. Although there is no classical acute inflammatory response or perivascular cuffing, astrocytes and microglia are activated, and cytokines

released, after PrP^{Sc} begins to accumulate and before neuronal apoptosis. It is still uncertain as to whether neuronal death is caused by PrP^{Sc} neurotoxicity, local depletion of the normal cellular isoform, PrP^C, or perturbation of signals from other affected cells.

The immune system and scrapie: pharmacological strategies for control

A functional immune system is required for efficient experimental transmission of scrapie and for TSE agents to cross the 'species barrier'. Outram and Dickinson have shown that immunologically immature, perinatal mice are less susceptible to scrapie infection than their 3-week-old littermates, and SCID mice are relatively refractory to infection by peripheral, natural, routes of infection. Various immunomodulators can affect the incubation period of peripherally-routed scrapie if administered at the time of infection. Phytohemagglutinin and methanol-extracted BCG increase susceptibility to infection, while prednisone acetate, dexamethazone, arachis oil and the antiviral agent HPA-23 have the opposite effect. However, interferon, antilymphocyte serum, cyclophosphamide, neonatal thymectomy or whole body irradiation fail to alter incubation period. The polyanions, dextran sulfate and pentosan polysulfate, increase survival time considerably, even when administered weeks after peripheral infection, and can completely protect against disease in some cases. They reduce the effective titer of infectivity and inhibit PrP^{Sc} accumulation, most probably by removing PrP^C from the cell surface by stimulating endocytosis, and are therefore of potential therapeutic value. Amphotericin B, a polyene antibiotic, is unique in delaying scrapie replication and PrP^{Sc} accumulation in some models infected by an intracerebral route. It is, however, highly toxic and has to be administered daily throughout pathogenesis. Safer derivatives are being developed.

See also: Neurological infections; Ovine immune system.

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TRANSPLANTATION

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No area of medicine has developed so rapidly in the past 40 years as that of transplantation. From the early pioneer attempts at renal transplantation in the 1950s, renal transplantation has now become the therapy of choice for the vast majority of patients with end-stage renal failure. So too have liver and cardiac transplantation in selected instances of end-stage disease of those organs. Combined heart and lung and single lung transplantation are being performed with increasing success and the pancreas is being transplanted into the insulin-dependent diabetic in the hope of halting the progression of the microangiopathic complications of diabetes. Corneal grafting is a routine procedure in the treatment of corneal opacification, while skin allografts have been used in the treatment of burns. As immunosuppression becomes more specific as well as more potent, not only will the short-term results of transplantation improve, but more importantly so too will the long-term results which still are generally poor.

Historical background

Experimental organ transplantation was reported at the beginning of this century, the most illustrious of

these early pioneers being Alexis Carrel who published extensively on renal allografts in dogs and cats. Skin allografts had been performed over the centuries with alleged success on occasions. However, the first human kidney transplants were performed by the Russian, Voronoy, in 1936, who carried out six such transplants in total but none probably functioned.

In the early 1950s renewed experimental interest in kidney transplantation by Simonsen in Denmark and Dempster in London led to a series of human cadaver transplants in Paris by Kuss, Servelle and Dubost and in Boston by David Hume. Some of these kidneys functioned for some weeks without immunosuppression. The first live related transplant, from mother to son, was reported from Paris in 1953 but the kidney was rejected at 3 weeks. And then in 1954 in Boston, Murray (awarded the Nobel prize in 1990 for his pioneer work in transplantation) performed a renal transplant between identical twins, one of whom had renal failure. Needless to say rejection did not occur, but this did also show that a transplanted kidney could function in a normal physiological manner. Total body irradiation was the only form of immunosuppression available at that time and, if given in sufficiently large doses to be immunosup-

pressive it inevitably resulted in pancytopenia and death from infection. In 1959 the immunosuppressive effect of the anticancer agent 6-mercaptopurine (6MP) was reported by Schwartz and Damashek from Boston, and soon after Calne in London and Zukoski, Lee and Hume in Richmond, Virginia showed that it suppressed rejection of kidney allografts in dogs. Elion and Hutchings of Burroughs Wellcome then developed a derivative of 6MP, azathioprine, which was perhaps less toxic, and this ushered in the modern era of transplantation.

In 1963 Starzl began the first liver transplantation program in Denver, Colorado, a field in which he, now in Pittsburgh, has continued to dominate. The first cardiac transplant was performed in 1968 by Barnard in South Africa, which was associated with a good deal of unfortunate publicity thereafter and led to many premature attempts all over the world at cardiac transplantation. Nevertheless Shumway at Stanford who had pioneered the experimental work in this field quickly entered the clinical arena and restored an air of respectability to the whole field in a program which established cardiac transplantation as a genuine therapy.

During that time immunosuppression was dependent essentially on azathioprine and prednisolone with the development of antilymphocyte globulin to treat resistant rejection; but then the arrival of cyclosporine, a new potent immunosuppressive agent, at the beginning of the 1980s, led to an explosion in organ transplantation. Today several new drugs – FK506, mycophenolate mofetil and rapamycin are available or about to come available, further enhancing the immunosuppressive armamentarium available to the clinician.

Donors

In general, the majority of donors of organs for transplantation are cadaveric donors, and the general acceptance of the criteria of brainstem death allowed hearts and livers to be removed before irreparable damage occurred from ischemia.

However, in the case of a paired organ, such as the kidney, donors from within the family of the patient may be used. Living related donors have the attraction of providing a better chance of long-term graft survival than a cadaver kidney, but do expose the donor to the risk of a major operation. More recently the use of living related donors of a segment of liver (for children) and of lung is being explored by a number of units.

Organ preservation

In the early days of kidney transplantation, the organ once removed, had to be implanted immediately. However, the development of first machine preservation and then later preservation by flushing the kidney with solutions of an intracellular electrolyte composition with storage in ice allows kidneys to be stored for up to 48 h. More recent developments in preservation solutions also allow livers to be stored in ice for up to 24 h, and similarly hearts and lungs can be preserved for up to 6 hours.

Matching for HLA (the MHC in humans)

Within a family the matching of a living related donor and recipient of a kidney is very precise and there is a good correlation between matching and graft outcome. However in cadaver renal transplantation the role of matching is controversial. Nevertheless there is considerable evidence that better graft survival is achieved by better matched kidneys, especially for HLA-DR, and more recently similar observations have been made in cardiac and pancreatic transplantation. Part of the matching of donor and recipient involves the detection of specific sensitization in the recipient against the donor, as evidenced by the presence of donor-specific cytotoxic antibodies. However, there are exceptions to this dogma that a positive cross-match between donor cells and recipient sera precludes transplantation when this is due to non-HLA antibodies.

Renal transplantation

There are virtually no contraindications to renal transplantation today and both the very young and the elderly may be transplanted successfully for all causes of end-stage renal failure. The kidney is generally placed in the iliac fossa retroperitoneally, the renal vessels being anastomosed to the iliac vessels and the ureter implanted into the bladder. The results of renal transplantation are excellent in the short-term with a 1-year patient and graft survival of 95% and 85% respectively being achieved. However, although 5-year patient survival is still around 90%, graft survival is only around 70%, there being a steady attrition of grafts from chronic rejection.

Liver transplantation

The major indications in the adult are the non-alcoholic cirrhoses while in the child biliary atresia is the most common indication. An increasing number of liver transplants are performed for acute

fulminating hepatitis while successful correction of metabolic defects, e.g. α_1 -antitrypsin deficiency, may also be achieved by liver transplantation. Technically liver transplantation is the most difficult of the organ transplants, the liver being placed in the bed of the patient's own liver which is first removed (orthotopic transplant). However, most of the technical problems have been resolved and 1-year graft survival both in adults and children is around 65%, with full rehabilitation of patients with a successful transplant being achieved.

Cardiac transplantation

Cardiac transplantation is performed for terminal heart disease in patients less than 55 years of age when the estimated life expectancy would be 6–12 months. The two major indications for cardiac transplantation are idiopathic cardiomyopathy and ischemic heart disease with left ventricular failure. The heart is placed orthotopically in the chest after removing the recipient's heart but leaving the back wall of the right and left atria with the atrial septum to which the appropriate part of the donor atria is anastomosed followed by the pulmonary artery and aortic anastomoses. The results of cardiac transplantation are very similar now to those of the kidney with around 80% 1-year graft survival and 60% at 5 years. Chronic rejection manifest by coronary atherosclerosis is a major problem in the medium term for many patients.

Combined heart-lung and lung transplantation

Combined heart-lung transplants are performed for intractable disease of the lungs either with or without associated cardiac damage. The commonest indications are Eisenmenger's syndrome, cystic fibrosis and primary pulmonary hypertension. In cystic fibrosis the heart is often not damaged and this does allow the recipient heart to be used for a patient needing just a cardiac transplant while the recipient is given a combined heart and lungs transplant (domino procedure). The other reason for using combined heart and lung transplants, even where the heart did not need to be replaced, was the unsatisfactory results of single or double lung transplants. However, more recent developments have led to very successful results of single lung transplants in patients with bilateral lung disease, e.g. end-stage obstructive airways disease. This approach represents a much more efficient use of organs and if successful in the medium term, the indications for combined heart and lung transplants will be con-

siderably reduced. However obliterative bronchiolitis, a manifestation of chronic rejection, is a major problem in some 30% of patients with a lung transplant.

Pancreas transplantation

The rationale of transplantation of the pancreas is to replace the endocrine pancreas, namely the islets of Langerhans, in insulin-dependent or type I diabetes with the expectation that this would halt the progress of the secondary microangiopathic complications of diabetes, e.g. retinopathy, nephropathy, peripheral arterial and ischemic heart disease. The pancreas is transplanted as a vascularized organ, either using the whole organ with the celiac artery and portal vein or a segment of the pancreas (tail and body) with the splenic vessels, the vessels in both instances being anastomosed to the iliac vessels of the recipient. The major problem of this procedure has been the exocrine drainage of the pancreas which has been dealt with by drainage of the pancreatic duct into the bladder or the small intestine, the former being now the most widely used technique.

The results have improved in recent years and some 70–80% of grafts are functioning at 1 year (as judged by insulin independence). However the procedure is associated with a high morbidity and so far there is little convincing evidence that the microangiopathic complications improve, perhaps because the procedure is only performed at a very advanced stage of the disease, generally in patients requiring a renal transplant for diabetic nephropathy at the same time.

A more attractive approach would be the transplantation of isolated pancreatic islets only. This has been performed successfully in experimental models of diabetes in the rodent for many years but a major obstacle to human application has been the lack of a satisfactory technique to prepare isolated islets from the large mammalian pancreas, including humans. However, these problems have been resolved to a large extent and clinical trials have commenced in several centers. The islet preparation is implanted in the liver via the portal vein, although the spleen or the kidney capsule may be alternative sites as demonstrated in experimental models. Although functional islet transplants have been achieved, as judged by normal C peptide levels, only a handful of patients have been rendered insulin independent.

Cornea

The major indication for corneal grafting is corneal opacification due to such problems as postinflam-

matory scarring, corneal dystrophies and endothelial dysfunction. It is performed as often or more than kidney transplantation. The cornea is a privileged site, presumably due to the lack of lymphatic drainage. For this reason primary grafts in a nonsensitized recipient fail only for technical reasons and immunosuppression is not required. However, in patients sensitized by a previous failed graft, the failure rate is high. Better results are achieved with secondary grafts in the presence of good HLA matching of donor and recipient or low doses of immunosuppressive drugs.

Skin

Skin allografts have been performed in patients with severe burns where inadequate donor skin sites are available. Although rejection does occur, it is delayed presumably because of the severity of the injury. Immunosuppression has also been used to prolong skin allograft survival in burn patients with some success but this adds to the risks of infection in severely injured patients who are already at risk.

Immunosuppression

Immunosuppression is used in clinical transplantation both to prevent rejection and also to treat rejection. Preventive immunosuppression therapies today are based on cyclosporine which may be used alone, with steroids, or with both azathioprine and steroids (triple therapy). Another very powerful induction therapy includes a course of a heterologous antilymphocyte globulin (ALG) or a pan T cell monoclonal antibody (e.g. OKT3) with azathioprine and prednisolone, introducing cyclosporine after 7 days (sequential therapy). Certainly in renal transplantation there is no convincing evidence in terms of graft survival that one immunosuppressive protocol is superior to another, although there is a lower incidence of rejection in sequential therapy protocols.

An acute rejection episode is treated with high-dose methylprednisolone, but if the rejection proves resistant to steroids then either ALG or OKT3 is generally introduced. Some 75% of steroid-resistant rejection episodes can be reversed with ALG or OKT3.

New immunosuppressive agents, FK506 (tacrolimus) and mycophenolate mofetil, have now undergone extensive investigation in large multicenter trials which have proven their value in liver and renal transplantation respectively. FK506, a macrolide antibiotic, has a very similar mechanism of action to cyclosporine and inhibits the activation of T lympho-

cytes and in particular the transcription of the genes for a number of cytokines, especially interleukin 2 (IL-2). However, it is a more potent agent on a weight basis, and does seem at least as effective as cyclosporine in liver transplantation and indeed may rescue livers that are being rejected on treatment with cyclosporine. Mycophenolate mofetil is an antiproliferative agent. It has been tested in renal transplantation as a replacement for azathioprine in association with cyclosporine and steroids, where it has been shown to reduce the incidence of acute rejection episodes substantially in the first 6 months after transplantation. Another new agent, rapamycin (sirolimus), also a macrolide antibiotic, which inhibits proliferation of activated T lymphocytes by a mechanism as yet unclear, is undergoing phase II trials at present in renal transplantation. Early results suggest that it is a potent immunosuppressive agent.

The development of monoclonal antibodies against various leukocyte subpopulations also provides the possibility of more specific immunosuppression. So far the only monoclonal antibody used extensively in clinical practice is OKT3, a pan T monoclonal antibody directed against the CD3 molecule. Trials of monoclonal antibodies against the IL-2 receptor (CD25), LFA-1, ICAM-1, CD4 and the T cell receptor have been, or are being, carried out using murine or humanized monoclonal antibodies. So far results available are disappointing, but expectation remains high that such antibodies will allow more specific immunosuppression and hence less of the potent nonspecific immunosuppressive drugs to be used. Certainly a number of very promising protocols have been developed in experimental models of transplantation which allow tolerance to be produced and which may be applicable in the clinic.

Complications

The complications of transplantation of all organs are either technical or related to the immunosuppressive therapy. Technical complications are relatively uncommon in renal and cardiac transplantation, but still are a feature of liver and especially pancreatic transplantation. The major complications related to immunosuppression, either directly or indirectly, are infection, ischemic heart disease and cancer. Viral infections, particularly cytomegalovirus (CMV) infection, remain the most prominent of the post-transplant infections, but more exotic infections are seen from time to time, e.g. *Aspergillus*, *Pneumocystis carinii*, tuberculosis, and clinicians must be aware of this possibility in these immunosuppressed patients.

Most types of cancer show a modest increase in

frequency in patients after transplantation but there is a marked increase in the frequency of lymphomas, cervical cancer, squamous cell cancers and Kaposi sarcomas, all tumors with a putative viral etiology. Ischemic heart disease is the major cause of death in patients with long-term functioning transplants. Hyperlipidemia and hypertension, two major risk factors associated with ischemic heart disease, are common in transplant patients and are probably related to the immunosuppressive drug therapy.

Xenografts

The possibility of transplanting organs from other species to humans in order to meet the unmet demand for organs remains an area of considerable research interest. However, a major immunological obstacle to xenografts is the presence of natural cytotoxic antibodies in humans directed against most species that might be used as a donor of organs, e.g. pig. Although kidney xenografts from chimpanzee to human were performed in the 1960s by Reemstma with function persisting for weeks to months with azathioprine/prednisolone, it is doubtful whether the use of chimpanzees would be ethically acceptable today (humans do not have natural cytotoxic antibodies reactive with chimpanzee). The natural cytotoxic antibodies in humans are, on the whole, directed against a xenoantigen, galactose ($\alpha 1,3$) galactose, which is expressed on most tissues in all species except humans and the Old World primates. Transplantation of an organ from a species such as a pig into a human would result in immediate hyperacute rejection mediated by a classical complement-dependent destruction of the target tissue by antibody. A variety of approaches to this problem have been investigated, e.g. depletion of antibody and/or complement, but one of the most promising has been the development of transgenic pigs expressing human complement inhibitors on endothelium as well as

other cells. In experimental models organs from such animals have been shown to be resistant to the immediate antibody and complement-mediated destruction. However, even if this approach proves to be successful, many other problems remain to be resolved such as suppression of the cellular immune response, both specific and nonspecific, the possibility of transferring potential pathogenic organisms to humans, and the metabolic and physiological behavior of an organ from another species in a human.

See also: Antilymphocyte serum; Hematopoietic stem cell transplantation; Graft rejection; Graft-versus-host reaction; Immunosuppression; Tissue typing; Xenotransplantation.

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TREPONEMA, INFECTION AND IMMUNITY

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The genus *Treponema* consists of pathogenic and nonpathogenic spirochaetes. Human diseases caused by the treponemes are syphilis (*Treponema pallidum* subsp. *pallidum*), endemic syphilis (*T. pallidum* subsp. *endemicum*), yaws (*T. pallidum* subsp.

pertenue) and pinta (*T. carateum*). While syphilis is a venereal disease, the remaining are nonvenereal diseases. These microorganisms are morphologically almost identical but exhibit different patterns of pathogenesis in humans and laboratory animals.

They cannot be detected in conventionally stained smears. They are visualized in wet films by dark-field microscopy, and in fixed preparations by immunofluorescent antibody technique or silver nitrate staining. The pathogenic treponemes are not cultivable *in vitro*, but can be propagated in animals (Table 1), with the exception of *T. carateum*. The nonpathogenic treponemes, which are numerous, can be cultivated *in vitro*. They colonize humans and animals.

Characteristics of *Treponema* and its antigens

Information on the morphology and molecular characterization were obtained using *T. pallidum* Nichols strain which is a reference strain of *T. pallidum* subsp. *pallidum*. Treponemes are spiral microorganisms ca. 0.1 μm thick and 5–20 μm long. They are helical in nature, having outer and cytoplasmic membranes, a thin peptidoglycan layer and periplasmic flagella. The latter are located between the cytoplasmic and the outer membranes. They extend from one to the other end of the microorganism allowing for the characteristic movements of the spirochetes. The flagellum consists of three structural components: the sheath, core and hook, and is composed of four major polypeptides. Treponemes also contain cytoplasmic filaments, which run in parallel to the periplasmic flagella. Their function is unknown. At least eight major membrane-associated lipoproteins have been identified in *T. pallidum*, but their precise location (internal versus external) has not been established. A unique structural feature of *T. pallidum* is the paucity of intramembranous protein particles in the outer membrane.

T. pallidum, *T. pertenue* and *T. endemicum* have over 95% homology by DNA–DNA hybridization. *T. pallidum* and *T. pertenue* vary at only one of 531 base pairs (bp), resulting in a single amino acid difference in the sequence. The DNA homology of *T.*

pallidum and nonpathogenic treponemes, e.g. *T. phagedenis*, varies from 20 to 80%, depending on the method used for comparison. *T. pallidum* has one of the smallest prokaryotic genomes (1000 kbp), which is in the form of a circular chromosome.

Twenty-seven polypeptides have been identified in *T. pallidum*. Those studied for potential practical use have a wide spectrum of molecular mass, representing surface-, membrane- and flagella-localized proteins. An increasing number of monoclonal antibodies to the various peptides are available but they are not species or subspecies specific.

Syphilis

In 1993 the World Health Organization reported 12 million new cases of syphilis worldwide. Of all known treponematoses, syphilis remains the focus of wide interest. Except for congenital syphilis, sexual contact accounts for virtually all syphilitic infection. The ID₅₀ for man is ca. 50 treponemes. The microorganisms enter the host through abraded skin or mucous membranes. Part of the inoculum spreads via lymphatics into regional lymph nodes, while the remaining organisms propagate at the site of entry, forming a characteristic lesion (chancre) in an average of 3 weeks (10–90 days). The course of infection in an untreated individual runs through stages: primary (6–8 weeks), secondary (a few weeks to a year), early latent (up to 2 years), late latent (several years) and tertiary (variable time). During primary and secondary stages the individual is infectious; in latent periods, except for a pregnant woman who can transmit the disease to the fetus, the individual is non-infectious. During spirochaetemia *T. pallidum* may invade many organs, including the central nervous system, causing a variety of neurologic symptoms. Clinical manifestations of tertiary syphilis occur in ca. 30% of untreated patients affecting various organs. Congenital syphilis is acquired by trans-

Table 1 Nonvenereal treponematoses

Organism	Disease	Production of antibodies ^a	Cross-immunity	Susceptible animals
<i>T. pallidum</i> subsp. <i>endemicum</i>	Endemic syphilis (Bejel)	Within a few weeks of infection	Protection against <i>T. pallidum</i> subsp. <i>pallidum</i>	Rabbit ^b Hamster
<i>T. pallidum</i> subsp. <i>pertenue</i>	Yaws (Frambesia)	Within a few weeks of infection	Protection against <i>T. pallidum</i> subsp. <i>pallidum</i>	Monkey Rabbit Hamster
<i>T. carateum</i>	Pinta (Carate)	Very slow seroconversion (3 months after primary lesion)	No protection against <i>T. pallidum</i> subsp. <i>pallidum</i>	Chimpanzee ^c

^aVDRL and treponemal.

^bRabbits respond differently to experimental infection from that with *T. pallidum*, Nichols.

^cDoubtful results.

placental passage of *T. pallidum* from an infected pregnant woman to her fetus. Depending on the time of infection and maternal immune status, stillbirth may occur, or a newborn may demonstrate characteristic symptoms immediately or within 2 years (early congenital), or after 2 years of birth (late congenital), or the newborn may be free of syphilis.

Syphilis has been frequently associated with AIDS. Severe courses of syphilis, accelerated development of neurosyphilis and syphilis masquerading as AIDS have been reported.

The antibiotic of choice for treatment of syphilis and nonvenereal treponematoses is penicillin. Individuals allergic to penicillin are treated with tetracycline or similar antibiotics.

Immune response in syphilis

T. pallidum infection evokes cellular and humoral immune responses. The humoral response manifested by production of phospholipid and treponemal antibodies is the basis for serodiagnosis of syphilis. The phospholipid or cardiolipin antibodies are detected by a variety of flocculation tests using a defined mixture of cardiolipin–lecithin–cholesterol as antigen (Venereal Disease Research Laboratory, VDRL antigen or VDRL test). A second type of antibody, known as cardiolipin F (detected by immunofluorescence) and directed against host-cell mitochondrial cardiolipin (autoantibody), is also associated with syphilis, but is not used for serodiagnosis. Treponemal antibodies, once detected by the cumbersome *T. pallidum* immobilization test (TPI) are now identified by immunofluorescence (FTA-ABS) or microhemagglutination test (MHA-TP). Treponemal antibodies appear after infection almost simultaneously with the cardiolipin antibodies. The treponemal antibodies persist in the host for many years even after treatment, whereas the cardiolipin antibodies parallel the dynamics of the infection and are useful in interpreting the efficacy of treatment. Autoantibodies to tissue phospholipids and other tissue constituents, to blood cells and serum globulins are also produced by *T. pallidum*-infected hosts.

Cell-mediated immune responses to infectious organisms involve activation of macrophages and the induction of CD8 T cells and helper CD4 T cells. The various subsets of CD4 T cells, and the cytokines they produce, play a crucial role in determining the outcome of infectious disease in terms of both protective immunity and immunopathology. Although a switch from T helper T_H1 to T_H2 immune response has been observed in the rabbit experimental model of syphilis, this remains to be further studied.

Immunity in syphilis

Correlation of the humoral and cellular immune responses with clinical manifestations of the disease in both natural and experimental infection seems to favor a cellular rather than a humoral mechanism in the acquisition of immunity, as deduced from the following information: 1) primary and secondary syphilis progresses despite a profuse humoral response; 2) passive immunization with immune serum has had only limited success; 3) immunization with killed *T. pallidum* does not protect recipients despite development of antibodies; 4) delayed hypersensitivity appears late in the course of infection; 5) tertiary lesions have a granulomatous appearance; 6) lasting immunity has been induced in animals only by infection with live or immunization with γ -irradiated *T. pallidum*; 7) adoptive transfer of *T. pallidum*-immune T lymphocytes into syngeneic naive recipients prevented the development of lesions after challenge and the hematogenous dissemination of *T. pallidum*, providing a longlasting protection against reinfection; 8) treponemal antibodies, i.e. TPI antibodies, are not associated with the protective mechanism(s) operative after challenge in adoptively immune animals.

Evasive strategies of *T. pallidum*

A large number of host macromolecules appear to be avidly associated with the outer envelope of *T. pallidum*. The biologic importance of this association, between host proteins and pathogen, seems to be considerable, as it may decrease the immunogenicity of the latter, promoting its survival and induction of autoimmune responses. It is also speculated that the immunologically inert surface of the *T. pallidum* (paucity of intramembranous proteins) contributes to the organism's evasion of the host inflammatory response.

Vaccine

Immunization protocols in experimental syphilis failed to produce protection from infection.

Nonvenereal treponematoses

Yaws and pinta are restricted to tropical countries but endemic syphilis has, besides Africa and Middle East, also been reported in Europe and North America. The nonvenereal treponematoses have been nearly eradicated through an intensive campaign by the World Health Organization. Only sporadic outbreaks or cases were reported. These diseases usually

occur in children living in poor sanitary conditions, and infection is due to contact with fluid-exuding lesions, and through usage of common utensils.

These diseases have several features similar to syphilis, i.e. the morphology of the treponemes, antibody production (VDRL, FTA-ABS, TPI), lesions, and cell infiltration in secondary and late active yaws. However, differences in the mode and severity of infection, host susceptibility, cross-immunity and time of antibody appearance exist (Table 1).

See also: Bacteria, immunity to.

Further reading

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TRICHURIASIS

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Trichuriasis is caused by infection with the nematode *Trichuris trichiura*, one of a group of worms, the soil-transmitted nematodes, that includes some of the commonest parasites of humans. Globally, some 800 million people harbour *T. trichiura*. The existence of such widespread and long-term infections raises a number of interesting questions about host resistance and susceptibility, some of which have been answered by studies in laboratory models.

Characteristics of the organism and its antigens

T. trichiura is a relatively long worm, measuring about 2 cm. It lives in the large intestine, occupying a superficial tunnel on the surface of the mucosa. The smaller larval stages live completely within such tunnels, which are produced as the worm moves through the epithelial cells, but at the adult stage only the anterior half of the body is embedded, the posterior lying free. The species has both males and females. When mature, the females release very large numbers of eggs, which pass out with fecal material, becoming infective after a period of development in the soil. Hosts are infected by accidental ingestion of eggs containing fully developed infective first-stage larvae, which then hatch in the small intestine.

Trichuriasis is common in countries where climatic and socio-economic conditions favor the survival

and transmission of infective eggs. Infections are most prevalent in children, where heavy infections may cause chronic diarrhea, anemia and retarded physical and educational development. Other *Trichuris* species occur in many animals. *T. suis*, for example, can cause a severe disease in pigs. *T. muris*, which infects mice, provides a useful experimental model for studying the immunobiology of this group of worms.

Trichuris worms belong to the same group as *Trichinella* and the body has the same overall organization. The anterior esophagus lies within glandular stichocyte cells, which are one of the major sources of antigens. Antigens are synthesized in the stichocytes, released into the esophagus and pass out of the mouth. Many are enzymes, which presumably facilitate feeding or mucosal penetration. The most important antigens are between 40 and 60 kDa, but certain molecules (47 kDa in *T. trichiura* and 43 kDa in *T. muris*) are immunodominant. Major antigens are also present within the pseudocoelomic fluid. Surface antigens are present on the cuticle but play only a minor role in immunity.

Immune responses of the host

In humans, infection elicits specific immunoglobulin G (IgG), IgA and IgE antibody responses. Levels of IgG increase with levels of infection, but raised levels

of IgA may correlate with reduced worm burdens, i.e. with resistance to infection. To date cellular responses have been little studied. The large intestine shows macroscopic inflammatory changes, which are associated with local increases in macrophages and mast cells. Macrophage release of cytokines (especially tumor necrosis factor α , TNF α) and an IgE-mediated release of mast cell mediators contribute to the inflammation and protein-losing enteropathy that are associated with heavy infection.

The immune response to *T. muris* in mice has been studied in detail. Infections elicit strong T cell responses both locally and in the draining node, and these lead to the premature expulsion of worms from the intestine. Exposure to primary infections can stimulate complete resistance to reinfection. Immunity can be transferred between animals using CD4⁺ T cells or immune sera. An IgA monoclonal antibody specific for *T. muris* antigen is also effective. Inflammatory responses (e.g. mucosal mastocytosis) occur but do not seem to contribute to the expression of immunity. How immunity is achieved is uncertain. Antibodies may combine with released molecules and interfere with feeding or movement; the role of T cells is unknown.

Immune responses to *T. muris* show considerable genetically determined variation, both major histocompatibility complex (MHC)-linked and background genes being involved. Variation affects the onset and degree of immunity, the level and specificity of antibody responses and the T cell response. Mouse strains that respond well and eliminate infection produce responses mediated by T_H2 cells; susceptible strains, that are unable to eliminate worms, show a switch to T_H1 subset responses.

Evasive strategies by the organism

It is characteristic of human infections with *Trichuris*, as with all helminths, that the infections are aggregated within the population. A majority of individuals have low-level infections, a few are heavily infected; children are the most heavily infected age group. Although differences in environmental exposure could be a major determinant of variations in the levels of infection, there are reasons to believe that differences in immune-mediated resistance could also be involved. Studies in mice, in addition to demonstrating the existence of similar genetic variation, have shown that the later stages of infection may be powerfully immunosuppressive and responsible for the switch to a T_H1 response that prevents effective

immune control. Thus, if for genetic reasons a host fails to control the early stages of infection, the later stages may inhibit the subsequent development of immunity, leading to the establishment of a chronic infection. In mice, at least, chronic infections are associated with a long-lasting inability to respond protectively, even when the initial infection is removed. Chronicity of infection and frequent reinfection of susceptible individuals are characteristic of trichuriasis in endemic areas. Both may therefore reflect the success of an evasion strategy based on the parasite's manipulation of the host's response.

Vaccines

No vaccine is available against *Trichuris* or indeed against any soil-transmitted nematode. The data from experimental studies suggest that vaccination could be achieved relatively easily, given that, in mice, identifiable antigens elicit high levels of immunity. Modern techniques of antigen purification and production could make such antigens available (the major antigen of *T. trichiura* has been cloned) but it seems unlikely that there will ever be sufficient demand for a vaccine against trichuriasis to be produced.

See also: Ascariasis; Hookworm disease; Parasites, immunity to; Strongyloidiasis.

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TRYPANOSOMIASIS, AFRICAN

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Characteristics of the organisms and their antigens

The tsetse fly-transmitted trypanosomes cause sleeping sickness in humans and a related disease, nagana, in domestic animals in Africa. These flagellated protozoan parasites multiply largely in the blood of the mammalian host but may invade extravascular sites (lymphatics, connective tissue, central nervous system), depending on the species of trypanosome.

Two types of sleeping sickness are recognized: a chronic West African form transmitted by riverine tsetse and caused by *Trypanosoma brucei gambiense*, and an acute East African form transmitted by savanna flies and caused by the morphologically identical *T. brucei rhodesiense*. A third 'subspecies', *T. brucei brucei*, cannot infect humans but causes nagana in animals. Other species of *Trypanosoma*, *T. congolense* and *T. vivax*, also cause nagana. All three species undergo a complicated cycle of development in the blood-sucking tsetse fly vector, details differing with the species. In all cases the end-result is the discharge of the mammal-infective metacyclic stage with the saliva when the fly takes a blood meal.

Sleeping sickness is usually fatal, unless treated with toxic drugs (e.g. suramin) and over 50 million people are at risk of acquiring infection. Cattle trypanosomiasis is widespread and has devastating effects on animal husbandry, preventing satisfactory stock rearing over 10 million km² south of the Sahara. Antelopes and other game animals serve as a reservoir of both human and animal trypanosomiasis, often showing no symptoms of disease. Outside the tsetse belt of Africa, *T. brucei* ('*T. evansi*') and *T. vivax* may be spread by tabanid and *Stomoxys* flies acting as flying syringes, transferring the infection directly from one host to another. The main victims of such mechanical transmission are the draught animals of unmechanized farming and transport in North Africa, South America and southern Asia – horses and camels in particular.

Because chemoprophylaxis has proved unsatisfactory and vector control raises major environmental problems, vaccination has long been regarded as a potentially ideal solution to the problem of African trypanosomiasis. Unfortunately it is an unattainable ideal, because although the antigen inducing a pro-

tective humoral response is well characterized as a surface glycoprotein covering the entire trypanosome, the parasite has the ability to change repeatedly the antigenic nature of this glycoprotein (the variant surface glycoprotein, VSG) and so avoid destruction by the host's immune response through antigenic variation.

The molecular basis of trypanosome antigenic variation has received a great deal of attention over the last 30 years so that we now know more about the trypanosome as antigenic challenge than we do about the response of the natural host to infection with the parasite. Most of our knowledge of immunity to trypanosomiasis is based on experimental infections in rodents. African trypanosomes usually infect laboratory animals readily, but repeated syringe passage results in increased parasite virulence with large numbers of trypanosomes in the blood, whereas in sleeping sickness patients and infected cattle or game animals, the parasitemia may be undetectable by microscopic examination of the blood.

In the mammalian host the VSG forms an enveloping 15 nm thick coat covering the entire trypanosome plasma membrane, body and flagellum, and is readily visualized by electron microscopy. In this monomolecular layer of a single species of glycoprotein (46–65 kDa, depending on the trypanosome species or variant), the immunogenic epitopes reside in the N-terminal domains distal to the membrane rather than in the C-terminal domains; each molecule is anchored to the membrane by a glycosylphosphatidylinositol (GPI) moiety at its C-terminus. Variant surface glycoproteins become N-glycosylated at one or more asparagine residues but the carbohydrate plays no part in the VSG immunogenicity. Dense packing of VSG homodimers in the exposed coat results in an effective macromolecular diffusion barrier and proteins as small as 50 kDa are unable to penetrate it. The coat thus protects the parasite against nonspecific attack by the alternative complement pathway. It also deters phagocytosis by macrophages. On entering the tsetse fly the VSG coat is shed and replaced by a different immunodominant protein called procyclin; by electron microscopy the various developmental stages in the tsetse gut and salivary glands or mouthparts appear uncoated, but

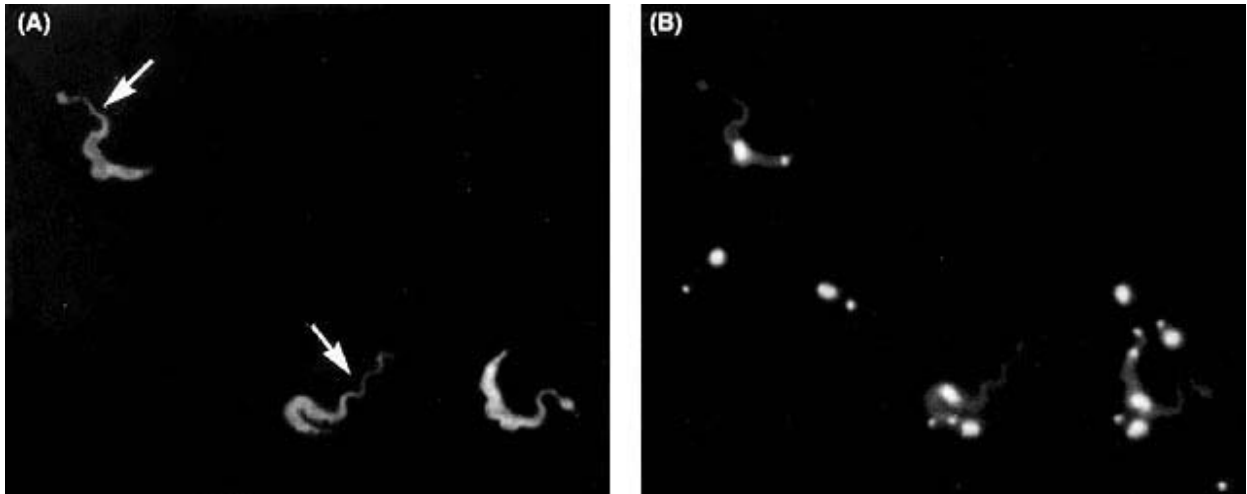


Figure 1 Blood smear showing trypanosomes undergoing antigen variation. (A) Three cells (two arrowed) are labeled with a VAT-specific antibody. (B) All parasites in the field of view are labeled, nucleus and kinetoplast, with DAPI.

the VSG coat is reacquired at the final metacyclic stage which is discharged in the fly's saliva. The metacyclic trypanosomes thus enter the mammal already protected against its host's nonspecific defences.

Antigen variation; the difficulties it poses for vaccine design

Only one VSG is expressed by a trypanosome at a time; trypanosomes expressing the same VSG are said to belong to the same variable antigenic type (VAT). In the blood of a chronically infected mammal, there is a hierarchical expression of VATs with some always being detected early in infection and others later. Protective immune responses of the host are VAT-specific and parasites expressing these VATs are cleared from the blood (through complement-mediated lysis and/or opsonization) and the parasitemia declines. Remission is invariably followed by recrudescence, however, as trypanosomes expressing other VATs multiply. Thus, the numbers of trypanosomes detectable in the blood fluctuate wildly such that the infection characteristically consists of a series of parasitaemic peaks.

Switching from one VAT to another occurs spontaneously; it is not induced by the host's immune response as it occurs in *in vitro* cultures of bloodstream stage trypanosomes. The rate of switching varies between 10^{-2} and 10^{-7} switches per cell generation and is higher in tsetse-transmitted than in syringe-passaged infections. Each VSG is encoded by a separate VSG gene and switching involves the expression of a different VSG gene at one of a few mutually exclusive chromosomal telomeric

expression sites. These are two principal mechanisms by which a VSG gene can be expressed: It can be activated *in situ* or a copy (the so called expression-linked copy or ELC) of a chromosomal internal gene (basic copy) may be inserted at a telomeric expression site, displacing the previously expressed gene. What controls the rate of switching or the order in which heterotypes grow up to become homotypes is not known.

Trypanosoma brucei appears to have about 1000 VSG genes underlying its VAT repertoire though *T. b. gambiense* and *T. vivax* may have a smaller number. Late in an infection new chimeric VSG genes may arise as a result of only partial replacement of the outgoing gene at the expression site by the incoming expression-linked copy. For a given trypanosome species or subspecies, a number of VAT repertoires (serodemes) can be discerned in different or in the same geographical areas. Despite the high immunogenicity of VSGs, the prospect of vaccinating potential hosts against all VATs in all serodemes is clearly hopeless. A more promising approach was thought to be to protect such hosts against tsetse-transmitted trypanosomiasis by rendering them solidly immune to the metacyclic VATs (M-VATs) that constitute a limited subset of the bloodstream repertoire (12 in *T. congolense*, ≤ 27 in *T. brucei*). Metacyclic VSG genes always reside at the telomeres of large chromosomes, are activated *in situ* and can be expressed at a lower temperature than bloodstream form VSGs. *T. brucei* has over 120 chromosomes, about 100 of which are 'minichromosomes' (average size 100 kb as compared with >1 Mb large chromosomes); the minichromosomes appear to house only VSG genes.

Although immunity against infection with metacyclic trypanosomes can be induced by allowing cattle to become infected by fly bites and then treating the resulting infection as soon as the parasitemia becomes patent, such immunity is usually short-lived. Breakthroughs in infection can occur as a result of replacement of a metacyclic VSG (M-VSG) gene with a bloodstream stage VSG as part of the process of antigenic switching occurring at a M-VSG gene expression site. The ability of the M-VAT repertoire of a given serodeme to evolve in this way ensures that hosts in a given area do not all become immune to a given serodeme. With the collapse of plans for vaccination based on M-VATs, attention has turned to the possibility of vaccination against nonvariable antigens of the parasite, that is those common to all trypanosomes irrespective of their VAT. Such common antigens are largely unexposed and so unable to react with host effector mechanisms, but the discovery that the trypanosome relies on receptor-mediated endocytosis to take up certain necessary host proteins (e.g. transferrin, low-density lipoprotein) from its flagellar pocket has raised hopes that the blocking of exposed receptors by host antibodies might halt or slow the infection. Alternatively, transmission blocking antibodies raised to procyclin or some other component of the trypanosome surface in early vector development might be used to limit the spread of trypanosomiasis by tsetse flies.

Immune responses of the host

Trypanosome infections elicit a strong humoral response. In the experimental mouse it has been established that the pronounced immunoglobulin M (IgM) and IgG responses which accompany the elimination of each VAT are responsible for killing of parasites and their clearance from the blood by two main effector mechanisms: activation of complement via the classical pathway and opsonization leading to uptake by Kupffer cells of the liver.

In bovines (including asymptomatic antelopes) IgM levels may increase ninefold over normal. This sustained macroglobulinemia is usually interpreted as being due to antibodies produced against successive trypanosome VATs, but heterophile antibodies have been demonstrated in trypanosome-infected hosts and taken to be a sign of polyclonal B cell activation. Elevated levels of IgM in the cerebrospinal fluid (5–10% of this IgM is 7S, possibly indicating failure to assemble the 19S product) is diagnostic of the disease.

The immune response in mice comprises both T-dependent and T-independent components. The classic evidence for the latter is the control of para-

sitemia in nude mice. With regard to T-dependent responses, there is no current consensus on either the nature of the T cells involved or the importance of their function. T_H1 type responses have been observed ($CD4^+$, $CD8^-$ cells secreting interferon γ ($IFN\gamma$) but not interleukin 4 (IL-4), antigen-specific, major histocompatibility complex (MHC) class II restricted) but these cells failed to mount proliferative responses and were found only in the peritoneum, not in the spleen or lymph node. $CD4^+$ knockout mice regulate parasitemias as well as control mice. Activation of $CD8^+$ cells secreting $IFN\gamma$ has also been reported but these cells unexpectedly, at least in some model systems, appeared to promote parasite growth and exacerbate infections. There is no evidence for $CD8^+$ cells modulating infections in bovines.

A profound depression of lymphocyte responsiveness is characteristic of trypanosome infections and, in mice, the onset of depression is very rapid – 5–7 days. This depression is caused in large part by suppressor macrophages which when elicited release nitric oxide and prostaglandins leading to a reduction in IL-2 production, IL-2 receptor expression and lymphocyte unresponsiveness.

Certain *Bos taurus* (humpless) breeds of domestic cattle, the N'dama and shorthorn (Muturu) of West Africa, are trypanotolerant, that is they can survive and be productive in tsetse-infected areas where other breeds succumb to trypanosomiasis in the absence of drug treatment. Trypanotolerance appears to be due largely to the superiority of the animal's acquired immune response, in ability both to respond to the trypanosome and to control the infection. The severity of the anemia, which often proves fatal, in cattle trypanosomiasis is broadly related to the level of parasitemia and its duration. Differences in anemia between N'dama and Zebu cattle appear to be due to the capacity to control parasitemia rather than differences in erythropoietic responsiveness. Other factors (food utilization, heat tolerance) may be important to innate trypanotolerance, and in game animals trypanocidal factors in the serum undoubtedly play a part. The inability of *T. brucei brucei* to infect humans is probably due to its susceptibility to lysis by human high-density lipoprotein; sleeping sickness trypanosomes resist such destruction.

Much of the pathology of African trypanosomiasis (anemia, circulatory disturbances, lymphoid cell proliferation, tissue destruction) has been attributed to the host's exaggerated response to the presence of the trypanosome. Trypanosomes expressing M-VATs multiply in the dermis at the site of an infected tsetse bite, inducing an inflammatory reaction (the chancre)

with redness, edema, and infiltration by neutrophils and small/medium lymphocytes. At the peak of the reaction, these cells are replaced by proliferating large lymphocytes and, progressively, plasma cells, numbers of the latter increasing as the chancre subsides in the third week. In sheep, T lymphocyte subpopulations (both CD4⁺ and CD8⁺) form a major component of the early mononuclear cell infiltrate, whereas after 5–7 days B cells dominate in the *T. congolense*-induced chancre. Another chancre does not form if the animal is challenged with the same trypanosome serodeme, suggesting immune neutralization of the challenge infections. Chancres never form in mice and rats.

Trypanosomes are found in the draining lymph of the chancre 6–7 days after infection and in the blood 3–5 days later. They switch from one M-VAT to another in the chancre: the first bloodstream VAT (B-VAT) to appear is that originally ingested by the tsetse fly (due to reactivation of the B-VSG gene *in situ*). After this the procession of B-VATs occurs as in syringe-passaged infections; early VATs appear to be coded by VSG-genes activated *in situ* at telomeric sites, later B-VATs the result of the ELCs of internal genes being transposed to an expression site.

The lymphoid proliferative phase of the disease starts with the first patent parasitemia and lasts for less than 2 weeks in mice, over 3 months in cattle. Enlargement of lymph nodes and spleen are striking, with a disproportionate increase in the size of B cell-dependent follicular areas, and germinal centers are usually well developed. After the proliferative phase a more protracted cellular depletion phase occurs in the lymphoid organs, supposedly due to clonal exhaustion.

Immune complex formation (especially due to the boosting of antibodies against common antigens as the infection advances) has been postulated to account for macrophage infiltration of tissues and Arthus reactions (tissue immune complexes), and anemia and kinin-induced vascular permeability (circulating complexes). These conclusions are based

on laboratory animals but the hematological changes observed in rodents appear similar to those in cattle where they pose the main clinical problem. In humans neurological changes are more prominent, hence the disease name 'sleeping sickness'. When trypanosomes invade the brain astrocytes assume an important role as antigen-presenting cells containing immune reactions to perivascular regions leading to vascular cuffing, the formation of a dense layer of lymphocytes, macrophages and plasma cells around blood vessels. Prostaglandin release by astrocytes may give rise to altered sleep patterns in advanced sleeping sickness patients. Intriguingly, the pathological symptoms of trypanosomiasis disappear rapidly after elimination of the parasite by drug treatment.

See also: Antigenic variation; Chagas' disease; Parasites, immunity to.

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TUMOR ANTIGENS

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The notion that tumor cells express target structures that can be recognized by the immune system was put forward a long time ago, but only recently was the molecular nature of tumor antigens rigorously defined, thanks to the tools of modern immunology combined with molecular biology.

Tumor antigens recognized by T cells

The study of animal models has clearly demonstrated that the immune system has the capacity to reject tumor cells and that T lymphocytes are instrumental in this rejection. In particular, the essential role of cytotoxic T lymphocytes (CTLs) was highlighted by adoptive transfer studies and by the analysis of tumor variants escaping rejection, which were found to have lost CTL epitopes. A number of mouse tumors were found to express one or several antigens recognized by CTLs derived from lymphocytes of mice having rejected these tumors. In humans, anti-tumor CTLs were obtained *in vitro* from the lymphocytes of cancer patients, and a number of antimelanoma CTL clones have been analyzed in detail. The study of melanoma variants that were resistant to some but not all of these CTL clones revealed that melanoma cells express several distinct antigens. The molecular definition of these antigens has afforded crucial insights into both the specificity of these tumor antigens and the mechanism of their expression by tumor cells.

The experimental approach that allowed the identification of most tumor antigens is a genetic approach aimed at cloning the gene that encodes the antigen. This is done by transfecting recombinant libraries into cells that are subsequently screened with the CTL for their expression of the antigen. As observed for viral CTL epitopes, most tumor antigens identified to date do indeed correspond to peptides derived from intracellular proteins and presented at the cell surface by major histocompatibility complex (MHC) class I molecules. Based on the pattern of expression of the parent protein, the antigens can be classified into four major groups: tumor-specific shared antigens, differentiation antigens, antigens resulting from mutations and viral antigens.

Tumor-specific shared antigens

These antigens are encoded by genes that are completely silent in most normal tissues but are activated in a number of tumors of various histological types. Prototype antigens of this group are those encoded by gene P1A in the mouse and by genes MAGE in humans. Additional human genes with a similar expression profile have been identified subsequently and are listed in **Table 1**. Antigenic peptides derived from the proteins encoded by these genes were defined and found to be presented to CTLs by specific MHC class I molecules (**Table 1**). With the exception of RAGE, the only normal cells where

Table 1 Tumor-specific antigens shared by different tumors

Gene	Normal expression	MHC	Peptide	Position
<i>Mouse</i>				
P1A	Testis	H-2 L ^d	LPYLGWLVF	35–43
<i>Human</i>				
MAGE-1	Testis	HLA-A1	EADPTGHSY	161–169
		HLA-Cw16	SAYGEPRKL	230–238
MAGE-3	Testis	HLA-A1	EVDPIGHLY	168–176
		HLA-A2	FLWGPRLV	271–279
		HLA-B44	MEVDPIGHLY	167–176
BAGE	Testis	HLA-Cw16	AARAVFLAL	2–10
GAGE-1/2	Testis	HLA-Cw6	YRPRPRRY	9–16
RAGE-1	Retina	HLA-B7	SPSSNRIRNT	11–20
GnTV ^a	None	HLA-A2	VLPDVFIIRC	38–64

^aAberrant transcript of *N*-acetylglucosaminyl transferase V (GnTV) that is found only in melanomas (50%).

significant expression of these genes was detected are testicular germ cells and placental trophoblasts. Since these cells do not express MHC Class I molecules, they do not present the peptide at their surface. Therefore such antigens can be considered as strictly tumor specific. The activation of the genes usually results from a demethylation of their promoter that correlates with a nonspecific genome-wide demethylation. Expression was detected in substantial fractions of human melanomas, head and neck, lung, bladder and breast carcinomas (Table 2). Because they are shared by tumors expressing the genes and bearing the appropriate MHC type, these antigens represent promising targets for cancer immunotherapy. About 60% of melanomas, 40% of head and neck tumors, 34% of non-small cell lung carcinomas and 28% of bladder tumors express at least one of the antigens identified to date.

Some CTLs-directed against breast, ovarian and pancreatic carcinomas recognize an epitope of mucin, a surface protein composed of multiple tandem repeats of 20 amino acids. Whereas in normal cells mucin is heavily glycosylated, in these tumors the peptide repeats are unmasked by underglycosylation, resulting in CTL recognition. Remarkably, this recognition, which depends on the presence of multiple repeats, occurs in the absence of HLA restriction. Mucin underglycosylation also occurs in breast duct epithelial cells during lactation, but only at the extracellular apical surface which normally is not accessible to T cells. These mucin antigens therefore appear to be very specific for tumor cells, and the lack of HLA restriction should facilitate therapeutic vaccination trials.

Lymphocytes infiltrating some HLA-A2⁺ ovarian carcinomas were found to recognize peptides derived from HER-2/neu, an oncogene expressed in normal

tissues at low levels and overexpressed in 30% of breast and ovarian carcinomas.

Differentiation antigens

A large number of CTLs directed against human melanoma were found to recognize not only a majority of HLA-A2⁺ melanomas but also HLA-A2⁺ normal melanocytes. The notion that such CTLs recognize melanocyte differentiation antigens was confirmed when the antigens were identified at the molecular level. Five genes encoding melanocyte differentiation antigens have been identified (Table 3). Most antigenic peptides are presented by HLA-A2, but other HLA-peptide combinations have been found. Two tyrosinase peptides are presented by HLA-DR4 to CD4 T cells. The gp75 peptide presented by HLA-A31 is peculiar in that it is translated from an open reading frame that is different from the one encoding the gp75 protein. Tyrosinase peptide 369–377 presents an interesting post-translational modification that was observed when the naturally occurring peptide eluted from an HLA-A2⁺ melanoma was analyzed (Table 3). The asparagine residue in position 3 is transformed into an aspartic acid residue, presumably because the asparagine was glycosylated and subsequently deglycosylated by an enzyme which removed the amino group with the glycan.

The role of CTLs against such antigens in melanoma rejection is not clear, but is supported by the reported association of vitiligo with prolonged survival and spontaneous regression of melanoma. Because melanocytes are also present in the choroid layer of retina, the potential ophthalmic toxicity of active immunotherapy targeted at these antigens needs to be evaluated.

Similar concerns about autoimmune side-effects

Table 2 Expression in tumor samples of genes encoding T cell antigens^a

Histological type	Percentage of tumors expressing:					
	MAGE-1	MAGE-3	BAGE	GAGE-1,2	RAGE-1	GnTV
Melanomas						48
Primary lesions	16	36	8	13	2	
Metastases	48	76	26	28	5	
Non-small cell lung carcinomas	49	47	4	19	0	0
Head and neck tumors	28	49	8	19	2	0
Bladder carcinomas	22	36	15	12	5	0
Sarcomas	14	24	6	25	14	0
Mammary carcinomas	18	11	10	9	1	0
Prostatic carcinomas	15	15	0	10	0	0
Colorectal carcinomas	2	17	0	0	0	0
Renal cell carcinomas	0	0	0	0	2	0
Leukemias and lymphomas	0	0	0	1	0	0

^aExpression was measured by RT-PCR on total RNA using primers specific for each gene.

Table 3 Differentiation antigens recognized by T cells on tumors

	Gene/protein	MHC	Peptide	Position
<i>Mouse melanoma</i>	TRP-2	H-2 K ^d	VYDFFVWL	181–188
<i>Human melanoma</i>	Tyrosinase	HLA-A2	MLLAVLYCL	1–9
		HLA-A2	YMNGTMSQV	369–377
			YMDGTMSQV ^a	
		HLA-A24	AFLPWHRLF	206–214
		HLA-B44	SEIWRDIDF	192–200
		HLA-DR4	QNILLSNAPLGQFP	56–70
			SYLQSDPDPSFQD	450–462
	Pmel17 ^{gp100}	HLA-A2	KTWGQYWQV	154–162
		HLA-A2	ITDQVPFSV	209–217
		HLA-A2	YLEPGPVTA ^a	280–288
		HLA-A2	LLDGTATLRL	457–466
		HLA-A2	VLRYGSFSV	476–485
	Melan-A ^{MART-1}	HLA-A2	(E)AAGIGILTV ^b	26–(7)-35
		HLA-A2	ILTVILGVL ^a	32–40
	gp75 ^{TRP-1}	HLA-A31	MSLQRQFLR	– ^c
	TRP-2	HLA-A31	LLGPGRPYR	197–205
<i>Other human tumors</i>	CEA	HLA-A2	YLSGANLNL	571–579

^aNatural peptide identified by elution from HLA-A2 molecules.^bDifferent CTLs preferentially recognize either the nonamer or the decamer.^cPeptide translated from an alternative open reading frame of the gp75 transcript.

apply to carcinoembryonic antigen (CEA), an oncofetal protein expressed in normal colon epithelium and in most gut carcinomas, that was recently shown to contain a peptide epitope recognized by T cells from immunized patients.

B cell lymphomas are monoclonal tumors derived from B cells. They express the idiotype corresponding to the antigen specificity of the parent B cell. Idiotypic immunoglobulins therefore represent a particular case of differentiation antigens that are unique to individual tumors. Such antigens, which need to be prepared individually for every patient, are being evaluated as vaccines for B cell lymphoma.

Antigens resulting from mutations

Antigens of the third category correspond to peptides derived from regions of ubiquitous proteins that are mutated in tumor cells (Table 4). The mutation either generates a new epitope in the peptide or enables its binding to the MHC molecule. In most cases the mutation is unique to an individual tumor, and therefore the antigen is not shared. Other mutations occur in several tumors that may then share the antigen. The mutations may affect genes involved in cell regulation, such as p53 and CDK4, and thereby play a role in carcinogenesis. The great majority of mouse tumor antigens identified to date result from mutations. This may be due to the fact that many mouse tumors were induced with carcino-

gens or radiation. However, antigens of this category were also found on spontaneous mouse tumors and on human tumors. Although their tumor specificity is optimal, the fact that these antigens are often individually unique prevents their use as components of generic cancer vaccines.

Viral antigens

Antigens derived from oncogenic viruses constitute another category of potentially useful tumor antigens. A number of viral antigens have been studied in detail on virally induced mouse tumors and shown to be relevant for tumor rejection. In humans, the best example is oncoprotein E7 of human papillomavirus 16 (HPV-16), which is present in most cervical carcinomas. Peptides derived from this protein and selected for their high binding affinity to HLA-A2 were used to stimulate CD8⁺ lymphocytes from normal individuals. CTL clones obtained against three of these peptides were found to recognize not only peptide-pulsed cells but also a cervical carcinoma line expressing HLA-A2 and HPV-16 E7, demonstrating that these peptides indeed correspond to genuine cervical carcinoma antigens.

Clinical perspectives

The next few years will see the evaluation of new clinical trials using some of these defined tumor antigens. Progress at three different levels is anticipated.

Table 4 Tumor antigens resulting from mutations

Gene/Protein	Tumor	MHC	Peptide ^a	Position
<i>Mouse</i>				
connexin-37	Lewis lung carcinoma 3LL (spontaneous)	H-2 K ^b	FEQ NTAQP	52–60
ribosomal protein L9	Skin tumor 6132A (UV-induced)	H-2 I-E ^k	LRRDFNHINVELSH LGKKKKRLRVD	34–58
gag IAP ^b	Leukemia LEC (spontaneous)	H-2 D ^k	RRKGKYTGL	88–96
gp70 env MuLV ^c	Colon carcinoma CT26 (carcinogen-induced)	H-2 L ^d	SPSYVYHQF	423–431
p53	Sarcoma MethA (carcinogen-induced)	H-2 K ^d	KYI CNSSCM	232–240
ras ^d	—	H-2 K ^b	L EEYSAM	61–67
ras ^d	—	H-2 K ^b	GK EEYSAM	60–67
ras ^d	—	H-2 I-A ^d	KLWVVGAV GVGKS	5–17
<i>Human</i>				
MUM-1	Melanoma	HLA-B44	EEKL/ VVLF	30–38
CDK4	Melanoma	HLA-A2	AC DPHSGHFV	23–32
β-catenin	Melanoma	HLA-A24	SYLDSGIH ^f	29–37
HLA-A2 ^e	Renal cell carcinoma	—	—	—
bcr-abl (b3a2)	Chronic myeloid leukemia	HLA-DR4	ATGFKQSSKALQRPVAS	—

^aThe residue modified by the mutation is italicized.

^bThe gag gene of an intracisternal A particle (IAP) is activated by transposition of the IAP.

^cThe peptide derives from the env gene product of MuLV that appears to be transcribed in several tumors.

^dPeptide selected from the ras sequence and used to stimulate CTLs *in vitro*.

^eThe mutation affects the HLA-A2 gene itself.

First, knowledge of the molecular nature of these antigens allows the selection of patients whose tumor actually expresses a given antigen. Secondly, it allows the rational design of highly specific vaccine preparations. These could consist of peptides mixed with appropriate adjuvants or used to pulse autologous antigen-presenting cells *ex vivo*. The availability of the genes encoding the antigens also allows the preparation of recombinant proteins that can be combined with adjuvants, the preparation of recombinant viral vectors that can be used for immunization, or the direct use of these genes in nucleic acid vaccination protocols. Thirdly, precise knowledge of the antigenic epitopes allows careful monitoring of the CTL response after vaccination. This should help to optimize the immunization procedure.

Since a number of tumors express several antigens, patients bearing these tumors could be immunized simultaneously against distinct defined antigens. In addition to being more effective at rejecting the tumor cells, this should reduce the emergence of escaping variants arising by loss of antigen expression, as it is unlikely that the same variant would simultaneously lose distinct nominal antigens.

Tumor antigens recognized by monoclonal antibodies

Considerable progress has been made in the identification of defined tumor-associated antigens recognized by monoclonal antibodies (mAbs). The process of identification and characterization of tumor anti-

gens recognized by mAbs involves careful serological analysis and rigorous immunohistochemistry screening of normal tissues and tumors. These methods differ from the genetic approach used to identify antigens recognized by CTLs, and the molecular nature of many of these antigens is therefore less well defined. Similarly, the function of many tumor-associated antigens (especially cell surface antigens in solid tumors) often remains poorly understood. The expression of antigens recognized by mAbs in tumors is often heterogeneous, and loss of expression may be observed in anaplastic transformation and as a result of immune escape. The classification of tumor antigens recognized by mAbs is generally performed on the basis of antigen structure, and the anatomical component that expresses the antigen within the tumor.

Differentiation antigens in hematologic malignancies

Leukemias and lymphomas are characterized by malignant cells that express immunophenotypes that are found in selected stages of hematopoietic differentiation. These antigens are usually associated with CD groupings, have a glycoprotein structure, but are not always strictly tumor specific or lineage specific. The ideal antigen target for mAb directed against leukemias or lymphomas has high expression in neoplastic cells, but not on normal cells or bone marrow progenitors. The antigens most successfully targeted by mAbs in clinical trials in leukemia and lymphoma are detailed in Table 5. The accessibility of leukemia

Table 5 Tumor-associated antigens recognized by monoclonal antibodies

<i>Categories</i>	<i>Examples of antigens</i>	<i>Tumor types commonly expressing the antigens</i>
Differentiation antigens	CD5 CD19, CD20, CD21, CD25, CD37 CD30 CD33, CD45 CAMPATH-1 (CDw52) HLA-DR	T cell leukemia/lymphoma B cell lymphoma Hodgkin's lymphoma Acute myelogenous leukemia Lymphoid malignancies (T and B cell) B cell lymphoma
Cell surface antigens		
Glycoproteins	CEA, TAG-72, Ep-CAM, MUC1 Folate-binding protein A33 G250 PSMA	Epithelial tumors (breast, colon, lung) Ovarian tumors Colorectal carcinoma Renal carcinoma Prostate carcinoma
Glycolipids	Gangliosides (e.g. GD2, GD3, GM2)	Neuroectodermal tumors
Carbohydrates	Le ^y CA-125	Epithelial tumors (breast, colon, lung) Ovarian carcinoma
Intracellular antigens	PSA Ferritin	Prostate carcinoma Hodgkin's disease, hepatoma
Growth factor receptors	EGFR p185 ^{HER2} IL-2 receptor	Lung, breast, head and neck tumors Breast, ovarian tumors T and B cell neoplasms
Mutated gene products	ΔEGFR	Glioblastoma multiforme, breast, lung cancer
Stromal/extracellular antigens	FAP α Tenascin Metalloproteinases	Epithelial cancers (breast, colon, lung) Glioblastoma multiforme Epithelial tumors
Endothelial/vascular antigens	Endosialin, VEGF, $\alpha_v\beta_3$	Tumor vasculature
Apoptosis	Apo-1	B cell neoplasms

and lymphoma cells to circulating mAbs provides distinct advantages in antigen targeting compared to solid tumors.

Cell surface antigens

Glycoproteins Glycoprotein antigens expressed on the cell surface of solid tumors are common targets for monoclonal antibodies. These antigens have variable expression patterns in normal tissues, ranging from extensive tissue distribution to restricted expression, and are predominantly found on tumors of epithelial origin (Table 5). The carcinoembryonic antigen (CEA), TAG-72 antigen, and the MUC1 gene product PEM (polymorphic epithelial mucin) have been the tumor antigens most studied in animal models and clinical trials with mAbs. All these antigens have variable expression in a broad range of epithelial tumors, and soluble forms of these antigens may be found in the serum of patients with these tumors. Both PEM and Ep-CAM (a 40 kDa glycoprotein targeted by the mAb 17-1A, and involved in cell adhesion) are expressed in epithelial tumors and a wide range of normal epithelial tissues. In most tumors expressing these antigens, downregulation of antigen in metastases compared to primary tumors has been reported. Glycoprotein antigens with distinct high tumor expression and restricted normal

tissue distribution include A33 and G250. The A33 antigen is a 43 kDa protein which has a restricted expression in human small and large intestine epithelial cells, and is expressed on over 95% of human colorectal carcinoma cells. Upon mAb binding to A33 antigen, the antibody-antigen complex is internalized and sequestered in vesicles. The unique structure of the A33 antigen has been shown to consist of a transmembrane glycoprotein with an extracellular domain structure related to the CD2 subgroup of the immunoglobulin superfamily. The function of this antigen is as yet undefined, but may be a novel cell surface receptor or involved in cell-cell adhesion. Another glycoprotein antigen with restricted normal tissue expression is G250, which is expressed in over 90% of clear cell renal carcinoma but not in normal kidney, and is also expressed in biliary epithelium. The precise structure of this antigen is undefined, but is thought to be related to carbonic anhydrase. mAbs directed against the A33 and G250 antigens have been studied in clinical trials, and have demonstrated excellent targeting characteristics indicating the suitability of these antigens for directed cancer therapy.

Glycolipids Gangliosides are glycolipids expressed in animal cells, and consist of a carbohydrate moiety

linked to a ceramide molecule which is anchored in the lipid bilayer of the cell surface membrane. Malignant transformation of cells may be associated with qualitative and quantitative changes in ganglioside expression on the malignant cell surface, which can be recognized immunologically. Ganglioside synthesis may be incomplete and result in precursor accumulation, e.g. GD2, GD3, GM2, GM3, which are found on tumors of neuroectodermal origin (melanoma, neuroblastoma, astrocytoma and small cell lung carcinoma). Modifications to the carbohydrate or ceramide portions may also be observed. The high expression of some gangliosides in tumor cell membranes may also lead to conformational changes of antigen epitopes which enhance monoclonal antibody binding. The function of gangliosides is unclear, but is thought to involve cell growth and differentiation.

Carbohydrates A range of carbohydrate determinants linked to either lipids or proteins have been identified by serological analysis of human cancer cells with mouse mAbs. Blood group-related antigens (e.g. Le^b and Le^y) have been the focus of much attention because of their high level of expression in epithelial tumors, although some expression in normal tissues is also present. The Le^y antigen is expressed in over 70% of epithelial cancers (including breast, colon, ovary and lung cancer), and is an attractive target for mAb-directed therapy. A number of mouse mAbs against Le^y antigen have been produced, although a consistent problem with these mAbs has been an element of cross-reactivity with Le^x and H-type 2 structures, and agglutination of human red blood cells. Meticulous serological analysis has been required to identify anti-Le^y mAb that have minimal or no cross-reactivity with other blood group-related antigens: a number have been studied in clinical trials and are the subject of ongoing research.

Growth factor receptors

Growth factors may be overexpressed in tumors, and the growth or survival of some tumors may partially depend on autocrine or paracrine production of epidermal growth factor (EGF) or transforming growth factor α (TGF α). These two factors share a common receptor, the EGF receptor, which is expressed in normal tissue (particularly the liver), but has increased expression in certain tumors (e.g. lung and breast). mAbs against the EGF receptor have been shown in *in vivo* models to inhibit growth of breast cancer xenografts, and clinical trials with anti-EGF receptor mAbs have also demonstrated specific targeting. A truncated mutant EGF receptor (δ EGFR) that contains a deletion of 267 amino acids from the

extracellular domain, and does not bind EGF, has also been identified in human gliomas. Expression of δ EGFR confers enhanced tumorigenicity to glioblastoma cells, and is associated with increased proliferation and reduced apoptosis. mAbs against the δ EGFR have been generated, and may represent an important new strategy for glioma therapy. An additional important receptor is the transmembrane glycoprotein receptor p185^{HER2} (encoded by the HER-2/neu oncogene). p185^{HER2} has partial homology with the EGF receptor, is overexpressed in 25–30% of breast cancers, and is associated with a worse prognosis in patients with primary disease involving axillary lymph nodes. mAbs directed against p185^{HER2} have been shown to inhibit the growth of tumors in animal models, and of transformed cells expressing high amounts of this receptor. Objective responses in patients with metastatic breast carcinoma overexpressing p185^{HER2} with mAbs directed against the extracellular domain have been reported, indicating the potential therapeutic use of growth factor-targeted therapy.

Stromal antigens

The human fibroblast activation protein α (FAP α) is a cell surface antigen expressed in reactive stromal fibroblasts of a number of epithelial cancers including colon, breast and lung. FAP α is absent or expressed in low levels in most adult tissues, but is seen in wound healing and transiently in some fetal mesenchymal tissues. FAP α has structural homology to CD26, but appears to be functionally distinct, and expression of FAP α can be induced *in vitro* on cultured fibroblasts by various growth factors (e.g. TGF β). The successful targeting of mAbs against FAP α expressed in tumor stroma has been demonstrated in clinical trials of patients with colorectal carcinoma. Another potential stromal antigen target is tenascin, an extracellular matrix glycoprotein present in developing tissue during the fetal period, and expressed in the stroma of many adult tumors, particularly glioblastoma multiforme.

Angiogenesis and apoptosis

Serological studies have identified a number of antigens expressed selectively on neovascular endothelium of tumors, including endosialin, vascular endothelial growth factor receptor (VEGFR), and the integrin $\alpha_v\beta_3$. Because of the restricted expression of these antigens in tumor vasculature, the potential anti-tumor effect of inhibitors of these antigens has been the subject of intense interest as a means of killing tumor cells without requiring specific tumor cell targeting. The role of apoptosis in mediating tumor

cell death is also being explored with mAbs against a variety of antigens including Fas (CD95, Apo-1).

Clinical perspectives

The clinical applications of tumor-associated antigens recognised by mAbs include both diagnosis and therapy. Diagnostic uses include serum detection of soluble antigen as a marker of disease activity (e.g. CEA, CA-125, PSA (prostate-specific antigen)), immunohistochemistry characterization of biopsy specimens for pathologic diagnosis, and the use of radiolabeled mAbs for tumor detection *in vivo* (e.g. anti-CEA, anti-TAG-72, anti-PSMA (prostate-specific membrane antigen) mAbs). Perhaps the most promising area of clinical development, however, is in mAb-targeted therapy. Based on the experience of clinical trials to date, the ideal therapeutic antigen target has a homogeneous expression in tumor, minimal expression in normal tissues, no soluble form of antigen to impede tumor binding, and easy accessibility to mAbs. Tumor antigen screening, and an understanding of the internalization characteristics and function of targeted antigen, will allow the optimal selection of mAbs for targeting. The range of tumor antigens recognized by mAbs and the expanding therapeutic strategies that can be employed will form the basis of continuing clinical research.

See also: Antigen presentation via MHC class I molecules; Antigen presentation via MHC class II molecules; Cytotoxic T lymphocytes; Embryonic antigens; Immune surveillance; Immunotherapy of tumors; Tumors, immune response to.

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TUMOR IMAGING

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Tumor imaging is a clinical and experimental medical technology designed to localize neoplastic lesions in a body using a labeled antibody or its fragment. The idea of localizing antitumor antibodies into human cancer originated in the 1890s with attempts to treat malignant diseases using antisera raised against human tumors in animals. These heterologous polyclonal antisera contained, by their nature, overwhelming amounts of antibodies reacting with species-specific tissue antigens, rather than those, if any, reacting with tumor-specific antigens. Thus, researchers in the earlier days tried to increase the specificity of their antitumor antisera by absorbing 'nonspecific reactivities' by incubation with normal human tissues. The problem of mixed specificity was overcome by the development of hybridoma technology that allowed continuous production of a homogeneous antibody reacting only with a single epitope. Production on a large scale, purification, enzymatic and genetic manipulation, and labeling of antibody molecules became easier after the introduction of hybridoma-derived monoclonal antibodies, and even further advances are currently taking place by the development of the phage display technique.

Tumor antigens

Tumor imaging depends on the presence of an antigen that is either exclusively expressed on the tumor or at least has an appreciable difference in its amount

and/or distribution between the tumor and normal tissues. Tumor antigens are usually separated into two categories: tumor-specific antigens and tumor-associated antigens. The former are expressed only in tumors and are never detectable in normal tissues. They are often unique to a single tumor and, thus, not shared by tumors of different individuals. Theoretically, an ideal tumor-specific antigen would be that associated with mutational change(s) of an oncogene or a tumor suppressor gene that caused the malignant transformation. Since peptides produced by enzymatic processing of intracellular proteins are displayed on major histocompatibility complex (MHC) class I molecules, T lymphocytes may recognize a mutated peptide associated with the oncogenesis. How professional antigen-presenting cells that are capable of activating naive T cells capture tumor-specific antigen peptides, which are produced only by the tumor cells but not by the antigen-presenting cells, is still unclear. In any case, most of these tumor-specific antigens are recognized by T cells, and it is generally difficult to imagine that antibodies reacting with an intracellular oncogene product accumulate in a small lesion filled with fully viable tumor cells.

On the other hand, tumor-associated antigens are expressed at an inappropriately high level in tumor cells, whereas the expression of the same antigen in normal tissues is very low or strongly restricted to a certain stage or site in tissue development. Many of

these tumor-associated antigens are detectable with heterologous and sometimes autologous antibodies. Notable examples are carbohydrate antigens including gangliosides and blood group antigens, carcino-embryonic antigen (CEA), α -fetoprotein, mucins, cell surface receptor-like molecules, and some hormones and enzymes. Among them, antibodies reacting with CEA, carbohydrate antigens and mucins have been most extensively studied for use in tumor imaging.

CEA is a cell surface glycoprotein of 180 kDa, and its expression is most frequently associated with adenocarcinomas of colon, breast and lung. CA19-9 or sialyl Le^a is a typical tumor-associated carbohydrate antigen. This modified blood group antigen is highly expressed on most gastrointestinal carcinomas including adenocarcinomas of pancreas, colon and stomach. Mucins are extremely high molecular weight glycoproteins that are abundantly expressed on the surface of breast, colon, pancreas, ovary and lung adenocarcinomas. Since the presence of mucins in normal tissues is confined to the apical surface of the ductal epithelial cells and in the ductal lumen, they are usually sequestered from the immune system. The unipolar and excessive nature of mucin production in tumor cells leads to the production of antibodies. Many monoclonal antibodies reacting with human tumor-associated mucins are generated by immunizing mice with tumor tissues. Human chorionic gonadotropin (hCG) secreted by choriocarcinoma was the first tumor antigen used for targeting.

The antibody and its fragments

The immunoglobulin (Ig) G molecule has a relatively large molecular weight of 150 kDa. Although IgG distributes throughout the body, its penetration into a tumor mass is slow, as demonstrated by autoradiography of tumor xenografts after intravenous injection of ¹²⁵I-labeled antitumor IgG. The number of grains in areas on autoradiography sections that are more than 30 μ m distant from vessel margins

were shown to be increasing even after 72 h postinjection in one study. The whole IgG can also bind to tissue macrophages and circulating leukocytes through Fc receptors. Since immunoglobulins are highly immunogenic in different species, injection of mouse antibodies raised against human tumors into humans results in the production of human anti-mouse Ig antibodies (HAMA). These antibodies accelerate the clearance of injected antitumor antibodies from the blood, and may cause a potentially fatal anaphylactic reaction and/or serum sickness. Replacement of mouse Ig constant domains with human counterparts by ligation of rearranged mouse Ig variable region with human Ig constant region genes results in the production of chimeric antibodies, which have much reduced immunogenicity.

Since Ig effector functions associated with the heavy chain constant region are not necessary for binding and accumulation of antitumor antibodies to neoplastic lesions, the use of smaller fragments of Ig that retain only the antigen-binding activity seems to be a reasonable way to reduce the immunogenicity and molecular mass of antitumor antibodies.

Enzymatic digestion of IgG with pepsin yields a $F(ab')_2$ fragment, which is still divalent because the two antigen-binding portions are linked by disulfide bonds at the remaining hinge regions (Figure 1). Reduction of the disulfide bonds and the blocking of them with iodoacetamide generates the monovalent fragment called Fab', composed of a single light chain and an N-terminal half of a heavy chain. Another proteolytic enzyme, papain, cuts IgG between the antigen-binding portion and the hinge, resulting in the generation of a slightly different monovalent fragment called Fab. Divalent $F(ab')_2$ usually retains the antigen-binding properties of the intact IgG, while binding affinities of monovalent Fab and Fab' are often much lower than those of the divalent counterparts. In a quantitative analysis, the affinity constant of Fab' was calculated to be one-seventh of those of $F(ab')_2$ and whole IgG. Plasma

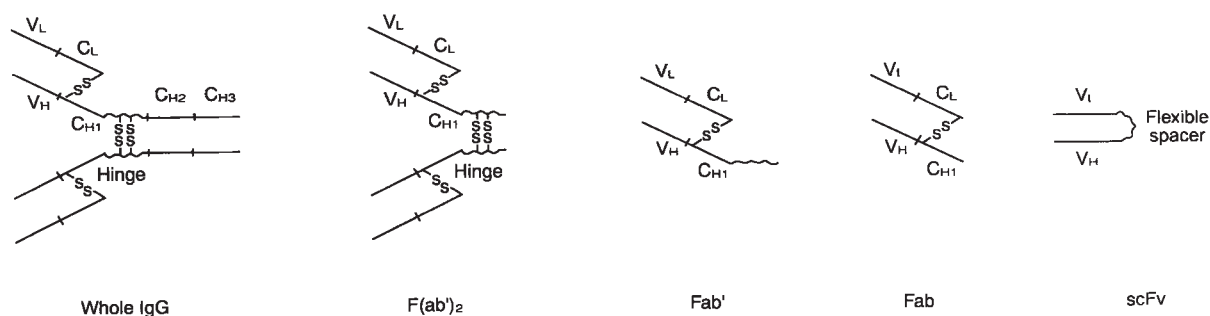


Figure 1 The structure of IgG and its fragments.

clearance of Fab and Fab' is also much faster than that of F(ab')₂ and whole IgG, leaving limited time for accumulation into the tumor. However, rapid clearance from the blood may conversely improve the contrast between the tumor and normal tissues. Because of the reduced binding affinity and rapid clearance, *in vivo* distribution of monomeric Fab is different from that of dimeric F(ab')₂ and whole IgG. In general, a higher percentage of injected dose per gram of tissue is achieved with IgG but at a much later point after injection than with Fab'. On the other hand, because of the smaller molecular mass of 50 kDa, Fab' penetrates into tumor mass much more rapidly and evenly than F(ab')₂ and whole IgG. Thus, tumor to normal tissue ratios of radioactivities are usually higher when a Fab or Fab' fragment is used as a radiolabeled antitumor targeting agent.

Single-chain Fv and phage display libraries

If monomeric Fab and Fab' fragments penetrate better into the tumor mass than their divalent counterparts, an even smaller fragment of Ig composed only of a single antigen-binding portion and devoid of constant domains may penetrate into the tissue even more rapidly. In addition, complete elimination of the constant regions may further reduce the immunogenicity. Thus, single-chain Fv (scFv) fragments composed of a combination of heavy and light chain variable regions linked with a flexible, synthetic spacer have been constructed by genetic engineering. The resultant scFv fragments have a molecular weight of 27 kDa, and penetrate into the tumor tissue much more rapidly, even in comparison with Fab'. One advantage of scFv technology is that this antigen-binding molecule can be expressed on the surface of filamentous bacteriophages that carry the specificity-encoding V_L and V_H genes. Thus, phages carrying and expressing a combination of high-affinity V_L and V_H genes are directly selected through binding assays. Since a large combinatorial library of V_L and V_H genes can be constructed from B lymphocytes derived from immunized mice, the step of making and selecting hybridoma cells can be skipped, thus

minimizing the time from immunization to isolation of a high-affinity antibody.

As mentioned above, the binding affinity of monovalent Fab' is usually weaker than that of whole IgG. If an scFv molecule is constructed by using a combination of V_L and V_H genes isolated from a hybridoma clone, its binding affinity cannot surpass that of the Fab' counterpart. However, because of the extremely large capacity of a phage display library, it is possible to access all the antibody specificities generated in an immunized animal. In contrast, it is practically impossible to screen more than 10³ clones of different specificities at one time by hybridoma technique. Thus, screening of a large phage display library can potentially result in clones of scFv with unusually high affinity, which is generally not registered by hybridoma technology. Such extreme high-affinity scFv may overcome the disadvantage of the monovalent nature of this fragment. Seemingly promising clinical applications of scFv have been reported.

The radionuclide and imaging technology

A variety of gamma-emitting radionuclides can be used to label antibodies and their fragments. Frequently used radionuclides are shown in Table 1 with their physical half-lives and γ energy. Because penetration and distribution equilibrium take 2–4 days when the whole IgG molecule is used as a labeled antibody, ¹³¹I with a long physical half-life was used in earlier studies. When separated from the antibody, a fraction of this radionuclide is taken up and retained in the thyroid. ¹²³I and ¹¹¹In are more efficient for γ -camera imaging than ¹³¹I, and have been used more frequently with recent advancements of Ig fragment preparation. ^{99m}Tc is most efficiently imaged by γ -camera, but its physical half life is very short. However, with rapid penetration of Fab' and scFv into tumors and improved methods of conjugation between ^{99m}Tc and proteins, this radionuclide has become more attractive for clinical use, especially because it is economical.

The technique of imaging neoplastic lesions *in vivo*

Table 1 Radionuclides used for immunoscintigraphy

Radionuclide	T _{1/2} (h)	Decay	γ energy (keV)	Sources
^{99m} Tc	6.0	Isomeric transitions	141 (89%)	X-ray generator
¹²³ I	13.2	Electron capture	159 (83%)	Cyclotron
¹³¹ I	193.0	β decay	364 (81%)	Nuclear reactor
¹¹¹ In	67.9	Isomeric transition	171 (90%), 245 (94%)	Cyclotron

after administration of a radiolabeled antitumor antibody is known as immunoscintigraphy. The distribution of radiolabeled antibody molecules in the patient's body is mapped using a γ -camera. Experimentally, this technique is often simulated by using human tumor xenografts in athymic mice. The statistical validity of γ -camera imaging depends on the level of radioactivity accumulated in the tumour, the time that a patient can remain still on the γ -camera couch during count acquisition, the physical decay properties of the radionuclide used, and the efficiency of detection. Thus, there are a lot of limitations especially in the clinical application of radioscinigraphy. To produce three-dimensional data, single photon emission computerized tomography (SPECT) has been developed, and is being more frequently reported in the literature. This method is, however, technically demanding. Hand-held γ -detecting probes are used for the detection of subcutaneous lesions including breast carcinomas and their lymph node metastases. They can also be utilized to define the extent of tumor infiltration and metastases during surgical operation (radioimmunoguided surgery).

Micromagnetic beads might be another possibility for labeling antibodies for tumor imaging. Immunomagnetic beads have been successfully utilized for depletion and enrichment of particular lymphocyte subpopulations and hematopoietic stem cells. In experimental models accumulation in carcinoma xenografts of antitumor antibodies labeled with micromagnetic beads was detected by magnetic resonance imaging. Although currently available detectors for magnetically labeled antibodies are unwieldy, if a small enough detector is developed, this method of labeling might be applicable to 'ferroimmunoguided surgery' in the near future.

Uses of antibody imaging

In experimental animals carrying human cancer xenografts tumor imaging has been successful with many tumor types. Clinical application has also been reported, but immunoscintigraphy is still not widely accepted as a prime method of cancer diagnosis. There are several reasons for this limited use of antibody-mediated tumor imaging. First, in many cases less than a few per cent of the injected dose localizes in the tumor, giving unsatisfactory and sometimes invalid data for γ -camera imaging. Second, repeated injection of labeled antibodies can induce the production of human anti-mouse Ig antibodies. This problem can be overcome by the use of humanized monoclonal antibodies or scFv. Third, other techniques used for morphological localization of tumorous lesions, conventional and enhanced CT scanning,

magnetic resonance imaging and ultrasonography, have become widely available, and they often give much better image quality and resolution than immunoscintigraphy. SPECT performed using rapidly penetrating scFv may give images with acceptable resolution, but spatial relationships between labeled lesions and normal organ structures are only seen in combination with conventional CT. Antibody imaging can sometimes surpass other imaging technology for localization of tumors in certain anatomical sites, such as the peritoneum, where other methods perform less effectively.

Thus, the use of immunoscintigraphy for initial diagnosis of tumors is limited to specific regions, such as ocular melanoma, in which biopsies are not possible. Immunoscintigraphy can also be useful for staging of cancer. In particular, immunoscintigraphy may be superior to ^{67}Ga scanning for the staging of malignant lymphoma. Useful application of immunoscintigraphy has been found in the detection and localization of recurrence in colon cancer, choriocarcinoma, germ-cell tumors, and thyroid carcinoma. Antibodies to CEA and CA19-9 are frequently reported to be used for radioimaging of metastatic and recurrent lesions. Hand-held γ -probes in combination with radiolabeled antibodies have also

been used for the staging of breast carcinoma.

Radioimmunoguided surgery performed using a hand-held γ -detector can be effective to determine the extent of tumor infiltration and to detect lymph node metastases during surgery. In some cases lymph node metastases were reported to be detectable with radiolabeled anti-CEA antibody in patients whose serum CEA was below the detectable level. However, there are always false negatives and positives when lymph nodes are histologically examined after radioimmunoguided surgery. Further improvements in labeling and imaging techniques are required for the clinical use of radiolabeled antibody methods.

Antibody targeted therapy

Antibody-radionuclide conjugates themselves may be used for the therapy of human cancers, as are antibody-toxin complexes. Immunoscintigraphy may also be useful to identify patients for therapy with an immunotoxin because efficient imaging with a radiolabeled antibody must correlate with efficient accumulation of the immunotoxin that shares the same specificity.

See also: Domains, immunoglobulin-type; Embryonic antigens; Monoclonal antibodies (mAbs); Phage display of antibodies; Targeting of immunological agents; Tumor antigens.

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TUMOR NECROSIS FACTOR α

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The remission of tumors after bacterial infections was first described in 1866. In 1893 Coley described tumor necrosis after treatment of patients with bacterial toxins from *Streptococcus* and *Serratia*, which were later named 'Coley's toxin'. Over 100 years after the first clinical observation, tumor necrosis factor α (TNF α) was described in 1975 by Carswell and colleagues. They reported on a soluble factor in the serum of endotoxin-treated bacillus Calmette-Guérin (BCG)-infected mice, which causes hemorrhagic necrosis of tumors.

TNF α has had a number of synonyms (Table 1). It is induced by all types of microorganisms and a number of their products (Table 2). The expression of TNF α has naturally counteracting cytokines (Table 2). Nearly all cell types are able to produce TNF α (Table 3) and respond to TNF α treatment

(Table 4). Over all the biological effects, the cytotoxic effects of TNF α and its induction of apoptosis seem to be the most important.

Table 2 Inducers and suppressors of TNF α production

Inducer	Suppressor
Allogenic cells	Cyclosporine
Bacteria	Glucocorticoids (i.e. dexamethasone)
Bacterial cell walls	
Complement	Interleukin 4
Cytokines (i.e. IL-1, GM-CSF)	Interleukin 10
Immune complexes	Interleukin 13
Lipid A	Lipo-oxygenase inhibitors
Lipopolysaccharide (LPS)	Metalloproteinase inhibitors
Lipoteichoic acid	(only for soluble TNF)
Mycoplasma membrane	Phosphodiesterase inhibitors
Mycoplasma components	Transforming growth factor β
Parasites	
Peptidoglycan	
Phorbol esters	
Reactive oxygen species	
Superantigens	
Tumor cells	
Viruses	
Yeast	
Zinc ions	
Zymosan	

Table 1 Synonyms for TNF α

Cachectin
Cytotoxin (CTX)
Differentiation-inducing factor (DIF)
Endogenous pyrogen (EP) (also synonym for interleukin 1)
Hemorrhagic factor
Macrophage cytotoxic factor (MCF)
Macrophage cytotoxin (MCT)
Necrosin
Tumor necrosis factor (TNF)

Table 3 TNF α -producing cells

Adipocytes
Antigen-presenting cells
Astrocytes
B cells
Breast cells
Epidermal cells
Fibroblasts
Glial tumor cells
Granulocytes
Macrophages
Mast cells
Monocytes
Neutrophils ?
NK cells
Smooth muscle cells
Synoviocytes
T cells

Table 4 Biological effects of TNF α

Antiviral activity
Chemotactic
Decreased lipoprotein lipase synthesis
Differentiation of monocytes
Enhanced antibody-dependent cellular cytotoxicity
Increased expression of adhesion molecules
Increased MHC class I and class II expression
Increased phagocytotic activity
Increased proliferation of fibroblasts
Induced adhesion to endothelium
Induced degranulation
Induced fever
Induced hypotension
Induction of apoptosis
Induction of granulocyte/macrophage-colony stimulating factor
Induction of interleukin 1 and 6
Induction of procoagulant activity
Induction of reactive oxygen species
Influence on hematopoiesis of myeloid cells
Inhibition of intracellular parasites and bacteria
Regulation of acute phase proteins
Regulation of extracellular matrix formation
Regulation of proliferation of T and B cells
Regulation of T and B cell functions

The TNF α gene

The TNF α gene (Figure 1, about 3.6 kbp) is separated from that of lymphotoxin α (LT α , alternatively known as TNF β) by 1.2 kbp within the major histocompatibility complex (MHC) locus on chromosome 6 between 6p21.1 and 6p21.3. The TNF α locus contains six microsatellites. Within the LT α (TNF β)

gene there are three TNF-related polymorphisms: a NcoI RFLP polymorphism, named TNFB*1 (rare allele with less TNF- α production) in the presence of the restriction site and TNFB*2 in its absence in the first intron, an EcoRI restriction site as the rare allele in the 5'-untranslated region (5'-UTR) and an AspHI restriction site as the rare allele in the intron of the 3'-UTR. In the TNF α promoter there are four G/A exchanges in positions -376, -308 (called TNF1 and TNF2), -238 and -163. TNF2 is the less frequent allele and seems to be associated with higher TNF α production. In the 5'-UTR of TNF α there is a cytosine-insertion polymorphism at position +70, resulting in an eight cytosine stretch in contrast to the normal seven cytosine stretch.

The promoter region contains the following binding sites: κ B enhancer 2/CK-1, κ B enhancer 3, TNF repressor site (TRS) showing a 10 bp sequence homologous to the AP-2 binding site, Y-box, an Ets binding element in direct juxtaposition to an AP-1/ATF-like palindromic position, NFAT/ κ 3 binding site of NF κ B, SP-1 and a TATA-box. TNF- α induction is mostly regulated by the κ 3 binding site of NF κ B. This κ 3 binding site also binds NFAT, resulting in a cyclosporine-sensitive TNF α induction. The basal 95 bp upstream of the transcription start are necessary for upregulation of TNF α expression by granulocyte-macrophage colony-stimulating factor (GM-CSF) and downregulation by interleukin 4 (IL-4). The TRS is regulated by TRS-binding proteins of 30–60 kDa.

TNF α transcription results in a primary transcript of 1.7 kbp. Regulation on the translational level predominantly occurs by controlling mRNA turnover by an UA-rich sequence in the 3'-UTR. The mRNA may be stabilized by a 38 kDa protein binding to the 3'-UTR and inhibiting translation. Furthermore, lipopolysaccharide (LPS)-stimulation downregulates this 38 kDa protein and independently enhances the initiation step of translation and formation of TNF α mRNA ribosome complexes. This or similar factors may be the reason for discrepancies observed in TNF α mRNA expression and protein release. It is not known how the elements for translational control are regulated, but endogenously increased nitric oxide (NO), cAMP or prostaglandins levels decrease TNF α production.

Biochemistry of TNF α

The mature human TNF α is a 157 amino acid protein of 17356 Da containing one disulfide-bond (Cys69–Cys101). It has an isoelectric point of pH 5.8. The highly proteolysis-sensitive protein is processed from a 26 kDa (233 amino acid) precursor

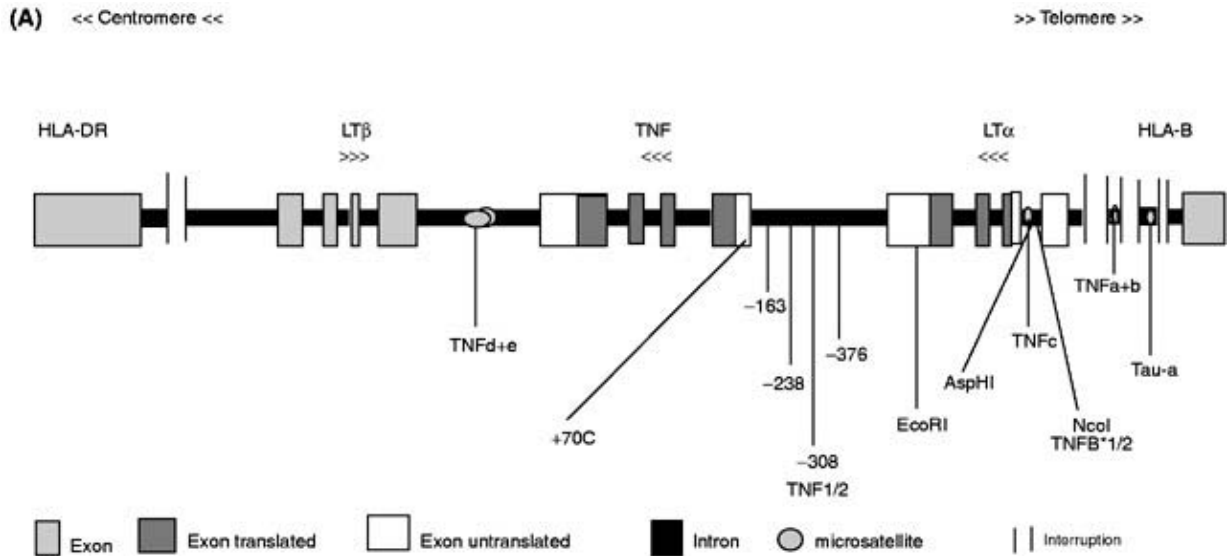


Figure 1 (A) One part of the MHC locus on chromosome 6, including the TNF gene complex. All TNF polymorphisms are included in the gene map: the (CT) n sequence microsatellites TNFb (7 alleles), TNFc (2 alleles), TNFd (7 alleles) and TNFe (3 alleles), the (CA) n sequence microsatellite TNFa (12 alleles), the microsatellite Tau-a (11 or 14 GTs), the restriction sites for EcoRI, AspHI and NcoI (TNFB*1/2), the single G/A exchanges at positions -163, -238, -308 (TNF1/2) and -376, and the C stretch (7 or 8 Cs) at +70.

(membrane-bound TNF α , a type II transmembrane protein) and shows no glycosylation. The leader sequence entails a linking domain (amino acids -1 to -20, unnecessary for TNF α function), a hydrophobic transmembrane domain (-21 to -46) and a cytoplasmic domain (-47 to -76). There is an alternative cleavage site at position 11.

Murine TNF α has only 156 amino acids derived from a 235 amino acid precursor and is glycosylated. Despite these differences, protein sequences from porcine, bovine, goat, feline, rabbit, rat, murine and human TNF α show homologies between 70 and 90%. TNF α shows no species specificity but some species preferences in cross-reactivity.

Natural TNF α is a trimeric molecule with an antiparallel β -sheet sandwich and an edge-to-edge packing structural motif. Transmembrane TNF α is cleaved by metalloproteases, neutral serine protease 3 (PR-3) and matrix metalloproteases. Metalloproteinase inhibitors are effective in the treatment of rat endotoxemia and autoimmune inflammatory diseases by blocking both processing of membrane-bound TNF- α and shedding of the 75 kDa TNF receptor. Membrane-bound TNF α seems to be the natural and effective ligand for the 75 kDa receptor.

TNF α develops an intermediate dimer after cleavage from the membrane before forming the natural trimer. The dimer accumulates first and trimerization is finished within 6 minutes. The existence of a TNF α

dimer may also be the natural correlate to TNF receptor dimerization.

The role of TNF α in development

TNF α is expressed during embryonic development. Some of the TNF α functions are important in normal embryogenesis, such as apoptosis. Furthermore, TNF α is important as a growth and differentiation factor, comparable to its function in hematopoiesis. In addition, TNF α plays a critical role in remodeling extracellular matrix and in the induction of cell adhesion molecules and integrins. TNF α promotes liver regeneration by promoting hepatocyte-specific gene expression. Despite this, TNF α is known to enhance ubiquitin.

The involvement of TNF α in development starts before embryogenesis. TNF α induces ovulation and enhances luteinizing hormone (LH)-induced ovulation. Soluble TNFR is increased in the blood of pregnant women and also in amniotic fluid and umbilical serum. However, not only does TNF α have an effect on reproduction but also sexual hormones have an effect on TNF α production. Estradiol and progesterone enhance TNF α release by macrophages, whereas testosterone has no effect. This may be one possible reason for the higher incidence of autoimmune diseases in women.

(B)

-618	TGT GAA TTC CGG GTG	ATT TCA CTC CCG GCT	GTC CAG GTC TGT CCT	-574
	κ B enhancer 2/CK-1			
-573	GCT ACC CCA CCC AGC	CTT TCC TGA GGC CTC	AAG CCT GCC ACC AAG	-529
-528	CCC CCA GCT CCT TCT	CCC CGC AGG ACC CAA	ACA GAG GCC TCA GGA	-484
-483	GTC AAC ACA GTC TTT	CCC TCC AAC CCG TTT	TCT CTC CCT CAA CGG	-439
-438	ACT CAG CTT TCT GAA	GCC CCT CCC AGT TCT	AGT TCT ATC TTT TTC	-394
-393	CTG CAT CCT GTC TGG	AAATA GAA GGA AAC	AGA CCA CAG ACC TGG	-349
	κ B enhancer 3			
-348	TCC CCA AAA GAA ATG	GAG GCA ATA GGT TTT	GAG GGG CAT GGA GAC	-304
-303	GGG GTT CAG CCT CCA	GGG TCC TAC ACA CAA	ATC AGT CAG TGG CCC	-259
-258	AGA AGA CCC CCC TCG	GAA TCAG GAG CAG GGA	GGA TGG GGA GTG TGA	-214
	TNF repressor site (TRS)			
-213	GGG GTA TCC TTG ATG	CTT GTG TGT CCC CAA	CTT TCC AAA TCC CCG	-169
-168	CCC CCAG CAG TGG AGA	AGA AAC CGA GAC AGA	AGG TGC AGG GCC CAC	-124
	Y-Box		Ets site	
-123	TAC CGC TTC CTC CAG	ATG AGC TCA TGG GTT	TCT CCA CCA AGG AAG	-79
	Ets site CK-1	AP-1/ATF	NFAT κ 3	
-78	TTT TCC GCT GGT TGA	ATG ATT CTT TCC CCG	CCC TCC TCT CGC CCC	-34
		SP-1		
-33	AGG GAC ATA TAA AGG	CAG TTG TTG GCA CAC	CCA $\downarrow$ GCC AGC AGA CGC	+12
	TATA-Box		transcription start	
+13	TCC CTC AGC AAG GAC	AGC AGA GGA CCA GCT	AAG AGG GAG AGA AGC	+57
-58	AAC TAC AGA CCC CCC ₇₀	CTG AAA ACA ACC CTC	AGA CGC CAC ATC CCC	+102
	-70C7(8)			
+103	TGA CAA GCT GCC AGG	CAG GTT CTC TTC CTC	TCA CAT ACT GAC CCA	+147
+148	CGG CTT CAC CCT CTC	TCC CCT GGA AAG GAC	ACC $\downarrow$ ATG AGC ACT GAA	-192
			translation start	

Figure 1 (B) The region from -618 to +192 of the TNF gene is shown in full sequence including the polymorphisms (G/A exchanges and C-stretch). The binding sites for transcription factors (bold letters) are underlined for each factor, overlapping binding sites for two factors are underlined twice. The promoter region contains the following binding sites: κ B enhancer 2/CK-1, κ B enhancer 3, TNF repressor site (TRS) showing a 10bp sequence homologous to the AP-2 binding site, Y-Box, an Ets binding element in direct juxtaposition to an AP-1/ATF-like palindromic position, NFAT/ κ 3 binding site of NF κ B, SP-1 and a TATA-box. TNF- α induction is mostly regulated by the κ 3 binding site of NF κ B. This κ 3 binding site also binds NFAT, resulting in a cyclosporin A sensitive TNF- α induction.

Autoimmune diseases

Rheumatoid arthritis (RA)

Elevated levels of TNF α are present in about 50% of samples of rheumatoid synovial fluid, particularly in those patients with more severe disease or with high white blood cell counts in synovial fluid. However, high levels of TNF α do not correspond to biological activity, because sTNFR is also enhanced in the fluids. *In vitro* it has been observed that antibodies to TNF α inhibit the spontaneous release of

IL-1 and GM-CSF in synovial cells. However, there is a strong synergism between TNF α and IL-1 in the induction of human articular cartilage degradation during prolonged culture.

In collagen-induced arthritis in mice and rats, the involvement of TNF α has been shown, in that application of rTNF α leads to an accelerated onset and a more severe course of the disease. Infusion of sTNFR or anti-TNF α reduces the frequency and severity of collagen-induced arthritis. TNF α serum levels in RA correlate positively with the number of activated T cells (CD3⁺/HLA-DR⁺ and CD3⁺/CD25⁻) and in acute rheumatic fever, the number of CD4⁺/CD25⁻ cells is enhanced. *Mycoplasma arthritidis*-derived superantigen (MAS), as a pathogenic factor of this arthritogenic organism, induced TNF α in human T cells and not in monocytes, corresponding to the detected activated T cells in RA.

Furthermore, IL-10 counteracts TNF α in RA, whereas TNF α counteracts TGF β to induce α 2(I) collagen gene transcription. This may contribute to the hypothesis that cartilage damage is a combination of destruction and ineffective rebuilding. It was shown that TNF α concentrations correlated with the extent of α ₁-antitrypsin in synovial fluid of RA patients.

In preclinical studies, anti-TNF α treatment of RA patients has a beneficial effect. This important role of TNF α in RA in combination with the defined HLA associations triggered the hypothesis of an association with some TNF α alleles. However, there are no associations between TNF β polymorphism and RA.

Insulin-dependent diabetes mellitus (IDDM)

TNF α is directly involved in the destruction of pancreatic β cells. Furthermore, in the IDDM animal models it is established that islet-infiltrating mononuclear cells produce TNF α . IDDM is associated with the haplotypes TNFB*2, TNFa2 and TNFa1b5. However, the associated TNF haplotypes are in linkage disequilibrium with HLA haplotypes which are established as being associated with IDDM.

Infectious diseases

Sepsis

TNF α plays a critical role in sepsis. In animal models anti-TNF α reduces mortality during sepsis and TNF α serum levels in septic patients correlate with mortality. However, TNF α is necessary in the host defense during experimental peritonitis, and pretreatment of mice with anti-TNF α prior to infection increases the mortality. All clinical trials to prevent negative TNF α effects with anti-TNF α , sTNFR or

TNF α reducing drugs showed no definitive benefit for septic patients. A comparative animal model study showed clearly that blockade of TNF α reduced endotoxemia lethality, but not the lethality of cecal ligation puncture. In abdominal sepsis, TNF α levels correlate positively with nitric oxide end products in the serum. Treatment of septic patients with pentoxifylline (PTX) reduces TNF α synthesis and ICAM-1 expression and produces an improvement of the APACHE II score. IL-13 reduces CD14 expression and blocks TNF α secretion by monocytes. High-density lipoproteins (HDLs) reduce LPS-stimulated TNF α production, whereas low-density lipoproteins (LDLs) enhance the capacity of LPS to induce TNF α . Therefore the balance of HDLs and LDLs in the serum may be critical for TNF α production during sepsis.

HIV

In symptomatic and asymptomatic human immunodeficiency virus (HIV)-positive patients there are elevated TNF α levels in the circulation, which increase during progression of the acquired immune deficiency syndrome (AIDS). The levels of sTNFR also increase and there is a strong correlation between their values and death. TNF α increases HIV replication, but treatment with PTX showed a reduction of TNF α production without reduction of HIV titers. However, PTX inhibits TNF α production but not that of IL-6, and IL-6 correlates, as TNF α did, with progression of AIDS and inversely with the CD4 cell counts. Interestingly, not only sTNFR increases during HIV infection but also the expression of TNF receptor (CD120), with a predominance of CD120b. Thus, it is unclear whether TNF α has more negative effects in AIDS or if the biological role of TNF α against infectious organisms is more important.

Tropical diseases

Strain variants of *Plasmodium falciparum* differ in their capacity to induce TNF α . TNF α overexpression during *Plasmodium* infection results in cerebral malaria, which can be treated with drugs that inhibit TNF α production. Interestingly, IL-10 inhibits *Plasmodium*-induced TNF α production.

Mucocutaneous leishmaniasis (*Leishmania braziliensis*) is accompanied by high levels of circulating TNF α and is associated with the TNF haplotypes TNF2 and TNFB*2 as high-risk factors. TNF α serum levels in African trypanomiasis (*Trypanosoma brucei gambiense*) correlate with the disease severity. However, *in vivo* studies using mice showed that TNF α mediates *Trypanosoma cruzi* killing by

activation of nitric oxide. Therefore, in tropical diseases TNF α has the same dual role as in sepsis.

Transplantation

TNF α and interferon γ (IFN γ) are more sensitive parameters in mixed lymphocyte culture than proliferation and may be possible prospective parameters in bone marrow and organ transplantation. Graft-versus-host disease significantly correlates with enhanced TNF α , but not IL-1 and IL-6 serum levels in bone marrow recipients, even in transplantation of bone marrow from siblings. During acute graft rejection of organs, there is an increase in plasma levels of TNF α . In heart transplantation TNFd3 (associated with high production of TNF α) seems to be a risk factor, whereas the -308 polymorphism is of no relevance, because this promoter region is negatively regulated by cyclosporine. During kidney rejection there are increased TNF α levels resulting from an imbalance between TNF α and sTNFR, which is not observed during cyclosporine nephrotoxicity. In a rat model, anti-TNF α treatment extends allograft survival. This effect is enhanced by a combination of anti-TNF α and anti-IFN γ .

Cancer

It is unclear if the presence of TNF α in tumor tissue indicates immune response against the tumor or promotes tumor progression. TNF α has been ineffective in the treatment of most tumors. However, isolated perfusions with high-dose rTNF α in combination with conventional chemotherapy showed high levels of complete response of melanoma. This therapy showed only moderate TNF α -specific side-effects because the amounts of TNF α entering the circulation were minimal. In a murine model of liver cancer, TNF α treatment increases the number of metastases and therefore the mortality. This showed once more that TNF α may have both tumor-necrotic and tumor-promoting activity.

Endothelial cells and the airway

TNF α induces hyperresponsiveness in the airway. In the lung, TNF α is performed and directly released upon stimulation from mast cells and produced by alveolar macrophages. Interestingly, TNF α inhibits surfactant protein C gene transcription, which may be a critical factor in a number of infections and diseases with production of high levels of TNF α . In asthma, TNF α is upregulated and can be detected in the sputum of patients with acute attacks. Influenza A viral pneumonia is mediated by TNF α , and anti-

TNF α treatment of mice prolongs survival without reducing the virus titer.

TNF α induces cell–cell dissociation of endothelial cells and reduces intracellular cAMP and glutathione levels, resulting in increased permeability of the endothelial cell barrier. TNF α also alters the responsiveness of airway smooth muscle cells.

See also: Acquired immune deficiency syndrome (AIDS); Apoptosis; Autoimmune diseases; Autoimmunity; Bacteria, immunity to; Cytokine assays; Cytokine genes, regulation of; Cytokine receptors, soluble; Cytokine receptors; Cytotoxicity, mechanisms of; Endotoxin (lipopolysaccharide (LPS)); Fas (CD95) and fas ligand; Graft rejection; Immunopathology; Insulin-dependent diabetes mellitus, animal models; Insulin-dependent diabetes mellitus, human; Macrophage activation; Malaria; Mixed lymphocyte reaction (MLR); Rheumatoid arthritis, animal models; Rheumatoid arthritis, human; Schistosomiasis; Septic shock; Superantigens; Transplantation; Lymphotoxin; TNF receptors; Tumors, immune response to.

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TUMORS, IMMUNE RESPONSE TO

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Around the turn of this century, Ehrlich postulated that cancer cells can be destroyed by immunological mechanisms, for example, by using antibodies as ‘magic bullets’. Cooley then started to treat cancer patients with bacterial toxins and obtained some clinical responses. Today this treatment would be attributed to the effects of ‘biological response modifiers’.

It was only when inbred strains of mice became available, however, that workers in this field could critically investigate whether tumors express antigens that serve as therapeutic targets. Using such mice, Gross, Foley, Prehn and others demonstrated in the 1940s and 1950s that tumors induced with chemical

carcinogens express antigens that can induce an immune response leading to the rejection of transplanted cells from the immunizing neoplasms. These antigens were referred to as tumor-specific transplantation antigens (TSTAs). Klein *et al* went one step further by showing that a chemically induced mouse tumor could be rejected also by the very animal in which it had originated, and that the antitumor immune response could be adoptively transferred with lymphocytes. There was little or no cross-reactivity between different neoplasms, even when they had the same morphology and had been induced with the same chemical agent. It is not surprising, therefore, that attempts to use

tumor cells to immunize against chemical carcinogenesis failed.

Another key discovery in tumor immunology was made in 1961 when Sjögren and Habel, working independently, reported that murine neoplasms induced with polyoma virus express TSTAs. Subsequently, TSTAs were detected among murine tumors induced by several other viruses and were found to be shared by tumors induced by the same virus and different among tumors induced by different viruses. Spontaneous tumors, in contrast, did not reveal any TSTA when tested by preimmunization with tumor cells that had been rendered incapable of dividing followed by challenge with live tumor cells. For example, Hewitt investigated 26 different spontaneous mouse tumors and failed to detect any tumor immunity at all; the spontaneous tumors were thus nonimmunogenic (under the conditions tested). Furthermore, the TSTAs in chemically induced rodent tumors were primarily seen in those neoplasms which had been induced by large doses of the carcinogens, while neoplasms induced by small doses either lacked detectable TSTAs or grew even better in immunized hosts than in untreated animals. This was discouraging, since most human tumors are 'spontaneous', and those which are caused by chemical agents probably result from exposure to low doses of such agents. It is also uncertain to what extent there are human neoplasms induced by viruses similar to those causing the expression of strong TSTAs in rodents.

Fortunately, there is now evidence that an immune response can be detected and/or induced against a large variety of tumors, including human tumors, and that the early failures to detect such a response was not due to a lack of tumor antigen, but rather related to the ways tumor immunity was induced. This raises the hope that immunological mechanisms can be utilized towards developing clinically beneficial immunotherapy in humans.

Antigens which can act as targets for tumor destruction can be detected in many different tumors

Immunologically based tumor rejection can, indeed, be detected against many mouse neoplasms previously believed to be nonimmunogenic. In order to achieve this it is necessary to employ techniques to either change the immunizing tumor antigens themselves or the mode by which they are presented. For example, after *in vitro* mutagenization of cells from some originally nonimmunogenic tumors, they could be used as immunogens to induce the rejection of cells from the respective nonimmunogenic 'wild-

type' tumors. Infection of tumor cells with certain viruses, such as vaccinia, sometimes accomplishes the same result. Another approach involves the transfection of tumor cells with genes encoding certain lymphokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), or of genes which encode molecules that can increase the level of immunological costimulation via 'second signals'. For example, transfection of a gene encoding a costimulatory molecule of the B7 family (B7-1 or B7-2) together with a gene encoding mouse CD48 (the ligand for CD2, which in humans is CD58) made both of two classically nonimmunogenic tumors rejectable by their host and usable as 'vaccines' against wild-type cells from the respective tumors.

Nature of the immune response to tumor antigens

Immunity to the TSTAs of murine tumors has been shown to be primarily mediated by T lymphocytes. Tumor immune T cells adoptively transferred from immune donors can localize in a tumor antigen-specific manner and they retain 'immunological memory' for a very long time (possibly for life).

Both CD8⁺ cytolytic T cells (CTLs) and CD4⁺ T helper (T_H) cells are important in this context. Their relative roles appear to vary between different systems, although CD8⁺ cells are involved almost always and their activity amplified as the result of lymphokines produced by T helper 1 (T_H1) cells. The CTLs can kill tumor cells with high efficacy and antigen specificity. However, as a result of the specificity, any existing antigen-negative tumor cell may escape from killing. Since the target epitope for CTLs needs to be presented at the cell surface together with class I molecules of the major histocompatibility complex (MHC), lack of such molecules will make a cell resistant to killing by CTLs, as will lack of molecules needed for antigen processing and transport within the cell.

T helper cells make lymphokines (such as interferon γ and interleukin 2 (IL-2) made by T_H1 cells). Some of these can activate macrophages and natural killer (NK) cells, in addition to T cells, so as to cause cytotoxic and/or inhibitory effects, others (such as tumor necrosis factor, TNF) can have a direct effect on tumor cells. Neoplastic cells appear to be more sensitive than normal cells towards exposure to activated macrophages and NK cells, and this can lead to their destruction as 'bystanders' also when they are antigen-negative. While T_H1 lymphocytes contribute to tumor destruction, T_H2 cells may act as 'suppressors' of an effective tumor immunity.

While the role of antibodies in tumor rejection may be less than that of immune cells, this role should not be ignored. In particular, antibodies which are directed against certain growth factors or growth factor receptors, such as receptors for epidermal growth factor or heregulin, can have inhibitory activity against certain tumors and they may potentiate the effects of anticancer drugs. Antibodies can also have antitumor effects by mediating antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In addition, antibodies can be used for the selective delivery to tumor cells ('targeting') of various anticancer agents. Monoclonal antibodies (mAbs) of mouse or human origin have been used for this purpose in many animal models, and promising clinical data are starting to emerge, particularly in B cell lymphomas.

Nature of the tumor antigens

The TSTAs of tumors induced by either the polyoma or the SV40 viruses were the ones first characterized at the molecular level. Both are encoded by genes of the respective viruses, localized to the nucleus and intimately associated with the initiation and maintenance of the neoplastic transformation. An analogous antigen in human neoplasms would provide an optimal target for immunotherapy since its loss is not compatible with a maintained neoplastic phenotype. It is of great interest, therefore, that most cervical carcinomas are associated with human papillomavirus (HPV) 16, and that the E6 and/or E7 genes of HPV-16 encode tumor antigens which can induce a CTL response detected *in vitro* as well as a CD8⁺ immune response leading to tumor rejection in animal models. Peptide targets of the CTLs have been identified both in animal models and, more recently, in human cervical carcinomas. Clinical trials with tumor vaccines have been started based on the latter findings.

Most neoplasms also express a variety of tumor-associated differentiation antigens (TADAs) together with their individually unique or shared TSTAs. Several of the TADAs have been studied in detail, primarily by using mAbs as tools for their isolation. Antibodies, T helper cells and CTL responses to TADAs have been demonstrated. In most cases, the cancer specificity is relative with some TADA expression also in normal cells from the adult host and often a high expression in some fetal tissues.

Immune responses to human neoplasms

The first evidence for immune responses to human cancers was published in the late 1960s. However,

this area remained controversial until technical advances made it possible to characterize the antigenic targets of the immune responses observed. Antigens recognized by patients' antibodies were identified first. While antibodies have been detected to a few antigens which were individually unique for each patient's tumor, most antibodies have defined antigens that are shared by many tumors, generally irrespective of their histological type.

Cell-mediated immune reactions are demonstrated in assays of either CTL activity or T helper cell responses, with most work nowadays concentrating on antigens recognized by CTLs. Since tumor cell killing by CTL is restricted by the ability of a given patient's MHC class I molecules to present target peptides, an antigen targeted by CTLs will appear as unique to each neoplasm, even if it is shared by many tumors, unless the tumor cells and the CTLs are matched for MHC class I. As a result of recent work taking this into account, as well as earlier studies studying the outcome of T helper cell activity, it has been established that the immune response to human neoplasms is primarily directed towards TADAs that are shared by many tumors. The MAGE group of tumor antigens discovered by Boon's group represents an interesting example. While MAGE antigens were first found in melanoma, they are shared by several other tumors as well. Their degree of tumor selectivity varies between different MAGE antigens but can be very high. Major efforts are presently given to identifying additional families of tumor peptides which can be recognized by human CTLs for use as therapeutic targets.

Much work is presently carried out attempting to devise strategies towards inducing a strong and clinically useful immune response, by developing various therapeutic tumor immunogens ('vaccines'). The logical first step in this direction is to define which tumor antigens are normally recognized by the human immune system or can be made to be so recognized. Progress has been made by working with antibodies in patient sera as well as with mAbs made by human hybridomas. For example, several gangliosides that are expressed on human melanomas are immunogenic in humans, and promising, albeit preliminary, clinical results have been obtained using either mAbs to such antigens or 'vaccines' consisting of antigen plus adjuvant.

Tumor vaccines in the form of recombinant viruses, tumor peptides combined with a suitable adjuvant, and anti-idiotypic antibodies are all of interest as other potential immunogens, as are tumor cells that have been modified so as to become more immunogenic, e.g. by transfection of genes encoding GM-CSF, B7-1 or some other lymphokine or

costimulatory molecule. However, 'vaccines' based on whole cells are less likely to become therapeutic products for various logistical reasons. Targeting of various immunomodulators such as lymphokines and anti-T cell antibodies is also of interest, using bispecific mAbs or tumor-reactive fusion proteins, with the goal to expand an ongoing antitumor immune response. Adoptive transfer of *in vitro* expanded, specifically immune lymphocytes provides a different approach. It may be applicable even in the face of a relatively large tumor load, while vaccines and targeted agents may be best confined for prolonging survival in patients with micrometastases.

Conclusions

Many experimentally induced rodent tumors express TSTAs which can induce an immune response that is capable of destroying neoplastic cells *in vivo*. Today there are also ways to induce a tumoricidal immune response against some tumors that are non-immunogenic when studied by the classical assays for TSTAs. T Lymphocytes play a key role in mediating the immunological destruction of most antigenic tumors. Antibody-mediated and cell-mediated immune reactions to human tumors have been identified and their targets characterized towards development of clinically beneficial immunotherapy.

See also: Antibodies, bispecific; Antibody-dependent cellular cytotoxicity; Cytokines; Cytotoxic T lymphocytes; Embryonic antigens; Epstein-Barr virus, infection and immunity; Helper T lymphocytes; Immunotherapy of tumors; Interleukin 2; Lymphokine-activated killer (LAK) cells; Natural killer (NK) cells; Papillomavirus, infection and immunity; Polyomavirus, infection and immunity; Stress proteins; Targeting of immunological agents; Tumor antigens; Tumor necrosis factor α ; Tumors, immunological escape of.

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TUMORS, IMMUNOLOGICAL ESCAPE OF

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Many experimentally induced rodent tumors express tumor-specific transplantation antigens (TSTAs). These are detectable by the rejection of tumor cells transplanted to immunized syngeneic hosts. Many spontaneous mouse tumors express antigens that

can, under certain circumstances, be recognized by the immune system. Human tumors can also induce an immune response which can be detected by assays for serum antibodies, T helper cell activity and/or the generation of cytotoxic T lymphocytes (CTLs), with

most of the targets being tumor-associated differentiation antigens.

Even those tumors that have strong TSTAs appear in immunocompetent hosts in which they grow progressively. A question that needs to be answered is, therefore, why are they not immediately eliminated by immunological mechanisms? Furthermore, immunological mechanisms which can kill tumor cells *in vitro* and reject transplants of small numbers of tumor cells *in vivo* commonly fail to destroy neoplasms which are more than a few millimeters in diameter. Even the transplantation immunity that can be induced against TSTA-positive tumors can be overcome by challenge with a 100- to 10 000-fold larger number of tumor cells than that needed for outgrowth in nonimmune animals. The next question is, therefore, what mechanisms make this possible? We shall discuss several such 'escape' mechanisms here.

Loss of TSTAs and/or their presentation

A simple explanation for the ability of an originally antigenic neoplasm to grow in an immune animal is that it has lost its TSTAs as a result of selection by the immune system. Since tumor rejection is most commonly mediated by CD8⁺ T cells with CTL activity, loss of major histocompatibility complex (MHC) class I molecules, and/or molecules involved in antigen processing and transport will produce the same result, i.e. lack of epitopes for CTLs to recognize.

There is, indeed, evidence that neoplastic cells can lose TSTAs and/or MHC class I antigens. However, the frequency of total loss of such antigens may be lower than that suggested by immunohistology. Furthermore, both TSTAs and MHC class I molecules are commonly expressed by tumor cells growing in immune animals, implying that a failed presentation of TSTA does not provide a general explanation as to why tumor immunity is not more effective. However, it is clearly one of the mechanisms that can lead to the selection of tumors that are not sensitive to immune destruction by CD8⁺ T cells.

Some tumor antigens, such as the TSTAs of polyomavirus-induced neoplasms, cannot be lost without loss of the neoplastic phenotype, since the molecule detected as a tumor antigen is involved in the neoplastic transformation. On the other hand, some tumor-associated differentiation antigens can be eliminated without loss of malignancy. It is noteworthy that polyomavirus-induced mouse tumors did retain their sensitivity to an immune response directed to the polyoma TSTA even after attempts to select for TSTA-negative cells by prolonged tumor

passage in immune animals, since this indicates that their ability to present the polyoma TSTAs via MHC class I molecules remained intact.

Substantially higher cell surface expression of MHC class I molecules is needed for a tumor cell to induce an immune response than to serve as its target, and there is evidence that tumor cells may still be killed by CD8⁺ lymphocytes even when their MHC class I expression is too low to be detectable by immunohistology. Furthermore, the expression of MHC class I molecules can be upregulated, e.g. following exposure to interferon γ . This suggests that immune T cells may be effective in a larger number of cases than might be indicated by the frequent downregulation of MHC during tumor progression. One should also bear in mind that tumor cells which have lost MHC class I molecules are likely to become more sensitive to destruction by natural killer (NK) cells.

Antigenic modulation

Some neoplastic cells can undergo antigenic modulation, i.e. they cease to express the particular tumor antigen as the result of exposure to specific antibodies. This event has been well demonstrated for certain antigens on leukemia cells, e.g. the TL antigen of mouse lymphomas. However, it appears to be less frequent for most TSTAs expressed on carcinomas or sarcomas.

Lack of costimulation

For an immune response to be induced, it is not sufficient that an antigen is presented to the T cell receptor via MHC class I or class II molecules. A second, costimulatory, signal is also required. Interaction between molecules of the B7 family (B7-1 or B7-2) on the antigen-presenting cells (APCs) and the CD28 counter-receptor on T lymphocytes provides probably the most important costimulatory signal. Antigen presentation in the absence of this signal does not induce an effective immune response but results in 'immunological ignorance', and can even lead to anergy. It is noteworthy, therefore, that most tumors fail to express B7-1 or B7-2 (the exception being some B cell lymphomas), i.e. they present their tumor epitopes in a way unable to cause a tumor destructive response. For an effective immune response to occur, tumor antigen needs to reach lymphoid tissues and be presented in the context of MHC class I or II molecules expressed on B7-positive APCs.

Transfection of B7-1 or B7-2 into tumor cells increases their immunogenicity and prevents outgrowth in immunocompetent syngeneic recipients.

Furthermore, tumors so transfected can be used as 'vaccines' to induce the rejection of wild-type cells from the respective neoplasms as well as the generation of tumor-specific CD8⁺ CTLs with high *in vitro* and *in vivo* potency. While transfection of genes encoding B7-1 and/or B7-2 vastly increases immunogenicity, it has failed to cause rejection of nonimmunogenic tumors, i.e. tumors which lack TSTAs according to conventional immunization challenge tests. However, when the B7-1 (or B7-2) gene was combined with a gene encoding a second costimulatory ligand, CD48, both of two classically nonimmunogenic mouse tumors became rejectable by the immune system, and the doubly transfected cells could be used as 'vaccines' to induce the destruction of wild-type cells from the respective tumors.

'Sneaking through'

This term implies that the immune response to a tumor appears too late to be effective, so that the tumor outgrows the ability of the immune system to keep up with it. Lack of costimulation may provide a simple explanation for this, since an immune response against wild-type, i.e. B7-negative, tumors will not be initiated until tumor cells, or released tumor antigen, have reached lymph nodes for antigen presentation in the presence of APCs providing the proper second signal.

'Blocking factors' and 'suppressor' cells

Circulating 'blocking factors' in the form of antigen-antibody complexes and free antigens were discovered in the late 1960s by their ability to inhibit ('block') the destruction of tumor cells by immune lymphocytes. This was demonstrated by using an assay which lasted 3-7 days and, therefore, included both lymphocyte activation and tumor cell killing. The presence of 'blocking factors' correlated with tumor growth in animal models.

The immune response to a growing tumor is less effective at the tumor site than systemically, and animals bearing immunogenic tumors are often able to reject a small number of cells transplanted from the same neoplasm; they display 'concomitant tumor immunity'. This suggests that the local concentration of 'blocking factors' is particularly high, and experimental data support this.

While part of the 'blocking' effect may be via competition for antibody molecules or effector cells, tumor antigen primarily inhibits the induction and/or manifestation of a tumoricidal host response in a more indirect way. It was originally suggested that

tumor antigen (and/or immune complex) can activate 'suppressor' T cells which downregulate the formation of a tumoricidal immune response. Using today's terminology, the phenomenon is explained on the basis of immune deviation, favoring a T helper 2 cell (T_H2) response over one mediated by T_H1 cells; a T_H2 response would disfavor CTL activity as a result of lack of interferon γ and IL-2. One may speculate that the immune deviation results from antigen presentation by B7-negative APCs which take up circulating tumor antigen-antibody complexes and present tumor epitopes in a way which induces anergy.

Suppression can be specific towards the antigens of a given tumor, but it can also occur against unrelated antigens once it has been induced. Treatment of tumor-bearing animals with certain immunosuppressive agents (e.g. cyclophosphamide) or by exposure to low doses of ionizing radiation, can inhibit tumor growth unless the treated animals are injected with lymphocytes with 'suppressor' activity.

Suppression by TGF β , p15E and other 'factors' made by the tumor

Destruction of immunogenic tumors by their hosts can also be thwarted by other molecules produced by the tumor and/or the host. The effect of such molecules is likely to contribute to the downregulated antitumor response at the tumor site, and it may explain why certain molecules involved in T cell signaling are underexpressed in T lymphocytes from tumor bearers. For example, many tumors produce transforming growth factor β (TGF β), which can effectively inhibit T cell-mediated responses, including those mediated by CTLs. Another factor is p15E, which is a peptide related to murine leukemia virus but expressed also on certain human tumors. Exposure of macrophages to p15E can abolish their ability to cause tumor destruction, and antibodies to p15E can facilitate tumor rejection under certain conditions. Sialomucin produced by neoplastic cells can provide yet another type of protection acting as a barrier against immunological destruction, and removal of the sialomucin by treatment with neuraminidase has increased both the immunogenicity and the immunosensitivity of some tumors in experimental animals.

Conclusions

Even those tumors that are immunogenic appear, and continue to grow, in immunocompetent syngeneic hosts, and the *in vivo* effectiveness of an anti-tumor immune response is rather modest. Escape

mechanisms must, therefore, exist which result in tumors evading the destruction of a tumoricidal response and/or make them incapable of inducing such a response. Failure of tumor antigen presentation by MHC class I molecules, lack of costimulation and downregulation of tumor-destructive immune responses by tumor antigens, immune complexes, as well as by molecules such as TGF β and p15E may all contribute to the inefficiency of tumor immunity.

See also: Antibody-dependent cellular cytotoxicity; Cytotoxic T lymphocytes; Effector lymphocytes; Embryonic antigens; Helper T lymphocytes; Immune complexes; Immune surveillance; Immunogen; Suppressor T lymphocytes; Transforming growth factor β (TGF β); Tumor antigens; Tumors, Immune response to.

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ULTRACENTRIFUGATION

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Construction of ultracentrifuges and development of the theoretical basis for ultracentrifugation was pioneered by The Svedberg and his collaborators in Uppsala, Sweden during the 1920s. At this time, ultracentrifugation was used only for analytical work. The principal methods were sedimentation

velocity and sedimentation equilibrium analysis. In sedimentation velocity experiments, the rate at which a particle sediments in solution is measured. This is expressed as the sedimentation coefficient of the particle. In sedimentation equilibrium studies, a particle suspension is centrifuged until equilibrium is reached

(i.e. when the process of sedimentation is balanced by that of diffusion). The distribution of the particles in the solvent can then be used to calculate their molecular weight. When measurements of the sedimentation coefficient and the molecular weight of a particle are combined with hydrodynamic theories for frictional resistance of particles as they migrate through aqueous solutions, the shape of a particle can be estimated. Major achievements within the field of protein chemistry were derived from analytical ultracentrifugation experiments, including the recognition of the discrete macromolecular nature of proteins, the association of proteins to form oligomers, and the conformational changes that accompany many enzyme-substrate interactions.

The earliest preparative rotors appeared around 1940, nearly two decades after Svedberg had built his first analytical ultracentrifuge. Two major advances contributed to the development of preparative ultracentrifugation: 1) the observation of the complex internal structure of cells made possible by electron microscopy generated a demand for techniques to isolate subcellular particles for biochemical investigations; and 2) the development of alloys with a high strength in relation to their density permitted construction of rotors withstanding the high *g*-forces generated during centrifugation. The earliest preparative studies were performed using fixed-angle rotors and the technique of differential pelleting. Density gradient centrifugation was introduced in 1951 when Brakke described the use of sucrose gradients.

Density gradient centrifugation can be performed either as rate zonal or as isopyknic centrifugation. In rate zonal centrifugation, the particles sediment as a narrow zone through a preformed gradient. This technique is used analytically for sedimentation rate measurements, and preparatively for separation of particles with different sedimentation coefficients. In isopyknic centrifugation, particles are separated purely on the basis of their density. Isopyknic centrifugation can be carried out both in preformed and in self-forming gradients. In self-forming gradients, the sample is mixed with the gradient medium, centrifuged, and as the solute molecules sediment to form the gradient, the sample molecules band at their isopyknic points. When preformed gradients are used, the size of the particles will affect the rate at which they reach their isopyknic positions.

A variety of gradient media have been used for centrifugal separations. For rate zonal centrifugation, the main function of the gradient medium is to stabilize the liquid column in the tube against convection currents. For most applications, sucrose is a satisfactory gradient medium. Glycerol has been used

among others for protein separations when preservation of enzymatic activity is important. For isopyknic separations, the maximum density of the gradient must be greater than that of the particle to be isolated. No single medium has proved satisfactory for all types of biological particles. High-density solutions of alkali metal salts (e.g. CsCl, CsSO₄, CsTFA, NaI, RbCl) are used for separation of proteins, nucleic acids and some viruses. For isopyknic separation of organelles and viruses, sucrose is the most widely used gradient medium, but colloidal silica suspensions (e.g. Percoll), polysaccharides (e.g. Ficoll) and iodinated compounds (e.g. Nycodenz) are in many cases superior, mainly because the osmotic pressure is lower in such gradients. The iodinated gradient media are, in addition, particularly well suited for fractionation of nuclei and native nucleoprotein structures.

Commercial production of ultracentrifuges started in 1948. The first instruments available were analytical centrifuges. Preparative centrifuges were released around 1960, and soon led to a widespread use of ultracentrifugation techniques. Different rotors were developed, including swing-out rotors, large capacity zonal rotors, and special rotor systems for continuous flow centrifugation. Ultracentrifugation has remained an essential method for purification of macromolecules, macromolecular complexes and subcellular particles such as organelles and viruses.

Analytical ultracentrifugation has partly been replaced by procedures such as gel electrophoresis or gel filtration for molecular weight determinations, but it is still an important tool, especially for studies of the interactions between macromolecules.

Application in immunology

Within the field of immunology, ultracentrifugation has been used extensively for studies of immune complexes and immune complex formation. Sedimentation velocity studies in analytical centrifuges, and rate zonal centrifugation in preparative swing-out or zonal rotors have revealed size distributions of immune complexes formed *in vivo* and *in vitro*, and have allowed measurements of association constants of complexes formed at various antigen:antibody ratios. Density gradient centrifugation has been used both for detection and for preparative isolation of immune complexes from biological fluids.

Centrifugation studies of immune complex formation *in vitro* have revealed that the outcome of antigen-antibody interactions is a mixture of compositionally different complexes, and not a single reactant as is otherwise typical of macromolecular reactions. The smallest complexes are formed in the

greatest amounts, and at a given antigen:antibody ratio, immune complex formation seems to be self-limiting with respect to the maximal size of the complexes. Association constants for immune complexes have been shown to depend on 1) antigen valence, 2) antibody affinity and 3) the concentration of antibody combined with 4) the degree of antibody excess. The association constants observed in antibody excess are higher than those recorded in antigen excess for the same set of antigen and antibody. These observations demonstrate that the interactions between antigens and antibodies are a unique set of biological reactions that are different from ordinary ligand binding reactions.

Rate zonal centrifugation in sucrose gradients has for many years been the 'gold standard' among procedures devised for the detection of immune complexes in biological fluids. After centrifugation, immune complexes are quantitated as immunoglobulins with increased sedimentation rates. The procedure is inconvenient for screening large numbers of samples, but has been used to confirm the presence of immune complexes in patients with a wide range of neoplastic and chronic inflammatory disorders.

Animal studies have shown that several physicochemical properties of immune complexes are important for the development of disease. When complexes are formed with positively charged antigen or antibody, or with antibodies of low affinity or avidity, there is increased risk for the development of glomerulonephritis. In addition, the size of the complex is important, but the critical size, as measured by rate zonal centrifugation, may vary between individual antigen-antibody complexes.

For analysis of the components present in immune complexes, preparative studies have been accomplished using rate zonal centrifugation in sucrose or in combined sucrose-polyethylene glycol gradients. DNA-antibody complexes were isolated by the former procedure from the serum of patients with systemic lupus erythematosus. The sedimentation coefficients of immune complexes varies, however, and rate zonal centrifugation is therefore not an ideal method for preparative work because the complexes will be distributed over several gradient fractions. In contrast, after isopyknic centrifugation, immune complexes can be recovered as sharp bands in the gradients. Analysis of immune complexes isolated by isopyknic centrifugation in sucrose gradients has indicated that antigens related to those present in a retrovirus-like particle may participate in immune

complex formation in patients with psoriasis and seronegative arthritis.

Recent developments within the field of ultracentrifugation include air-cooled, benchtop centrifuges and a novel analytical centrifuge. The benchtop centrifuges are ideal for rapid, preparative work in small volumes and have also been used for analytical studies. Compared to the previous generation of analytical ultracentrifuges, the new centrifuge permits analytical centrifugation to be carried out with considerably increased sensitivity. Although ultracentrifugation has to some extent been replaced by other techniques, it still has great potential, both in analytical and preparative work, and remains an invaluable tool for the immunologist.

See also: Affinity; Immune complexes; Protein separation techniques; Valency of antigens and antibodies.

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UNGULATE IMMUNE SYSTEMS

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The immune system of ungulates, a group of species including sheep, goats, deer and cattle, has been widely studied. This is not only because of their importance as food animals but also because they are available in large numbers and are a convenient alternative to laboratory animal species for the study of immune function, especially where large sample size and access to anatomical compartments are required.

Structural and cellular components of immunity

Structural elements of the immune system in ungulates is similar to that in most other mammalian species, with the thymus and bone marrow functioning as primary lymphoid tissue for generation of T and B cells, respectively. However, a feature which appears to be unique to ruminant ungulates is the high contribution to primary B cell development made by the ileal Peyer's patch, which in this species occurs as a single patch of up to 1 m in length located in the terminal ileum. Whereas B cells originate from both bone marrow and ileal Peyer's patch, T cells, as in other species, emanate from the thymus, especially in late gestation and early postnatal life. As in other species, B cells committed to immunoglobulin G (IgG) production reside predominantly in systemic compartments such as spleen and lymph nodes, but those committed to IgA preferentially localize at submucosal compartments but have the ability to traffic between mucosal sites. This ensures that the antigenic experience at one mucosal site can be reflected in IgA antibody production at a remote site, the principle often referred to as common mucosal immunity.

The T cell profile of ruminants is unusual in that a higher proportion of γ/δ TCR⁺ cells occurs than in other species. Whereas about 2–3% of peripheral blood lymphocytes in rodents and humans are γ/δ TCR⁺, in ruminants around 60% carry the γ/δ T cell receptor. Also in contrast to other species where γ/δ TCR⁺ cells have a restricted V region repertoire, in ruminants they have a diverse array of antigen specificities and are of much greater functional significance than in other species, although they display the same anatomical localization preference for skin and mucosal tissues.

Ungulate immunoglobulins

Ungulate immunoglobulins have been identified and classified according to their analogy with immunoglobulins in other species on the basis of similarities in amino acid sequence, immunological cross-reaction, physicochemical properties and biological function. In sheep and cattle, the most widely studied of the ruminant species, analogs of IgG, IgM, IgA and IgE have been identified, with structure and function characteristics as defined in Table 1. In contrast to other species there are only two subclasses of IgG, designated IgG1 and IgG2, and, also in contrast to other species, IgA in serum exists predominantly as a dimer, originating from IgA plasma cells at mucosal surfaces rather than from bone marrow.

For many years controversy persisted over the nature of the reaginic or homocytotropic antibody (i.e. the analog of IgE) in ruminants, with some evidence supporting the existence of a minor IgG subclass accounting for these effects. However, more recent work has clearly revealed the existence of a true IgE isotype which can be identified by mono-

Table 1 Structure and function of ungulate immunoglobulins

Immunoglobulin isotype	Molecular weight kDa	Structure	Complement fixation	Serum half-life (days)	Sedimentation coefficient
IgG1	150	 Monomeric	+	9.5	6.5–7.0
IgG2	150	 Monomeric	+/-	10–15	6.5–7.0
IgM	900	 Pentameric with J chain	+	6	19
IgA (serum)	350	 Dimeric	–	3	10
IgA (secretory)	400	 Dimeric with J chain	–		11
IgE	150	 Monomeric	–	?	7.0

clonal antibodies specific for the IgE heavy chain determinant.

Secretory or mucosal surfaces in ruminants, as in other species, are characterized by the predominance of IgA antibodies. These antibodies are synthesized by plasma cells underlying the mucosal epithelia and are secreted in dimeric form with subunit elements held together by a joining chain (J chain). These are transported into external secretions by association with the polymeric immunoglobulin receptor (pIgR or secretory component) expressed on the basal membrane of the epithelial cells. The molecule is secreted as a dimer complexed to a component of the extracellular domain of the pIgR. Dimeric IgA which escapes secretion is returned via draining lymphatics to the blood circulation, from which it may be re-secreted at remote mucosal surfaces, representing an additional dimension of the common mucosal immune system. The extent to which plasma-derived IgA contributes to the total IgA content of external secretions is inversely proportional to the amount of local production, a result of competition between locally-produced and serum-derived IgA molecules for the pIgR molecules on the epithelial cell basal membrane.

The mammary gland in ruminants produces milk with a vastly different immunoglobulin profile to that in most other species. As a consequence of the lack of immunoglobulin transport across the placenta in ruminants (see section on acquisition of passively transferred antibodies), colostrum contains extremely high levels of IgG1 and very little IgA. At this time most of the IgA in milk is plasma derived. As lactation progresses, and particularly during involution, the selective transport of IgG1 into milk declines and there is an increased number of IgA plasma cells contributing to local IgA production, with relatively less serum-derived IgA.

The existence of a biliary pump to remove IgA from serum for delivery via bile to the gut lumen, a feature which is well developed in rodents, has been identified in ruminants, but only small amounts of serum IgA are processed by this route.

Ontogeny of immune function in ungulates

The first lymphoid organ to appear in the developing fetus is the thymus, which is the main component of mammalian primary lymphoid apparatus. In the fetal lamb, it initially consists of epithelial cells arising from pharyngeal pouches and is first observed macroscopically after about 35 days of gestation, at which time small lymphocytes begin to appear in the thymic tissue in the lamb, but this does not occur

until around 60 days of gestation in the calf. Distinct differentiation into cortex and medulla appear by about 50 days in the fetal lamb and the organ reaches its maximum size relative to body weight just before term. In fetal lambs, lymph nodes begin to appear at about 50–60 days of gestation and the spleen is first visible after 55–60 days but organizational structure does not occur until around 75 days. Mucosal lymphoid structures such as Peyer's patches do not appear until around 80–90 days of gestation and lymphocytes are not present in the lamina propria of the gut until around 120 days.

All the immunologic tissues are well developed before birth but differentiation of the peripheral organs does not proceed to any extent until the animal experiences the antigenic stimulation normally associated with extrauterine life. This results in the appearance of follicles and germinal centers, especially in the gut-associated lymphoid tissue, and mitotic figures and plasma cells are seen in the medullary tissues of lymphoid organs at this time.

Acquisition of passively transferred immunity

In all higher vertebrates mechanisms have evolved to ensure that passive immune protection is provided to the neonate to assist in control of microbial challenge after birth until the endogenous immune mechanisms of the newborn animal have achieved sufficient functional maturity. The extent to which this occurs before or after birth differs vastly between species, an adaptation which is related to the type of placentation (Table 2). Ungulates obtain no immunoglobulin prenatally from the maternal circulation and are born agammaglobulinemic, but large quantities of maternal IgG are selectively transported into the mammary gland just prior to parturition and are ingested and absorbed intact into the circulation by the suckling neonate. The intestine in newborn ruminants is consequently adapted for large-scale uptake of intact immunoglobulin across the gut into the neonatal circulation in the first 24–28 hours of life, during which time the large, pale staining vacuolated cells of the intestinal epithelium of the newborn ruminant are replaced by cells characteristic of the adult intestine to achieve gut closure.

In humans, where IgG is transported placentally, there are very low levels of IgG in colostrum but relatively higher levels of IgA, whereas in ruminants the reverse applies, with high levels of IgG1 in colostrum and relatively low levels of IgA, but a higher ratio of IgA:IgG occurs in later lactation and in involution secretion.

Soluble factors other than immunoglobulins in

Table 2 Placentation type and mode of passive antibody transfer from mother to young in various species

Species	Placentation	Transmission of passive immunity				
		Prenatal		Postnatal		
		Proportion	Route	Proportion	Duration	Route
Cattle, sheep, goat, horse, pig	Epitheliochorial	0	None	+++	36 hours	Gut
Dog	Endotheliochorial	+	Transplacental	++	10 days	Gut
Mouse	Hemochorial	+	Transplacental	++	16 days	Gut
Rat	Hemochorial	+	Transplacental	++	20 days	Gut
Guinea pig	Hemochorial	+++	Transplacental	0	—	None
Rabbit	Hemochorial	+++	Transplacental	0	—	None
Man	Hemochorial	+++	Transplacental	0	—	None

Adapted from Brambell (1958).

milk are also important effectors of passive immune protection, and part of the protective capacity of milk is attributable to the nonimmunoglobulin fraction. Antibacterial substances such as lactic acid, interferon, lysozyme, lactoferrin and the complement components C3 and C4 have been demonstrated in both human and ruminant milk and have been shown to affect the establishment of pathogenic bacteria, either through inherent bactericidal activity or in conjunction with specific immune responses.

While antibodies in colostrum are the predominant immune effectors transferred to the ruminant neonate, cells and other soluble factors in milk play an important role in terms of passive protection. There are normally large numbers of viable cells in bovine milk, varying from 5×10^4 to 2×10^6 cells ml^{-1} , although substantially higher numbers may occur if the gland becomes infected or inflamed. After milk ingestion by the suckling ruminant neonate some of these cells are taken up intact across the duodenal mucosa and are transported via lacteal lymph and mesenteric lymph nodes, indicating the potential for cells of maternal origin to have functional relevance in passive protection of the neonate.

The efficacy of passive transfer of antibody via colostrum in ruminants in protecting the neonate from specific diseases has been well documented. Calves which do not receive colostrum usually succumb to disease at an early age, or if they do survive have restricted growth. Deliberate passive immunization against selected diseases has been highly effective and calves fed colostrum antibodies from immunized cows have been demonstrated to be resistant to specific challenge with a range of selected pathogens.

Despite the beneficial effect of maternal antibody transfer, it is well documented that maternal anti-

body acquired postnatally via colostrum has a suppressive effect on the development of endogenous mucosal immune responses in the young animal and the endogenous production of serum IgA occurs earlier in calves deprived of colostrum.

See also: CD antigens; Immunoglobulin, evolution of; Mucosa-associated lymphoid tissue (MALT); Ovine immune system; Phylogeny of the immune response; Porcine immune system; Tolerance, peripheral.

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URINARY TRACT INFECTIONS

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The term urinary tract infection (UTI) describes clinical conditions caused by the presence of microorganisms (usually bacteria but occasionally fungi or, more rarely, viruses or schistosomes) in the urinary tract. The severity of infection depends on the virulence of the infecting strain and responsiveness of the infected host. Urinary tract infections may therefore be seen as a spectrum of host responses to microorganisms of different virulence. The diagnostic categories used in clinical practice correspond to different parts of this spectrum: acute pyelonephritis is a febrile infection involving the kidney with signs of local and systemic inflammation as well as renal involvement. Acute cystitis is characterized by lower urinary tract symptoms with pain, frequency of micturition, and local inflammation but typically these patients lack signs of systemic infection. Screening of covert bacteriuria is used to describe patients in whom bacteriuria gives rise to few symptoms and is therefore only detected at screening.

UTIs are among the most common bacterial infections. The most reliable frequency estimates, based on extensive screening of different age groups, show that about 1% of schoolgirls, 2–11% of pregnant women and $\geq 10\%$ of elderly women carry $\geq 10^5$ bacteria ml^{-1} of urine. Men mostly acquire bacteriuria during the first year and the last decades of life. The cumulative frequency before 11 years of age of symptomatic infections is about 3.5% in girls and 1% in boys. While acute cystitis is common in women of all ages, the frequency estimates of acute pyelonephritis in adults vary extensively depending on underlying disease conditions.

Bacteria

Escherichia coli, *Proteus*, *Klebsiella*, *Enterobacter* and *Staphylococcus saprophyticus* are the most common etiologic agents in UTI. *E. coli* dominate in both outpatient and hospitalized populations and are the most extensively characterized regarding mechanisms of virulence.

The population structure of *E. coli* is clonal, i.e. genetic elements occur in nonrandom, relatively stable combinations. Uropathogenic *E. coli* are a selected subset of the *E. coli* flora at large. The pyelonephritogenic clones are identified by a limited number of surface antigen serotypes (O:K:H:F = lipopolysaccharide (LPS), capsular polysaccharide, flagellar and fimbrial antigens) and intracellular iso-

enzyme patterns (electrophoretic types), and by the expression of molecules which damage the host tissues. Adherence is mediated by bacterial surface lectins, which may be associated with fimbriae. The lectins bind the bacteria to host receptor molecules consisting of oligosaccharide sequences in glycoproteins or glycolipids. The P fimbrial lectins recognize the Gal α 1–4Gal β and GalNAc epitopes within the globoseries of glycolipid receptors. S Fimbrial lectins recognize sialic acid residues, and type 1 fimbriae bind mannose residues found, for example, on the Tamm Horsfall protein and secretory immunoglobulin A (IgA), and connective tissue proteins such as collagen and laminin.

LPS is the endotoxin of *E. coli*, which contains the lipid A moiety responsible for eliciting a wide array of inflammatory host responses. Hemolysin participates in the destruction of host cells through its lytic activity. The capsular polysaccharides protect bacteria against attack by complement and neutrophils, and aerobactin facilitates the trapping of free iron required for the bacterial oxidative metabolism, especially in environments with limited oxygen supply. These characteristics are all designated as virulence factors, since they occur more frequently in acute pyelonephritis than in screening bacteriuria strains.

Pathogenesis

Uropathogenic *E. coli* first become established at a site outside the urinary tract (the large intestine, the introital or preputial areas). Colonization at these sites is influenced by adherence through P and type 1 fimbriae and by the receptor repertoire of the host. The uropathogenic clones enter the urinary tract and establish bacteriuria via the ascending route. The bacterial properties that determine this initial establishment are still poorly understood. The bacteria resist elimination with the urine flow at voiding and the killing by bacteriocins in the mucosa.

Acute pyelonephritis is caused by bacteria that reach the kidney and trigger local and systemic inflammation. The bacteria activate cells at the site of infection to secrete cytokines and other proinflammatory mediators. The molecular mechanisms through which uropathogenic bacteria elicit cytokine responses have been studied *in vitro*. Attachment to uroepithelial cell receptors activates transmembrane signaling events that upregulate cytokine mRNA

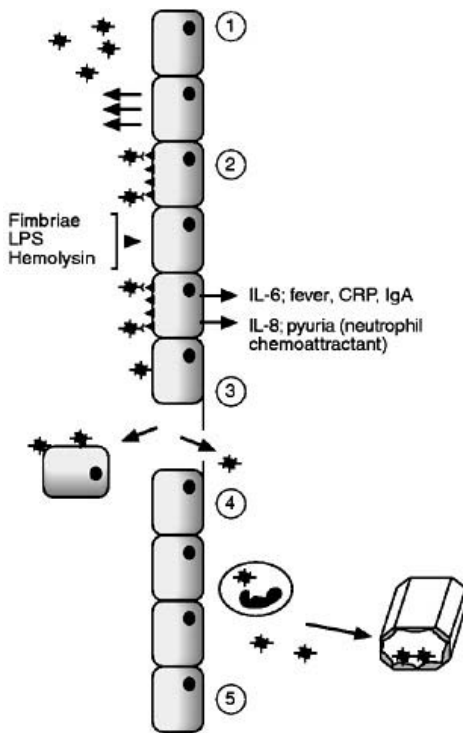


Figure 1 Early events in the pathogenesis of urinary tract infection. 1: Bacteria enter the urinary tract. Persistence is determined by (a) resistance to bacteriocins and (b) adherence factors suitable for host receptors. 2: Bacteria induce the production of inflammatory mediators by the mucosa (cytokines) which trigger local and systemic inflammation. 3: Bacteria induce cell detachment and cross the epithelial barrier. 4: Phagocytic cells eliminate bacteria. 5: Bacteria reach the bloodstream and cause urosepsis. (Drawn by W Agace.)

levels and protein synthesis, and results in cytokine secretion. The transmembrane signaling pathway is influenced by the fimbrial receptor specificity.

The release of cytokines and other inflammatory mediators elicits the chain of events that leads to the classical inflammatory changes associated with acute pyelonephritis (fever, elevated acute phase responses, local inflammation and loss of tubular function). The rapid influx of neutrophils (PMNs) into the urine is partly explained by the local production of interleukin 8 (IL-8), a neutrophil chemoattractant. In response to mucosal IL-8, PMNs from the circulation migrate to the epithelium, and cross the epithelial barrier into the urine. This process is also influenced by epithelial cell adhesion molecules such as ICAM-1 that are upregulated by the presence of bacteria, and bind the CD11b/CD18 neutrophil ligands. The febrile response to UTI and elevation of circulating acute phase reactants like C reactive protein may be explained by the release of IL-6 from the site of infection. Further studies are required to define the *in vivo* relevance of the cytokines as mediators of disease.

In about 30% of patients with acute pyelonephritis bacteria reach the bloodstream and cause urosepsis. This involves the disruption of the epithelial barrier and survival of bacteria in the bloodstream. P Fimbriated *E. coli* are more common in urosepsis than in acute pyelonephritis. The role of mucosal and systemic immunity or inflammation for this phase of infection is not well understood.

Host resistance to UTI

The urine flow is a major host defense mechanism as shown by the drastically increased frequency of UTI in patients with catheters or other mechanical defects.

Individuals prone to UTI have an increased tendency to carry uropathogenic *E. coli* in the intestinal flora and introital area. Epithelial cells from these sites have a higher density of receptors than cells from healthy controls. The establishment of bacteria in the urinary tract is also thought to be influenced by the host expression of receptors for bacterial adhesins. The P blood group determines expression of the globoseries of glycolipids; P_1 individuals run an 11-fold increased risk of suffering from recurrent pyelonephritis compared to P_2 individuals. The mucosal expression of A, B, H determinants is influenced by the ABH blood group and secretor state. Bacteria which recognize the globo A receptor infect individuals of the A_1P_1 blood group, who express this receptor in the urinary tract. Blood group ABH nonsecretors have increased inflammatory reactions to UTI and are enriched in renal scarring.

The establishment of bacteriuria is counteracted by secreted receptor analogs or free oligosaccharides that inhibit bacterial attachment; the Tamm Horsfall glycoprotein which binds type 1 and S fimbriae, and secretory IgA which trap type 1 fimbriated *E. coli*. Secreted Gal α 1-4Gal β -containing oligosaccharides or glycoconjugates that inhibit the attachment of P fimbriated bacteria have not been demonstrated. The tissue affinity of the Gal α 1-4Gal β -specific adhesins combined with the lack of inhibitors may explain their association with disease.

The acute inflammatory response to infection is a key to the symptoms, and to the clearance of bacteria from the urinary tract. Resistance mechanisms have mainly been studied in experimental UTI models. The inflammatory response initiated by bacterial contact with the mucosa mediates clearance of infection. In the absence of this response, e.g. in animals with genetic defects or treated with anti-inflammatory drugs, bacteria are not cleared from the kidneys. In contrast, defects in specific immune response did not influence the natural resistance to UTI in mice.

Animals with B lymphocyte defects or severe combined immunodeficiency, cleared experimental *E. coli* infections as efficiently as their normal counterparts.

The results from the experimental studies cannot immediately be translated to patients suffering from various forms of UTI but some parallels may be pointed out. There is no evidence that specific immune mechanisms determine the resistance to sporadic UTI. Patients with immunodeficiency states do not appear to have an increased susceptibility to UTI. Evidence for immune-mediated protection against recurrent UTI in humans is also lacking.

Patients prone to UTI may have defects in the acute inflammatory response leading to defective clearance of infection but this has not been demonstrated. Defective clearance of bacteria from the tissues combined with an abnormal inflammatory response has been suggested to occur in patients with renal scarring.

See also: *Bacteria, immunity to; Escherichia coli, infection and immunity; Klebsiella, infection and immunity; Proteus, infection and immunity; Staphylococcus, infection and immunity.*

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V

VACCINATION, METHODS OF ADMINISTRATION

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Routes of antigen administration

There are two general routes of antigen administration – parenteral and mucosal. The former refers to routes which involve penetration of the skin; the latter refers to absorption of antigen through a mucosal layer.

Parenteral administration

A number of routes are used in experimental work, including intradermal (i.d.), subcutaneous (s.c.), intramuscular (i.m.), intraperitoneal (i.p.) and intravenous (i.v.) administration. Three routes are mainly used in human administration. The smallpox (vaccinia) vaccine was routinely given by skin scarification.

Most vaccines given parenterally are by s.c. or i.m. administration, the choice largely depending upon the need for an alum adjuvant, when i.m. is the route of choice.

The last decade has seen a range of new preparations which, though not yet licensed as vaccines, hold considerable promise. DNA in the form of a plasmid is injected i.m. and this results in a long-lived immune response (antibody and cytotoxic T lymphocyte (CTL) formation). Rather large amounts are given (100 µg) by this route, but much smaller amounts (<1 µg) if adsorbed to tiny gold beads and delivered by a 'gene gun' into the mouse skin, can also give a good response. In the latter situation, examination has shown that some of the particles are

taken up by Langerhans cells and carried off to the draining lymph nodes.

Mucosal administration

The area of mucosal surface available for infection by infectious agents and for antigen presentation is far greater than the skin area. Correspondingly, the mucosal surface is very well endowed with lymphoid tissue, in the gut by intraepithelial lymphocytes and Peyer's patches. The gut-associated lymphoid tissue (GALT) has been characterized in more detail than other sites. The major routes available for administration are oral, intranasal, ocular, intrarectal and vaginal (Table 1). These are anatomically discrete lymphoid compartments but there is a link between them which is referred to as 'the common mucosal system', whereby immunocytes sensitized at one site, particularly GALT, may transfer immunity after traveling to other mucosal and glandular sites.

Because of this relationship and the ease of administration, it is surprising that the oral route of administration of vaccines has not been more widely used other than for those vaccines which protect against diseases caused by agents, such as certain viruses and bacteria, which normally infect by this route. One reason is the need to avoid positive immune responses in the gut to food antigens, so that suppressor mechanisms are often activated. Second, for many antigens, much higher doses are required to achieve a desired response after oral compared to parenteral administration. Third, feeding an antigen orally does not necessarily result in a good immune response at all other mucosal sites. For example, there are only a few examples where oral administration of an antigen formulation has resulted in protection against a vaginal challenge.

Oral administration of live agent vaccines is effective for the prevention of enteric infections, such as poliomyelitis and typhoid fever and would be used for rotavirus and cholera vaccines. The adenovirus

vaccine, a tissue culture produced live preparation with no markers of attenuation, has been administered to defense personnel in the USA for many years and protects against a respiratory infection by the wild-type agent. The virus is thought to replicate in the GALT underlying the M (microfold) cells which line the walls of the intestine. The rectal route is used extensively in the poultry industry, partly because of the close location of the bursa of Fabricius, where B lymphocytes differentiate. In mammals, there is a high concentration of lymphoid tissue located near the rectum. The ocular route is not used at present, but it would probably be used if a vaccine became available to prevent trachoma caused by *Chlamydia* infections.

The vaginal route becomes increasingly important when one considers the number of sexually transmitted diseases, not the least of which is human immunodeficiency virus (HIV) infection. However, immunization by this route with a noninfectious antigen usually results in a poor and short-lived response. There is little organized lymphoid tissue adjacent to the vagina and the reason for this may be to minimize the risk of an antibody response to sperm. A small proportion of women become infertile for this reason. In experimental work with monkeys, injection of antigens adjacent to lymph nodes which drain the vaginal area has given good vaginal responses. Again experimentally, intravaginal administration of chimeric herpesvirus has resulted in a good response to the encoded antigen.

Immunopotentiality – the need for adjuvants

The term adjuvant is widely used to describe potentiating effects that may be specific or nonspecific. The term vehicle is sometimes used to describe the way in which the antigen is presented. The final mode of presentation has been called the antigen formulation. The only adjuvant licensed for general medical use is alum, which was first used over 50 years ago.

A great variety of materials have been shown to have adjuvant activity, but with increasing knowledge of immune mechanisms and especially the role of lymphokines, it became clear that an adjuvant should preferentially have three functions:

- the formation of a depot at primarily the site of application;
- the presentation or delivery of antigen to cells involved in the immune response, principally antigen-presenting cells; and
- the synthesis and secretion of cytokines which

Table 1 Mucosal routes of administration for current and some future vaccines

Route	Organisms
Oral ^a	Poliovirus (OPV), rotavirus, <i>Salmonella</i> , cholera, adenovirus
Rectal	Many vaccines administered to poultry
Ocular	<i>Chlamydia</i> (trachoma), herpesvirus
Vaginal	Sexually transmitted disease (STD) organisms, including HIV, HTLV, herpesvirus
Intranasal ^a	Live attenuated influenza, measles?, DNA?, some STD organisms?

^aSome antigens administered by this route have given a vaginal response.

Table 2 Some adjuvant formulations under trial and their activities in terms of antigen depot, delivery to antigen-presenting cells (APCs) and factor activation

Preparation	Depot	Delivery	Factor activation
Alum	+	?	?
Algamulin	+	?	+
Mineral oils	+		
Nonionic block polymers ^a	+	+	+
Controlled-release devices ^a	+	?	+
Liposomes ^a	+	+	+
ISCOMS ^b	+	+	+
VLPs ^c	+	?	+
Bacterial products		+/-	+

^aCytokines can be incorporated.^bImmunostimulatory complexes, includes an adjuvant.^cDNA sequences inserted into yeast retro-transposon, which forms virus-like particles.

would act principally on other cells in the immune system, mainly T cells.

Next to alum, Freund's complete adjuvant (FCA), containing mineral oil and inactivated tubercle bacillus was initially used widely and was regarded as the 'gold standard' but fell into disuse because it formed a granuloma. The active principle of the bacillus is muramyl dipeptide (MDP) and many derivatives of this were made and some have been used in clinical trials.

Table 2 lists some of the different preparations now under trial and the extent to which they fulfill the above roles. A second very important aspect is that these different formulations frequently will pre-

ferentially stimulate a CD4⁺ T cell type 1 or type 2 response which results in the secretion of different patterns of cytokines. Furthermore, it is now possible in some situations to selectively induce a CD8⁺ T cell response. In other words, it is becoming increasingly feasible to manipulate the immune system much more than was originally thought possible.

See also: Adjuvants; Antigen, entry into the body; Immunopotentiality; ISCOM (immuno-stimulating complex); Liposomes; Mucosa-associated lymphoid tissue (MALT); Muramyl dipeptide; DNA vaccines; Skin, contribution to immunity; Vaccines; Vaccines, adverse reactions to.

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VACCINES

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It was noted in ancient times that those who survived an infection seldom suffered a second, similar sickness. Some people clearly suffered less than others and it might have been observed that this could be related to the site of the infection. Smallpox was particularly feared and the mildness of some infections led to the deliberate induction of disease by the process of variolation, by the cutaneous route as practised in India or by the nasal route in China. It was a hazardous procedure,

with a case fatality of 0.5-2%, but this compared favorably with the rate of 20-30% from the natural infection.

The observation that milkmaids rarely suffered smallpox led to the deliberate inoculation of people with cowpox, but it was Jenner who, in 1796, carried out the critical experiment of inoculating a boy, James Phipps, first with cowpox material and then with smallpox. The prior 'vaccination' protected Phipps from disease. Vaccination with cowpox

rapidly replaced variolation as a prophylactic measure and it became legal in some countries in the early nineteenth century.

Pasteur was the second great contributor to this area, now called vaccinology. He developed (initially by accident) the means of changing the properties of microbes so that their potential for causing disease (the property of virulence) was greatly reduced. This process is called attenuation and is still used in the development of some viral vaccines. Attenuation occurred if the infectious agent was maintained under adverse conditions or, more regularly, by serial passage in an unrelated host or in tissue culture.

The isolation of toxins from tetanus and diphtheria organisms soon followed, and shortly thereafter, the use of the adjuvant, alum, was introduced by the English scientist, Glenny, as a means of delaying the disappearance of antigen from the injection site. Glenny also noted differences in the rate of the antibody response to a toxin in nonimmune compared with immune animals, i.e. the differences between a primary and a secondary antibody response to an antigen. This difference lies at the very heart of vaccination.

Current vaccines and vaccines under trial

Viral vaccines

Many of the current most successful vaccines are composed of attenuated live viruses (Table 1). The

Table 1 Viral vaccines

		Agents
	Current	Under trial
Live attenuated	Vaccinia	Cytomegalo
	Measles	Hepatitis A
	Mumps	Influenza
	Rubella	Dengue
	Yellow fever	Rota
	Polio (OPV)	Respiratory syncytial
	Adeno	
	Varicella zoster	Parainfluenza Japanese encephalitis
Inactivated	Polio (IPV)	Hepatitis A
	Influenza	
	Rabies	
	Japanese encephalitis	
Subunit	Hepatitis B	
	Influenza	
Combination	Measles, mumps, rubella (MMR)	

introduction of tissue culture technology after World War II greatly facilitated this development. Most of these live virus vaccines are highly effective, inducing long-lasting immunity – sometimes life-long – after one or two administrations.

Disease eradication by vaccination A worldwide program of vaccination with the smallpox vaccine (vaccinia virus) organized by the World Health Organization (WHO) led to the eradication of smallpox (achieved in 1977 and certified in 1980). WHO aims to eradicate poliomyelitis by the year 2000: this disease was declared eliminated from the Americas in 1994 due to the tremendous effort of the Pan American Health Organization (PAHO). PAHO now aims to eliminate measles from the Americas by the year 2000. Eradication is possible for these three diseases because humans are the only host for the viruses. A combination vaccine, measles, mumps and rubella (MMR, Table 1) is widely used and using a two-dose administration of MMR, Finland declared these infections eliminated from that country in 1995.

Inactivated whole virus also forms the basis of some vaccines, most of which are effective although larger doses given more frequently are required. The influenzavirus vaccine is less effective, partly because of the considerable antigenic variation of the envelope proteins, the hemagglutinin and neuraminidase, of this virus.

Vaccines may also be composed of part of an infectious agent, usually the antigen(s) recognized by antibody which can neutralize the viral infectivity. The most successful viral vaccine in this category is the surface antigen, HBsAg, of the hepatitis B virus (HBV). As this virus is infectious only in higher primates, the first vaccine was prepared from the blood of infected people, and is now at last being used in developing countries. A second-generation vaccine, produced by yeast transfected with DNA coding for HBsAg, is in general use in developed countries.

The process of attenuation of viruses has been so successful that most viral vaccines in late-stage clinical trials are of this type. However, many of the new viral candidate vaccines under early development, such as HIV, have followed the earlier lead of the hepatitis B vaccine. Other approaches use some current viral vaccines as vectors of DNA coding for protective antigens of other infectious agents such as HIV, as is discussed later.

Bacterial vaccines

Table 2 lists current bacterial vaccines and those in late-stage clinical trials. In contrast to the viral vaccines, BCG (bacille Calmette–Guérin) was for many

Table 2 Bacterial vaccines

Type	Agent	
	Current	Under trial
Live, attenuated	BCG	<i>V. cholerae</i>
	<i>S. typhi</i> (Ty21a)	<i>S. typhi</i> (Aro A)
Inactivated	<i>V. cholerae</i>	<i>V. cholerae</i> plus B subunit of CT ^a
	<i>B. pertussis</i> (P)	<i>M. leprae</i> , with/without BCG
	<i>S. typhi</i>	
Subunit	<i>H. influenzae</i>	<i>S. typhi</i> (Vi polys.)
	<i>N. meningitidis</i>	
	<i>S. pneumoniae</i>	
	<i>B. pertussis</i> (acellular)(aP)	
Conjugates	<i>H. influenzae</i> (Hib)	Pneumococcal
		Meningococcal
Toxoids	Tetanus (T)	
	Diphtheria (D)	
Combinations	Diphtheria, tetanus, pertussis (DTP or DTaP)	

^aCT, cholera toxin.

years the only attenuated bacterial vaccine. It has given very variable results in several large trials on adults, but is still used in the WHO Expanded Program for Immunization (EPI) of the world's infants. BCG is not administered in the USA, but recently antibiotic-resistant strains have arisen, and this has emphasized the urgent need for a more effective vaccine against tuberculosis. BCG has strong adjuvant activity and can nonspecifically stimulate other immune responses. More recently, an attenuated form of *Salmonella typhi* (Ty21a) has been licensed and has replaced earlier inactivated preparations.

There are several inactivated whole bacterial vaccines. The *Bordetella pertussis* vaccine against whooping cough is effective and is administered as part of a trivalent vaccine, diphtheria-tetanus-pertussis (DTP), with the two toxoids, the detoxified secreted toxins of the tetanus and diphtheria bacteria. The pertussis component is considered responsible for some side-reactions given by this combination vaccine, and recently this component has been replaced by a less reactogenic acellular preparation in a combination called DTaP.

In addition to the two toxoids, there is another form of subunit bacterial vaccines. 'Encapsulated' bacteria have a coating of usually complex polysaccharides which act as protective antigens. They often occur in a variety of serotypes. For example, the pneumococcal vaccine (*Streptococcus pneumoniae*)

contains the carbohydrate epitopes of 23 different serotypes and is administered to the young and the elderly. Generally, children younger than 2 years respond poorly to such preparations, but this can be remedied by conjugating to a carrier protein such as a toxoid or another bacterial protein. The conjugate of the *Haemophilus influenzae* carbohydrate and protein (Hib) is highly effective against meningitis in very young children.

Other vaccines

Other infectious diseases

There is no antiparasite vaccine made by traditional methods currently in use. Q Fever is a debilitating disease particularly prevalent in abattoir workers, and is caused by *Coxiella burnetii*. Inactivated whole organisms caused reactions in those previously sensitized by a natural infection. The vaccine is now administered to nonsensitized personnel and appears to give complete protection.

Anti-insect vector vaccines

During feeding on mammals, most antigens of insect vectors do not come into contact with the host's immune system. If the host was preimmunized with these antigens, however, feeding with uptake of host blood might damage sensitive tissues of the vector. This approach has been tried successfully with the cattle tick, *Boophilus microplus*. Immunization with a tick gut protein results in the destruction of the feeding tick. It would be a great boon if this approach could be successfully adapted to other vectors such as mosquitoes and tsetse flies.

Fertility-control vaccines

Many consider that the greatest threat to the planet is the rapid increase in population, so new approaches to help control this growth are sought. In view of the general acceptance of vaccination as a public health measure, the possibility of using such an approach for fertility control is being evaluated. The finding that some women become infertile because they make antibody to their partner's sperm gives hope to this approach. There are two major targets for such vaccines – gamete antigens and the very early products of conception, and the hormones which control the reproductive cycle. Success has been achieved with some animal models, e.g. the guinea pig, but there is still some way to go with the development of vaccines for human use. The most advanced is based on human chorionic gonadotropin (hCG), a transiently expressed hormone required for implantation of the fertilized egg. In the long run, a

mixture of several gamete antigens may be the more effective approach.

There are also recent advances in the experimental control of noncommunicable diseases such as cancer and autoimmune diseases. Vaccination in these situations is usually referred to as immunotherapy.

The immune response to infections

The development of vaccines has been based on two requirements: safety – the product should not induce undue morbidity; and efficacy – immunization should prevent clinical disease on subsequent exposure to the wild-type agent. Other than measurement in relatively recent times of specific antibody levels and particularly a functional test such as neutralization of infectivity, generally no other parameters of the immunogenicity of the product were considered. Most current vaccines are to 'acute' infections, i.e. those in which the agent replicates for a few days, replication is halted and then cleared without persistence of the infectious agent. Diseases caused by agents which cause chronic infections, i.e. the immune response is subverted in some way, pose a greater challenge to vaccine development. Many of the diseases for which vaccines are now needed are in this category.

Our knowledge of the different responses generated and their role during infection has come from the study of model systems, using laboratory animals. The immune response can be divided into four stages: 1) prevention of infection; 2) limitation of infection; 3) recovery from infection; and 4) the generation of immunological memory.

Stage 1

The only mechanism for specifically preventing infection is antibody directed towards certain epitopes present on 'protective' antigens of the infectious agent. In the case of viruses, usually one or two surface proteins/glycoproteins are important; with bacteria, proteins or oligo/polysaccharides are important. An *in vitro* test which measures the neutralization of infectivity is used to predict the protective effect of an antibody preparation. The epitope(s) recognized may be a linear sequence, e.g. the outer membrane protein of *Chlamydia*, or discontinuous but adjacent sequences, as with many viruses, such as influenza or polio, so that conformation is critical.

Stage 2

If present, pre-existing specific antibody will also greatly limit infection. In its absence, a variety of 'nonspecific' effects are helpful and, for bacteria, may

be critical. These include different cell types (natural killer (NK) cells, neutrophils, macrophages/monocytes, etc.) and factors such as interferons and complement.

Stage 3

Antibody, via mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent lysis and nonspecific factors, can contribute to recovery from infection. For extracellular infections (some bacteria and parasites), antibody is critical. There are several special cases where antibody has been shown to clear a viral infection, but for intracellular infections in general, cell-mediated immune (CMI) responses, consisting of CD4⁺ T_H1 cells and/or CD8⁺ T cells are critical. For most viruses, some bacteria and parasites, CD8⁺ cytotoxic T lymphocytes (CTLs) are the more important because they may lyse infected cells and liberate important cytokines such as interferon γ . CD4⁺ T_H1 cells are usually noncytolytic but secrete cytokines which mediate delayed-type hypersensitivity. The cytokines (interleukins) mediate inflammation, the extent depending upon the amounts produced and the extent of the infection.

Both infectious and noninfectious preparations induce CD4⁺ class II major histocompatibility complex (MHC)-restricted T cell responses. Generally, class I MHC-restricted CTL responses are best induced by infectious agents as the newly synthesized antigens provide peptides in the cytoplasmic pathway which bind to class I MHC antigen. Techniques which permit entry of an antigen preparation into this pathway result in CTL formation, but many infectious agents generate strong CTL responses.

Stage 4

Surprisingly little is known about the pathways leading to the generation of memory B and T cells. It is generally found that the requirements for activation of memory T cells are less demanding than for naive cells and they express quantitatively more of certain markers (though no marker seems to be unique for these cells). Memory B cells, which require T cell help for their generation, express immunoglobulin (Ig) isotypes other than IgM and IgD as receptors. After immunization or infection, the number of specific T or B cells is larger compared to the naive situation.

Immunological requirements/expectations of a vaccine

Vaccines may be administered to populations where

the disease is endemic and a recipient already infected. Both specific and nonspecific immune responses may then have a therapeutic effect and contribute to the control of an existing infection. In most situations, however, vaccination is planned to protect from an infectious agent to which the recipient may be exposed at some variable time in the future. In this case, the requirements differ and depend entirely on the specific components. Because the life of naive lymphocytes is quite short (from days to a couple of weeks), a vaccine should: 1) generate a large pool of memory T and B cells; 2) cause antigen to persist as antigen:antibody complexes on follicular dendritic cells so that memory B cells are formed and constantly recruited to become antibody-secreting cells and thus provide a continuing supply of antibody of increasing affinity; and 3) provide appropriate T cell epitopes to overcome the genetic restriction to this response in an outbred population.

Newer approaches to vaccine development

There are a great variety of diseases – viral, bacterial and parasitic – for which no vaccines are available. The need is great: five parasitic diseases alone affect about one-quarter of the world's population. Reasons such as the inability to grow sufficient of the organism either *in vitro* or in an animal, the complex nature of the infectious process (often including substantial suppressor effects), a lack of understanding of the basis of many chronic and persisting infections, all contribute to the lack of progress in vaccine development. Fortunately, techniques have become available which may overcome some of these restrictions.

Peptide-based preparations

In association with class I or II MHC antigens, specific linear amino acid sequences from foreign proteins may be recognized by the T cell receptor (TCR). By using a sufficient range of such sequences most people in an outbred population should respond. Peptide-based vaccines provide the opportunity to eliminate sequences which may cause immunosuppression or cross-react with self protein. There is great interest in this approach, but to date, no candidate vaccine based on peptides has been licensed. A putative vaccine to prevent malaria and containing several peptides from plasmodial proteins has shown moderate potential in some field trials, but was inactive in a recent trial in The Gambia.

There are fairly severe restrictions on this approach as any B cell epitopes would need to be

continuous sequences and several T helper and CTL epitopes would need to be included so that most people would respond. Special methods of presentation would be needed if a strong CTL response was required. Generally, oligo- or polypeptides are poorly immunogenic, so usually a powerful potentiator, called an adjuvant, must be used to ensure an immune response.

Anti-idiotypic preparations

A number of such preparations have been made and tested in different animal models to control either viral, bacterial or parasitic infections, and many have been relatively successful. But despite the obvious attractions of the approach, e.g. to mimic discontinuous epitopes or those composed of carbohydrate, no candidate vaccine has reached the licensing stage, and in view of the continuing developments with recombinant DNA technology, it seems unlikely that this approach will fulfill its earlier promise.

Approaches using recombinant DNA technology

The ability to manipulate DNA has transformed this area of biology. There are three main approaches with an extra one waiting in the wings. These are as follows.

1. Transfection of cells with DNA coding for the antigen(s) in question. Three classes of cells have been used – bacteria, lower eukaryotes such as yeast, and mammalian cells.
2. The construction of chimeric viruses or bacteria which, upon infection of cells, will express the inserted foreign DNA.
3. Direct immunization with a plasmid containing the foreign DNA.
4. The possibility of producing the antigens in DNA-transfected plants, such as banana, potato or tobacco.

Transfection of bacteria is appropriate for generating relatively simple proteins, such as occur in bacteria and some parasites, but limitations on post-translational processing restrict this approach. Transfected yeast, the source of the first genetically engineered vaccine, the hepatitis B viral vaccine composed of the surface antigen (HBsAg), has also considerable promise for some other products. For some preparations, e.g. viral glycoproteins, transfected mammalian cells – either primary cells (e.g. monkey kidney), cell strains (a finite capacity to replicate) or cell lines (immortalized cells) – offer great promise. All have been used successfully for veterinary vaccines, and will find increasing use in the future for medical products.

Chimeric live vectors In this approach, DNA coding for the antigen is inserted into the genome of an appropriate live virus or bacteria (frequently an existing vaccine). Upon infection, the DNA is expressed with the production of the foreign antigen either in the chimeric virus-infected cell or in the bacterium. Table 3 lists some of the vectors used successfully for this purpose. Each has its advantages and disadvantages. Vaccinia virus has mainly been used and the list of antigens expressed by different vaccinia constructs is now quite long. Though none is yet licensed for medical use, two veterinary vaccines are in use: a vaccinia-rabies glycoprotein construct used in the field to immunize foxes and similar pests, and a vaccinia-rinderpest antigen construct used in Africa to vaccinate cattle. Several vaccinia constructs have successfully undergone clinical trials.

DNA (genetic) immunization When mice are injected intramuscularly with plasmids containing DNA coding for foreign antigens, long-lived immune responses, including both antibody and CTL, occur after a single inoculation. A modification which uses much less antigen has been to coat gold microbeads with the plasmid and inject into skin epithelia with a 'gene gun'. This technique holds great promise in many ways, e.g. the plasmids should be inexpensive to make and effective in the presence of antibody to the donor agent. The next few years should see just how good this approach may be in humans.

Plants as sources of future vaccines? There are encouraging early experiments showing that some antigens can be produced by transfected plants, and even molecules as complex as antibodies have been prepared in this way.

Prospects for the future

One might think from the above that from now on it should be possible to make a vaccine to any infectious agent. But there are many other factors which

Table 3 Main candidates for use as live vectors

<i>Virus</i>	<i>Bacteria</i>
Vaccinia virus	BCG
Fowlpox virus	<i>Salmonella</i>
Canarypox virus	<i>Escherichia coli</i>
Herpesvirus	
Adenovirus	
Poliomyelitis virus	
Mengo virus	
Baculovirus	

Table 4 Factors favoring infectious agent control or eradication by vaccination

1. Only one or a small number of causative strains
2. Agent is only moderately infectious
3. Absence of subclinical cases and a carrier state
4. Infectious process is acute: agent does not persist
5. Generates both antibody and full CMI response
6. Viral DNA is not integrated into host cell genome

Eradication

1. Infectious agent is limited to human host - no animal or environmental reservoir
2. Infection causes a characteristic clinical disease
3. There is a simple marker of successful vaccination

can influence at least the rate at which a vaccine is developed. Table 4 lists some factors which favor vaccine development. It will be noticed that many of the agents for which vaccines are already available fulfill many of these criteria. In contrast, a variety of factors can militate against rapid vaccine development (Table 5). For example, many of these later ones apply to HIV.

Table 6 gives an estimate of the vaccines which may become available within the next 10–15 years. One development which will need to accompany the release of many new vaccines is the need to make combinations. A major cost in this area is the delivery of vaccines to the populace. The current combinations, MMR and DTP, have been highly successful. Determining those vaccines which might be added to existing combinations, such as hepatitis B and *H. influenzae* type B, has become a major activity for vaccine manufacturers.

See also: Adjuvants; Attenuated organisms as vaccines; Bacteria, immunity to; BCG; Carbohydrate antigens; Cell-mediated immunity; Humoral immunity; Idiotypic; Immunogen; Parasites, immunity to; T cell vaccination; Vaccination, methods of adminis-

Table 5 Contrary factors to the control of an infectious disease by vaccination

1. Antigenic variation - many serotypes (antigenic drift shift)
2. Integration of viral DNA/cDNA into the host cell genome
3. Infection may be transmitted by cells which are latently infected
4. Agent grows in monocytes/macrophages; antibody to surface antigens may enhance infection of these cells
5. Agent infects cells in immunoprivileged sites in the host
6. Crucial cells of the immune system are infected and destroyed or their function impaired
7. Failure to induce protective antibody or appropriate CMI responses

Table 6 Some disease agents targeted for vaccine development for use in three age groups

Infants	Adolescents	Adults
Dengue virus	<i>Chlamydia</i>	<i>Helicobacter pylori</i>
Hepatitis A virus	HIV-1,2	HIV-1,2
Influenza	Hepatitis C virus	Influenza
Respiratory syncytial virus	Epstein-Barr virus	Malaria
Rota virus	Cytomegalovirus	Leishmaniasis
Parainfluenza	Human papillomavirus	Leprosy
Malaria ^a	Herpesvirus type 2	<i>Shigella</i>
	<i>Gonococcus</i>	
	<i>Treponema</i>	

^aIn tropical countries.

tration; Vaccines, adverse reactions to; Viruses, immunity to.

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VACCINES, ADVERSE REACTIONS TO

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Vaccines developed from the need to prevent the continuing morbidity and mortality from infectious diseases. The earlier attempts included variolation, the practice of deliberately inducing disease by transmission of material from smallpox pus or scabs which was practised in China and India and spread to the Middle East and thence to Europe and America. Despite the high risk of variolation (0.2–2% mortality), it was much less than the risk of death from being infected during a smallpox epidemic. Benjamin Franklin stated in his autobiography, 'In 1736, I lost one of my sons (Francis Folger), a fine boy of four years old by the smallpox. I long regretted bitterly and still regret that I had not given it to him by inoculation. This I mention for the sake of parents, who omit the operation on the supposition that they should never forgive themselves if a child died under it; my example shows the regret may be the same either way, and that therefore the safer should be chosen.'

The history of vaccination has many dark episodes and it has been vigorously opposed by different groups during the 200 years since Edward Jenner showed the value of 'vaccinating' with material from cowpox in protecting from a subsequent attack of smallpox. The value of vaccination has always been a balance between benefit and risk.

For livestock, the benefit outweighs the risk; for medical use, safety has increasingly become the major consideration. Rigorous criteria for the assessment of a candidate vaccine's safety and efficacy now exist, particularly in a number of industrialized countries. Prior to human use, regulatory authorities, such as the Food and Drug Administration (FDA) in the USA, require extensive animal tests for toxicity and sometimes for teratogenicity, and trials for efficacy in lower primates. The primate may or may not be an entirely relevant model for the human disease. For example, neither the chimpanzee for human immunodeficiency virus (HIV) nor different monkeys

for *Plasmodia* reproduce the human disease symptoms, but at least the animals sustain an infection.

Clinical trials in humans occur in three or four stages which may vary according to the preparation. In a phase 1 trial, the candidate vaccine is administered to human volunteers to test mainly for safety but also where practical for an indication of immunogenicity. If a vaccine is produced in an industrialized country, it is usual for the trial to be carried out in that country, even though the vaccine may be destined for use in a developing country. Phase 2 trials are conducted to test further safety and immunogenicity, but in addition, an indication of the efficacy of the product in those who may be exposed to the infectious agent. Phase 3 trials are carried out in the field on a much larger number of participants, perhaps up to 100 000 depending on the prevalence of the disease in the population. Phase 3 trials in particular may be very expensive, sometimes running into millions of dollars. Finally the vaccine is licensed or registered for use but where possible, a large number or a cohort of vaccinees should be followed in a postregistration program.

A candidate vaccine may run into difficulties at any of these stages or after registration, depending on the frequency of occurrence of the side-effects. Several examples are now quoted which illustrate past or present experiences.

Respiratory syncytial virus

A member of the pneumovirus genus, respiratory syncytial virus (RSV) is a highly important cause of lower respiratory tract disease in infants. It replicates at that site in several species, but the mouse and cotton rat are the main models, though neither accurately demonstrate the symptoms of the human disease. Shortly after its discovery, virus grown in African green monkey kidney tissue culture was inactivated to form a vaccine. The rationale for this approach was the prior experience with both the influenza and poliovirus vaccines which had been prepared using similar approaches, though an early batch of the latter had been found to be incompletely inactivated. However, the candidate RSV vaccine not only failed to confer protection against a subsequent RSV infection, but the disease which RSV vaccinees experienced on subsequent infection with the wild-type virus was unexpectedly severe, many vaccinees developing pneumonia or bronchiolitis with some deaths. Both humoral and cell-mediated reactions may have contributed to this result. Many attempts to make an effective subunit vaccine have since been made, but currently, a live, attenuated vaccine may turn out to be the best preparation.

Influenzavirus vaccines

To avoid a possible outbreak in 1976 of swine influenza (the viral subtype which caused the 1918 influenza pandemic in which an estimated 20 million people died), a rush program was instituted in the USA to vaccinate many millions of US citizens with a vaccine containing this serotype. The vaccine went through the usual tests during which it was administered to many volunteers. But in a total of 41.5 million people who finally received the vaccine, about 500 cases of Guillain-Barré syndrome occurred in vaccinees within a few weeks of vaccination, an incidence of about 1/80 000 recipients. The chances of detecting such a relationship in clinical trials is negligible. This relationship had not been seen previously or since, and it has been speculated that such an effect might only be seen in an extremely large, rushed program.

***Bordetella pertussis* vaccination**

The whole-cell (inactivated) pertussis vaccine is the most reactive component of the triple antigen diphtheria-tetanus-pertussis (DTP) vaccine. Several categories of reaction are recognized. Some of these, like protracted bouts of crying with unusual shock-like states or minor local and systemic effects, do not have long-term consequences. Another category includes more major neurological reactions which may lead to permanent disability. It has been extraordinarily difficult to establish a causal relationship between such injuries and vaccination. For example, the frequency of encephalopathy with residual brain damage one year after DTP administration was estimated to be 1/300 000 cases, but the correct figure could be between 1/54 000 and 1/5 300 000 cases.

A study by the Institute of Medicine (IOM) in the USA concluded that the pertussis component of DTP is responsible for rare instances of encephalopathy, but not for other disabling effects. Some countries stopped vaccination with this vaccine and in the UK this had disastrous effects following adverse publicity in the media in the mid-1970s (Figure 1). Two severe outbreaks of disease occurred with 29 deaths, and hundreds of cases of serious complications. Following a government publicity campaign, public acceptance of the vaccine increased. But there was obviously a need for a less reactogenic vaccine, and recently, an acellular preparation was licensed for use in the USA, as part of a trivalent combination, DTaP.

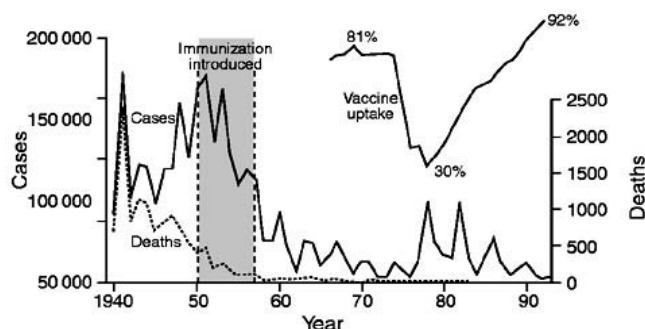


Figure 1 Whooping cough notifications: cases and deaths, England and Wales 1940–1993 (Source: OPCS. Prepared by CDSC).

Other childhood vaccines

More recently, the IOM has published findings on adverse events associated with a number of other childhood vaccines, to see whether any evidence bearing on causality could be established. Reactions to the diphtheria and tetanus toxoids, measles, mumps, polio, hepatitis B, and *Haemophilus influenzae* type B (Hib) vaccines, were examined. Evidence for neurological disorders (both demyelinating and nondemyelinating diseases) and a series of immunologic reactions (anaphylaxis, Arthus reaction, delayed-type hypersensitivity and autoimmunity in particular) was sought. After an exhaustive review of the available data, the findings suggested that these vaccines are remarkably safe.

Chimeric virus vectors

One of the first fruits of the biological revolution brought about by recombinant DNA technology was the concept of using some current viral vaccines as vectors of DNA coding for antigens from other infectious agents. Much work has been carried out using vaccinia virus (the smallpox vaccine) as the vector. But the use of this vaccine on very large numbers of people during the smallpox eradication campaign revealed that there was an unacceptably high incidence of undesirable reactions (Table 1), so that it is unlikely that these days a medical vaccine based on the use of this virus would be approved. Two approaches have been used to overcome this. One was to selectively delete particular DNA sequences from the viral DNA. The resulting virus, called NYVAC, was shown to be much less reactogenic but to retain the immunogenicity of the parent virus. A second approach was to use a poxvirus – fowlpox or canarypox viruses – which undergo only abortive infections in mammalian cells. These have given encouraging early results and should be safer than vaccinia virus.

Table 1 Reported cases (deaths) per million in the United States following smallpox vaccination

Reported complications	Primary vaccination (5 959 000)
Encephalopathy	6.5
Encephalitis	2.4
Progressive vaccinia	0.9
Eczema vaccinatum	10.4
Generalized vaccinia	23.4
Accidental infection	25.4
Other complications	11.8
Total	80.8

There was a much lower frequency of complications after revaccination and occasionally, complications occurred in contacts of vaccinated people.

Groups at risk from vaccination

Certain groups have a higher morbidity and mortality rate to natural infections compared to the normal population. This includes diabetics (influenza and pneumococcal pneumonia), immunocompromised or immunodeficient people. Pregnant women represent a special case because of the potential harm to the embryo. Live agent vaccines should be withheld from such groups. On the other hand, maternal immunization to protect the fetus and mother from certain diseases, e.g. tetanus, is the aim of the World Health Organization.

As vaccine development becomes more highly specialized with the great potential for peptide and subunit preparations, for recombinant live vectors and for direct immunization with nucleic acids, the risk of side-effects following vaccination should decrease. It may never be possible however to completely eliminate all hazards but these are likely to occur at such a low frequency that they are detectable only after mass immunizations. Though society in the long run must make the decisions, the eradication of smallpox, the elimination of poliomyelitis from the

Americas and of measles from Finland and from some Caribbean communities argues strongly in favor of vaccination.

See also: **Attenuated organisms as vaccines; *Bordetella*, infection and immunity; Immunodeficiency, primary; Maternal antibodies; Influenza virus (*orthomyxovirus*), infection and immunity; Paramyxoviruses, infection and immunity; Vaccines.**

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VALENCY OF ANTIGENS AND ANTIBODIES

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Valency is an intrinsic property of both antigens and antibodies, the term being adopted early in the description of these molecules to carry the concept of binding properties and to define its numerical complexity. Confirmation of the structural basis of valency came with the subsequently acquired knowledge of the nature of binding sites of antibodies (paratopes) and of antigenic determinants (epitopes) of antigens.

Valency is usually considered in operational (or effective) terms, the valency of an antigen being a statement of the number of paratopes with which each antigen molecule can (or does) combine under particular conditions and, likewise, the valency of an antibody is the number of epitopes with which an antibody can (or does) combine. Native antibody molecules carry a theoretical (structural) valency of two (divalent, with two binding sites per molecule) or a multiple of two, according to the degree of polymerization of the antibody involved (see below: Valency of antibodies). Subunits of antibodies carrying a single binding site have a valency of one. Operationally, native antibodies may express a valency less than that defined by structure for several reasons outlined below.

Antigen molecules carry, by definition, at least one epitope. Monovalent antigens are rare in nature but can be produced as peptides and chemical haptens and these have been used extensively to study antibody valency, specificity, and affinity. Most native

antigens are multivalent and often carry a mosaic of structurally different epitopes. Multivalency in both antigen and antibody has important consequences in terms of how these molecules interact; it is the basis for the formation of immune precipitates (or agglutinates) which consist of a complex lattice of bound antigen and antibody molecules. Through this the physiological host defense purpose of the antibody response is achieved as it leads, through various pathways, to the neutralization and removal of bacterial and other toxins, the inhibition of virus entry to host cells and of adhesion of bacteria to tissues, the fixation and activation of complement, efficient phagocytosis of microorganisms and the removal of foreign molecules from the circulation. Precipitation and agglutination have been exploited as *in vitro* reactions for diagnostic purposes and to measure antibodies and antigens.

Valency has been applied in some circles to describe the specificity of antisera. In some branches of serology antisera are referred to as monovalent or polyvalent to define the extent of their reactions with a range of antigens. A good example of this other usage of antibody valency came from the production of anti-snake venom antisera for therapeutic use in some parts of the world. Antisera reacting with (neutralizing) only a single (immunizing) species venom are described as monovalent, whereas others, either cross-neutralizing other species, or raised deliberately to a mixture of species' venoms are

described as polyvalent. This practice persists although it is tending to be replaced by descriptions of specificity.

Valency of antigens

Monovalent antigens are molecules which, with respect to antibody, carry a single epitope. Antigens of this structural simplicity are not normally immunogenic – i.e. they cannot individually induce an antibody response. However, antibodies to small chemical groups (haptens) were the basis of much important classical early work that revealed the remarkable diversity potential of antibodies and the structural features and limitations of antigenic determinants. The limitations to immunogenicity of simple chemical groups was overcome by coupling (as haptens) to larger carrier molecules; some of the antibodies then induced are specific to the hapten and bind to soluble monovalent hapten in solution, the latter behaving as a homogeneous monovalent antigen.

The reacting system of antibody and hapten is sufficiently homogeneous to allow a good estimate of antibody binding affinity. Antibodies binding to monovalent antigens in solution carry no potential for immune complex lattice formation and the measurement of binding requires certain strategies. Haptens in free solution will cross a dialysis membrane into an antibody solution and binding affinity of the antibody can be measured by means of equilibrium dialysis in which, in effect, the capacity of the antibody to hold diffusible hapten in its compartment is a measure of its affinity. High-affinity antibodies have been used since the early 1960s in radioimmunoassays to measure the concentration of small peptide and steroid hormones in the blood which behave as monovalent antigens. The antibodies are usually raised by using hormone-carrier conjugates as immunogens, and the binding of free hormone in the assay is measured after precipitation of soluble complex, usually by addition of divalent antiglobulin.

Monoclonal antibodies have homogeneous specificity towards a single epitope of complex antigens and are thus very different from conventional antisera raised to the same antigen. Except where epitopes are repeated on the molecule, monoclonal antibodies treat a target molecule as a monovalent antigen. Mixing monoclonal antibodies to different epitopes of a complex antigen has shown that an antigen needs to express a valency of four to achieve precipitation. The reaction of a monoclonal antibody with a hapten in solution represents the most homogeneous system for measuring antibody affinity.

Polyvalent antigens are structurally complex molecules with intrinsic immunogenicity that elicit complex antibody responses. The reaction with antibody *in vitro* of polyvalent antigens is a correspondingly complex phenomenon with a mixture of antibody specificities binding to different epitopes with different affinities. No true affinity measurement can be achieved in such a heterogeneous system but the overall binding capacity of the antibody mixture (antiserum) for the antigen can be measured and more generally described as the avidity.

Under appropriate conditions antibodies will operate as bivalent molecules and this leads to complex precipitation. The precipitation phenomenon with polyvalent antigens has been studied throughout the twentieth century, although an adequate theoretical and mathematical model of precipitation has yet to be formulated. The first precipitin equation was put forward by Arrhenius on observations of precipitation of serum proteins by antiserum (1907). He assumed that the soluble reactants and the insoluble product were in equilibrium according to the law of mass action.

By the 1930s it had been shown by Heidelberger that precipitation could be used to measure absolute amounts of antibody nitrogen in antisera to polyvalent pneumococcal polysaccharide and a further set of classical experiments was performed with egg and serumal bumin. Antibody measurement depended upon the critical observation that precipitation occurred at optimal proportions of antibody and antigen, with greater amounts of reactants remaining in solution in conditions of increasing antibody and antigen excess on each side of the equivalence zone. At equivalence, antibody is brought completely out of solution and the zone ends as conditions move into mild antigen excess. Heidelberger's data on the content of antibody and antigen in complexes formed at different molar ratios have been extensively analyzed. At equivalence the effective valency of antibodies to albumin approaches a figure of two and the valency of egg albumin is five in regard to one studied antiserum. The composition of the precipitate is thus of 2–3 antibody molecules per antigen molecule, with most antibodies utilizing both binding sites in the complex.

In contrast the precipitate formed in conditions of antibody excess contains a predominance of antibodies with an effective valence of one, the degree of precipitation depending upon the extent to which some antibodies can still operate as divalent molecules to bridge between antigens. In growing antigen excess, the precipitate again begins to break down with more antigen in solution. The antigen component still involved in precipitation dwindles in

proportion to antibody and the effective valency rises, becoming, for egg albumin, eight when the total amount of the antigen in the system is twice that of equivalence. In conclusion, it has to be recognized that valency expressed by multivalent antigens, and by antibodies, is a property that is variable according to the conditions of interaction and this in turn affects the nature and properties of the product. Physiologically, immune complexes formed in antibody excess are more efficiently removed and are less pathogenic, whereas small soluble complexes formed in antigen excess are less readily removed and may be deposited in tissues to cause inflammatory, tissue-damaging reactions.

Valency of antibodies

Most antibodies in the serum of hyperimmune animals, i.e. of the sort used in the original precipitation studies which measured their valency properties, are known to consist predominantly of immunoglobulin G (IgG) which, as described above, exhibits at equivalence a valency approaching two consistent with its determined structure. While all antibodies have a basic unit structure similar to IgG, some other classes of antibody exist in polymeric form and in consequence may express a greater valency.

IgA can exist in polymers of various sizes, the most common being found in secretions as a dimer with the two units joined end-to-end by a J chain. Such molecules have a structural valency of four. This is of little significance in considering antibodies in serum as most IgA in serum of normal individuals is monomeric. However, the polymeric secretory IgA active at mucosal surfaces may owe its protective properties in part to the distribution of binding sites and polyvalent nature.

Serum IgM antibodies are constructed on a pentameric form with ten combining sites. In some circumstances, especially in primary responses, IgM may be a significant component of specific serum antibody and its multivalency offers certain distinct advantages in efficient bacterial and viral agglutination and complement fixation. In practice steric hindrance

between binding sites and the local availability of antigen epitopes for all binding sites may reduce the effective valency of IgM to less than that predicted by structure. In reactions of pure IgM antibody with soluble antigen a valency of five is given as the normal property of this molecule.

The special properties of IgE antibodies depend upon it first attaching through receptors to the surface of mast cells and basophils. In this position they are known to bind antigen (allergen) to initiate the release of mediators of immediate hypersensitivity. Evidence suggests that the trigger mechanism for mediator release depends upon the cross-linking of IgE molecules on the cell membrane by allergen, and the divalent property of IgE would be an essential feature of this mechanism.

Recent studies suggest that antigen valency may also be a critical factor in mast cell degranulation. In an experimental system with rat leukemic mast cells, a conjugate of dinitrophenyl (DNP) hapten with a carrier molecule, with high DNP valency (DNP32-HSA) gave a much greater degranulation response by anti-DNP IgE-coated mast cells than the same allergen design with lower DNP valency.

See also: Affinity; Agglutination; Allergens; Antigen-binding site; Antigens; Antiserum; Epitopes; Hapten; IgA; IgE; IgG; IgM; Immunogen; Immunoglobulin structure; Joining J chain; Precipitation reaction.

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VARICELLA-ZOSTER VIRUS, INFECTION AND IMMUNITY

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Varicella-zoster virus (VZV) is one of seven human herpesviruses. The other six are herpes simplex virus 1 (oral), herpes simplex virus 2 (genital), cytomegalovirus, Epstein-Barr virus, human herpesvirus 6, and human herpesvirus 7. An eighth herpesvirus-like DNA sequence has been discovered recently in tissues obtained from Kaposi sarcoma; this agent has been designated Kaposi sarcoma herpesvirus. VZV is the etiologic agent of two distinct illnesses. The primary infection is called chickenpox or varicella. Virus reactivation in adulthood causes the dermatomal exanthem shingles or herpes zoster.

Characteristics of the virus and its antigens

The human herpesviruses share a common morphology and genome structure. The VZV virus is about 200 nm in diameter and includes an inner nucleocapsid surrounded by a lipid envelope from which protrude the immunogenic viral glycoproteins. The DNA molecule is housed within the nucleocapsid. The sequence of the genome contains nearly 125 000 base pairs, including 71 open reading frames which may encode viral proteins. Six of the open reading frames specify viral glycoproteins; these have been designated gB, gC, gE, gH, gI and gL (Figure 1).

The glycoproteins are important structural components of the viral particle; in addition, they are inserted into the plasma membrane of the infected cell. As such, they are important antigens by which the host regulates the immune response after infection. The predominant viral glycoprotein is gE, a

98 kDa product which is the main component within the membrane of the infected cell. This glycoprotein is easily identified in cells removed from a chickenpox or zoster vesicle. Therefore, it is an ideal antigen for rapid diagnosis of VZV infection with a monoclonal antibody probe. Monoclonal antibodies to this antigen have neutralization activity only in the presence of an exogenous source of complement.

Varicella-zoster virus gB is a 140 kDa product which is the second most prominent viral glycoprotein constituent of the infected cell membrane. Antibodies to this glycoprotein exhibit complement-independent neutralization as evidenced by their ability to reduce VZV plaque formation. The same antibodies inhibit VZV-induced cell-to-cell fusion in infected monolayers. The third glycoprotein, VZV gH, has a molecular weight of 118 kDa. This glycoprotein is barely detectable in the membrane of infected cells. However, of the three major glycoproteins, it appears to possess the most immunogenic neutralization epitope. Monoclonal antibodies to VZV gH have the ability to inhibit plaque formation at dilutions of 1:40 000. Glycoprotein gL forms a complex with gH and, likewise, gI forms a complex with gE. The gE/gI complex can bind to the Fc portion of immunoglobulin G (IgG) and both components of the complex contain phosphorylation sites in their cytoplasmic tails. In addition to the glycoproteins, other virally encoded proteins are important antigens, but they have been less well characterized.

Immune responses of the host to chickenpox

In order to properly understand the immune response of the host, it is first necessary to visualize the pathogenesis of primary VZV infection. A schema is shown in Figure 2. This model includes two viremic stages, with the characteristic chickenpox exanthem appearing after the second viremia. Chickenpox is acquired from small virus-laden droplets which are carried by air currents from an infected child to a nonimmune (susceptible) individual. The site of infection is probably the conjunctivae or the

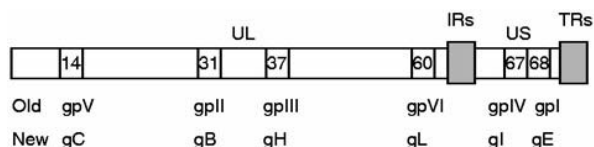


Figure 1 Diagram of the VZV genome. The VZV genome contains about 71 open reading frames. The locations of the glycoprotein genes are designated by their gene numbers. Both the old and the new nomenclatures are listed. UL, unique long sequence; US, unique short sequence; IRs, internal repeated sequence; TRs, terminal repeated sequence.

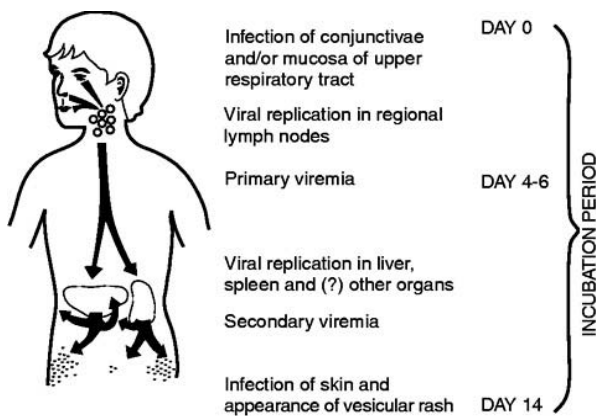


Figure 2 Schema for pathogenesis of chickenpox. This scheme is explained in greater detail in Grose (1981). (Reproduced by permission of the American Academy of Pediatrics.)

nasal mucosa, as illustrated in **Figure 2**. An IgM immune response is detectable by the end of the incubation period, when the vesicular rash first appears. One of the better tests to measure VZV-specific antibodies is by fluorescence antibody to membrane antigen (FAMA); this indirect fluorescence method detects antibodies to VZV antigens present in the outer membrane of live VZV-infected cells. The first antibodies to appear are often directed to VZV gB, followed in rapid succession by antibodies to gH and gE. The infected host also develops antibodies to certain nonglycosylated structural components of the virion.

In addition to the humoral response, individuals with chickenpox mount a cellular immune response. Among the important targets for cellular immunity following VZV infection are the same viral glycoproteins that are recognized by antibodies, as well as the regulatory protein called IE62. When measured by lymphocyte proliferation assays, most healthy individuals with chickenpox develop proliferative responses to VZV gE, gI, gB, gH and IE62 antigens. The responder cell frequency has been estimated at 1:39 000 in adults who had chickenpox as children. In cytotoxicity assays, both CD8⁺ class I-restricted T cells and CD4⁺ class II-restricted T cells mediate VZV-specific cytotoxicity. The mean precursor frequency for T lymphocytes that recognize gE is 1:121 000; the precursor frequencies were similar for CD4⁺ and CD8⁺ populations. The VZV cellular responses appear to limit the severity of the chickenpox exanthem in normal hosts.

Immune response following VZV reactivation

Most children with chickenpox develop a latent VZV

infection, i.e. the virus remains dormant in the dorsal root ganglia for decades. However, in late adulthood, the virus occasionally reactivates and causes herpes zoster (shingles). Waning immunity over a lifetime is considered to be an important factor in reactivation. In the weeks prior to the onset of zoster, very little anti-VZV glycoprotein antibody is present in the serum. Cellular immunity is similarly diminished; in particular, the frequency of cells producing interferon γ (T_H1 cells) declines more than those producing interleukin 4 (T_H2 cells). Within a few weeks after appearance of shingles, there is a dramatic anamnestic humoral response, resulting in high titers of VZV-specific antibody. Likewise, there are heightened lymphoproliferative responses to VZV antigens shortly after onset of herpes zoster.

Immune globulin and vaccines

Chickenpox can be prevented by administration of high-titer human varicella-zoster immune globulin (VZIG) by intramuscular injection. In order to be effective, the globulin must be given within 3–4 days after exposure to chickenpox. Based on the schema of pathogenesis (**Figure 2**), the most likely explanation is that antibody to VZV abrogates the primary viremia and thereby inhibits spread of virus throughout the host.

The medical indications for VZIG include prevention of chickenpox in VZV seronegative children with cancer who are exposed to chickenpox; VZIG is also administered to newborns, especially premature newborns, who are exposed to chickenpox in the postpartum period. Some experts recommend VZIG administration to VZV seronegative pregnant women who are exposed to chickenpox, but there are conflicting data about VZIG efficacy in adults. The data are difficult to interpret because the clinical studies have been performed with two different VZIG preparations (one produced in the USA and another made in Europe). Not only are the VZIG preparations prepared in different facilities, the recommended dosages differ between the European and North American preparations. In the USA, one infant with congenital varicella syndrome has been born to a pregnant woman who received VZIG but still developed gestational chickenpox. These issues have been recently reviewed (see Further reading).

A live attenuated varicella vaccine was developed in the 1970s and is currently licensed in Japan, Europe and the USA. The vaccine virus was designated Oka strain after the name of the 3-year-old boy with chickenpox from whom it was isolated. The first human studies of the VZV vaccine were carried out in Japan with subsequent clinical trials in Europe and

the US. Because of their increased susceptibility to severe complications of chickenpox, the initial recipients of the vaccine were children with leukemia. Initially, one vaccination was given. However, because of low seroconversion rates the number of vaccine doses was raised to two injections. Under the latter regimen, 95% of vaccine recipients developed antibody; 66% remained seropositive 3 years after immunization. A small number of vaccine recipients have developed clinical chickenpox after exposure in the community; this disease is called 'breakthrough chickenpox'. Usually the disease has been mild.

Based on efficacy data derived from the large clinical trials in the USA, the vaccine is estimated to be 80% effective in children with leukemia. In other clinical trials, the vaccine was administered to healthy children in Japan and the USA. Healthy children require only a single immunization. Immunized children have been followed for several years; over 90% of the vaccinees showed persistence of both cellular and humoral immune responses. Like the children with leukemia, however, a few healthy vaccinees with declining immunity have occasionally contracted breakthrough chickenpox following a community exposure.

Healthy young adults who failed to contract chickenpox as children have been immunized in clinical trials. Two doses of vaccine were required to achieve a seroconversion rate of 92%. Even after two doses, the cellular immune responses of immunized adults were significantly lower than those of immunized children. Furthermore, at 1 year after immunization, the lymphocyte proliferation indices continued to be lower in the adult population.

Finally, varicella immunization has been investi-

gated in seropositive adults older than 55 years of age, who had a history of wild-type chickenpox as children. Both cellular and humoral immunity was boosted by immunization with live attenuated varicella vaccine. Future studies will investigate whether shingles can be prevented by booster immunization of the elderly.

See also: *Attenuated organisms as vaccines; Cytomegalovirus, infection and immunity; Epstein-Barr virus, infection and immunity; Herpes simplex virus, infection and immunity; Herpesvirus-6, infection and immunity; Vaccines; Viruses, immunity to.*

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VENOMS

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Historical background

The immunological effects of envenomation have been known since ancient times. Ancient Egyptian hieroglyphics indicate that the Pharaoh Menes of Memphis died 5000 years ago from the sting of a great wasp. In modern times, Waterhouse first distinguished between allergic reactions to insect venoms and the natural toxic effects in 1914. Then Braun introduced skin testing with extracts prepared

from honeybee abdomens in 1925. The first treatment with pure venoms was reported by Loveless in 1956, and in the 1970s Lichtenstein, Valentine, Sobotka and coworkers conclusively demonstrated that Hymenoptera venoms were the materials of choice for diagnosis and treatment of allergic reactions to stings. The insect venom model has been extensively studied in attempts to understand the mechanism by which immunotherapy with allergen reduces clinical hypersensitivity.

Insects

Allergic reactions to the venoms of social Hymenoptera are relatively common. Estimates of the prevalence of stinging insect allergy by history range from 1 to 10% of the population. A random screen of industrial employees in the mid-Atlantic USA found that 26% exhibited evidence of sensitization by being either skin test or radioallergosorbent test (RAST or specific immunoglobulin E (IgE) antibody measurement) positive. Highly exposed populations have been found with over half of the subjects positive. The major insects causing reactions are those of the genera *Apis* (honeybees) and *Bombus* (bumblebees) in the family Apidae; *Vespula* (yellowjackets), *Dolichovespula* and *Vespa* (hornets) and *Polistes* (paper wasps) of the family Vespidae; and a wide variety of stinging ants of the superfamily Formicoidea, especially the genera *Solenopsis* (fire ants) and *Pogonomyrmex* (harvester ants). Many of the venoms have been extensively characterized and the allergens identified. Honeybee venom protein allergens include phospholipase A₂, hyaluronidase, acid phosphatase, allergen C and the aggregated peptide melittin. The vespid wasp venoms contain hyaluronidase, a phospholipase A₁B unrelated to the bee phospholipase, an invertebrate neurotoxin called antigen 5 and at least two minor proteins. Bumblebee venoms contain a tryptic amidase. Venoms from ants of the genus *Solenopsis* are over 90% water-insoluble piperidine alkaloids, but contain nanogram amounts of three or four allergenic proteins de-

pending on the species. *Pogonomyrmex* venom contains several enzymes including a phospholipase. Ponerine ant venoms are as yet uncharacterized. Australian bulldog and jack-jumper ants (*Myrmecia* spp.) contain a series of apparently unique allergens. Immunologic cross-reactions are commonly seen among the vespid venoms and between bee and vespid hyaluronidase. Allergen Sol i and r I from *Solenopsis* cross-reacts with vespid wasp venoms. Well-characterized insect venom allergens are summarized in Table 1.

There is good evidence that almost all severe reactions to insect venoms are IgE mediated. These range from extensive subcutaneous swelling contiguous with the sting site (large local reactions) to fatal anaphylaxis. In several studies specific IgE antibodies against venom were demonstrated in blood from all victims of fatal sting anaphylaxis. Uncommon reactions to venoms include a serum sickness-like syndrome, recurrent cold urticaria, and a nonimmunologic mediator release that mimics anaphylaxis in patients with mast cell disorders like urticaria pigmentosa. IgE antibodies in patients can be detected by either intradermal skin testing with 1 µg ml⁻¹ or lower concentrations of venom or by *in vitro* tests for specific IgE antibodies. High concentrations of venom may give nonspecific skin reactions because of the mediator and basic peptide contents of venoms. Sting challenge is used as a research tool for diagnosis and evaluation of immunotherapy. Approximately 50–60% of untreated patients with a

Table 1 Characterized insect venom allergens

Allergen	From	Molecular weight	Related to	Male related
Phospholipase A ₂	Bees	16 000 14 500 (–CHO)	Vertebrate secretory phospholipase A ₂ s	+
Hyaluronidase	Bees, wasps	43 000 39 000 (–CHO)	Vertebrate hyaluronidases	+
Acid phosphatase	Bees	48 000 (monomer)	Prostatic acid phosphatases	+
Melittin	Honeybees	11 360 (tetramer)		
Tryptic amidase	Bumblebees	27 250	Acrosin, clotting factors	+
Phospholipase A ₁ B	Wasps, ants	33 500	Lipoprotein lipases, vitellogenins	
Antigen 5	Wasps, ants	23 000	CRISPs, Helminth secretory proteins, plant pathogenesis-related proteins, human glioma protein	+
Sol 2	Fire ants	26 432 (dimer)	Sol i 4	
Sol i 4	Fire ants	13 340	Sol 2	
Myr p 1	Jack-jumper and bulldog ants	9 103 (precursor)	Melittin precursor	
Myr p 2	Jack-jumper and bulldog ants	8 144 (precursor)		

CRISP, Cysteine-rich secretory proteins; –CHO, protein without carbohydrate.

recent history of a prior systemic reaction will have a systemic reaction to a live sting challenge. Extensive studies using sting challenges have demonstrated a 90–98% efficacy of venom immunotherapy in protecting from systemic reactions.

The mechanisms of protection in venom immunotherapy have been extensively studied. It was first noted that beekeepers who are frequently stung suffer systemic reactions very rarely, while those that are stung once or twice a year have a high incidence of reactions. Laboratory studies showed comparable prevalence and levels of specific IgE antibodies in both groups, but very high levels of specific IgG antibodies in the frequently stung group. A group of volunteers who were sting challenge positive were administered IgG fractions prepared from beekeepers with a high level of specific IgG antibody. On rechallenge all experienced either no reaction or much milder symptoms. It is proposed that the IgG acts as a blocking antibody, preventing the venom allergens from combining with mast cell-bound IgE. During the first years of immunotherapy there is a good correlation between increased specific IgG levels and a state of protection. Long-term studies of patients on immunotherapy have shown that virtually all patients can stop receiving venom after 5 years and that they remain protected for at least 2 additional years, even though the specific IgG has significantly declined and the specific IgE is still present. The mechanism of this effect is not known. Studies of the natural history of venom allergy have shown that children tend to lose their specific IgE antibodies and reactivity naturally; however many adults remain sensitive for 20 years or more.

Ants of the genus *Pseudomyrmex* have a venom that inactivates C2, C3, C4, B and I components of complement. It also contains a trypsin-like enzyme. *In vivo* this venom has a significant anti-inflammatory effect.

Arachnids

Over 1000 people die annually from scorpion stings. Scorpion venoms contain neurotoxins, cardiovascular toxins and toxins that induce necrotic inflammation, e.g. pancreatitis. The neurotoxins have been extensively studied and are generally basic polypeptides of 60–65 amino acids with four disulfide bonds. The α -toxins slow down the inactivation of the sodium channel and the β -toxins bind synaptosomes at a distinct site and are voltage independent. Many of the neurotoxins have been sequenced and their immunology extensively studied. The toxins show more antigenic cross-reactivity between species than between types and subtypes of toxins. Scorpions can

also project venom, which can cause severe eye damage. A variety of antivenoms are prepared in France, Egypt, Mexico, Brazil, Britain and other countries. Some cross-neutralization has been found between antivenom for the North African *Buthrus* and North American *Centruroides*, but it is best to use the antivenom preparation raised against the appropriate species.

All spiders can inject venom when they bite. Severe reactions usually fall into two patterns: lathrism and loxoscelism. Lathrism consists of excruciating cramp-like pain, chest tightness and various other symptoms. It is primarily mediated by a neurotoxin and follows bites of the genera *Latrodectus*, black widows and *Atrax*. Loxoscelism is a necrotic type of reaction, many times exhibiting repeated waves of partial healing followed by expanding necrosis. Hemolytic anemia is the most common systemic symptom. Occasionally a slow progressive deterioration develops, ending in death. Bites of spiders of the genus *Chiracanthium* may lead to reactions that are both neurotoxic and locally necrotic. Antivenom prepared in horses against *Latrodectus* is commercially available in the USA. Antivenom against *Loxosceles* is only useful when administered within 36 h of the bite.

Reptiles and amphibians

Poisonous snakes belong to four families, Viperidae or vipers with two subfamilies Viperinae and Crotalinae, Elapidae or fixed front-fanged snakes, Hydrophidae or sea snakes including some Australian snakes, and Colubridae or back-fanged snakes. The crotalid venoms are usually toxic to tissues, although there are some species that have venoms with primary neurotoxic activity. The elapid venoms function primarily as neurotoxins; some contain factors that react with complement components (e.g. cobra venom factor). In various venoms cytotoxic, cardiovascular, anticoagulant, hemorrhagic, myotoxic, nephrotoxic, autopharmacologic or neurotoxic effects may dominate. Direct host immunologic reactions to reptile and amphibian venoms are unusual. There are now a number of reports that convincingly demonstrate IgE-mediated allergic reactions to reptilian venoms. As can all foreign proteins, venoms induce antibody formation. Individuals who suffer repeated envenomations develop high levels of protective IgG antibodies against the venoms. This has been best studied in those who milk snake venom for commercial producers.

Symptomatic envenomation is treated by injection of passive antibody, usually of horse or goat origin. Antivenom is given until the progression of symp-

toms is halted or reversed. The antivenom for crotalids is polyvalent and is known to be effective against venoms from *Crotalus*, *Agkistrodon*, *Sistrurus*, *Bothrops* and *Lachesis* species. Antivenoms against elapid, hydrophid and colubrid venoms are much more specific and are generally available in only the countries in which the particular snake is found. In the USA many snake envenomations are the result of keeping exotic species as pets. Immunologic reactions to antivenom are common. IgE-mediated allergic reactions to the foreign serum proteins can be ruled out by performing an intradermal skin test with diluted antivenom before administration. Individuals with positive skin tests need to be desensitized by having antivenom administered starting with a very small amount and doubling the dose every 15 min. Serum sickness, both primary and accelerated, is a common complication of antivenom therapy. Its incidence and severity increase in a dose-dependent manner.

Immunoassays are used in the diagnosis of snake bite, especially in Australia, where a commercial kit is available.

Marine animals

Approximately 100 species of fish inject venoms dangerous to humans. These include some sharks, rays, catfish, weever fish, scorpion fish and star-gazers. The most dangerous are *Scorpaena* (scorpion fish) and *Synanceja* (stonefish). Stings cause excruciating pain and extensive multisystemic symptoms. Antivenoms are commercially available from Australia.

Many marine coelenterates have venomous nematocysts on their tentacles or spines. The venoms generally cause direct toxic symptoms and release mediators including histamine and prostaglandins. Local treatment with 4–6% acetic acid usually reduces symptoms significantly. Allergic reactions,

presumably IgE-mediated, from *Physalia* (the Portuguese man-of-war) have been reported. The sea wasp (*Chironex fleckeri*) can deliver life-threatening stings and an antivenom is available from Australia.

See also: Allergens; Anaphylatoxins; Cobra venom factor; Cutaneous anaphylaxis; Hypersensitivity reactions; IgE; Serum sickness; Toxins.

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VETO CELLS

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Veto cells were initially proposed on theoretical grounds as cells which could inactivate mature T lymphocytes that recognize them. One could regard them as antigen-presenting cells that inactivate rather than stimulate T cells that recognize them. Cells with this property could play a useful role in the establishment and maintenance of T cell tolerance to self anti-

gens, and in the downregulation of an immune response. Veto cells are readily distinguishable from T suppressor cells, which are thought to have a specific receptor; they regulate T cells they recognize via this receptor. With veto cells recognition is the other way. They carry specific antigen and regulate T cells on being recognized.

There is a growing body of experimental evidence for the existence of a veto cell that can delete cytotoxic T lymphocyte (CTL) precursors reactive against allogeneic class I major histocompatibility complex (MHC) molecules or against antigens presented in association with self class I MHC. Certain cells in bone marrow and in fetal liver, as well as CTLs themselves, can act as veto cells. Note that when any cell (including a CTL) acts as a veto cell, specificity is determined by the class I MHC molecule and associated peptide on the veto cell surface rather than the specificity of any T cell receptor which might be present on that cell.

Veto activity was first described in *in vitro* mixed lymphocyte cultures (MLC). Letting A, B and C be three MHC different inbred mouse strains, veto cells from strain A block CTL precursor activation when included in a B anti-A MLC but not when included in an A anti-B or B anti-C MLC. Studies in this system have led to the following conclusions:

1. Veto cells must carry the antigenic determinant (including the class I MHC restricting element) against which the response is being suppressed.
2. Veto cells act early in an immune response and produce functional deletion of CTL precursors capable of recognizing them. Deletion is probably physical, with death occurring by apoptosis. Veto cells are ineffective against mature CTLs.
3. Veto activity can occur in the presence of appropriate stimulatory antigen-presenting cells and lymphokines such as interleukin 2 (IL-2).
4. Cells carrying CD8 can act as veto cells. CD8 on the veto cell interacts with the α_3 domain of the class I MHC on the recognizing T cell. This, in

conjunction with the signal through the T cell receptor, leads to the death of the T cell.

What appears to be veto activity has also been demonstrated *in vivo*. CTL precursors reactive against allogeneic class I MHC molecules or against minor H antigens can be inactivated *in vivo* by injecting the host with viable CD8⁺ T cells carrying those antigens. Survival of subsequent grafts carrying the same antigens as the injected T cells is greatly enhanced.

There are conflicting data as to whether there are veto cells capable of inactivating T cells restricted by or reactive against class II MHC. Note that if such veto cells do exist, they must necessarily carry class II MHC products and it is these products that would determine their specificity.

The *in vivo* role of veto cells remains unclear but they may be important in establishing/maintaining peripheral T cell tolerance and in immunoregulation.

See also: Cytotoxic T lymphocytes; Mixed lymphocyte reaction (MLR); T lymphocytes; Tolerance, peripheral.

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VIABILITY, METHODS OF ASSESSING LEUKOCYTE

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The advent of cell culture methodology in the early twentieth century imposed a requirement for rapid and reliable methods to assess the viability of cells maintained *in vitro*. In 1914, by establishing a positive correlation between the capacity of intact cells to exclude exogenous dyes and their viability, Evans and Schuleman laid the foundation of all viability assays based on the dye exclusion principle. Soon thereafter Pappenheimer determined the viability of

isolated thymocytes by microscopic enumeration of trypan blue stained and unstained cells. Although many dyes have been used for viability assays, the anionic dye trypan blue (961 Da) is the one most frequently employed.

The viability of leukocytes as assessed by trypan blue exclusion is usually determined by suspending the cells either in a balanced salt solution or in phosphate-buffered saline. After diluting the cell suspen-

sion with the corresponding salt solution, it is mixed with 0.4% w/v freshly filtered trypan blue dissolved in an identical salt solution. Thereafter, leukocytes are counted on a hemacytometer, and the percentage viability is calculated by dividing the number of viable (unstained) cells by the number of all counted cells (stained and unstained). Until the middle of the twentieth century, microscopic assessment of dye exclusion was the sole practical method of determining viability. Rapid strides in particle counting, computing and ancillary technologies made in the 1960s spawned a number of commercially produced, versatile flow cytometric systems. In contrast to microscopic viability assays, numerically large cell samples can be analyzed by flow cytometry within minutes. In this case the fundamental principle of viability assessment, i.e. viability implies plasma membrane integrity, remains inviolate. In flow cytometric procedures, fluorochromes such as fluorescein diacetate, 4'-6-diamidino-2-phenylindole, acridine orange and propidium iodide are commonly used instead of non-fluorescent dyes employed in microscopic assays.

All cell populations assayed for viability invariably contain dead and dying cells. Regardless of whether necrosis or apoptosis is the underlying cause of cell death, dead and dying cells undergo progressive degradation of their macromolecular components, such as RNA, and consequently lose their affinity for supravital dyes. For this reason any leukocyte population to be assayed for viability must be regarded from the onset as consisting of viable dye excluding cells, morphologically intact nonviable stained cells, and dead chromophobic cells. The failure to include all three categories of cells in viability determinations may yield a significantly higher viability index than its true value.

The identification of nonviable chromophobic cells is practically unattainable with a hemacytometer. They can, however, be identified and counted in fixed leukocyte smears after staining at acidic pH with a cationic dye such as toluidine blue. Dead chromophobic cells in these smears are recognized by the absence of cytoplasmic and/or nuclear basophilia.

Analogous determinations can be performed more expeditiously by flow cytometric methods. In such cases, unfixed cells stained with acridine orange and ethidium bromide are quantified by single-laser multiparametric flow cytometry. Moreover, cells with damaged plasma membranes (nonviable)

undergoing progressive structural degradation (apoptosis) have a lower refractive index than viable cells. As a consequence, optical properties and especially forward light scatter of such cells differ from their viable counterparts. These differences may also be used for flow cytometric assessment of cell death by forward and right-angle light scatter.

Viability assays performed with a hemacytometer and by flow cytometry usually yield higher viability values with the latter method. This quantification disparity is generally attributed to the higher sensitivity of flow cytometric systems as compared with visual observation. A real possibility that laser illumination of cells stained with photosensitizers may inflict lethal photosensitization of analyzed cells is essentially ignored in reporting results. For all practical purposes there is no ideal method of cell viability determination. Therefore, a choice of a specific assay procedure is frequently a matter of personal preference and the accessibility of requisite instrumentation and facilities.

See also: Apoptosis; Cell separation techniques; Cytotoxicity, assays for; Flow cytometry; Leukocyte culture.

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VIBRIO CHOLERAЕ, INFECTION AND IMMUNITY

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Vibrio cholerae is the causative agent of the acute diarrheal disease cholera. It is a complex pathogen expressing a variety of virulence factors necessary for adherence to and colonization of the gut, as well as a variety of toxins including the potent cholera enterotoxin and other secreted proteins. A number of protective antigens have been defined and considerable progress has been made along the path of vaccine development. However, matters have been complicated by strain variation and the ability to change, apparently under immune pressure, which only in 1992 led to a newly evolved serotype being associated with the disease.

Characteristics of the organism and the disease

Cholera is a term from the ancient Greek and the early writings could have equally well referred to dysentery and diarrhea which are associated with other enterobacterial pathogens such as *Shigella* spp., enteropathogenic *Escherichia coli* and some *Salmonella* spp. However, although Indian writings had various names for the disease, descriptions from as early as the time of Lord Buddha (500–400 BC) basically describe the disease of cholera as we know it. Cholera was probably endemic to the Ganges delta and its spread from this region corresponds well with the development of travel.

Seven great pandemics have been described, beginning in 1817. During this time cholera has produced mortality and morbidity of immense proportions to humanity, reaching every continent with the probable exception of Antarctica. The Russian composer Peter Ilich Tchaikovsky is reported to have died, under suspicious circumstances, of 'the cholera' in the outbreak of 1891–92 which was brought to European Russia by pilgrims returning from Mecca.

In 1854, John Snow brought notoriety to the Broad Street pump by correlating deaths due to cholera with the source of water used in different households, and so providing the first explanation for the etiology of cholera. In the same year Pacini described the comma-shaped organism now known as *Vibrio cholerae*, which subsequently was shown to be the causative agent by Koch in 1883.

V. cholerae is a gram-negative facultative bac-

terium with a single polar sheathed flagellum (Figure 1), with the sheath being essentially an extension of the outer membrane lipid bilayer containing lipopolysaccharide (LPS). There are more than 139 O serogroups (variant forms of LPS) and prior to 1992 only organisms of the O1 serotype were responsible for epidemic cholera in humans. Non-O1 or non-cholera-vibrios (NCVs) or *V. cholerae* which do not agglutinate (NAGs) with O1 antisera were not associated with cholera although they could cause acute diarrheal disease. However, in 1992 a new serotype designated O139 (Bengal) arose on the Indian sub-continent and rapidly replaced the O1 strains as the epidemic agent, but after about 2–3 years the O1 strains re-emerged as the major form.

O1 strains can also be grouped into two biotypes: classical and El Tor. The El Tor biotype was first isolated at an Arabian field station of the same name in the early part of this century. These strains could be differentiated by their ability to produce a soluble hemolysin, for sheep and goat erythrocytes, not detected in classical isolates. More recently, this property has been shown to be variable amongst El Tor

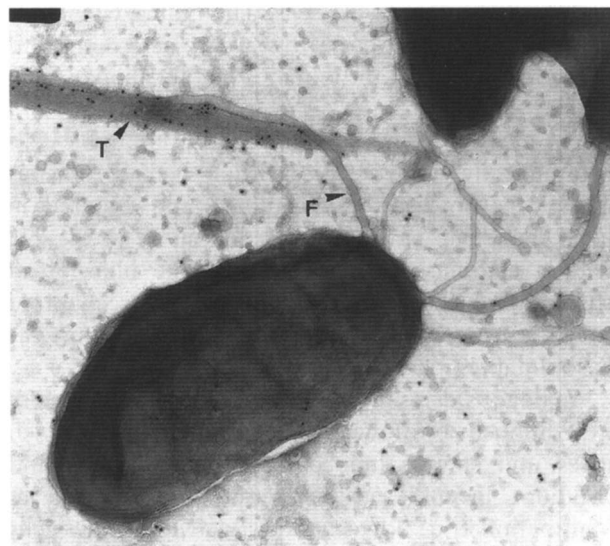


Figure 1 Electron micrograph of cells of *Vibrio cholerae* stained with uranyl acetate. The cells were grown under conditions to optimize the expression of TCP (T) which have been identified by immunogold labeling. The polar sheathed flagellum (F) can also be seen.

strains and other methods such as sensitivity of the classical biotype to the antibiotic polymyxin B and the type IV phages of Mukerjee are used for biotype differentiation. El Tor strains also produce a positive Voges-Proskauer reaction and can agglutinate chicken erythrocytes.

The O groups are associated with the O antigens of the lipopolysaccharides (LPS). O1 strains can be subdivided into Inaba and Ogawa serotypes and a third form, Hikojima, would appear to be a regulatory variant of Ogawa. *V. cholerae* O1 can undergo a form of antigenic variation involving serotype switching from Inaba to Ogawa or the reverse and genetic and chemical analyses have demonstrated that Ogawa O antigen is a methylated form of Inaba, and that Inaba strains are mutants in the 'methylase'. Thus, switching from Ogawa to Inaba involves selection of a mutation in the methylase and the reciprocal switch involves reversion of a mutation. The selection pressure for the switch is thought to be provided by the immune response. The new O139 (Bengal) serotype has been shown to be essentially an O1 El Tor strain in which the region of the chromosome encoding O antigen biosynthesis was replaced by genes encoding both the O139 O antigen and an antigenically related capsule. The genetic means by which this event has occurred are somewhat obscure but there is evidence for the involvement of an insertion sequence (IS1358).

Pathogenesis

V. cholerae typically infects by the oral-fecal route, primarily via contaminated food and water supplies. The bacteria are very sensitive to stomach acid, which provides a natural barrier. However, food particles in particular may provide sufficient protection for an infectious dose to reach the small intestine where the organism must adhere, multiply and colonize the mucosal surface. The vibrios remain attached to the intestinal epithelium throughout the course of the infection. They do not invade or cross the epithelium although some local damage can be detected and this is most marked with El Tor strains. Whilst in association with the small bowel, the vibrios secrete, besides cholera toxin, a number of extracellular products which facilitate the colonization process and are responsible for the net secretion of fluids into the intestinal lumen resulting in the typical rice-water stools. The extent of fluid loss in the diarrhea is variable but can approach one-third of body weight per day.

Thus, the symptoms of cholera are essentially the result of extreme fluid loss and a relatively simple treatment has been developed. This involves fluid

replacement therapy based upon a defined solution of salts and glucose designed to facilitate restoration of the fluid and electrolyte balance. This treatment does not reduce the amount of purging, and antibiotic therapy, usually with tetracyclines, may be additionally used.

Toxins

The *V. cholerae* enterotoxin, cholera toxin (CT), is one of the best studied bacterial toxins both in terms of structure, function and genetics. CT is comprised of one A and five B subunits and the A subunit is nicked and activated by either an endogenous *V. cholerae* protease or one encountered in the gut, to give the A₁ and A₂ chains which are held together via a disulfide bridge. The N-terminal A₁ subunit possesses the ADP-ribosylating activity which catalyzes the transfer of NAD to the G_s regulatory component of the adenylate cyclase, resulting in increased activity of the enzyme. The direct consequence of an elevation of the intracellular cAMP concentration is an increase in specific ion (primarily Cl⁻) efflux and a decrease in specific ion (primarily Na⁺) absorption which provides a potent osmotic driving force for the loss of water. Hence, the watery diarrhea ensues.

Extensive genetic analysis has shown that the cholera toxin genes *ctxA* and *ctxB* exist as an operon under the control of a positive regulatory environmental sensory system which also coregulates the expression of several other virulence determinants including a critical colonization factor, the toxin-coregulated pilus (TCP). The *ctxAB* operon is contained within a virulence cassette associated with transposon-like repeat sequences which are capable of mediating the tandem multiplication of the operon. Under the appropriate selection conditions, such as *in vivo* passage, this can lead to many copies of the CT genes within the cell and consequently increased capacity to produce cholera toxin. The B subunits are essential for the secretion of CT and also provide the binding component to permit it to interact with its receptor, GM₁ ganglioside on the surface of the eukaryotic cell.

The genes for two other toxins, ACE (accessory cholera enterotoxin) and ZOT (zonula occludens toxin), are contained within the same virulence cassette as the CT genes. However, their functions in pathogenesis are less well-defined than CT, except that ACE has enterotoxin activity and ZOT is reported to destroy the tight junctions in the intestinal epithelium.

Other toxins have been reported in *V. cholerae* but the most significant is the El Tor hemolysin which

has both enterotoxic (cytolytic) and cytotoxic activities. It is under different genetic control to CT and classical strains possess a small deletion within the *hlyA* structural gene rendering them nonhemolytic. Elimination of the hemolysin from El Tor strains markedly changes the pathology of the tissue at the site of infection, as well as the appearance of the diarrheal stool.

Adherence and colonization

Numerous components have been implicated in the processes of adherence and colonization. These include surface components such as LPS, outer membrane proteins, the flagellum and pili or fimbriae, as well as extracellular products like DNAases, proteases and neuraminidase. Mutants in the production of TCP or a mannose–fucose-resistant hemagglutinin are dramatically affected in their ability to colonize presumably because of a defect in adherence. Mutations eliminating LPS O antigen biosynthesis are also attenuating but this is probably due to pleiotropic effects including preventing the assembly of TCP. The mannose-sensitive hemagglutinin is also a pilus adhesin and was initially thought to be an El Tor equivalent to TCP, but its role in colonization of the gut is questionable.

The neuraminidase is capable of converting higher gangliosides to GM₁, effectively increasing the availability of receptors for CT. Under appropriate conditions a significant reduction in virulence can be detected in neuraminidase-less mutants. Although other components may contribute to adherence and colonization, the corresponding mutants are not necessarily attenuating. However, as was the case with the neuraminidase, due to insufficient knowledge, the conditions under which these components are critical for virulence may not have been used for comparing the parent and mutant strains. It is also clear that there are factors which may be only expressed *in vivo* and have not been detected by conventional experimental protocols.

Protective antigens and immunity

Protection against cholera requires mucosal immunity optimally mediated via locally produced secretory IgA antibodies. Since *V. cholerae* is a noninvasive pathogen, it is thought that these antibodies function to block adherence and inhibit colonization, and possibly to either neutralize the toxins or prevent them from binding to their specific receptors. The immune response in the intestine is initiated in the lymphoid follicles which can occur in isolation or as clusters known as Peyer's patches. The mononuclear

cells of this tissue are responsible for the phagocytic sampling of antigen from the intestinal lumen. Live *V. cholerae* organisms are a more efficient antigen for stimulating an immune response than killed organisms.

Epidemiological data suggest that an initial cholera infection provides long-lasting immunity and this agrees with studies performed on human volunteers. The most significant protective antigen appears to be the O antigen of the LPS, however, it is clear from animal model studies that antibodies to TCP are protective but biotype restricted due to biotype-specific antigenic variation in the major structural subunit. The B subunit of CT is the best-documented other protective antigen, but there also appears to be biotype-specific epitope differences.

The ability of the newly evolved O139 serotype to replace O1 as the epidemic strain heightens the importance of anti-LPS immunity, since O139 strains possess all of the known virulence attributes of O1 El Tor strains. The incidence of O139 was the same throughout all age groups suggesting that acquired immunity to non-LPS antigens was far less significant than to the specific LPS type. The available data also imply that non-LPS antigens are less immunogenic. O139 strains are also novel in that they produce a capsule, but because of its antigenic similarity to the O139 LPS, the relative protective activity of antibodies to these two structures is not clear.

Vaccine development

No effective cholera vaccine has yet been put into widespread use. Immunity to cholera can occur in the absence of antibodies to CT, but experimental studies suggest that antibacterial and antitoxic immunities can act in synergy. A vaccine based on killed whole cells and purified CT B subunit has shown promise in extensive field trials in Bangladesh and elsewhere, but the presence of B subunit in the preparation only seems to be important for immunity in the first year or two.

The incorporation of the B subunit may have other benefits since CT has been demonstrated to have adjuvant activity, and at least the B subunit is critical. However, recent data indicate that this can be further enhanced by using whole CT in which the A subunit has been mutated to eliminate its ADP-ribosylating activity.

Much attention has been given to constructing attenuated *V. cholerae* strains in which specific genes have been deleted or their expression modified. Various strains have been constructed in which one or both of the *ctx* genes have been deleted, but all still produce a mild diarrheal response in a significant

proportion of volunteers. This is in part correlated with the colonizing potential of the vaccine strain. One construct, CVD103Hg^R, based upon a poorly colonizing strain in which *ctxA* has been deleted, as well as several other modifications, seems to be efficacious and is being evaluated in the field. There is also considerable variation in the success of this approach and this again appears to be strain-dependent, as was observed with O139 strains into which the same mutations were introduced.

A further approach has been to express the genes for the protective antigens in heterologous vaccine strains such as attenuated *Salmonella typhi*. Experiments with strains expressing the *V. cholerae* O antigens show that construction of such chimeric vaccine strains has potential.

Environmental *V. cholerae* O1

The so-called environmental *V. cholerae* O1 El Tor strains commonly isolated from surface waters have a number of features which distinguish them from the epidemic cholera strains. They are referred to as nontoxigenic in that they lack the *ctxA,B* genes; however, they invariably express the El Tor hemolysin. These strains also lack the genes encoding the biosynthesis of the colonization pilus, TCP, but although other pili have been identified, there are no convincing data on their role in colonization. The mechanism by which these organisms cause disease appears to differ from toxigenic epidemic O1 and O139 strains.

See also: Bacteria, immunity to; Endotoxin (lipopolysaccharide (LPS)); Gastrointestinal tract infections; IgA; Toxins; Vaccines.

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VIRUSES, IMMUNITY TO

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Viruses are obligate intracellular parasites which can cause a spectrum of disease from asymptomatic persistent infection to fatal illness. The need to resist virus infection has been a major driving force in the evolution of the immune system, in particular of those facets of the immune response involved in the recognition of foreign antigens on the surface of cells. From the time of Jenner's observation that exposure to cowpox gave protection, or immunity, against smallpox, the study of viruses has prompted research in immunology, and the disciplines of virology and immunology continue to be closely interrelated. For instance, a considerable amount of

work in immunology is still driven by the need to produce vaccines to protect against current pathogens, such as human immunodeficiency virus (HIV). Detailed studies of individual viruses (such as influenza) have made important contributions to the understanding of molecular recognition of antigen by T cells, and recombinant vaccinia viruses provide a research tool for introducing foreign antigens into cells. As well as producing tissue damage by direct cytopathic effects, viruses may also evoke immune responses which are harmful to the host; these may include responses directed primarily against virus-infected cells (in which uninfected bystander cells

may also be damaged), and autoimmune responses against uninfected cells.

Viruses as antigens

Viruses consist of a central core of nucleic acid which may be RNA or DNA, and single- or double-stranded. This is surrounded by a coat (capsid) of repeating protein subunits (capsomers). In most viruses, the capsomers are arranged symmetrically around the nucleic acid, either as an icosahedron or as a helix. Some viruses also have a lipid envelope derived from the host cell membrane into which viral glycoproteins are inserted. The structure of some smaller viruses (such as polio), and component proteins of more complex viruses (such as influenza hemagglutinin, HA) has been solved at high resolution by X-ray crystallography. Viruses are classified according to the type of nucleic acid making up the genome, whether the genome is segmented or not, and the presence or absence of an outer envelope. Viral genomes vary in size from very small (such as parvovirus with 5 kb of single-stranded DNA) to large (such as poxviruses with up to 280 kb of double-stranded DNA).

Viral infection begins with the attachment of the virus to a component of the cell membrane which acts as its specific receptor, such as the CD4 molecule on T cells to which HIV binds, or sialic acid for the influenza virus hemagglutinin. Enveloped viruses may penetrate the cell by fusion with the cell membrane (mediated by fusogenic viral glycoproteins, e.g. Sendai virus) or by endocytosis after binding to their specific receptor (e.g. influenza). In the latter case, the virus then enters the cytoplasm by fusion with the endosomal membrane following an acidic pH-dependent cleavage of viral glycoproteins. Viruses are then uncoated allowing transcription and translation of viral nucleic acids (single positive-stranded RNA viruses are translated directly). In more complex viruses, some of the viral proteins expressed in the infected cell do not form part of the virion; these nonstructural proteins serve functions such as regulating the transcription of other viral and cellular genes, and shutting down synthesis of host cell proteins. Other viral proteins are structural and are incorporated into new virions. Following viral nucleic acid replication, virus particles are assembled by aggregation of structural proteins around the nucleic acid. Nonenveloped viruses may be released from the cell by lysis, whereas enveloped viruses insert their viral glycoproteins into the host cell membrane and bud from the cell surface.

The immune response to viruses

Studies of patients with isolated immunodeficiencies give an insight into the relative importance of the different components of the immune response. Individuals with isolated defects of cell-mediated immunity develop severe, sometimes fatal viral infections such as measles and chickenpox. A single case of natural killer (NK) cell deficiency has been reported, with increased susceptibility to severe primary herpesvirus infections. Those subjects with isolated immunoglobulin deficiency recover normally from most viral infections, except enteroviruses (which may cause chronic central nervous system infection). Such 'experiments of nature' coupled with an extensive body of experimental work in animal models permit the following generalizations.

Antibodies appear to act principally by neutralizing virions (i.e. rendering virions noninfectious), whereas cell-mediated responses are directed against virus antigens present in the infected cell. Antibody is important in preventing primary infection and reinfection, by neutralizing viruses on mucosal surfaces and limiting their dissemination in body fluids, while cell-mediated immunity seems to be responsible for eliminating intracellular infection and limiting reactivation of persistent viruses.

The immune response may be divided into three phases (see Table 1) and into humoral and cell-mediated components, which include specific and nonspecific mechanisms.

Nonspecific immunity (natural killer cells and interferons)

Natural killer (NK) cells are large granular lymphocytes (CD16⁺, CD56⁺ but without an expressed T cell receptor or CD3), which are able to kill virus-infected cells without prior exposure to that virus. NK cell recognition of virus-infected cells differs

Table 1 Phases of the immune response against virus infection

Phase	Characteristics	Mechanism
Immediate <4 h	Nonspecific, innate No memory No specific T cells	NK cells
Early 4–96 h	Nonspecific, inducible No memory No specific T cells	IFN α , IFN β IFN-activated NK cells
Late >96 h	Specific, inducible Memory Specific T cells	Antibody Cytotoxic T cells Cytokine-secreting T cells

IFN, interferon.

from the classical major histocompatibility complex (MHC) restriction of T cells, but is affected by MHC molecule expression; the lower the level of MHC class I expression on the surface of the target cells, the greater their susceptibility to killing by NK cells. This is because specific MHC class I alleles on the surface of target cells can *inhibit* killing by defined NK cell clones, and thus normal levels of expression of certain autologous MHC class I molecules may protect uninfected host cells from being killed by NK cells.

NK cells are activated by interferon α (IFN α) and IFN β released by virus-infected cells, and by certain viral glycoproteins, e.g. mumps hemagglutinin. Virus-infected cells show increased susceptibility to NK cell lysis compared to uninfected cells. The reasons for this are not fully understood, although several mechanisms have been proposed. These include enhanced binding of NK cells to virus-infected cells; increased susceptibility of infected cells to NK killing due to inhibition of host cell RNA and protein synthesis; altered surface expression of host cell proteins (e.g. reduced surface expression of MHC class I); and the protective effects of IFNs on noninfected cells. Natural killer lysis is not prevented by blocking the synthesis or surface expression of structural viral proteins.

It is thought the NK cells are an important defense mechanism early in the course of viral infection, before specific cytotoxic T lymphocytes (CTLs) and antibody responses have fully developed. In experimental models, NK cells seem to be more important in resistance to certain viral infections, such as herpesviruses, than to others. In mice, for example, NK cells protect against murine cytomegalovirus (MCMV), but not against lymphocytic choriomeningitis virus (LCMV).

Type I interferons (IFN α and IFN β) are proteins produced by a wide variety of cell types in response to virus infection and other stimuli such as double-stranded RNA. IFN α and IFN β bind to a common receptor on the surface of uninfected cells and produce an 'antiviral state' by stimulating synthesis of cellular proteins which interfere with viral RNA transcription and translation. Type I IFNs are completely nonspecific, and are produced in response to, and against, a wide range of viruses. The importance of IFN α and IFN β in host resistance to viruses is clearly illustrated by transgenic mice which lack a functional type I IFN receptor; these animals show greatly increased susceptibility to acute virus infection, leading to death within 3 days of infection associated with very high titers of virus in many organs. The type II interferon IFN γ is produced by NK cells and by T cells on exposure to specific anti-

gen, and activates monocytes and macrophages, enhancing their ability to process and present antigen. IFNs also activate NK cells and the effector cells responsible for antibody-dependent cellular cytotoxicity (ADCC) and give rise to many of the systemic symptoms associated with viral infections, such as fever, malaise and myalgia. Tumor necrosis factor α (TNF α) also has an antiviral effect which may be synergistic with that of IFNs.

Humoral response

The major class of virus-specific antibody is immunoglobulin G (IgG). IgM is also produced during the early phase of primary infection, and secretory IgA is important in prevention of viral entry via mucosal surfaces. Virus-specific antibodies may bind to viral epitopes on the surface of the virion, or to viral glycoproteins expressed on the surface of the infected cell. In order to neutralize the virion, a specific antibody must bind with high avidity to particular epitopes on viral proteins that interact with cell surface receptors. While antibodies can be raised against individual small linear peptides derived from a viral glycoprotein such as influenza hemagglutinin, these antibodies are frequently non-neutralizing; many or most neutralization epitopes are probably conformational ones. Antibody may also limit the spread of infection by agglutination of virions (reducing the number of infectious units), and by enhancing the phagocytosis of virions by mononuclear phagocytes (opsonization).

Antibody may also recruit other effector systems. *In vitro*, antibody attached to viral glycoproteins may lead to complement-mediated lysis of the infected cell and of enveloped virions. Certain cells with Fc receptors (CD16⁺ cells such as NK cells and monocytes) may bind to the Fc portion of virus-specific antibodies bound to viral glycoproteins on the cell surface and subsequently lyse the infected cell by ADCC. The precise importance of ADCC and complement-mediated lysis of virus-infected cells *in vivo* is unclear.

Complement enhances the effect of neutralizing antibody by coating the surface of sensitized virions preventing their interaction with cellular receptors. Complement also acts as an opsonin for phagocytic cells. Some viruses may activate complement directly by the alternative pathway.

Specific cell-mediated responses

Virus-specific CTLs are predominantly CD8⁺ in phenotype, although approximately 10% of human peripheral blood CD4⁺ T cells are potentially cyto-

toxic and capable of giving rise to virus-specific CTLs. CTLs recognize viral antigens which have been processed and presented as peptides on the surface of infected cells by the appropriate MHC molecules; class I in the case of CD8⁺ T cells, class II for CD4⁺ T cells. Processing and presentation of viral antigen allows viral proteins not otherwise expressed on the cell surface to be exposed to T cells, e.g. influenza nucleoprotein. Hence, an infected cell can be recognized and killed by T cells before the virus can replicate, thereby limiting the spread of the infection. Whilst recognition of viral antigen by T cells requires interaction between the T cell receptor and the MHC-antigen complex, interactions between accessory molecules including LFA-1 and ICAM-1, and LFA-3 and CD2 facilitate effector T cell binding to a virus-infected cell. With a few exceptions such as reovirus, CTLs are not serotype specific, i.e. they do not recognize the same epitopes as virus-specific antibodies. The ability of CTLs to confer protection against viral disease can be demonstrated by adoptive transfer in many experimental virus models; for example, naive mice can be protected against lethal influenza A by prior transfer of an influenza A-specific CTL clone. The T cell response to virus infection includes cytotoxic effector T cells and an expanded population of virus-specific memory T cells. After clearance of an acute virus infection (e.g. influenza, in which viral antigen is completely eradicated), effector T cells rapidly disappear, and the size of the memory T cell population may gradually diminish over time. In contrast, in many persistent virus infections (e.g. Epstein-Barr virus (EBV), human cytomegalovirus (HCMV)), virus-specific memory CTL precursors remain at high frequency for many years, and probably play a role in controlling the reactivation of persistent virus and maintaining the normal equilibrium between virus and host.

CD4⁺ T cells may recognize multiple epitopes within a given viral protein, such as influenza HA, and these epitopes may differ from those recognized by antibody. CD4⁺ T cells augment T cell, B cell and NK cell responses to viral proteins through secretion of cytokines including IL-2, IL-12 and IFN γ . Cytokines secreted by subpopulations of virus-specific CD4⁺ T cells and CD8⁺ CTLs contribute to the generation of the delayed-type hypersensitivity (DTH) response, an inflammatory reaction characterized by a mononuclear cell infiltrate of activated lymphocytes and macrophages. In some experimental virus infections, DTH responses can confer resistance to infection; in response to local infection with herpes simplex virus (HSV) inoculated into the ear pinna in mice, CD4⁺ T lymphocytes from the draining lymph nodes proliferate in response to HSV antigens,

mediate DTH and upon adoptive transfer can mediate protection against HSV in naive mice. However, DTH can sometimes be harmful. Mice which have previously been exposed to noninfectious influenzavirus suffer increased mortality when they are subsequently challenged with live influenzavirus: adoptive transfer experiments have shown that induction of strong DTH (by prior exposure to non-infectious virus) may be responsible for this observed increase in mortality.

In vivo, the different T cell populations obviously interact together rather than producing effects in isolation, and can show considerable plasticity. Transgenic mice that lack β_2 -microglobulin (which results in grossly impaired CD8⁺ T cell development), are able to resist infections by induction of increased numbers of virus-specific CD4⁺ CTLs.

Persistent viral infections

Many viruses are able to maintain long-term infections which are often symptomless. This may involve chronic production of infectious virus, or latent infection with intermittent reactivation leading to virion production. Certain viruses may exhibit both patterns dependent on host cell type – for example, hepatitis B virus appears to latently infect lymphocytes but produces infectious virus in hepatocytes. In order to establish a successful persistent infection, a virus should minimize damage of host cells. Certain noncytopathic viruses such as the murine arenavirus LCMV replicate with little apparent disruption of cell function. Other viruses have evolved different strategies that can reduce their lytic potential. These include restricted virus gene expression (in the herpesviruses during their latent nonlytic infection of neuronal or lymphoid cells); the generation of mutant viral strains which do not cause cell lysis (in reovirus after *in vitro* passage in fibroblast lines); and infections where only a small percentage of cells are infected at any one time (in lactic dehydrogenase virus in mice).

The persistent virus also has to evade detection and elimination by the host immune system. Various mechanisms have been described for this, although their relative importance is not known. Some viruses exhibit antigenic variation which presents the immune system with a continually moving target, best demonstrated in chronic infections by the lentivirus equine infectious anemia virus. An excess of non-neutralizing antibody has been found in some chronic viral infections, which may interfere with neutralizing antibody function. A number of viruses are now recognized to encode genes which downreg-

ulate the surface expression of class I MHC molecules through a variety of mechanisms (see Table 2).

Persistent viruses frequently infect immunocompetent cells, thereby compromising their function and preventing elimination of the virus, for example HIV infection of CD4⁺ T cells and dendritic cells. Certain viruses are more likely to establish persistent infection if infection occurs very early in life when the immune system is immature. Infections *in utero* or at birth in mice may be associated with selective impairment of virus-specific immune responses, e.g. neonatal infection with LCMV is accompanied by a defect in the LCMV-specific CD8⁺ CTL response, that can be restored by adoptive transfer of CD8⁺ CTLs from LCMV immune normal adults.

It is likely that several mechanisms for avoiding the host immune response are active in any given persistent virus infection.

Viral immunopathology

The immune response to viruses may also inflict damage on host cells, as a result of immune responses directed against virus-infected cells, or as a result of indirect mechanisms that damage uninfected cells. In addition, a number of viruses can alter immune responses by infecting immunocompetent cells.

In immunocompetent adult mice that are infected

with noncytopathic LCMV, virus-specific cytotoxic T cells induce immunopathology by attacking virus-infected cells in the brain, causing a lethal choriomeningitis; the meningitis can be prevented by immunosuppression with cyclophosphamide. Virus-specific CTLs may contribute to the liver damage seen in chronic hepatitis B virus infection (although in this infection, immunosuppression *worsens* the clinical outcome). Mice experimentally infected with coxsackie B virus develop inflammatory myocarditis which is reduced by T cell depletion, and is abolished in transgenic mice lacking the proinflammatory C-C chemokine MIP-1 α . In addition, DTH cells have been shown to enhance tissue damage in mice infected with influenza-virus.

It is believed that the adverse reactions evoked by certain killed virus vaccines when the vaccine recipients encountered the natural infection (particularly respiratory syncytial virus and measles virus) may have resulted from an immunopathological mechanism such as DTH or Arthus reaction.

The generation of non-neutralizing antibodies to certain viruses may have a pathological effect. Non-neutralizing antibody to flaviviruses such as dengue virus can facilitate their entry into macrophages via Fc receptors, where they replicate. Immune complexes are frequently found in many viral infections, e.g. in acute hepatitis B virus infection, immune complexes may be responsible for the arthralgia and rash seen in 10–20% of cases. Immune complexes also occur in chronic hepatitis B virus infection, although only a small proportion of these patients develop vasculitis or chronic glomerulonephritis. It has been suggested that certain human chronic diseases with immunological abnormalities, e.g. chronic immune complex diseases of unknown etiology and multiple sclerosis, might have an underlying viral etiology.

There has been much speculation that viruses may initiate or promote autoimmune disease. Several mechanisms by which this may occur have been suggested. These include generation of anti-idiotypic antibodies against antiviral antibodies which could react with host cells; molecular mimicry, where virus and host share antigenic determinants; promotion of endogenous antigen presentation in virus-infected cells; and impairment of normal immune response suppressor mechanisms. In general there is little direct evidence in support of any of these theories to date. However mice infected with reovirus develop transient diabetes and weight loss in association with autoantibodies to islet and anterior pituitary cells, and these abnormalities can be prevented by immunosuppression with cyclophosphamide.

Table 2 Examples of viral molecules which modulate host immune responses

Virus	Gene product	Mechanism
<i>Interference with antigen presentation</i>		
HSV	ICP 47	Inhibition of peptide transport by TAP1/TAP2 complex
HCMV	US 3	Retention of class I molecules in endoplasmic reticulum
Adeno	E3	Retention of class I molecules in endoplasmic reticulum
<i>Virus-encoded cytokines</i>		
EBV	BCRF-1	Homology to IL-10
H. saimiri	HVS-13	Homology to IL-16 (CTLA8)
Vaccinia	VGF	Homology to epidermal growth factor
<i>Modulation of host cytokines</i>		
Vaccinia	B18R	Soluble receptor for IFN α , IFN β
Vaccinia	E3L	Inhibition of activation of IFN-inducible cellular kinase DAI
Vaccinia	K3L	Competitive inhibition of cellular kinase DAI
HCMV	US 28	Membrane-bound receptor for C-C chemokines

DAI, double-stranded RNA activated inhibitor of translation.

See also: **Adenovirus, infection and immunity: Anti-**

body-dependent cellular cytotoxicity; Arenavirus, infection and immunity; Arthus reaction; Auto-immune disease, pathogenesis; *Bunyavirus*, infection and immunity; Calicivirus, infection and immunity; Coronavirus, infection and immunity; Hepatitis B virus, infection and immunity; Cytomegalovirus, infection and immunity; Cytotoxic T lymphocytes; Delayed-type hypersensitivity; Epstein-Barr virus, infection and immunity; Filoviruses (Marburg virus and Ebola virus) infection and immunity; Hepatitis A virus, infection and immunity; Hepatitis C virus, infection and immunity; Hepatitis E, F and G viruses, infection and immunity; Herpes simplex virus, infection and immunity; Herpesvirus-6, infection and immunity; Human immunodeficiency viruses; Interferon α ; Interferon β ; Interferon γ ; Iridovirus, infection and immunity; Transmissible spongiform encephalopathies, infection and immunity; Leukemia viruses; Natural killer (NK) cells; Opsonization; Influenza virus (*orthomyxovirus*), infection and immunity; Papillomavirus, infection and immunity; Paramyxoviruses, infection and immunity; Parvovirus, infection and immunity; Phagocytosis; Picor-

navirus, infection and immunity; Polyomavirus, infection and immunity; Poxvirus, infection and immunity; Reovirus, infection and immunity; Retrovirus, infection and immunity; Rhabdovirus, infection and immunity; Togavirus, infection and immunity; Tumor necrosis factor α ; Vaccines; Varicella-Zoster virus, infection and immunity; Viruses, infection of immune cells by.

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VIRUSES, INFECTION OF IMMUNE CELLS BY

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Most viruses can infect cells of the immune system, and certain viruses display clearly defined though seldomly exclusive tropism for specific leukocyte subsets. Infection of leukocytes is often relatively innocuous to the host, as usually only a small percentage of the total leukocytes are infected, and most of the cells of the immune system can readily repopulate. In other cases, however, viral infections of immune cells may lead to severe immunodeficiency and/or lymphocytic or myelogenous malignancies.

T and B lymphocytes

The immune suppression associated with human immunodeficiency virus (HIV) infection has focused attention on viral infections that target lymphocytes as host cells. The outcome of the virus–lymphocyte interaction is sensitive to multiple restrictions that are imposed at many levels by the biology of the tar-

get lymphocyte and that effectively limit virus replication. The requirement for an appropriate receptor and accessory molecules on the cell surface for initial virus entry is the first level of restriction. This explains the tropism of HIV, which requires the CD4 molecule found on T cells for attachment and a coexpressed member of the chemokine receptor family for entry into the cytoplasm. Similarly, the B cell tropism of Epstein–Barr virus (EBV) is a result of attachment and entry being mediated by the complement receptor CR2, which is found on B cells. Other viruses using more ubiquitous receptors can target many types of host cells in addition to lymphocytes.

Virus entry *per se* does not necessarily lead to a productive infection, which is usually dependent on the activation state of the lymphocyte. The majority of peripheral lymphocytes, both virgin and memory, are resting cells in the G₀ stage of the cell cycle. Their scant cytoplasm and quiescent metabolism make

resting lymphocytes inefficient hosts for high-level virus production, whereas lymphocytes activated by antigen or mitogen stimulation readily support a productive infection by many viruses. The finding that inactivated virions and/or isolated glycoproteins from measles, Sendai, Newcastle disease and influenza viruses are polyclonal lymphocyte mitogens suggests that viruses may in some cases indirectly or directly cause the generation of inductive signals promoting their replication. One illustration of this is the life-cycle of mouse mammary tumor virus (MMTV). Virus-free newborn pups ingest MMTV present in the milk of the mother. MMTV infects resting B cells in the mouse gut, but this infection is not productive. Infected B cells display the MMTV superantigen, provoking recognition by T cells with antigen receptors of the appropriate V_{β} subgroup. The infected B cells recirculate, and a superantigen-specific cognate T cell:B cell interaction activates the B cell in a CD40-dependent fashion to produce and disseminate infectious virus.

The transition from the nonpermissive to permissive state accompanying the activation of resting lymphocytes has been studied *in vitro* and occurs in discrete activator-dependent stages. Vesicular stomatitis virus (VSV) infection of small B lymphocytes does not lead to a productive VSV infection, but primary viral transcripts are readily detected within 8 h of infection of resting B cells. Primary viral transcription is enhanced when infected B cells are cultured with T cell-derived cytokines, including interleukin 4 (IL-4), but viral proteins are not produced, even though the viral mRNA is associated with polysomes. This translational block can be relieved when infected B cells are activated polyclonally with anti-immunoglobulin or phorbol ester. Although all viral proteins and the positive strand of VSV (the replicative intermediate) are synthesized, no infectious particles are produced. Virus assembly and/or release is effected by simultaneous exposure to multiple B cell activators. Thus primary viral transcription, protein synthesis, and assembly and/or release are all found to be activator-dependent regulated processes that can be separated from B lymphocyte proliferation and differentiation to antibody synthesis.

The release of large amounts of infectious virus is associated with death of the host cell. Acutely infected lymphocytes are no exception, and they die by either apoptotic or necrotic mechanisms. Virus infection can induce apoptosis directly through the activation of the cell death program or indirectly by the induction of the Fas (CD95) molecule through which an apoptotic signal can be delivered. Apoptosis following Fas cross-linking is enhanced in

both $CD4^{+}$ and $CD8^{+}$ T cells taken from HIV-infected donors, and the Fas ligand is upregulated on HIV-infected macrophages. This raises the possibility that the depletion of T cells during HIV infection may be in part due to Fas-mediated apoptosis. The programmed cell death pathway in infected host cells can be suppressed by a number of DNA viruses, including adenovirus, cowpox and EBV. The virus-encoded gene products E1B 19K of adenovirus, and *crmA* of cowpox directly suppress the apoptosis pathway, whereas the LMP product of EBV works indirectly by inducing the synthesis of endogenous antiapoptotic proteins such as Bcl-2. Suppression of apoptosis promotes the completion of the replicative cycle, allowing either cell transformation or a larger burst of infectious viral particles.

Examples of retroviruses and large DNA viruses that have developed strategies to disseminate and perpetuate the viral genome by insertion into the host's DNA are well known. The activation-dependence for full viral replication in infected lymphocytes, however, can make resting lymphocytes hosts for persistent infections by RNA viruses that normally induce acute lytic infections. Both VSV and measles virus can infect and remain dormant in resting lymphocytes. The persistence results from the stability of the virus nucleocapsid. Primary VSV mRNA transcripts are readily detected for up to 5 weeks in VSV-infected resting B lymphocytes taken from Bcl-2 transgenic mice and maintained in culture. As was seen in the case of acute infections, no VSV protein or infectious VSV particles were detected throughout the culture of unactivated cells. Infectious particles were readily induced when the cells were activated appropriately. The relatively long lifespan of resting lymphocytes, the stability of the viral nucleocapsid in the cytoplasm of resting cells, and the ability to recirculate and release fully infectious particles upon appropriate cellular activation, combine to make resting lymphocytes ideal reservoirs for RNA virus infection.

Natural killer (NK) cells

In contrast to nonproliferating T and B lymphocytes, NK cells have large, active cytoplasm capable of supporting virus growth. Little is known about the frequency or significance of virus infections of NK cells *in vivo*, but cytomegalovirus (CMV), measles and influenza viruses infect human NK cells *in vitro*. These infections are not lytic, and influenza virus does not inhibit NK cell-mediated cytotoxicity; in contrast, both CMV and measles virus inhibit the ability of NK cells to mediate natural cytotoxicity but do not impair their ability to mediate antibody-

dependent cellular cytotoxicity (ADCC). The reason for this selective inhibition in lytic function is not known, but it is of interest that some strains of HIV can infect NK cells and that NK cells isolated from patients infected with HIV similarly display a selective defect in natural cytotoxicity but not ADCC. Also during HIV infection there is a selective loss of CD8⁺ NK cells; it has been suggested that HIV infection of CD8⁺ NK cells leads to tumor necrosis factor α (TNF α) production, which activates HIV synthesis, resulting in lysis of the cell, whereas the predominant cytokine made in CD8⁻ NK cells is interferon α (IFN α), which inhibits viral synthesis and protects the cell. The cytotoxic mechanism of NK cells can be triggered directly by treatment with glycoproteins isolated from a variety of viruses, including mumps, measles, influenza, Sendai, and lymphocytic choriomeningitis viruses, and this may provide a means by which NK cells can selectively lyse virus-infected targets.

Monocytes and macrophages

By secreting antiviral cytokines such as IFN and TNF α and by serving as antigen-presenting accessory cells which stimulate specific immune responses, monocytes and macrophages play fundamental roles in resistance to viruses. Intravenous injection of mice with radiolabeled virus results in a rapid clearance of the label from the bloodstream and concentration by the liver Kupffer cell macrophage population. The ability of viruses to replicate in monocytes, macrophages, and the more differentiated macrophage-like histiocytes such as Kupffer cells is thought to be an important virulence factor. This concept is supported by the fact that nearly all persistent virus infections involve extensive infections of macrophages.

Many viruses replicate in macrophages, but few viruses have macrophage-specific tropism or bind to macrophage-specific receptors. Lactic dehydrogenase (LDH) virus, which causes a persistent infection in mice, has a predilection for infecting macrophages expressing major histocompatibility complex (MHC) class II antigens and may use these molecules as receptors. Some macrophages express CD4, which leave them permissive to HIV. Some viruses enter macrophages by an aberration of opsonization known as 'immune enhancement'; macrophages have high-affinity receptors for complement and for the antibody Fc region, which facilitate the adsorption, penetration and productive infection by some antibody- and/or complement-coated viruses, such as dengue virus and HIV. More conventionally, antibody-coated viruses are suitably disposed of. Infection of macrophages by vaccinia virus normally

results in entry of virus into the cytoplasm, followed by productive replication; antibody-coated vaccinia virus suffers a different fate, as it is engulfed in a phagolysosome and degraded.

Viral infections of macrophages may be abortive, productive, latent and/or transforming, and the outcome of infection commonly depends on the macrophage activation state. Many viruses, such as herpes simplex virus (HSV) and influenza, grow poorly in activated macrophages, but others, such as murine CMV or LDH virus may grow better in activated macrophages. The reasons for these responses vary with the virus, but abortive infection in activated macrophages may be due in part to the lack of requisite host cell factors not found in nonreplicating, poorly differentiated cells. The ability of viruses to grow in macrophages is extensively influenced by cytokines. Genetically determined resistance of mouse macrophages to influenza virus has been shown to be due to an IFN-induced Mx gene product, which is a protein essential for resistance to influenza virus but not to VSV. IFN γ , produced by NK cells or T cells, has been shown to induce nitric oxide synthase in macrophages, and the liberated nitric oxide inhibits the replication of several viruses, including HSV-1, vaccinia virus, murine CMV, and VSV.

Dendritic cells and Langerhans cells

Epidermal Langerhans cells and follicular dendritic cells play important roles in antigen presentation and may be an important leukocyte source for virus-induced IFN α . Hence, infection of these cells may be advantageous to the host, by stimulating T cell responses and facilitating the activation of NK cells and other IFN-dependent mechanisms of natural resistance. These cells express some CD4 and complement receptors, and it is of interest that they can be infected with HIV *in vivo*. In animals, viruses such as murine retroviruses, Aleutian disease of mink virus, and bovine lentivirus, have been shown to replicate in dendritic cells.

Granulocytes

Infection of granulocytes with viruses occurs but is seldom of great significance, because mature granulocytes are so short-lived. However, granulocytes harboring viruses such as CMV may be a source for transmission of viruses during blood transfusions.

Virus-induced transformation of lymphoid cells

Tumors of the immune system are commonly caused by retroviruses and herpesviruses. Avian, murine, and feline leukemia virus-induced lymphomas and leukemias correlate with virus-induced mutations in host cell genes that control cell growth and differentiation (proto-oncogenes). The insertions of retrovirus DNA (the provirus) into the host chromosomal DNA represent mutations, most which are benign. However, occasionally an insertion mutates the activity of a proto-oncogene such that the gene becomes an oncogene. The genes that viruses mutate to cancer-causing genes are remarkably specific; for example, mutations in *c-myc* are associated with IgM⁺ B cell lymphomas. The frequency at which tumors are brought about by insertional mutagenesis correlates with the level of retrovirus replication in a tissue, with, for example, murine leukemia virus variants which grow well in the thymus being more likely to cause thymomas, because of the increased likelihood for generating the appropriate mutagenic event.

In contrast to the above, bovine and human T cell leukemia virus (HTLV)-induced cancers do not correlate with viral mutations of specific host sequences. These cancers appear to result from a viral gene product (Tax) altering the expression of host genes (including IL-2 and the IL-2 receptor) in such a way that the cells are under an intermediate level of growth control. These partially destabilized cells are at a greater than normal risk for a mutation stimulating an oncogenic conversion. Such a situation also occurs in cells immortalized by the herpesvirus EBV; here, lymphoma induction correlates with a mutation of the *myc* proto-oncogene in the form of a chromosomal translocation which puts this growth-promoting gene under the influence of immunoglobulin transcriptional control regions.

Many viruses encode proteins which inhibit the ability of the cell to abort the transformation process by undergoing apoptosis. One EBV gene product, BHRF1, has homology with the cellular anti-apoptosis gene Bcl-2; a second EBV-encoded gene product, LMP1, can induce the expression of cellular Bcl-2. A third EBV gene product, EBNA5, binds to and inactivates the tumor suppressor gene products

Rb and p53, which regulate cell growth and apoptosis. By usurping these normal cellular regulatory mechanisms successful transformation events can take place.

See also: Acquired immune deficiency syndrome (AIDS); CD antigens; CD4; Chromosome translocations of immune genes; Complement receptors; Cytomegalovirus, infection and immunity; Epstein-Barr virus, infection and immunity; Herpes simplex virus, infection and immunity; Herpesvirus-6, infection and immunity; Immunodeficiency, secondary; Interferon γ ; Leukemia; Leukemia viruses; Lymphoma; Influenza virus (*orthomyxovirus*), infection and immunity; Paramyxoviruses, infection and immunity; Retrovirus, infection and immunity; Retroviral vectors; Togavirus, infection and immunity; Tumor necrosis factor α .

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VITAMIN A AND THE IMMUNE SYSTEM

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Worldwide, it is estimated that vitamin A deficiency affects over 200 million individuals. It is the leading cause of blindness in children, a major cause of child mortality, and a key determinant of measles severity and mortality. Infants, preschool children, pregnant women and women of childbearing age are at greatest risk for developing vitamin A deficiency.

Vitamin A is a fat-soluble vitamin essential for immunity, cellular differentiation, growth, reproduction and vision. It is found as preformed vitamin A in foods such as liver, cod-liver oil, eggs and butter, or as provitamin A carotenoids in foods such as dark green leafy vegetables, carrots, mangos and papayas. About 90% of the vitamin A in the body is stored in the liver, and most of the rest circulates in the blood bound to retinol-binding protein (RBP) and transthyretin (TTR). After entering cells, vitamin A is converted to retinoic acid, its active metabolite, and within the nucleus, retinoic acid influences gene activation via specific receptors known as retinoic acid receptors (RAR) and retinoid-X receptors (RXR). These nuclear receptors are in the thyroid and steroid hormone receptor superfamily. Vitamin A, through its active metabolite retinoic acid, acts as a powerful gene regulator. Vitamin A deficiency has major consequences for immunity and the clinical course of many infectious diseases.

Vitamin A deficiency and infection

Vitamin A deficiency is associated with a variety of infectious diseases, including diarrheal and respiratory disease, measles, and human immunodeficiency virus type 1 (HIV-1) infection. Epidemiological studies demonstrate that preschool children with moderate vitamin A deficiency, i.e. nightblindness and/or Bitot's spots, have a higher risk of subsequently developing diarrheal disease or respiratory infection. Likewise, children suffering from diarrheal disease or respiratory infection have a higher risk of subsequently developing mild vitamin A deficiency. These data suggest that vitamin A deficiency and infection are related in a vicious cycle, which may result in increased mortality. Longitudinal studies in Indonesia show that mild vitamin A deficiency is linked with a fourfold increased risk of death in preschool children.

The severity of measles, a disease characterized by immunosuppression and infectious complications, is related to the degree of vitamin A deficiency. In a large study of children with acute measles in Zaire, those with the worse vitamin A status had the highest mortality. During HIV infection, another disease characterized by immunosuppression, vitamin A deficiency has been associated with higher mortality. There may be a specific relationship between vitamin A deficiency and mother-to-child transmission of HIV-1. Studies from Malawi, Haiti, Kenya and Rwanda suggest that HIV-infected women with vitamin A deficiency during pregnancy have a higher risk of transmitting HIV infection to their infants. The increased transmission of HIV may possibly be due to vitamin A-related immune compromise in mother and infant, increased chorioamnionitis, and higher HIV viral burden in breastmilk. Infants born to women who are vitamin A-deficient during pregnancy have lower birthweight, child growth retardation and higher mortality.

Infections may predispose to vitamin A deficiency because of low intake of vitamin A-rich foods, anorexia, malabsorption, increased utilization of vitamin A, inability to mobilize vitamin A from the liver, and accelerated depletion of vitamin A. During the acute phase response, retinol-binding protein can cleave away from the circulating RBP-TTR-retinol complex, allowing high, abnormal urinary losses of vitamin A. An episode of infection, such as chickenpox, has been shown to hasten the depletion of liver vitamin A stores in preschool children. Vitamin A deficiency during infection has widespread implications for innate and adaptive immunity.

Vitamin A and innate immunity

Vitamin A is essential for maintenance of mucosal surfaces, the first line of defense against infection. Vitamin A deficiency causes squamous metaplasia, loss of ciliated epithelium, and loss of goblet cells and mucus in the respiratory tract, as well as loss of microvilli, goblet cells and mucus in the gastrointestinal tract. The mucosae of the genitourinary tract and eye also exhibit pathological alterations. Immune effector cells which mediate nonspecific immunity include polymorphonuclear cells, macro-

phages and natural killer (NK) cells. In experimental animals, phagocytosis by neutrophils is compromised during vitamin A deficiency. In the vitamin A-deficient host, viral infections may be more severe because of increased viral replication, reduced cytolytic activity by NK cells, and increased damage to the mucosal epithelia. Vitamin A supplementation has been associated with increased circulating NK cells in both animal models and in children.

Vitamin A and acquired immunity

Vitamin A deficiency compromises acquired (adaptive, antigen-specific) immunity. Vitamin A deficiency has been associated with atrophy of the thymus, spleen, lymph nodes and Peyer's patches, suggesting major alterations in the microenvironment of immune effector cells. *In vitro* studies demonstrate that vitamin A and its metabolites are essential for growth, differentiation and activation of human T cells and B cells. Lymphopenia and T cell subset alterations, including a lower proportion of circulating CD4 T cells, have been reported in vitamin A-deficient children. The production of cytokines may be altered during vitamin A deficiency. In vitamin A-deficient mice, immune effector cells produce less interleukin 4 (IL-4) and IL-5 but more IL-12 and interferon γ , suggesting that vitamin A deficiency may be involved in the regulation between T helper type 1- and type 2-like responses. Vitamin A also seems to influence the expression of IL-2 receptor.

A hallmark of vitamin A deficiency is the impaired ability to generate an antibody response to T cell-dependent antigens (tetanus toxoid, proteins, viral infections, etc.) and T cell-independent type 2 antigens (pneumococcal polysaccharide, meningococcal polysaccharide). Vitamin A-deficient animals are generally less able to mount an antibody response against a wide variety of protein antigen challenge. Children supplemented to normal vitamin A status have higher immunoglobulin G (IgG) antibody responses after challenge with tetanus toxoid compared to children who are clinically or subclinically deficient in vitamin A. The observations of compromised innate and adaptive immunity during vitamin A deficiency may explain the increased severity of infections and higher mortality observed in both experimental animal models of infection and in epidemiological studies in humans.

Vitamin A as an immune enhancer

Vitamin A, in the form of liver oils, has been used to treat various infections since antiquity. Clinical trials conducted in the 1930s showed that cod-liver oil therapy reduced mortality in children with measles and reduced morbidity in women with puerperal sepsis (the infectious complications of delivery and postpartum period). In the last decade, clinical trials have shown that periodic, high-dose vitamin A supplementation can reduce child mortality in developing countries by 20–50%. Vitamin A supplementation reduces morbidity and mortality primarily by reducing the severity of diarrheal disease but seems to have little effect upon acute lower respiratory infections. Vitamin A supplementation is now standard therapy for infants and children with acute measles. Many developing countries have adopted periodic vitamin A supplementation to reduce morbidity and mortality in preschool children. The use of vitamin A for disease-targeted therapy, i.e. HIV infection, diarrheal disease, and as a means to enhance immune responses to vaccination are currently under investigation.

See also: Immunodeficiency, secondary; Nutrition and the immune system.

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VITAMIN B GROUP AND THE IMMUNE SYSTEM

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The B-complex vitamins differ markedly in chemical structure and biological function. Pyridoxal phosphate (PLP), the predominant coenzyme form of vitamin B₆, has a paramount role in the metabolism of amino acids and protein, including protein synthesis. Folate coenzymes function in the transport of single carbon moieties and are essential for the synthesis of DNA. Folate deficiency results in impaired cell division and altered protein synthesis, particularly of rapid growing tissues. Vitamin B₁₂ is needed for the utilization of folate and the synthesis of DNA. Thiamin performs its coenzyme function as thiamin pyrophosphate in metabolism of 2-ketosugars and α -keto acids. Riboflavin and niacin are precursors of coenzymes involved in biological oxidations. Pantothenic acid functions in acyl-group activation reactions as a component of coenzyme A. Biotin is involved in biochemical reactions which add carboxyl groups to molecules.

Vitamin B₆ deficiency produces profound changes in immune function in animals. The thymus is the lymphoid tissue most severely affected. Thymic hormone activity is decreased and lymphopenia is observed. Vitamin B₆ deficiency impairs delayed cutaneous hypersensitivity responses, primary and secondary T cell-mediated cytotoxicity and rejection of skin grafts. Impaired humoral responses in vitamin B₆-deficient animals are indicated by poor antibody production after primary or booster immunization. Both B and T lymphocytes from vitamin B₆-deficient animals have depressed proliferative responses to *in vitro* antigen or mitogen stimulation. Vitamin B₆ deficiency, however, does not affect phagocytosis by macrophages or activity of natural killer (NK) cells. Increased amounts of vitamin B₆ do not enhance immune function in mice that are not vitamin B₆-deficient.

Humans depleted of vitamin B₆ have fewer circulating lymphocytes. Vitamin B₆ deficiency, produced by a vitamin B₆ antagonist, mildly impaired humoral antibody responses to tetanus toxoid and typhoid vaccine. In patients with Hodgkin's disease or renal failure, in whom both biochemical vitamin B₆ deficiency and impaired immune responses were observed, supplementary vitamin B₆ normalized vitamin B₆ status and improved results of various immunological tests. In elderly subjects who had no

evidence of any underlying systemic disease, vitamin B₆ supplementation improved *in vitro* lymphocyte blastogenesis in response to various mitogens. Lymphocyte blastogenesis after vitamin B₆ supplementation was higher in subjects whose presupplement vitamin B₆ status was subnormal than in those whose presupplement vitamin B₆ status was normal. Vitamin B₆ supplementation in the elderly also resulted in increased proportions of CD3⁺ and CD4⁺ cells, but not CD8⁺ cells. A vitamin B₆ intake slightly higher than the US recommended dietary allowance (RDA) improved *in vitro* lymphocyte proliferation and interleukin 2 (IL-2) production in young and elderly adults.

The biochemical lesion(s) underlying the detrimental effects of vitamin B₆ deficiency on the immune system are not well understood. Early studies suggested that synthesis of DNA is diminished through a decrease in one-carbon pools resulting from the depressed PLP-dependent conversion of serine to glycine in vitamin B₆ deficiency.

Folate and vitamin B₁₂ deficiencies are associated with reduced host resistance and impaired lymphocyte function. Folate-deficient animals have a smaller thymus, a decrease in T cells, as well as impaired cytotoxicity and *in vitro* lymphocyte response to mitogens. Additionally, folate deficiency results in reduced complement-fixing antibodies and impaired response to murine rotavirus. Patients with megaloblastic anemia due to folate deficiency had impaired neutrophil phagocytosis. Patients with low serum concentrations of vitamin B₁₂ also had impaired neutrophil function, which was corrected by vitamin B₁₂. In animals, vitamin B₁₂ improves antibody function and mitogenic responses.

Pantothenic acid deficiency suppresses humoral antibody responsiveness to antigens in animals. In humans, when a combined deficiency of pantothenic acid and vitamin B₆ was induced by antagonists of these vitamins, antibody responses to tetanus toxoid and typhoid antigen were almost completely inhibited and hypogammaglobulinemia developed. Antibody response, however, to polio vaccine immunization was normal.

Deficiencies in thiamin, biotin and riboflavin interfere moderately with immunologic function. Riboflavin deficiency decreases the ability to produce

humoral antibodies in response to antigens. Rats deficient in thiamin, riboflavin or biotin had a reduced development of plaque-forming cells in response to immunization. Infants with multiple biotin-dependent carboxylase deficiencies showed delayed cutaneous hypersensitivity, impaired *in vitro* lymphocyte response to antigens and depressed antibody formation response to immunization.

Although isolated B-complex vitamin deficiencies have been found to have a detrimental effect on immune functions, the problem in reality is more complex. The metabolism of this group of vitamins along with other micronutrients is interconnected and multiple nutritional deficiencies occur in humans. Current research suggests that an intake of micronutrients, including the B-complex vitamins, two to three times higher than the US RDA, is necessary to obtain optimal immune function in healthy older adults. In placebo-controlled, double-blind studies in older adults a micronutrient supplement which included B-complex vitamins significantly enhanced NK cell cytotoxicity, lymphocyte proliferation and antibody titers to influenza vaccine, and reduced the number of sick days. Research is needed

to determine the amounts of B-complex vitamins, as well as other micronutrients, needed for optimal immune function in humans and the mechanisms by which these micronutrients function in the immune system.

See also: Aging and the immune system; Humoral Immunity; Lymphoma; Nutrition and the immune system; Thymic hormones and peptides.

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VITAMIN C AND THE IMMUNE SYSTEM

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Ascorbic acid (vitamin C) is required for the optimal functioning of all cells, tissues, and organs. It is the most important water soluble antioxidant and is a cofactor in many hydroxylating reactions. Man, primates, guinea pigs and a few other species lack the capability to synthesize ascorbic acid, for them ascorbic acid is an essential nutrient. There is abundant evidence from animal and human studies that the immune system is sensitive to the level of intake of vitamin C.

Ascorbate concentration in plasma and leukocytes

All leukocytes maintain high levels of ascorbate which are correlated with their functions. In normal healthy individuals the ascorbate levels are 1.0–1.4 mM in neutrophils and even higher in monocytes, B lymphocytes and T lymphocytes, reaching about 6.0–7.0 mM. Thus, compared with physiological plasma ascorbate concentrations of 0.03–0.06 mM, the leukocyte levels are on average 20- to 100-fold

greater than in plasma. The uptake occurs primarily by a stereospecific active transport which is temperature and energy dependent. Many infections and inflammatory diseases lower plasma and neutrophil ascorbate levels.

Nonspecific immune functions

Natural barriers

The skin and the epithelial linings of the bodily openings provide the front-line protection against infectious organisms. Collagen is an essential component of these natural barriers, as it forms the matrix for the dermal and epidermal layers and is a constituent of basement membranes which serve as attachment for epithelial cells. Functionally intact collagen occurs in the form of fibrils composed of collagen molecules cross-linked via hydroxylated prolyl and lysyl residues. Ascorbate is a cofactor in the hydroxylation reactions of proline and lysine and is therefore involved in the maintenance of the natural barrier.

Neutrophil function

Clinically an association exists between recurrent infections and impairment of neutrophil function. As opposed to a vitamin C deficiency state, adequacy of ascorbate nutrition improves several aspects of neutrophil function, specifically chemotaxis, and motility.

Neutrophils from vitamin C-deficient guinea pigs have depressed chemotaxis and bactericidal activities. Vitamin C can enhance the chemotactic response and motility of neutrophils from healthy individuals and has also been reported to normalize the reduced chemotactic and bactericidal activities of neutrophils from individuals with an inherited phagocytosis dysfunction.

Humoral defense

In the guinea pig, tissue saturating intakes of vitamin C produce higher plasma concentrations of the hydroxyproline-rich complement protein C1q than lower intakes. In man the role of ascorbate in maintaining complement levels is controversial. While complement levels have been found unaltered during scurvy and in volunteers taking 1 or 3 g vitamin C, and no relationship between blood vitamin C levels and complement titers was apparent in patients with acute infections, some studies reported increases in serum complement component C3 following vitamin C supplementation.

Ascorbic acid enhances the production of interferon in cultured mouse cells and in mice *in vivo*, and in human fibroblasts infected with some viruses but not with others.

Specific immune functions

Antibody production

Significant increases in serum immunoglobulin G (IgG) and IgM were found in institutionalized women when given 200 mg vitamin C twice daily for 4 months; this was also found in middle-aged people. However, in another study on volunteers, the levels of IgA, IgG and IgM were not affected by 1 or 3 g vitamin C.

Lymphocyte proliferation and transformation

The proliferative response of lymphocytes from vitamin C-deficient monkeys is significantly depressed *in vitro*, but the findings in guinea pigs are not consistent.

A number of investigators have found that vitamin C supplementation (1 g day^{-1}) enhanced *in vitro* lymphocyte proliferative responses in adults. In

elderly individuals, tissue saturating doses (500 mg, intramuscularly, daily for 1 month) enhanced cell-mediated immune function as manifested by enhanced T cell lymphocyte proliferative responses *in vitro* and tuberculin hypersensitivity *in vivo*.

After high doses of vitamin C, increased neutrophil motility associated with an increased T lymphocyte transformation was observed. The effect was explained by the prevention of induction of suppressor activity in human mononuclear leukocytes or protection of essential oxidizable groups in the monocyte membrane of ascorbate.

Delayed-type hypersensitivity

Depressed delayed-type hypersensitivity skin reactions as a result of inadequate vitamin C intake are restored following supplementation in guinea pigs. Normal delayed-type hypersensitivity responses were enhanced following megadose supplementation of vitamin C to young adults and after injections of 500 mg day^{-1} of vitamin C in a study in an elderly population. However, in another study, oral supplementation of 2 g day^{-1} vitamin C in an elderly population did not affect delayed-type hypersensitivity responses.

Antimicrobial and antiviral effects

Deprivation of vitamin C leads to decreased cellular immune functions and increased susceptibility to microbial infections. The effect of vitamin C on leukocytic microbicidal activity is controversial. No effect was found *in vitro* and a connection between the antimicrobial activity of phagocytes *in vitro* and the vitamin C status of healthy individuals could not be established. However, it was shown to exist in patients with Chediak-Higashi syndrome, where the microbicidal activity of the neutrophils against *Staphylococcus aureus* could be restored by giving the patients 50 mg kg^{-1} body weight of ascorbic acid daily. In general, orally administered high-dose vitamin C seems to have little effect on the course of infectious disease.

Leukocyte ascorbate levels rapidly decrease following a virus infection and return to normal after recovery. *In vitro* and in some animal experiments, ascorbic acid has displayed antiviral activity. In humans, in a very careful, placebo-controlled, double-blind study it was shown that vitamin C not only lessened the severity of rhinovirus cold but also reduced the potential for virus transmission.

Mechanism of action

There is now good evidence which indicates that part of the immunostimulatory properties of vitamin C are related to the antioxidant activity of ascorbate. During the activation of neutrophils, reactive oxidants are generated for the killing of phagocytosed microorganisms. Neutrophils are susceptible to damage by these self-produced oxidants. Protection against autooxidative neutrophil dysfunction is provided by ascorbate together with other antioxidants. The protection afforded by ascorbate does not compromise the bactericidal activity of neutrophils. Clearly, it is crucial that leukocytes possess antioxidant mechanisms to protect themselves from auto-oxidation during mobilization of host defenses.

Indirect effects via maintenance of vitamin E

Apart from direct effects, vitamin C may improve immune responses by protecting the antioxidant capacity of vitamin E, a potent immunoenhancing nutrient in its own right. Chronic vitamin C deficiency lowers the vitamin E levels in tissues and plasma. The free radical scavenging action by vitamin E is associated with the production of a vitamin E radical. Vitamin C reacts with the free radical of vitamin E and regenerates the reduced form, while being oxidized. The net result is a decrease in ascorbate and a maintenance of the antioxidant vitamin E level.

Summary

The high concentration of ascorbate in leukocytes and its rapid expenditure during infection and phagocytosis suggests a role for vitamin C in the immune process. Vitamin C enhances neutrophil chemotaxis and migration, stimulates the production of interferon, maintains the integrity of the mucous membranes and is involved in the induction and expression of delayed-type hypersensitivity. Its effects on complement and antibody production are controversial. Increased T and B lymphocyte proliferation following high-dose vitamin C supplementation is likely. Vitamin C reduces nonspecific extracellular free radical damage and autotoxicity following the oxidative burst activity of stimulated neutrophils. Vitamin C may enhance immune function indirectly by maintaining optimal vitamin E levels. Clinically, vitamin C has a role in the compensation of increased metabolic demands during infections and in prophylaxis, especially in individuals prone to infections because of functionally defective neutrophils.

See also: Antioxidant micronutrients and the immune system; Microbicidal mechanisms, oxygen-dependent; Nutrition and the immune system; Vitamin E and the immune system.

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VITAMIN D AND THE IMMUNE SYSTEM

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Vitamin D₃ is hydroxylated in the kidney to its physiologically active form, 1 α ,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃), known as calcitriol. Substantial evidence is accumulating that the hormonal form of vitamin D, calcitriol, can regulate the differentiation, growth and function of a broad range of cells or organs that are not directly involved in calcium homeostasis. In particular, calcitriol plays an important regulatory role in cell differentiation and proliferation of the immune system. It mediates its effects via specific intracellular vitamin D₃ receptors (VDRs). Malignant cell lines of the myelomonocytic and lymphocytic cell lineage, as well as normal peri-

pheral blood monocytes and tissue macrophages express VDRs similar to those found in classical target tissues of calcitriol. These receptor proteins bind the ligand with high affinity (K_d 10⁻¹⁰ M).

Some of the key findings with calcitriol *in vitro* and *in vivo* are shown in Table 1.

Effects of calcitriol on a variety of cells mediated by specific receptors

Accumulated evidence has established that the biological actions of calcitriol on calcium metabolism are mediated by binding to specific receptors in the

Table 1 Effects of calcitriol *in vitro* and *in vivo* on the immune system

In vitro studies:

Monocytes/Macrophages

- Induction of monocyte differentiation to macrophages
- Increased expression of MHC class II antigens induced by IFN γ
- Enhancement of chemotaxis, phagocytosis and cytotoxic functions
- Increased production of hydrogen peroxide
- Modulation of cytokine production

T lymphocytes

- Decreased proliferation of activated lymphocytes (antigen-, mitogen- or cytokine-induced)
- Inhibition of IL-2 production by activated T lymphocytes
- Inhibition of IFN γ and GM-CSF production by activated T lymphocytes
- Decreased proliferation of IL-1/PHA-activated thymocytes

B lymphocytes

- Inhibition of IgM and IgG synthesis mediated by an inhibitory action on the helper function of T lymphocytes

In vivo studies:

- Inhibition of cytokine release from monocytes from human volunteers
- Inhibition of autoimmune encephalomyelitis and thyroiditis in mice
- Prolongation of skin allograft survival in mice
- Potential of primary immune responses and inhibition of hyperimmune responses in mice
- Restoration of defective macrophage and lymphocyte functions in vitamin D-deficient rats and in patients with vitamin D-resistant rickets
- Restoration of lymphocyte proliferation and IL-2 production in patients on hemodialysis

intestine, bone and kidney. It has been shown that after binding to its receptor in the intestinal epithelial cells, calcitriol can induce the synthesis of calcium-transporting proteins, named calbindins, that mediate the uptake of calcium from the gut into the organism. In the last decade, calcitriol has been shown to exert biological activities far beyond its effects on calcium uptake, control of serum calcium levels and regulation of bone mineralization. The specific calcitriol receptor has been found in many cells and tissues not traditionally involved in the regulation of calcium metabolism, including various cancer cell lines, skin cells, muscle cells, hematopoietic cells and also in the pancreas, brain and pituitary. These ubiquitously expressed proteins appear to function as transcription factors mediating effects of calcitriol on a range of genes.

Initial observation in mouse myeloid leukemia cells suggested that calcitriol possessed a number of cell-regulating properties in differentiation and proliferation. Since then a large number of undifferentiated or low-differentiated cancer cells have been shown to differentiate towards the normal phenotype under the influence of calcitriol. At the same time, their rate of proliferation was reduced markedly. The cells of the upper layer of the skin, the basal epithelial keratinocytes, also responded to calcitriol *in vitro* by inhibition of growth, coupled with an increased rate of terminal differentiation. Among the cells of the immune system, monocytes and macrophages have been found to express the calcitriol receptor constitutively, whereas lymphocytes require prior activation with antigen or mitogen before expressing the receptor. The proliferation of activated T lymphocytes *in vitro* was found to be inhibited profoundly by calcitriol.

The immunological role of calcitriol

The secretory role of macrophages for calcitriol

In contrast to endocrine synthesis of calcitriol in the kidney, normal macrophages have also been shown to synthesize calcitriol when activated by agents such as interferon γ and lipopolysaccharide. Production of the hormone may thus act as part of the normal immune response and induction of calcitriol synthesis in response to infection may stimulate the synthesis of other inflammatory mediators such as interleukin 1 (IL-1), which will in turn affect the level of lymphocyte activity. Initial evidence for the interaction of calcitriol with the immune system arose from studies of the disease sarcoidosis, a chronic granulomatous disorder often associated with hypercalcemia and elevated circulating levels of calcitriol. The latter have been attributed to ectopic synthesis

of calcitriol by immune cells associated with sarcoidosis, such as alveolar macrophages. The production of the hormone by sarcoid macrophages appears to be insensitive to feedback control by calcitriol itself, as well as other regulators such as calcium and parathyroid hormone.

Effect of calcitriol *in vitro* on monocytes and macrophages

Monocytes and macrophages from both human and animal sources constitutively express the calcitriol receptor. Upon incubation with calcitriol, monocytes acquire characteristics of functionally mature macrophages, and a general enhancement of the antigen-presenting cell function is observed. Calcitriol also enhances the antibody-dependent cytotoxic function of monocytes/macrophages, and calcitriol-treated human monocytes exhibit enhanced phagocytosis and killing of mycobacteria. Other studies have shown that the hormone increases Fc receptor expression on monocytes. Reports have also demonstrated that calcitriol treatment of peripheral blood monocytes increases the production of hydrogen peroxide and of heat shock proteins. Several studies indicate that calcitriol downregulates cytokine production (IL-1, IL-6, tumor necrosis factor α (TNF α)) by antigen or lipopolysaccharide (LPS)-stimulated monocytes, whereas others have found unchanged or increased production, depending on the eliciting stimulus and the culture conditions.

Macrophages are derived from less mature myeloid stem cells which originally have a common pluripotent cell origin and many laboratories have examined the effect of vitamin D on stem cell development, in particular the mononuclear phagocyte system. These studies have been greatly facilitated by the development of leukemic cell lines from myeloid precursor cells. It is not yet clear at which point in the stem cell pathway VDR induction occurs, but more mature cell lines such as HL-60 and U937 express these receptors and are responsive to calcitriol, which stimulates differentiation towards macrophage-like cells. In this respect the antiproliferative effects of calcitriol are similar to those observed with agents such as retinoic acid, dimethylsulfoxide and phorbol esters. These *in vitro* effects of calcitriol are observed at physiological concentrations of 10^{-10} – 10^{-8} mol l $^{-1}$.

Effect of calcitriol *in vitro* on lymphocytes

Calcitriol has also been shown to act on T and B lymphocytes, and antibody-producing function of lymphocytes (Table 1). Studies *in vitro* have suggested that the main effect of calcitriol is to inhibit the proliferation of activated T cells which, unlike

resting T cells, express VDR. Lymphocytes require activation in order to express the calcitriol receptor. Following this initial activation induced by antigen, cytokine or mitogens, incubation with calcitriol inhibits T lymphocyte proliferation. IL-2 production by activated T lymphocytes is strongly inhibited by calcitriol and this seems to be one of the crucial events in the immunoregulatory activities of calcitriol, linking it to the immunosuppressive properties exerted by known immunosuppressants such as cyclosporine.

Calcitriol also inhibits interferon γ synthesis by T cells, and this may act as part of the control of calcitriol synthesis by macrophages which produce the hormone (calcitriol) when stimulated with interferon γ .

T cell development takes place in the thymus with VDR being expressed in medullary cells, but not in the less mature cortical cells. However, the precise role of calcitriol in T lymphocyte development is unclear as VDR expression is lost after the cells leave the thymus. VDR are also present in natural killer cells, indicating that calcitriol may have a role in modulating the immune response to viral infection and neoplastic development. The interaction of vitamin D with B cells is less clear than its effects on T cells, and although calcitriol inhibits immunoglobulin production by B cells this appears to be due to indirect suppression of the antibody-stimulating activity of T helper cells rather than a direct effect on B cells.

Effect of calcitriol on immune functions *in vivo*

Administration of calcitriol can prevent the development of experimental autoimmune encephalomyelitis and thyroiditis in mice and prolong the survival of transplanted skin allografts in mice. In normal mice, administration of 1α -hydroxyvitamin D_3 [$1\alpha(OH)D_3$], a synthetic vitamin D analog that is converted to calcitriol in the organism, leads to an increased primary antibody response, but the same treatment suppresses the hyperimmune response induced by concomitant administration of colchicine. Reports have demonstrated a reduction in antibody formation in ricketic rats challenged with diphtheria toxin or Sendai virus, a more fulminant spread of pneumococcal and *Klebsiella* infection when ricketic rats were inoculated with these organisms, and a decrease in antibody-forming cells in the spleen from ricketic rats. Several studies have shown that treatment with calcitriol or $1\alpha(OH)D_3$ can restore deficient macrophage and lymphocyte functions in vitamin D-deficient rats and patients with vitamin D-resistant rickets. Calcitriol blocks the development of renal disease and improves survival in the MRL

mouse model of autoimmunity. These findings suggest a role for calcitriol under physiological conditions as an important factor in sustaining normal functions of the immune system (which can be compromised by vitamin D deficiency).

Clinical studies

Nutritional vitamin D deficiency has been shown to be associated with impaired immune responses such as decreased leukocyte chemotaxis, and has also been reported to increase susceptibility to infection and predisposition to disseminated tuberculosis. Calcitriol or $1\alpha(OH)D_3$ can restore deficient macrophage and lymphocyte functions in patients on hemodialysis. The contribution of immune effects of calcitriol on mineral homeostasis have yet to be studied in detail, but may have a significant effect on calcium mobilization. Calcitriol stimulates monocyte secretion of IL-1 and prostaglandin E_2 , both of which induce bone resorption but inhibit lymphocyte production of interferon γ , a suppressor of calcium mobilization.

Because a large number of undifferentiated or low differentiated cancer cells have been shown to differentiate toward normal cell phenotypes, efforts to treat cancer with calcitriol have been developed. A potentially important clinical application of the anti-proliferative effects of calcitriol on monocytes has been in the development of antileukemic agents. The use of vitamin D metabolites in myeloproliferative disorders *in vivo* has, so far, been less successful than related agents such as retinoic acid. This idea has stimulated an interest in the possible use of calcitriol in the treatment of other proliferative diseases such as psoriasis and also in the treatment of autoimmune disorders and in the prevention of graft rejection. However, the therapeutic application of calcitriol is limited by its potent effects on calcium metabolism which may result in development of hypercalciuria, hypercalcemia and soft tissue calcifications. A wide range of new vitamin D analogs are currently being investigated with the aim of retaining the sensitive differentiating properties of calcitriol with a decreased risk of induction of the dangerous hypercalcemic side-effects. The interested reader is referred to the comprehensive review by Binderup concerning structural and clinical aspects of various calcitriol analogs. Recent studies have also shown that calcitriol inhibits both CD4 expression and human immunodeficiency virus (HIV) infection of human monocytes, and this offers another potential application in the development of new vitamin D analogs for the treatment of immunodeficiency.

There is now accumulating evidence for the contribution of endocrine systems to the immune response,

and calcitriol may have a particularly versatile role in this interaction. Localized production of the hormone can benefit the immune environment in several ways. First, by stimulating phagocytic and antigen-presenting actions, calcitriol may help to promote initial immune responses. This would include the recruitment of additional monocytes by promotion of stem cell differentiation, but may also involve activation of T cells via antigen presentation and cytokine secretion. These stimulatory effects are counterbalanced by the ability of the hormone to inhibit T cell proliferation and thereby act as part of the feedback control of the immune response. Vitamin D may be seen as an addition to the long list of cytokines which have been reported in recent years. However, its close association with immune disorders and potential therapeutic applications have made a particularly important contribution to these studies.

The role of vitamin D-binding protein in the immune system

Vitamin D-binding protein is a precursor for the macrophage-activating factor

Inflammation results in chemotaxis and activation of macrophages. Inflamed tissues release lysophospholipids. Administration of lysophosphatidylcholine (lyso-Pc) or other lysophospholipids to mice activates macrophages for phagocytic and superoxide gener-

ation capacities. This inflammation-primed macrophage activation process is the major macrophage-activation cascade, which requires serum vitamin D₃-binding protein (DBP; human DBP is known as Gc protein) and participation of B and T lymphocytes. Stepwise incubation of inflammation-primed (or lyso-Pc-treated) B cells and T cells in a medium containing purified human Gc protein generates a potent macrophage-activating factor. However, stepwise cultivation of inflammation-primed T cells and B cells in a medium containing purified human Gc protein generates no macrophage-activating factor.

Analysis of intercellular signal transmission among these lymphocytes revealed that inflammation-primed (or lyso-Pc-treated) B cells modify Gc protein to yield the macrophage-proactivating factor which can be converted by T cells to the macrophage-activating factor. Gc glycoprotein (mol. wt 52 000; 458 amino acid residues) carries a trisaccharide composed of *N*-acetylgalactosamine with dibranched galactose and sialic acid termini. The generation process of the macrophage-activating factor was also demonstrated by stepwise incubation of Gc protein with an inducible membranous β -galactosidase of B cells and the membranous sialidase (Neu-1) of T cells to yield the macrophage-activating factor (MAF), a protein with *N*-acetylgalactosamine as a remaining sugar. Thus, Gc protein (or DBP) is a precursor for MAF as shown in Figure 1A. Conversion of Gc protein to macrophage-activating factor requires a sequential

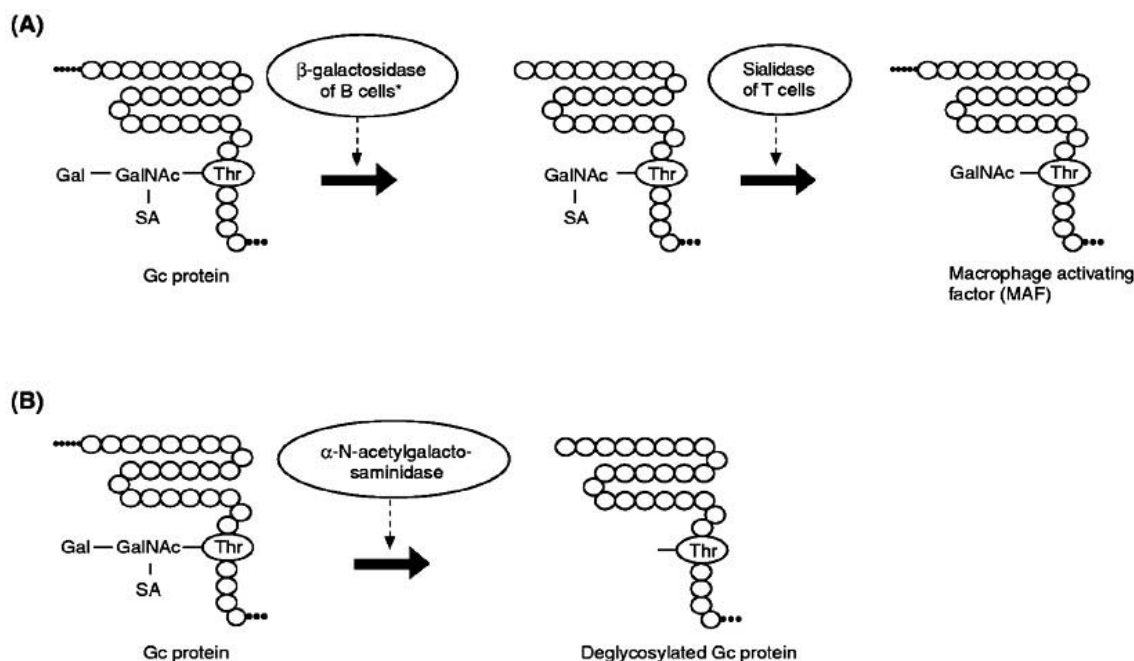


Figure 1 Schematic illustration of formation of macrophage-activating factor (A) and deglycosylation of Gc protein (B). *. lyso-Pc-treated B cells.

contact of Gc protein with the inducible β -galactosidase of B cells and the Neu-1 sialidase of T cells. These glycosidases are present in limiting amounts on the plasma membranes. Native Gc protein must first interact with B cells and, through the action of β -galactosidase, have a galactose moiety removed. This yields a proactivating factor whose structure allows it to then interact with T cells. The T cell sialidase cleaves off the sialic acid residue, producing a macrophage-activating factor. Thus, the specific oligosaccharide sugars of the native Gc protein and its derivatives would appear to recognize the respective lymphocyte receptors. The affinity studies of the membranous receptors for Gc derivatives revealed that the native Gc protein binds B lymphocytes while the proactivating factor binds T lymphocytes exclusively. Further, the macrophage-activating factor binds macrophage exclusively. However, there is no evidence that these Gc derivatives or macrophage-activating factors, transmit calcitriol or its precursors to lymphocytes and macrophages during these processes.

Enzymatic conversion of vitamin D-binding protein to MAF

In vitro incubation of Gc protein with a mixture of commercial β -galactosidase and sialidase generates a

potent macrophage-activating factor, termed GcMAF. For clinical application of GcMAF, immobilized glycosidases should be used to prevent contamination of enzymes. Stepwise incubation of Gc protein with immobilized β -galactosidase and sialidase revealed that either sequence of hydrolysis of Gc glycoprotein by these glycosidases yields a remarkably potent GcMAF (Figure 2). A 3 h incubation of mouse peritoneal macrophages or human peripheral blood monocytes/macrophages with 4–10 $\mu\text{g ml}^{-1}$ of GcMAF results in greatly enhanced (5- to 7-fold) phagocytic capacity and a 15-fold increase in superoxide generation. Administration of 4–10 $\mu\text{g/mouse}$ (10–100 ng/human) of GcMAF also results in a greatly enhanced (5- to 7-fold) phagocytic and 15-fold increased superoxide-generating capacities of macrophages.

Administration to mice of GcMAF (3–50 $\mu\text{g/mouse}$) and sheep red blood cells simultaneously produces a large number of antibody-secreting splenic B cells (8×10^4 to 24×10^4 cells per spleen) in 2–4 days. Thus, the structurally well-defined macrophage-activating factor has a potent adjuvant activity for immunization of mice with sheep erythrocytes.

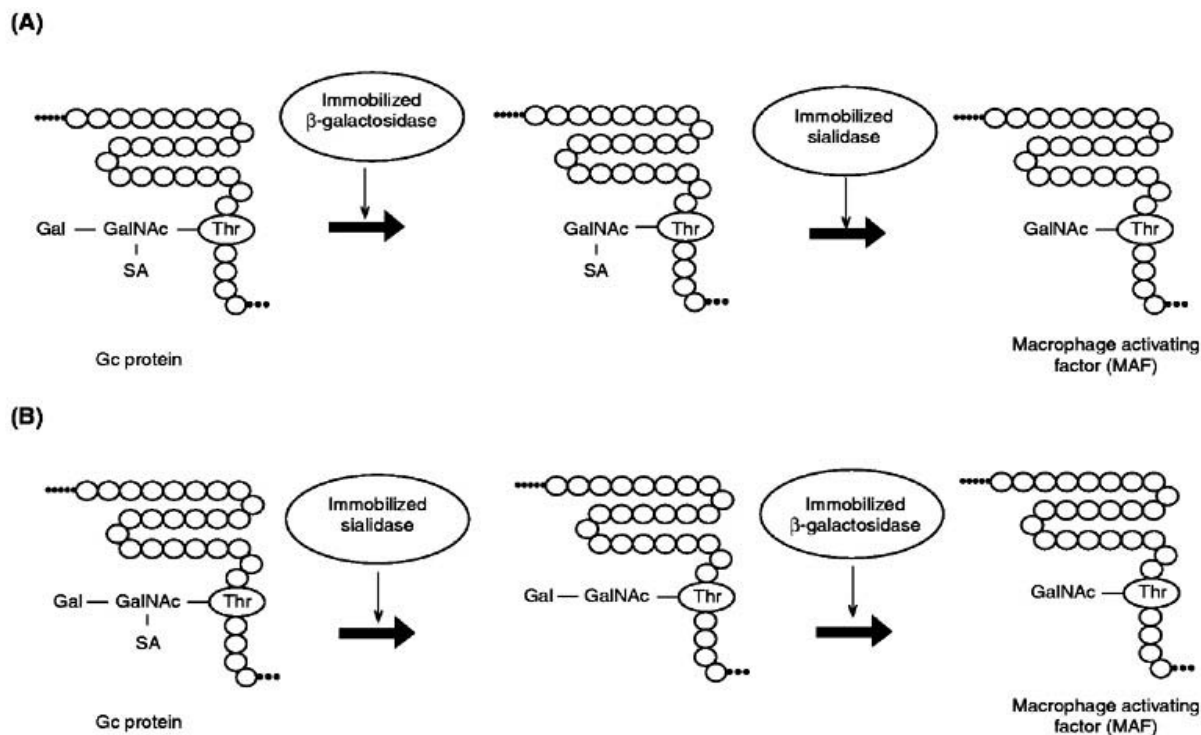


Figure 2 Two stepwise enzymatic pathways of Gc protein with immobilized glycosidases to yield the macrophage-activating factor (GcMAF). Stepwise hydrolysis of Gc protein by β -galactosidase and sialidase (A). Stepwise hydrolysis of Gc protein by sialidase and β -galactosidase (B).

Clinical significance of GcMAF

Vitamin D-binding protein, the precursor protein for the macrophage-activating factor, is abundant in the serum because Gc protein circulates in remarkably high concentration (250–350 $\mu\text{g ml}^{-1}$) in the blood-stream. Only 5% of Gc protein carries 25-hydroxy-vitamin D_3 which is a reservoir form of vitamin D. Gc protein has a strong affinity for 25-hydroxyvitamin D_3 but a weak affinity for calcitriol. This differential affinity of vitamin D metabolites allows Gc protein to transport and transmit the vitamin D metabolites to target cells. However, purified Gc protein, free of 25-hydroxyvitamin D_3 , is enzymatically converted to GcMAF. Addition of vitamin D metabolites during the above GcMAF generation process has no effect on generation rate or potency of GcMAF.

The membranous glycosidases of inflammation-primed lymphocytes rapidly convert serum Gc protein to the potent macrophage-activating factor. This inflammation-primed macrophage-activation process appears to be the major macrophage-activation cascade which is shared by other phagocytes such as osteoclasts. A defect in the inducible β -galactosidase of B lymphocytes in the macrophage-activation cascade produces dysfunctional macrophages and osteoclasts which manifest osteopetrosis in newborn. GcMAF can bypass the defective lymphocyte function and act directly on phagocytes. *In vitro* incubation of human osteoclasts with GcMAF efficiently activates osteoclasts as determined by superoxide generation. GcMAF has a capacity to rectify the osteopetrotic disorder as demonstrated by increased bone resorption and marrow formation when administered to osteopetrotic mice.

Another defect in the macrophage-activating cascade can be caused by deglycosylation of serum Gc protein by α -N-acetylgalactosaminidase secreted into the bloodstream from cancer cells or HIV-infected cells, as shown in Figure 1B. Deglycosylated Gc protein can not be converted to macrophage-activating factor, leading to immunosuppression. An impairment of immune function of the host will enhance opportunities for survival and proliferation of neoplastic cells.

See also: Cytokines; Leukemia; Macrophage activation; Monocytes; Neuroendocrine regulation of immunity; Nutrition and the immune system.

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VITAMIN E AND THE IMMUNE SYSTEM

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Vitamin E refers to a group of eight naturally occurring compounds: α -, β -, γ - and δ -tocopherols and tocotrienols, with the naturally occurring D- α -tocopherol having the highest biological activity. Rich sources of vitamin E include vegetable oils and products made from these oils, with smaller yet appreciable levels found in nuts, green leafy vegetables and wheat germ. Meats, fruits and vegetables seem to have little vitamin E.

Vitamin E is the major chain-breaking antioxidant in body tissues, protecting cell membranes at an early stage of free radical attack through its free radical-quenching activity. Singlet oxygen (an energized form of oxygen) is produced as a result of an oxidation process, damaging cell membranes and chromosomal material as well as producing free radicals. In addition, environmental pollutants such as smog, radiation, cigarette smoke, pesticides, herbicides and many drugs can react with tissues to produce free radicals, which react very quickly causing damage to membranes, enzymes, nucleic acids and inactive proteins. Specific damage to cells' RNA or DNA may result in uncontrolled cellular transformation and cancerous cell growth. Unless quenched by an antioxidant like vitamin E, highly unstable free radicals attack the polyunsaturated fatty acids of membrane phospholipids.

Vitamin E is also effective in preventing biological damage by immunoenhancement. High doses of vitamin E decrease CD8⁺ T cells and increase CD4⁺:CD8⁺ T cell ratio, increase total lymphocyte count, and stimulate activity of cytotoxic cells, natural killer (NK) cell activity, phagocytosis by macrophages and mitogen responsiveness. The immunostimulatory nature of vitamin E provides a basis for its use in the therapeutic modulation of immune functions in humans. Vitamin E supplementation is nontoxic, and may provide an effective therapy for prevention of several different diseases.

Substantial evidence from tissue culture and animal studies show that vitamin E is required by the immune system. In experimental models resistance to infections, antibody production in response to antigens, delayed cutaneous hypersensitivity skin tests, *in vitro* mitogenic responses of lymphocytes, phagocytic index and reticuloendothelial system clearance can be suppressed by vitamin E deficiency. Conversely, vitamin E has been found to stimulate helper

T cells, NK cell cytotoxicity, cytotoxic T lymphocyte activity, antibody response, macrophage phagocytosis, T and B cell mitogen responsiveness, and resistance to infection in animals.

The effect of vitamin E on the immune system depends on interaction with other antioxidant and pro-oxidant nutrients, polyunsaturated fatty acids, and other factors modulating the immune response, such as age and stress. The extent to which these vitamin E-induced immunological changes are associated with meaningful clinical outcomes has not been well studied. However, high plasma vitamin E levels are associated with a low incidence of infectious disease and colon cancer.

As immune responsiveness declines with age, with an increased incidence of infectious disease and tumors, vitamin E stimulation of immunity may be particularly applicable to the elderly. There was a significant increase in helper T cells and lymphocyte proliferation responses in institutionalized elderly patients given vitamins A, C and E for 28 days. In a community-based survey of 100 healthy subjects over 60 years of age, plasma vitamin E levels were positively correlated with delayed hypersensitivity responses to diphtheria toxoid, *Candida* and *Trichophyton*. Positive correlations were also observed between vitamin E levels and the number of delayed hypersensitivity responses in men; subjects with tocopherol levels greater than 135 mg l⁻¹ were found to have higher CD4:CD8 cell ratios.

Vitamin E may be beneficial to host defense mechanisms by preventing the infection-induced increases in tissue prostaglandin production from arachidonic acid.

Regulation of cytokine production by vitamin E could have major consequences on the prevention of disease development via modulation of immune responses. Vitamin E supplementation to levels 15-fold over standard mouse diets significantly increased interleukin 2 (IL-2) and interferon γ production by mitogen-stimulated splenocytes *in vitro*. Vitamin E at physiological concentrations (1–5 μ g ml⁻¹) *in vitro* significantly stimulated the production of IL-2 and interferon γ by mitogen-stimulated lymphocytes. Although vitamin E supplementation (800 IU D, α -tocopheryl acetate for 48 days before exercise) of elderly human subjects did not affect the basal production of IL-1 or tumor necrosis factor, it prevented

the exercise-induced rise in IL-1, and inhibited the production of IL-6. IL-1 and IL-6 have been implicated in exercise-induced muscle proteolysis and damage.

In the murine acquired immune deficiency syndrome (AIDS) vitamin E supplementation stimulates retrovirus-suppressed production of T_H1 cytokines and cell-mediated immunity, whereas it inhibits the excessive secretion of T_H2 cytokines and the hypergammaglobulinemia induced by the retrovirus. Thus, the normalization of T_H1/T_H2 cytokine secretion by vitamin E provides a mechanism for resolution of hypergammaglobulinemia and restoration of the cell-mediated immune response during murine AIDS.

In patients with human immunodeficiency virus (HIV) infection, there are high serum levels of IL-6 and tumor necrosis factor α (TNF α). Excessive amounts of these cytokines are also produced by mitogen-stimulated splenocytes or macrophages from LP-BM5 retrovirus-infected mice. Increased IL-6 and TNF α production would explain the hypergammaglobulinemia and global B cell dysfunction seen with both retrovirus infections. Dietary vitamin E significantly reduced the increased production of IL-6 and TNF α by mitogen-stimulated splenocytes, perhaps providing an explanation for the vitamin E-mediated alleviation of hypergammaglobulinemia and global B cell dysfunction in murine AIDS.

A dietary supplementation of 2–10 times higher than currently recommended levels of vitamin E significantly increased humoral and cell-mediated immune responses and phagocytic functions in laboratory and farm animals. It also increased their resistance to infectious diseases, and may counteract the immunosuppressive effect of stress in animals. Other antioxidant nutrients can interact with vitamin E in these processes.

A principal function of vitamin E is the prevention

of lipid peroxidation of cell membranes, which may form part of the mechanism of enhancing immune responses and phagocytosis. The rapidly proliferating cells of the stimulated immune and phagocytic systems are particularly prone to peroxidative damage by free radicals, peroxides and superoxides. The antioxidant effect also modulates the biosynthesis and activity of important cell regulators including prostaglandins, thromboxane and leukotrienes.

See also: Antioxidant micronutrients and the immune system; Nutrition and the immune system.

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VITILIGO

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Vitiligo is a syndrome of depigmentation of the skin caused by damage to epidermal melanocytes. This may occur as a component of autoimmune syndromes, as a polygenic disorder, or sporadically. Melanocyte damage in vitiligo is believed to be caused by a number of cytotoxic stimuli: specific immune reactions, accumulation of toxic products of melanization, biochemical imbalances which favor cellular destruction, or neural stimuli.

Characteristics of vitiligo

Vitiligo is a syndrome characterized by acquired loss of pigmentation in a usually symmetrical but 'spotty' distribution, commonly involving the central face and lips, genitalia, hands and extremities. Pigmentation in the skin is produced by melanin, a heteropolymer synthesized in melanocytes by a complex biochemical pathway controlled by the rate-limiting

enzyme tyrosinase. Melanin is packaged in melanosomes which are transferred to keratinocytes to provide broad-spectrum photoprotection to the skin.

Jarrett and Szabo first proposed that vitiligo was characterized by two stages that might be sequential: type I vitiligo with decreased melanocyte tyrosinase activity, and type II vitiligo in which melanocytes were destroyed. In established vitiligo, cells with ultrastructural and immunohistochemical features of melanocytes are lost from the basal layer of the epidermis. Vacuolar damage to both basal layer melanocytes and keratinocytes can be found in association with infiltrates composed of monocytes and lymphocytes. Melanocytes and keratinocytes in the active border of vitiligo lesions show increased ICAM-1 expression, suggesting local effect of cytokines and potentiation of immunologic cytotoxicity.

Proposed pathological mechanisms

Several theories have been proposed to explain the functional inhibition and melanocyte destruction in vitiligo: the autocytoxic hypothesis, the neural hypothesis and the autoimmune hypothesis. In the autocytoxic hypothesis, toxic melanin intermediates are produced in excess or imbalance, causing autocytoxicity. The neural hypothesis is based on a proposed adrenergic/cholinergic imbalance in some vitiligo patients, and was promoted as a cause for segmental vitiligo. Schallreuter and Wood have proposed a variant of this hypothesis, that vitiligo is a disease of the entire epidermis in which heightened adrenergic responses lead to biochemical imbalances which inhibit melanization, but do not necessarily destroy melanocytes. They have demonstrated a number of biochemical abnormalities which induce decreases in antioxidant defenses and increases in tetrahydrobiopterin, which are proposed to inhibit melanization or to bleach melanin. The autoimmune hypothesis is based on the association of vitiligo with numerous autoimmune syndromes. Melanocyte-specific autoantibodies have been consistently found in modern studies of vitiligo, and these antibodies have been proven to induce cytotoxic damage of melanocytes.

Melanocytes are abnormally susceptible to immunologic cytotoxicity and to cytotoxicity induced by reactive oxygen species, in part because of defective antioxidant defenses. Abnormalities observed in cultured melanocytes from the margins of vitiligo lesions have led to the proposal that there are intrinsic melanocyte defects in vitiligo. It is accepted that multiple factors may influence melanocyte destruction in vitiligo, leading to a common

endpoint of melanocyte inhibition, then cytotoxic destruction. However, immunologic damage to melanocytes in vitiligo is still the most convincing mechanism of melanocyte damage, supported by extensive immunopathology and immunocytotoxicity experiments.

Autoimmune features of vitiligo

Vitiligo can be found in autoimmune families in association with Addison disease, alopecia areata, pernicious anemia, or mucocutaneous candidiasis, with an incidence 10–15 times higher than in the general population. Antibodies to thyroid, adrenal gland and gastric parietal cells can be demonstrated in vitiligo patients; using modern techniques, antibodies reactive with melanocytes have been consistently found in vitiligo patients' sera. The levels of these antibodies are greatest in patients with actively progressing disease and in vitiligo patients with other autoimmune disease. Both melanocyte-specific antibodies and antibodies reactive to other cells in addition to melanocytes have been identified. Some of these antibodies are specific for proteins involved in melanization such as tyrosinase and tyrosinase-related protein 1. Antibodies to a melanocyte-specific 85 kDa antigen have been described in dogs, cats and horses with vitiligo. Similar antibodies are found in a chicken model of vitiligo; bursectomy in these animals delays onset of vitiligo.

The functional significance of these autoantibodies was demonstrated when Norris and colleagues showed that cytotoxicity of cultured human melanocyte targets could be induced by antibodies in vitiligo patients' sera by either complement-mediated lysis or antibody-dependent cellular cytotoxicity (ADCC). Other investigators have subsequently verified that autoantibodies in vitiligo patients' sera can induce cytotoxic damage of melanocytes by multiple antibody-dependent mechanisms. In a nude mouse model, injection of sera from vitiligo patients induced depigmentation of human skin grafted onto the nude mouse. Cytotoxicity induced by autoantibodies is the only proposed mechanism in vitiligo in which a specific factor isolated from vitiligo patients has been directly demonstrated to induce melanocyte destruction both *in vitro* and *in vivo*.

The factors inducing melanocyte destruction may differ in individual vitiligo patients. Members of autoimmune families are predisposed to produce autoantibodies, and melanocyte-specific autoantibodies are likely to be the major determinant of melanocyte cytotoxicity in these families. In other vitiligo patients it is possible that autoimmune melanocyte destruction may represent a later stage of

disease, following melanocyte destruction by auto-cytotoxicity or other factors. However, once melanocyte-specific antibodies are produced, it is likely that these produce additional melanocyte damage and the depletion of melanocytes in the basal layer of the epidermis.

See also: Adrenal autoimmunity; Antibodies, specificity; Antibody-dependent cellular cytotoxicity; Antigens, cell surface; Autoimmune diseases; Cytotoxicity, mechanisms of; Polyendocrine autoimmunity; Skin, autoimmune diseases.

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WESTERN BLOTTING

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Western blotting (immunoblotting, protein blotting) is a sensitive immunological method for detecting electrophoretically separated proteins. The technique was developed independently by several groups in 1979 and was later termed 'western blotting' because of its analogy to Southern and northern blotting. Its characteristic feature is the transfer of a complex protein pattern resolved by slab gel electrophoresis to a membrane which serves as a carrier for all subsequent immunochemical reactions. Major fields of application in basic research are determination of molecular weights of protein antigens, detection of antigens in crude mixtures of proteins, and assessment of the quality of animal antisera. Western blotting has found important clinical applications in autoimmune disease, allergy, infectious diseases and others.

Steps of western blotting experiments

Basically, western blotting experiments consist of five steps (cf Figures 1 and 2 and Table 1):

1. Sample preparation and electrophoretic separation in a slab gel.
2. Transfer to a membrane with high protein binding capacity.
3. Blocking (quenching) of the residual binding capacity of the membrane.
4. Incubation with antibody.
5. Detection of bound antibody with secondary reagents.

The procedure combines elements of immunoelectrophoresis, enzyme-linked immunoassay, and – with respect to the localized dye formation – immunohistochemistry. The deposition and immobilization

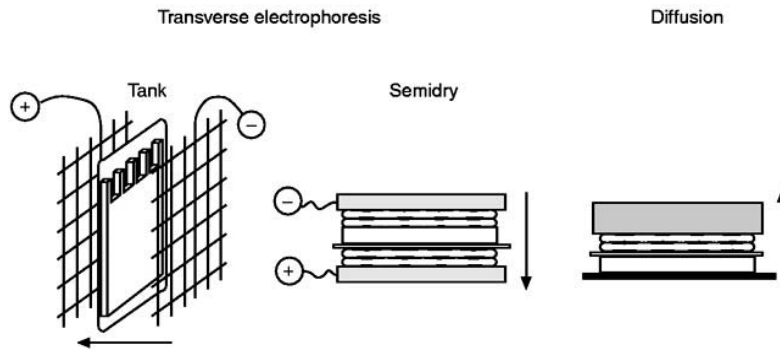


Figure 1 Common methods of transferring proteins from a slab gel to an immobilizing membrane. Two methods rely on transverse electrophoresis either by a buffer tank device (buffer tank, plastic pads and supports not shown) or by a semidry blotting apparatus where transfer is mediated by filter paper soaked in buffer. The third method, transfer by diffusion, is particularly useful for thin plastic supported gels. The arrows indicate the direction of the movement of the proteins from the gel towards the immobilizing membrane.

of the proteins on a membrane greatly facilitates access of large molecules (antibodies) to the antigen as well as all washing and any further incubation steps. Western blotting is particularly valuable for characterizing protein antigens which are difficult to analyze by immune precipitation, e.g. antigens not soluble in buffers compatible with immunological reactions or antigens that cannot be radioactively labeled (e.g. complex allergens, protozoa, food components, etc.).

Electrophoretic separation

The numerous variations possible at each step render western blotting a versatile analytical tool. For separation, electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) is usually preferred because this detergent solubilizes virtually all proteins and because the system yields information on the molecular weight of the proteins. Western blot-

ting is also easily adapted to agarose gels (e.g. isoelectric focusing) or to electrophoresis in buffer systems containing urea or physiological buffers.

Transfer

Several methods have been devised to transfer the resolved proteins to membranes. Replicas may be produced by letting the proteins diffuse to a membrane placed on one – or both sides – of the gel. However, diffusion from thick polyacrylamide gels is slow and inefficient. To speed up elution transverse electrophoresis is usually employed (Figure 1). An electric field is applied perpendicularly to the gel surface. The immobilizing membrane and the gel are held in place by plastic sponges and plastic grids to form a rigid sandwich assembly which is placed into a chamber containing buffer and plate electrodes. Alternatively, a horizontal setup is used where gel and membrane are sandwiched between several

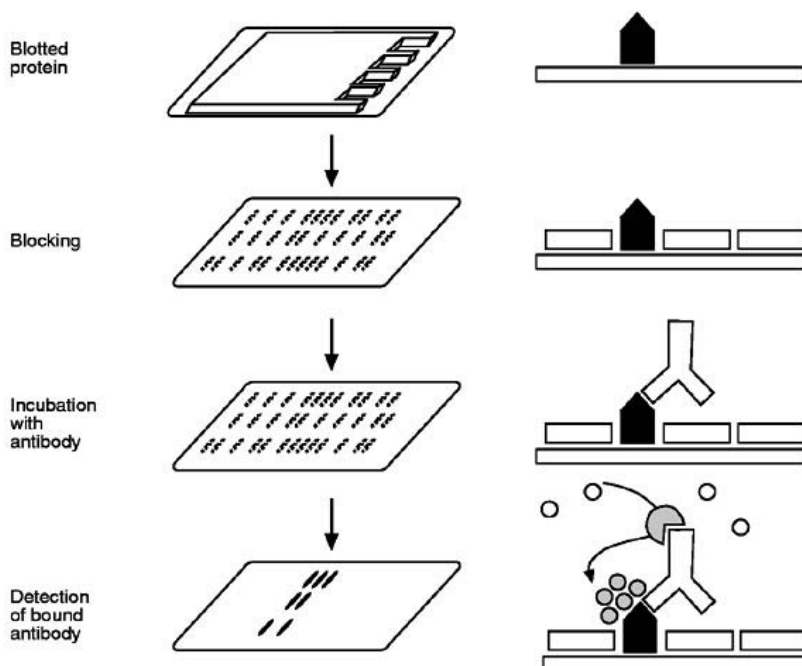


Figure 2 Scheme of a typical immunological detection procedure for protein antigens transferred by western blotting. Microscopic views of the membrane are shown on the right.

Table 1 Commonly used components in western blotting

Transfer methods	Transverse electrophoresis Tank system Semidry system Diffusion Vacuum induced flow
Membranes	Nitrocellulose (NC) Polyvinyl difluoride (PVDF) Nylon derivatives
Blocking solutions	Bovine serum albumin Defatted dry milk Nonionic detergents
Primary ligands	Animal or human serum Affinity purified antibodies Monoclonal antibodies
Secondary reagents	Antibodies Protein A or protein G [as enzyme conjugated reagents (alkaline phosphatase, peroxidase) or as iodinated reagents]
Detection principles	Color formation Radioactivity Chemiluminescence Fluorescence

layers of filter paper imbibed with buffer (semidry blotting). The buffer for electrophoretic elution has to be chosen in such a way that all proteins move into the same direction. In order to retain the negative charges as well as to keep the proteins soluble, electroelution from SDS-gels is done in the presence of a low concentration (0.01%) of SDS. An excess of the detergent prevents adsorption to the membrane.

Membranes

Immobilizing membranes are extremely porous and provide a large internal surface. The binding capacity of a membrane is in the range of 100-fold higher than a flat surface. Nitrocellulose is one of the earliest types of membrane used for western blotting and still enjoys wide popularity. The forces retaining proteins on nitrocellulose are believed to be hydrophobic, mainly. This view is supported by the observation that nonionic detergents may release adsorbed proteins. Novel, sturdier membranes, for example based on nylon or other synthetic polymers, have been developed to suit the needs of blotting techniques. In particular, polyvinyl difluoride (PVDF) membranes have found extensive use. Substitution with strongly positively charged groups has further improved the binding capacity of membranes, presumably by adding ionic binding forces to the hydrophobic interaction. Furthermore, several types of chemically activated membranes are in use which allow covalent binding of the transferred protein.

Visualization of total protein pattern

In order to assure that the proteins are transferred and retained on the membrane the protein pattern can be visualized by a range of protein-staining methods. Several dyes (e.g. Ponceau S) allow reversible staining and for these antigenicity is usually not impaired. Molecular weight standards covalently derivatized with a dye are also useful for monitoring the efficiency of the transfer and, in addition, later serve to position immunostained bands relative to marker proteins on the membrane.

Blocking

The high capacity to absorb proteins nonspecifically interferes with ensuing steps which rely on specific binding interactions. In order to avoid nonspecific sticking of the antibody probe, the excess binding capacity of the chosen membrane has to be saturated. Blocking can be achieved by concentrated solutions of cheap proteins (bovine serum albumin, an irrelevant serum, milk proteins, etc.). As nonionic detergents do not usually interfere with antibody binding, they can be used alone or as additions to protein-based blocking solutions. Blocking with detergents allows staining of the total protein pattern after visualization of immunologically stained bands, an aspect particularly important for two-dimensional separations.

Probing with specific antibodies

Western blotting may be performed with both polyclonal and monoclonal antibodies. In most respects, considerations given to solid phase immunoassays in microtiter plates also apply to western blotting. Hence, the signal ultimately generated will depend on the interplay of numerous factors such as affinity, concentration, duration of incubation, nonspecific binding, etc. As a rule of thumb, solutions of $1 \mu\text{g ml}^{-1}$ specific antibody incubated for several hours is a good starting point for optimizations. Only a small fraction of the antibody in the incubation mixture will be bound to the immobilized antigen, even if monospecific antibodies are used. Due to the thickness of the membranes, incubations and washing steps are often longer than for solid phase immunoassays on nonporous surfaces such as microtiter plates. Before the next step (detection of bound antibody), nonbound primary antibody has to be washed out thoroughly.

Detection of bound antibody

It is possible to directly label the primary antibody, for example by radioiodination. However, it is the large choice of secondary reagents which accounts

for the flexibility of western blotting and the large signal amplification that often is needed. There is virtually no limitation to the variations at this step and any of the labels (radioactivity, enzymes, fluorescence, colloidal gold or dyes) may be combined with antibody-binding reagents (antibodies directed against the primary antibody, protein A, protein G). The avidin-biotin system with all of its variations has also been introduced for blotting applications. Some of the staining protocols have in part been adapted from immunohistochemistry where localized enzymatic color formation is the final readout, as in western blotting. When monitoring patient sera it is often important to assess the class of the reactive immunoglobulins (IgE in allergy, IgM/IgG in infectious diseases, etc.). With commercially available specific reagents this can be readily achieved on western blots. Sensitive detection in the range of picograms of antigen is accomplished with chemiluminescent enzyme substrates where the products decay under light emission. Bands are made visible by exposing the blot to photographic films or sensitive cameras for a few seconds up to several minutes.

Quantification

Blots may be quantitatively analyzed by reflectance densitometry of enzymatically stained blots of autoradiographs as well as by directly counting excised areas of a radiolabelled blot. Usually by comparing with appropriate standards, the amount of antigen or the potency of an antiserum can be measured.

Antigenicity of blotted proteins

The sample treatment before electrophoresis usually affects the conformation of proteins and influences their antigenicity. Even mere adsorption to solid phases may lead to loss or gain of antigenicity, be it due to interactions between the solid phase and the antigenic region of the protein or due to conformational changes associated with adsorption. If polyclonal antibodies are used as probes on western blots, sample treatment is often less critical than for monoclonal antibodies. The phenomenon may be explained by considering that various types of antibody may be present in a polyclonal serum: 1) antibodies recognizing the native molecule exclusively, 2) antibodies recognizing native as well as denatured proteins, and 3) antibodies reacting with denatured proteins, exclusively. The antibody spectrum present in the serum is, of course, dependent on the immunization schedule as well as on the properties of the protein of interest. Many examples are known where sera raised against peptides of a protein react much better with a blotted protein than with the native protein. In the case of monoclonal antibodies, the

range of antigenic conformations tolerated is often much narrower and an antibody selected for binding the native molecule may completely fail to react on western blots.

However, even after boiling in SDS and blotting, proteins may refold to some extent. Refolding can be promoted by certain detergents or heating during the blocking step, or be initiated during the transfer step.

Special techniques

Affinity purification of antibodies from blots

Small amounts of specific antibodies can be recovered from excised bands of a blot. The antibodies can serve as reagents in immunohistochemistry or for clarifying suspected cross-reactions between protein mixtures. In order to deposit a large amount of antigen on the blot the sample is applied across the whole width of a slab gel.

Protein sequencing

In this technique proteins are transferred from SDS-gels to glass fiber filters or membranes resisting the organic solvents and reagents used for sequencing. Proteins, visualized by a general protein stain, are excised and directly inserted into an automatic sequencer. The technique is used for small amounts of protein which are difficult to purify by other methods.

Ligand blotting

Many solubilized receptor proteins will specifically bind their ligands and do so even after western blotting if harsh treatments (heating in SDS, reduction of disulfide bonds) are avoided at all steps. The ligand-receptor pairs analyzed by this procedure encompass a wide variety of molecules (steroids, peptide hormones, toxins, adhesion molecules). Even live cells (bacteria, eukaryotic cells) have been found to interact specifically with immobilized blotted adhesion proteins.

Fingerprinting of pathogens

This is useful for epidemiological studies of bacteria and viruses. Pathogens are cultured by usual microbiological techniques and isolates are subjected to SDS-gel electrophoresis and blotting. The strips are incubated with a standard antiserum and developed with suitable secondary reagents. The banding pattern characterizes the strain and distinguishes it from other strains.

Immunization

Proteins attainable only in small amounts are difficult to purify by liquid chromatography but may be separated by gel electrophoresis. The small amounts of pure proteins in a resolved band often suffice to immunize experimental animals. As an alternative to homogenizing the relevant slices of polyacrylamide (which leads to quite voluminous slurries) the blotted protein band may be implanted or injected into an animal.

Dot immunobinding

Other names for dot immunobinding are dot blotting and dot assay. The high binding capacity of blotting membranes can also be utilized for immunoassays where the electrophoretic separation of western blotting is not required. The antigen solution is spotted on to the membrane as a drop of 1 µl volume or smaller, resulting in a dot of a few millimeters diameter. Larger volumes may be deposited in a filtration manifold where proteins adsorb to the membrane. The technique is useful for adsorbing relatively impure antigens where the reactive components only constitute a small fraction of the total protein. The investigator is free to deposit different antigen and control preparations on the same piece of membrane. Further processing for antigen detection is analogous to western blotting.

HIV-1

A diagnostic test based on blotted viral proteins is commercially produced to confirm presence of antibodies against human immunodeficiency virus 1 (HIV-1). To perform the test the prefabricated strip has only to be incubated with test serum and developing reagents. About nine viral proteins are discernible on blots. Certain empirically found constellations of banding patterns confirm the primary test.

See also: Affinity; Antibodies, detection of; Antigen-binding site; Autoantibodies, tests for; Enzyme-linked immunosorbent assay (ELISA); Epitopes; Immunoabsorbents; Immunoassays; Immuno-electrophoresis; Isoelectric focusing; Northern blotting; SDS-polyacrylamide gel electrophoresis (SDS-PAGE); Southern blotting.

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XENOTRANSPLANTATION

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Xenotransplantation is the transplantation of tissues between members of different species. The term 'heterotransplantation' appeared in earlier literature before the words autologous, syngeneic, allogeneic and xenogeneic were commonly accepted to describe the genetic differences between donors and recipients. Xenogeneic transplantation can involve 'concordant' or 'discordant' donors, roughly according to the phylogenetic distance between the species involved. Discordant species combinations are those in which the recipient has natural preformed antibodies against donor endothelial antigens which cause hyperacute rejection of most vascularized organ transplants.

Xenogeneic transplantation elicits an immune response which is fundamentally similar to that which occurs in allotransplantation. Unique features of xenotransplantation stem partly from the greater antigenic disparities involved and partly from the physiologic incompatibilities which occur when mixing tissues from different species.

Brief clinical history

Clinical interest in xenogeneic transplantation has stemmed from the shortage of organs available for human transplantation, originally because cadaver donors were not often available before the concept of brain death was accepted in the 1960s, and more recently because the extraordinary success of clinical allotransplantation has again created a demand which exceeds the supply of organs.

Reemtsma was the first to report a technically successful xenotransplant to a human patient in 1963. He performed a series of kidney transplants from chimpanzees, one of which survived for 9 months with good renal function before the patient died from complications of immunosuppression. Over the next several years, Starzl and several others performed kidney, liver and heart transplants from

baboons, chimpanzees and pigs to human recipients without long-term success.

Clinical xenotransplantation began again with a heart transplant from a baboon to 'Baby Fac' in 1985. Since then there have been two baboon-to-human liver transplants, a series of islet transplants from fetal pigs to humans, transplantation of fetal pig neural cells to patients with Parkinson disease, and at least one bone marrow transplant from a baboon to a patient with the acquired immune deficiency syndrome (AIDS). In addition, livers from pigs and other species have been used to maintain patients with fulminant hepatic failure by intermittent *ex vivo* perfusion of an animal organ, and there has been at least one effort at 'bridge' transplantation in which a pig liver was transplanted to a critically ill human patient as a first step toward subsequent replacement by an allogeneic organ. At this point, however, no clinically successful xenotransplant has been reported in the sense of achieving patient survival for one year based on the ongoing physiologic function of the transplanted tissue.

Choice of donor species

Clinical efforts at xenografting have used both concordant (chimpanzee and baboon) and discordant (pig) donors. Although there are clear immunologic advantages to the use of concordant donors, practical issues will prevent the large-scale use of non-human primates for human transplantation. Chimpanzees are an endangered species. Baboons, while available in larger numbers, are too small for many types of transplantation. In addition, the need to exclude many potential pathogens in baboons, their long gestation time, and their slow maturation make it unlikely that large numbers of transplants from baboons to humans will ever be performed. Thus, concordant clinical xenotransplantation will prob-

ably occur only as a step toward more clinically useful discordant transplantation.

While many discordant species are available for humans, research efforts have focused almost exclusively on pigs. They are abundantly available, of appropriate size, demonstrate some important physiologic similarities to humans, and have short gestation times and large litters. These last features make it possible to accomplish genetic modifications of pigs over reasonable periods of time.

Immunologic barriers and types of rejection

Hyperacute rejection

Hyperacute rejection of primarily vascularized organ transplants is characterized by vascular disruption leading to hemorrhage and thrombosis within minutes of transplantation. The essential elements leading to hyperacute rejection include the expression of foreign determinants on donor vascular endothelium, the presence of preformed antibodies in the recipient which bind to these determinants, the activation of complement, and the subsequent activation of donor endothelium.

The primary pig endothelial determinant recognized by human recipients is a galactose- α 1,3-galactose sugar linkage formed by the action of a galactosyltransferase enzyme expressed by pigs but not by higher order primates. This determinant is present on many cell surface glycoproteins, especially endothelial adhesion molecules. There are undoubtedly other novel determinants on pig endothelium which are not expressed at sufficient density to cause hyperacute rejection consistently.

The human preformed antibodies which bind the pig α -Gal determinant are similar to blood group antibodies within a species. They are thought to arise from cross-reactions with bacterial carbohydrate antigens which are common in the environment. Thus, although they are called 'natural' antibodies, their presence actually reflects previous exposure. Although both immunoglobulin M (IgM) and IgG natural antibodies exist, only IgM antibodies cause hyperacute rejection of pig organs by primates. This reflects the low binding affinity of natural antibodies with their antigens such that hyperacute rejection is only triggered when the foreign determinant is expressed at high density and polyvalent antibodies are involved.

Sufficient binding of natural antibodies to pig endothelium activates complement by the classical pathway. Complement then becomes the principal mediator of hyperacute rejection. In some species combinations, complement activation can also occur

by the alternative pathway leading to hyperacute rejection even when natural antibodies have been removed. However, this does not occur in the pig-to-human combination.

Complement activation is normally controlled by a series of complement regulatory proteins including decay-accelerating factor (DAF), CD59 and membrane cofactor protein (MCP). Despite the function of these proteins, hyperacute rejection can occur in allogeneic transplantation when preformed antibodies are present. In xenogeneic transplantation, however, the process is magnified considerably because some of the complement regulatory proteins show species-restricted function. For example, the pig DAF protein does not interact with molecules of the human complement system, allowing complement activation to occur without its ordinary feedback inhibition.

The target of complement activation in hyperacute rejection is the donor endothelium. Evidence suggests that it is not the death of endothelial cells which is important, but rather their activation triggered by binding of the complement membrane attack complex. Endothelial activation causes changes in the shape of endothelial cells, creating intercellular gaps, and the loss of heparan sulfate, which normally enhances antithrombin III activity and inhibits coagulation and complement activation. The outcome of endothelial activation is the escape of blood components, exposure of thrombogenic surfaces and, therefore, interstitial edema, hemorrhage and thrombosis in the graft.

Acute vascular or delayed xenograft rejection

Concordant xenografts do not suffer hyperacute rejection. In addition, this form of rejection can be prevented in discordant combinations by inhibition of complement activation or by depletion of preformed natural antibodies. In both cases, however, xenografts are still rejected more rapidly than allografts by a process which has been termed 'acute vascular rejection' or 'delayed xenograft rejection' by different investigators. This rejection appears to be independent of complement, but is associated with the early appearance of antibodies directed primarily at donor endothelial glycoproteins. Thus, this early antibody response may reflect pre-existing 'natural' antibodies which were not detectable prior to transplantation or which were only transiently removed to avoid hyperacute rejection. Although this accelerated form of xenograft rejection can sometimes be prevented by immunosuppressive agents directed at B cells, it is not clear that the process is entirely dependent on antibodies.

'Acute vascular' or 'delayed xenograft' rejection

involves endothelial activation, but the process is slower than in hyperacute rejection and requires the synthesis of new endothelial proteins. Thus, this has been referred to as type II endothelial activation. Changes in the endothelium include the upregulation of adhesion molecules (including P- and E-selectin and VCAM-1), loss of fibrinolytic and anticoagulant activity, and the release of cytokines such as interleukin 1 (IL-1) and IL-8 which promote inflammation. In some cases, species-restricted function of some of the molecular interactions which regulate inflammatory responses may augment the consequences of this endothelial activation, just as the failure of complement regulatory proteins enhances hyperacute rejection.

Cell-mediated xenograft rejection

Because the humoral rejection of xenografts is so powerful, it has been difficult to study the cell-mediated rejection which would play a role if both hyperacute and delayed xenograft rejection were adequately controlled. However, cellular xenografts, including cultured pancreatic islets, do not express donor endothelium and are revascularized by recipient vessels. Thus, these types of xenotransplants may not undergo hyperacute rejection and acute vascular rejection, both of which depend on endothelial activation. The difficulty in achieving prolonged survival of xenogeneic cellular transplants and the rapid rejection of all types of xenografts by B cell-deficient mice indicate that cellular immunity causes powerful xenograft rejection.

In vitro studies of xenogeneic cellular immunity show different results from allogeneic responses depending on the species combination. In some cases, primary *in vitro* responses are weak or absent, while in others (including the pig-to-human combination) they are intact and include an additional natural killer (NK) response which is not seen in allogeneic cultures. Molecular incompatibilities between species again appear to be responsible for these unusual results. In some cases, incompatibilities between the accessory molecules involved in T cell activation and their ligands on xenogeneic stimulating cells weaken the T cell response. In others, the inability of NK cell inhibitory receptors to recognize xenogeneic class I molecules may increase this form of cellular immunity. After xenograft rejection, *in vitro* assays show strong responses which often depend on the indirect pathway by which T cells are stimulated by self antigen-presenting cells (APCs) presenting peptides of the xenoantigens.

In vivo studies of cell-mediated xenograft rejection have suggested that CD4⁺ T cells are especially important in initiating rejection. The cytokines pro-

duced by these T cells are probably required to activate macrophages, eosinophils or NK cells, but the actual effector mechanisms of cell-mediated xenograft rejection are poorly understood.

Chronic rejection

Few xenografts have survived long enough *in vivo* to determine their susceptibility to chronic rejection. However, their greater antigenic disparity and the importance of their vascular endothelium as a target for other types of rejection suggest that xenografts will be even more susceptible to chronic rejection than allografts.

Achieving prolonged survival to xenografts

The immunologic barriers to xenografts are stronger than for allografts. Strategies to overcome these barriers include efforts to reduce the recipient's immune response, to modify the donor animals, and to induce tolerance to xenogeneic antigens.

Suppression of the recipient's response

To avoid hyperacute rejection, natural antibodies have been removed from recipients by plasmapheresis, by pretransplant organ perfusion, and by column absorption of the critical $\alpha 1,3$ -Gal-binding antibodies. Complement inhibition has been achieved using cobra venom factor and by treatment with soluble complement receptor 1 (sCR1). To avoid the subsequent vigorous antibody response to xenografts, recipients have been treated with B cell-targeted immunosuppression, including drugs (such as cyclophosphamide, methotrexate, or 1,5-deoxyspergualin), IgM-specific antibodies to deplete B cells, and splenectomy. Additional immunosuppressive agents such as cyclosporine or tacrolimus have been used to suppress T cell immunity.

Accommodation

While it would seem unlikely that recipient antibody responses could be permanently eliminated by immunosuppression, some evidence suggests that this may be unnecessary. Human renal allografts transplanted between ABO incompatible individuals have been shown to survive even if recipient blood group antibodies are allowed to return several weeks after transplantation. The term 'accommodation' has been used to describe the changes in donor endothelium which prevent hyperacute rejection despite the expression of antigens and the presence of recipient antibodies reactive with them. Several examples of apparent accommodation have been demonstrated in xenogeneic transplantation.

Modifications of donor animals

One of the advantages of xenogeneic transplantation is that donor animals can be genetically modified to diminish the rejection of their organs. For pigs, it is not yet possible to eliminate gene expression by targeted gene disruption, but it is possible to generate transgenic animals. Efforts are therefore underway to diminish the antigenicity of pigs, to eliminate the physiologic incompatibilities which promote xenograft rejection, and to generate animals which actually suppress the immune response.

The α 1,3-galactosyltransferase gene has been knocked out in mice. While this is not yet possible in pigs, overexpression of the gene for α 1,2-fucosyltransferase has been found to compete during glycosylation, leading to greatly diminished expression of the α -Gal determinant in favor of a nonantigenic one. Transgenic pigs have also been generated which express genes encoding human DAF or CD59. High-level expression of these complement regulatory proteins not only corrects the physiologic incompatibilities of the xenogeneic molecules, but also appears to provide supraphysiologic protection against hyperacute rejection. Further efforts are underway to generate transgenic pigs which might prevent T cell activation or induce peripheral tolerance by the elimination of donor-reactive lymphocytes.

Tolerance induction

Because of the greater antigenic disparities and the additional immunologic barriers associated with xenografting, more immunosuppression has been required to achieve survival of xenografts comparable to that of allografts. The induction of tolerance would eliminate this serious disadvantage of xenografts for clinical transplantation. Genetic modifications of donor animals may help to achieve tolerance by peripheral mechanisms. In addition, efforts are underway to achieve deletional tolerance by generating mixed bone marrow chimeras. Small animal models have shown that xenogeneic mixed bone marrow chimeras can be generated in closely related species combinations. However, achieving engraftment of xenogeneic marrow requires more vigorous conditioning of the recipient than for allogeneic marrow and also appears to require the provision of species-specific growth factors in more distantly related species combinations. Thus, physiologic incompatibilities again play a role in the induction of tolerance for xenografts.

Limitations of physiologic organ function in xenografting

Physiologic incompatibilities are an important aspect of the immune response to xenografts. It is likely that physiologic incompatibilities of organ function will also become important in xenografting, although at present too few functioning xenografts have survived long enough to examine this issue. It does appear that pancreatic islets from one species can regulate glucose levels satisfactorily in another species. In addition, humans have survived for many months with renal function provided by a chimpanzee kidney. On the other hand, patients with baboon livers were found to have vastly reduced blood levels of uric acid and cholesterol because of differences in hepatic physiology. Thus, it is almost certain that important physiologic incompatibilities will become apparent when it is possible to transplant complex organs across wide species differences.

Xenozoonoses

The possibility that animal organs could be used for human transplantation has raised concern that infections from animals might be transmitted to human recipients. The term 'xenozoonoses' has been developed to describe this possibility. While many potential human pathogens have been identified in animals, screening of potential animal donors and their breeding in specific-pathogen-free facilities can reduce the likelihood of disease transmission. Indeed, from the point of view of the individual recipient, the infectious risk of xenotransplantation is likely to be less than for cadaveric allotransplantation, where donor screening must be done rapidly, focusing only on the most serious pathogens. On the other hand, cross-species transplantation raises the possibility that endogenous retroviruses of the donor might recombine in human recipients to generate as yet unknown pathogens.

While the risk of such an event is small for an individual recipient, the possibility raises a public health concern for society as a whole. This fear is heightened by the belief that AIDS in humans developed as a result of cross-species transmission of a retrovirus from nonhuman primates. Despite these theoretical concerns, national agencies responsible for public health safety have determined that it is reasonable to proceed with clinical attempts at xenotransplantation as long as donor animals are suitably screened and recipients monitored for evidence of disease transmission.

See also: Carbohydrate antigens; Chimerism, hema-

topoietic; Cobra venom factor; Complement, alternative pathway; Complement, classical pathway; Complement, membrane attack pathway; Complement receptors; Decay-accelerating factor (CD55); Endothelium; Graft rejection; Tolerance, peripheral; Transgenic animals; Transplantation.

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YERSINIA, INFECTION AND IMMUNITY

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Yersinias are gram-negative rods and belong to the family Enterobacteriaceae. The three *Yersinia* species virulent for humans and rodents are *Y. pestis*, *Y. pseudotuberculosis* and certain serotypes of *Y. enterocolitica*. They all show tropism for lymphoid

tissue. However, yersiniosis, the disease caused by *Y. pseudotuberculosis* and *Y. enterocolitica* is very different from bubonic plague or 'black death' caused by *Y. pestis*. Yersiniosis is characterized in the acute phase by gastroenteritis with inflammation of the ter-

minal ileum and mesenteric lymph nodes, and it is sometimes followed by postinfection complications, but life-threatening infections are exceptional. In contrast, as recently as in 1988, 13 of 1363 reported cases of bubonic plague were fatal. Interestingly enough, *Y. pestis* and *Y. pseudotuberculosis* show an extremely high degree of homology at the DNA level.

The virulence of *Yersinia* species depends on a 70–80 kb virulence plasmid, but chromosomal genes also contribute to virulence. Serotyping of *Yersinia* species is based on lipopolysaccharide. At least 57 serotypes of *Y. enterocolitica* and 13 serotypes of *Y. pseudotuberculosis* have been determined, and certain serotypes are clearly more virulent than others. Nevertheless, the role of lipopolysaccharide in the virulence of *Yersinia* has not been established.

Immune responses of the host

Yersiniosis elicits both humoral and cell-mediated immune responses in the host. It appears that most of the antibody response is directed toward the lipopolysaccharide. Follow-up studies have, however, demonstrated that patients with *Yersinia* infection produce antibodies directed towards a multitude of antigenic determinants, both plasmid and chromosomally encoded ones. The antibodies often persist for several months and in the case of postinfection complications even years. As yersiniosis is fairly common in some parts of the world it is thus not surprising that, for example in northern Europe, the sera of 10–30% of healthy blood donors indicate previous *Y. enterocolitica* or *Y. pseudotuberculosis* infection. A remarkable feature of *Yersinia* infection is that it may pass quite unnoticed. This is illustrated by occasional severe reactions upon transfusion of blood, the donor of which, unaware of it, has carried *Yersinia* in the blood. During preservation at refrigerator temperature the psychrophilic yersinias multiply. Furthermore, epidemiologic studies have demonstrated that many individuals with verifiable infection remain symptomless.

Reactive arthritis, a common postinfection complication of yersiniosis, belongs to the seronegative spondyloarthropathies strongly associated with possession of the class I major histocompatibility complex (MHC) antigen HLA-B27. Approximately 80% of patients with *Yersinia*-triggered reactive arthritis are B27-positive. *Yersinia*-triggered uveitis and urethritis are also associated with B27 antigens but erythema nodosum is not. Of the seronegative spondyloarthropathies, ankylosing spondylitis shows the strongest association to HLA-B27 (95%), and hypotheses regarding the pathogenesis of *Yersinia*-triggered reactive arthritis and ankylosing spondylitis

go along similar lines. It has been suggested but not proven that molecular mimicry between antigens of the *Yersinia* microorganism and the B27 antigen leads to the development of reactive arthritis. Molecular mimicry might either cause ineffective elimination of the microorganism due to tolerance or, on the other hand, trigger autoimmunity. Extensive studies regarding the peptide-binding groove of the HLA-B27 molecule and of the bound peptides have not led to an explanation of this disease association. However, a surface component of *Y. enterocolitica* shares certain structural similarity with the human thyrotropin receptor.

There is evidence that patients with *Yersinia*-triggered reactive arthritis differ in their immune functions from yersiniosis patients with uncomplicated disease. The former tend to have a weaker *Yersinia*-specific cell-mediated immune response but a stronger and more persistent immunoglobulin A (IgA) antibody response against the microbe. At the early phase of infection those with reactive arthritis show more *Yersinia*-specific opsonic antibodies, and they have more *Yersinia*-specific immune complexes. In addition, B27-positive individuals may have exaggerated inflammatory responsiveness, such as stronger chemotactic function and production of some inflammatory mediators. It is probable that the immune response against the causative microorganism has an important role in the pathogenesis of *Yersinia*-triggered reactive arthritis. *Yersinia* antigens have been detected in synovial membrane and in synovial fluid cells in patients with reactive arthritis, and the longstanding antibody response may be a reflection of the continuous stimulation by the microbes hiding in, for example, gut-associated lymphoid tissues.

Evasive strategies by the organism

Yersinias are regarded as facultatively intracellular microorganisms, but this notion is currently questioned, especially regarding *Y. enterocolitica* and *Y. pseudotuberculosis*. Yersinias can obviously stay viable and even multiply in macrophages. In calcium-deficient conditions at 37°C, the growth of virulence plasmid-positive yersinias is greatly retarded and they express several outer membrane proteins, which are also released to the culture medium. This phenomenon is called calcium dependency or low calcium response. It is currently believed that low calcium content and the temperature of 37°C signal to the microbe that it is in an intracellular compartment.

Y. pestis has, in addition to the virulence plasmid coding for low calcium response, other plasmids

coding for the plague murine toxin, pesticin, plasminogen activator and coagulase. In addition to plasmid-encoded genes, chromosomal genes are needed for full virulence. For example, some invasion and iron metabolism-associated structures necessary for virulence are chromosomally encoded. This is also illustrated by the fact that the avirulent *Yersinia* species do not gain virulence even if they are provided with the plasmid. The role of iron is also indicated in the clinical setting, as iron overload predisposes to severe septic forms of yersiniosis.

Virulence-determining *Yersinia* plasmid-encoded outer membrane proteins (YOPs) of *Y. enterocolitica* and *Y. pseudotuberculosis* mediate attachment of the bacteria to epithelial cells, cytotoxicity *in vitro* for mouse macrophage cell lines and resident mouse peritoneal macrophages, resistance to the complement-mediated bactericidal activity of serum and resistance to opsonization and phagocytosis. A few of these virulence-associated functions have already been attributed to specific YOPs. Invasiveness, a chromosomally encoded factor, is understood to some extent at the molecular level and involves a molecule termed 'invasin'. The entry of yersinias into mammalian cells is initiated by specific binding of bacteria to cell receptors belonging to the integrin family of cell adhesion molecules. A *Yersinia* adhesin (YadA) has been demonstrated to bind specifically to joint collagens and other extracellular matrix molecules and it has also been found to contribute to the arthritogenicity of *Y. enterocolitica* 0:8 in a rat experimental model. Furthermore, yersinias produce interesting superantigens, stimulating and expanding T cells.

Vaccines

Effective vaccines against yersiniosis have yet to be developed. An effective plague vaccine would naturally be valuable. *Yersinia* gastroenteritis as such is rarely a problem for patients, but the postinfection complications may cause a great amount of pain and inconvenience. A vaccine against yersiniosis might be useful for individuals prone to reactive arthritis. Relatively little is known about the role of immune responses in protection against *Yersinia* infections. Some studies have indicated that cell-mediated reactions are associated with long-term immunity to plague. This is compatible with the fact that *Y. pestis* is a pathogen capable of intracellular growth. That cell-mediated immune responses are elicited during yersiniosis is quite clear, but their role in protection against infection remains unclear. Most of the evidence supports the role of humoral immunity in protection against yersiniosis.

To obtain protection against the many serotypes of pathogenic *Yersinia* species the putative vaccine should be directed against a determinant found in all of them. Such a determinant could be one of the YOPs or invasin. However, more information regarding the pathogenesis of *Yersinia* infections is needed if effective vaccines are to be developed.

See also: Bacteria, immunity to; Bacterial cell walls; Molecular mimicry.

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ZINC AND THE IMMUNE SYSTEM

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Zinc, an essential nutrient, is an element of profound importance in many functions of the immune system and other host defense mechanisms. And uniquely, through the action of proinflammatory cytokines, immunological responses can acutely alter the body distribution of zinc.

Since zinc is in the active site of more than 200 metalloenzymes, it is not surprising that it is of widespread metabolic importance, contributing to the proper functioning of all body cells, including those of the immune system. Zinc is an essential nutrient during cell replication, because zinc metalloenzymes are required for nucleic acid synthesis, with DNA and RNA polymerases, reverse transcriptase and nucleoside phosphorylase serving as examples.

But molecular mechanisms by which zinc influences the immune system are not limited to enzymatic functions. Zinc may also influence nucleic acid metabolism by altering the binding of F1 and F3 histones to DNA, so as to affect RNA synthesis. Zinc appears to modify and stabilize cell membranes, forming mercaptides with thiol groups of proteins, and interacting with phosphate groups of phospholipids and with carboxyl groups of sialic acid. Zinc may inhibit some calmodulin-stimulated functions. In another important role, zinc molecules in plasma must bind to newly synthesized thymulin and other thymic hormones in order to activate them, and allow these hormones to stimulate T lymphocytes throughout the body.

Zinc deficiency and immunity

Severe, or even marginal, deficiencies of zinc lead to reversible dysfunctions of human T lymphocytes.

Zinc deficiency is easily produced in laboratory animals, with a prompt development of numerous abnormalities in immune functions. Zinc deficiency consistently leads to atrophy of the thymus and other lymphoid organs, to reduced lymphocyte numbers in tissues (especially in the T cell areas) and to lymphopenia. Zinc deficiency is accompanied by anergy, manifested predominantly by dysfunctions of cell-mediated immunity, along with some depression in the production of T cell-dependent antibodies and splenic plaque-forming cells.

Zinc deficiency initiates a selective decrease in CD4⁺ helper cell numbers. *In vitro* testing reveals a markedly reduced proliferative response by these cells to phytohemagglutinin and other mitogens. Natural killer cells also show decreased activity and T cell-mediated cytotoxicity is reduced. In zinc deficiency, lymphocytes have a reduced ability to produce interleukin 2 (IL-2) *in vitro*. These dysfunctions could be due to direct effects of zinc deficiency on individual lymphocytes and their metabolic mechanisms. But some dysfunctions, especially in T cells, could also be due to a concomitant reduction in the synthesis and zinc-dependent activation of thymic hormones. Zinc deficiency also has adverse effects on B cell functions, although not as marked as those seen in T cells.

Zinc deficiency also affects many nonspecific aspects of host defense. The production of monocytes and macrophages is reduced, and granulocytes exhibit impaired chemotaxis. Wound healing and tissue integrity become defective, with the development of extensive mucosal and dermal lesions, especially at mucocutaneous borders.

Dysfunctions in host defense mechanisms during

zinc deficiency thus include the immune system, monocyte/macrophages, granulocytes and the breakdown of anatomical surface barriers. Such combined problems make the zinc-deficient host extremely susceptible to infectious diseases, and greatly increases the severity of infections that do occur.

Effects of acute phase reactions on the distribution of body zinc

The body distribution of zinc responds in a unique and unusual manner during proinflammatory cytokine-induced acute phase reactions which accompany generalized infections, trauma or endotoxemia. Zinc concentrations in plasma show an abrupt decline, falling to about half of their normal values. This suddenly developing hypozincemia occurs because of a sequestration of zinc within hepatocytes, thymocytes and marrow cells. Zinc sequestration then persists throughout the duration of illness. The sequestration of zinc is the secondary consequence of the induced expression, caused by IL-1 and other proinflammatory cytokines, of metallothionein 1 and 2 genes within these responding cells. Newly synthesized intracellular metallothionein proteins generate binding sites for zinc, and lead to the hypozincemia which characterizes acute phase reactions. Biological reasons for this unique sequestration of zinc remain unknown, although macrophage activation may be enhanced by low plasma zinc values.

The broad spectrum of zinc deficiency

Zinc deficiency is generally present whenever protein-energy malnutrition is seen. The losses of body nitrogen that occur during the acute phase reactions to febrile illness or severe trauma, measured by negative balances, are accompanied by concomitant, comparable losses of body zinc (as well as by the previously described sequestration of zinc). Zinc deficiency can also occur as an isolated nutritional problem if zinc intakes are low, or if there is an inherited defect in intestinal zinc absorption.

Since the intestinal absorption of zinc is an active process, zinc deficiency can occur if absorption is inhibited. Phytates and some dietary fibers can bind zinc within the intestinal tract and thereby impair its absorption. The congenital disease acrodermatitis enteropathica is due to a defective intestinal absorption of zinc. Infants with this disease exhibit exten-

sive mucocutaneous lesions, immunological defects and severe infections which lead to an early death. Fortunately, an increase in oral zinc intake can usually reverse all manifestations of this disease. A similar congenital problem, seen in one variety of Friesian cattle, is also reversible with dietary zinc supplements. Zinc deficiency is also a factor in congenital epidermolysis bullosa.

Reversibility of zinc deficiency

The immunological aspects of zinc deficiency are, as a rule, easily and rapidly restored to normal by the oral administration of sufficient zinc. Excessive supplementation of diets with zinc, may, on the other hand, lead to immune system suppression. In the nutritional rehabilitation of children with severe protein-energy malnutrition (marasmus or kwashiorkor), supplementation with oral zinc may be necessary in order to restore the thymus to its normal size. Some studies in laboratory animals suggest that zinc malnutrition during fetal, and even neonatal life may result in a long-term impairments in the development and function of the immune system.

See also: Nutrition and the immune system; Polyclonal activators.

Further reading

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